

Coaggregation of Oral *Bacteroides* Species with Other Bacteria: Central Role in Coaggregation Bridges and Competitions

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Seventy-three freshly isolated oral strains representing 10 *Bacteroides* spp. were tested for their ability to coaggregate with other oral gram-negative and gram-positive bacteria. None coaggregated with any of the gram-negative strains tested, which included *Capnocytophaga gingivalis*, *C. ochracea*, *C. sputigena*, and *Actinobacillus actinomycetemcomitans*. Strains of *Bacteroides buccae*, *B. melaninogenicus*, *B. oralis*, and *B. gingivalis* failed to coaggregate with any of the gram-positive strains tested. However, six *Bacteroides* spp. coaggregated with one or more species of gram-positive bacteria. Most isolates of *B. buccalis*, *B. denticola*, *B. intermedius*, *B. loescheii*, *B. oris*, and *B. veroralis* coaggregated with strains of *Actinomyces israelii*, *A. viscosus*, *A. naeslundii*, *A. odontolyticus*, *Rothia dentocariosa*, or *Streptococcus sanguis*. The strongest coaggregations involved *B. denticola*, *B. loescheii*, or *B. oris*; 22 of 25 strains coaggregated with *A. israelii*. Only *B. loescheii* interacted with certain strains of *S. sanguis*; these coaggregations were lactose inhibitable and were like coaggregations between *A. viscosus* and the same strains of *S. sanguis*. In fact, *B. loescheii* and *A. viscosus* were competitors for binding to *S. sanguis*. Many bacteroides also acted as coaggregation bridges by mediating coaggregations between two noncoaggregating cell types (e.g., *S. sanguis* and *A. israelii*). Evidence for binding-site competition and coaggregation bridging involving noncoaggregating cell types from three different genera provides support for the hypothesis that these intergeneric cell-to-cell interactions have an active role in bacterial colonization of the oral cavity.

The oral bacteroides have been recognized as prominent members of maturing dental plaque for at least 20 years (4, 18, 30, 33). Although samples taken from the surface of a freshly cleaned tooth contain a low proportion (often zero) of bacteroides, samples removed from diseased sites of patients with moderate periodontitis and severe generalized periodontitis contain many *Bacteroides* spp. (24, 26). Some species, such as *Bacteroides intermedius* 4197 and *B. gingivalis* may be potential etiological agents of severe periodontitis (26, 28), whereas others, like *B. loescheii* and *B. oralis*, occur in similar numbers in healthy persons and in patients with moderate periodontitis (24).

We became interested in this group of bacteria as part of our study of cell-to-cell recognition systems among oral bacteria. The systems studied to date include the actinomyces (*Actinomyces viscosus* and *A. naeslundii*) and streptococci (*Streptococcus sanguis*, *S. mitis*, and *S. morbillorum*), which exhibit an elaborate network of highly specific, nonrandom cell-to-cell interactions (3, 14-16). The specificity of these coaggregations is manifested by the ability of lactose to inhibit these interactions (22) and by the effect of heat or protease treatment of the partner strains, which gives unimodal or bimodal status to coaggregations (10). All (40 of 40 strains tested) of the *A. viscosus*, 50 of 64 *A. naeslundii*, and 57 of 117 *S. sanguis*, *S. mitis*, and *S. morbillorum* strains exhibit lactose-sensitive coaggregations.

Although widespread coaggregations occur among these actinomyces and streptococci, *A. israelii* does not participate in any of them (3; unpublished observations). Instead, it appears that several strains of *A. israelii* do coaggregate with certain gram-negative oral bacteria such as *Capnocytophaga* spp. (13, 20) and the *Bacteroides* spp. described here. *B.*

gingivalis (*B. melaninogenicus* subsp. *asaccharolyticus*), *B. intermedius* (*B. melaninogenicus* subsp. *intermedius*), and *B. melaninogenicus* (*B. melaninogenicus* subsp. *melaninogenicus*) (6-8) were reported to adhere to a variety of gram-positive bacteria, *A. viscosus*, *A. naeslundii*, *A. israelii*, *S. sanguis*, and *S. mitis* (29).

