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Chemical Characterization of Extracellular Polysaccharides Produced by *Actinomyces viscosus* T14V and T14Av

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The human isolates *Actinomyces viscosus* T14V and T14Av produced extracellular polysaccharides in the absence of sucrose. In contrast to strain T14V, strain T14Av produced abundant viscous slime polysaccharide in the culture supernatant fluids when grown in a chemically defined medium containing glucose. After resolution of the T14Av polysaccharides into seven fractions, it was demonstrated that two of these exhibited viscous properties and lacked methylpentose and muramic acid. The major slime polysaccharide purified by gel filtration and ion-exchange column chromatography contained 39% (moles percent carbohydrate) galactose, 37% *N*-acetylglucosamine, 19% glucose, and 5 % mannose. Only trace amounts of protein and phosphorus were detected in this preparation. On the other hand, strain T14V produced negligible slime polysaccharide under the same culture conditions. The major extracellular polysaccharide fraction from this strain contained methylpentoses, hexoses, hexosamines, muramic acid, protein, and phosphorus, suggesting that this fraction might be derived from the cell wall.

Actinomyces viscosus and *A. naeslundii* are capabe of inducing both root surface caries and periodontal bone loss in rodents fed with sucrose (10, 12, 24). However, previous investigations with rodents monoinfected with *A. viscosus* or *A. naeslundii* have demonstrated that periodontal disease was induced even when sucrose was replaced with glucose or corn starch (12, 13). Several strains of *A. viscosus* isolated from rodents or humans produce extracellular slime polysaccharide in the presence of glucose as the sole carbohydrate (15, 20, 23, 28), and the potential role of the extracellular slime polysaccharide in plaque formation has been suggested (10, 23, 26, 27). However, recent results have demonstrated that the extracellular slime polysaccharide produced by *A. viscosus* T14Av inhibits the colonization of this strain to rat teeth or saliva-coated hydroxylapatite beads (4, 28).

Recently, Ooshima and Kuramitsu reported that the human oral isolate *A. viscosus* T14Av (avirulent strain) produced abundant extracellular slime polysaccharide in chemically defined medium containing glucose as a sole carbohydrate and that *A. viscosus* T14V (virulent strain) produced only low levels of slime (19). In regard to the structure of slime polysaccharides, Brecher et al. reported that a partially purified slime fraction of strain T14Av was an *N*-acetylglucosamine-rich polysaccharide with small amounts of other sugars (4). In addition, Rosan and Hammond (22) and Van der Hoeven (26) reported that analogous slime polysaccharide preparations obtained from rodent strains of *A. viscosus* contained large amounts of glucosamine and lower levels of neutral sugars. However, detailed information concerning the composition of purified slime polysaccharides produced by human strains of *A. viscosus* has not yet been reported. Therefore, this investigation was initiated to purify and chemically characterize the slime polysaccharides produced in the presence of glucose by the human oral isolates *A. viscosus* T14V and T14Av.

MATERIALS AND METHODS

Microorganisms and culture conditions. *A. viscosus* T14V and T14Av were kindly provided by F. C. McIntire (University of Colorado, Denver) and S. Brecher (Forsyth Dental Center, Boston, Mass.), respectively. Each organism was maintained in Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) at 4°C and routinely transferred monthly in the same medium.

To prepare crude slime polysaccharide, each strain was cultured in 250 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C for 18 h, and the resulting cells were harvested, washed with sterile saline, inoculated into 5 liters of chemically defined medium containing 44 mM sodium bicarbonate (19) with a 5% inoculum, and grown at 37°C aerobically as static cultures for 48 h.

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Purification of slime polysaccharide. Crude extracellular slime polysaccharide was isolated from culture supernatant fluids according to the procedure of Rosan and Hammond (22). The crude slime samples were then purified by column chromatography as previously described (19), with the indicated modifications. The lyophilized crude slime samples (10 to 15 mg, dry weight) were fractionated initially by gel filtration chromatography on Sepharose 4B. The column (1.5 by 85 cm) was developed at 4°C with 0.05 M phosphate buffer (pH 7.0) at a flow rate of 10 ml/h. The fractions (5 ml) were assayed for the presence of neutral sugars, amino sugars, and protein. The fractions containing neutral sugars and amino sugars were pooled, dialyzed against distilled water, lyophilized, and subjected to ion-exchange chromatography on DEAE-Bio-Gel A columns (1.5 by 30 cm). Samples were eluted with a gradient from 0 to 1 M NaCl in 0.05 M Tris-hydrochloride buffer (pH 7.5) at a flow rate of 30 ml/h. The fractions (5 ml) were assayed for carbohydrate as described below. The concentration of NaCl in the gradient was measured in a refractometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Chemical analyses. Total neutral sugar was determined by the phenol-sulfuric acid method (8), and hexosamine was measured according to Rondle and Morgan (21). Uronic acids were estimated by the carbazole method (7), muramic acid was assayed according to Barker and Summerson (2), and protein was determined as previously described (16). Total phosphate in each fraction was estimated according to Bartlett (3).

