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Characterization of Lectinlike Surface Components on *Capnocytophaga ochracea* ATCC 33596 That Mediate Coaggregation with Gram-Positive Oral Bacteria

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The interactions between *Capnocytophaga ochracea* ATCC 33596 and *Streptococcus sanguis* H1, *Actinomyces naeslundii* PK984, or *Actinomyces israelii* PK16 are dependent on specific recognitions between heat-sensitive adhesins on *C. ochracea* and heat-stable structures (probably carbohydrate-containing receptors) on the surfaces of these gram-positive coaggregation partners. The coaggregation of *C. ochracea* with each of these three organisms was inhibited by L-rhamnose and D-fucose and to a lesser extent by β-methyl-galactoside. The reaction with *S. sanguis* was the most sensitive, while the coaggregation with *A. israelii* was the least sensitive and was only partially inhibited by each of the sugars that were considered to be effective inhibitors. A more effective inhibition of the coaggregation between *C. ochracea* and *A. israelii* was achieved by adding a combination of the 6-deoxysugars and *N*-acetylneuraminic acid. To further characterize the coaggregations, naturally occurring coaggregation-defective (Cog^-) mutants of *C. ochracea* were obtained from several different selections. Three phenotypically distinct groups of mutants were isolated. Type 1 mutants failed to coaggregate with *S. sanguis* only. Type 2 mutants lost ability to interact with both *S. sanguis* and *A. naeslundii*. Type 3 mutants failed to coaggregate with all three coaggregation partners. Characterization of the Cog^- mutants by sugar inhibition studies made it possible to distinguish three classes of adhesin activity.

Many recent studies have confirmed the original observation of Gibbons and Nygaard (2) that oral bacteria from different genera and species participate in coaggregation reactions (1, 4, 6–14). Of the three species that comprise the genus *Capnocytophaga*, *Capnocytophaga ochracea* ATCC 33596 exhibits the most diversified coaggregation profile. This gram-negative gliding bacterium can coaggregate with representatives of five species of oral gram-positive bacteria (6), including *Streptococcus sanguis*, *Actinomyces viscosus*, *Actinomyces naeslundii*, *Actinomyces israelii*, and *Rothia dentocariosa*. Preliminary characterizations of these interactions demonstrated that, in all instances, *C. ochracea* was the protease- and heat-sensitive partner; the gram-positive bacteria were resistant to both treatments (6). Further testing revealed that coaggregation between *C. ochracea* and *S. sanguis* was inhibited by the disaccharide lactose.

In the present study, a series of 36 sugars and sugar derivatives were tested to determine whether the inhibition of the *C. ochracea-S. sanguis* interaction was specific for lactose and whether the coaggregations between the gram-negative glider and representatives of two other species, *A. naeslundii* and *A. israelii*, were sensitive to sugars other than lactose. A series of coaggregation-defective (Cog^-) mutants were used to distinguish differences among several adhesins. This information serves as a useful first step in differentiating adhesins on the surface of *C. ochracea* and provides some insight into the nature of the receptors on the gram-positive partners.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All bacterial strains used in this study have been described elsewhere (6). Cells were grown at 37°C under an atmosphere of N_2-H_2-CO_2 (80:10:10; GasPak Anaerobic System; BBL Microbiology Systems, Cockeysville, Md.) in commercial Schaedler broth (BBL), harvested, washed with coaggregation buffer (0.001 M Tris, 10^{-4} M $CaCl_2$, 10^{-4} M $MgCl_2$, 0.15 M NaCl, 0.02% NaN_3 [adjusted to pH 8.0 with HCl]), and stored at −20°C until used.

Isolation of Cog^- mutants of *C. ochracea*. Spontaneously occurring Cog^- mutants of *C. ochracea* ATCC 33596 were isolated by using the selection procedure of Kolenbrander (5). The parent strain was made resistant to gentamicin and rifamycin at concentrations of 500 and 25 μg/ml, respectively. Freshly harvested cells were suspended in coaggregation buffer without azide and used immediately. To avoid loss of viability, the selection procedure was completed within 3 h. Suspected Cog^- mutants were tested for resistance to both antibiotics to verify their origin from the parent strain.

