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Regulation of Extracellular Slime Production by *Actinomyces viscosus*

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Extracellular slime polysaccharides produced by two *Actinomyces viscosus* strains, T14V and T14Av, were compared. In various media containing glucose, T14Av produced abundant extracellular viscous slime polysaccharide, whereas T14V produced lower levels. Furthermore, fractionation of these polysaccharides showed that the two extracellular polysaccharides differed in molecular size and net charge. Since there was a significant difference in the relative abilities of chemically defined medium and chemically defined tissue culture medium to support slime production by T14Av, the nutritional factors influencing the production of extracellular slime were examined. Sodium bicarbonate was demonstrated to stimulate both cellular growth and the production of extracellular slime. In chemically defined medium with and without sodium bicarbonate, strain T14Av produced large quantities of viscous slime in glucose and sucrose media. In contrast, relatively low levels of slime were produced in fructose, lactose, raffinose, and inositol media, even though sodium bicarbonate stimulated the growth of T14Av in these latter media.

Actinomyces viscosus has been shown to produce extensive plaque deposition, root surface caries, and massive bone loss characteristic of periodontal disease when inoculated into experimental animals (6, 8). Some strains of *A. viscosus* produce extracellular viscous slime polysaccharide, and a role for the extracellular polymer in plaque formation has been proposed (7, 12, 14-16). In a recent investigation using both virulent strain T14V of *A. viscosus* and avirulent strain T14Av, it was demonstrated that T14Av (which produces abundant extracellular slime) colonized rat teeth very poorly, whereas T14V (a poor producer of slime) could readily colonize tooth surfaces of the animals (1). Furthermore, this difference in the ability of the two strains to colonize tooth surfaces may result from the presence of a thick slime layer covering the surface of strain T14Av which is absent in strain T14V (1, 2). The cell-associated slime layer may interfere with the recently proposed fibril-mediated attachment of *A. viscosus* to saliva-coated tooth surfaces (18). Previous investigations with *A. viscosus* strains isolated from rodents have demonstrated that two of these strains, T6 (14) and Ny1 (15), produce abundant extracellular slime. Chemical analysis of partially purified slime preparations revealed that these high-molecular-weight polymers contain large amounts of glucosamine with lower levels of other sugars (14, 15). However, a comparable analysis of slime polysaccharides produced by human oral isolates of *A. viscosus* has yet to be reported. The present investigation was initiated to compare extracellular polysaccharide production by the *A. viscosus* strains T14V and T14Av, which are of human origin. In addition, the regulation of extracellular hexosamine-rich polysaccharide formation by these two organisms was examined to identify the environmental factors affecting slime production.

MATERIALS AND METHODS

Organisms and media. *A. viscosus* T14V and T14Av were kindly supplied by F. C. McIntire (University of Colorado, Denver) and S. Brecher (Forsyth Dental Center, Boston), respectively. Each organism was maintained in Trypticase soy broth (TS) (BBL Microbiology Systems, Cockeysville, Md.) at 4°C and routinely transferred biweekly in TS broth.

Batch cultures of the organisms were grown in TS broth, Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.), brain heart infusion broth (Difco), chemically defined medium (5) supplemented with 0.01% inositol (CD medium), and Dulbecco tissue culture (TC) medium (GIBCO Laboratories, Grand Island, N.Y.). The organisms from stock cultures were inoculated into 10 ml of TS broth and grown for 18 h at 37°C. These cultures were harvested, washed with saline, and used to inoculate the indicated media with a 2% inoculum.

Growth conditions. The cultures were normally incubated at 37°C aerobically as static cultures for the indicated time intervals. Anaerobic growth conditions were obtained by placing the cultures in the GasPak anaerobic system (BBL).

Cellular concentrations were estimated by measuring cell turbidities with a Klett-Summerson colorime-

ter (no. 54 filter). Viscous polysaccharide did not interfere with the turbidimetric estimation of bacterial growth, since the turbidities of culture samples were almost the same as those of the same cell suspensions washed with 1 M NaCl (three times) and suspended in the original volume of medium. pH adjustments of the media were carried out by adding 1 N NaOH or 1 N HCl.

