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Actinomyces Tissue Specificity May Depend on Differences in Receptor Specificity for GalNAcβ-Containing Glycoconjugates

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Actinomyces naeslundii* 12104 and *A. viscosus* LY7 were compared for receptor specificities and adherence properties because these relate to their oral colonization sites. Both strains bind GalNAcβ-containing glycosphingolipids (GSLs) in a GalNAcβ1-3GalαOethyl-sensitive fashion but differ with respect to the number of cells bound to GSLs and the effect of neighboring sugar groups on the binding. Their hemagglutination and saccharide inhibition profiles confirm the existence of two receptor specificities (for example, when GalNAcβ1-3GalαOethyl is multivalently conjugated to albumin, its inhibitory activity increases fourfold toward strain 12104 but decreases fourfold toward strain LY7). Trypsin or chymotrypsin treatment of human erythrocytes, which possess receptor GSLs, improves their hemagglutination with strain 12104. In contrast, the same treatment of chicken erythrocytes, which lack receptor GSLs, abolishes their hemagglutination. These findings suggest that both GSLs and glycoproteins act as functional receptors on eukaryotic cells. The strains also differ with respect to the following GalNAcβ1-3GalαOethyl-sensitive adherence properties: (i) strain LY7 adheres somewhat better than does strain 12104 to buccal epithelial cells; (ii) in spite of their similar overall coaggregation patterns with streptococci, strain 12104 coaggregates with *Streptococcus oralis* MPB1 but strain LY7 does not; (iii) strain 12104 alone shows GalNAcβ-sensitive saliva aggregation and adherence to saliva-coated hydroxyapatite. The GSL binding patterns of fresh *Actinomyces* isolates reveal a high prevalence of LY7-like specificities among buccal isolates, whereas 12104-like specificities are most prevalent among plaque isolates. These findings strongly suggest that fresh *Actinomyces* isolates use fine specificity for GalNAcβ-containing glycoconjugates in recognition and subsequent colonization of specific oral surfaces.

Cell surface carbohydrates act as receptors for bacterium-host and interbacterial adherence and are thus complementary to the bacterial adhesins (36). Numerous adhesins have distinct receptor structures, for example, mannose receptors for type 1 fimbriae of enterobacteria (15) and Galα1-4Gal receptors for P fimbriae of uropathogenic *Escherichia coli* (3, 23, 28). However, various enterobacteria express type 1 fimbriae with different detailed sugar specificities (15), and otherwise similar P-fimbrial adhesins show variant sugar specificities in response to the receptor mosaics at different colonization sites (42, 43).

Actinomyces viscosus and *A. naeslundii* are prominent oral bacteria (13, 14) that colonize tooth and mucosal surfaces by expressing two antigenically and funtionally distinct fimbriae designated types 1 and 2 (7–9). Type 1 fimbriae mediate attachment to salivary pellicles formed on apatitic surfaces (9–11) by interacting with the acidic proline-rich proteins (PRPs) and statherin in a protein-protein interaction (17, 18). Type 2 fimbriae, on the other hand, mediate attachment to plaque streptococci (7, 26, 33), oral epithelial cells (4, 5), and salivary pellicles (38, 45) through an assumed protein-carbohydrate interaction involving β-linked GalNAc or related structures, such as lactose (6, 21, 29, 41). Both glycosphingolipids (6, 41) and glycoproteins (4) have been implicated as the receptors for type 2 fimbriae on epithelial cells, whereas the pellicle receptors involve various salivary glycoproteins (38). The receptor structure on streptococci has been traced to repeating hexasaccharide units of their capsular polysaccharides (1, 2, 30), exemplified by GalNAcα1-3Rhaβ1-4Glcβ1-6Galβ1-6GalNAcβ1-3Gal on *Streptococcus sanguis* Ss34 (30). However, no studies have investigated whether *Actinomyces* strains use type 2 fimbriae of slightly different sugar specificities for their interactions with these diverse receptor molecules.

In this study, we examined the receptor specificities of GalNAcβ-sensitive type 2 fimbrial lectins of *A. naeslundii* 12104 and *A. viscosus* LY7 and compared them with those of fresh *Actinomyces* isolates. We found two distinctly different GalNAcβ-binding specificities that influence the adherence properties of *Actinomyces* strains. The expression of the two binding specificities among wild-type *Actinomyces* strains appeared to be dependent on the oral tissue site from which they were isolated.

MATERIALS AND METHODS

Bacterial strains, culturing, and labeling. *A. viscosus* ATCC 19246 and *A. naeslundii* ATCC 12104 were obtained from the culture collection at the National Bacteriological Laboratory, Stockholm (41). *A. naeslundii* 12104 (CCUG 2238) and *Streptococcus mitis* 24892 were obtained from the culture collection at the Department of Clinical Bacteriology, University of Göteborg. *A. viscosus* LY7 (17) was a gift from R. J. Gibbons. Most streptococcal strains and *Actinomyces* strains B74 and 8AO6 were provided by Bo Krasse from the culture collection at the Department of Cariology, University of Göteborg. Fresh *Actinomyces* isolates were obtained by scraping plaque, buccal, and tongue surfaces of one individual and then culturing of the samples on *Actino-*

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myces selective agar plates (47) for 3 days in 5% H_2–10% CO_2–85% N_2. Gram-positive colonies were selected and cultured to homogeneity. The species identity was established based on catalase activity, carbohydrate fermentation profiles, and gas chromatographic analyses of metabolic end products. The *Actinomyces* strains were maintained on blood-agar plates and analyzed for adherence properties before lyophilization. The bacteria were cultured on blood-agar plates at 37°C in N_2 for 24 h (streptococci) or 48 h (actinomycetes). Cells were metabolically labelled by adding 10 μl of [^{35}S]methionine (1,245 Ci/mmol; Amersham) to bacteria suspended in 100 μl of phosphate-buffered saline (PBS) (pH 7.2) and then grown on blood-agar plates.

HA and saccharide inhibition studies. For hemagglutination (HA), 10 μl each of fresh bacteria (10^9 cells per ml) and erythrocytes (4%), both suspended in PBS (pH 7.2), were mixed on a glass slide. The occurrence of agglutination was examined visually during gentle mixing for 5 min at room temperature. Human erythrocytes depleted of sialic acid were obtained by incubating washed erythrocytes with *Clostridium perfringens* neuraminidase (Sigma, St. Louis, Mo.) at a concentration of 0.2 U/ml for 20 min at 37°C.