Visual coaggregation assay. Coaggregation of the Cog^- mutants was determined by the visual assay of Cisar et al. (1). All cell suspensions were adjusted to an optical density of 260 Klett units (5×10^9 cells per ml) with a Klett-Summerson colorimeter containing a 660-nm (red) filter (Klett-Summerson Manufacturing Co., New York, N.Y.), and 100 μl each of the Cog^- mutant suspension and a coaggregation partner suspension were vortexed for 10 s. A visual rating scale of 0 through 4 was used to grade the reactions. The visual coaggregation assay was also used for the preliminary screening of potential sugar inhibitors.

Spectrophotometric assay for coaggregation. The modification of Kagermeier et al. (3) of a technique originally described by McIntire et al. (12) was used to quantitate the extent of coaggregation of *C. ochracea* or its Cog^- mutants and each of the coaggregation partners (*S. sanguis* H1, *A.*

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TABLE 1. Coaggregation reactions of *C. ochracea* and its Cog⁻ mutants with gram-positive oral bacteria[a]

Strain[b]	Selection partner strain	Mutant type[c]	Coaggregation score with:		
			S. sanguis H1	*A. naeslundii* PK984	*A. israelii* PK16
Cog⁻ mutant					
PK1860	H1	1 (4)	0	3	3
PK1861	H1	3 (3)	0	0	0
PK1865	PK984	2 (1)	0	0	3
PK1866	PK984	3 (2)	0	0	0
PK1862	PK16	3 (2)	0	0	0
PK1863	PK16	3 (3)	0	0	0
C. ochracea (parent)			4	4	4

[a] Coaggregation scores were estimated by visual assay as described in Materials and Methods. Four rounds of selection were done with coaggregation partners *S. sanguis* H1, *A. naeslundii* PK984, or *A. israelii* PK16.
[b] Strains listed are selected phenotypic examples from each set of mutants.
[c] Values in parentheses represent the number of mutants isolated from that particular round of selection.

naeslundii PK984, or *A. israelii* PK16). The same assay was used to quantitate the inhibitory effect of the various sugars. Coaggregation buffer (100 μl) was added to test tubes containing 200 μl of *C. ochracea* cell suspension (either the parent strain or its Cog⁻ derivatives), and the tubes were incubated at room temperature for 30 min. When additives were used, they were prepared in the coaggregation buffer and adjusted to a pH of 7.4. A 200-μl volume of the respective coaggregation partner was added, vortexed vigorously for 10 s, and allowed to stand at room temperature for 10 min before being centrifuged at 600 × *g* for 1 min. Supernatants were carefully removed, and the A_{600} was determined with a recording spectrophotometer (2400S; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Controls consisted of suspensions of *C. ochracea* cells, suspensions of the respective coaggregation partner, or a mixture of the type 3 mutant and the relevant gram-positive partner. Percent coaggregation was calculated as follows: % coaggregation = $\{[(A_{600}$ control) − $(A_{600}$ experimental)] × 100$\}/A_{600}$ control. Calculations by this method showed that 80 to 85% of the added cells participated in the coaggregations involving the parent and the various partners, while coaggregations between the respective Cog⁻ mutants and their reactive partners as described below yielded values of 49 to 54% participation of the total amount of added cells. The inhibitory effect was expressed as the percent decrease in coaggregation relative to the coaggregation in the absence of inhibitor (normalized to 100% coaggregation). All experimental points were determined in triplicate and are expressed as the average of the three values. The 50% inhibition value was estimated from the inhibition curves.