Isolation of extracellular slime. Extracellular slime was isolated according to the procedures of Rosan and Hammond (14) with slight modifications. The cells were grown in complex medium (TS, brain heart infusion, or Todd-Hewitt broth) at 37°C for 24 h, and the culture supernatant fluids were collected after centrifugation at 10,000 × *g* for 10 min. Three volumes of acetone was added to the culture supernatant fluids, and the precipitates formed were allowed to settle at 4°C for 18 h. The precipitates were then collected by centrifugation and suspended with 10% trichloroacetic acid for 4 h at room temperature. The suspensions were next centrifuged at 10,000 × *g* for 10 min, and an equal volume of acetone was added to the supernatant fluids. The precipitates that formed were again allowed to settle at 4°C overnight and collected by centrifugation. The precipitates were dissolved in distilled water and dialyzed against distilled water at 4°C for 18 h. The slime solutions were finally lyophilized, and the dry weights were determined. In chemically defined (CD and TC) media, slime was isolated by modifying the standard procedure; i.e., 3 volumes of acetone was added to the supernatant fluids of the 10% trichloroacetic acid suspension, since little precipitate was obtained when an equal volume of acetone was added.

Procedures for viscosity measurement. The viscosities of culture supernatant fluids were measured by using a Cannon-Fenske viscometer with a centistoke range of 0.8 to 4 in a water bath at 18°C. Efflux times were measured three times for each sample with a stopwatch. There was little variation in flux rates for a given sample. The increase in viscosity was calculated by subtracting the viscosity value (centistokes) of noninoculated medium from that of the culture supernatant fluid.

Fractionation of extracellular slime. Samples (15 mg of dry weight) of the lyophilized slime were fractionated initially by gel filtration chromatography on a Sepharose 4B column. The column (1.5 by 90 cm) was developed at 4°C with 0.01 M phosphate buffer (pH 7.0), and 4.5-ml fractions were collected. The hexosamine-rich fractions (T14Av, fractions 6 through 13; T14V, fractions 14 through 20) were then pooled, dialyzed against distilled water, and lyophilized. To further purify and compare the polysaccharide fractions, samples (10 mg of dry weight) of the partially purified slime were subjected to ion-exchange chromatography on diethylaminoethyl-Bio-Gel A (1.5 by 30 cm). Samples were eluted with a gradient from 0 to 0.5 M NaCl in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.5). All fractions were assayed for hexosamine, hexose, and protein content.

Chemical analyses. The total hexoses were determined by the anthrone procedure (19); protein was measured according to Lowry et al. (11). Hexosamine content was assayed according to Levy and McAllan (10). The optimal conditions for release of hexosamine from the slime samples were determined to be incubation with 2 N HCl at 100°C for 3 h.

RESULTS

Measurement of slime production. To develop a convenient assay for *A. viscosus* slime production, we examined initially the relationship between the increase in viscosity of the culture fluids and the levels of hexosamine in acetone-precipitated slime samples (Table 1). This relationship was previously suggested by using *A. viscosus* Ny1 (15). The amounts of hexosamine in the precipitated slime samples from strains T14Av and T14V were measured and correlated with viscosity changes of the supernatant fluids. In strain T14Av, the increase in hexosamine in the slime correlated well with the increase of viscosity of the corresponding culture fluids. However, although strain T14V produced hexosamine-containing precipitable material, there was essentially no increase in the viscosity of the culture fluids. Thus, the acetone-precipitable polysaccharide made by strain T14V differed markedly from that produced by the avirulent strain T14Av. In addition, these results indicated that measurements of culture fluid viscosity could be related to extracellular slime production by strain T14Av but not by strain T14V.

