Multigeneric Aggregations among Oral Bacteria: a Network of Independent Cell-to-Cell Interactions

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A radioactivity-based assay was developed to define the participation of radioactively labeled cell types within the milieu of unlabeled partners in multigeneric aggregates. The cell types in these multigeneric aggregates consisted of various combinations of 21 strains representing five genera of human oral bacteria. The coaggregation properties of each cell type, when paired individually with various strains, were delineated and were unchanged when the microbes took part in the more complex multigeneric aggregations. Competition between homologous labeled and unlabeled cells for binding to a partner cell type was achieved only when the homologous cells were mixed together before the addition of their partner cells. Attempts to displace a labeled cell type from an aggregate by subsequent addition of a large excess of the same unlabeled cell type were unsuccessful, which suggested that the forces that bound different cell types together were very strong and the cell-to-cell interactions were stable. However, a cell type that exhibited only lactose-reversible coaggregations with partners was easily and selectively released by the addition of lactose to multigeneric aggregates otherwise consisting solely of lactose-nonreversible cell-to-cell interactions. This not only indicates the independent nature of individual coaggregations but also suggests the involvement of lectinlike adhesins in these sugar-inhibitable coaggregations. Although the molecular mechanisms responsible for multigeneric aggregations are unknown, the principle of a common partner cell type serving as a bridge between two otherwise noncoaggregating cell types was firmly established by the observation of sequential addition of one cell type to another. Thus, competition, bridging, coaggregate stability, independent nature of interactions, and partner specificity are the key principles of adherence that form the framework for continued studies of multigeneric aggregates. While the human oral cavity is a prime example of a complex microbial community, collectively the community appears to consist of simple and testable individual interactions.

Intergeneric coaggregations among pairs of human oral bacteria have been investigated in several laboratories, and the results of these surveys all indicate that interactions between coaggregating partners are not random (7, 9, 15, 18, 24, 35, 38). On the contrary, specific coaggregating pairs were observed, and on the basis of this specificity, coaggregation groups of certain oral streptococci (Streptococcus sanguis and S. morbillorum) and actinomycyces (Actinomyces naeslundii and A. viscosus) were delineated (4, 20–22). Over 100 fresh isolates and stock culture strains of both streptococci and actinomycyces have been examined. The coaggregation patterns (a combination of partner specificity, reversibility of coaggregation by simple sugars and chelating agents, and inactivation of partners by prior treatment with heat or protease digestion) of about 95% of each of the two bacterial types are represented by six streptococcal coaggregation groups (groups 1 through 6) and six actinomycyces coaggregation groups (groups A through F) (20). Taken together, these properties suggest that coaggregations among oral bacteria are mediated by complementary surface structures on the partner cell types and that these structures are lectin-carbohydrate in nature as first proposed by McIntire et al. (29).

Other microbial ecosystems also exhibit both specificity of cell-to-cell recognition and similar inhibitions by simple sugars. Rhizobium trifolii adheres specifically to clover root hairs, and these interactions are prevented by 2-deoxyglucose (5) but not by related sugars. Adherence of some enterobacteria to erythrocytes (6) or epithelial cells (33) is mediated by a D-mannose-sensitive mechanism involving type 1 fimbriae, while other strains like the uropathogenic Escherichia coli adhere via P-fimbriae interacting with galactose-containing sugars on the animal cell (14, 23). Mycoplasma pneumoniae possesses a distinct tiplike organelle that adheres to a sialic acid-containing receptor on the eucaryotic host cell (1). L-Fucose inhibits the hemagglutinating property of Vibrio cholerae (11). With the exception of conjugation systems, reports of bacteria-bacteria interactions outside of the oral microflora are few and have not described the adherence properties of the participating cell types (2, 8, 10, 34).

All of the studies of the adherence properties of oral bacteria as well as the other microbial partnerships described above were conducted with pairs of cell types. Our aim here is to take advantage of the widespread intergeneric recognition systems (17) to investigate the relationship between multigeneric aggregations and the complex microbial mass found in samples of dental plaque (30–32). Factors such as (i) stability of coaggregating pairs, (ii) competition among cell types, (iii) bridging noncoaggregating cell types together by a common partner, and (iv) independent but cooperating coaggregations were examined in an effort to describe adherent microbial communities that may be active in a dynamic human oral ecosystem.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains were of human origin, and their identity and source are given in the legend to Fig. 2. The growth medium was modified Schaedler broth (3). Cells were harvested at late exponential
to early stationary phase of growth from either screw-cap tubes (16 by 125 mm) or bottles (100- to 500-ml capacity) incubated at 37°C under anaerobic atmosphere containing H₂, CO₂, and N₂ (10:10:80) by using GasPaks (BBL Microbiology Systems, Cockeysville, Md.).

