Effects of Metabolic Inhibitors on Extracellular Fructosyltransferase Production in *Actinomyces viscosus*

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Extracellular fructosyltransferase (levansucrase; EC 2.4.1.10) production in *Actinomyces viscosus* T14AV was demonstrated to occur concomitantly with cellular growth. The inhibition of both cellular ribonucleic acid and protein synthesis resulted in no further accumulation of enzyme activity. The antibiotic sodium clofibrate differentially inhibited the production of fructosyltransferase by strain T14AV. Furthermore, the antibiotic preferentially inhibited [14C]acetate incorporation into cellular lipid, but did not affect protein synthesis. In addition, no inhibition of fructosyltransferase production was observed upon the addition of the fatty acid synthesis inhibitor cerulenin. On the other hand, extracellular fructosyltransferase production was apparently stimulated in the presence of the cell wall synthesis inhibitors penicillin, amphomycin, and tunicamycin. These results are discussed in terms of the mechanism of extracellular protein production in *A. viscosus*.

*Actinomyces viscosus* is an oral microorganism which has been implicated in the etiology of the human oral tissue diseases gingivitis (18) and periodontitis (19). Studies with germfree animal model systems have demonstrated that monoinfection of oral cavities with *A. viscosus* results in extensive plaque formation, root surface caries, and alveolar bone loss typical of periodontal disease (17, 19). Based on recent investigations, immunological injury has been proposed as a major factor in the pathogenesis of *A. viscosus* infection (2, 26). Both specific and nonspecific activation of lymphocytes by *A. viscosus* have also been demonstrated (8, 10, 26). It is possible that some cellular product of the organism may serve as a direct irritant of the adjacent tissue or as a mitogen for local lymphoid elements. However, these putative pathogenic factors have yet to be conclusively identified.

Extracellular levan (polyfructose) has been reported to be synthesized by *A. viscosus* (24). Since bacterial polysaccharides appear to act as B-lymphocyte mitogens (15), it is possible that gingival inflammation after *A. viscosus* infection may represent the result of the blastogenic activity of levan molecules produced by the organism. In addition, the release of extracellular levan may play an important role in other aspects of the pathogenicity of *A. viscosus*. The presence of levan may also promote plaque formation by inducing aggregation between *A. viscosus* and other bacteria (17, 19). Furthermore, extracellular levan may serve as a carbon source for plaque microorganisms capable of hydrolyzing levan molecules (9, 23).

Fructosyltransferase (FTF; levansucrase; EC 2.4.1.10) catalyzes the formation of levan by the following reaction: sucrose + acceptor → levan + glucose. The presence of FTF activity has been demonstrated both in the growth medium and on the cell surface of *A. viscosus* (24). On the other hand, no large pool of intracellular (compartment within the cell wall) FTF has been reported (24). Recently, FTF from the human oral isolate *A. viscosus* T14V has been extensively purified and characterized by Pabst (24). However, the regulation of extracellular FTF production has not been investigated in detail.

The present investigation was undertaken to examine the mechanism of FTF secretion in *A. viscosus* in light of recent studies on procaryotic extracellular protein secretion (14, 27, 29, 31, 33). Because of the possible role of fatty acid synthesis in this process (7, 20), the effects of cerulenin and another recently described inhibitor of bacterial lipid synthesis, sodium clofibrate (the sodium salt of chlorophenoxyisobutyrate [NaCPIB] [22]), on FTF synthesis were investigated. A recent study has demonstrated that the addition of penicillin to growing cultures of *Streptococcus mutans* results in the apparent stimulation of glucosyltransferase production, due to the increased secretion of putative amphipathic activators (16). It was therefore of...
interest to investigate the possible analogous release of FTF activators in *A. viscosus* as a result of the inhibition of cell wall synthesis. The effects of cell wall synthesis inhibitors on extracellular FTF production in *A. viscosus* were assessed by the addition of penicillin, amphotericin, and tunicamycin to growing cell cultures. These results are discussed relative to the mechanism of FTF production (synthesis plus secretion) in *A. viscosus*.