From an ecological viewpoint, cells with the ability to coaggregate with or to attach to plaque bacteria have a great advantage over noncoaggregating cells which would be removed from the oral environment by salivary flow. The potential significance of the already-recognized, highly specific coaggregations described above to the development of microbial communities increases as other groups of bacteria are examined for coaggregation ability. It is with this ecological model in mind that we examined the oral bacteroides and found that they also are specific in their choice of coaggregation partners.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains used in this study were from humans and were grown (except *B. gingivalis*) in complex broth medium consisting of brain heart infusion broth supplemented with yeast extract, vitamin K₁, cysteine, and hemin (5). The strains of *B. gingivalis* were grown in chopped meat-carbohydrate medium (Carr Scarborough Microbiologicals, Inc., Decatur, Ga.). The reagent strains used to test coaggregation properties of fresh bacteroides isolates were those characterized in previous studies and are listed in a footnote to Table 1. Reagent strains were also grown in the defined medium RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.).

The isolates examined here were obtained by procedures previously described (25, 26). All were from subgingival sites. Isolates were characterized and identified by morpho-

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TABLE 1. Coaggregation reactions between *Bacteroides* spp. and other oral bacteria

<i>Bacteroides</i> sp. strain	No. that coaggregate with:						No. of strains that coaggregate/no. of strains tested	
	<i>A. israelii</i> ^a	<i>A. odontolyticus</i> ^b	<i>A. naeslundii</i> ^c	Reagent actinomyces ^d	Reagent streptococci ^e	<i>R. dentocariosa</i> ^f		Gram-negative strains ^g
<i>B. loescheii</i>	8	5	0	0	8 ^h	0	0	8/8
<i>B. loescheii</i> (DIC-20 group)	5	2	0	4	0	3	0	5/5
<i>B. oris</i>	5	5	0	0	0	0	0	5/6
<i>B. denticola</i>	4	0	3	3	0	2	0	4/6
<i>B. intermedius</i>	2	0	2	2	0	2	0	2/4
<i>B. intermedius</i> (8944 group)	4	0	4	4	0	4	0	4/7
<i>B. veroralis</i>	1	0	1	1	1	1	0	2/5
<i>B. buccalis</i>	1	0	1	1	0	1	0	1/6
<i>B. buccae</i>	0	0	0	0	0	0	0	0/6
<i>B. gingivalis</i>	0	0	0	0	0	0	0	0/4
<i>B. melaninogenicus</i>	0	0	0	0	0	0	0	0/6
<i>B. melaninogenicus</i> (9343 group)	0	0	0	0	0	0	0	0/4
<i>B. oralis</i>	0	0	0	0	0	0	0	0/6

^a The strains of *A. israelii* used were ATCC 29322, ATCC 10048, PK16, W855, CROB 2030, ATCC 12103, and ATCC 23860.

^b *A. odontolyticus* ATCC 17982.

^c *A. naeslundii* CROB 2056.

^d The reagent actinomyces strains were *A. viscosus* T14V, *A. naeslundii* I, *A. naeslundii* PK947, *A. naeslundii* PK606, *A. naeslundii* PK984, and *Actinomyces* sp. strain WVa 963 (VPI D33C-25), which represent actinomyces coaggregation groups A through F, respectively.

^e The reagent streptococcus strains were *S. sanguis* DL1 (NCTC 7868), H1, 34, and J22, *S. morbillorum* PK509, and *S. sanguis* (VPI E1A-1A), which represent streptococcus coaggregation groups 1 through 6, respectively.

^f *R. dentocariosa* ATCC 17931.

^g The gram-negative strains were *C. ochracea*, *C. sputigena*, *C. gingivalis*, *C. gingivalis* (*Cytophaga* sp. strain DR2001), *C. gingivalis* (*Cytophaga* sp. strain DR2002), *C. gingivalis* (*Cytophaga* sp. strain DR2012), and *Actinobacillus actinomycetemcomitans* strain Y4 and N27.

