Surface Localization of Sialic Acid on *Actinomyces viscosus*

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This study reports the presence of sialic acid in *Actinomyces viscosus* strains T14V and T14AV. Mild acid hydrolysis of whole organisms released a compound which reacted positively in the periodate–thiobarbituric acid, direct Ehrlich's and resorcinol assays, and which co-chromatographed on paper with authentic *N*-acetylneuraminic acid. Strain T14V contained 10-fold greater concentrations of sialic acid than did strain T14AV. Sialic acid content was dependent upon the stage of growth of the culture, reaching a maximum in early stationary phase. Epifluorescence microscopy of fluorescein isothiocyanate (FITC)-conjugated *Limulus polyphemus* agglutinin (LPA), a lectin specific for sialic acid, revealed a uniform distribution of bound lectin on the surfaces of strains T14V and T14AV. Additional evidence for surface localization was obtained by demonstration of whole-cell agglutination of both strains with LPA. All LPA interactions with *A. viscosus* were inhibited by the presence of 0.1 M-*N*-acetylneuraminic acid. Neuraminidases from *Clostridium perfringens*, *Arthrobacter ureafaciens* and *Vibrio cholerae* did not release detectable amounts of sialic acid, but the extracellular enzyme from *A. viscosus* cleaved amounts equivalent to those obtained by acid hydrolysis. Other laboratory strains (W1053, M100, W859, 5-5S, RC45, ATCC 19246, and 'binder') as well as recent clinical isolates of *A. viscosus* were agglutinated by LPA and released sialic acid upon mild acid hydrolysis. Surface-available sialic acid has been implicated in the inhibition of alternative complement pathway activation and subsequent opsonophagocytosis. Thus the occurrence of surface sialic acid in *A. viscosus* may represent a mechanism of pathogenesis for this oral bacterium.

INTRODUCTION

Sialic acids are ubiquitous among vertebrates, do not occur in plants, and are of limited distribution among bacteria. These nine-carbon amino sugar acids are named as derivatives of neuraminic acid; both the generic and trivial names reflect sources rich in this carbohydrate, namely saliva and brain gangliosides. In mammals, sialic acids are constituents of membrane glycoproteins which are important determinants in many cell–cell interactions and function in the specificity of macromolecular binding to cell surface receptors in many systems (Schauer, 1982). Strains of *Escherichia coli* (Barry, 1959), *Neisseria meningitidis* (Apicella & Robinson, 1970; Liu *et al.*, 1971) and group B *Streptococcus* (Baker & Kasper, 1976a) produce capsular polysaccharides which contain terminal sialic acid residues. *E. coli* and *N. meningitidis* produce homopolymers or simple alternating copolymers, whereas the group B streptococci synthesize

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Abbreviations: NeuAc, *N*-acetylneuraminic acid; NeuLac, *N*-acetylneuraminlactose; BSM, bovine submaxillary mucin; LPA, *Limulus polyphemus* agglutinin; LFA, *Limax flavus* agglutinin; WGA, wheat germ agglutinin; FITC, fluorescein isothiocyanate; PTA, periodic acid–thiobarbituric acid; KDO, 3-deoxyoctulosonic acid.

more complex carbohydrate structures bearing terminal sialic acid residues. Also, sialic acid has been demonstrated in *Salmonella* species (Barry *et al.*, 1962; Kedzierska, 1978), *Citrobacter freundii* (Barry *et al.*, 1962) and *Corynebacterium parvum* (Dawes *et al.*, 1974).

The common denominator among these micro-organisms is that all represent members of the normal human flora which can infect as opportunistic pathogens. In particular, *E. coli*, *N. meningitidis* and group B *Streptococcus* strains encapsulated by sialic-acid-containing polymers are the most common causative agents of meningitis among infants and septicaemia in immunocompromised patients (Mulder & Zanen, 1984; Pangburn & Muller-Eberhard, 1978; Robbins *et al.*, 1974). Polysaccharide material on these organisms may be a significant virulence factor, since it correlates with a decrease in opsonophagocytosis and enhanced invasiveness (Bortolussi *et al.*, 1979; Schauer, 1982; Varki & Diaz, 1984).

Actinomyces viscosus is an important member of the oral flora in several respects. The organism is numerically common in established supragingival (Syed & Loesche, 1978) and subgingival plaque (Newman & Sims, 1979), is associated with the development of gingivitis (Loesche & Syed, 1978; Syed & Loesche, 1978), causes periodontal disease in animal models (Brecher *et al.*, 1978; Jordan *et al.*, 1972) and occasionally behaves as an opportunistic pathogen in lung infections.

