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Evidence for the Participation of *N*-Acetylated Amino Sugars in the Coaggregation Between *Cytophaga* Species Strain DR2001 and *Actinomyces israelii* PK16

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Coaggregation between *Cytophaga* sp. strain DR2001 and *Actinomyces israelii* PK16 was partially inhibited by certain *N*-acetylated amino sugars (*N*-acetylneuraminic acid, *N*-acetylgalactosamine, and *N*-acetylglucosamine) and was completely inhibited by the trisaccharide neuraminin-lactose. The monosaccharides exerted their effect at concentrations between 30 to 100 mM, whereas the trisaccharide was an effective inhibitor at significantly lower concentrations. Outer membrane preparations caused *A. israelii* PK16 cells to aggregate; however, vesicles released from the cell envelope during growth failed to do so. Adherence studies with a non-coaggregating mutant of the cytophaga suggest that the spheroidal hydroxyapatite attachment sites and coaggregation receptors are separate entities.

The list of coaggregating pairs of oral bacteria is being enlarged constantly; there are now documented examples of interactions between gram-positive and gram-positive (4, 5, 13), gram-positive and gram-negative (7, 9, 14, 15), and gram-negative and gram-negative bacteria (B. C. McBride and H. Merilees, J. Dent. Res. Special Issue A **60**:549 abstr. 957, 1981). In some instances, the high degree of specificity exhibited by these respective coaggregating pairs of bacteria in vitro is believed to be ecologically significant in vivo (9). However, with the exception of the lactose reversible coaggregation of *Streptococcus sanguis* and *Actinomyces viscosus* (12), almost nothing is known of the biochemical nature of the interacting ligands found on the cell surfaces of other coaggregating pairs of bacteria.

Recently, Kolenbrander and Celesk (9) described a series of highly specific coaggregations that occur between certain strains of *Actinomyces israelii* (gram positive) and *Cytophaga* sp. (gram negative). Since both types of organism are known to occur in periodontal pockets and both are capable of affixing themselves to hydroxyapatite-containing surfaces, the potential exists for the creation of a well-anchored subgingival community. This study describes the characteristics of the coaggregation and presents information regarding the chemical nature of the interacting ligands.

MATERIALS AND METHODS

Bacterial strains. The oral isolate *Cytophaga* sp. strain DR2001 was carried in modified Schaedler broth (2). *A. israelii* PK16 (9) was maintained in National Institutes of Health thioglycollate broth (10). Batch cultures of both organisms were grown in 500-ml or 1-liter bottles of modified Schaedler broth containing 0.2% glucose as static cultures in air at 37°C.

Cells used in the coaggregation assay were harvested, washed twice with phosphate-buffered saline (PBS; 0.02 M sodium phosphate buffer [pH 7.2] containing 0.78% NaCl), suspended in PBS containing 0.02% sodium azide (PBSA), stored at 4°C, and used within 24 h to 48 h of harvesting.

Isolation of COG$^-$ mutants of *Cytophaga* sp. strain DR2001. Spontaneously occurring mutants of the cytophaga were isolated by the enrichment procedure of Kolenbrander (8). The parent strain was made resistant to streptomycin (Sm^r) and subsequently cultivated in 100 ml of Schaedler broth containing 4 mg of streptomycin per ml. After aseptic harvesting, the cytophaga cells were washed three times with sterile PBS, and the cell density was adjusted to 260 Klett units (1.9×10^9 cells per ml) measured with a Klett-Summerson colorimeter with a red (660-nm) filter. A 2-ml sample of this suspension was mixed with 2 ml of *A. israelii* cells adjusted to 260 Klett units (1.5×10^9 cells per ml) in a sterile screw-top test tube. After stirring by Vortex mixer, the suspension was allowed to stand for 5 to 10 min, and the contents were centrifuged at $600 \times g$ for 5 min. Nonaggregated cells in the supernatant fluid were decanted, centrifuged at $13,000 \times g$ for 15 min, and suspended in 2 ml of sterile PBS. The procedure was repeated once more with the addition of fresh *A. israelii* cells. After the coaggregated cells were removed, nonaggregated cells were collected by centrifugation, and the resultant pellet was resuspended in 1 ml of sterile PBS and serially diluted. Samples of 0.1 ml were spread onto Schaedler agar plates containing 4 mg of streptomycin per ml. Plates were incubated at 37°C for 48 or 72 h. Isolated colonies were replicated onto Schaedler agar plates containing 4 mg of streptomycin per ml and were tested for the ability to coaggregate after growth had occurred. Coaggregation assays were carried out in microtiter plates by the method of Kolenbrander (8). Presumptive coaggregation-negative (COG$^-$) mutants were cloned on Columbia blood agar plates and retested for coaggregation. Of the 186 clones selected at random from 10^{-3} or 10^{-4} dilution plates, 8 clones retained the COG$^-$ characteristic through several successive transfers in Schaedler broth. Thus, the enrichment procedure produced a population containing roughly 4% stable COG$^-$ mutants. The mutation had no effect on the size or morphology of the mutants, and, at equivalent optical densities, the cell numbers of the mutant and the wild-type parent were essentially identical. The mutant strain designated *Cytophaga* sp. strain DR2001/1-7 was used as a control in the ensuing studies.