Screening of inhibitors of coaggregation. The visual assay was used to examine the potential inhibition of coaggregation by the following carbohydrates at a final concentration of 100 mM: adonitol, allose, galactose, 2-deoxygalactose, galactosamine, galacturonic acid, glucose, glucosamine, glucuronic acid, 6-deoxyglucose, D-fucose, L-fucose, lactose, lactulose, maltose, D-mannose, L-mannose, melezitose, melibiose, L-rhamnose, ribose, 2-deoxyribose, sucrose, talose, xylitol, xylose, α-methyl-galactoside, β-methyl-galactoside, *N*-acetylgalactosamine, *N*-acetylglucosamine, *N*-acetylmannosamine, *N*-acetylneuraminic acid (NeuAc), *N*-acetyllactosamine, *N*,*N*′-diacetylchitobiose, *N*-acetylneuramin-lactose, and 3-*o*-β-D-galactopyranoside-arabinose. All sugars were purchased from Sigma Chemical Co., St. Louis, Mo., except for 2-deoxygalactose, which was obtained from Pfanstiehl Laboratories, Inc., Waukegan, Ill. Solutions of amino sugars, N-acetylated amino sugars, and sugar acids were adjusted to a pH of 7.4 with either 1 N HCl or 1 N NaOH.

RESULTS

Properties of Cog⁻ mutants of *C. ochracea*. Fifteen naturally occurring Cog⁻ mutants of *C. ochracea* were obtained from four rounds of selection (Table 1). The use of *S. sanguis* H1 as the coaggregation partner in the first round of selection resulted in the isolation of two phenotypically distinct types of mutants. Type 1 mutants (four mutants represented by PK1860) failed to coaggregate with *S. sanguis* H1, but they maintained the coaggregation properties of the parent strain with *A. naeslundii* PK984 and with *A. israelii* PK16. Type 3 mutants (three mutants represented by PK1861) failed to coaggregate with any of the three gram-positive coaggregation partners. In the second round of selection, *A. naeslundii* PK984 was used as the partner to obtain Cog⁻ isolates. Two types of mutants were obtained from this selection. The Cog⁻ type 2 mutant (one mutant PK1865) failed to coaggregate with *S. sanguis* H1 and with *A. naeslundii* PK984 but coaggregated with *A. israelii* PK16, and Cog⁻ type 3 mutants (two mutants represented by PK1866) failed to coaggregate with any of the three coaggregation partners. Two additional selections performed in the presence of *A. israelii* PK16 yielded type 3 mutants only (five mutants represented by PK1862 and PK1863). No other types of mutants were isolated during any of the four rounds of selection. The relative strengths of the coaggregations of parent or mutant with the three partners are summarized in Table 1.

Inhibition of coaggregation by simple sugars. Of 36 sugars tested for inhibition of coaggregation between *C. ochracea* and *S. sanguis* H1, the hierarchy of inhibition was as follows: L-rhamnose or D-fucose > β-methyl-D-galactoside > D-galactose or lactose > α-methyl-D-galactoside (Table 2). Only 3 of 36 sugars gave 50% inhibition in *C. ochracea-A. naeslundii* PK984 or *C. ochracea-A. israelii* PK16 coaggregations (Table 2). L-Rhamnose was the most potent inhibitor of the interactions of *C. ochracea* with all three coaggregation partners. The relative potency of the other inhibitory sugars as compared with rhamnose (on a molar concentration basis) was similar in each of the three different

TABLE 2. Inhibition of coaggregation between *C. ochracea* and its coaggregation partners by different sugars

Sugar	50% Inhibitory sugar concn (mM) with coaggregation partner[a]:		
	S. sanguis H1	*A. naeslundii* PK984	*A. israelii* PK16
L-Rhamnose	1 (1)	8 (1)	18 (1)
D-Fucose	1 (1)	10 (0.8)	18 (1)
β-Methyl-galactose	7 (0.14)	50 (0.16)	80 (0.22)
D-Galactose	15 (0.07)	—[b]	ND
Lactose	16 (0.06)	—	—
α-Methyl-galactose	18 (0.05)	—	—