Infrared spectrometry. A 1- to 2-mg dried sample was mixed with 300 mg of potassium bromide, and a disk (13 mm in diameter and approximately 0.8 mm thick) was prepared by utilizing a Perkin-Elmer potassium bromide die at an 18,000-lb (ca. 8,165-kg) total load for 2 min. Infrared spectrometry was performed on a Perkin-Elmer 700 spectrophotometer.

Acid hydrolysis. The optimal conditions for hydrolysis of the polysaccharide fractions to release neutral sugars and amino sugars were examined by paper chromatography and chemical analyses of hydrolysates of a purified slime fraction from strain T14Av (fraction A-2); these conditions were used for all other samples. The optimal conditions for release of neutral sugars were determined to be hydrolysis with 4 N H_2SO_4 at 100°C for 8 h. Hydrolysis for 8 h in 4 N HCl at 100°C produced optimal results for amino sugars. HCl hydrolysates were neutralized with sodium hydroxide, and H_2SO_4 hydrolysates were neutralized with saturated barium hydroxide or barium carbonate; they were then filtered through glass fiber filters (Whatman, GF/A) and lyophilized.

Paper chromatography. Samples were spotted on paper (Whatman no. 1) and developed by ascending chromatography, using a mixture of *n*-butanol–pyridine–water (6:4:3, by volume) at 50°C. Reducing sugars were detected with acetone-silver nitrate reagent, and amino sugars were detected with ninhydrin and Elson-Morgan reagent (21).

Gas-liquid chromatography. Neutral sugars and amino sugars were identified simultaneously as alditol acetate derivatives (18, 23). Briefly, neutralized hydrolysates containing inositol as an internal standard were initially reduced by sodium borohydride at room temperature for 2 h. After decomposing excess sodium borohydride by adding acetic acid, the remaining boric acid was removed by codistillation with methanol (five times), and the resulting alditols were then acetylated with 0.5 ml of acetic anhydride at 100°C for 2 h. To the acetylated alditols, 3 ml of methylene chloride was

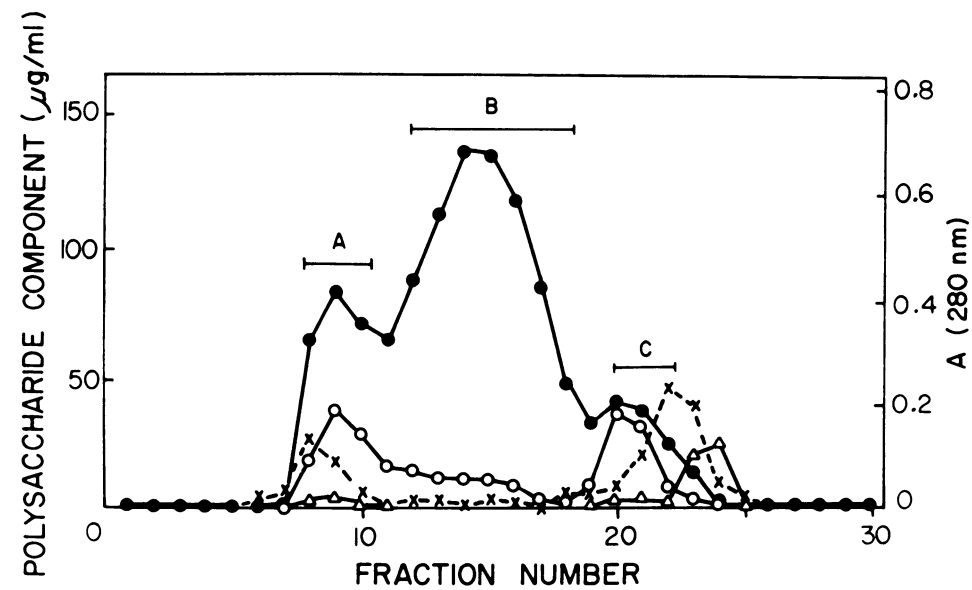

FIG. 1. Gel filtration chromatography of crude slime polysaccharide of *A. viscosus* T14Av on Sepharose 4B. The crude slime polysaccharide was obtained from the culture supernatant fluid of 48-h cultures in chemically defined medium supplemented with 1% glucose. Conditions for elution are described in the text. Symbols: (●) neutral sugar; (○) amino sugar; (△) protein; (×) optical density (absorbance [A]) at 280 nm.