The inhibitory activities of various saccharides and glycoproteins were determined by preincubating the bacteria with the substance for 30 min before adherence tests. GalNAcβ1-3GalαOethyl, GalNAcβ1-3Galα–bovine serum albumin (BSA), and lactose-BSA were purchased from Sockerbolaget, Arlöw, Sweden, and the other saccharides were obtained from Sigma and Biocarb (Lund, Sweden).

Glycolipids. Glycosphingolipids (GSLs) were purified and isolated as described previously (41). This included extraction of tissues with chloroform-methanol, treatment of the lipid extract with 0.2 M KOH in methanol followed by dialysis to degrade and remove phosphoglycerolipids, and silicic acid column chromatography to remove nonpolar lipids. Neutral and acidic GSLs were separated by DEAE-cellulose chromatography, and then sphingomyelin was removed from the neutral fraction by acetylation and a second silicic acid column chromatography. Individual GSLs were isolated by repeated chromatography on a column containing Iatrobeads (6RS-8060, Iatron laboratories Inc., Tokyo, Japan), applied for both acetylated and native derivatives with continuous gradients of chloroform-methanol (0 to 4%) and chloroform-methanol-water (65:24:4 to 50:40:10). The structures were established by nuclear magnetic resonance spectroscopy, mass spectrometry, and gas chromatography-mass spectrometry as described previously (41).

For the isolation of GSLs from erythrocytes (43), plasma membranes were isolated by continuously shaking fresh erythrocytes washed in saline with toluene (65 ml of toluene and 30 ml of water per 100 ml of erythrocyte concentrate) and then centrifuging (10,000 × *g* for 5 min). The membranes (10 g) at the toluene-water interface were extracted three times with methanol (100 ml each time) and once with chloroform-methanol (2:1; 100 ml) for 25 min at 70°C. Total neutral and acidic GSL fractions were prepared as described above and characterized by immunostaining with monoclonal antibodies (43).

Glycolipid binding assay. The chromatogram binding assay was performed as described previously (20, 24). GSL chromatograms on high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) were treated with 0.5% polyisobutylmethacrylate (P28; Merck) in diethyl ether for 1 min, dried, and soaked with 2% BSA in PBS for 2 h. The plate was then overlaid with 2 ml of ^{35}S-labelled bacteria (10^7 cpm/ml, 10^8 cells per ml), washed five times with PBS, dried, and exposed to X-ray film for 70 h to reveal attached bacteria.

The microtiter well assay (24) was performed as follows. Serial dilutions of GSLs in methanol (50 μl per well) were dried overnight in microtiter wells (Cooks M24, Nutacon, Holland), and then blocked with 2% BSA in PBS (pH 7.3) for 2 h. The wells were incubated with [^{35}S]methionine-labelled bacteria (50 μl per well with 10^9 cells per ml; 10^7 cpm/ml) for 4 h, washed five times with PBS, and dried, and radioactivity was measured in a scintillation counter.

Treatment of erythrocytes with trypsin and chymotrypsin. Trypsin and chymotrypsin treatment of erythrocytes was performed essentially as described previously (12, 31). The enzymes, stored as stock solutions (10 mg/ml), were added to erythrocyte suspensions (20%) in concentrations of 0, 0.05, 0.1, 0.2, 1.0, and 5.0 mg/ml. After incubation at 28°C for 2 h, the digestions were terminated by adding 1 mM phenylmethylsulfonyl fluoride–0.3 U of aprotinin–5 mM EDTA. After incubation of the samples for 15 min, the erythrocytes were washed 5 times (10 volumes per wash) with ice-cold 5 mM Tris (pH 7.4)–150 mM NaCl–1 mM EDTA–1 mM phenylmethylsulfonyl fluoride and used the same day.

Gel filtration chromatography of parotid saliva. After citric acid stimulation, parotid saliva was collected on ice by using Lashley cups. Protease inhibitors were immediately added in the following final concentrations: 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium bisulfite, 2 mM benzamidine-HCl, 2 μg of pepstatin per ml, 1 μM leupeptin, 50 IU of penicillin per ml, and 50 μg of streptomycin per ml. The saliva sample was adjusted to 20 mM Tris-HCl (pH 8)–0.5 M NaCl and separated on a Sephacryl S-200 column (200 by 5 cm) in 20 mM Tris-HCl (pH 8.0)–0.5 M NaCl–penicillin (50 IU/ml)–streptomycin (50 μg/ml) with an elution rate of 5 ml/h. The protein profile was established by using the Pierce bicinchoninic acid protein assay and UV A_{280}. The protein composition of each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (38).

Coaggregation assays. Coaggregation was measured by mixing equal volumes (0.5 ml) of freshly prepared cell suspensions (5×10^9/ml) of streptococci and actinomycetes. The occurrence of coaggregation was determined by visual inspection at 5, 10, and 30 min. The reactions were also measured by pelleting the coaggregates in a centrifuge (500 × *g* for 10 min) and determining the A_{550} of the supernatant.

Cell adhesion assays. Oral epithelial cells were obtained by scraping the buccal and tongue mucosal surfaces of several individuals with a cotton bud. The epithelial cells were dislodged in PBS, washed twice by centrifugation (250 × *g*, 10 min), and incubated overnight in Eagle's minimal essential medium containing amoxicillin (150 μg/ml; Sigma). The epithelial cells were then washed three times and resuspended in PBS to a certain concentration by relating optical density to epithelial cell number based on microscopic counts. To measure adherence, 0.5 ml each of epithelial cells (10^5/ml) and ^{35}S-labelled bacteria (10^9/ml, 10^6 cpm/ml) was incubated together for 90 min in a 37°C water bath under gentle shaking and split into duplicate fractions. One cell fraction was washed three times with 2 ml of PBS by filtration through a 12-μm-pore-size polycarbonate filter (Millipore), a procedure that arrests epithelial cells but not unbound bacteria. The number of attached bacteria was determined by scintillation counting. The other cell fraction was washed in a centrifuge, and the number of attached bacteria on 30 randomly selected cells was counted in a

microscope after staining with crystal violet. Epithelial cells incubated in the absence of bacteria were included to give background values for binding of indigenous bacteria.

Adherence to saliva-coated hydroxyapatite. Bacterial adherence to experimental salivary pellicles was measured as previously described (16, 17). Samples (40 mg) of spheroidal hydroxyapatite (BHD Chemicals Ltd., Poole, England) that had been equilibrated overnight with adhesion buffer (50 mM KCl, 2 mM KPO_4, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$ [pH 6.8]) were incubated with 0.5 ml of adhesion buffer and 0.5 ml of various parotid salivary fractions by continuous inversion for 45 min at room temperature. After two washes with adhesion buffer, the saliva-treated and untreated control beads were incubated with 1.0 ml of 0.5% BSA in adhesion buffer for 30 min. The beads were washed three times with adhesion buffer and then incubated with 1.0 ml of metabolically ^{35}S-labelled bacteria in adhesion buffer (10^5 cpm/ml, 5×10^8 cells per ml) for 45 min. The number of bacteria that remained attached after three washes was determined by scintillation counting.