[a] Concentration values were calculated from inhibition curves and are rounded to the nearest whole number. Numbers in parentheses indicate the inhibition potency relative to L-rhamnose as a standard of reference on a molar basis. The relative potency values were calculated separately for each coaggregation partner. ND, Not done.
[b] At a concentration of 80 mM, the inhibition value calculated was <50%.

coaggregation interactions (Table 2, footnote *a*). For example, in the coaggregation between *C. ochracea* and *S. sanguis* H1, inhibition by β-methyl-galactoside and lactose was 0.14 and 0.06 as effective, respectively, as that by L-rhamnose. The efficacy of the rhamnose inhibition of the coaggregation between the parent and each of the three partners is depicted in Fig. 1. Coaggregation with *S. sanguis* H1 as a partner was clearly the most sensitive to the 6-deoxysugar, while that with *A. israelii* PK16 was the least sensitive, with a maximal inhibition of 75%.

Inhibition of coaggregation by combinations of two or more sugars. Testing combinations of rhamnose, fucose, or β-methyl-galactoside in pairs or triads in coaggregations with the three partners yielded inhibition values that were equal to the sum of the individual inhibitors; no synergistic effect was observed (data not shown). In a previous study (3) the coaggregation of a related microorganism, *Capnocytophaga gingivalis* DR2001, with *A. israelii* PK16 was inhibited by N-acetylated amino sugars. Since the interaction between *C. ochracea* and *A. israelii* PK16 was not inhibited completely by L-rhamnose (75% inhibition at 80 mM L-rhamnose concentration) (Fig. 1), we tested the combined effect of rhamnose with different N-acetylated amino sugars. *N*-Acetylgalactosamine, *N*-acetylglucosamine, *N*-acetylmannosamine, *N*-acetyllactosamine, NeuAc, and *N,N'*-diacetylchitobiose were screened at concentrations of 100 mM in the presence of 5 mM L-rhamnose by the visual assay. Only *N*-acetylgalactosamine and NeuAc acted synergistically with L-rhamnose to inhibit the coaggregation of *C. ochracea* with *A. israelii* PK16, with NeuAc being more effective than *N*-acetylgalactosamine (data not shown). A quantitative analysis of the combined effect of L-rhamnose and NeuAc was conducted by preincubating *C. ochracea* cells with 5 mM L-rhamnose (Fig. 2), to which increasing concentrations of NeuAc were added. A synergistic effect was observed at each of the concentrations of NeuAc tested. For example, at a concentration of 40 mM NeuAc alone, 10% inhibition was observed, whereas this same concentration in combination with 5 mM L-rhamnose produced 62% inhibition.

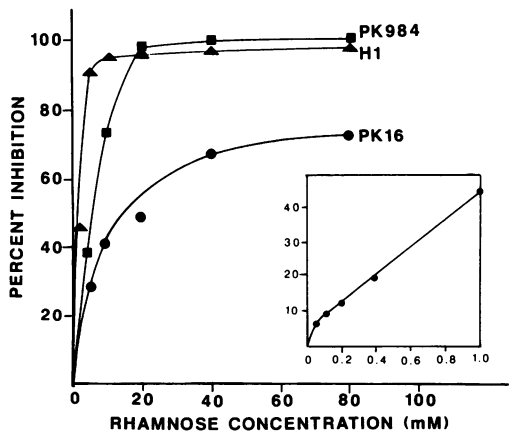

FIG. 1. Inhibition of coaggregation between *C. ochracea* and its gram-positive coaggregation partners by L-rhamnose. Symbols: ▲, *C. ochracea-S. sanguis* H1; ■, *C. ochracea-A. naeslundii* PK984; ●, *C. ochracea-A. israelii* PK16. Each point represents the average value of triplicate samples. (Inset) Inhibition of coaggregation with *S. sanguis* H1 at concentrations of between 0.005 and 1 mM. The extent of coaggregation in the absence of inhibitors was 80, 83, and 82% for *C. ochracea-S. sanguis* H1, *C. ochracea-A. naeslundii* PK984, and *C. ochracea-A. israelii* PK16 interactions, respectively.