Visual coaggregation assay. For the preliminary screening of potential inhibitors, the visual assay of Cisar et al. (4) was used. A 200-μl sample of a cytophaga cell suspension adjusted to an optical density of 260 Klett units (red filter)

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was preincubated for several minutes with 100 μl of a potential inhibitor added to a final concentration of 50 or 100 mM. Coaggregation was estimated after the addition of 200 μl of an *A. israelii* cell suspension brought to a density of 260 Klett units; a visual rating scale of 0 through 4+ was used (4).

Spectrophotometric assays for coaggregation. The technique described by McIntire et al. (12) was used to quantify the extent of the *Cytophaga* sp.-*A. israelii* coaggregation. After establishing that the optimal ratio of *Cytophaga* sp. to *A. israelii* was between 1:1 and 2:1, all suspensions were made up to an optical density of 260 Klett units. Experiments were performed in 12- by 75-mm glass test tubes containing 200 μl of each bacterial suspension and 100 μl of PBSA. Where additives were used, they were prepared in PBSA and adjusted to a pH of 7.2. Controls consisted of either individual suspensions of the actinomyces and parent strain of cytophaga or a mixture of the COG^- mutant and the actinomyces. Tubes were mixed vigorously for 10 s on a Vortex-type agitator, allowed to stand at ambient for 10 min, and centrifuged at 600 × *g* for 5 min. Variations in gravity forces resulting from the use of the low-speed setting made it essential that controls be centrifuged with the experimental tubes. Supernatants were carefully removed by capillary pipette, and the absorbancy at 600 nm (A_{600}) was determined in 0.5-ml cuvettes with a Gilford model 2400S recording spectrophotometer. Differences in the A_{600} between noncoaggregating controls and coaggregating sets ranged from 0.20 to 0.48 absorbance units: percent coaggregation = $[(A_{600}$ control) − (A_{600} experimental)] × $100/(A_{600}$ control), where the controls were either the sum of the A_{600} of the two individual suspensions after centrifugation or A_{600} of the mutant-*A. israelii* combination. Calculating percent coaggregation by this method yields values of 30 to 50. The numbers are lower than those reported by McIntire et al. (12) because the increased rate of centrifugation speed employed in these experiments automatically reduced the number of potential participants in the coaggregation reaction by the number of control cells removed in the centrifugation process. All experimental points were determined in triplicate and are expressed as the average of the three values.

Heat and protease treatments. To determine the extent of heat lability of the receptors on the cytophaga, 2-ml samples of a cell suspension (260 Klett units) were exposed to temperatures ranging from 56 to 64°C for 15 min. Afterwards, the samples were cooled rapidly in ice, and the cells were tested for their ability to coaggregate.

Protease treatments were carried out in PBS at 37°C. Cell suspensions (260 Klett units) were treated with 1 mg each of proteinase K (E.M. Biochemicals), *Tritirachium* protease (Sigma Chemical Co.), pronase (Calbiochem-Behring), trypsin (Sigma), or chymotrypsin (Sigma) per ml for periods of 0.5 to 3 h. Enzyme units are reported below. After incubation, the proteolytic enzymes were removed from the cell suspensions by two successive washes in 5 ml of PBS. The cells were suspended to their original cell density and assayed for coaggregation. None of the treatments affected the morphology of the cells.