Saliva aggregation assay. The ability of parotid saliva to induce bacterial aggregation was studied by mixing 0.6 ml of concentrated saliva with 1.2 ml of bacteria (5×10^9 cells per ml) suspended in PBS at room temperature. Aggregation was measured by visual inspection.

RESULTS

Variant GSL binding specificities of *A. naeslundii* 12104 and *A. viscosus* LY7. When several laboratory *Actinomyces* strains were screened for GalNAcβ1-3GalαOethyl-sensitive adherence to neuraminidase-treated human erythrocytes and *Streptococcus oralis* 24892, GalNAcβ-sensitive lectinlike activities were demonstrated for *A. naeslundii* 12104 and B74 and *A. viscosus* LY7 and 8A06 (Table 1).

To investigate these *Actinomyces* strains for their detailed carbohydrate specificities, naturally occurring GalNAcβ-containing GSLs were separated on thin-layer plates and incubated with [^{35}S]methionine-labelled bacteria (Fig. 1 and Table 1). Consistent with its reported specificity (6, 41), strain 12104 binds strongly to numerous GSLs with GalNAcβ as a common structure. Strains LY7, B74, and 8A06 differ from strain 12104 in their weaker and variant pattern of binding to GalNAcβ-containing GSLs. Binding of metabolically ^{35}S-labelled bacteria to serial dilutions of GSLs coated on microtiter wells confirmed the weaker binding of strain LY7 compared with that of strain 12104 (Fig. 2). In this assay, binding of strain LY7 required 100-fold more GSL than did binding of strain 12104 and the resultant binding was less prominent than that of strain 12104. Pretreatment of strains 12104 and LY7 with GalNAcβ1-3GalαOethyl (2 mg/ml) prevented their binding to GSLs immobilized on thin-layer plates and microtiter wells (Fig. 1 and 2), verifying the involvement of lectinlike activities specified by GalNAcβ or related structures. Strains LY7 and 12104 were selected for comparative studies because they are used frequently in many laboratories (6, 17, 41).

The GSL structures that mediate binding of strain LY7 contain Galβ1-3GalNAc (GSLs 2, 6, and 7 in Table 1), GalNAcβ1-3Gal (GSL 3), or GalNAcβ1-3GalNAc (GSL 5) in terminal positions. Structures lacking the GalNAcβ sequence do not mediate binding. The GalNAcβ terminus of GSL 1 does not mediate binding of strain LY7 unless substituted with Galβ1-3 (GSL 2). The common Galβ1-3Gal sequence of the active GSLs (GSLs 2, 3, and 5 through 7) may thus specify binding. The finding that strain LY7 does not bind to GlcNAcβ1-3Gal (GSL 13) may indicate that the axial hydroxyl at C-4 of GalNAc/Gal residues is crucial for binding, as suggested previously for strain 12104 (41). Strain LY7 was also similar to strain 12104 in not tolerating terminal substitutions on GSL 2, such as Fucα1-2 (GSL 8) or $SO_3$1-3 (GSL 9), whereas its internal GalNAc may be substituted with NeuAc at C-6 (GSL 7). Strain LY7 differs distinctly from strain 12104 in its lack of binding to GSL 1 and GalNAcβ1-3Gal linked α1-4 instead of α1-3 to the GSL core (compare GSLs 4 and 3).

TABLE 1. Binding of metabolically ^{35}S-labelled *A. naeslundii* 12104 and *A. viscosus* LY7 to GSLs immobilized on a thin-layer plate[a]

GSL no. and structure[b]	Binding[c]	
	12104	LY7
1. GalNAcβ1-4Galβ1-4GlcCer	++	–
2. Galβ1-3GalNAcβ1-4Galβ1-4GlcCer	++	+
3. GalNAcβ1-3Galα1-3Galβ1-4GlcCer	++	+
4. GalNAcβ1-3Galα1-4Galβ1-4GlcCer	++	–
5. GalNAcβ1-3GalNAcβ1-3Galα1-4Galβ1-4GlcCer	++	+
6. Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4GlcCerh	++	(+)
7. Galβ1-3(NeuAcα2-6)GalNAcβ1-4Galβ1-4GlcCer	++	+
8. Fucα1-2Galβ1-3GalNAcβ1-4Galβ1-4GlcCerh	–	–
9. SO_3-3Galβ1-3GalNAcβ1-4Galβ1-4GlcCerh	–	–
10. GalCer(h)	–	–
11. Galβ1-4GlcCer	–	–
12. Galα1-4Galβ1-4GlcCerh	–	–
13. GlcNAcβ1-3Galβ1-4GlcCer	–	–
14. NeuAcα2-3Galβ1-4GlcCer(h)	–	–
15. Galβ1-3GlcNAcβ1-3Galβ1-4GlcCerh	–	–
16. Fucα1-2Galβ1-4GlcNAcβ1-4Galβ1-4GlcCerh	–	–
17. GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4GlcCer	–	–
18. NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcCer	–	–
19. Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcCerh	–	–
20. GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4GlcCerh	–	–
21. NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer	–	–

[a] *A. naeslundii* B74 and *A. viscosus* 8A06 exhibit GSL binding patterns similar to that of strain LY7. All strains including 12104 show strong HA (3+) of neuraminidase-treated human erythrocytes and coaggregation reactions (3+) with *S. oralis* 24892, all of which are prevented by preincubation of the *Actinomyces* strain with GalNAcβ1-3GalαOethyl (2 mg/ml).

[b] Cer, ceramide-containing nonhydroxy fatty acid and dihydroxy long-chain base; Cerh, presence of hydroxy fatty acid and/or trihydroxy base; Cer(h), both ceramide types.

[c] ++, strong darkening of the autoradiogram; +, soft darkening; (+), binding required optimal binding conditions for detection (Fig. 1); –, no darkening. For all assays, 1 to 2 μg of GSL (Fig. 1) was used.