In this study, *A. viscosus* strains T14V and T14AV were utilized to investigate the occurrence of sialic acid on this oral bacterium. Strain T14AV (avirulent) is a spontaneous laboratory variant of strain T14V (virulent) which produces large amounts of an extracellular polysaccharide material, is unable to colonize the teeth of gnotobiotic rats (Brecher *et al.*, 1978), and shows markedly decreased adherence to saliva-treated hydroxyapatite (Moncla *et al.*, 1985). The polysaccharide produced by T14AV probably accounts for its limited ability to colonize the oral cavity. Several experimental techniques were employed to demonstrate the presence of sialic acid on the surface of both strains T14V and T14AV. This observation was extended to other isolates of *A. viscosus*, suggesting that surface sialic acid is a general trait of serotype 2 strains of this species, and by analogy to other systems, may be an important virulence factor. A preliminary report of this study has appeared (Jones *et al.*, 1983).

METHODS

Materials. Tryptic soy broth and yeast extract were from Difco. *N*-Acetylneuraminic acid (NeuAc), *N*-acetylneuraminlactose (NeuLac), bovine submaxillary mucin (BSM), *Clostridium perfringens* neuraminidase, and lectin from *Limulus polyphemus* were obtained from Sigma. Neuraminidase from *Arthrobacter ureafaciens* and *Vibrio cholerae* were purchased from Calbiochem-Behring. Affinity-purified lectins from *Limulus polyphemus* (LPA), *Limax flavus* (LFA) and *Triticum vulgaris* (wheat germ agglutinin, WGA), as well as fluorescein isothiocyanate (FITC)-conjugated LPA and FITC-conjugated rabbit antiserum to LPA, were obtained from E-Y Laboratories, San Mateo, Calif., USA.

Source of cultures. Laboratory strains T14V and T14AV of *A. viscosus* and clinical isolates UF620, UF636 and UF810 were from our stock culture collection. Laboratory strains W1053 and W859 of *A. viscosus* were obtained from Sandra Bragg, CDC, Atlanta, Ga., USA. *A. viscosus* strains M100, 5-5S and 'binder' were from Howard Bladen, NIDR, Bethesda, Md., USA, and RC45 was from Arthur D. Eisenberg, Eastman Dental Center, Rochester, NY, USA. Strain ATCC 19246 of *A. viscosus* was provided by one of us (B. J. M.). Clinical isolates GB3 and GB4 were from George Bowden, University of Manitoba, Winnipeg, Canada. Group B, type III *Streptococcus* was obtained from Steve Mattingly, University of Texas Health Science Center, San Antonio, Tx., USA.

Media and growth conditions. Bacteria were grown in tryptic soy broth supplemented with 0.5% (w/v) glucose and 0.1% (w/v) yeast extract, and buffered with 15 mM-sodium bicarbonate, pH 7.4 (TSBS). Cultures were inoculated (0.1%, v/v) from stock suspensions of bacteria stored at -70 °C in 20% (v/v) glycerol, sparged for 30 s with N₂/CO₂ (95:5, v/v), and incubated at 37 °C on a gyratory shaker at 250 r.p.m. Growth was monitored turbidimetrically with a Klett-Summerson colorimeter (no. 66 filter). Cultures were harvested by centrifugation at 10000 g and washed twice with 0.01M-sodium phosphate/0.15 M-NaCl (PBS). The pH of the washing medium was adjusted to the final culture pH, and ranged from pH 6.7 for early exponential phase cultures to pH 4.9 for stationary phase cultures.

Hydrolysis conditions. A wide range of hydrolysis conditions based upon published methods were examined (Schauer, 1982). The concentrations of acids employed were 0.01-1.0 M-H₂SO₄, 0.005-0.1 M-HCl and 0.175-0.7 M-acetic acid for times from 15 min to 4 h at 0, 25, 37 and 85 °C. Also, cells were extracted at 85 °C in distilled water. Hydrolyses were performed with weighed pellets of washed bacterial cells suspended in 10 vols distilled water per

unit of wet weight. A sample was taken at this point for dry weight measurement as needed. The cell suspension was adjusted to the desired acid concentration, hydrolysed, cooled and centrifuged for 10 min at 10000 *g*. The supernatant fluids were stored at -20°C until assayed. The positive control group B type III *Streptococcus* was hydrolysed in 0.35 M-acetic acid at 85°C for 25 min. Uninoculated medium was hydrolysed by addition of acetic acid to 0.35 M and incubation for 45 min at 37 and 85°C .

