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Clinical Isolates of *Streptococcus mutans* Serotype *c* with Altered Colony Morphology Due to Fructan Synthesis

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Streptococcus mutans MT6801, MT6861, and MT6879, which form large mucoid colonies on mitis salivarius agar, were isolated from a mother and her two daughters. These isolates were identified as serotype *c* by immunodiffusion with serotype-specific antisera. The large colonies formed on sucrose-containing agar were found to contain water-soluble fructan. The cell-free fructosyltransferase (FTase) activity of the strains which formed large colonies was five to eight times higher than that of serotype *c S. mutans* which produced small, rough colonies typical of this serotype. Furthermore, greater quantities of fructan were synthesized from sucrose by growing cells of MT6801 when compared with MT8148, a typical serotype *c S. mutans*. Glucosyltransferase and FTase could be isolated by chromatofocusing from culture supernatants of MT6801 and MT8148. The FTase activity of both strains was eluted at pH 4.5, and glucosyltransferase was released by elution with an NaCl linear gradient. The eluted FTase activity of MT6801 was significantly higher than that of MT8148. Strains MT6861 and MT6879 were also found to possess a similar property in terms of FTase activity. These results suggest that formation of large mucoid colonies by these strains is a consequence of high FTase activity.

Streptococcus mutans, a known causative agent of dental caries, is able to adhere in vitro to the smooth surface by synthesizing insoluble glucans from sucrose (17). In addition, *S. mutans* produces acids by fermenting various sugars, including sucrose. These properties make *S. mutans* an important pathogenic bacterium in the development of dental caries.

S. mutans can be immunologically classified into seven serotypes, *a* to *g* (2, 25). Among these serotypes, serotype *c* is the most frequently isolated organism from Japanese children (13, 14) and also from North Americans and Europeans (3, 25). Serotype *c S. mutans* usually forms small (0.5- to 1-mm diameter), raised, rough, pale blue colonies on mitis salivarius (MS) agar, which has been employed for separation and identification of streptococci (9, 15). In this regard, it has been noted that serotype *c* strains appear to be relatively sucrose independent in in vivo colonization of *S. mutans*, whereas serotype *d* and *g* strains seem sucrose dependent (10).

Recently, we have isolated serotype *c S. mutans* strains that produce large mucoid colonies on sucrose-containing agar media, similar to that of *Streptococcus salivarius*, but different from typical serotype *c S. mutans* colonies. Preliminary studies showed that the large colonies of these strains might be a consequence of increased synthesis of fructans. These strains may be useful to investigate the role of fructan in the development of dental caries.

The purpose of the present study is to characterize biologically these clinical isolates with unique colony morphology.

MATERIALS AND METHODS

Bacterial strains. *S. mutans* MT6879, MT6801, and MT6861 were isolated from a mother and her two daughters, respectively, who visited the pedodontic clinic of Osaka University Dental School Hospital. The isolation procedure of these strains was based upon the previous report of Hamada et al. (14). Laboratory-maintained serotype *c S. mutans* MT8148, Ingbritt, C67-1, OMZ 70, MT6R, and MT12 were selected from the stock culture collection in the Department of Dental Research, the National Institute of Health, Tokyo, Japan, and employed as "typical" serotype *c* reference strains. *S. salivarius* HT9R (24) was used to obtain a streptococcal fructan.

Antiserum and antigens. The antiserum specific for serotype *c S. mutans* was prepared by immunizing rabbit with merthiolate-killed *S. mutans* MT8148 (serotype *c*) whole cells as previously described (14). Autoclaved extracts of the organisms (RR antigens) were obtained by the method of Hamada et al. (12).

Biological characteristics. MS agar (Difco Laboratories, Detroit, Mich.) was used to check the colony morphology of the test strains. Sugar fermentation abilities were determined in phenol red broth base (Difco) containing 1% of the sugar to be tested: sorbitol, mannitol, raffinose, inulin, or melibiose. Arginine hydrolysis was determined by the method of Niven et al. (23). Hemolytic activity was tested on brain heart infusion (BHI; Difco) agar containing 5% defibrinated sheep blood. The adherence of growing cells to a glass surface was determined in BHI broth containing 1% sucrose as described previously (20).

