Does an Increase in Membrane Unsaturated Fatty Acids Account for Tween 80 Stimulation of Glucosyltransferase Secretion by *Streptococcus salivarius*?

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When *Streptococcus salivarius* was grown in batch culture in the presence of various Tween detergents, the fatty acid moiety of the detergent was incorporated into the lipids of its membrane. Tween 80 (containing primarily oleic acid) markedly stimulated the production of extracellular glucosyltransferase and also increased the degree of unsaturation of the membrane lipid fatty acids. The possibility that an increase in membrane unsaturated fatty acids promoted extracellular glucosyltransferase production was examined by growing cells at different temperatures in the presence or absence of Tween 80. The membrane lipids of cells grown at 30 °C, 37 °C and 40 °C without Tween 80 exhibited unsaturated/saturated fatty acid ratios of 2.06, 1.01 and 0.87 respectively. A significant increase in the production of extracellular glucosyltransferase was observed at 30 °C compared to cells grown at 40 °C. However, cells produced much more exoenzyme at all temperatures when grown with Tween 80. The results indicated that an increase in the unsaturated fatty acid content of the membrane lipids was not by itself sufficient to account for the stimulation of extracellular glucosyltransferase production by Tween 80, but that the surfactant also had to be present.

INTRODUCTION

The ability of certain oral streptococci to synthesize water insoluble glucans is related to their colonization and establishment on tooth surfaces (Gibbons & Banghart, 1967; Gibbons & Fitzgerald, 1969; Gibbons & van Houte, 1975). However, the mechanism of control over the production of the extracellular glucosyltransferase enzymes responsible for the formation of glucan remains to be elucidated. The amount of glucosyltransferase produced by oral streptococci varies markedly depending upon the composition of the growth medium (Carlsson & Elander, 1973; Schachtele *et al.*, 1976; Janda & Kuramitsu, 1976; Umesaki *et al.*, 1977; Wittenberger *et al.*, 1978), the growth rate, and the carbohydrate available to the organism in continuous culture (Carlsson & Elander, 1973; Hamilton *et al.*, 1979; Hardy *et al.*, 1981). In particular, the enhancement of glucosyltransferase synthesis and secretion by the non-ionic surfactants Emanon 4115 (polyethylene glycol mono-oleate) and Tween 80 (polyoxyethylene sorbitan mono-oleate) (Umesaki *et al.*, 1977; Wittenberger *et al.*, 1978) has implicated the cell membrane as one possible controlling factor in the production of the extracellular enzyme. In

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Abbreviation: U/S, ratio of unsaturated to saturated.

the case of *Streptococcus salivarius* (Jacques, 1983) and *S. mutans* (Umesaki *et al.*, 1977), Tween 80 has been shown to increase the octadecenoic acid content of the lipid bilayer and it has been suggested that an increase in fluidity of the membrane lipids might facilitate glucosyltransferase secretion, thereby enhancing its rate of synthesis (Umesaki *et al.*, 1977; Wittenberger *et al.*, 1978). In this paper it will be shown that simple enrichment of *S. salivarius* membrane lipids with unsaturated fatty acids is not the sole factor in the Tween 80 mediated stimulation of extracellular glucosyltransferase production.

METHODS

Organism and medium. *Streptococcus salivarius* ATCC 25975 was used throughout these studies and was grown in a chemically-defined medium (Wittenberger *et al.*, 1978). Stock cultures were stored at 4 °C in defined medium containing excess CaCO₃ (20 mg ml⁻¹). Stocks were transferred to fresh medium every month. Glucose (0.4%, w/v) served as the fermentable carbon source in all experiments.

Materials. [U-glucosyl-¹⁴C]sucrose was purchased from New England Nuclear; BF₃ in methanol, fatty acid standards and the gas-liquid chromatographic column packing (10% (w/w) SP-2330 on 100/200 Chromosorb) were obtained from Supelco, Bellefonte, Pa., USA. Tweens 20, 40, 60 and 80, containing dodecanoic, hexadecanoic, octadecanoic and octadecenoic acid substituents respectively, were purchased from Sigma.

Growth measurements. *S. salivarius* was grown at 37 °C in screw cap bottles containing 100 ml defined medium. Growth was monitored with a Klett-Summerson colorimeter equipped with a 660 nm filter (Klett Manufacturing Co., New York, NY, USA); 100 Klett units were equivalent to a cell dry weight of 0.36 mg ml⁻¹. When cells were cultured at different temperatures, the inoculum was first grown through one transfer at the desired temperature except where otherwise indicated in the text.