Preparation of neuraminidase from *A. viscosus* and neuraminidase assays. Crude soluble neuraminidase was prepared from *A. viscosus* T14V by incubation of bacterial cells in PBS overnight at 37°C (Costello *et al.*, 1979). Strain T14V was harvested at late exponential phase (20 h), washed three times in PBS containing 0.2% sodium azide, and suspended in buffer to the original culture volume. After overnight extraction at 37°C in a shaking incubator at 250 r.p.m., the supernatant fluid was vacuum dialysed against 5.5 l PBS at 4°C overnight to a final protein concentration of $150\text{ }\mu\text{g ml}^{-1}$ in a MicroProDiCon (BioMolecular Dynamics, Beaverton, Ore., USA) with a molecular mass cut-off of 10 kDa. The dialysed and concentrated buffer extract was assayed for both protease and neuraminidase activities. Protease activity was measured as TCA-soluble products of casein hydrolysate as determined by the Lowry method, and was absent from the enzyme preparation. Neuraminidase activity of the preparation was assayed in PBS at 37°C (Costello *et al.*, 1979) and compared with *C. perfringens* neuraminidase under the same conditions using NeuLac and BSM as substrates. The assay consisted of 15 μg protein of the crude *A. viscosus* enzyme preparation or 0.1 units of the commercial enzyme in 0.6 ml total reaction volume containing 100 μg NeuLac or BSM. Both enzymes released sialic acid from the control substrates in 1 h incubations. Specific activities (expressed in $\text{nmol }\mu\text{g}^{-1}\text{ h}^{-1}$) for the *A. viscosus* enzyme were 1 and 4 for BSM and NeuLac, respectively, and 16 and 45 for the same substrates with the *C. perfringens* neuraminidase, as assayed by the periodic acid-thiobarbituric acid (PTA) assay method (Skoza & Mohos, 1976).

Assays of neuraminidase sensitivity of the sialic acid from *A. viscosus* cells were performed at least three times with 0.1 unit of enzyme from *C. perfringens*, *Ar. ureafaciens* or *V. cholerae*, or with 15 μg (total protein) of the *A. viscosus* enzyme. The reaction mixture contained 200 μl of T14V or T14AV harvested in early exponential phase, washed three times in PBS and suspended in buffer to 200 Klett units. All neuraminidase assays were performed in PBS at pH 7 (Costello *et al.*, 1979) for 1 and 24 h at 37°C , except for *V. cholerae* neuraminidase which was assayed in 0.1 M-sodium acetate/1 mM- CaCl_2 /0.15 M-NaCl, pH 7.0. The low pH (4.5) routinely used for neuraminidase assays resulted in release of sialic acid from *A. viscosus* controls without added enzyme. At the end of the incubation period, the cells were sedimented and the supernatant fluids were assayed for released sialic acid by the PTA method. Enzyme activity was routinely assayed with 100 μg BSM as a control substrate. Controls consisted of bacterial cells incubated in the appropriate buffer.

Colorimetric assays. The PTA assay of Skoza & Mohos (1976) was used, and the absorption spectrum from 500 to 600 nm was determined on a Beckman model 25 spectrophotometer.

The direct Ehrlich's reaction of Werner & Odin (1952) was scaled down to one-tenth of the original volumes. The absorption spectrum from 500 to 600 nm was used to confirm the presence of sialic acid.

The resorcinol method of Svennerholm (1957) was performed with 1 ml of aqueous sample, and the absorption spectrum of the extract was determined from 550 to 650 nm.

Values calculated from the standard curves of colorimetric assays were determined by linear regression analysis of duplicates.

Paper chromatography. Acid hydrolysates of *A. viscosus* strain T14V harvested at late exponential phase (20 h) from two individual cultures were examined by paper chromatography in two different solvent systems. Descending paper chromatography was performed on Whatman no. 1 paper with ethyl acetate/pyridine/water (60:25:20, by vol.) for 4 h (system 1) (Baker & Kasper, 1976a), or with 1-butanol/1-propanol/0.1 M-HCl (1:2:1, by vol.) for 16 h (system 2) (Svennerholm & Svennerholm, 1958). Chromatograms were stained with alkaline silver nitrate (Trevelyan *et al.*, 1950) or with resorcinol (Svennerholm & Svennerholm, 1958). Authentic NeuAc was used as a standard, 5 μg for silver nitrate staining and 10 μg for resorcinol staining. Acid hydrolysates, containing comparable amounts of sialic acid (as determined by the PTA method) were utilized for analyses.