TABLE 1. *Relationship between the increase of culture fluid viscosity and the amount of hexosamine in polysaccharide samples produced by A. viscosus T14Av and T14V*[a]

Incubation time (h)	T14Av		T14V	
	ΔViscosity (centistokes)[b]	ΔHexosamine (μg/ml)[c]	ΔViscosity (centistokes)	ΔHexosamine (μg/ml)
0	0	0	0	0
3	0.03	ND	−0.01	0.35
6	0.05	0.48	−0.01	0.43
9	0.07	0.72	0	0.63
12	0.16	1.23	−0.01	0.36
15	0.18	1.81	−0.01	0.76
18	0.22	2.68	−0.01	1.13

[a] Each organism was grown at 37°C, and 10-ml portions were removed at the indicated time intervals. Measurements of viscosity and the amount of hexosamine in supernatant fluids were carried out as described in the text.

[b] All values were calculated by subtracting the viscosity value (centistokes) of noninoculated medium from those of the culture supernatant fluid.

[c] Acetone precipitates of culture supernatant fluid (all values corrected for hexosamine present in acetone precipitates from sterile medium). ND, not determined.

Growth of *A. viscosus* and extracellular slime production in TS broth. To assess the role of cellular growth on slime production, cell concentrations of strains T14V and T14Av were measured, and the production of extracellular viscous slime was followed by measuring the viscosity of culture supernatant fluids (Fig. 1). In TS broth, strain T14Av produced an abundance of slime during the logarithmic phase of growth, but T14V produced no detectable viscous slime at any time. Furthermore, slime production by strain T14Av was coincident with growth and ceased when the cells entered the early stationary phase.

Slime production in various media. Strain T14Av produced significant levels of viscous slime in all media used, with the lowest yields in the CD medium (Table 2). By both the criteria of hexosamine-containing polysaccharide precipitation and viscosity changes, strain T14V produced little slime in any medium (data not shown). In each medium, cultures of T14V and T14Av reached nearly maximal cell density at the time of harvest. Changes in medium viscosity produced by growth of strain T14Av did not always correspond directly with hexosamine-containing polysaccharide precipitation.

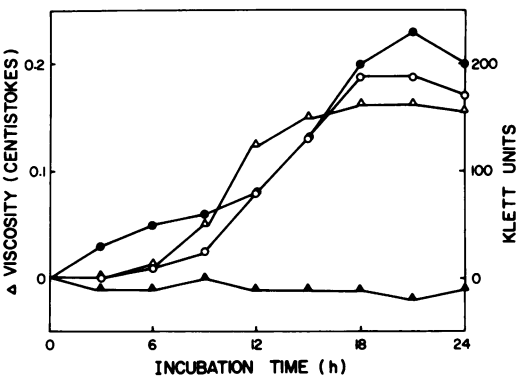

FIG. 1. *Growth of A. viscosus and extracellular slime production in TS broth. The cells were cultured in TS broth (100 ml), and cell concentration and the viscosity of culture supernatant fluids were estimated as described in the text. Symbols: (●) Viscosity of T14Av culture supernatant fluids; (○) cell growth of T14Av; (▲) viscosity of T14V culture supernatant fluids; (△) cell growth of T14V.*

TABLE 2. *Slime production by A. viscosus T14Av in various media*[a]

Medium	ΔViscosity (centistokes)[b]	ΔHexosamine (μg/ml)[c]
TS broth	0.22	1.89
Todd-Hewitt broth	0.42	2.80
Brain heart infusion broth	0.13	3.81
CD medium	0.03	1.68
TC medium	0.50	3.66

[a] Growth conditions, measurements of viscosity, and determination of hexosamine were carried out as described in the text.

[b] Change in culture viscosity relative to sterile uninoculated media.

[c] Increase in culture fluid hexosamine in acetone precipitates relative to sterile uninoculated media.