Assay of glucosyltransferase. Total extracellular glucosyltransferase activity was measured in samples taken from growing cultures. Cells were removed by vacuum filtration through 0.45 µm Nalgene filter units (Nalge Company) and the filtrates were assayed with [U-glucosyl-¹⁴C]sucrose according to a modification (Wittenberger *et al.*, 1978) of the procedure described by Robrish *et al.* (1972). One unit of enzyme activity (U) was defined as the amount of glucosyltransferase that catalysed the incorporation of 1 µmol of the glucose moiety of sucrose into 75% ethanol-insoluble polysaccharide min⁻¹. In all cases, the specific activity of glucosyltransferase was expressed as U (mg dry wt)⁻¹.

Preparation and analysis of fatty acids. Samples of cells for fatty acid analysis (10 to 50 ml cultures) were harvested by centrifugation (10000 g, 4 °C, 10 min), washed and resuspended in distilled water (2.5 ml). The membrane lipids were extracted according to the method of Bligh & Dyer (1959), and methyl esters were prepared (Jacques, 1982) and analysed as reported previously (Jacques, 1983).

RESULTS

Influence of Tween detergents on the membrane fatty acids

The fatty acid profile of the membrane lipids of *S. salivarius* was significantly altered when cells were grown in the presence of Tween 20, 40, 60 or 80 (Table 1). In all instances except one, the major increase in the membrane fatty acids corresponded to the fatty acid component of the added detergent. The one exception to this pattern occurred with cells grown in the presence of Tween 20, where the dodecanoic acid substituent of the surfactant was not detected in the membrane lipids and may have been elongated to hexadecanoic acid (Table 1). Organisms grown in detergents containing a saturated fatty acid substituent (Tween 20, 40 and 60) also possessed decreased levels of octadecenoic acid. While these results do not preclude *de novo* synthesis by the organism of specific fatty acids corresponding to those in a given Tween detergent, it is more likely that the fatty acid substituent of the detergent was released by hydrolysis and incorporated directly into the membrane lipids. It was also shown that direct adsorption of the detergents to the cells did not occur. When cells were grown in medium without added surfactants and then incubated at 4 °C for 30 min with the various Tweens, no alterations in the fatty acid profile were observed. Moreover, analysis of the fatty acid profiles of cells sampled throughout exponential growth in the presence or absence of Tweens revealed that the pattern of the fatty acid profile was established in late lag/early exponential phase and was thereafter constant throughout growth (data not shown).

Table 1. *Fatty acid profile of S. salivarius grown in the presence and absence of various Tween detergents*

Cells were grown at 37 °C in the absence of Tween detergents and were inoculated (10%, v/v) into 100 ml volumes of medium and grown to late exponential phase. The values in bold type correspond to the fatty acid present in the Tween detergent added to the culture medium. In the case of Tween 20, the fatty acid substituent dodecanoic acid was not detected in the membrane lipids. The Tweens were each present at a final concentration of 0.05% (v/v). The standard error for any given fatty acid was 0.1% (three analyses).

Fatty acid	Fatty acid composition (% of total)				
	No Tween	Tween 20	Growth condition: Tween 40*	Tween 60	Tween 80
Dodecanoic (C _{12:0})	—	—	—	—	—
Tetradecanoic (C _{14:0})	5.1	8.6	3.2	5.6	3.5
Hexadecanoic (C _{16:0})	39.4	56.5	69.5	47.3	28.6
Hexadecenoic (C _{16:1})	10.9	7.7	6.1	10.1	11.8
Unknown	—	—	1.9	1.2	1.0
Octadecanoic (C _{18:0})	5.2	7.1	4.3	20.7	3.8
Octadecenoic (C _{18:1})	29.9	17.9	11.3	11.4	42.7
Eicosenoic (C _{20:1})	9.0	2.2	2.2	1.9	7.7

—, Not detected.

* Growth in the presence of Tween 40 was slow. The values given are for cells grown for 24 h.

Table 2. *Extracellular glucosyltransferase production in the presence or absence of various Tween detergents*

Cells were grown at 37 °C in 100 ml cultures to the late exponential phase of growth in all cases, except for the culture to which Tween 40 had been added where growth was much slower and cells were harvested after 24 h. All Tweens were present at a concentration of 0.05% (v/v).

Addition to growth medium	Total growth time (min)	U/S* ratio	Extracellular glucosyltransferase [mU (mg dry wt) ⁻¹]
No Tween	180	1.01	31.8
Tween 20	200	0.39	96.3
Tween 40	1440	0.26	194.4
Tween 60	360	0.34	315.0
Tween 80	200	1.65	636.0

* Ratio of unsaturated to saturated fatty acids in membrane lipids (calculated from the data in Table 1).

