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Structural Studies of the Serotype-f Polysaccharide Antigen from *Streptococcus mutans* OMZ175

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The serotype f antigen of *Streptococcus mutans* has been described as a rhamnose-glucose polysaccharide associated with the bacterial cell wall. In this study, the structure of serotype f polysaccharide was examined by analyses of the methylated derivatives of the antigen and the periodate-oxidized antigen. Methylated derivatives were characterized with a gas chromatograph-mass spectrometer. The polysaccharide appeared to have a backbone of alternating 1,3- and 1,2,3-linked rhamnose units. Branching occurred at the 3-position of the 1,2,3-linked rhamnose. Side chains were composed of terminal alpha-linked glucose units. A small proportion of longer side chains containing 1,2- and 1,6-linked glucose units were noted in some preparations; however, these determinants were not reactive with serotype f antisera.

The serotype c, e, and f antigens of *Streptococcus mutans* are rhamnose-glucose polysaccharides covalently bound to the cell wall peptidoglycan (8, 12, 15). In vitro studies in the 1970s demonstrated that antibodies to the serotype antigens interfered with the adherence of *S. mutans* to hard surfaces (6). In vivo studies by Michalek et al. (14) have correlated induction of salivary antibodies to polysaccharides of *S. mutans* with reduction in the recovery of this organism in plaque and protection against caries.

The serotype c and e antigens of *S. mutans* have been well characterized chemically (7, 10, 19, 20) and structurally (12, 15). The serotype f polysaccharide was purified and chemically characterized by Hamada et al. (5). Pritchard et al. (15) suggested that the f antigen had a polyrhamnose backbone similar to that of the serotype e polysaccharide with side-chains of single α-D-glucosyl units. However, these authors were unable to clearly deduce the backbone linkages from their data or distinguish the structure of the serotype f antigen from that proposed for the serotype c polysaccharide (12). This paper attempts to clarify the structure of the serotype f antigen by using analyses of methylated derivatives of the polysaccharide and periodate-oxidized polysaccharide.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. mutans* OMZ 175-H was originally obtained from S. Hamada in 1975 and maintained as lyophilized stock cultures with one transfer. *S. mutans* OMZ175-B was obtained from D. Bratthall in 1984. Cultures were grown in a dialysate of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1.8% glucose, 0.8% $NaHCO_3$, 0.3% NaCl, and 0.15% K_2HPO_4, Cells were incubated at 37°C for 18 h, harvested by centrifugation, washed twice with distilled water, and lyophilized.

Purification of the serotype antigen. Lyophilized cells of *S. mutans* OMZ175-H and OMZ175-B (5 and 6 g, respectively) were suspended at 60 mg/ml in 0.01 M Tris hydrochloride buffer (pH 7.0). The cells were digested with 5 mg of mutanolysin per ml, which is a muramidase from *Streptomyces globisporus* 1829 (21), for 18 h at 37°C. The digests were incubated at 70°C for 10 min to inactivate the mutanolysin and then centrifuged at 27,000 × *g* for 20 min. The supernatants were dialyzed extensively against distilled water and lyophilized.

The concentrated cell digests were fractionated on columns of DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) as described by Hamada et al. (5). The dialyzed digest (1 g [dry weight]) was dissolved in 0.01 M Tris hydrochloride buffer (pH 8.2) and applied to a DEAE Sephadex A-25 column (2.5 by 30 cm; Pharmacia) that had been equilibrated in the same buffer. The column was eluted with 100 ml of the equilibration buffer and developed with 300 ml of a linear gradient between 0.05 and 1.0 M NaCl in buffer. Column fractions (5 ml) were monitored for protein and nucleotides at 280 nm and assayed for carbohydrates by the phenol-sulfuric acid method (2). Samples containing carbohydrate peaks were pooled, lyophilized, and further fractionated on a column (1.5 by 60 cm) of Sephadex G-100 (Pharmacia) with 0.01 M Tris hydrochloride (pH 8.2) as the eluting buffer. Fractions comprising the major carbohydrate peak were pooled, dialyzed against distilled water, and lyophilized.