Different saccharide inhibition profiles of strains 12104 and LY7. Various saccharides were compared for their abilities to inhibit the HA of strains 12104 and LY7 (Table 2). GalNAcβ1-3GalαOethyl is a severalfold more potent inhibitor of strains 12104 and LY7 than are lactose and other sugars. Strains 12104 and LY7 differ in their responses to GalNAcβ1-3Galα conjugated to albumin (25 mol of sugar per mol of protein). Although the GalNAcβ1-3Galα–BSA conjugate is fourfold more potent than the free disaccharide as an inhibitor of strain 12104, it is fourfold less potent than the free disaccharide in blocking the HA of strain LY7. In addition, whereas lactose has the same inhibitory effect on both strains, only strain 12104 is inhibited by Gal, GalNAc, and GalNAcβO-*p*-nitrophenyl.

HA profiles of strains 12104 and LY7. HA is a simple method of studying bacteria-cell interactions (43). For this

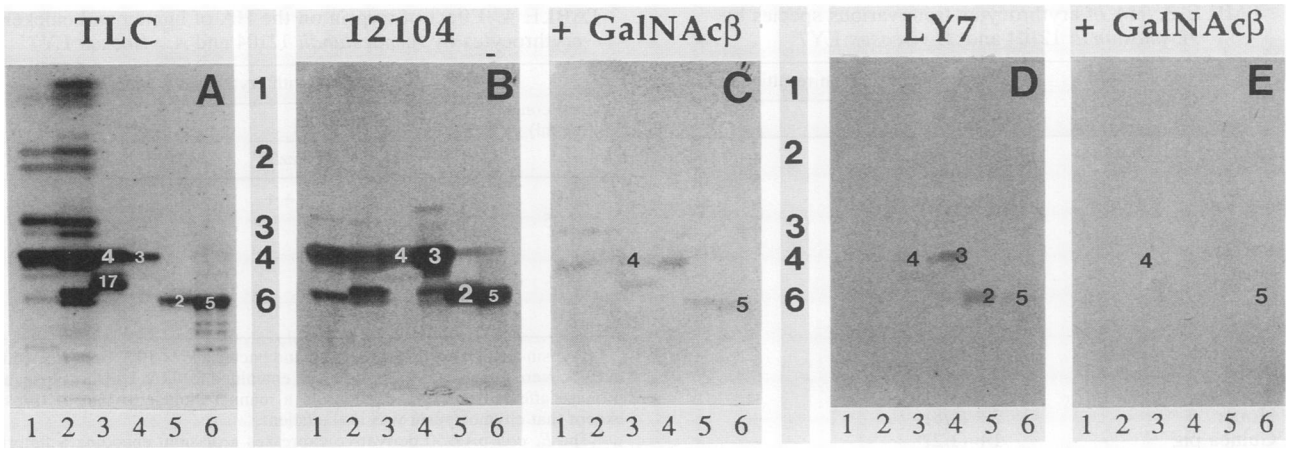

FIG. 1. Binding of metabolically ^{35}S-labelled *A. naeslundii* 12104 and *A. viscosus* LY7 to GSL chromatograms. Six lanes are shown after spray detection with anisaldehyde (A) and after autoradiography (70 h) from an overlay with [^{35}S]methionine-labelled bacteria (B and D) (see Materials and Methods). Lanes: 1 and 2, total neutral GSLs (20 to 40 μg) from human erythrocytes (lane 1) and Vero cells (lane 2); 3 through 6, approximately 2 μg each of GSLs 4 and 17 (lane 3), GSL 3 (lane 4), GSL 2 (lane 5), and GSL 5 (lane 6). The numbers on GSL spots refer to those in Table 1, and the vertically aligned numbers indicate the numbers of sugars in the GSLs. In panels C and E, binding to GSLs (lanes 1 through 6) was monitored after preincubation of the bacteria for 1 h in the presence of 2 mg of GalNAcβ1-3GalαOethyl per ml.

reason, a panel of erythrocytes from various species was tested for HA, and the erythrocytes were grouped into three different classes with respect to their reactivities with strains 12104 and LY7 (Table 3). Class 1 erythrocytes are agglutinated strongly by strains 12104 and LY7, whereas class 3 cells are negative with both strains. Class 2 erythrocytes are agglutinated only by strain 12104. Preincubation of strains 12104 and LY7 with lactose (2 mg/ml) or GalNAcβ1-3GalαOethyl (2 mg/ml) prevents all HA reactions (Table 3).

GSLs versus glycoproteins as functional receptors. The receptor GSL compositions of erythrocytes were investigated by binding strains 12104 and LY7 to thin-layer chromatograms of total neutral erythrocyte membrane GSLs (Fig. 3). Strain 12104 binds strongly to the major GSL species of several agglutinating cells, for example, globoside (GSL 4), of porcine and human erythrocytes (Fig. 3, lanes 2 and 4). However, in spite of the abundance of high-affinity GalNAcβ1-4Galβ1-4GlcCer (GSL 1) receptors for strain 12104 on guinea pig erythrocytes (Fig. 3, lanes 8), these erythrocytes are only weakly agglutinated by strain 12104 (Table 3). Also, the strongly agglutinating chicken erythrocytes lack isoreceptor GSLs for strain 12104 (Fig. 3, lanes 1). An even greater discrepancy between HA and the presence of isoreceptor GSLs is observed for strain LY7, which did not bind to any GSLs of the agglutinating class 1 erythrocytes, including chicken, porcine, and goat cells (Fig. 3, lanes 1 through 3).

To investigate further the nature of the erythrocyte membrane receptors, human and chicken erythrocytes were treated with trypsin and chymotrypsin and the effect on HA was monitored (Table 4). In control experiments, both

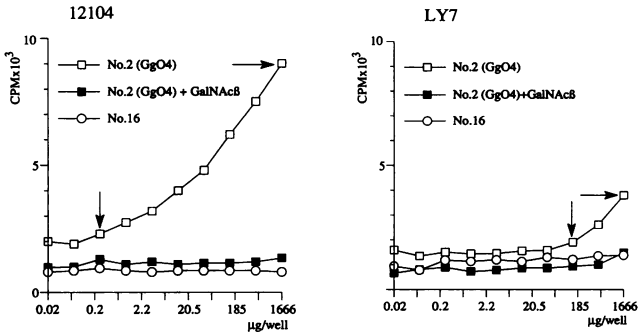

FIG. 2. Quantitative binding curves of *A. naeslundii* 12104 and *A. viscosus* LY7 to GSLs coated in microtiter wells. The GSLs (2 and 16 in Table 1) were added to the wells in amounts indicated on the horizontal scale (nanograms of GSL in 50 μl of methanol). The wells were incubated with ^{35}S-labelled bacteria (see Materials and Methods), and bacteria bound to microtiter wells are given as counts per minute. Bacterial binding to GSL 2 was also measured after preincubation of the bacteria for 1 h with 0.5 mg of GalNAcβ1-3GalαOethyl per ml. The arrows mark the amounts of GSL necessary to induce binding and the resultant binding at the highest amount of GSL. The data are expressed as mean values of triplicate determinations (standard deviations were less than 15% of the means).