Epifluorescence detection of lectin binding. For direct FITC-LPA binding, cultures were harvested at 16 h by centrifugation, washed twice with PBS, pH 7.2, washed once with LPA buffer (0.05 M-Tris/HCl, pH 7.8, 0.01 M- CaCl_2 , 0.15 M-NaCl), and suspended to 250 Klett units. The conjugated lectin, as supplied, binds as large irregular masses of fluorescent material. More uniform FITC-LPA binding to *A. viscosus* T14V was obtained by prior dissociation of the aggregated lectin by addition of EDTA (0.01 M final concentration). Buffer (1.0 ml) containing 100 μg FITC-LPA was added to 200 μl bacterial suspension. The lectin binding reaction was initiated by restoration of Ca^{2+} to 10 mM. After incubation at 37°C for 15 min, glutaraldehyde was added to 1.25% (v/v) final concentration. The bacteria were centrifuged immediately for 3 min at 11 200 *g* in a Beckman Microfuge 11 and then washed three times in LPA buffer to remove unbound FITC-LPA and glutaraldehyde. Glutaraldehyde fixation contributed low background fluorescence to the bacterial cells, but this was not sufficiently bright to expose photographic film under the conditions utilized. Sedimented bacteria were suspended in 200 μl LPA buffer and examined by phase-contrast and epifluorescence microscopy. Representative fields were photographed on

Kodak Panatomic X and/or Kodak Kodachrome II film. Group B type III *Streptococcus* was utilized as the positive control for FITC-LPA binding, and an oral isolate of *Streptococcus faecalis* as the negative control.

An indirect method was also employed to demonstrate lectin binding. Washed bacterial cells were treated with 100 µg LPA as described above. After fixation with glutaraldehyde and three washes in buffer, the bacterial pellet was suspended in 200 µl LPA buffer, pH 7.4, and incubated at 37 °C for 1 h in the presence of 0.5 ml FITC-labelled rabbit anti-LPA (0.9 mg ml⁻¹). The treated cells were washed three times with LPA buffer and examined by phase-contrast and epifluorescence microscopy as above.

Competitive inhibition studies were performed as described above except that solid NeuAc was added to the reaction mixture to 0.1 M final concentration.

All epifluorescence experiments were repeated on at least three occasions.

Lectin agglutination. Cultures of *A. viscosus* strains T14V and T14AV were harvested and washed as described above. Cell suspensions were prepared in appropriate buffers to 60 Klett units. The lectins tested for interactions were LPA, which binds sialic acid, LFA, which also recognizes sialic acid, and WGA, which interacts with both sialic acid and *N*-acetylglucosamine- β -1,4-*N*-acetylglucosamine. LPA and LFA were used with LPA buffer and WGA was examined in PBS. Autoaggregates were dispersed by brief sonication (5 s) prior to the assay. Each reaction mixture consisted of 0.1 ml bacterial suspension plus 0.1 ml lectin solution containing 10, 15 or 100 µg lectin. The suspensions were placed into O-ring plates and agitated on a rotary platform at 120 r.p.m. for 10 min at room temperature. Agglutination was scored with lectin-treated suspensions compared to controls without added lectin. The extent of agglutination was scored from 0 to 4+ relative to the agglutination of the group B type III *Streptococcus* control. Agglutination of the latter with 5 µg LPA was assigned a value of 1+. Inhibition of lectin interactions was performed by the addition of solid NeuAc (final concentration, 0.1 M) to the bacterial suspensions prior to the addition of lectin. Other *A. viscosus* strains were tested with 25 µg LPA as described for strains T14V and T14AV. Agglutination reactions were reproducible from at least three cultures of each strain.

RESULTS

Release of sialic acid from *A. viscosus*. In order to determine the optimal condition for this system, sialic acid release from *A. viscosus* strains T14V and T14AV was measured by the PTA method under various conditions of mild acid hydrolysis as outlined in Methods. Strong mineral acids and high temperatures, usually employed for sialoglycoprotein and glycolipid hydrolyses (0.1 M, 1–2 h, 80–85 °C), yielded no sialic acid from *A. viscosus* cells. Reduction of mineral acid concentration to 0.01 M and of temperature to 0 or 37 °C improved recovery from strain T14V, but not from strain T14AV. Approximately the same amount of sialic acid was recovered from strain T14V by extraction in both distilled water and in 0.35 M-acetic acid for 60 min at 37 °C. Only the latter condition was appropriate for recovery of sialic acid from strain T14AV.