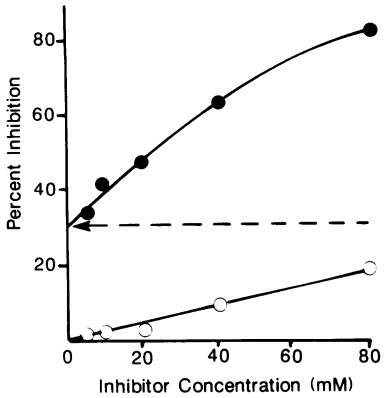

FIG. 2. Synergistic effect of L-rhamnose and NeuAc on the inhibition of coaggregation between *C. ochracea* and *A. israelii* PK16. – – –, 30% Inhibition by 5 mM L-rhamnose, which was held constant. Symbols: ●, 5 mM L-rhamnose and increasing concentrations of NeuAc; ○, NeuAc. Each point represents the average value of triplicate samples. The extent of coaggregation in the absence of inhibitors was 80%.

Effect of sugars on the coaggregation of Cog⁻ mutants of *C. ochracea* with *A. naeslundii* PK984 and *A. israelii* PK16. The inhibitory effect of rhamnose on Cog⁻ type 1 mutant-*A. naeslundii* PK984 (Fig. 3A) and Cog⁻ type 1 mutant-*A. israelii* PK16 interactions (Fig. 3B) was similar to the effect of rhamnose on the parent strain coaggregation with these two coaggregation partners (Fig. 1). Moreover, the effect of NeuAc on the Cog⁻ type 1 mutant-*A. israelii* PK16 interaction (Fig. 3B) was the same as its effect on the parent-*A. israelii* PK16 interaction (Fig. 2). Little or no effect on the Cog⁻ type 1 mutant-*A. naeslundii* PK984 interaction was observed with NeuAc alone (Fig. 3A), a finding which was in agreement with the visual examination of the parent-*A. naeslundii* PK984 interaction in the presence of 100 mM NeuAc (data not shown).

In contrast, L-rhamnose had almost no effect on the Cog⁻ type 2 mutant-*A. israelii* PK16 coaggregation, whereas NeuAc had a pronounced effect on this interaction (Fig. 3C). Nearly total inhibition (90%) was observed with 80 mM NeuAc. A comparison of the NeuAc inhibition curve for the parent strain-*A. israelii* PK16 (Fig. 2) with that for the Cog⁻ type 2 mutant-*A. israelii* PK16 (Fig. 3C) demonstrated that the modification or loss of the rhamnose-sensitive sites had two related effects. Not only was the extent of inhibition measurably increased, but the efficacy of the NeuAc inhibition, as evidenced by the change in the slope of the latter curve (*m* value), was significantly greater (1.9 compared to 1.35).

DISCUSSION

The sugar inhibition studies suggested that the coaggregations between *C. ochracea* ATCC 33596 and *S. sanguis* H1, *A. naeslundii* PK984, or *A. israelii* PK16 are probably mediated by a lectinlike surface component(s) on the gram-negative organism. Among the monosaccharides tested, L-rhamnose, D-fucose, and to a lesser extent β-methyl-galactoside were the most effective inhibitors of all three interactions. The *C. ochracea-S. sanguis* coaggregation was the most sensitive to the 6-deoxysugars, rhamnose and fucose; an inhibition of 50% was observed at a concentration