Immunodiffusion was performed with 1.5% Noble agar (Difco) in veronal buffer (0.05 M, pH 8.6).

Quantitative determination of glucan and fructan. Polysaccharides synthesized by colonies grown on Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.) agar containing 5% sucrose were quantitated as follows. After 72 h of incubation at 37°C on sucrose agar, 5 or 10 colonies and associated zooglea were collected in a test tube with a sterile spatula and suspended in 1 ml of deionized water. After

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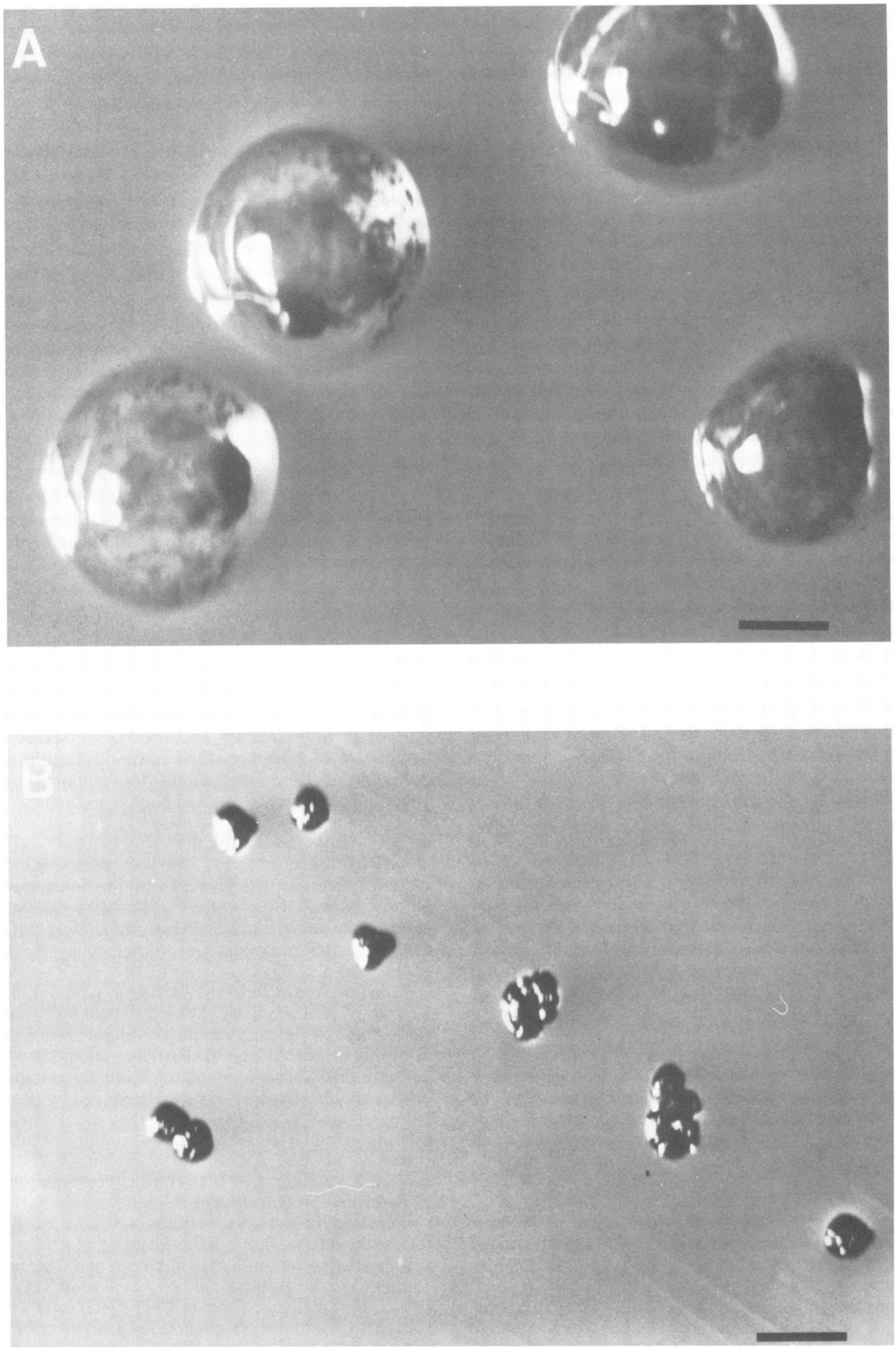