Nutritional factors influencing the production of slime. Since there was a significant difference in the relative ability of CD and TC media to support slime production by strain T14Av (Table 2), it was of interest to determine the molecular basis for this observation. Unlike TC medium, CD medium contained no $CaCl_2$, KCl, $NaHCO_3$, choline chloride, or sodium pyruvate and relatively low levels of NaCl, pantothenate, folic acid, and pyridoxine. Since any one of these components could be responsible for the difference in the relative capacities of the two media to support slime production, each was added separately to CD medium, and slime production was measured. Sodium bicarbonate was demonstrated to markedly stimulate both the growth of T14Av and the production of extracellular slime in CD medium (data not shown). None of the other potential nutrients produced significant increases in slime production. In addition, we examined cell growth and slime production in CD medium containing 44 mM $NaHCO_3$ to determine whether slime production was influenced by the growth stage in this defined medium. As in TS broth, slime production paralleled growth in CD-$NaHCO_3$ medium (data not shown). The increase in hexosamine-containing polysaccharide was also paralled by an increase in culture viscosity. Since the presence of $NaHCO_3$ stimulated both growth and slime production, the effects of other carbonate salts (Na_2CO_3 and K_2CO_3) on cell growth and slime production were evaluated (Table 3). At low levels of $NaHCO_3$ (<9 mM), both cell growth and slime production were stimulated. However, at higher concentrations of $NaHCO_3$ (>18 mM), slime production appeared to be differentially stimulated. Addition of Na_2CO_3 to CD medium stimulated both cell growth and slime production at lower concentrations. However, at high concentrations of either Na_2CO_3 or K_2CO_3 (>18 mM), strain T14Av did not grow well. Addition of K_2CO_3 also stimulated cell growth at low concentrations, but the stimulatory effects on slime production were less striking than those of $NaHCO_3$ and Na_2CO_3. These results suggested that pH or the presence of CO_2, or both, stimulated cell growth and slime production. Subsequently, we examined the effects of pH in CD medium without the carbonate salts under aero-

bic and anaerobic conditions (Table 4). Under anaerobic conditions, cell growth and slime production were both stimulated, and pH 6.9 appeared to be optimal for cell growth and slime production. When CD media containing equal concentrations of $NaHCO_3$, Na_2CO_3, or K_2CO_3 were adjusted to pH 6.9, maximal cell growth and slime production occurred in the presence of $NaHCO_3$. Media containing Na_2CO_3 or K_2CO_3 at this pH displayed similar levels of growth, but more slime was produced in the presence of Na_2CO_3 than K_2CO_3 (data not shown).

Slime production in the presence of various sugars. To determine the effects of various carbon sources on slime production, strain T14Av was grown in CD medium with and without 44 mM $NaHCO_3$ in the presence of 1% glucose, sucrose, fructose, lactose, galactose, raffinose, and inositol (Table 5). In glucose- and

TABLE 3. *Effects of $NaHCO_3$, Na_2CO_3, and K_2CO_3 on slime production and cell growth of A. viscosus T14Av*[a]

Addition	Concn (mM)	pH of medium	Cell growth (ΔKlett units)	Slime production (Δcentistokes)
None		6.60	110	0.03
$NaHCO_3$	4	6.65	355	0.12
	9	6.80	390	0.16
	18	6.85	450	0.26
	26	6.90	500	0.32
	35	7.00	550	0.36
	44	7.05	550	0.40
Na_2CO_3	4	6.90	410	0.19
	9	7.20	465	0.29
	18	7.80	350	0.04
K_2CO_3	4	6.90	380	0.06
	9	7.25	350	0.09
	18	7.80	120	0

[a] Strain T14Av cells were grown in 10 ml of CD medium at the indicated initial pH values for 18 h.

TABLE 4. *Effects of pH on slime production and cell growth in CD medium without bicarbonate under aerobic and anaerobic conditions*[a]

pH of medium	Cell growth (ΔKlett units)		Slime production (Δcentistokes)	
	Aerobic	Anaerobic	Aerobic	Anaerobic
6.60	110	186	0.03	0.09
6.75	110	215	0.04	0.10
6.90	107	240	0	0.10
7.05	70	160	0	0.04
7.30	48	125	0	0.04
7.70	35	80	0	0.03
8.10	30	65	0	0.02

[a] Strain T14Av cells were grown in 10 ml of CD medium for 18 h at the indicated initial pH values.