**MATERIALS AND METHODS**

**Organisms.** *A. viscosus* T14AV and T14V, human oral isolates, were kindly supplied by S. Brecher (Forsyth Dental Center, Boston, Mass.) and F. McIntire (University of Colorado, Denver, Col.), respectively. Growth medium. The organism was routinely grown in a static culture at 37°C in actinomyces broth (BBL Microbiology Systems, Cockeysville, Md.) which contained the following: brain heart infusion, trypicase (BBL), peptone, yeast extract, cysteine, sodium phosphate, ammonium sulfate, magnesium sulfate, and calcium chloride. The broth was also supplemented with 1% glucose.

**Production of FTF.** For each experiment, 10 to 20 ml of actinomyces broth was inoculated with a 1% (vol/vol) inoculum of *A. viscosus* T14AV from cultures grown for 12 h. After growth for an additional 12 h, the cells were harvested by centrifugation at 1,000 x g for 15 min at 22°C. The cells were washed twice with saline (0.9% NaCl) and resuspended in the actinomyces broth so that the initial turbidity yielded a Klett reading of approximately 75 in a Klett-Summerson colorimeter (no. 54 filter). At 0 time and at various time intervals during logarithmic-phase and early stationary-phase growth, turbidity readings were recorded, and 1.5-ml samples of the cultures were withdrawn and placed in centrifuge tubes. The samples were then centrifuged immediately at 10,000 x g for 10 min (Sorvall RC-2B centrifuge) at 4°C, and the culture supernatants were removed with Pasteur pipettes. The supernatant samples were dialyzed against 2 liters of 0.005 M potassium phosphate buffer (pH 6.0). The dialyzed samples were then assayed for FTF activity as described below.

**FTF assay.** The enzyme activities were determined by a modification of the isotope procedure previously described (24) involving measurement of the incorporation of [3H]fructose from [3H]fructose-sucrose into methanol-insoluble poly saccharide. The standard incubation mixture contained 0.02 M potassium phosphate buffer (pH 6.0), 7.3 mM [3H]fructose-sucrose (0.75 μCi/ml), water, and enzyme in a total volume of 0.5 ml. After incubation for 1 h at 37°C, the reactions were terminated by the addition of 8.0 ml of methanol. The samples were then filtered through glass fiber filters (Whatman GF/A) and washed twice with 8.0 ml of methanol. The filters were dried in an oven and suspended in 8.0 ml of toluene-based Liquiflor (New England Nuclear Corp., Boston, Mass.) scintillation fluid. The samples were then counted in a Packard liquid scintillation spectrometer (model 3385). One unit of enzyme activity was defined as the amount of enzyme required to incorporate 1.0 μmol of fructose from sucrose into methanol-insoluble fructan per minute under standard assay conditions.

**Macromolecular synthesis.** Cells were grown in 15 ml of actinomyces broth containing [14C]acetate (0.33 μCi/ml) for the measurement of lipid synthesis, [14C]Juracil (0.656 μCi/ml) for the determination of ribonucleic acid synthesis, or [3H]-amino acids mixture (1.3 μCi/ml) for the measurement of protein synthesis. At the indicated time intervals, 1.0-ml portions were removed and added to an equal volume of cold 10% trichloroacetic acid. The suspensions were then filtered through glass fiber filters (Whatman GF/A), and the filters were washed twice with 2.0 ml of cold trichloroacetic acid. After drying, the filters were counted as described previously.

**Materials.** [3H]fructose-sucrose (10 Ci/mmol), [14C]Juracil (58 mCi/mmol), and [3H]-amino acids mixture (0.35 to 60 Ci/mmol) were obtained from New England Nuclear Corp. [14C]acetate (56.1 mCi/mmol) was obtained from ICN Pharmaceuticals, Inc., Irvine, Calif. Cerylnein, penicillin G, chlomphenicol, and rifamycin were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium chloride was generously provided by Averst Laboratories, Montreal, Canada. Tunicamycin was kindly supplied by G. Tamura (Tokyo University, Japan). Amphotericin was a gift from W. F. Minor (Bristol Laboratories, Syracuse, N.Y.).