Visual assay for coaggregation. Cells were washed by three cycles of centrifugation (10,000 × g for 10 min) and suspension of the pellet in coaggregation buffer, which consisted of the following dissolved in 0.001 M Tris adjusted to pH 8.0: CaCl₂ (10⁻⁴ M), MgCl₂ (10⁻⁴ M), Na₂SO₄ (0.025%), and NaCl (0.15 M). The final cell density was about 10⁵ cells per ml (Klett value of 260 with a 660-nm [red] filter in a Klett-Summerson colorimeter; Klett Manufacturing Co., Inc., New York, N.Y.). The number of cells was determined by two methods: (i) direct count with a Petroff-Hauser counting chamber and (ii) viable count by plating dilutions of freshly harvested cells suspended in growth medium. The numbers of cells obtained by the two methods were in general agreement where the direct count of total cells was the same to twofold higher than the viable count, since all of the bacteria studied here form some small clumps (actinomyces) or short chains (streptococci and bacteroides). Coaggregation of potential partners was monitored by mixing equal volumes (0.15 ml) of cell suspensions (4). The change from an evenly turbid cell suspension before mixing to formation of visible aggregates consisting of both cell types (coaggregates) occurred immediately during mixing. A coaggregation score ranging from 0 (no change in turbidity and no visible coaggregates) to +4 (maximum coaggregation where large coaggregates settled immediately leaving a water-clear supernatant) was given for each pair. A score of +3 indicated the formation of large settling coaggregates but a slightly turbid supernatant; a +2 score was given when definite coaggregates were visible but did not settle immediately. Pairs that exhibited weak coaggregations which appeared as finely dispersed aggregates in a turbid background (+1 score) were not included in this study.

Radioactivity labeling of bacterial cells. Either [³H]thymidine (80 Ci/mmol; New England Nuclear Corp., Boston, Mass.) or [¹⁴C]uracil (55 mCi/mmol; New England Nuclear Corp.) at 10 or 2 μCi, respectively, per ml of growth medium was used to label cells. After four to six cell doublings, cells were harvested and washed (as above) by centrifugation, suspended in coaggregation buffer, and stored at 4°C (specific radioactivity about 10³ bacteria per cpm). Radioactivity measurements were made by using a LS 6800 scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.) after pipetting a portion (not more than 400 μl) into a scintillation vial, adjusting the volume to 400 μl with coaggregation buffer if necessary, and adding 10 ml of counting solution for aqueous samples (Hydrofluor; National Diagnostics, Somerville, N.J.).

Radioactivity assay for coaggregation. (i) Cell preparation. If the labeled cells were stored at 4°C overnight or longer, they were washed twice with coaggregation buffer (10,000 rpm for 5 min) (Microfuge 12; Beckman Instruments). The amount of radioactivity remaining in the supernatant after washing the cells was less than 3% of the total and was probably due to a combination of leaching of the radiolabel and nonsedimented cells.

(ii) Assay protocol. The radioactive cell suspension was mixed with a suspension(s) of an unlabeled potential partner strain(s) in a polypropylene tube (capacity, 0.5 ml; Brinkmann Instruments, Inc., Westbury, N.Y.) that was coated with a solution of 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) and dried at 55°C before use. When lactose was tested, it was added before (inhibition) or after (reversal) the appropriate partner strain(s). The volume was adjusted to 200 μl with coaggregation buffer and the suspension was vortexed for 5 s. Coaggregates were pelleted by low-speed centrifugation (1,000 rpm for 1 min) in a Microfuge 12. A portion of 100 μl of supernatant was transferred to a scintillation vial, and the radioactivity in the sample was measured as described above. The remaining 100 μl and pellet in the tube were centrifuged at 10,000 rpm for 5 min to pellet all cells, free and coaggregated. A 50-μl portion of the supernatant was removed and counted as above to determine the amount of non-cell-associated radioactivity (presumed to be due to leaching). The non-cell-associated value was subtracted from the total counts per minute in the supernatant, and after adjusting for volume differences (multiplying by 2), the percentage of input counts per minute remaining in the supernatant was calculated. Since this reflected the noncoaggregated cells, this value was subtracted from 100% to yield the percentage of input counts per minute in coaggregates.