Preparation of antisera. Antisera to cells of *S. mutans* OMZ175-H were prepared in New Zealand White rabbits. Cells were suspended in phosphate-buffered saline containing 0.5% Formalin for 16 h at room temperature (RT). After washing, the Formalin-killed cells were suspended in phosphate-buffered saline containing 0.01% Formalin at a final cell concentration of 10 mg/ml (dry weight). Rabbits were immunized via their footpads with 1 ml of an emulsion of the cell suspension with incomplete Freund adjuvant. Animals were boosted at 2 weeks. When antisera with high titers to *S. mutans* OMZ175-H were detected, the animals were exsanguinated by cardiac puncture. Antisera were made serotype f specific by adsorption with heat-killed cells of *S. mutans* Ingbritt serotype c (20 mg of cells per ml of serum) at 4°C for 18 h.

Serological assays. In the quantitative precipitin assay, portions (20 μl) of antiserum were incubated with increasing

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TABLE 1. Chemical composition of polysaccharide antigens from *S. mutans* OMZ175-H

Component	Antigen I (mg/100 mg [dry wt])	Antigen II (mg/100 mg [dry wt])
Carbohydrate		
Rhamnose	37 (13)[a]	43 (14)[a]
Glucose	36 (13)	22 (7.2)
Peptidoglycan		
Glutamic acid	2.4 (1.0)	2.6 (1.0)
Alanine	4.9 (3.4)	5.5 (3.6)
Lysine	2.0 (0.9)	2.6 (1.0)
Glucosamine	3.8 (1.1)	3.3 (0.9)

[a] Molar ratio with respect to glutamic acid is indicated in parentheses.

concentrations of antigen in saline containing 0.001% Merthiolate (final volume, 100 μl) for 1 h at RT and overnight at 4°C. Precipitates were collected by centrifugation in a Microfuge B (Beckman Instruments, Inc., Fullerton, Calif.) and washed twice with 1-ml portions of phosphate-buffered saline. The washed precipitates were dissolved in 1 ml of 2% Na_2CO_3 in 0.1 N NaOH and assayed for antibody protein by the method of Lowry et al. (13). For inhibition assays, the antiserum and inhibitor were incubated for 15 min at RT before the antigen was added. In cross-precipitin assays, the initial supernatant, recovered after centrifugation of the overnight reaction, was incubated with the second antigen for 1 h at RT and overnight at 4°C. Antigen-antibody precipitates were collected and processed as described. Cross-precipitin assays and quantitative inhibition assays were performed in triplicate.

Periodate oxidation. Antigen I from strain OMZ175-H was subjected to periodate oxidation. The polysaccharide (10 mg) was dissolved in 5 ml of 0.08 M sodium acetate buffer (pH 4.5) and reacted with sodium *meta*-periodate (50 mg) at 4°C for 18 h. The reaction was terminated with the addition of a twofold molar excess of ethylene glycol versus the periodate. The sample was reduced with sodium borohydride, neutralized, dialyzed, and lyophilized. The reduced sample was subjected to controlled acid hydrolysis with 0.05 N hydrochloric acid at 80°C for 1 h. After titration to pH 7, the sample was filtered on a column (1.5 by 60 cm) of Sephadex G-15 (Pharmacia), and the void volume sample was subjected to the methylation analysis.

Analytical methods. Total sugars were assayed by the phenol-sulphuric acid method (2). Rhamnose was measured by the methylpentose assay of Gibbons (3) with thioglycolic acid substituted for cysteine-hydrochloride. Protein was measured by the method of Lowry et al. (13). Amino acids and amino sugars were characterized with a Beckman 6300 amino acid analyzer. Samples for amino acid analysis were hydrolyzed in constantly boiling HCl under N_2 at 105°C for 28 h. For analysis of amino sugars, samples were hydrolyzed in 2 N HCl at 100°C for 6 h.

Quantitative determination of specific sugars was performed with a model 2740 gas chromatograph (Varian, Palo Alto, Calif.). Samples were subjected to methanolysis in 0.5 N HCl–methanol at 78°C for 20 h. The HCl-methanol was removed under vacuum, and Tri-Sil Z (Pierce Chemical Co., Rockford, Ill.) was used as the silylation reagent. Sugars were characterized with appropriate standards on a column of OV-101 (Alltech Associates, Inc., Applied Science Div., State College, Pa.) over a temperature range of 140 to 200°C, increasing at 2°C per min.