**RESULTS**

**Growth and production of extracellular FTF.** The rate of extracellular FTF production constituted the major concern in the selection of organisms for the investigation of FTF secretion by *A. viscosus*. Compared with the virulent strain T14V, the avirulent strain T14AV (6) accumulated significantly higher levels of FTF activity (data not shown). Therefore, T14AV was selected as the organism to be examined in the present study.

Growth resumed immediately when T14AV cells grown as a preinoculum for 12 h were washed and suspended in fresh actinomyces broth (Fig. 1). The production of extracellular FTF was concomitant with the growth of the cells. FTF activity appeared in the medium during early logarithmic-phase growth, and further accumulation ceased when the cells reached the stationary phase of growth. In some, but not all experiments, a small decline in FTF activity was apparent early in the stationary phase.

A significant proportion of FTF activity produced in *A. viscosus* T14AV is associated with the cell surface (24). In addition, cell-associated enzyme has been suggested to be identical to the extracellular enzyme (25). Under the present conditions, cell-associated FTF activity represented approximately 70% of the total culture activity. However, as shown in Table 1, changes in extracellular FTF activity in the presence of inhibitors were paralleled by alterations in cell-associated activity.


Effects of chloramphenicol on extracellular FTF production. For investigating the secretion mechanism of extracellular FTF, it was important to determine whether the production of FTF is tightly coupled to cellular protein synthesis or whether intracellular pools of FTF are involved in this process. T14AV cells actively secreting FTF and had been growing for 2 h were divided into two portions, and chloramphenicol (100 μg/ml) was added to one culture. Cellular protein synthesis was immediately inhibited upon the addition of chloramphenicol (data not shown). At the same time, the production of extracellular FTF was completely inhibited in the chloramphenicol-treated sample, whereas secretion continued in the control medium (data not shown). These results are compatible with the absence of significant intracellular pools of preformed FTF molecules.

Effects of rifamycin on extracellular FTF production. Previous reports (5, 11) have suggested that certain microorganisms continue to secrete extracellular proteins after ribonucleic acid synthesis is inhibited. This has been rationalized by postulating the existence of stable messenger ribonucleic acid for extracellular proteins or the synthesis of an overabundance of messenger ribonucleic acid which is able to sustain protein synthesis for a period of time after the termination of transcription. To determine whether the production of extracellular FTF requires concomitant ribonucleic acid synthesis, we examined the effects of rifamycin on extracellular FTF synthesis. T14AV cells were divided into two portions after 2 h of growth, and rifamycin (10 μg/ml) was added to one culture.

[14C]Uracil incorporation into trichloroacetic acid-insoluble material was monitored during growth in parallel cultures, and rapid inhibition of uracil incorporation took place after the addition of rifamycin (Fig. 2A). Under these conditions, the antibiotic also inhibited further growth and protein synthesis. Moreover, the treated sample yielded no further accumulation of extracellular FTF activity (Fig. 2B), and a decrease in activity, which may represent proteolysis, was observed.

Effects of cerulenin on extracellular FTF production. One approach toward determining whether de novo lipid biosynthesis is involved in extracellular protein secretion has been to use the antibiotic cerulenin. This antibiotic specifically inhibits the condensation reaction between acyl and malonyl thioesters by acting as a competitive inhibitor of the enzyme catalyzing fatty acid chain elongation (32). Several reports have suggested that the secretion of some extracellular proteins involves a cerulenin-sensitive step (1, 7, 20).

When T14AV cells were incubated with cerulenin (20 μg/ml), growth was inhibited approximately 40%. That such inhibition resulted from an inhibition of fatty acid synthesis is indicated by the reduction of the differential rate of [14C]acetate incorporation in the presence of the antibiotic (Fig. 3B). However, during the time interval when differential fatty acid synthesis was inhibited approximately 42%, the differential rate of appearance of FTF activity was virtually the same in the presence or absence of cerulenin (Fig. 3A). These results suggest that de novo fatty acid synthesis is not required for the synthesis or secretion of FTF or both.