Being an indirect measure of the percent coaggregation of the labeled cell type, it was important to establish the validity of sampling the supernatant. First, the radioactivity in the coaggregates was measured directly by suspending the pellet, and this value was compared with that obtained by the indirect method (Fig. 1). The saturation curves shown here reflect the saturation and resultant coaggregation of a labeled cell type (Bacteroides loescheii PK1295) by the addition of increasing numbers of unlabeled partner strain (Actinomyces israelii PK14). Both methods yielded the same results which demonstrated the saturaibility of the system, whereby all of the labeled cell type could be found in the pellet (direct measure) or absent from the supernatant (indirect measure).
The second point to validate was the ability to recover 100% of input radioactivity when both pellets and supernatants were measured. In the above experiment (Fig. 1), the average recovery was 97%, and the range of values was 95 to 100%. This point was examined further by investigating several parameters testable by this assay. Four conditions of centrifugation were chosen (1,000 rpm for 1 min, 2,000 rpm for 1 min, 1,000 rpm for 2 min, and 2,000 rpm for 2 min). Two bacterial strains labeled with either $[^{3}H]$thymidine (Actinomyces serovar WVa 963 strain PK1259) or $[^{14}C]$uracil (A. naeslundii PK29) were tested with and without partner strains S. sanguis C104 and H1, respectively. The range of values of total recovery of input cpm with the $[^{3}H]$labeled cell type was 96 to 107% with an average of 103%. With the $[^{14}C]$labeled cell type, the range was 94 to 101% with an average of 97%. Since $\pm 3\%$ was within the experimental range of values (see below) and 100% of input radioactivity was recovered, measurements of percent coaggregation by the difference of cpm in supernatants (noncoaggregated cells) of controls and experimental coaggregation mixtures seemed validated and was used to obtain the results presented here. A tube containing only buffer and the radioactive cell type was centrifuged as above and served as the control (no coaggregation). The buffer control value of total noncoaggregated cells was about 90% of the input radioactivity (determined by sampling directly from the radioactively labeled cell suspension into a scintillation vial). In some experiments, where the size of the coaggregates was small, the conditions of centrifugation were 2,000 rpm for 2 min. In these circumstances, the buffer control value of noncoaggregated cells was about 80% of the input radioactivity.

(ii) Precision. To test assay repeatability, radioactively labeled ($[^{14}C]$uracil) A. naeslundii PK29 cells were mixed with unlabeled partner strain S. sanguis J22 in seven identical tubes and centrifuged at 1,000 rpm for 1 min. When equal portions of the seven supernatants were measured, the range

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of values was 1,948 to 2,154 cpm, the median value was 2,120 cpm, and the overall average of the seven values was 2,076 cpm, indicating an assay variability of ±5% of the average value.

RESULTS

Intergeneric coaggregation of bacterial pairs. The coaggregation reactions of the various strains (cell types) used in this study are given in Fig. 2. Many were negative, and reactions of this kind were used as controls in experiments designed to test competition or trapping of cells in multigeneric aggregates (see below). The others were strong coaggregations, where visually all of the cells were in large flocculent coaggregates, and the supernatant was clear (coaggregation score of +4). Some of these coaggregations were reversed by adding lactose (circled coaggregation scores), and this property was used to determine the independent nature of lactose-reversible and -nonreversible coaggregations (see below). Some strains recognized only one or a few of those listed which allowed the study of sequential addition or coaggregate accretion (see below). Based on the results of these pairwise coaggregations and the overall coaggregation patterns of the strains, experiments were designed to test potential kinds of interactions between pairs within a network of multigeneric aggregations.

Stability of coaggregation bonds. One of the factors that could affect interpretation of the properties of multigeneric aggregates is the strength of the adherence between cells in the aggregates. If the forces of interaction were weak, bound cells would readily be released by the addition of other partner cell types. This would lead to competition for attachment between the weakly attached cells and free cells of either the same or different cell types. With the exception of the addition of lactose to release cells that coaggregate by lactose-reversible mechanisms, no evidence of release of cells already attached was observed. Even so, we tested the stability of coaggregation under rigorous conditions designed for.