Release of sialic acid from *A. viscosus* by commercial neuraminidase preparations was unsuccessful. Washed early exponential phase cells of strains T14V and T14AV were incubated for 1 and 24 h at 37 °C with 0.1 unit of neuraminidase from *C. perfringens*, *Ar. ureafaciens* or *V. cholerae*. The sialic acid content of the enzyme reaction supernatant fluids and control values of bacteria incubated in buffer alone were below the limit of detection for the PTA assay (0.01 µg). However, the crude preparation of the neuraminidase from *A. viscosus* T14V did cleave sialic acid from whole organisms. The amount of sialic acid released was equivalent to that obtained by acid hydrolysis.

Colorimetric assays. Fig. 1 shows the absorption spectra of authentic *N*-acetylneuraminic acid and the mild acid hydrolysate of *A. viscosus* T14V produced by the PTA assay, the direct Ehrlich's reaction and the resorcinol method. The profiles of the bacterial hydrolysate and the standard are comparable in all three tests, and indicate a spectrum of reactivity consistent with the presence of sialic acid in *A. viscosus* strain T14V. Similar results were obtained for strain T14AV (data not shown).

Paper chromatography. In order to confirm the results obtained by colorimetric derivatization, paper chromatographic separation was utilized. The mild acid hydrolysate of *A. viscosus* strain T14V harvested at late exponential phase (20 h) showed co-chromatography with authentic NeuAc in both system 1 (R_F = 0.245) and system 2 (R_F = 0.478). System 1 revealed an array of compounds which might obscure NeuAc or give a false positive; system 2 separated NeuAc well away from contaminating compounds. Visualization of the chromatogram obtained from

Fig. 1. Comparison of absorption spectra obtained for *N*-acetylneuraminic acid (NeuAc) and the hydrolysate from *A. viscosus* T14V in the PTA, direct Ehrlich's and resorcinol assays. In the top row, the amount of authentic NeuAc is indicated in the upper right corner of each panel. The bottom row shows the spectra produced from *A. viscosus* strain T14V harvested at 20 h and hydrolysed in 0.35 M-acetic acid (HAc) for 45 min at 37 °C. The top line of each panel represents 0.25 absorbance units. Arrows at the bottom of the figure indicate the absorbance maxima for the sialic acid product of each colour reaction.

Fig. 2. Sialic acid contents of *A. viscosus* T14V in batch culture as a function of incubation time. ▲, Sialic acid content; ○, cell viability; □, cell density. The pH of the culture at each time point is indicated on the graph. Cells were hydrolysed in 0.35 M-acetic acid for 45 min at 37 °C, and the sialic acid released was determined by the PTA method. All values represent means obtained from nine separate experiments.

system 2 with resorcinol confirmed that the spot which co-migrated with authentic NeuAc reacted to give the same violet-coloured derivative.

Production of sialic acid in batch culture. The sialic acid content of *A. viscosus* strain T14V was examined as a function of stage of growth (Fig. 2). The amount of sialic acid per g dry weight of cells increased threefold during the exponential phase of growth to a maximum during early stationary phase. Sialic acid content thereafter decreased in concert with the decrease in cell

Fig. 3. Phase-contrast (*a, c, e*) and epifluorescence (*b, d, f*) photomicrographs of *A. viscosus* cells reacted with LPA. Direct staining with FITC-LPA is shown for strains T14V (*a, b*) and T14AV (*c, d*). Strain T14V was also reacted with LPA followed by FITC-conjugated rabbit anti-LPA (*e, f*). Magnification approx. $\times 1600$.

viability. The maximum content of sialic acid in strain T14V was 0.1% of the dry weight. Dry weights varied from 148 mg per g wet weight in the early exponential phase to 184 mg per g wet weight in the early stationary phase.

Epifluorescence microscopy. Surface localization of sialic acid on *A. viscosus* strains T14V and T14AV was visualized by the use of FITC-conjugated LPA, a lectin specific for glycosidic sialic acid, and by an indirect method with FITC-labelled rabbit anti-LPA. Lectin-agglutinated cells of both strain T14V (Fig. 3*a, b*) and strain T14AV (Fig. 3*c, d*) displayed a uniform distribution of bright green fluorescence.