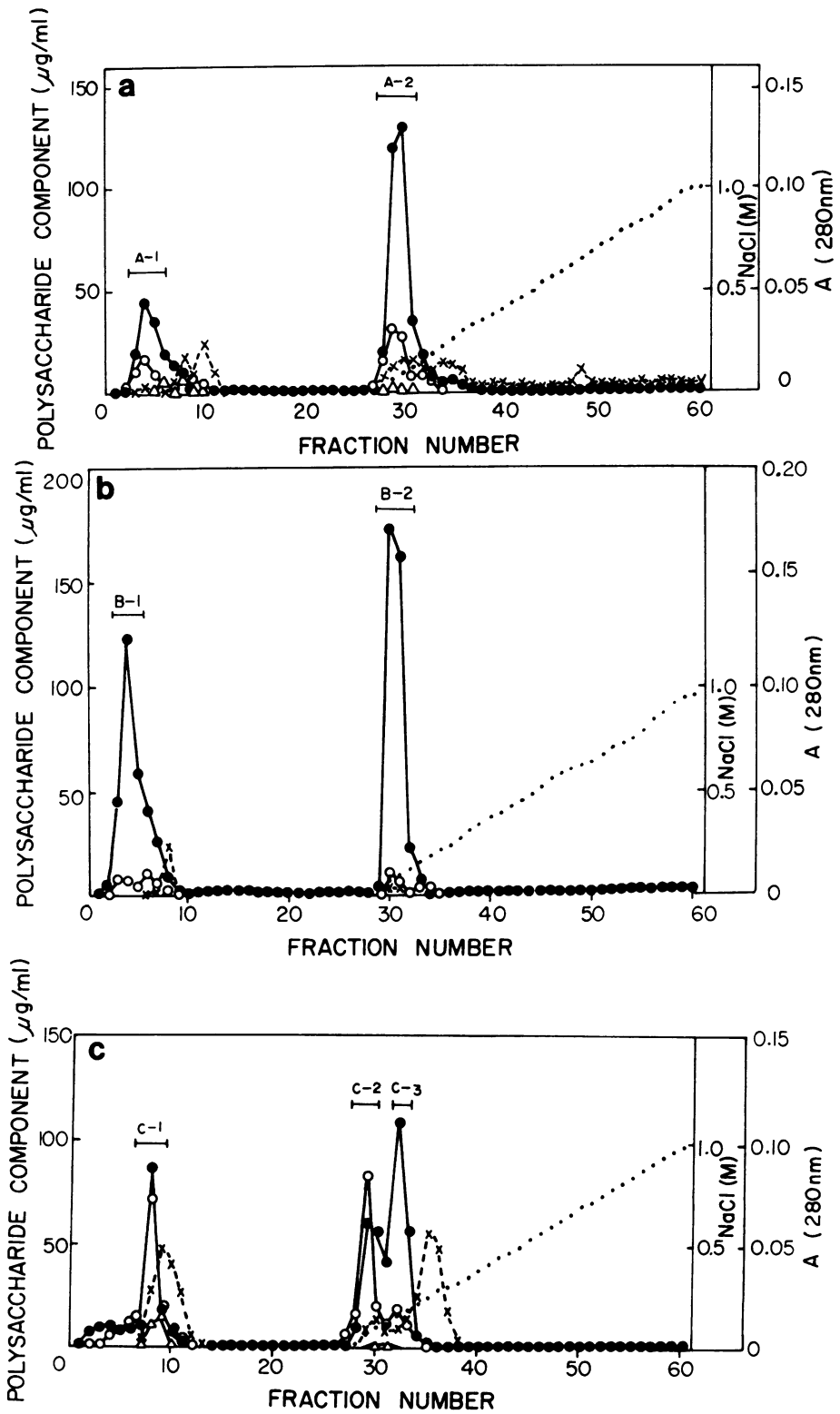

FIG. 2. Ion-exchange chromatography of partially purified polysaccharide fractions A (a), B (b), and C (c) obtained after Sepharose 4B gel filtration chromatography. The conditions for chromatography are described in the text. Symbols: (●) neutral sugar; (○) amino sugar; (△) protein; (×) optical density (absorbance [A]) at 280 nm.