FIG. 1. Colony morphology of *S. mutans* MT6801 (A) (a large colony-forming strain) and MT8148 (B) (a typical serotype *c*) on MS agar (Difco). *S. mutans* MT6801 and MT8148 were incubated at 37°C for 48 h and then at room temperature for 72 h. Bar indicates 1 mm.

mixing, the test tube was centrifuged (3,000 × *g* for 20 min) to remove the insoluble materials, and ethanol was added to the supernatant to a final concentration of 75%. The precipitated materials were collected by centrifugation, washed twice with 75% ethanol, and then mixed with 1 ml of 1 M NaOH to dissolve water-soluble glucan and fructan. The remaining insoluble material was washed twice with deionized water, followed by extraction with 1 ml of 1 M NaOH for 1 h at 37°C. The 1 M NaOH-soluble and -insoluble fractions were separated by centrifugation (3,000 × *g* for 20 min). The insoluble fraction was washed twice and resuspended in 1 ml of 1 M NaOH. The glucan and fructan contents of these three fractions were quantitated differentially by the anthrone method (18), using dextran T2000 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and inulin (Wako Pure Chemicals, Osaka, Japan) as the standards.

Glucosyltransferase (EC 2.4.1.5; GTase) and fructosyltransferase (EC 2.4.1.9; FTase) activities were measured by the method of Koga and Inoue (21), using [*U*-^{14}C]sucrose. Culture supernatant (10 μl) from *S. mutans* grown in BHI broth was mixed with 10 μl (7.2×10^4 cpm) of 20 mM [*U*-^{14}C-fructose]sucrose or [*U*-^{14}C-glucose]sucrose (0.25 mCi/mol; New England Nuclear Corp., Boston, Mass.) in potassium phosphate buffer (0.2 M, pH 6.8), and the reaction mixture was kept at 37°C for 4 h. For the assay of cell-bound enzyme activities, washed cells from 7 ml of the BHI broth culture were resuspended in 0.5 ml of potassium phosphate buffer. The cell suspension (20 μl) was then mixed with 10 μl of [^{14}C]sucrose and incubated for 10 h at 37°C. The reaction mixture was spotted on a piece of filter paper (no. 514; Toyo Roshi, Tokyo, Japan) and washed three times with methanol, and the incorporation of [^{14}C] into glucan or fructan was measured with a liquid scintillation spectrometer (20).

Polysaccharide synthesis by growing cells was determined as follows. *S. mutans* MT6801 or MT8148 was cultured in BHI broth containing 10% sucrose for 24 h at 37°C. The organisms were collected by centrifugation at 3,000 × *g* for 20 min and washed twice with deionized water. The centrifuged pellet was suspended in 1 M NaOH and allowed to stand for 1 h with occasional mixing. Further centrifugation separated the 1 M NaOH-soluble and -insoluble fractions. The polysaccharide content of three fractions (water-soluble, 1 M NaOH-soluble, 1 M NaOH-insoluble) was determined by the anthrone method as noted above.

Determination of linkage type of fructan. Twenty colonies of *S. mutans* MT6801 developed on MS agar were collected and suspended in 0.5 ml of deionized water. After standing for 2 h at room temperature, the suspension was centrifuged at 5,000 × *g* for 10 min. The supernatant was dialyzed extensively against deionized water, and the fructan concentration of the dialysate was adjusted to 10 mg/ml. Fructan was similarily extracted from the colonies of *S. salivarius* HT9R grown on MS agar. Reactivity of these fructan preparations with 5 mg of concanavalin A (Con A; grade 3, Sigma Chemical Co., St. Louis, Mo.) per ml was confirmed in 1% agar gel (0.01 M phosphate-buffered saline [pH 7.2]) (5). Inulin (10 mg/ml; Wako Pure Chemicals) and levan from *Aerobacter levanicum* (10 mg/ml; Sigma) were employed as standards.