Effect of Tweens on extracellular glucosyltransferase production

Tweens 20, 40 and 60 stimulated extracellular glucosyltransferase production and this stimulation appeared to be related to the chain length of the fatty acid moiety of the surfactant. Tween 80, however, was clearly the most effective in enhancing glucosyltransferase production and it was also the only detergent that increased the unsaturated/saturated (U/S) fatty acid ratio of the cell membrane (Table 2). The increase in the U/S ratio appeared to be due to the incorporation of the octadecenoic acid moiety of Tween 80 into the membrane lipids at the expense of hexadecanoic and octadecanoic acids (Table 1).

Effect of temperature on the membrane fatty acids and glucosyltransferase production

The possibility was investigated that the increase in the unsaturated fatty acid content of the membrane lipids of cells grown with Tween 80 was directly responsible for the stimulation of extracellular glucosyltransferase production. In *Escherichia coli*, it is known that the membrane lipids are enriched with unsaturated fatty acids when the organism is grown at suboptimal temperatures (Marr & Ingraham, 1962). Accordingly, *S. salivarius* was grown at 30 °C, 37 °C, and 40 °C in the presence and absence of Tween 80. The fatty acid composition of the membrane

Table 3. *Effect of temperature on the membrane lipid fatty acids of S. salivarius grown in the presence or absence of Tween 80*

Cells grown in the absence of Tween 80 at the appropriate temperature were inoculated (10%, v/v) into 100 ml of medium and grown to late exponential phase. The standard error for any given fatty acid was 0.1% (three analyses). Where indicated, Tween 80 was present at 0.05% (v/v).

Fatty acid	Fatty acid composition (% of total)					
	Growth condition:					
	30 °C		37 °C		40 °C	
	No Tween	Tween 80	No Tween	Tween 80	No Tween	Tween 80
Tetradecanoic (C _{14:0})	0.8	0.6	5.1	3.5	3.9	1.4
Hexadecanoic (C _{16:0})	26.1	17.7	39.4	28.6	42.1	33.5
Hexadecenoic (C _{16:1})	7.3	7.4	10.9	11.8	6.3	6.4
Unknown	—	0.9	—	1.0	—	0.7
Octadecanoic (C _{18:0})	5.8	2.6	5.2	3.8	7.6	6.1
Octadecenoic (C _{18:1})	43.9	58.0	29.9	42.7	29.9	42.0
Eicosenoic (C _{20:1})	16.0	12.7	9.0	7.7	10.2	9.8
U/S* ratio	2.06	3.74	1.01	1.65	0.87	1.42

—, Not detected.

* Ratio of unsaturated to saturated fatty acids in membrane lipids.

Table 4. *Effect of temperature on the unsaturated/saturated fatty acid ratio of the membrane lipids and the amount of glucosyltransferase produced in the presence and absence of Tween 80*

Growth temp.	Tween 80 (0.05%, v/v)	U/S* ratio	Extracellular glucosyltransferase† [mU (mg dry wt) ⁻¹]
30 °C	—	2.06	67.9
30 °C	+	3.74	309.6
37 °C	—	1.01	31.8
37 °C	+	1.65	641.7
40 °C	—	0.87	13.2
40 °C	+	1.42	1235.0

* Ratio of unsaturated to saturated fatty acids in membrane lipids (data from Table 3).

† Samples from cultures described in Table 3.

lipids was then analysed and the amount of glucosyltransferase present in the culture supernatants was assayed. Cells grown at 30 °C without Tween 80 had a membrane lipid fatty acid profile very similar to that found in cells grown at 37 °C or 40 °C in the presence of Tween 80 (Table 3). All three growth conditions resulted in an increase in the U/S fatty acid ratio of the membrane lipids compared to cells grown at 37 °C or 40 °C without Tween 80 and in all three cases this was due largely to an increase in octadecenoic acid and a decrease in hexadecanoic acid. Although cells grown at 30 °C without Tween 80 (U/S = 2.06) produced significantly more extracellular glucosyltransferase than cells grown without Tween 80 at 37 °C (U/S = 1.01) or 40 °C (U/S = 0.87), they produced much less exoenzyme than cells grown in its presence at all three temperatures (Table 4). This suggests that simple enrichment of the membrane lipids with unsaturated fatty acids was not the sole explanation for the dramatic stimulation of extracellular glucosyltransferase production by Tween 80.

DISCUSSION

Prior reports have shown that Tween 80 markedly stimulates the production of extracellular glucosyltransferase by *S. salivarius* (Wittenberger *et al.*, 1978) and by *S. mutans* (Umesaki *et al.*, 1977). The mechanism by which this occurs, however, has not been resolved. In the case of *S. salivarius*, it is not exclusively a consequence of the surfactant acting directly on the cell membrane to facilitate export of the enzyme. Other Tweens were far less effective than Tween 80 in stimulating glucosyltransferase production (Table 2) and detergents such as Triton X-100,

when added to the medium at sub-growth inhibitory concentrations, were ineffective in promoting the production of this enzyme (Wittenberger *et al.*, 1978).