The serotype polysaccharides were methylated according to the procedure of Hakomori (4). The permethylated oligosaccharides were converted to partially methylated hexitol acetates and 6-deoxy-hexitol acetates by the acetolysis-hydrolysis method outlined by Stellner et al. (16). The derivatives were analyzed with a model 5992 gas chromatograph-mass spectrometer (Hewlett-Packard Co., Palo Alto, Calif.) with a glass column (0.2 by 200 cm) packed with 3% OV-225 (17). Derivatives were identified by their elutions with respect to terminal glucose and by their mass spectra.

RESULTS

Preparation of the serotype f antigen. Serotype antigen was solubilized from cells of *S. mutans* OMZ175-H and OMZ175-B by digestion with mutanolysin. Fractionation of the digests on columns of DEAE-Sephadex A-25 (Pharmacia) yielded two major carbohydrate peaks that reacted with serotype-specific antiserum. These peaks corresponded to Ag1 and Ag2 described by Hamada et al. (5). Fractions within these peak areas were pooled, concentrated, and further purified on columns of Sephadex G-100 (Pharmacia). The resulting samples were designated antigen I and antigen II. On chemical analysis, the carbohydrate antigens were found to be associated with peptidoglycan fragments (Table 1). For the samples from strain OMZ175-H, antigen I had a rhamnose-to-glucose ratio of 1:1, whereas antigen II had a rhamnose-to-glucose ratio of 2:1. Antigens I and II from strain OMZ175-B had rhamnose-to-glucose ratios of 2:1 (data not shown).

Serological characterization of the serotype f antigen. A cross-precipitin reaction was performed to examine the immunological cross-reactivity between antigens I and II of strain OMZ175-H. After incubation of antigen II with serotype f-specific antiserum, the reaction was centrifuged and the supernate containing excess antibodies was reacted with 2 μg of antigen I. After a second incubation period, the reaction was centrifuged and both precipitates were assayed for antibody protein (Fig. 1). As increasing concentrations of antigen II were tested, less antibody was available for the second reaction with antigen I, suggesting that antigen I was

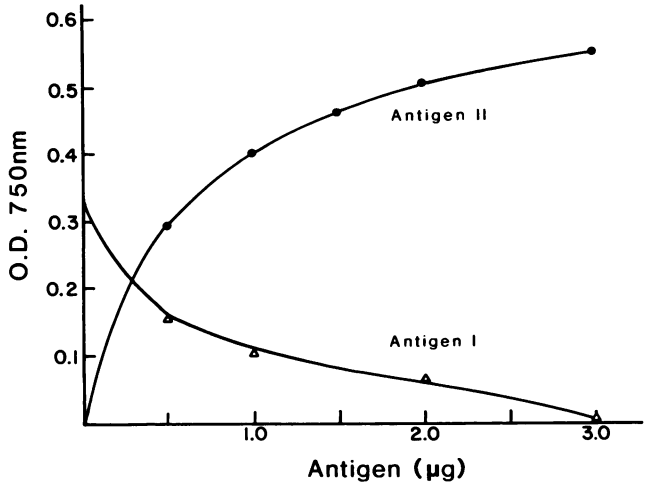

FIG. 1. Quantitative cross-precipitin reaction with antigens I and II from *S. mutans* OMZ175-H. Increasing concentrations of antigen II were reacted with anti-serotype f serum for 1 h at RT and 4°C overnight (●). After centrifugation to remove antigen-antibody complexes, the supernatants were reacted with 2 μg of antigen I (△).

TABLE 2. Inhibition of the precipitin reaction between serotype f antiserum and antigens from *S. mutans* OMZ175-H

Inhibitor	Concn (μg)	Inhibition (%)	
		Antigen I	Antigen II
α-Methyl glucopyranoside	100	42	46
	250	76	60
β-Methyl glucopyranoside	100	8	16
	250	11	20
Isomaltose (Glc-α-1,6-Glc)[a]	100	40	28
	250	59	52
Gentiobiose (Glc-β-1,6-Glc)	100	3	8
	250	12	14
Rhamnose	100	5	20
	250	13	20

[a] Glc, Glucose.

reacting with the same population of antibodies as was antigen II.