TABLE 1. Sugar composition of various polysaccharide fractions of *A. viscosus* T14Av and T14V

Sugar[a]	%, dry wt							
	T14Av							T14V
	A-1	A-2	B-1	B-2	C-1	C-2	C-3	V-1
Rhamnose	0	0	0	29.6	5.8	10.3	0	10.3
6-DOT	0	0	0	0	13.5	0	0	20.0
Mannose	0	4.1	2.5	18.7	4.4	6.8	0	3.2
Galactose	0	31.7	1.7	21.8	2.0	14.3	45.6	0.5
Glucose	53.0	15.2	88.7	2.3	16.1	19.3	10.6	10.0
Glucosamine	23.7	29.4	1.8	0.8	5.5	3.6	6.6	8.3
Galactosamine	0	0	0	0.8	32.7	36.8	0	21.2
Muramic acid	0	0	0	4.4	0	0	0	6.0

[a] Individual sugar components were quantitated as described in the text.

added, and the methylene chloride phase was washed with an equal volume of saturated sodium bicarbonate (three times) and water (three times). The sample was then evaporated by flushing with nitrogen gas and dried completely in a desiccator over phosphorous pentoxide. The resulting alditol acetate derivatives were dissolved in acetone and then injected onto the column of a Hewlett-Packard 7610A gas chromatograph. A glass column (4 mm by 180 cm) containing 3% ECNSS-M on Gas-Chrom Q (100 to 120 mesh) was used. Nitrogen was used as a carrier gas at a flow rate of 40 ml/min. The temperatures of the injection port and flame ionization detector were 250 and 270°C, respectively. The oven was temperature programed from 185 to 205°C at 1°C/min increments after 4 min of running at 185°C. When it was necessary to separate methylpentoses, the sample was initially chromatographed isothermally at 160°C. After the separation of methylpentoses, the oven temperature was increased as mentioned above.

The determination of acetyl groups in slime polysaccharide was also performed by gas chromatography (14, 17). The sample was hydrolyzed in 2 N HCl at 100°C for 2 h with propionic acid as an internal standard. The hydrolisates (1 to 3 μl) were directly injected onto the column (4 mm by 170 cm) packed with 20% Tween 80–2% H_3PO_4 on Chromosorb W (80 to 100 mesh) and chromatographed isothermally at 120°C.

RESULTS

Purification of T14Av and T14V extracellular polysaccharide. The viscous crude extracellular polysaccharides produced by strain T14Av in the culture supernatant fluid of chemically defined medium were initially fractionated by gel filtration chromatography on Sepharose 4B (Fig. 1). In contrast to earlier attempts to fractionate the polysaccharides (19), the crude extracellular polysaccharide was separated into three fractions based on the contents of neutral and amino sugars. The amino sugar-rich fractions were present in the void volume of the column (fraction A) and a lower-molecular-weight peak (fraction C). Fraction A exhibited viscous properties (20), whereas fractions B and C were nonviscous. Fractions A, B, and C were then dialyzed against distilled water, lyophilized, and subjected to ion-exchange chromatography on DEAE–Bio-Gel A (Fig. 2). Fraction A was separated into two fractions (Fig. 2a): fraction A-1, which passed through the column in the void volume; and the main fraction (A-2), which eluted from the column with approximately 0.05 M NaCl. Both fractions were viscous and contained high concentrations of neutral sugar and lower concentrations of amino sugar (Table 1). Fraction B was also separated into two subfractions, and each contained large amounts of neutral sugar and only small amounts of amino sugar (Fig. 2b). From fraction C, three fractions were obtained and two of these were rich in amino sugar (Fig. 2c).

In contrast, only one major polysaccharide fraction was obtained from the culture supernatant fluids of strain T14V after gel filtration chromatography on Sepharose 4B and ion-exchange chromatography on DEAE–Bio-Gel A, confirming a previous report from this laboratory (19). This lower-molecular-weight fraction (V-1) was slightly retarded on ion-exchange columns and was eluted at approximately 0.05 M NaCl (data not shown).