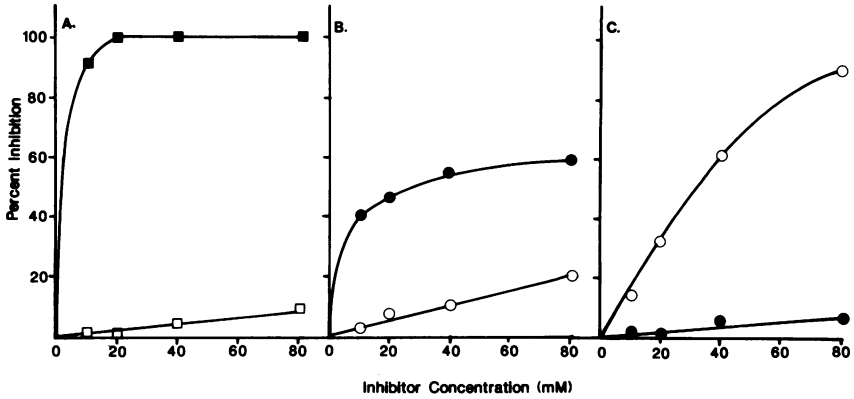

FIG. 3. Inhibition of coaggregation between Cog⁻ type 1 (strain PK1860) and *A. naeslundii* PK984 (A) or *A. israelii* PK16 (B) and between Cog⁻ type 2 (strain PK1865) and *A. israelii* PK16 (C) by L-rhamnose (■ and ●) or NeuAc (□ and ○). Maximal values for coaggregations were as follows: *C. ochracea* PK1860-*A. naeslundii* PK984, 50%; *C. ochracea* PK1860-*A. israelii* PK16, 52%; and *C. ochracea* PK1865-*A. israelii* PK16, 54%. Each point represents an average value of triplicate samples.

of 1 mM. The *C. ochracea-A. naeslundii* interaction was 8- to 10-fold less sensitive to the same sugars, whereas the inhibition of the coaggregation with *A. israelii* was even less sensitive and did not exceed 75% inhibition when a high concentration of rhamnose (80 mM) was used. Kagermeier et al. (3) have shown that the coaggregation of *C. gingivalis* DR2001 with *A. israelii* PK16 was inhibited by N-acetylated amino sugars. We found that the same compounds were only slightly inhibitory when tested in the *C. ochracea-A. israelii* system. However, the combination of rhamnose and NeuAc in the *C. ochracea-A. israelii* interaction acted synergistically, increasing the efficacy of NeuAc as an inhibitor. Other N-acetylated sugars were less effective than NeuAc in the presence of rhamnose. Thus, at least two structurally distinct monosaccharides, a 6-deoxysugar and an N-acetylated amino sugar, appear to play a role in inhibiting the various *C. ochracea* coaggregations.

A study of Cog⁻ mutants derived from *C. ochracea* underscored the specificity and differences of the recognition sites on the adhesin(s). The 15 naturally occurring Cog⁻ mutants isolated from four rounds of selection fell into one of the following three phenotypic groups. Type 1 mutants failed to coaggregate with *S. sanguis*, type 2 mutants lost the ability to interact with both *S. sanguis* and *A. naeslundii*, and type 3 mutants were negative with the three partners. The partner used to select the various mutants appeared to dictate the type of mutant isolated. For example, selection with *S. sanguis* H1 produced type 1 and type 3 mutants, and selection with *A. naeslundii* yielded type 2 and type 3 mutants, while two separate rounds of selection with *A. israelii* produced only type 3 mutants. However, the following Cog⁻ mutant phenotypes were not isolated during the four rounds of selection: (i) positive with *S. sanguis* and *A. naeslundii* but negative with *A. israelii*, (ii) positive with *S. sanguis* and *A. israelii* but negative with *A. naeslundii*, (iii) positive with *S. sanguis* and negative with both *A. naeslundii* and *A. israelii*, and (iv) positive with *A. naeslundii* but negative with *A. israelii* and *S. sanguis*. The observations that only three of the seven possible types of mutants were isolated, that the preponderance of isolates were type 3 mutants, and that the dependence of the type of mutant obtained upon the partner used in the selection all suggest that the natural mutations affecting the adhesin(s) synthesis may be ordered events.