The specificities of the determinants on antigens I and II were examined by hapten inhibition of the quantitative precipitin assay (Table 2). Isomaltose and α-methyl glucopyranoside were the most effective inhibitors, whereas β-haptens and rhamnose were poor inhibitors. The results suggested that the determinant of the serotype f polysaccharide possessed a terminal α-1-glucosyl residue.

Structural characterization of the serotype f antigen. The methylated derivatives of the polysaccharides from strains OMZ175-H and OMZ175-B were prepared and analyzed by a gas chromatograph-mass spectrometer (Table 3). Glucose was the only terminal carbohydrate noted. The only branch point detected was 1,2,3-linked rhamnose. Also present were 1,3-linked rhamnose and trace amounts of 1,2-linked rhamnose. Antigen I from strain OMZ175-H appeared to have significant quantities of 1,6- and 1,2-linked glucose; these residues were not detected in antigen II. Antigens I and II from strain OMZ175-B demonstrated small amounts of 1,2-linked glucose but no 1,6-linked glucose. The gas chromatography-mass spectrometry data suggested that antigens I and II had backbones of alternating 1,3- and 1,2,3-linked rhamnose units.

To verify this backbone structure and attempt to identify the branch site on the 1,2,3-linked rhamnose unit, antigen I was subjected to oxidation by sodium *meta*-periodate. After reduction and mild hydrolysis, the polysaccharide was filtered on Sephadex G-15 (Pharmacia) (Fig. 2). Only a void volume carbohydrate peak was detected. The recovered polymer represented a third of the initial carbohydrate and was composed of 94% rhamnose. The methylation data are shown (Table 4) in relation to the recovery of 1,3-rhamnose because this moiety was resistant to the periodate oxidation reaction. The methylation analysis revealed a decrease in recovery of 1,2,3-linked rhamnose with a corresponding increase in recovery of 1,2-linked rhamnose. The 1,2- and 1,6-linked glucose units were destroyed in the oxidation reaction, and only trace amounts of terminal glucose were recovered. The rhamnose polymer was not reactive with serotype f antiserum (data not shown).

DISCUSSION

In their studies reporting the original purification of the serotype f antigen, Hamada et al. (5) used a hot acid extraction technique to solubilize the polysaccharide. For structural studies, an enzyme digestion technique was chosen to avoid random hydrolysis of the antigen. The mutanolysin digests yielded two carbohydrate peaks on DEAE-Sephadex chromatography similar to the two antigen fractions resolved after the acid extraction technique (5). Antigens I and II from strain OMZ175-H had rhamnose-to-glucose ratios of 1:1 and 2:1, respectively (Table 1). These ratios agree with the carbohydrate compositions of Ag1 and Ag2 in the earlier study (5). In contrast, antigens I and II from strain OMZ175-B both had rhamnose-to-glucose ratios of 2:1; Hamada et al. (5) reported a similar carbohydrate content for the serotype f antigen purified from *S. mutans* MT557. In the studies by Pritchard et al. (15), mutanolysin and nitrous acid extracts of a serotype f organism yielded one rhamnose-containing peak on DEAE-cellulose chromatography. This antigen had a rhamnose-to-glucose ratio of 2:1, the strain was not identified (15). Antigens prepared by muramidase digestion were covalently bound to remnants of the peptidoglycan (8, 15). In the present study, peptidoglycan components constituted 15 to 20% of the purified preparations.

In spite of the apparent differences in carbohydrate contents, cross-precipitin reactions indicated that antigen I was reacting with a population of antibodies that also reacted with antigen II (Fig. 1). In agreement with Hamada et al. (5), hapten inhibition studies suggested that the determinant on the serotype f polysaccharide possessed a terminal α-1-glucosyl residue (Table 2). Hamada et al. (5) reported cross-reactivity of their Ag2 preparation with anti-teichoic acid serum. None of the serotype f antigen preparations in this study reacted with anti-teichoic acid or antidextran sera (data not shown).