FIG. 3. Stability of coaggregation formation between S. sanguis C104 and B. loescheii PK1295. (A) Addition of increasing numbers of unlabeled S. sanguis C104 to a constant number of radioactively labeled B. loescheii PK1295 saturates the bacteroides resulting in 100% of the bacteroides found in coaggregates. Arrow indicates that all bacteroides are in coaggregates (i.e., excess streptococci) at a ratio of bacteroides to streptococci of 1:8. (B) Homologous competition by unlabeled S. sanguis C104 against labeled C104 for coaggregation with B. loescheii PK1295. Increasing numbers of unlabeled C104 were added to a constant number and ratio of labeled C104 to unlabeled PK1295 of 8:1. In the absence of unlabeled competitor, the percentage of input labeled C104 in coaggregates was 80% (C). (C) Addition of decreasing numbers of unlabeled B. loescheii PK1295 to a constant number of radioactively labeled S. sanguis C104 indicates an excess of streptococci at a ratio of 1:8 of bacteroides:streptococci (arrow). (D) Attempt to release labeled S. sanguis C104 from coaggregates formed with B. loescheii PK1295 by the addition of increasing numbers of unlabeled competitor C104. At a ratio of 8:1 of labeled C104 to unlabeled PK1295, 79% of the input C104 coaggregated (in agreement with 80% in B and 71% in C). Ten identical vials containing the coaggregates were centrifuged at 2,000 rpm for 2 min, and the coaggregates were washed three times with coaggregation buffer equal to the original volume. The amount of radioactivity in portions of the supernatant was determined (wash numbers 1 to 4) where wash number 1 represents the original supernatant. Increasing numbers of unlabeled competitor C104 were then added to the 10 vials to give 10 different ratios of bound-labeled C104 to unbound-unlabeled competitor C104. The background radioactivity (---) was determined from portions of the supernatants after centrifugation at 10,000 rpm for 5 min to pellet free cells as well as coaggregates. The total percent recovery of input radioactivity was 94%.
to detect the release of radiolabeled cells from preformed coaggregates in the presence of an excess of homologous unlabeled cells.

A coaggregating pair exhibiting complete saturation as defined by complete removal of one cell type (B. loescheii PK1295) by increasing numbers of the partner cell type (S. sanguis C104) was chosen (Fig. 3A). At a ratio of eight C104 cells to one PK1295 cell (arrow), it is clear that all of the bacteroids were coaggregated by streptococci. Another requirement of this test was that, in this example, the radioactively labeled streptococcus must be sensitive to competition by unlabeled homologous competitor cells (Fig. 3B). At a ratio of 1:4 of labeled to unlabeled cells, 90% of the amount of labeled cells coaggregated in comparison with the control value. At a ratio of 1:8 only 22% (about 75% competition) of the labeled cells was found in the coaggregates. Thus, the latter ratio should be sufficient to dislodge labeled streptococcal cells if preformed coaggregates were unstable. On the other hand, if intercellular binding forces were strong and resultant coaggregates were stable, then release of bound C104 should not occur even in the presence of excess homologous cells. To make certain that the labeled C104 cells behaved similarly to unlabeled C104 in the saturation of bacteroids cells with streptococci, increasing numbers of unlabeled PK1295 were added to a constant number of radioactive C104 cells (Fig. 3C). At a ratio of 1:2 (bacteroids to streptococci), maximum percent binding (96%) was observed. As the relative number of streptococcal cells increased (by decreasing the number of PK1295), the percentage of input cpm bound decreased indicating that at a 1:8 ratio, the bacteroids were saturated with streptococci (arrow). In fact, it is clear that saturation occurred at a ratio of 1:4 where the initial indication of a reduced percentage of input cpm in coaggregates was first evident (see also the first indication of saturation at a ratio of 1:4 in Fig. 3A).

Thus, the coaggregation between C104 and PK1295 was saturable and sensitive to homologous competition, which allowed us to test the stability of these coaggregates (Fig. 3D). A ratio of 1:8 unlabeled PK1295 to radioactive C104 was used to make certain that all of the bacteroids were coated with labeled streptococci. The coaggregates were washed three times with buffer to remove excess unbound C104 followed by the addition of increasing numbers of unlabeled C104 competitor cells. There was no measurable release of bound, labeled C104 during the three washes (the first wash is the original supernatant) as shown by the precipitous drop to background levels of radioactivity in the values obtained for the last two washes. Clearly evident is the fact that there was also no detectable displacement by unlabeled competitor cells of labeled C104 from the preformed coaggregates with partner PK1295. Thus, it appears that this coaggregating pair is stably formed with strong bonding. Although the other coaggregations studied here were not as thoroughly examined, we observed no evidence of weaker bonding in any of them.