^h Coaggregations were reversed by 0.06 M lactose (final concentration).

logical, biochemical, chromatographic, and electrophoretic methods already described in detail (5, 23).

Coaggregation assays. (i) **Visual assay.** Each of the bacteroides isolates was checked by a visual assay (3) for its ability to coaggregate with a battery of reagent strains as well as with the other bacteroides isolates. Reagent strains are those whose coaggregation properties have been thoroughly investigated by procedures outlined in earlier reports (3, 13–16). Each reagent strain represents a larger number of strains with identical coaggregation properties. The visual assay involves a scoring system of 0 (for no coaggregation) to 4 (for maximum coaggregation). A score of 4 is given when large coaggregates are formed immediately after dense cell suspensions (about 5×10^9 cells per ml) of the two partner strains are mixed. The coaggregates settle immediately to the bottom of the tube and leave a clear supernatant. Reversal or inhibition of coaggregation by lactose or EDTA was monitored by adding these compounds to a final concentration of 0.06 M and 0.6 mM, respectively. The effect of heat on the coaggregation properties of cells was determined by heating a cell suspension at 85°C for 30 min before mixing it with heated or unheated cells of a partner strain. Protease digestion of cells was done at 50°C for 60 min at a concentration of 1 mg of protease (no. 537088; Calbiochem-Behring, La Jolla, Calif.) per ml of cell suspension (5×10^9 cells per ml) in coaggregation buffer (0.001 M Tris adjusted to pH 8.0 and containing 10^{-4} M CaCl₂, 10^{-4} M MgCl₂, 0.15 M NaCl, and 0.02% NaN₃).

(ii) **Radioactivity assay.** Cells radioactively labeled with [³H]thymidine (New England Nuclear Corp., Boston, Mass.) were used to determine the ability of those cells to coaggregate with appropriate partner strains. The procedure is described in detail elsewhere (P. E. Kolenbrander and R. N. Andersen, manuscript in preparation), but briefly, coaggregates of radioactively labeled cells (specific radioactivity,

about 10^3 bacteria/cpm) and unlabeled partner strains were pelleted by low-speed centrifugation (1,000 rpm for 1 min) (Microfuge 12; Beckman Instruments, Inc., Palo Alto, Calif.). The amount of radioactivity contained in an aliquot of the supernatant (half the total volume) was determined by liquid scintillation counting after mixing the aliquot with a counting solution for aqueous samples (Hydrofluor; National Diagnostics, Somerville, N.J.). A polypropylene tube (capacity, 0.5 ml; Brinkmann Instruments, Inc., Westbury, N.Y.) containing only buffer and the radioactive cell type and centrifuged as above served as the control for no coaggregation. The control value indicated recoveries between 95 and 100% of the input radioactivity (determined by sampling directly from the radioactively labeled cell suspension into a scintillation vial). After the radioactivity lost by leaching (radioactivity remaining in the supernatant after high-speed centrifugation; 10,000 rpm for 5 min) was subtracted, the percent radioactivity that was part of the coaggregates was determined by the difference of 100% of input minus the radioactivity measured in the low-speed supernatant. The reproducibility of triplicate samples was $\pm 4\%$ of the average value.

RESULTS

Coaggregation properties of fresh isolates of *Bacteroides*. An average of six strains in each of 13 DNA-DNA homology groups of bacteroides (6, 7) was tested for ability to coaggregate with a variety of other oral bacteria as well as with each of the 73 bacteroides isolates (Table 1). The 26 strains of *B. buccae*, *B. gingivalis*, *B. oralis*, and the two homology groups of *B. melaninogenicus* did not coaggregate with any of the strains tested. Only 3 of 11 isolates of *B. buccalis* and *B. veroralis* exhibited coaggregation; of the remaining six groups, 50% or more of the isolates coaggregated. *A. israelii* seemed to be the preferred coaggregating partner, since with