TABLE 5. *Slime production by A. viscosus T14Av grown in CD medium in the presence of various sugars*[a]

Sugar	$NaHCO_3$ (44 mM)	Incubation time (h)	Cell growth (ΔKlett units)	Slime production viscosity (Δcentistokes)	Hexosamine (Δμg/ml)
Glucose	+	24	430	0.43	6.93
	−	24	83	0.02	1.05
Sucrose	+	24	430	0.38	5.57
	−	24	72	0.03	1.05
Fructose	+	24	55	0.02	0.41
	−	24	60	0.02	0.51
	+	72	242	0.03	2.00
	−	72	130	0.02	0.68
Lactose	+	24	20	−0.01	0.38
	−	24	21	0.01	0.63
	+	72	310	0.01	1.43
	−	72	135	0	0.82
Galactose	+	24	10	0.03	0.01
	−	24	13	0.01	0.11
	+	72	338	0.04	1.24
	−	72	135	0.01	0.09
Raffinose	+	24	44	0.04	0.44
	−	24	42	0	0.53
	+	72	108	0.12	1.83
	−	72	91	0.09	1.05
Inositol	+	24	25	0.01	0.34
	−	24	25	0.02	0.38
	+	72	150	0.09	0.92
	−	72	37	0.04	0.51

[a] Measurements of cell concentration, viscosity, and the amount of hexosamine precipitable with acetone were carried out as described in the text.

sucrose-containing media, large quantities of viscous slime were produced. In addition, the cells reached maximal growth within a 24-h period. Sodium bicarbonate stimulated the growth of T14Av in media containing relatively poor carbon sources (lactose, galactose, raffinose, and inositol), but the cells produced relatively low levels of slime. These results suggest that the presence of glucose (either as free glucose or as glucose derived from sucrose) in the growth medium is required for maximal production of extracellular slime. Therefore, it was of interest to determine the optimal concentration of glucose required for slime production in CD-$NaHCO_3$ medium. Slime production measured as a function of glucose concentration revealed that optimal growth and slime production of strain T14Av were observed at approximately 0.6% glucose (data not shown). Above this level, no increase in growth was observed, and only a small increase in slime production was detected.

Preliminary fractionation of T14Av and T14V slime polysaccharides. The previous results (Table 1) demonstrated a direct correlation between the increase in culture supernatant viscosity and the amount of hexosamine measured in T14Av slime samples in TS broth culture. However, no such direct relationship was established for T14V hexosamine-containing polysaccharide. Therefore, we examined the potential differences between the T14Av and T14V polysaccharides by purification of both fractions. Gel filtration chromatography of T14Av slime revealed that hexosamine-containing fractions were found predominantly in the void volume of the column, whereas hexosamine-containing material from the T14V polysaccharide (peak at fraction 16) was retarded by the column (Fig. 2). After ion-exchange chromatography, the hexosamine-rich polysaccharide from strain T14Av passed directly through the column with no significant retention, whereas the corresponding fraction from T14V was retained and could only be eluted by increasing concentrations of NaCl (Fig. 3).

DISCUSSION

The present investigation has demonstrated that *A. viscosus* T14Av produces abundant viscous extracellular slime polysaccharide containing hexosamine in a variety of growth media containing glucose. Under most conditions (Table 2), slime production could be correlated with an increase in the viscosity of culture fluids. Slime production also paralleled the growth of strain T14Av and ceased in the early stationary