Effects of NaCPIB on extracellular FTF

**TABLE 1. Effects of metabolic inhibitors on cell-associated activity**

<table>
<thead>
<tr>
<th>Metabolic inhibitor</th>
<th>Increase in FTF activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.38 (100)</td>
</tr>
<tr>
<td>NaCPIB (4 mg/ml)</td>
<td>3.25 (35)</td>
</tr>
<tr>
<td>Penicillin (0.5 μg/ml)</td>
<td>15.0 (160)</td>
</tr>
<tr>
<td>Cerulenin (20 μg/ml)</td>
<td>12.5 (154)</td>
</tr>
</tbody>
</table>

* T14AV cells were incubated with the indicated concentrations of inhibitors as described in the text. At various intervals, samples were removed, and the cells were suspended after centrifugation. The supernatant fluids were treated as described in the figure legends. The washed cells were assayed for FTF activity in the presence of 0.3% NaF.

* Data represent the change in cell-associated FTF activity (in milliunits per milliliter) per 100 Klett units after 1 h of incubation at 37°C. The numbers in parentheses represent the percent increase in FTF activity per 100 Klett units relative to the control.
EXTRACELLULAR FRUCTOSYLTRANSFERASE

FIG. 2. (A) Effects of rifamycin on uracil incorporation. [3H]uracil incorporation was measured as described in the text. Rifamycin (10 µg/ml) was added after 1.5 h of growth. Symbols: •, uracil incorporation in the absence of rifamycin; ○, uracil incorporation in the presence of rifamycin. (B) Effects of rifamycin on extracellular FTF production. Cells grown for 12 h were washed and suspended in fresh medium. Samples were removed at the indicated intervals and assayed for FTF activity. Rifamycin (10 µg/ml) was added after 2 h of growth. Symbols: •, enzyme activity in the absence of rifamycin; ○, enzyme activity in the presence of rifamycin.

FIG. 3. (A) Effects of cerulenin on extracellular FTF production. Preinoculum cells were suspended in fresh medium without or with cerulenin (20 µg/ml). Samples were removed at the indicated intervals and assayed for FTF activity. Symbols: •, enzyme activity in the absence of cerulenin; ○, enzyme activity in the presence of cerulenin. (B) Effects of cerulenin on lipid synthesis. Cells were grown in the presence and absence of cerulenin. [14C]acetate incorporation was measured as described in the text. Symbols: •, acetate incorporation in the absence of cerulenin; ○, acetate incorporation in the presence of cerulenin.

production. NaCPIB is a water-soluble derivative of the mammalian phosphoglyceride inhibitor chlorophenoxyisobutyrate ethyl ester (22). A previous report has suggested that NaCPIB inhibits lipid synthesis in S. mutans but does not affect the secretion of extracellular glucoamylase-transferase (20). Therefore, it was of interest to investigate the effects of NaCPIB on lipid biosynthesis and FTF secretion in T14AV cells.

Washed preinoculum cells were initially suspended in fresh medium in the presence and absence of NaCPIB (4 mg/ml). NaCPIB apparently inhibited the growth of T14AV cells (approximately 25% inhibition under these conditions) by preferentially inhibiting [14C]acetate incorporation into cellular lipids (Fig. 4A). In addition, extracellular FTF production was differentially inhibited by NaCPIB (Fig. 4B). Nevertheless, this inhibition of enzyme production could reflect a concomitant inhibition of cellular protein synthesis by the antibiotic. This possibility was ruled out by the observation that differential 3H-amino acid incorporation into cellular protein was unaffected by NaCPIB (Fig.
Moreover, the inhibition of FTF secretion was directly related to the inhibition of \([^{14}\text{C}]\text{acetate incorporation. No differential inhibition of extracellular FTF production was achieved with lower concentrations of NaCPIB, which also had little effect on lipid synthesis.}