one exception (strain VPI E8A-20 of *B. veroralis*) all of the *Bacteroides* spp. that coaggregated also coaggregated with several strains of *A. israelii*. None of the *Bacteroides* spp. coaggregated with other gram-negative strains tested or with other bacteroides. As was found in earlier studies with fresh isolates of other oral bacteria (13-16), the bacteroides also showed a high degree of specificity for coaggregation partners. With one exception (*B. veroralis* VPI E8A-20), only *B. loescheii* coaggregated with reagent streptococci. Even the closely related D1C-20 group of *B. loescheii* did not coaggregate with streptococci, but, along with *B. denticola* and the two groups of *B. intermedius*, this group coaggregated with certain reagent actinomyces. Another example of partner specificity is that *B. denticola* and the two groups of *B. intermedius* coaggregated with *A. naeslundii* CROB 2056 but not *A. odontolyticus* ATCC 17982, whereas *B. oris* and the two groups of *B. loescheii* exhibited the opposite coaggregation pattern. Finally, excepting the *B. loescheii* D1C-20 group, those bacteroides that coaggregated with *Rothia dentocariosa* ATCC 17931 also coaggregated with *A. naeslundii* CROB 2056 and reagent actinomyces. In contrast to the two homology groups of *B. intermedius*, which are identical in their coaggregation patterns, the two homology groups of *B. loescheii* are distinct. In fact, the coaggregations between reagent streptococci and *B. loescheii* were the only coaggregations that were lactose inhibitable (see below). Although a few coaggregates appeared to be inhibited by EDTA, most were not (data not shown).

Heat treatment and protease digestion of bacteroides and coaggregation partners. The partners in each coaggregating pair were heated at 85°C for 30 min to determine whether one (unimodal coaggregation) or both (bimodal coaggregation) (10) were heat inactivated. The heat-inactivated partner in all pairs was the *Bacteroides* sp., which indicated that all of these coaggregations were unimodal. To further characterize the surface structures that mediated coaggregation, each partner of several representative pairs was treated with protease (1 mg of protease per ml of cell suspension incubated at 50°C for 1 h). Heating alone at 50°C for 1 h had no effect, and protease treatment of partner strains had no effect, but incubation of the *Bacteroides* spp. with protease eliminated coaggregation activity.

***B. loescheii* VPI 12530 acting as a coaggregation bridge.** With the discovery that *B. loescheii* coaggregated with both *A. israelii* and *S. sanguis* cells, it became possible to test the potential for bacteroides to serve as a bridge between the other two cell types. Visual examination of the mixed cell suspension containing *S. sanguis* 34 and *A. israelii* ATCC 10048 revealed no coaggregation. Radioactively labeled *S.*

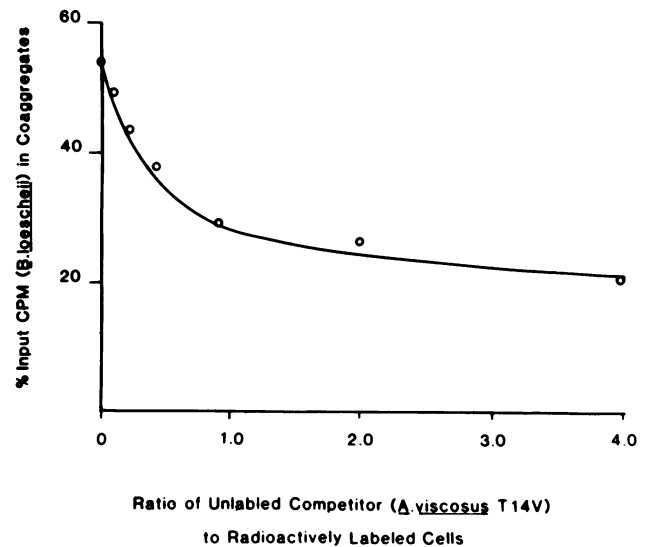


FIG. 1. Competition between unlabeled *A. viscosus* T14V and radioactively labeled *B. loescheii* VPI 12530 cells for coaggregation with *S. sanguis* 34. Competitor cell types were mixed before the addition of *S. sanguis* 34. Coaggregates were separated from free cells by low-speed centrifugation as described in the text.