Gross chemical composition of various polysaccharide fractions. The general chemical composition of each fraction was determined and the results are summarized in Table 2. The major component in each fraction was carbohydrate, and only small amounts of protein were detected except for fraction C-1 of strain T14Av and fraction V-1 of strain T14V. Fraction C-3 of strain T14Av contains relatively large amounts of phosphorus, but all of the other fractions contain very low levels.

Infrared pattern of purified polysaccharides. To characterize the nature of the chemical bonds present in the polysaccharides, several purified polysaccharide fractions were subjected to infrared spectrometry. Figure 3 shows a spectrum of the purified major viscous polysaccharide (frac-

TABLE 2. Gross chemical composition of extracellular polysaccharide fractions of *A. viscosus* T14Av and T14V

Fraction	%, dry wt		
	Carbohydrate	Protein	Phosphorus
T14Av			
A-1	76.7	2.5	1.1
A-2	80.4	0.4	1.0
B-1	94.7	0.3	0.3
B-2	78.4	1,1	0.1
C-1	80.0	7.8	3.3
C-2	91.1	3.6	2.2
C-3	62.8	1.5	16.8
T14V			
V-1	79.5	9.8	0.6

tion A-2). The absorption peak around 1,620 cm^{-1} indicates the absorption of amide I and is probably associated with the presence of acetyl groups of amino sugars (1). The absorption shoulder at 1,550 cm^{-1} (amide II) and the peak at 1,320 cm^{-1} (amide III) were also clearly detected, and these absorptions reflect the presence of N-H and C-N bonds in the polymer chain. The absorption peaks at 890 and 840 cm^{-1} suggest the presence of β- and α-linkages, respectively. The presence of S=O structure in the polysaccharide was not detected since a strong absorption peak around 1,240 cm^{-1} was not observed.

Sugar composition of various polysaccharide fractions. All of the polysaccharide fractions were hydrolyzed, reduced, acetylated, and then subjected to gas-liquid chromatography. Both neutral sugars and amino sugars were detected simultaneously. A typical gas chromatogram pattern of the major viscous polysaccharide (fraction A-2) was found to contain mannose, galactose, glucose, and glucosamine (Fig. 4). The percentages of each sugar in the individual polysaccharide fractions are shown in Table 1. The muramic acid and uronic acid compositions were determined chemically. In comparison with fraction A-2, the major component of the other viscous fraction, A-1, is glucose. Mannose and galactose were not detected in this fraction. Moreover, muramic acid, uronic acid, and fructose were not observed in either viscous fraction A-1 or A-2. In fractions B-1 and B-2 only trace amounts of amino sugars were detected. Since neither fraction is viscous and the percentages of neutral sugars are different from fractions A-1 or A-2, the two B fractions might not be related to the slime polysaccharides. In addition, fraction B-2 contained the cell wall component rhamnose, which was also detected in fractions C-1, C-2, and V-1. The presence of another *A. viscosus* cell surface component (9), 6-deoxytalose (6-DOT), was observed in fractions C-1 and V-1, and muramic acid was detected in fractions B-2 and V-1. In addition to glucosamine, galactosamine was also detected in fractions B-2, C-1, C-2, and V-1 and is a major component of fractions C-1, C-2, and V-1. The degrees of acetylation of hexosamines in fractions A-2 and V-1 were determined to be 96 and 80%, respectively, but the other fractions were not examined in this regard because of insufficient quantities available.

DISCUSSION

The extracellular polysaccharides produced by strain T14Av could be resolved into several distinct fractions. Of these fractions, fractions A-1 and A-2 were very viscous and behaved as