Preparation of vesicles and membranes. Both vesicle and membrane preparations were derived from the same 20-liter batch of *Cytophaga* sp. strain DR2001. Vesicles were collected from the culture supernatant after the removal of intact cells and purified by a previously published procedure (2). Membranes were obtained by ultrasonic disruption of a 30-ml suspension containing 6 g (wet weight) of cells in 0.02 M Tris-hydrochloride buffer (pH 7.3). The cells were disrupted with a Branson model 350 sonifier operating at 70% of peak power for 3 min; the cell suspension was maintained at 10°C during the operation. Intact cells were removed by centrifugation at 28,000 × *g* for 20 min, and the amber, opalescent supernatant was decanted and centrifuged at 195,000 × *g* for 2 h. The gelatinous pellet was suspended in 20 ml of PBSA and centrifuged again. After the washing, the membrane pellet was suspended in PBSA. A microscopic examination established that the preparation was free of intact cells. A 6-ml portion of the membrane preparation was treated with 100 μg each of RNase and DNase per ml for 1 h at 37°C, and 2-ml samples were layered onto discontinuous gradients consisting of 15 ml of a 70% solution of sucrose in PBS under 15 ml of a 60% solution of sucrose in PBS (1). Centrifuging the gradients at 68,000 × *g* for 18 h separated the preparation into three distinct bands with mobility coefficients of 0.13, 0.18, and 0.44, respectively. The lower most band (R_f 0.44) exhibited the greatest capacity to coaggregate *A. israelii* cells. The R_f 0.44 bands from the three gradients were pooled and washed twice as described above to remove the sucrose. Washed membranes were suspended in 5 ml of PBSA and stored at 4°C until used.

Adherence assays. The ability of *Cytophaga* sp. strain DR2001, its Sm^r derivative, and the COG^- mutant to attach to spheroidal hydroxyapatite (SHA) was measured by a previously published procedure (2).

Screening of inhibitors of coaggregation. The visual assay was used to determine which, if any, simple carbohydrate or carbohydrate-like compounds would inhibit coaggregation. The following were tested as potential inhibitors of coaggregation at concentrations of 100 mM: fructose, fucose, galactose, glucose, mannose, α-methylglucoside, methylmannoside, rhamnose, tagatose, cellobiose, lactose, melezitose, melibiose, raffinose, trehalose, gluconic acid, glucuronic acid, galacturonic acid, galactitol, mannitol, sorbitol, galactosamine, glucosamine, mannosamine, *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GluNAc), *N*-acetyllactosamine, *N*-acetylmannosamine, *N*-acetylneuraminic acid (NeuAc), NeuAc-lactose, and *N*-acetylmuramic acid.

Chemicals and protein determinations. Amino sugars, N-acetylated amino sugars, NauAc-lactose and *N*-acetyllactosamine were purchased from Sigma. Protein concentration was determined by the biuret (6) or Bio-Rad (Bio-Rad Laboratories) procedure.

RESULTS

Effects of metals, EDTA, saliva, and serum on the coaggregation reaction. Omitting both $CaCl_2$ and $MgCl_2$ had no effect on aggregation (Table 1). The addition of 10 mM EDTA to the coaggregation buffer also had no effect on the reaction, suggesting that soluble metal ions in general were not required (Table 1).

Since both members of the coaggregating pair inhabit an environment containing serum, saliva, or a mixture of the two, it was essential to determine whether either component would affect their interaction. The addition of equal volumes of serum or clarified saliva to suspensions of the two organisms before mixing neither enhanced nor inhibited coaggregation (Table 1). Extending the normal preincubation period from 10 min to 2 h likewise produced no effect.

Destruction of the lectin-like activity on the *Cytophaga* sp. cell surface by heat and proteases. The presumed lectin sites on the cell envelope of the cytophaga were sensitive to heat. Heating a suspension of cells at 60°C for 15 min reduced coaggregation by 41%; heating at 64°C for 15 min completely abolished all detectable coaggregation. Treating the intact

TABLE 1. Effect of various additives on the coaggregation between *Cytophaga* sp. strain DR2001 and *A. israelii* PK16

Component added	A_{600} difference[a]	% Coaggregation
None	0.218 ± 0.004	28
0.1 mM $CaCl_2$ plus 0.1 mM $MgCl_2$	0.218 ± 0.002	28
10 mM EDTA	0.239 ± 0.006	31
Saliva[b]	0.212 ± 0.004	27
Human serum[c]	0.239 ± 0.008	31

[a] Difference after centrifugation between uncombined suspensions of each member (nonaggregating control) and combined cell suspensions (aggregating control).
[b] Sample contained a total of 184 μg of protein.
[c] Sample contained a total of 6.1 mg of protein.

cells with a variety of proteases also reduced coaggregation (Table 2). Neither member of the coaggregating pair appeared to be susceptible to neuraminidase treatment (4.5 units per reaction mixture incubated for 2 h at 37°C) as no decrease in coaggregation was observed after treatment with the enzyme (data not shown).