sanguis 34 cells also failed to coaggregate with *A. israelii* ATCC 10048 (Table 2). However, when *B. loescheii* VPI 12530 cells were added to the mixed-cell suspension, 96% of the input radioactivity was found in the coaggregates. This was not due to simple pairing of *S. sanguis* and uncoaggregated *B. loescheii*, since *B. loescheii*-*A. israelii* coaggregates washed free of uncoaggregated cells also bound more than 90% of the input *S. sanguis* cells (data not shown). Conversely, when the *B. loescheii* suspension by itself was washed identically (i.e., by low-speed centrifugation, with the supernatant discarded to remove uncoaggregated cells [in this case the entire suspension]), no *S. sanguis* cells were bound (data not shown). Although *S. sanguis* had become part of a triad, the interaction between *S. sanguis* and *B. loescheii* remained lactose inhibitable (Table 2). This indicated that coaggregation between *B. loescheii* and *S. sanguis* operated independently and distinctly from the coaggregation between *B. loescheii* and *A. israelii*, which was not lactose inhibitable (53 versus 45% input cpm in coaggregates in the absence and presence of lactose, respectively).

Competition between *A. viscosus* T14V and *B. loescheii* VPI 12530 for binding to *S. sanguis* 34. The only lactose-inhibitable coaggregations between bacteroides and other oral bacteria were those involving *B. loescheii* and certain reagent streptococci (Table 1). The streptococci were either *S. sanguis* (coaggregation groups 3 and 4) or *S. morbillorum* (coaggregation group 5). In each pair, only the bacteroides were heat inactivated and protease sensitive. The similarity of the properties of these coaggregations to those of the *S. sanguis* 34-*A. viscosus* T14V coaggregating pair (22) prompted us to test the possibility that *A. viscosus* and *B. loescheii* are competitors for coaggregation with *S. sanguis*. Indeed, such competition was observed when radioactively labeled *B. loescheii* VPI 12530 cells were mixed with *S. sanguis* 34 in the presence of increasing numbers of *A. viscosus* T14V cells (Fig. 1). In the absence of competitor *A. viscosus* T14V cells, 54% of the input radioactivity was recovered in coaggregates. However, the amount of radioactivity in coaggregates rapidly decreased to only 20% of

TABLE 2. Coaggregation between *S. sanguis* 34 and *A. israelii* ATCC 10048 mediated by coaggregation bridge bacterium, *B. loescheii* VPI 12530

Cell type added			Other additions		% of input cpm in coaggregates
<i>A. israelii</i>	<i>B. loescheii</i>	<i>S. sanguis</i>	Buffer	Lactose	
+		+ ^a	+		0
+	+	+ ^a	+		96
+	+	+ ^a		+	6
+	+ ^a		+		53
+	+ ^a			+	45
	+ ^a	+	+		81
	+ ^a	+		+	0

^a Radioactively labeled cells added.

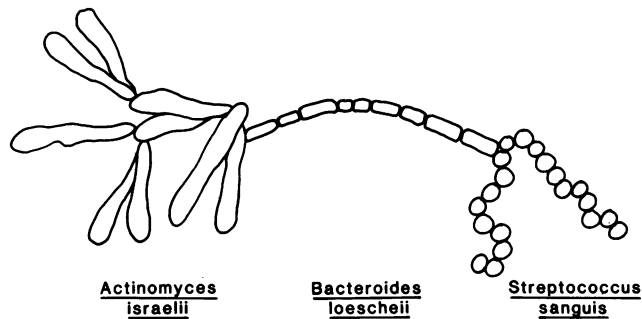


FIG. 2. Model depicting *B. loescheii* VPI 12530 acting as a coaggregation bridge between two noncoaggregating cell types, *A. israelii* ATCC 10048 and *S. sanguis* 34. Coaggregation between the bridge bacterium and *S. sanguis* is lactose reversible, but coaggregation with *A. israelii* is not.

input radioactivity in the presence of increasing competitor concentration.