Chemical modification of a lectin to produce a fluorescent derivative could possibly alter its binding specificity. To confirm that the specific lectin-carbohydrate interaction had occurred, unmodified LPA was incubated with a cell suspension of strain T14V, and then stained with FITC-conjugated rabbit antiserum to LPA. Comparable results were obtained (Fig. 3*e, f*), demonstrating that both the lectin and its conjugate bind a cell surface component. Both LPA and FITC-LPA binding were inhibited by 0.1 M-NeuAc. Fluorescence of T14V and T14AV was greatly reduced by incorporation of the competing sugar into the reaction mixture.

Lectin agglutination. Surface localization of sialic acid in *A. viscosus* strains was further supported by bacterial cell agglutination with specific lectins. Strains T14V and T14AV were tested with affinity-purified lectins reported to bind sialic acid residues. Agglutination of strain T14V could be achieved with as little as 1 μ g LPA. Maximum agglutination (4+) occurred over a range of 50–100 μ g lectin under the assay conditions employed, but ordinarily was evaluated at 25 μ g (3+). Agglutination of strain T14AV with LPA was less by one unit than that obtained with strain T14V at the same lectin concentrations (see Table 1). LPA-mediated aggregation of both strains was inhibited by 0.1 M-NeuAc. Late stationary phase cells, which possessed no sialic acid, did not agglutinate with LPA.

Table 1. Content of sialic acid in *A. viscosus* strains

Colour assays and lectin agglutinations were performed on three individual 20 h cultures grown on separate occasions. Standard deviations were calculated from the mean of duplicate values obtained from each hydrolysate of the three individual cultures.

Strain of <i>A. viscosus</i>	Sialic acid content as determined by:		
	PTA [$\mu\text{g NeuAc (g wet wt)}^{-1} \pm \text{SD}$]	Direct Ehrlich's	Lectin (LPA) agglutination
T14V	192 $\pm$ 26	215 $\pm$ 17	+++
T14AV	16 $\pm$ 1	23 $\pm$ 4	++
W1053	323 $\pm$ 7	323 $\pm$ 21	++
W859	61 $\pm$ 3	40 $\pm$ 15	+
M100	22 $\pm$ 2	32 $\pm$ 1	+++
5-5S	46 $\pm$ 12	27 $\pm$ 6	+
RC45	30 $\pm$ 10	25 $\pm$ 2	+
ATCC 19246	62 $\pm$ 20	58 $\pm$ 26	+
'Binder'	31 $\pm$ 19	23 $\pm$ 5	+
GB3	67 $\pm$ 4	72 $\pm$ 4	++
GB4	63 $\pm$ 3	64 $\pm$ 1	+
UF620	14 $\pm$ 4	11 $\pm$ 1	++++
UF633	13 $\pm$ 3	12 $\pm$ 2	++++
UF810	27 $\pm$ 2	24 $\pm$ 2	++
Group B type III <i>Streptococcus</i>	1200	720	+

* Agglutination with group B type III *Streptococcus* was determined at 5 μg instead of 25 μg LPA.

Agglutination of *A. viscosus* T14A and T14AV with other sialic acid lectins was not comparable to that achieved with LPA. The sialic-acid-specific lectin LFA agglutinated cells of strain T14V weakly, was inhibited by 0.1 M-NeuAc, and did not react with T14AV. WGA has been reported to interact with sialic acid (Bhavanandan & Katlic, 1979), but did not aggregate *A. viscosus* strains T14V and T14AV.

Other strains of A. viscosus. Each strain was harvested in late exponential phase (20 h), tested for whole-cell agglutination with LPA, and assayed by the PTA and direct Ehrlich's methods. All laboratory strains and clinical isolates were positive for sialic acid by the three criteria tested (Table 1). The large standard deviations reported in Table 1 probably reflect the great variation in sialic acid content as a function of stage of growth. This is consistent with our observations with strain T14V, where small differences in the time of cell harvest result in large differences in sialic acid content (see Fig. 2).

DISCUSSION

The establishment of appropriate hydrolysis conditions for quantitative release of sialic acid from *A. viscosus* was essential for chemical demonstration, but was hampered by the acid lability of the compound. Use of the conventional techniques for glycoproteins and glycolipids (Schauer, 1982) with *A. viscosus* produced little or no detectable sialic acid from T14V and none from T14AV. Substitution of acetic acid for strong mineral acids and reduction of temperature achieved adequate recovery from this organism. Acetic acid hydrolysis minimizes destruction of sialic acids (Varki & Diaz, 1984).