Inhibitors of coaggregation. Since coaggregation between the cytophaga and actinomyces appears to be a lectin-carbohydrate interaction (9) similar to that reported for *S. sanguis* and *A. viscosus*, a series of inhibition studies was conducted to determine the nature of the participating carbohydrate. All potential inhibitors were preincubated with cell suspensions of *Cytophaga* sp. strain DR2001 before adding *A. israelii* cells. A survey that included the monosaccharides, disaccharides, trisaccharides, amino sugars, *N*-acetylated amino sugars, and aldonic and uronic acids listed above revealed that only five of the compounds tested, NeuAc, GalNAc, GluNAc, *N*-acetyllactosamine, and NeuAc-lactose, were effective in partially inhibiting coaggregation. Surprisingly, *N*-acetylmannosamine, a precursor of NeuAc, had no effect on the interaction.

Quantitative inhibition studies. Before the COG^- mutant *Cytophaga* sp. strain DR2001/1-7 could be used as a control to quantify the *N*-acetylated amino sugar-mediated inhibition of coaggregation, it was necessary to establish that the mutant strain was indeed incapable of interacting with the actinomyces. Visually, no coaggregation could be observed (Fig. 1). In the quantitative assay, the resultant cell density of a mixture of mutant plus actinomyces equalled the additive cell densities of the appropriately diluted controls of the wild-type cytophaga and actinomyces after centrifugation (data not shown). Thus, within the limits of detection *Cytophaga* sp. strain DR2001/1-7 did not coaggregate with *A. israelii* PK16.

In the quantitative inhibition system, the *N*-acetylated amino sugars did not effectively inhibit coaggregation at concentrations below 30 to 40 mM. Figure 2 shows that distinctly sigmoidal or autocatalytic-type curves were obtained with increasing concentrations of NeuAc and GalNAc. GluNAc produced an inhibition curve similar to that of GalNAc, but the maximum inhibitory level was half that of NeuAc (data not shown). However, when NeuAc and GalNAc were added to the cytophaga cell suspension simultaneously, the curve became less sigmoidal, and the degree of inhibition was increased (Fig. 2). Hyperbolic saturation curves were observed when (i) all three inhibitors were added simultaneously at equimolar concentrations or (ii) the ratio of GalNAc to NeuAc was adjusted to 3:2 (Fig. 3). The importance of adding the inhibitors in the appropriate ratios is also evident from Fig. 3. Reversing the ratio by the addition of NeuAc and GalNAc in a 3:2 ratio produced a complex biphasic curve suggestive of a less efficient inhibition at the higher inhibitor concentrations.

When the inhibitors were added in pairs or triads the effects were greater than the sum of the individual inhibitors (Table 3). At the lower concentrations, between 5 and 20 mM, the degree of inhibition was 3 to 5 times greater than that observed with the individual *N*-acetylated amino sugars.

The most effective inhibitor of coaggregation was the trisaccharide neuraminin-lactose [*N*-acetylneuraminyl (2→3) β-D-galactopyranosyl (1→4)-D-glucopyranose], which produced an inhibition of between 20 and 40% at concentrations below 10 mM (Fig. 2). Although the initial curve was hyperbolic, the inhibition produced a complex biphasic curve over the range of 20 to 60 mM. In contrast to the *N*-acetylated amino monosaccharides, NeuAc-lactose completely inhibited coaggregation when present at concentrations below 100 mM. The simultaneous addition of equimolar amounts of NeuAc plus lactose or GalNAc plus lactose did not mimic the inhibition produced by NeuAc-lactose (data not shown). *N*-Acetyllactosamine was also inhibitory, but the inhibition did not exceed 15% at concentrations of 50 mM or less.

In repeating the experiments described above with different batches of cells, little or no difference was observed in the pattern of inhibition with NeuAc-lactose. The inhibition curves produced by NeuAc, GalNAc, and GluNAc were shifted to the right when using cells from slow-growing cultures or older cultures (96 h or greater). And, although an inhibitor concentration of 60 to 75 mM was needed to produce a half-maximal inhibition, the sigmoidal character of the respective curves was maintained (data not shown).