TABLE 2. Inhibitory effect of saccharides and neoglycoproteins on HA by *A. naeslundii* 12104 and *A. viscosus* LY7[a]

Inhibitor	Concn (μg/ml) of inhibitor that abolished HA[b]	
	12104	LY7
Galβ1-4Glc	500	500
Galβ1-4GlcbOethyl	500	1,000
(Galβ1-4GlcβO)$_{25}$–BSA	–	–
GalNAcβ1-3GalαOethyl	62.5	125
(GalNAcβ1-3GalαO)$_{25}$–BSA	15.6	500
Glc	–	–
GlcNac	–	–
Gal	1,000	–
GalNAc	500	–
GalNAcβO-*p*-nitrophenyl	500	–
Galα1-4GalOethyl	500	1,000
Manα1-3Manβ1-4GlcNAc	–	–

[a] HA of chicken erythrocytes was measured as described in Materials and Methods.

[b] The minimal concentration abolishing HA was established by preincubation of the bacteria with serial dilutions of the compounds for 30 min prior to HA. –, no inhibitory effect at 2,000 μg/ml of substance.

TABLE 3. HA of erythrocytes from various species by *A. naeslundii* 12104 and *A. viscosus* LY7[a]

Erythrocyte source	HA[b] (lowest dilution resulting in HA)	
	12104	LY7
Human[c]	4+ (1/32)	4+ (1/32)
Class 1		
Chicken	4+ (1/32)	4+ (1/32)
Porcine	4+ (1/32)	4+ (1/32)
Goat	2+ (1/8)	2+ (1/8)
Class 2		
Human	2+ (1/8)	–
Sheep	3+ (1/16)	–
Bovine	2+ (1/8)	–
Horse	2+ (1/8)	–
Guinea pig	1+ (1/2)[d]	–
Class 3		
Rat	–	–
Mouse	–	–
Rabbit	–	–

[a] HA was measured by mixing equal volumes of bacteria (10^9/ml) and erythrocytes (4%), both suspended in PBS, on a glass slide.
[b] HA was estimated both by visual examination (scored from – to 4+) and by reciprocal dilution of the bacteria. Lactose (2 mg/ml) and GalNAcβ1-3GalαOethyl (2 mg/ml) abolish the HA by strains 12104 and LY7.
[c] Neuraminidase-treated human erythrocytes.
[d] Whereas the strong HA reactions (2+ to 4+) were reproducible, the weak HA reaction of guinea pig erythrocytes was not.

cytes by the pAzz50 recombinant *E. coli* strain expressing S fimbriae with specificity for glycophorin (31). Similarily, trypsin (0.05 mg/ml) or chymotrypsin (0.2 mg/ml) abolishes the HA of chicken erythrocytes by strains 12104 and LY7. In contrast, treatment with trypsin and chymotrypsin increases the HA of human erythrocytes by strain 12104, probably by removing glycoproteins and thereby unmasking its high-affinity globoside isoreceptor (GSL 4) on these cells.

Comparison of strains 12104 and LY7 for adherence reactions relevant to the oral environment. Both strains 12104 and LY7 adhere strongly to buccal epithelial cells in a GalNAcβ1-3GalαOethyl (2 mg/ml)-sensitive fashion but only weakly to tongue epithelial cells (Fig. 4). However, strain

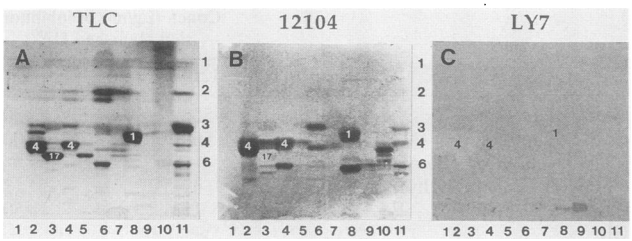

FIG. 3. Detection of receptor GSLs for *A. naeslundii* 12104 and *A. viscosus* LY7 on various erythrocyte species (Table 3) by binding of metabolically ^{35}S-labelled bacteria to GSL chromatograms. Autoradiograms after binding of the bacteria (B and C) and the total GSL pattern after spray detection with anisaldehyde (A) are shown (see Materials and Methods). Total neutral GSL fractions (20 to 40 μg) used were from erythrocytes of the following species (lanes): 1, chicken; 2, pig; 3, goat; 4, human; 5, sheep; 6, bovine; 7, horse; 8, guinea pig; 9, rat; 10, mouse; 11, rabbit. The vertically aligned numbers denote the approximate numbers of sugars in the GSLs, and those on GSL spots refer to the numbers in Table 1.

TABLE 4. Effect of trypsin on the HA of human and chicken erythrocytes by *A. naeslundii* 12104 and *A. viscosus* LY7[a]

Trypsin concn (mg/ml)	HA with erythrocytes from:					
	Human			Chicken		
	12104	LY7	pAzz50[b]	12104	LY7	pAzz50[b]
0.0	+	–	+++	+++	+++	+++
0.05	++	–	–	++	–	–
0.1	++	–	–	+	–	–
0.2	++	–	–	+	–	–
1.0	+++	–	–	(+)	–	–
5.0	+++	–	–	–	–	–

[a] Trypsin-treated erythrocytes (4%) and bacteria (5×10^8), both suspended in PBS, were mixed on a glass slide and examined for HA. Chymotrypsin and pronase affected the HA reactions in a manner similar to that of trypsin, except that chymotrypsin was less efficient.
[b] The *E. coli* pAzz50 derivative expresses a plasmid encoding S fimbriae that binds to trypsin-sensitive receptors on glycophorin (31).

LY7 adheres consistently somewhat more efficiently than does strain 12104 to the buccal epithelial cells.

Strains 12104 and LY7 show similar overall coaggregation properties with various *S. sanguis* and *S. oralis* strains but does not coaggregate with any of the *S. mitis*, *Streptococcus salivarius*, *S. mutans*, or *S. sobrinus* strains (Table 5). However, although strain LY7 reacts weakly with *S. sanguis* GVE1 and OPA1 and not at all with *S. oralis* MPB1, strain 12104 reacts strongly with all three streptococcal strains. Preincubation of the *Actinomyces* strains with GalNAcβ1-3GalαOethyl (2 mg/ml) prevents the coaggregation reactions.