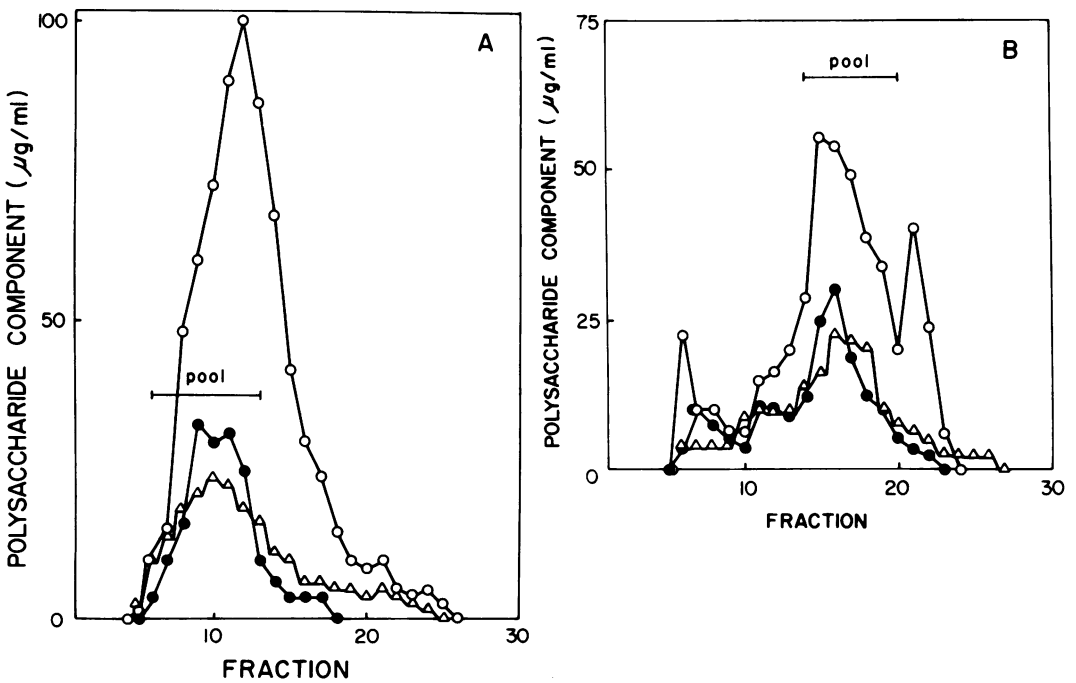

FIG. 2. *Gel filtration chromatography on Sepharose 4B of acetone-precipitated polysaccharides of A. viscosus T14Av and T14V. Acetone-precipitated polysaccharides from T14Av (A) and T14V (B) were obtained from culture supernatant fluids of 24-h cultures in TC medium. Samples (15 mg) were applied as described in the text. Symbols:* (●) *hexosamine;* (○) *hexose;* (△) *protein.*