Preliminary evidence for the existence of distinctive *N*-acetylated amino sugar receptors. The synergistic fashion in which the three *N*-acetylated amino sugars interacted to inhibit coaggregation suggested that two and possibly three structurally distinct binding sites participate in the reaction. The physical and chemical treatments described above were used in an attempt to produce a differential destruction of one of the three binding sites. Resting cells heated at 61°C for 15 min to produce a 50% reduction in coaggregation were tested for sensitivity toward the three *N*-acetylated amino sugars. The inhibitors were effective to the same degree as the unheated controls, indicating that no differential destruction of the binding sites had occurred. However, pronase treatment appeared to preferentially degrade GalNAc- and GluNAc-sensitive lectin activity. In the experiment summa-

TABLE 2. Abolition of coaggregating activity by treatment of *Cytophaga* sp. strain DR2001 suspensions with proteases

Protease tested[a]	Enzyme units added	% of cells in coaggregation	% Decrease
None		47	
Proteinase K	20	7 ± 3	85
Tritirachium sp. protease	16	13 ± 4	72
Pronase	6	20 ± 6	56
Trypsin	59	31 ± 4	36
Chymotrypsin	11,110[b]	31 ± 2	36

[a] All cells were incubated for 2 h at 37°C.
[b] BTEE units (*N*-benzoyl-L-tyrosine ethyl ester; 1 μmol/min = 1 U).

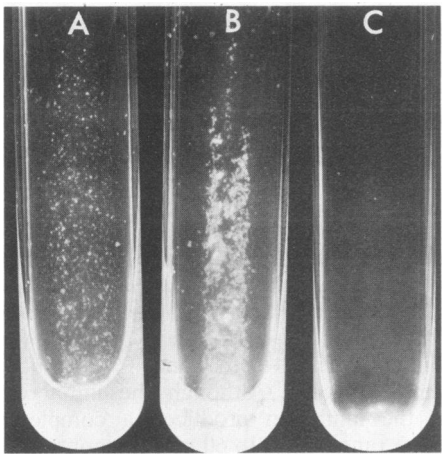

FIG. 1. Differences in the abilities of the three derivatives of *Cytophaga* sp. strain DR2001 to coaggregate with *A. israelii* PK16 as determined by the visual assay. A, *Cytophaga* sp. strain DR2001; B, *Cytophaga* sp. strain DR2001 Sm^r; C, *Cytophaga* sp. strain DR2001/1-7 (COG$^-$ mutant).

rized in Fig. 4A, *Cytophaga* sp. strain DR2001 cells were treated with an amount of pronase that reduced coaggregation at a rate of roughly 25% per 30 min. The degree of inhibition of coaggregation produced by the respective *N*-acetylated amino sugars at concentrations of 50 mM was determined over this period and compared with the zero time value, which was arbitrarily made to 100%. It can be seen that NeuAc inhibition remained unaltered during the 90-min pronase treatment, whereas sensitivity to GluNAc and GalNAc decreased, the latter at a rate commensurate with the decrease in coaggregation.

Aging wild-type *Cytophaga* sp. cell suspensions at 4°C in PBSA for 14 days not only reduced total aggregating ability by 25 to 30%, but also preferentially reduced the NeuAc-sensitive inhibition of coaggregation. As shown in Fig. 4B, the aging process produced two discernible alterations in the pattern of inhibition. First, the sigmoidal inhibition curves observed with freshly harvested cells now appear as linear slopes over the range of inhibitors tested. Second, at concentrations of 100 mM the ability of NeuAc to inhibit coaggregation was reduced 50% as compared with a 9% reduction for GalNAc inhibition.

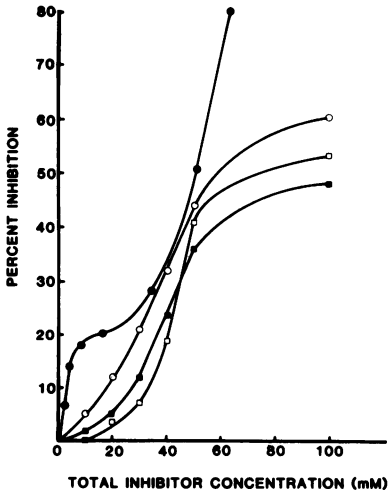

FIG. 2. Inhibition of coaggregation by *N*-acetylated amino sugars and NeuAc-lactose. Symbols: ●, NeuAc-lactose; □, NeuAc; ■, GalNAc; ○, NeuAc plus GalNAc. The variations in percent coaggregation for the first three points of the NeuAc and GalNAc experiments are shown in Table 3; the remainder of the points in the two series did not vary by more than 8%. Experiments with NeuAc-lactose and GluNAc gave very similar results.