The major difference between Tween 80 and the other surfactants in this series is that Tween 80 contains a mono-unsaturated fatty acid (oleate). The increase in unsaturated fatty acids (predominantly C_{18:1}) in the membrane lipids that accompanied growth of *S. salivarius* in medium containing Tween 80 (Table 1) appears to be a contributing factor in the stimulation of extracellular glucosyltransferase production (Table 2). The membrane lipids of cells grown at 30 °C without detergent were also enriched with unsaturated fatty acids (again, predominantly C_{18:1}) and these cells too produced more extracellular glucosyltransferase than cells grown at 37 °C or 40 °C without Tween 80 (Table 4). The increase in octadecenoic acid that occurred in the temperature shift-down experiments without Tween 80 may have been due to an increase not in oleic acid but in its isomer, *cis*-vaccenic acid. This has been reported to occur in *E. coli* (Marr & Ingraham, 1962; Kito *et al.*, 1975; Evkokimova *et al.*, 1978; de Mendoza & Cronan, 1983). Moreover, the predominant C_{18:1} fatty acid normally found in streptococcal membrane lipids is *cis*-vaccenic acid (Hoffmann & Tausig, 1955). The analytical procedures used in this study could not distinguish between the octadecenoic acid isomers. It remains to be established, therefore, whether the isomeric configuration of the octadecenoic acid species in the *S. salivarius* membrane lipids is also important in the synthesis and export of glucosyltransferase.

However, the simple enrichment of the *S. salivarius* membrane lipids with unsaturated fatty acids, and the assumed attendant increase in membrane fluidity (Kimura & Izui, 1976; Cronan & Gelmann, 1975; Evkokimova *et al.*, 1978; Nesmeyanova, 1982), is not the sole requirement for maximum production of extracellular glucosyltransferase. Cells grown at 30 °C without Tween 80 had a U/S ratio (2.06) greater than cells grown at 37 °C or 40 °C with the detergent (1.65 and 1.42; Table 3), yet produced only about one-tenth as much extracellular glucosyltransferase (Table 4). Cells grown in the presence of Tween 80 produced much more glucosyltransferase at all three temperatures than cells grown without the detergent.

The conditions, therefore, that gave maximal glucosyltransferase production at all temperatures were those in which the cell membrane was enriched with unsaturated fatty acids and Tween 80 was also present in the growth medium. One interpretation of these results is that Tween 80 serves two functions. First, it is a source of oleic acid which, after hydrolysis from the detergent, is incorporated directly into the membrane bilayer. Second, by virtue of its amphiphilic properties, the surfactant might then interact physicochemically with the altered membrane so as to facilitate glucosyltransferase translocation. Ample precedent exists for amphiphilic compounds interacting directly with bacterial membranes to alter membrane functions (Fishman *et al.*, 1980; Petit-Glatron & Chambert, 1981; Jacques, 1983). A prediction of this hypothesis is that surfactants other than Tween 80 might stimulate maximal glucosyltransferase production in *S. salivarius* cells whose membrane lipids were previously enriched with oleate. We have found that washed cell suspensions, prepared from cells grown at 37 °C in defined medium containing Tween 80 (U/S = 2.45) and incubated in a non-growth-supporting medium, produced nearly equal amounts of extracellular glucosyltransferase in response to Tween 40, Tween 60 or Tween 80 (unpublished observation). We have not determined, however, whether any alterations in the fatty acid composition of the lipid bilayer occurred during the incubation period with Tweens containing the saturated fatty acids.

It remains to be established whether the postulated dual role of Tween 80 explains the enhanced production of extracellular glucosyltransferase by *S. salivarius*. The cell membrane potential is important in the maturation of exported proteins in *E. coli* (Enequist *et al.*, 1981; Daniels *et al.*, 1981) and depolarization of the membrane prevents export of these proteins (Pages & Lazdunski, 1982). Keevil *et al.* (1984) have also shown that in *Streptococcus sanguis*, the modulation of protonmotive force across the energized membrane by varying the Na⁺/K⁺ ratio significantly affects the secretion of glucosyltransferase. It is interesting to speculate that enrichment of the *S. salivarius* lipid bilayer with unsaturated fatty acids, and the subsequent physicochemical interaction of the bilayer with Tween 80, might affect the electrochemical gradient, or a specific combination of lipid interactions and the gradient, so as to facilitate the translocation of glucosyltransferase.

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