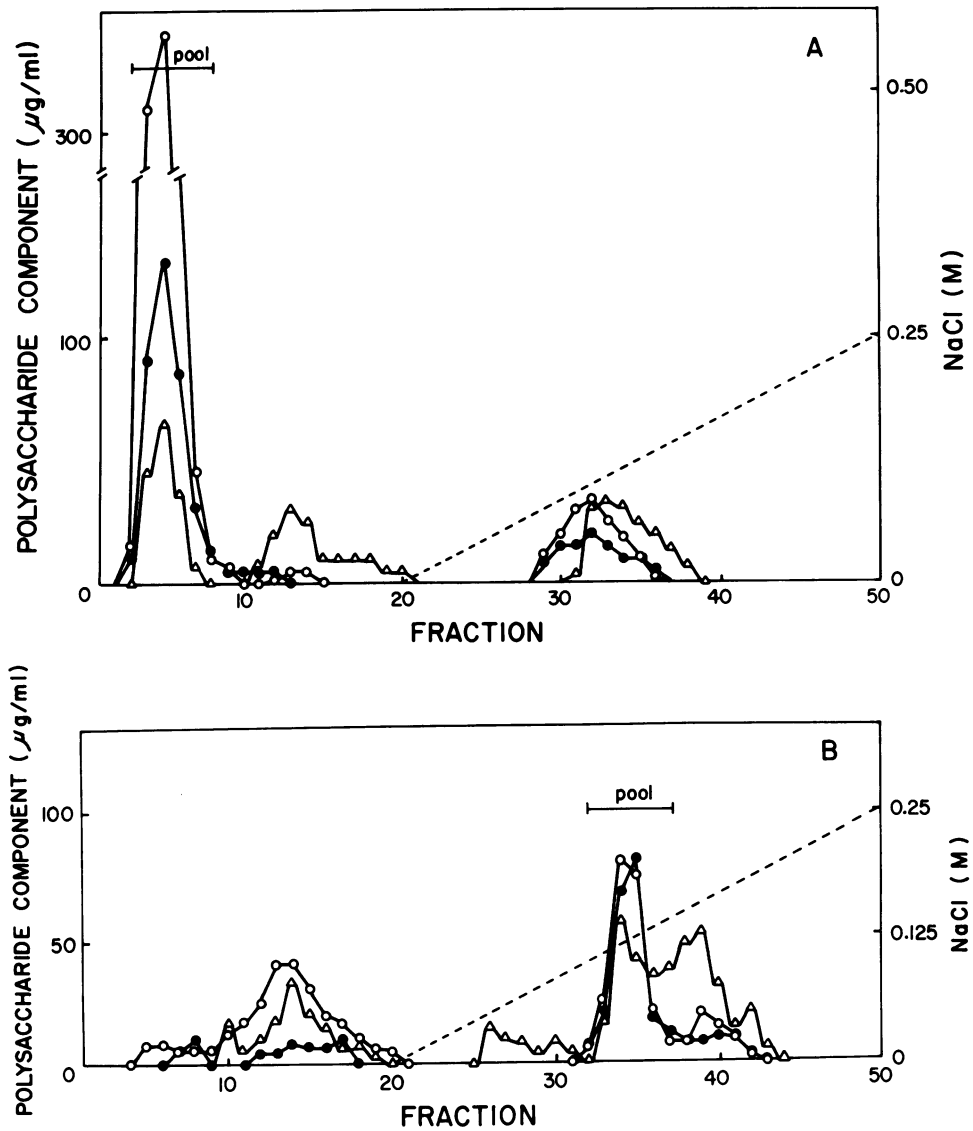

FIG. 3. *Fractionation of hexosamine-rich polysaccharides of T14Av and T14V on diethylaminoethyl-Bio-Gel A resin. Partially purified polysaccharide fractions obtained after Sepharose 4B gel filtration chromatography from T14Av (A) and T14V (B) were chromatographed as described in the text. Symbols:* (●) *hexosamine;* (○) *hexose;* (△) *protein.*

phase of growth (Fig. 1). In contrast, virulent strain T14V produced lower levels of acetone-precipitable polysaccharide containing hexosamine which did not affect the viscosity of the culture fluids (Table 1). Comparison of the partially purified polysaccharide fractions produced by the two human oral strains revealed that the T14Av slime possessed a higher molecular weight than the comparable T14V polysaccharide. In addition, the T14V product behaved like a strongly anionic component, whereas that of strain T14Av appears to be a neutral or basic polysaccharide. Based on preliminary chemical characterization of the two polysaccharides, it is not yet possible to account for the differences in net charge detected after ion-exchange chromatography (Fig. 3). In addition, it is not clear whether the purified fractions are each homogeneous or consist of mixtures of polysaccharide molecules. Further chemical characterization of the products will be required to explain the chromatographic differences observed.

Previous results (14) indicated that the extracellular slime polysaccharide produced by *A. viscosus* T6, a rodent strain, consisted primarily of *N*-acetylglucosamine with lesser proportions of other sugars. In addition, another rodent strain of *A. viscosus*, Ny1, was demonstrated also to produce a comparable polysaccharide in the presence of glucose (15). These rodent slime polysaccharides are apparently similar in composition and properties to that of the human oral isolate strain T14Av. However, since none of the polysaccharides have been extensively characterized, differences in structure may occur.