**Effects of penicillin on extracellular FTF production.** A previous report has suggested that extracellular glucosyltransferase production in *S. mutans* is enhanced by penicillin treatment (16). The addition of penicillin (0.5 µg/ml) to T14AV cells during the logarithmic phase of growth resulted in a 50% inhibition of the growth rate (data not shown). However, cells grown in penicillin-containing medium produced extracellular FTF at a significantly faster rate than cells grown in the absence of the antibiotic (Fig. 5). Moreover, the differential rate of cellular protein synthesis did not appear to be increased by the addition of penicillin (data not shown). Direct addition of penicillin to FTF preparations did not result in any detectable stimulation of the enzyme activity. Therefore, the apparent stimulation of extracellular FTF production by penicillin may also be related to the effects of penicillin on cell wall synthesis.

**Effects of tunicamycin and amphomycin on extracellular FTF production.** Tunicamycin is an antibiotic which has been suggested to inhibit the incorporation of glucosamine into macromolecules (3). In *Bacillus subtilis*, tunicamycin inhibits cell wall synthesis, subsequently inducing the formation of spherical or tadpole-shaped cells and subsequent cell lysis (28). Therefore, it was of interest to study the effects of tunicamycin on extracellular FTF production in *A. viscosus* T14AV. Cells grown in fresh medium in the presence and absence of
tunicamycin (4 µg/ml) were monitored for growth and FTF production. The addition of tunicamycin resulted in a very slight inhibition of cell proliferation (data not shown). Extracellular FTF production did appear to be stimulated in the presence of tunicamycin (Fig. 6) although not to the same degree as in the presence of penicillin. Amphotericin, another polypeptide antibiotic which inhibits peptidoglycan synthesis in Bacillus subtilis (30), also produced a similar stimulatory effect on the differential production of extracellular FTF (data not shown) under conditions in which growth was partially inhibited.

Effects of metabolic inhibitors on cell-associated FTF activity. When cell-associated FTF activity was measured at different time intervals in the presence of inhibitors, alterations similar to those for extracellular activity were observed (Table 1). Penicillin produced an apparent increase in cell-associated FTF activity, whereas cell-associated activity in the presence of NaCPIB was severely depressed. On the other hand, cerulenin did not inhibit the increase in cell-associated FTF activity within the initial 1-h incubation period and actually produced some stimulatory effects. Indeed, as was observed with extracellular FTF activity (Fig. 3), cell-associated FTF activity accumulated under conditions in which fatty acid synthesis was strongly inhibited.

DISCUSSION

The appearance of extracellular FTF activity in A. viscosus T14AV appeared to be closely related to cellular growth. No obvious lag was observed between the appearance of extracellular FTF and the growth of strain T14AV (Fig. 1). In addition, further accumulation of FTF activity ceased when the cells reached the stationary phase of growth (Fig. 1). The apparent decline in FTF activity noted in some experiments in the early stationary phase may represent the induction of fructanase activity (9) or the release of proteolytic activity. The release of potential inhibitors of FTF activity during the stationary phase of growth could not be detected in mixing experiments. Furthermore, extracellular FTF production was immediately inhibited upon the addition of the protein synthesis inhibitor chloramphenicol. This latter observation is consistent with the absence of large pools of intracellular FTF, although it is possible that intracellular pools requiring protein synthesis for secretion are present. However, disruption of T14AV cells yielded no significant release of FTF activity (data not shown).

The existence of stable messenger ribonuclease acids for extracellular FTF could not be demonstrated in the present investigation (Fig. 2B). In this regard, the production of extracellular FTF was concomitant with ribonuclease acid synthesis. However, since the production of FTF was measured at 30-min intervals, these results do not rule out the possible existence of FTF messenger ribonuclease acids with half-lives longer than the 1- to 2-min half-lives of most bacterial messenger ribonuclease acids (4, 5, 11).

Relative to the results in other bacterial systems secreting extracellular proteins (1, 7, 20), the addition of growth-inhibiting concentrations of cerulenin did not appear to affect the differential rate of production of extracellular FTF (Fig. 3A). This suggests that de novo fatty acid synthesis in strain T14AV is not specifically required for the expression of extracellular FTF activity. Alternatively, if fatty acids are in fact required for such expression, T14AV cells may contain significant pools of these fatty acids under the growth conditions used in the present investigation.