Parotid salivas aggregate strains 12104 and LY7 by different mechanisms (Table 6). Whereas strain 12104 is strongly aggregated in a lactose-sensitive fashion, strain LY7 is weakly aggregated in a lactose-insensitive manner. Parotid saliva, a high-molecular-weight fraction, and a PRP-contain-

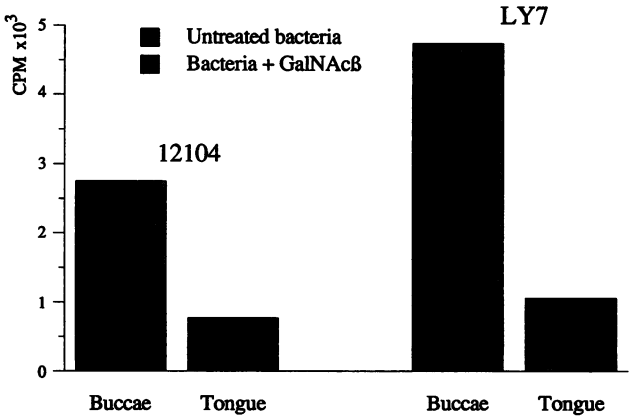

FIG. 4. Adherence of *A. naeslundii* 12104 and *A. viscosus* LY7 to human buccal and tongue epithelial cells. ^{35}S-labelled bacteria were incubated at 37°C with 10^5 epithelial cells in the absence or presence of 2 mg of GalNAcβ1-3GalαOethyl per ml. An estimate of adherence was obtained by scintillation counting and direct microscopic enumeration of attached ^{35}S-labelled bacteria (the input of strains 12104 and LY7 was the same; see Materials and Methods). Strains 12104 and LY7 show the same degree of bacterial aggregation when stained with crystal violet and viewed under the microscope (data not shown). Carbohydrate inhibition tests were only performed for bacterial binding to the buccal cells. The data represent the means of duplicate determinations (standard deviations were less than 25% of the means).

TABLE 5. Coaggregation of *A. naeslundii* 12104 and *A. viscosus* LY7 with oral streptococci[a]

Species and strain(s)	Coaggregation with:	
	12104	LY7
S. sanguis		
KPD1:2	2+	3+
LVG1	2+	2+
PPX1	4+	2+
GVE1	4+	1+
OPA1	4+	1+
KPD1:1	3+	1+
ATCC 10556, MPD1, HPC1, KPB1, MPC1, GSG2R, PPE21, KPD1, HPA1, MPH1, KPB2	−	−
S. oralis		
MPB1	3+	−
CCUG 24892 (ATCC 10557)	3+	3+
9811	4+	3+
S. mitis		
Nt63, MCP9, Mt61, NV71, MCP1, HV81, Nt61, MPF1	−	−
S. salivarius MSS2, 9759	−	−
S. mutans and *S. sobrinus*		
IngBritt, B13, GW12, LK9, LK50, OMZ 175, OMZ 176	−	−

[a] Equal volumes (0.5 ml, 5 × 10^9 cells) of actinomycetes and streptococci were mixed, and coaggregation was scored − to 4+ by visual examination. The results remained essentially the same when the coaggregation was measured spectroscopically after removal of coaggregates by centrifugation (data not shown). Preincubation of strains 12104 and LY7 with lactose (2 mg/ml) prevents all reactions; mannose (2 mg/ml), in contrast, has no effect.

TABLE 6. Ability of parotid saliva from four individuals to induce aggregation of *A. naeslundii* 12104 and *A. viscosus* LY7[a]

Subject no.	Treatment	Aggregation of:	
		12104	LY7
I	PBS	3+	(+)
	Lactose	−	(+)
II	PBS	4+	1+
	Lactose	−	1+
III	PBS	5+	2+
	Lactose	−	2+
IV	PBS	1+	(+)
	Lactose	−	(+)

[a] Parotid saliva and bacterial suspensions (10^9 cells per ml) were mixed in 1:1 proportions, and aggregation was scored by visual examination. The effect of lactose on saliva aggregation was established by preincubation of the bacteria with 2 mg of the saccharide per ml. *A. viscosus* 19246, which is devoid of GalNAcβ-sensitive lectinlike activities, produces an aggregation pattern virtually identical to that of strain LY7.

saliva, a high-molecular-weight fraction, and a PRP-containing fraction obtained by fractionation on a Sephacryl S-200 column were coated onto hydroxyapatite and tested for adherence-promoting activities (Fig. 5). All three fractions promote strong GalNAcβ1-3GalαOethyl-sensitive adherence of strain 12104. In contrast, only whole parotid saliva and the PRP fraction mediate efficient binding of strain LY7 that cannot be prevented by GalNAcβ1-3GalαOethyl, a result which is consistent with the binding specificity of strain LY7 for PRP (17). Collectively, these findings suggest that strains 12104 and LY7 exhibit different adherence recognition properties for GalNAcβ-sensitive salivary receptor sites.

Expression of the strains 12104 and LY7 binding modes among fresh *Actinomyces* isolates from different oral sites. To investigate the presence of strain 12104- and LY7-like specificities among fresh *Actinomyces* isolates, 33 *Actinomyces* strains were isolated from tongue, buccal, and plaque sites of one individual and tested for GalNAcβ-sensitive lectinlike activities measured as binding to neuraminidase-treated human erythrocytes and coaggregation of *S. sanguis* GVE1 (Table 7). The GalNAcβ binding property is common among fresh plaque isolates, less common among strains from buccal sites, and nearly absent among tongue strains. When the positive strains were tested for binding to GSLs on thin-layer plates (Fig. 6), 5 of 13 plaque isolates showed strain 12104-like specificities in their binding to globoside (GSL 4) and GSL 2; 3 of the remaining 8 isolates showed strain LY7-like specificities in binding to Galβ1-3GalNAcβ1-4Galβ1-4GlcCer (GSL 2) but not to globoside (GSL 4), whereas the other strains did not bind to any GSLs (data not shown). Five of six positive buccal strains show binding characteristics similar to those of strain LY7 (Fig. 6). The fresh plaque isolates with *A. naeslundii* 12104-like specificities were all species typed as *A. viscosus* (Table 7), indicating that the two binding modes occur independently of species. None of the tongue strains showed binding to GSLs.