A diagrammatic representation of a functional model is depicted in which the adhesins on the surface of *C. ochracea* bear several L-rhamnose- and NeuAc-sensitive sites (for simplicity, one NeuAc and two L-rhamnose sites are shown; Fig. 4). According to this model, *S. sanguis* interacts with the terminal L-rhamnose-sensitive site, whereas *A. naeslundii* interacts with both of the L-rhamnose-sensitive sites, which may explain why a higher concentration of this sugar is required to inhibit the latter interaction. The loss of the terminal L-rhamnose-sensitive site, because of a mutational event (Cog⁻ type 1 mutant), results in the failure of *C. ochracea* to interact with *S. sanguis* only.

Since the terminal L-rhamnose-sensitive site participates in the coaggregation with the two other partners, i.e., *A. naeslundii* and *A. israelii*, its loss may affect these interactions by decreasing the extent of coaggregation in the absence of inhibitor from roughly 80% when the parent is used to 50% with the Cog⁻ mutants. This observation is confirmed by the visual coaggregation assay, in which the interactions of the mutants with the gram-positive partners exhibited reduced scores, decreasing from a +4 with the parent to a +3 with the type 1 and type 2 mutants (Table 1). In losing both L-rhamnose-sensitive sites, the type 2 mutant could coaggregate only with *A. israelii*, and again a reduced extent of coaggregation was observed. This interaction be-

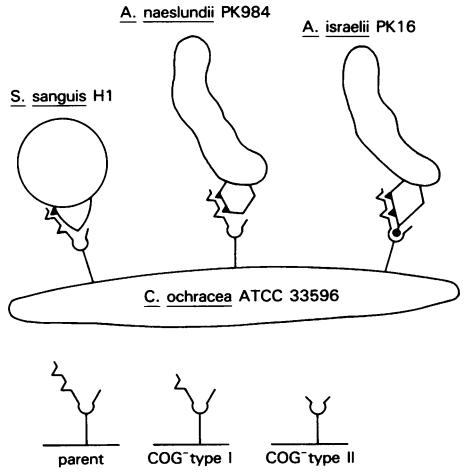

FIG. 4. Diagrammatic representation of the three proposed *C. ochracea* adhesin sites. Adhesin sites for Cog⁻ mutant types 1 (I) and 2 (II) are indicated. Symbols: ▲, L-rhamnose-containing compound; ●, NeuAc-containing compound.

came NeuAc sensitive, in contrast to the parent strain-*A. israelii* or the Cog$^-$ type 1 mutant-*A. israelii* coaggregations, in which NeuAc and L-rhamnose acted synergistically to inhibit the process.

It is unclear whether all adhesins are present on a single structure on the outer surface of the gram-negative microorganism or are part of several protein complexes, since no attempt was made to isolate or locate the *C. ochracea* adhesins. Interpreting our model in a structural fashion can aid in visualizing the sequential loss of adhesin activities in the different types of Cog$^-$ mutants. Thus, the loss of the distal L-rhamnose-sensitive site produced the type 1 mutant, the loss of both L-rhamnose-sensitive sites gave rise to the type 2 mutant, and the loss of all three adhesins resulted in the type 3 mutant. The single-structure hypothesis, as depicted in the model, also accounts for the failure to isolate mutants other than the types described above and explains why the type of mutant obtained from each selection depended on the coaggregation partner used. For example, using *A. naeslundii* as the coaggregation partner in the mutant selection should result in the isolation of type 2 and type 3 mutants. It follows that using *A. israelii* as a selection partner should yield only type 3 mutants. The results of these two mutant selections are in complete accord with the model described here. However, it is not yet clear why only type 1 and type 3 mutants were isolated when *S. sanguis* was used as a selection partner; this selection should have yielded all three types of mutants. Perhaps, if a larger number of mutants had been screened, type 2 mutants would have been found. Overall, the proposed functional model appears to be the simplest one that is concordant with the results reported in this study. A primary goal of future studies will be to identify and isolate the surface proteins of *C. ochracea* that mediate the coaggregation with the three gram-positive partners and to determine whether they exist as a single protein complex.

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