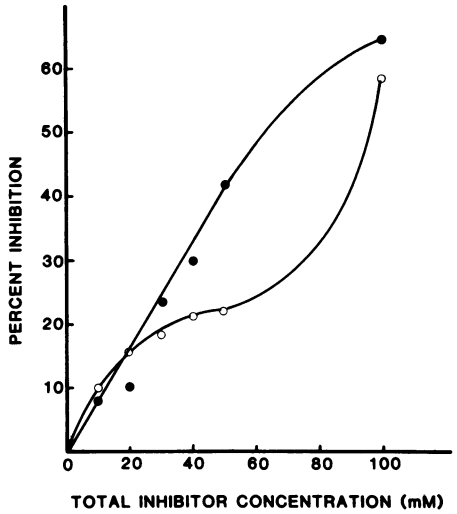

FIG. 3. Alteration of inhibitory response as a function of the following GalNAc/NeuAc ratios: 3:2 (●), 2:3 (○). The variation in percent coaggregation was between 2 and 4% at concentrations of inhibitor below 40 mM and no greater than 6% at concentrations above 40 nM.

Outer membrane-mediated coaggregation. Purified preparations of membranes and outer envelope vesicles derived from the same batch of cells and adjusted to comparable protein concentrations were tested for their ability to mediate the aggregation of *A. israelii* PK16 cells. At the lowest amount tested, 109 μg of protein (Fig. 5), the membrane preparation produced a fine aggregate, which became coarser as the amount of membrane was increased. Surprisingly, the vesicle preparation did not mediate the aggregation of *A. israelii* cells (Fig. 5), even after prolonged incubation (24 h). The *N*-acetylated amino sugars (NeuAc, GalNAc, and GluNAc) also inhibited membrane-mediated inhibition of aggregation.

TABLE 3. Enhancement of inhibition of coaggregation produced by the simultaneous addition of inhibitors

Concn of Inhibitors (mM)		% Inhibition	Additive (expected) inhibition
NeuAc	GalNAc		
10		0	
20		5 ± 2	
30		7 ± 5	
	10	2 ± 1	
	20	4 ± 3	
	30	12 ± 7	
5	5	5 ± 2	0
10	10	12 ± 4	2
15	15	21 ± 6	5[a]

[a] This value is an estimate derived from Figure 3.

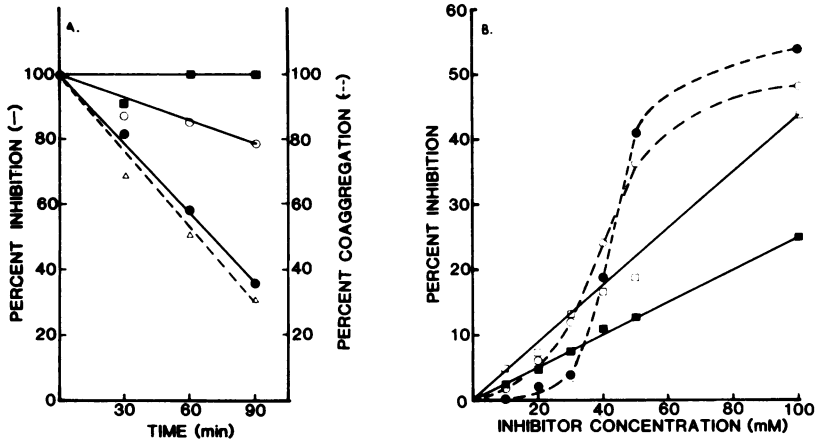

FIG. 4. Differential destruction of binding sites by pronase treatment or aging. A, Time course of pronase destruction of GalNAc-inhibitable site. Inhibition produced by 50 mM NeuAc (■), 50 mM GluNAc (○), and 50 mM GalNAc (●), and loss of toal coaggregation ability (△). B, Loss of NeuAc sensitivity with aging of the cell suspension. NeuAc inhibition of fresh cells (●), and 14-day-old cells (■), GalNAc inhibition of fresh cells (○), and 14-day-old cells (□). Variations in percent coaggregation between replicate samples were no greater than 5%.