Bacterial sialic acid residues are often resistant to the action of neuraminidases due to O-acetylation or intrachain lactone formation (Liu *et al.*, 1971; McGuire & Binkley, 1964; Shigeoka *et al.*, 1983). The commercial enzymes utilized in this study collectively cleave α -2,3, α -2,6 and α -2,8 ketosides on glycoprotein, glycolipid and oligosaccharide substrates, including colominic acid (Corfield *et al.*, 1981). Each enzyme varies in activity towards specific glycosidic

linkages and is influenced by adjacent glucose units as well as the extent of sialic acid derivatization. These considerations limit any structural conclusions which might be made as to the exact nature of the residue and its chemical environment.

A. viscosus T14V has been described as possessing both a cell-associated and an extracellular form of neuraminidase (Costello *et al.*, 1979). Both the cell-bound and soluble enzymes were active with glycoproteins in solution, but only the extracellular form cleaved surface sialic acid from human erythrocytes. The same pattern of activity was observed when *A. viscosus* T14V was used as the neuraminidase substrate. Although both the soluble neuraminidase and bacterial cell suspensions released sialic acid from BSM, control reactions of *A. viscosus* cells yielded no free sialic acid. Addition of the extracellular neuraminidase to a suspension of mid-exponential phase cells did release sialic acid.

Sialic acids may be assayed by several methods which vary in specificity, sensitivity and appropriateness for use with complex samples. No one colorimetric assay is sufficiently specific to demonstrate the presence of sialic acid unequivocally. Those selected in this study were chosen on the basis of adequate sensitivity for the material tested and mutual elimination of potential cross-reacting substances (Schauer, 1982). The most sensitive and specific method in common use is the PTA assay, which requires non-glycosidic sialic acid as a substrate. The most common interference is from the deoxyribose chromophore ($\lambda_{\max}$ 532 nm) which can obscure the sialic acid peak ($\lambda_{\max}$ 549 nm). The only group of compounds which produce the same chromophore as sialic acids are 3-deoxyaldulosonic acids, such as 3-deoxyoctulosonic acid (KDO) (Kuwahara, 1980). The position of the $\lambda_{\max}$ in absorption spectra from 500 to 600 nm can be used to determine the relative contributions of the 2-deoxyribose and sialic acid chromogens. The direct Ehrlich's reaction is characteristic of sialic acids and does not require prior hydrolysis, but it is comparatively insensitive and forms interfering chromophores from hexosamines, neutral sugars and lysine. The resorcinol method also may be performed on unhydrolysed material and is more sensitive than the Ehrlich's reaction, but serious interference from proteins, pentoses and hexoses can occur (Schauer, 1982; Werner & Odin, 1952). The sialic acid chromophore of the Ehrlich's reaction has two absorption maxima, 565 and 530 nm; the resorcinol product has a maximum at 580 nm. Analysis of these colour reactions by absorption spectra was also used to evaluate colour contributions of possible interfering compounds. Neither the direct Ehrlich's nor resorcinol methods are subject to interference by 3-deoxyaldulosonic acids, the only potential cross-reactants in the PTA assay. The combination of characteristic colour reactions and co-chromatography on paper with NeuAc support the conclusion that sialic acid occurs on *A. viscosus*.

The variation of sialic acid content with stage of growth in batch culture is similar to that observed for group B type III streptococci (Baker & Kasper, 1976*b*; Jennings *et al.*, 1980). Sialic acid content increases through exponential growth, reaches a maximum in early stationary phase, and is affected by the pH of the culture medium. Not only does the amount of cell-associated sialic acid rise during the exponential phase of growth, but the hydrolysable sialic acid content of the culture supernatant fluid also increases. This probably occurs as a result of shedding, and not the extracellular neuraminidase, as free sialic acid was not detected in culture supernatant fluid (unpublished observations).

The LPA lectin for sialic acid shows the greatest specificity towards glycoproteins with NeuAc in α -2,3 or α -2,6 linkage to *N*-acetylgalactosamine. *N*-Acetylhexosamines show no activity with the lectin (Roche & Monsigny, 1978). LPA also recognizes glucuronides (Vaith *et al.*, 1978), gangliosidic *N*-glycolylneuraminic acid (Maget-Dana *et al.*, 1978) and LPS-associated KDO (Rostam-Abadi & Pistole, 1982). The binding of LPA to *A. viscosus* strain T14V could be inhibited by addition of NeuAc to 0.1 M, even in the presence of glutaraldehyde. This observation argues against nonspecific lectin binding as a consequence of fixation. Furthermore, the chemical tests performed exclude glucuronate and KDO as potential candidates for the site of lectin binding. Both solvent systems utilized for paper chromatography can resolve *N*-glycolylneuraminic acid from the *N*-acetyl derivative; therefore, the *N*-glycolyl form is also unlikely to be the ligand. Bacterial cell agglutination with LPA in the absence of glutaraldehyde supports the specific nature of the lectin interaction. The sialic acid lectin LFA,