The methylation analysis studies suggested that the f antigen had a backbone polymer of alternating 1,3- and 1,2,3-linked rhamnose units (Table 3). This finding was in contrast to the study of Pritchard et al. (15) which reported high concentrations of 1,2- and 1,2,3-linked rhamnose. The presence of high concentrations of 1,3-linked rhamnose in the backbone was confirmed by the periodate oxidation studies on antigen I. After periodate oxidation, over 60% of

TABLE 3. Analysis of methylation derivatives of polysaccharide antigens from *S. mutans* OMZ175

Derivative	Substitution site(s)	Relative peak area[a]			
		OMZ175-H		OMZ175-B	
		AgI	AgII	AgI	AgII
1,2,3,5-Tetra-*O*-acetyl-4-*O*-methyl rhamnitol	1, 2, 3	1.0	1.0	1.0	1.0
1,3,5-Tri-*O*-acetyl-2,4-di-*O*-methyl rhamnitol	1, 3	0.82	0.80	0.77	0.78
1,2,5-Tri-*O*-acetyl-3,4-di-*O*-methyl rhamnitol	1, 2	0.18	Tr	0.13	0.12
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol	1	1.0	0.85	0.90	1.1
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucitol	1, 6	0.74	ND	ND	Tr
1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methyl glucitol	1, 2	0.26	ND	0.20	0.35

[a] AgI, Antigen I; AgII, antigen II; ND, not detected.

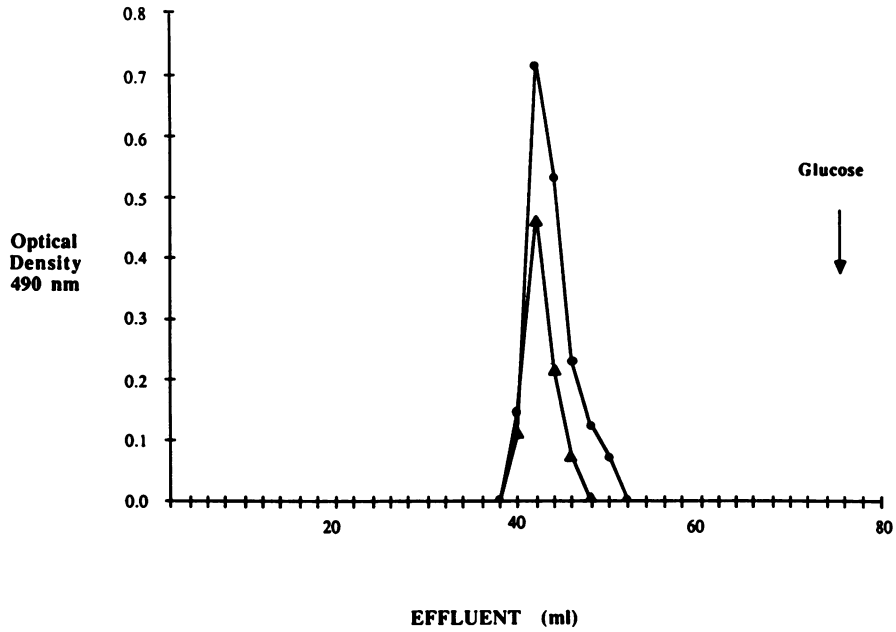

FIG. 2. Chromatography of antigen I from *S. mutans* OMZ175-H on a column of Sephadex G-15 (Pharmacia) before (●) and after (▲) periodate oxidation. The column (1.5 by 60 cm) was eluted with H_2O, and the fractions were assayed for total carbohydrate. The polysaccharides were eluted at the void volume. For reference, the elution volume for a glucose standard was indicated.

the original rhamnose and less than 4% of the glucose were recovered in the void volume peak on Sephadex G-15 (Pharmacia) filtration (Fig. 2). If 1,2-linked rhamnose or glucose units were major components of the backbone structure, only short rhamnose fragments would have been recovered. The increased recovery of 1,2-linked rhamnose and the corresponding decrease in recovery of 1,2,3-linked rhamnose after periodate oxidation, reduction, and controlled hydrolysis suggested that the terminal glucose units were bound to the 3-position of the 1,2,3-linked rhamnose (Table 4). This position for the glucose units is in agreement with the work of Pritchard et al. (15) who used partial hydrolysis techniques with recovery and characterization of a glucose-1,3-rhamnose disaccharide.