In contrast to the secretion of glucosyltransferase in S. mutans (20), the production of extracellular FTF was shown to be differentially inhibited by the addition of NaCPIB (Fig. 4B). Furthermore, a direct correlation between the inhibition of FTF secretion and [14C]acetate incorporation was indicated (Fig. 4A and B). This observation is compatible with the role of de novo lipid biosynthesis in extracellular protein production. However, the inhibitory effects of NaCPIB in bacterial systems have not been studied extensively, and further investigation is required to depict the specific action of NaCPIB on biochemical processes in T14AV cells. Therefore, the divergent effects of NaCPIB and cerulenin on FTF expression probably reflect different sites of action of the two antibiotics in lipid
biosynthesis. In this regard, it is of interest that cerulenin, but not NaCPIB, differentially inhibits extracellular glucosyltransferase expression by S. mutans GS-5 (20). For clarifying the role of lipid synthesis in these systems, it will be necessary to specifically identify the site of action of NaCPIB in bacterial systems and to determine the role of the molecules in extracellular protein secretion. Efforts in this regard are currently in progress in this laboratory.

Elevated FTF activities were also demonstrated in the presence of several inhibitors of cell wall synthesis with different sites of action (Fig. 5 and 6). Therefore, it is unlikely that these elevated activities occur as a result of secondary effects at non-cell wall target sites. These effects were immediate, and no enhancement of activity could be detected by the direct addition of cell wall synthesis inhibitors to FTF preparations. Since the surface barriers of the cells are altered by these antibiotics, it is possible that the stimulation of extracellular FTF production may occur subsequent to the release of some amphiphatic cellular molecules (phospholipids, teichoic acid-like molecules) which are able to promote or stabilize enzyme activity by interacting with the enzyme secreted during growth. A recent study has suggested that extracellular glucosyltransferase from S. mutans interacts with amphiphatic molecules secreted during growth, resulting in the stabilization of enzyme activity (21). Furthermore, the release of lipid material has been demonstrated to be enhanced in the presence of the cell wall inhibitor penicillin in S. mutans (13). Therefore, it is possible that an analogous release of molecules which are capable of enhancing FTF activity may take place in T14AV cells upon the addition of inhibitors of cell wall synthesis. Moreover, FTF activity has been demonstrated to be affected by the direct addition of lipid-containing detergents to the enzyme preparations; i.e., FTF activity was stimulated roughly twofold in the presence of Tween 80. This may indicate that FTF molecules are able to interact with certain lipid-containing substances, resulting in a stimulation of enzyme activity. In addition, direct alteration of the cell surface after antibiotic treatment may provide an alternate explanation for the stimulation of FTF activity. Since the cell wall represents the final barrier to extracellular enzyme secretion, it is conceivable that the distortion of the surface structures of the cells may account for the stimulation of extracellular FTF activity. However, no large pools of precursor extracellular protein molecules have been demonstrated to exist between the membranes and cell walls of gram-positive bacteria. Therefore, it is unlikely that the enhancement of FTF activity is solely caused by changes in the permeability of the cell surface.

The results of the present study represent an initial investigation of the regulation of extracellular FTF secretion in A. viscosus T14AV. Further investigation is required for a detailed understanding of the mechanism of FTF secretion. In this regard, it will be of interest to investigate the possible existence of a membrane-associated FTF precursor in this organism. Relative to the apparent increase in extracellular enzyme activity in the presence of cell wall synthesis inhibitors, the possible release of putative lipid-containing activators after antibiotic treatment should also be examined. Furthermore, the present results indicate that strain T14AV produces higher levels of FTF than does strain T14V. However, it is not clear whether the greater production of slime polysaccharide by the former strain contributes to this difference. It will also be of interest to extend these studies to strain T14V as well as rodent strains of A. viscosus. These, as well as other, approaches should provide the basis for further investigation into the mechanism of FTF secretion in A. viscosus.

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LITERATURE CITED