which recognizes both *N*-acetyl- and *N*-glycolylneuraminic acids (Miller *et al.*, 1981), does not react as strongly as LPA with *A. viscosus*, but the results with this lectin may be interpreted as corroborating the data obtained with LPA. WGA binds to the type-specific polysaccharides of the group B streptococci (Gray *et al.*, 1984), but it did not agglutinate *A. viscosus*.

In order to determine whether surface sialic acid is a widespread trait of *A. viscosus* serotype 2 strains, or whether it is restricted to the strains T14V and T14AV, several laboratory strains and clinical isolates were tested for the presence of this compound. The lectin agglutination assay was selected as the method for demonstrating surface localization among the strains tested. The PTA and the direct Ehrlich's assays were chosen on the basis of sensitivity and overlapping specificity, as the only compound which gives a positive reaction in both tests is sialic acid. There was no correlation between the extent of lectin agglutination and sialic acid content. This could reflect the availability of terminal sialic acid residues for binding, steric hindrance, or the influence of adjacent residues. The values obtained by the two colorimetric methods were not always in agreement, due either to the presence of cross-reacting compounds released by acid hydrolysis or to the variable PTA absorption coefficients obtained for different sialic acid derivatives. Even in the case of the group B type III *Streptococcus*, sialic acid contents estimated by the PTA and direct Ehrlich's methods were not equivalent.

The potential significance of surface-available sialic acid in *A. viscosus* remains to be evaluated. However, a consideration of some aspects of pathogenesis of sialidated *E. coli* strains and group B type III *Streptococcus* indicates that the primary effects are to inhibit alternative complement pathway activation and thereby thwart subsequent opsonophagocytosis and to enhance invasiveness (Bortolussi *et al.*, 1979; Fearon, 1978). Sialidated *E. coli* strains do not produce capsular material when grown below 30 °C and are readily opsonized. Cells grown at 37 °C produce sialic acid and are not opsonized (Bortolussi *et al.*, 1983). No serotype of the group B type III streptococci examined activates the alternative complement pathway to any significant extent, and that activation can occur only when specific antibody titres toward the capsule are quite high (Edwards *et al.*, 1980). Enzymic and chemical modification of capsular sialic acid in type III *Streptococcus* results in a marked increase in consumption of complement factor C3 (Edwards *et al.*, 1982). These findings are consistent with the hypothesis that the amount of surface-available sialic acid may regulate the initiation of activation of the alternative complement pathway (Fearon, 1978; Kazatchkine *et al.*, 1979; Pangburn & Muller-Eberhard, 1978). Enhanced invasiveness appears to be conferred by the sialic acid heteropolymer of group B type III streptococci. Mutants deficient in either production or cell wall linkage of the type-specific polysaccharide are cleared rapidly when injected into mice, whereas the wild-type strain disseminates quickly to other tissues (Yeung & Mattingly, 1983, 1984). The sialidated capsular material of *E. coli* strains associated with neonatal meningitis is antigenically and biochemically identical (except for polymer length) to a cell surface sialic acid homopolymer that occurs on neuronal cells of chick embryos and newborn rats (Vimr *et al.*, 1984). Antisera to group B meningococci cross-react with isolated polysialosyl glycopeptides from both human and rat foetal brain tissues (Finne *et al.*, 1983). These findings suggest that host antigen mimicry may be an additional virulence factor for these micro-organisms.

The occurrence of sialic acid on a numerically important member of the normal oral flora may have certain implications for its ability to colonize and to evade the host defence mechanisms available in the gingival crevice. It has been reported that certain strains of *Streptococci sanguis* are agglutinated with saliva and serum components in a sialic-acid-dependent fashion (Levine *et al.*, 1978; Morris & McBride, 1983). *S. sanguis* strain KS32AR possesses a lectin specific for NeuAc in a trisaccharide sequence consistent with the carbohydrate composition of a purified salivary mucin (Murray *et al.*, 1982). These observations suggest that sialic-acid-containing polymers are an important determinant in the establishment and maintenance of the normal oral flora. Studies to demonstrate the significance of sialic acid on *A. viscosus* would require careful attention to the physiological state of the organism and its surface content of sialic acid.

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