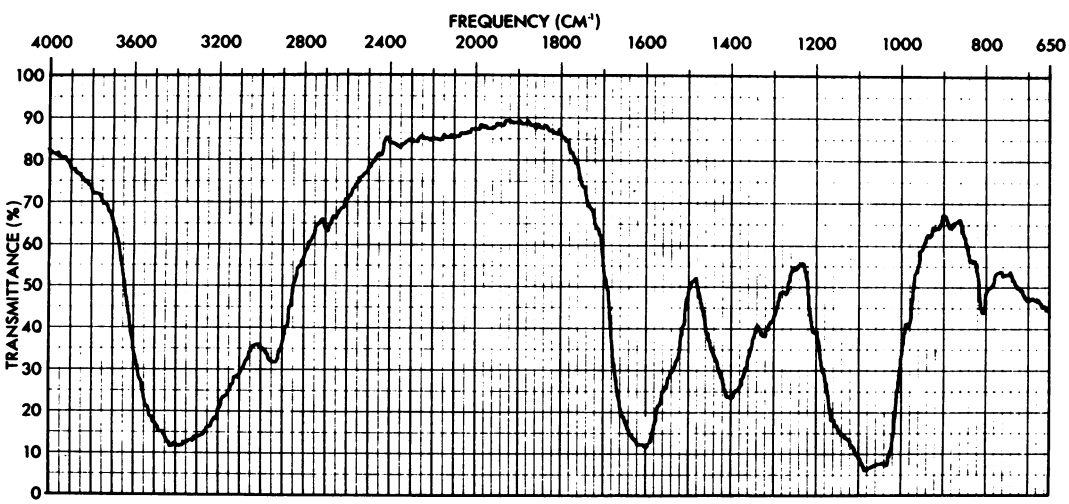

FIG. 3. Infrared spectrum of slime polysaccharide (fraction A-2) or *A. viscosus* T14Av.

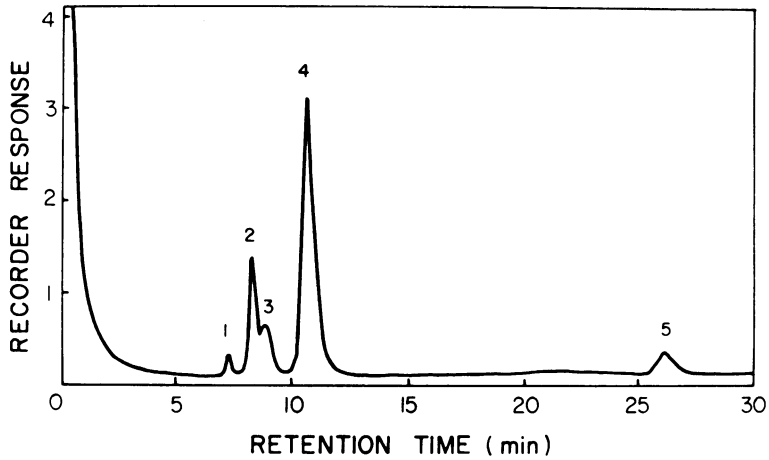

FIG. 4. Gas-liquid chromatogram of alditol acetate derivatives derived from slime polysaccharide (fraction A-2) of *A. viscosus* T14Av. Alditol acetates (1) mannose, (2) galactose, (3) glucose, (4) inositol (internal standard), and (5) glucosamine were separated on glass columns as described in the text.

high-molecular-weight polymers. Since both fractions contain only trace amounts of phosphorous, there is little possibility that the fractions contain phospholipid, which was detected in the cell wall of *A. viscosus* T6 (20). Furthermore, Hammond et al. (9) reported that the cell wall of *A. viscosus* T14V contains an unusual sugar, 6-DOT, which is absent in strain T14Av, and that 6-DOT is a major component and the immunodominant component of the virulent antigen from Rantz-Randall extracts of strain T14V. However, Tylenda et al. (25) demonstrated that both strains T14Av and T14V contain equal contents of 6-DOT in the cell walls by examining the enzyme systems for the synthesis of 6-DOT. Brown et al. (5, 6) also reported that cell walls of strains T14Av and T14V both contain 6-DOT. In our laboratory, 6-DOT was also detected in the cell walls of both strains of T14Av and T14V (data not shown) and in the extracellular polysaccharides of each (Table 1). Since the viscous polysaccharide fractions A-1 and A-2 of strain T14Av do not contain 6-DOT, rhamnose, and muramic acid, neither fraction is contaminated by cell wall material. In addition, both fractions contain only trace amounts of protein. Therefore, the purified major viscous fraction A-2 and the minor viscous fraction A-1 can be designated as highly purified slime polysaccharide fractions. It is also clear that the major viscous fraction A-2 is not identical to fraction A-1 since fraction A-2 contains galactose, *N*-acetylglucosamine, glucose, and mannose as sugar components whereas fraction A-1 contains only glucose and glucosamine. The compositions of the A fractions suggest that one may serve as an intermediate in the synthesis of the other or, alternatively, could result from degradation of the other (A-2 may be degraded to A-1). Fraction A-1 was eluted in the void volume of a DEAE–Bio-Gel A column, whereas fraction A-2 was weakly retained on the column (Fig. 2a). However, it is not clear why fraction A-2 was slightly retarded on the ion-exchange column. Fraction A-2 apparently lacks negatively charged groups since uronic acid was not detected chemically and the infrared spectrum of this fraction never revealed the strong absorption peak around 1,240 cm^{-1} which signifies the existence of the S=O bond in polysaccharides. Thus, the retardation of fraction A-2 on the column may result from interactions between the resin matrix and the polysaccharide. It is also of interest that the slime polysaccharide of *A. viscosus* Ny1 does not contain uronic acid or sulfate and was also slightly retarded on a DEAE-Sephadex A-50 column despite the virtual lack of negatively charged groups (26).