Separation of GTase and FTase by chromatofocusing. Culture supernatants of *S. mutans* MT6801 or MT8148 grown in BHI broth were concentrated about 10 times with a Centriflow CF25 (Amicon Corp., Lexington, Mass.) and dialyzed against 0.025 M imidazole-hydrochloride buffer (pH 7.4). The sample was applied to a polybuffer exchanger PBE 94 (Pharmacia) column (0.7 by 15 cm) equilibrated with 0.025 M imidazole-hydrochloride buffer (pH 7.4). The column was first eluted with polybuffer 74 (pH 4.0; Pharmacia), followed by an NaCl gradient elution (22). GTase and FTase activities were measured by the use of [*U*-^{14}C-glucose]sucrose or [*U*-^{14}C-fructose]sucrose as mentioned above. Protein was monitored by measuring the optical density at 280 nm.

RESULTS

During epidemiological surveys of the dental plaque samples from a single family, we found many morphologically unusual colonies on MS agar. These clinical isolates, designated as MT6801, MT6861, and MT6879, were obtained from plaque samples of a mother and her two daughters. These isolates formed large mucoid colonies covered with soft gelatinous materials. Although bacteriological examinations revealed that these strains were serotype *c S. mutans*, the size of the colonies was three to five times larger than that of typical serotype *c S. mutans* (Fig. 1). The colony morphology of these isolates was not altered by monthly laboratory transfers of these organisms for more than 5 years. All of the strains examined fermented mannitol, sorbitol, raffinose, inulin, and melibiose, did not hydrolyze arginine, and were gamma-hemolytic on sheep blood agar. The growing cells of these strains adhered strongly (>90%) to the surface of a culture tube in the presence of sucrose. RR extracts of strains MT6801, MT6861, and MT6879 reacted with antiserum specific for serotype *c*, and the precipitin band of the RR extract fused with the reference strains of serotype *c S. mutans*, MT8148 and Ingbritt.

Chemical analyses were carried out with the polysaccharides extracted from the colonies formed on 5% sucrose-containing Trypticase soy agar. The colonial polysaccharides of strain MT6801 were found to contain large quantities of soluble fructan, whereas *S. mutans* MT8148 produced little fructan (Table 1). The large colonies formed on MS agar were also found to be rich in fructan. In addition, no significant change in colony morphology was found when filter-sterilized *Spicaria violacea* dextranase (16) was incorporated into MS agar (data not shown).

To determine whether the fructan synthesized by *S. mutans* MT6801 was an inulin type or a levan type, a precipitin reaction with Con A was attempted. The fructan extracted from large colonies on MS agar of *S. mutans* MT6801 did not react with Con A, whereas the fructan from *S. salivarius* HT9R formed a precipitin band (Fig. 2). In this

TABLE 1. Chemical analysis of the polysaccharide extracted from zooglea of the colonies produced by *S. mutans* MT6801 and MT8148[a]

Polysaccharide	MT6801	MT8148
Water-soluble		
Fructan	407	0.2
Glucan	ND[b]	1.1
1 M NaOH-soluble		
Fructan	14.6	11.3
Glucan	18.2	38.8
1 M NaOH-insoluble		
Fructan	5.0	5.1
Glucan	38.8	20.0

[a] Colony appeared on 1% Trypticase soy agar containing 5% sucrose. Amounts of glucan and fructan (in micrograms per colony) were determined differentially by the anthrone method.

[b] ND, Not detected (<0.1 μg per colony).