DISCUSSION

A. viscosus and *A. naeslundii* show an oral epithelium and tooth surface specificity that is thought to be due to the

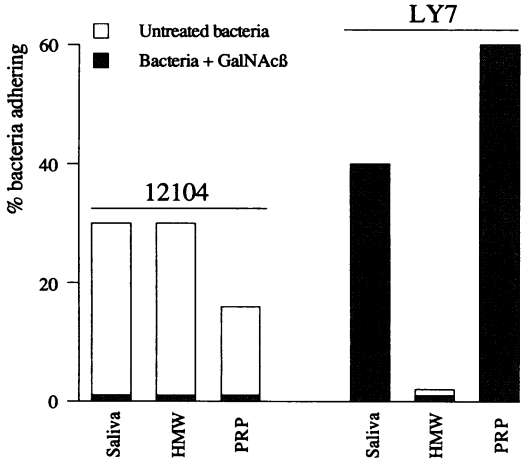

FIG. 5. Attachment of ^{35}S-labelled *A. naeslundii* 12104 and *A. viscosus* LY7 to experimental pellicles formed on hydroxyapatite beads (see Materials and Methods). Unfractionated parotid saliva, a high-molecular-weight (HMW) fraction, and a PRP-containing fraction obtained by Sephacryl S-200 column chromatography of parotid saliva were used. The data represent the adherence in the absence of soluble receptors (untreated bacteria) and after preincubation of the bacteria with 2 mg of GalNAcβ1-3GalαOethyl per ml. The bars overlap each other almost completely in the case of strain LY7 with saliva and PRP. Strains 12104 and LY7 show the same degree of intrabacterial aggregation when stained with crystal violet and viewed under the microscope (data not shown). The data represent the means of duplicate determinations (standard deviations were less than 5% of the means).

TABLE 7. Expression of the GalNAcβ-binding property among *Actinomyces* isolates from different oral sites of one human subject[a]

Strain	Species		HA[b]	Coaggregation[c]
	A. viscosus	*A. naeslundii*		
B-1		+	–	–
B-2		+	–	–
B-3	+		3+	3+
B-4	+		3+	1+
B-5	+		3+	1+
B-6	+		4+	1+
B-7	+		3+	2+
B-8	+		–	–
B-9		+	–	–
B-10	+		3+	1+
T-1		+	–	–
T-2		+	–	–
T-3		+	–	–
T-4		+	–	–
T-5		+	–	–
T-6		+	–	–
T-7		+	3+	3+
T-8		+	–	–
T-9		+	–	–
T-10		+	–	–
P-1	+		3+	2+
P-2	+		3+	2+
P-3	+		4+	4+
P-4	+		3+	4+
P-5	+		3+	4+
P-6	+		4+	4+
P-7	+		4+	1+
P-8	+		3+	1+
P-9	+		4+	2+
P-10		+	3+	4+
P-11	+		3+	2+
P-12	+		3+	2+
P-13	+		3+	4+

[a] The actinomycetes were isolated and characterized as described in Materials and Methods. Strain prefixes indicate ecological sites as follows: B, buccae; T, tongue; P, plaque.
[b] HA was measured with neuraminidase-treated human erythrocytes.
[c] Coaggregation was measured with *S. sanguis* GVE1.

expression of type 1 and 2 fimbriae. Type 1 fimbriae mediate adherence to salivary pellicles, and type 2 fimbriae are mediators of multiple adherence reactions, including adherence to buccal epithelia, salivary pellicles, and oral streptococci. These reactions involve protein-carbohydrate interactions of type 2 fimbriae with GalNAcβ or related structures in mucosal GSLs and glycoproteins, salivary proteins, and streptococcal capsular polysaccharides. The present study shows that *A. naeslundii* 12104 and *A. viscosus* LY7 possess variant GalNAcβ receptor specificities that dictate their adherence properties in vitro. These two specificities are nonrandomly distributed among *Actinomyces* wild-type strains from buccal epithelia and plaque, suggesting that the tissue specificity of *Actinomyces* strains depends on fine differences in the sugar specificity of the GalNAcβ-binding type 2 fimbrial adhesin.

The conclusion that *A. viscosus* LY7 and *A. naeslundii* 12104 express slightly different GalNAcβ receptor specificities is supported by the following observations. (i) Strains 12104 and LY7 differ in both their binding affinities (Fig. 2) and preferences (Table 1) for GalNAcβ-containing GSLs. (ii) Although both strains mediate strong and GalNAcβ-sensitive HA and coaggregation reactions, strain 12104 shows a broader reactivity pattern than does LY7 (Tables 1 through 3; Fig. 3). (iii) When GalNAcβ1-3Galα, a potent inhibitor of strains 12104 and LY7, was multivalently conjugated to albumin, its blocking ability increased fourfold for strain 12104 but decreased fourfold for strain LY7 (Table 2). This is consistent with the proposal that carbohydrate receptor functions may largely be affected by the structure of their internal carrier portions (32, 42, 44). The Galβ/GalNAcβ unit may be sufficient to induce binding of strain 12104 to GSLs (41), whereas strain LY7 appears to require the Galβ1-3Gal sequence (Table 1). The strains may therefore recognize different epitopes on the conserved GalNAcβ1-3Gal/Galβ1-3GalNAc portion of the repeating hexasaccharide units of the streptococcal capsule (1, 30).

Previous studies have indicated that *Actinomyces* strains may utilize both GSLs (6, 41) and glycoproteins (4) as functional receptors on oral epithelial cells. However, in spite of the abundance of receptor GSLs on human (globoside) and guinea pig (GalNAcβ1-4Galβ1-4GlcCer) erythrocytes (Fig. 3), strain 12104 fails to agglutinate these cells strongly (Table 3). These observations and the finding that enzymatic digestion of human erythrocytes improves their HA by strain 12104 (Table 4) are consistent with the cryptic behavior of GSLs (19, 27). In contrast, the finding that the strain 12104- and LY7-induced HA of chicken erythrocytes (which are devoid of receptor GSLs) is abolished by enzymatic treatment (Table 4) suggests that glycoproteins are the functional receptors on these cells. These findings, taken together with the exclusive expression among fresh *Actinomyces* strains from the buccal mucosae of the strain LY7 specificity that binds poorly to GSLs (Fig. 2 and 6), could be interpreted to mean that glycoproteins rather than GSLs constitute functional receptors on the oral mucosal epithelium.