DISCUSSION

As a group, the oral *Bacteroides* spp. are heterogeneous in their ability to coaggregate with other oral bacteria. In contrast, each DNA homology group exhibits a high degree of coaggregation partner specificity. *B. loescheii* is the only group that exhibits lactose-reversible coaggregation with *S. sanguis* (Table 1). It does not coaggregate with reagent actinomyces, but a closely related group (*B. loescheii* D1C-20 group) does. Numerous examples of such group specificity were found (Table 1), which suggests that DNA homology is correlated with specificity of surface structures that mediate highly organized coaggregations.

In the two previous surveys of gram-negative oral bacteria (11, 13) and in the current one, *A. israelii* was a common coaggregation partner. Unlike many of the coaggregations between *S. sanguis* and *A. viscosus* or *A. naeslundii* (3, 14-16), none of the coaggregations between the bacteroides and *A. israelii* were lactose-inhibitable. However, it was recently shown that the *C. gingivalis* (*Cytophaga* sp. strain DR2001)-*A. israelii* PK16 pair was inhibited by certain *N*-acetylated amino sugars, including *N*-acetylneuraminic acid, *N*-acetylgalactosamine, and *N*-acetylglucosamine (9). It would be of interest to test the coaggregations with the bacteroides reported here for inhibition by *N*-acetylneuraminic acid (sialic acid) in view of this and another recent report that sialic acid inhibits certain *S. sanguis*-*A. naeslundii* coaggregations (12). In fact, a sialic acid-sensitive lectin was found on *S. sanguis* (27) and the presence of sialic acid on certain *A. viscosus* strains was noted (A. H. Jones, R. Marroquin, and D. C. Birdsell, *J. Dent. Res.* 62A:657, 1983). Thus, sialic acid-sensitive coaggregations may be widespread among oral bacteria. Certainly, abundant sources of sialic acid residues are exposed in the oral cavity in the form of sialoglycoproteins and eucaryotic cell surface glycoproteins (17).

By virtue of an apparent preference for strains of *A. israelii* as coaggregation partners, the bacteroides have the potential for playing a central role in microbial colonization by acting as a coaggregation bridge. A coaggregation bridge is defined here as a bacterium that coaggregates with noncoaggregating partner strains that belong to different genera. Very few bacteria are known to coaggregate with both *A. israelii* and reagent actinomyces (*A. viscosus* and *A. naeslundii*) or reagent streptococci (*S. sanguis* and *S. morbillorum*).

However, almost all of the bacteroides that coaggregate exhibit such bridging coaggregations (Table 1). Besides mediating interactions between noncoaggregating partners, the bridge organism coaggregates with its two partners by nonidentical mechanisms (Fig. 2). In this model, the coaggregation between *B. loescheii* VPI 12530 and *S. sanguis* 34 is lactose reversible, whereas the coaggregation between the bridge organism and *A. israelii* ATCC 10048 is not. Without the bridge, *S. sanguis* 34 and *A. israelii* ATCC 10048 do not coaggregate (Table 2). This example is just one of many in the oral microflora: *B. intermedius* coaggregates with both *A. israelii* and *R. dentocariosa*; *B. oris* coaggregates with both *A. israelii* and *A. odontolyticus* (Table 1).