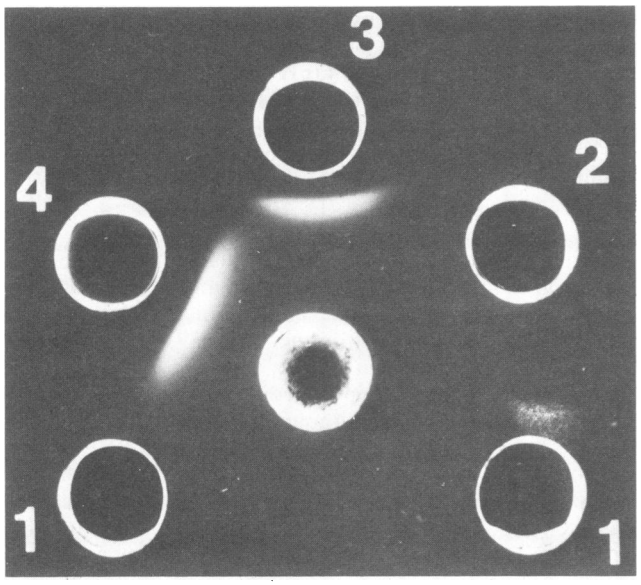

FIG. 2. Precipitin reaction of fructan with Con A in agar gel. Wells: 1, fructan extracted from the colonies of *S. mutans* MT6801; 2, inulin; 3, fructan extracted from the colonies of *S. salivarius* HT9R; and 4, levan. The concentration of each fructan preparation was 10 mg/ml. The central well was filled with Con A (5 mg/ml).

regard, the glucan content of these two fructan preparations was found to be less than 2% by the anthrone method. It should be noted here that Con A reacted with levan to form a precipitin band but did not react with inulin (Fig. 2).

Polysaccharides synthesized by cell-free and cell-bound GTase and FTase of various strains of serotype *c S. mutans* grown in BHI broth were measured (Table 2). The cell-free FTase activities of MT6801, MT6861, and MT6879 were several times higher than those of reference strains.

Analysis of polysaccharides synthesized by cells grown in sucrose broth revealed that MT6801 synthesized large amounts of soluble fructan (1.95 mg/ml of culture) in addition to the soluble glucan, the polysaccharides synthesized by the strain MT8148 were primarily soluble and insoluble glucan (4.74 mg/ml of culture), and the amount of soluble fructan was only 0.8 mg/ml (Table 3).

Cell-free enzymes of strains MT6801 and MT8148 synthesizing glucan and fructan were isolated by chromatography on a polybuffer exchanger PBE 94. In both cases, FTase was eluted at about pH 4.5 during the pH gradient, and GTase was released by linear gradient elution with NaCl. FTase and GTase could be separated almost completely by this method (Fig. 3). It was also found that the FTase activity of MT6801 eluted at pH 4.5 was significantly higher than that of MT8148.

DISCUSSION

Serotypes *c*, *e*, and *f S. mutans* have been shown to give rise to small, rough, raised colonies on MS agar plates (15), whereas *S. salivarius* produces large, smooth, dome-shaped colonies on the same medium. In this study, we examined three serotype *c S. mutans* strains that formed colonies with altered morphology. These strains were isolated by chance from a mother and two daughters of a single family. The colony morphology of these strains was large and smooth, similar in appearance to that of *S. salivarius*. The fact that these unusual strains had been isolated only from a single family strongly indicates that an intrafamilial transmission of *S. mutans* has occurred, as has been suggested by others (1, 26).

S. mutans synthesized primarily glucans as well as various amounts of fructans from sucrose (17, 28). Most of the glucans synthesized by GTase are water insoluble and adhere to solid surfaces, including teeth, glass, and plastic, but the fructans synthesized by FTase have been demonstrated to be easily degraded by many oral bacteria in dental plaque (6, 29). In this regard, Donkersloot et al. (7) carried out a chemical analysis of zooglea produced by *S. mutans* SL-1 colonies on sucrose-containing agar medium. They showed that the zooglea was composed of glucan, sucrose, and fructose. In contrast, *S. salivarius* produces large quantities of fructan from sucrose, accounting for the typical colony morphology of this species on sucrose-containing agar media, such as MS agar (19, 24, 27).