To exclude the possibility that the vesicles were too small to effectively bridge the distance between the *A. israelii* cells, the vesicles (1.5 mg of protein) were preincubated with the actinomyces cells in an attempt to block or mask the binding ligands. The treated *A. israelii* cells coaggregated with cytophaga cells even when the vesicles were not removed from the cell suspension of the former (data not presented).

Differentiation of coaggregation receptors from SHA binding sites. In an earlier publication (2), it was reported that *Cytophaga* sp. strain DR2001 readily adsorbed to SHA. To determine whether the coaggregation receptor sites play an important role in the attachment to SHA, the ability of the COG^- mutant to bind to SHA was compared with that of the wild type and its more immediate Sm^r parent. Table 4 clearly shows that the COG^- mutant possesses the same number of binding sites (*N* value) as the wild type and the Sm^r parent and that there is little or no difference in the respective affinities (K_a) for SHA. Thus, the mutation responsible for the loss of the coaggregation receptors had no discernible effect on SHA attachment.

DISCUSSION

The *Cytophaga* sp.-*A. israelii* coaggregation differs from the lactose-reversible *S. sanguis*-*A. viscosus* coaggregation in several major respects. First, *N*-acetylated amino sugars participate in the former reaction, whereas lactose or terminal galactosyl residues take part in the latter interaction. In addition, lactose completely reverses the coaggregates formed by the two gram-positive microorganisms, but mixtures of *N*-acetylated amino sugar only partially reverse the coaggregates produced by *Cytophaga* sp. and *A. israelii*. Last, the reaction between *Cytophaga* sp. and *A. israelii* requires no divalent metal ions and was therefore not affected by EDTA (9). In contrast, both lactose-sensitive and lactose-insensitive coaggregations between *S. sanguis* and *A. viscosus* were inhibited by EDTA (12).

Some insights into the chemical composition of the carbohydrate ligand on the *A. israelii* cell surface and the conformational specificity of the lectin of *Cytophaga* sp. were provided by the inhibitor studies. Only three *N*-acetylated amino sugars were capable of inhibiting the coaggregation when they were preincubated with suspensions of the cytophaga. The fact that deacylated amino sugar derivatives had no effect on coaggregation suggests that one of the recognition domains within the binding site(s) of the lectin is specific for the *N*-acetyl group. Similarly, the failure of *N*-acetylmannosamine to significantly inhibit coaggregation indicates that some portion of the amino sugar molecule is also recognized by the binding site. However, the efficacy with which NeuAc-lactose inhibits coaggregation and its ability to completely inhibit coaggregation at concentrations below 100 mM has furnished the most accurate estimate yet of the actual configuration of one of the *A. israelii* carbohydrate ligands. The complex biphasic inhibition curve produced by

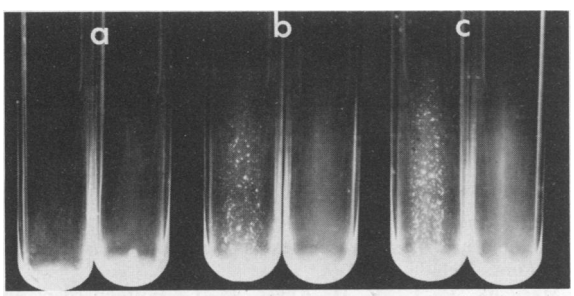

FIG. 5. Comparison of vesicle and membrane preparations as mediators of *A. israelii* PK16 coaggregation. Membranes plus actinomyces cells on left of pair, vesicles plus actinomyces cells on right of pair. Tubes contain (A) 109 μg of protein, (B) 218 μg of protein, and (C) 426 μg of protein in a total volume of 500 μl.

TABLE 4. SHA-binding characteristics of *Cytophaga* sp. strain DR2001 wild-type and Sm^r isolates and *Cytophaga* sp. strain DR2001/1-7

Derivative of *Cytophaga* sp.	Maximum no. of binding sites[a] (*N*)	Affinity constant (K_a)	Correlation coefficient
Wild type	3.3×10^7	4×10^{-8}	0.98
Sm^r wild type	4.7×10^7	2×10^{-8}	0.96
COG^- mutant	4.6×10^7	3×10^{-8}	0.99

[a] Binding sites per 20 mg of SHA.

this inhibitor is probably a manifestation of interactions with at least two different binding sites or two distinct lectins. The initial slope of the biphasic curve most probably represents insertion of the trisaccharide into a high-affinity site that specifically recognizes both NeuAc and lactose or NeuAc and galactose. The second sigmoidal slope may represent an interaction between the trisaccharide and a site of lower affinity such as the GalNAc or GluNAc binding site. Covalent bonding of NeuAc to lactose appears to be necessary, because the simultaneous addition of NeuAc and lactose did not produce an inhibition greater than that observed with NeuAc alone, and *N*-acetyllactosamine was only slightly inhibitory.