We propose that this kind of coaggregation bridging is of critical importance in the development of microbial plaque. Løe et al. (18) reported 20 years ago that the bacterial populations changed from primarily gram-positive bacteria to more complex populations containing gram-negative bacteria in plaque from volunteers participating in an experimental gingivitis study. Since then, workers in many laboratories have investigated this population change and found that streptococci (e.g., *S. sanguis* and *S. mitis*) and actinomyces (e.g., *A. viscosus* and *A. naeslundii*) were the predominant early colonizers on freshly cleaned tooth surfaces (1, 19, 21, 25, 31, 32). Only at later sampling times (several days later) did *Bacteroides* spp. and *A. israelii* constitute a substantial proportion of the cultivable flora. Bacteria like *S. sanguis* adhere to and colonize a cleaned tooth surface. Microcolonies are formed and provide a new surface that can be recognized by other bacteria such as *Bacteroides* spp., which in turn are potential attachment sites for a third kind of bacterium like *A. israelii*. Bacteria must adhere to a fixed surface to prevent washout from the oral cavity, because the dilution resulting from salivary secretions and swallowing would exceed the growth rate of oral bacteria (34). Substrates and environmental conditions (e.g., pH and redox potential) change and are in a dynamic state which may favor or discourage the growth of particular organisms. If during their favored period of growth they can adhere to developing plaque, then they have an additional advantage over nonadherent organisms.

Although coaggregation bridging occurs by different mechanisms of coaggregation between the bridge organism and its partners, another kind of multiple-cell interaction, namely competition, involves similar or perhaps identical mechanisms. We have studied only the lactose-reversible multiple-cell type competitions. When the lactose-reversible coaggregation between *S. sanguis* 34 and *B. loescheii* VPI 12530 was discovered, it was clear that there were certain similarities to the coaggregation between *S. sanguis* 34 and *A. viscosus* T14V (22). During the course of studying the streptococcal surface receptor for the lactose-reactive lectin on *A. viscosus* T14V, Cisar isolated a coaggregation-defective mutant of *S. sanguis* 34 that was unable to coaggregate with *A. viscosus* T14V (2). When its ability to coaggregate with *B. loescheii* VPI 12530 was tested by Kolenbrander and Andersen, no coaggregation was detectable (12). These results suggested that the two coaggregations were at least similar and could be competitive. When tested experimentally, T14V was an effective competitor of the *B. loescheii* VPI 12530-*S. sanguis* 34 pairing (Fig. 1). A model depicting such a competition is presented in Fig. 3. Although the complementary symbols are shown as identical, they are intended only to indicate functional identity, i.e., lactose-reversible coaggregations. Coaggregation-defective mutants of *A. viscosus* T14V (or *A. naeslundii* ATCC 12104) that do not

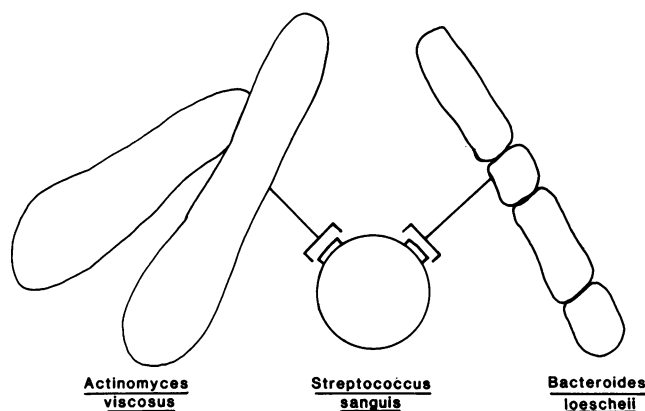


FIG. 3. Model depicting competition for binding sites on *S. sanguis* 34. These coaggregations are lactose reversible, and although the same symbols are used to represent interactions between different cell types, identity of structures is not intended. Rather, it is likely that these structures are just part of a larger network of functionally similar, lactose-reversible coaggregations among oral bacteria.

exhibit lactose-reversible coaggregation with *S. sanguis* 34 are not competitors (12; unpublished results). Furthermore, results from a recent study of lactose-reversible coaggregations among certain reagent streptococci indicated that they also were competitors against *B. loescheii* VPI 12530 (12).

Thus, *B. loescheii* VPI 12530 could be in a dynamic multigeneric association with many other oral bacteria that recognize certain oral streptococci by a mechanism that is sufficiently similar to be functionally competitive. Also, the bacteroides as a group have the potential to act as coaggregation bridges and recognize a variety of other oral bacteria.

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