We therefore compared the chemical composition of polysaccharides obtained from colonies with unusual morphology (*S. mutans* MT6801) and from those with colonies typified by reference strain MT8148. Colonies of MT6801 contained considerable quantities of fructan, whereas the MT8148 colonies contained glucan with trace amounts of fructan

TABLE 2. Glucan and fructan production by various strains of serotype *c*. *S. mutans*

Strain	Polysaccharide synthesized (cpm/μl of culture per h)[a]				Cell growth[b]	pH of culture[c]
	Cell-free		Cell-bound			
	Glucan	Fructan	Glucan	Fructan		
Ingbritt	106.6	22.2	7.6	0.9	3.2	5.1
C67-1	59.4	27.8	5.9	0.6	2.6	5.1
OMZ 70	52.7	30.6	3.5	1.9	3.1	5.2
MT6R	72.4	24.8	5.7	2.4	2.6	5.1
MT12	71.7	23.1	3.1	0.5	3.1	5.1
MT8148	123.6	28.6	7.8	0.7	2.9	5.1
MT6801	36.5	157.7	6.1	1.4	2.9	5.1
MT6861	43.9	168.7	7.4	1.5	3.1	5.1
MT6879	39.1	161.3	7.0	1.6	2.9	5.1

[a] The radioactivity of [^{14}C]glucan or [^{14}C]fructan synthesized from [*U*-^{14}C-glucose]sucrose or [*U*-^{14}C-fructose]sucrose was measured as described in the text. For determination of cell-free activity, overnight broth cultures of each strains were centrifuged, and the supernatants were used for the assay. For cell-bound activity, cells collected by centrifugation were resuspended in 0.5 ml of potassium phosphate buffer and used for the assay.

[b] Values represent the absorbance at 550 nm of the BHI broth culture at the end of incubation.

[c] pH of the BHI broth culture after the incubation. The initial pH of BHI broth was 7.3.

(Table 1). Furthermore, the culture supernatants of BHI-grown cells of the strains MT6801, MT6861, and MT6879 were found to possess higher enzyme activity synthesizing fructan from sucrose, as compared with that of many reference serotype *c S. mutans* strains (Table 2). The MT6801 cells grown in a sucrose broth medium produced large amounts of soluble fructan, whereas the MT8148 cells produced mostly water-soluble or -insoluble glucans (Table 3). These results suggest that MT6801, MT6861, and MT6879 may be mutant strains of serotype *c S. mutans* with high FTase activity, and the unique colonial morphology of these strains may be a consequence of the production of large quantities of fructan. It is of interest to note that the fructan produced by MT6801 might be an inulin type but not a levan, because Con A reacted selectively with levan (11) (Fig. 2). This result agrees with the finding by Ebisu et al. (8) that the fructan synthesized by *S. mutans* is an inulin type, whereas that of *S. salivarius* is a levan type.

Since the interest of investigators has been focused on the GTases which play an important role in cellular adherence and, hence, caries development, there are a few reports on the purification and characterization of FTase from *S. mutans* (4). But considering the fact that both FTase and GTase use the same substrate, sucrose, the role of FTase and its product on cellular adherence and caries induction must not be ignored. Since these enzymes easily aggregate together with other extracellular proteins or lipoteichoic acids to form high-molecular-weight materials, the purification of FTase and GTase of *S. mutans* has proven to be difficult. We found here that chromatofocusing the culture supernatants of MT6801 and MT8148 separated FTase and GTase (Fig. 3). The chromatofocusing method has also been employed for the purification of insoluble glucan synthesizing GTase and soluble glucan synthesizing GTase of serotype *d S. mutans* (22). Thus, this method should be useful in preparing various enzymes synthesizing water-soluble and -insoluble glucans, and fructans. It is of interest to note that the strains forming large colonies on MS agar plates were found to possess high FTase activity, and thus, the culture supernatant of these strains may be a good starting material for the purification of FTase.