It is not yet clear why a sigmoidal response to the respective *N*-acetylated amino sugars was observed in experiments performed with suspensions of freshly harvested cells or why sigmoidicity decreased markedly when two or more of the inhibitors were introduced simultaneously. An enzyme that produces a sigmoidal curve when its reaction rate is plotted against an increasing amount of inhibitor is said to possess multiple sites for the inhibitor which interact in a cooperative fashion (16). After the first inhibitor molecule binds to the protein, a conformational change occurs within the enzyme that facilitates the binding of other inhibitor molecules, and the enzyme becomes a less efficient catalyst at relatively low inhibitor concentrations. The nature of the inhibition produced by *N*-acetylated amino monosaccharides cannot be interpreted as easily or as clearly because coaggregation appears to be a complex interaction between large numbers of multivalent, low-affinity lectins and their corresponding acceptor molecules (3). For example, if the efficacy of inhibition is a measure of the affinity of an inhibitor for a binding site, the K_a of NeuAc-lactose for the lectin binding site is significantly greater than that of NeuAc or GalNAc. Yet no apparent cooperativity was noted in the initial phase of the trisaccharide inhibition; rather, it was observed only when using low-affinity inhibitors at comparatively high concentrations. Furthermore, the monosaccharides were incapable of producing inhibitions greater than 60% even when added in pairs or triads. These observations do not fit the classical definition of cooperativity (16). Indeed, in this instance, a decrease in cooperativity signaled a more effective inhibition. Unless it can be demonstrated that the lectin-like molecules on the cytophaga outer membrane possess more than one binding site, any analogy to an allosteric enzyme becomes very imprecise. The kinetics of inhibition must remain descriptive until the receptors are purified and an accurate estimate of the number of binding sites is made.

McIntire et al. (11) recently tested a number of β-D-galacto-oligosaccharides to determine which specific configurations most effectively inhibited coaggregation between certain *Actinomyces* species and *S. sanguis* 34. Galactosyl-*N*-acetyl galactosamine disaccharides were up to 50 times more inhibitory than galactose alone. A similar result was observed during studies reported here. The trisaccharide NeuAc-lactose was far more inhibitory than NeuAc. Interestingly, the most effective configuration for an inhibitor in the McIntire study was galactose-GalNAc, whereas in this work the inverse configuration, an *N*-acetylated amino sugar linked to galactose proved to be the most effective inhibitor tested. However, other disaccharides consisting of various *N*-acetylated monosaccharides plus galactose must be tested to confirm this supposition.

Studies with the cytophaga mutant have established that loss of the coaggregation receptors does not affect the organism's ability to attach to SHA. However, because attachment to SHA appears to be a comparatively nonspecific interaction mediated by both hydrophilic and hydrophobic cell wall components (2), it is possible that the loss of a relatively minor component would not have produced a measurable effect on SHA attachment. In this regard, it is also worth noting that vesicles desquamated into the culture medium during growth of *Cytophaga* sp. strain DR2001 blocked attachment of this gram-negative bacterium when the SHA beads were pretreated with them (2). Surprisingly, purified vesicle preparations neither mediated aggregation nor blocked the reaction when they were preincubatd with *A. israelii* cell suspensions. Only the purified preparation of cell membranes served as a bridging agent that caused cell suspensions of *A. israelii* to aggregate. As with the intact cells, aggregation was inhibited by the three *N*-acetylated amino sugars (data not shown). The fact that vesicles appear to arise from almost all portions of the cytophaga outer membrane (2) makes the preceding observation difficult to reconcile. Even if the assumption is made that the vesicles do carry the lectin-like protein(s) and that an inversion of the vesicle has most of the receptors facing inward, a sufficient number of these structures should have been in the appropriate orientation to produce a limited aggregation. Alternative explanations require that (i) the receptors be located in areas of the outer membrane that do not give rise to vesicles or (ii) the receptor is anchored to the peptidoglycan layer or cytoplasmic membrane of this bacterium. Membrane and vesicle analyses already in progress should resolve this enigma.

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