Several possibilities may explain the strain 12104 and LY7 specificities. First, in contrast to the species-specific variant mannose (15) and lactose specificities (37, 39, 40, 41, 44), the difference between strains 12104 and LY7 does not appear to be species dependent, since the fresh *Actinomyces* isolates with specificities similar to that of *A. naeslundii* 12104 all belong to the *A. viscosus* species (Table 7). Second, since strains 12104 and LY7 differ somewhat in their interactions with certain *S. sanguis* strains and markedly in their interactions with *S. oralis* MPB1 (Table 5), they may serve to generate different microbial ecosystems. It should be noted that *Veillonella* species form coaggregation pairs only with streptococci that inhabit the same ecological niche (22, 25) and that several *Actinomyces*-streptococcus coaggregation groups have been described (25). Third, as with the receptor-binding variants of influenza viruses (34, 35, 46), the two specificities may have arisen from selective pressures placed on the *Actinomyces* strains by variations in host-specified glycoconjugates. However, since four individual saliva samples induce GalNAcβ-sensitive aggregation of strain 12104 alone (Table 6) and since both specificities are present among *Actinomyces* isolates from one subject (Fig. 6), such selective pressures at an individual level appear less likely. Instead, the two specificities may have arisen in response to variations in receptor saccharides in different colonization sites. In fact, whereas strain LY7 adheres somewhat more strongly than does strain 12104 to buccal cells, strain 12104 alone mediates GalNAcβ-sensitive adherence to salivary pellicles (Fig. 5). The interaction of strain 12104 with both salivary pellicles and plaque streptococci may confer a

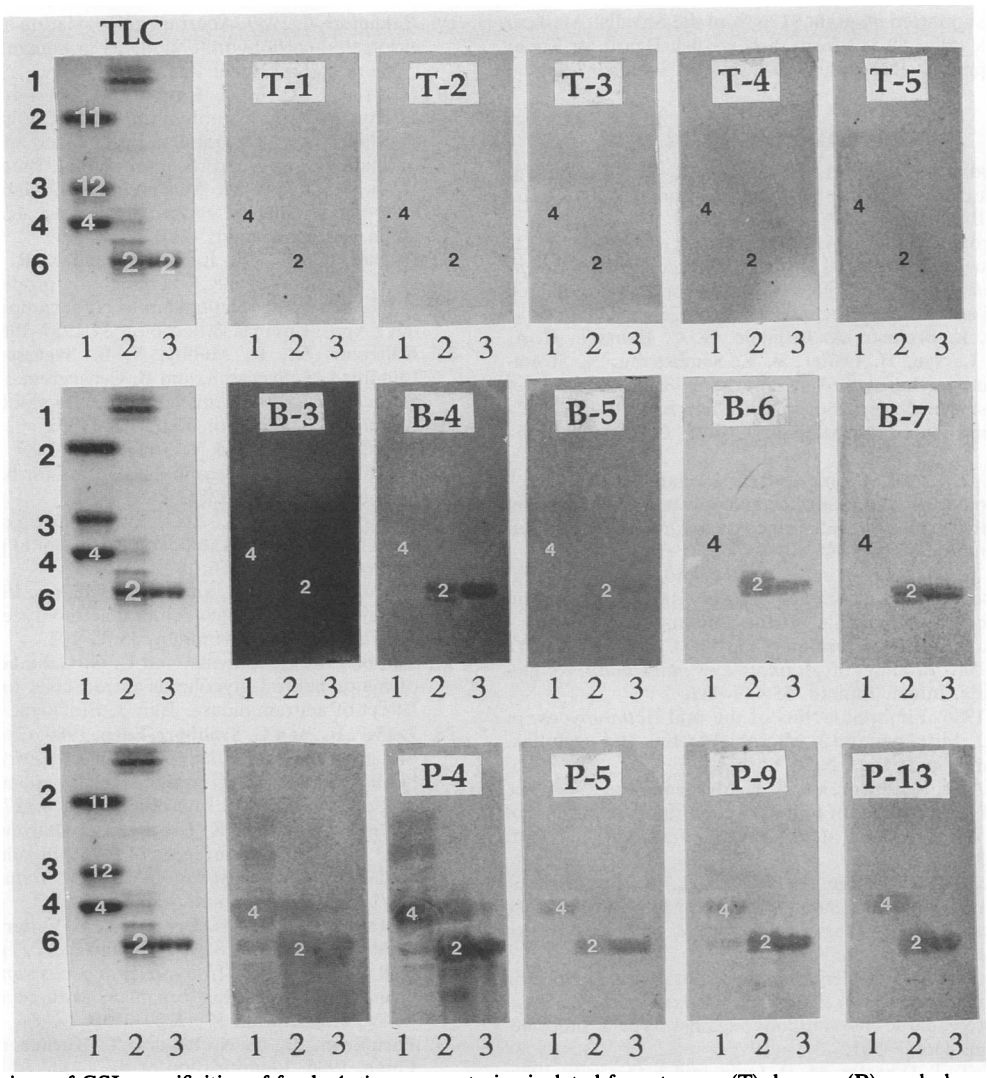

FIG. 6. Comparison of GSL specificities of fresh *Actinomyces* strains isolated from tongue (T), buccae (B), and plaque (P) by binding of metabolically ^{35}S-labelled bacteria to GSL chromatograms (see Materials and Methods). Shown are autoradiograms (70 h) after overlay with radiolabelled actinomycetes. The total GSL pattern as visualized by spray detection with anisaldehyde is shown to the left. Lanes: 2, total neutral GSL from mouse small intestine (20 μg); 1 and 3, approximately 2 μg each of GSLs 4, 11, and 12 (lane 1) and GSL 2 (lane 3). The numbers used to label GSL spots refer to those in Table 1, and the vertically aligned numbers refer to the numbers of sugars in the separated GSLs.

selective advantage in colonizing tooth surfaces. The high prevalence of strain 12104-like specificities among *Actinomyces* plaque isolates (Table 7) and strain LY7-like specificities among isolates from buccal epithelia supports the idea that the two binding modes determine the tissue specificity of *Actinomyces* strains for buccal and tooth surfaces. However, since both binding modes exist among plaque isolates and since they match different glycosylation patterns of parotid and submaxillar salivas (38), they may also be involved in recognizing different tooth sites. These various possibilities agree with recent studies showing that P fimbriae of *E. coli* undergo variation in receptor specificities to colonize the uroepithelia of different animal hosts and different tissue sites in one individual (42, 43). Different foldings of Galα1-4Gal-containing isoreceptor GSLs at the host mucosal membranes may explain this variation in P-fimbria receptor specificity (43). Whether similar conformational or structural differences of receptor glycoconjugates at the various colonization sites underlie the *Actinomyces* receptor-binding variants remains to be determined.

In conclusion, like other microbial adhesins, the *Actinomyces* GalNAcβ-binding adhesin is subject to variation in fine receptor specificity that affects the tissue specificity of this organism. The ease of sampling oral epithelial cells, salivas, and oral microbiota makes *Actinomyces* spp. a promising model for studying the extent and background of variations in receptor specificities of bacterial adhesins. Such studies will increase our understanding of microbial ecology and are prerequisite for future attempts to use synthetic receptor analogs in novel antimicrobial strategies.

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