More recently, we have isolated several mutants from strain MT6801 which lack the ability to develop large colonies on sucrose-containing agar plates and have markedly reduced FTase activity. The mechanism of the synthesis and structure of fructan by these *S. mutans* will be reported in detail elsewhere.

In summary, the present results indicate (i) that colony morphology on MS agar is not always an adequate means for distinguishing between oral streptococcal species, (ii) that the ability to produce fructan may be elevated without a loss in ability to remain part of the oral flora, and (iii) that extracellular polysaccharide production is not a consistent characteristic among *S. mutans* strains but transmitted strains may maintain a particular property.

TABLE 3. Glucan and fructan synthesized by growing cells of *S. mutans* MT6801 and MT8148[a]

Polysaccharide	MT6801	MT8148[b]
Water-soluble		
Fructan	1.95	0.80
Glucan	2.40	4.40
1 M NaOH-soluble		
Fructan	0.05	0.02
Glucan	0.07	0.07
1 M NaOH-insoluble		
Fructan	0.02	0.03
Glucan	0.08	0.27

[a] Cells were grown in BHI broth containing 10% sucrose for 24 h at 37°C. Amounts of polysaccharides (in milligrams per milliliter of culture) were determined by the anthrone method.

[b] MT8148 is a typical strain of serotype *c S. mutans*.

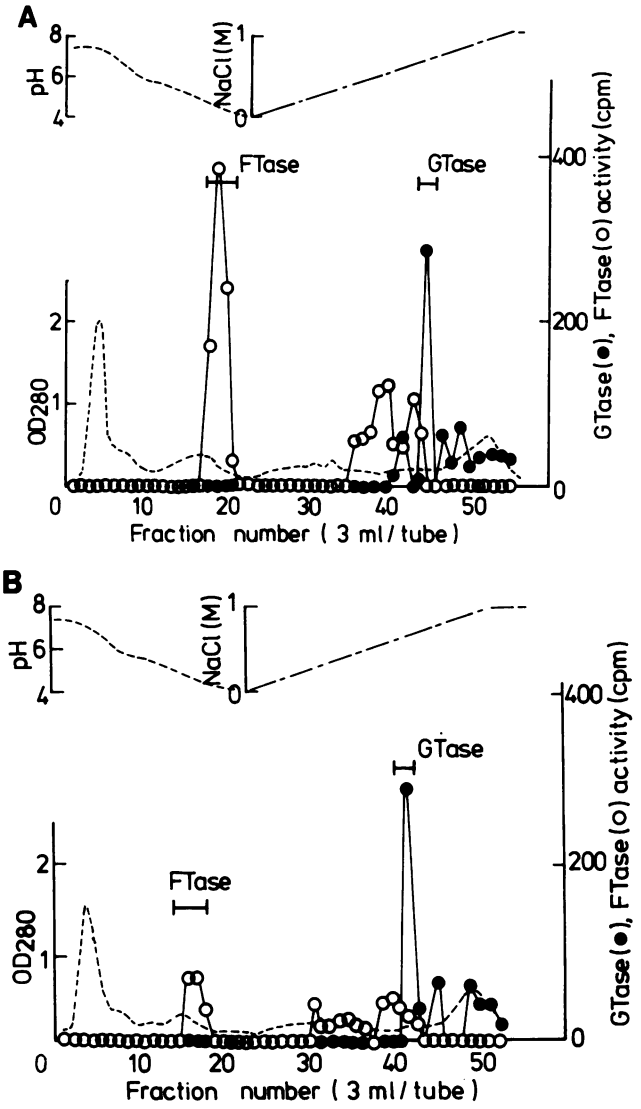

FIG. 3. Separation of GTase and FTase by the chromatofocusing method. A polybuffer exchanger column (0.7 by 15 cm; Pharmacia) was first eluted with polybuffer (pH 4.0) to obtain a pH gradient, followed by an NaCl gradient (0 to 1 M). (A) Culture supernatant of *S. mutans* MT6801; (B) culture supernatant of *S. mutans* MT8148.

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