The proposed structure for the determinant of the serotype f antigen is as follows (Rha, rhamnose; Glc, glucose):

$$\rightarrow 3\text{Rha}1\rightarrow 2\text{Rha}1\rightarrow 3\text{Rha}1\rightarrow 2\text{Rha}1\rightarrow$$
$$\qquad\qquad\uparrow \alpha\text{-}1,3 \qquad\qquad \uparrow \alpha\text{-}1,3$$
$$\qquad\qquad \text{Glc} \qquad\qquad \text{Glc}$$

The configuration of the backbone polymer distinguishes the serotype f antigen from the *S. mutans* serotype c and e polysaccharides. The backbones of the latter polysaccharides are composed of alternating 1,2- and 1,2,3-linked rhamnose units with branching at the 2-position of the 1,2,3-linked units (12, 15). A backbone similar to that of the f antigen has been described for the Lancefield group A, AV, and C polysaccharides by Coligan et al. (1). The Lancefield A and C antigens have branches of terminal amino sugars consisting of β-1,3-linked *N*-acetylglucosamine in the A antigen and α-1,3-linked di-*N*-acetylgalactosamine in the C antigen. The AV antigen is unbranched.

The specificity of the serotype f determinant appeared to be related to the terminal α-1-linked glucose unit on the rhamnose backbone. Inhibition studies (Table 2) found significantly greater inhibition by α-methyl glucopyranoside and isomaltose than by the β-linked haptens. This confirmed the work of Hamada et al. (5). However, those investigators suggested that a terminal α-1,6-linked diglucose unit was part of the determinant. Structural studies of antigen II of strain OMZ175-H and antigens I and II from strain OMZ175-B revealed that these antigens did not possess α-1,6-diglucose units, although they did react with the serotype-specific antiserum (Fig. 1). Therefore, although some longer side chains of 1,6- and 1,2-linked glucose units were present on antigen I, they did not appear to react with the antiserum tested here. Variations in side chain composition may be

TABLE 4. Analysis of methylation derivatives of antigen I before and after periodate oxidation

Derivative	Substitution site(s)	Relative peak area[a]	
		Pre	Post
1,3,5-Tri-*O*-acetyl-2,4-di-*O*-methyl rhamnitol	1, 3	1.0	1.0
1,2,3,5-Tetra-*O*-acetyl-4-*O*-methyl rhamnitol	1, 2, 3	1.2	0.53
1,2,5-Tri-*O*-acetyl-3,4,-di-*O*-methyl rhamnitol	1, 2	0.22	0.79
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol	1	1.2	0.10
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucitol	1, 6	0.9	ND
1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methyl glucitol	1, 2	0.32	ND

[a] Pre, Before periodate oxidation; post, after periodate oxidation; ND, not detected.

related to repeated laboratory subculturing or variations in growth conditions. Variations in side chain composition were observed in chemostat studies of the rhamnose-glucose polysaccharide antigen of *Streptococcus sobrinus* B13 (9), formerly *S. mutans* serotype d. In those studies, organisms grown at pH 7.5 had a higher ratio of rhamnose to glucose in their rhamnose-glucose polysaccharide antigen than did cultures grown at pH 6.5. The rhamnose-glucose polysaccharide antigen has a polyrhamnose backbone similar to the serotype c and e antigens of *S. mutans*. Side chains are composed of 1,2- and 1,6-linked glucose (11). Structural studies demonstrated that the 1,6- and 1,2-linked glucose units had been lost from the side chains of antigen prepared from cultures grown at pH 7.5 (12). In the present study, strains OMZ175-H and OMZ175-B were grown under identical conditions; therefore, the variations in side chain composition noted cannot be related to culture conditions.

In addition to their usefulness in epidemiological studies, the serotype polysaccharides are also of interest because of their immunoprophylactic potential. Wachsmann et al. (18) reported the preparation of a bivalent vaccine consisting of the purified polysaccharide antigen from *S. mutans* OMZ175 covalently coupled to a cell wall protein. Oral administration of their conjugate in liposomes induced a local salivary immunoglobulin A response directed against both the polysaccharide and the protein. Understanding the structure of the serotype polysaccharides of *S. mutans* may contribute to the development of protective multivalent vaccines.

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