Recently, it was reported that strain T14Av produced little slime polysaccharide when the organism was cultured in Socransky chemically defined medium (13). However, the present investigation revealed that slime polysaccharide was produced in comparable amounts when the organism was cultured in CD-$NaHCO_3$ or TC medium (Tables 2 and 3). The demonstration that the addition of $NaHCO_3$ to CD medium stimulated both growth and slime production by strain T14Av suggests that a component present in CD medium may be limiting in Socransky defined medium for slime synthesis. Similar but weaker effects on slime synthesis were also obtained after addition of Na_2CO_3 or K_2CO_3 (Table 3). Strain T14Av did not grow in CD medium containing these carbonates at higher concentrations (>26 mM), since these concentrations produced relatively high pH levels which retarded the growth of the organism. That the buffering action of $NaHCO_3$ plays an important role in stimulating cellular growth and slime production was further suggested by the observation that the growth of strain T14Av was markedly improved between pH 6.6 and 8.1 when the cells were incubated anaerobically in the GasPak system (Table 4). Since this anaerobic system continually generates CO_2, it is suggested that the conversion of CO_2 to HCO_3^- may aid in buffering the growth media. However, these results do not rule out a direct stimulatory effect of CO_2 on the growth of strain T14Av. These results further indicate that maximum growth and slime production are observed near pH 7.0 (Tables 3 and 4). Therefore, the major effect of $NaHCO_3$ addition to CD medium appears to be the stimulation of cellular growth, probably as a result of the buffering capacity of this compound. Thus, increased production of slime polysaccharide after $NaHCO_3$ addition is primarily a secondary effect of the increase in the growth rate.

Howell and Jordan (4) earlier reported that the addition of $NaHCO_3$ to the growth medium stimulated both growth and levan synthesis by rodent strains of *A. viscosus*. An earlier investigation (15) suggested that the production of slime polysaccharide by *A. viscosus* rodent strains was increased in the presence of glucose. However, since this suggestion was based on experiments involving complex growth medium, the effects of glucose on slime polysaccharide were not clearly delineated. The present results with CD medium have demonstrated the requirement for glucose (either as the free sugar or derived from sucrose) in the production of slime polysaccharide by strain T14Av (Table 5). Glucose can be produced from sucrose in *A. viscosus* as a result of the action of fructosyltransferase (EC 2.4.1.10) (12, 17) or invertase (EC 3.2.1.26) (9). However, it is not clear why growth of T14Av in the presence of lactose yielded little extracellular slime, since *A. viscosus* strains (9) have β-galactosidase activity which should cleave lactose to glucose and galactose. One possible explanation for this observation is that extracellular free glucose (derived from added glucose or produced from sucrose by the action of extracellular fructosyltransferase) is a direct precursor of slime polysaccharide and not intracellular glucose (produced from lactose by intracellular β-galactosidase). In this regard, we are currently investigating the synthesis of slime polysaccharide in cell-free extracts of strain T14Av to determine the nature of the precursors involved in slime synthesis.

Since strains T14V and T14Av are similar in many biochemical properties (2, 12), it is of interest to examine the relationship between these two human oral isolates. Since T14Av produces abundant slime polysaccharide and T14V does not, it is difficult to envision how a spontaneous mutation in strain T14V results in mutants (T14Av) which synthesize abundant polysaccharide (3). Moreover, recent results suggest that strain T14Av possesses at least one membrane-associated enzyme activity involved in slime polysaccharide synthesis which is absent in strain T14V extracts (T. Ooshima and H. K. Kuramitsu, unpublished data). Thus, T14Av and T14V may represent two different strains of *A. viscosus* containing multiple genetic and biochemical differences, or T14V may be a spontaneous mutant of T14Av selected for its ability to colonize tooth surfaces more effectively (1). Further investigation will be required to distinguish between these possibilities.

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