Previously, Rosan and Hammond (22) demonstrated that the crude slime polysaccharide isolated from the culture fluids of the rodent strain *A. viscosus* T6 was composed of *N*-acetylglucosamine (62%, dry weight), galactose (7%), glucose (4%), uronic acid (3%), phosphorus (5%), and lower amounts of glycerol, rhamnose, arabinose, and xylose. In addition, Van der Hoeven (26) reported that another rodent strain, *A. viscosus* Ny1, produced an extracellular slime containing two major components with distinctly different chemical compositions when grown in dialyzed actinomyces broth containing glucose. Glucosamine, *N*-acetyl groups, glucose, other carbohydrates, and protein were found in one partially purified slime fraction. However, in the other fraction, glucose was predominant without other neutral sugars and

with only trace amounts of amino sugars, suggesting that this fraction might be intracellular polysaccharide released from lysed cells. The major sugar component of the slime polysaccharides from both rodent strains is *N*-acetylglucosamine, but in the human strain, T14Av, almost equal amounts of *N*-acetylglucosamine (36%, dry weight) and galactose (32%) were detected in the major slime polysaccharide fraction and 24% glucosamine was found in the minor slime fraction (Table 1). It was previously reported that the extracellular polysaccharide (viscous gel), partially purified by gel filtration chromatography, of *A. viscosus* T14Av contained higher amounts (approximately 45%) of *N*-acetylglucosamine (4). In addition, the content of *N*-acetylglucosamine in the slime polysaccharides from rodent strains (22, 26) was reported to be much higher than that of human strain T14Av. This discrepancy could be attributed to inherent differences in the strains and to the relative purity of the slime polysaccharides examined. The resolution of the strain T14Av slime polysaccharide in the present investigation (Fig. 2) appears to represent improved purification of this component relative to earlier investigations (4, 22). However, it is possible that further resolution of the slime polysaccharide may be obtained with other procedures.

Fraction B-1 is composed predominantly of glucose with only small amounts of other sugars. Since this fraction lacks methylpentoses, it might not be derived from the cell wall of strain T14Av but may represent intracellular polysaccharide which may have been released after cell lysis during growth. This fraction is also similar to an extracellular glucose-rich polysaccharide fraction of *A. viscosus* Ny1 (26). The galactose-rich fraction C-3 might be an intermediate in slime polysaccharide synthesis since its sugar composition is similar to that of fraction A-2. In addition, this fraction contains relatively large amounts of phosphorus (Table 2) which could represent phosphorylated intermediates in slime synthesis. All of the other fractions from strain T14Av contain methylpentose(s). Fraction C-1 of strain T14Av and fraction V-1 of strain T14V contain especially large amounts of 6-DOT. Since the sugar composition and weight ratio of each component of both fractions C-1 and V-1 are similar to those of cell walls prepared from both strains of T14Av and T14V except for galactosamine (5, 6), fractions C-1 and V-1 might be derived from the cell wall. However, the possibility that both fractions C-1 and V-1 are extracellular polysaccharides or glycoproteins distinct from cell wall polysaccharide cannot be excluded. The chemical composition of the extracellular polysaccharides synthesized by virulent strain T14V (Table 1) reveals no apparent relationship with the slime polysaccharides (fractions A-1 and A-2) of avirulent strain T14Av. In addition, the sugar composition of fraction V-1 suggests that a large portion of this fraction is derived from or related to the cell wall of strain T14V. Therefore, it will be necessary to investigate the molecular mechanism of slime polysaccharide biosynthesis to determine the genetic basis for the difference in slime formation between strains T14V and T14Av. Such an approach has been recently initiated in this laboratory.

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