**Multigeneric aggregation without trapping nonreactive cells.** Like the pairwise coaggregates, on visual examination, all of the multigeneric aggregates described here exhibited large flocculent masses of cells. On microscopic viewing, these flocs contained morphological representatives of all included cell types. In contrast to pairwise coaggregations, where, by definition, both cell types participated, it was not known whether multigeneric aggregates consisted of both active and passive participants or just the former. It was critical to establish that the radioactivity assay was not subject to misinterpretation due to random trapping by...
nonreactive, free cells into multigeneric aggregates (Table 1). Eight different actinomycoses and two streptococci were chosen to test this possibility; none of the 10 strains coaggregated with members of their respective genus. Seven of the actinomycoses coaggregated with both streptococcal strains, but *A. naeslundii* PK91 coaggregated only with *S. sanguis* H1. Thus, if radioactively labeled PK91 were found in the multicellular aggregates when J22 was mixed with the actinomycoses, then it would be likely that trapping free cells of PK91 had occurred. No trapping was detectable since no radioactivity was found in the aggregates when *S. sanguis* J22 was used. However, 72% of the PK91 cells were found in the aggregates when *S. sanguis* H1 was substituted for J22. Five of the remaining seven actinomycoses were individually labeled and tested, and in each case, the radioactivity was primarily located in the aggregates with either streptococcal strain. These results are depicted diagrammatically in Fig. 4 and illustrate the fact that nonreactive cells do not contribute to multicellular aggregates.

**Lactose-reversible multigeneric aggregates.** Since many of the pairwise coaggregations were reversed by adding lactose (60 mM, final concentration), it was of interest to determine if these intergeneric partnerships would remain lactose reversible when they form a multigeneric aggregate (Fig. 5). The unlikely possibility existed that different or additional kinds of adherent mechanisms might be expressed in complex cellular aggregates that were undetectable in simple partnerships. *S. sanguis* PK488, *Capnocytophaga ochracea* ATCC 33596, *A. naeslundii* PK29, *B. loescheii* PK1295, and *S. sanguis* J22 were used to test this possibility. Each of the latter three strains was radioactively labeled and monitored in the multigeneric aggregates with unlabeled partner cell types in the presence and absence of lactose (100 mM, final concentration). In the absence of lactose, 40, 51, and 93% of input radioactivity was found in the multigeneric aggregates for J22, PK1295, and PK29, respectively. In the presence of lactose, none of the radioactivity for any of the three strains was associated with aggregates. In fact, there were no visible aggregates. These results are shown as a proposed model in Fig. 5 where they depict the principle that complex cellular aggregates do not change the lactose reversibility of the individual constituent coaggregations. The reversal by lactose was not due to a nonspecific effect, since 100 mM sucrose had no inhibitory effect on the coaggregations in this aggregate.

**Independent nature of lactose-reversible and -nonreversible coaggregations in multigeneric aggregates.** While lactose-reversible coaggregations did not appear to be altered in the presence of other partnerships that were also sensitive to lactose, their fate in a complex network composed primarily of lactose-nonreversible coaggregations was unknown. The key to establishing the independent nature of individual partnerships in a larger cellular aggregate was the use of a strain like *S. sanguis* J22 that exhibited both lactose-reversible and -nonreversible coaggregations with different partners. Coaggregations between J22 and three of the four actinomycoses tested, *Rothia dentocariosa* PK44, *A. naeslundii* PK984, and *A. israelii* ATCC10048, were lactose nonreversible (Fig. 2). The other streptococcal strain used, *S. sanguis* H1, behaved identically to J22 with these three partners, and the lactose-nonreversible interactions among these five strains formed the core of the multigeneric aggregate. In addition to these coaggregations, J22 recognized *A. naeslundii* PK947 which in turn coaggregated with *S. sanguis* C104. Both of these interactions were lactose reversible. The latter two participants, as well as *R. dentocariosa* PK44, *S. sanguis* J22, and *A. naeslundii* PK984, were tested for selective release following the addition of lactose (Table 2). It is important to point out that only *S. sanguis* J22 exhibited both a lactose-reversible (with *A. naeslundii* PK947) and -nonreversible (with the other three actinomycoses) coaggregations. Accordingly, after the addition of lactose to the complex cellular aggregate, J22 remained part of the aggregate, but PK947 was released. Furthermore, C104 was released from the aggregate after adding lactose, but the other cell types were not. The independent nature of lactose-reversible and -nonreversible coaggregations is represented graphically in Fig. 6. Although...
S. sanguis J22 exhibited both kinds of coaggregations, it remained as part of the multicellular aggregate composed of other strains that interacted solely by lactose-nonreversible adherence. However, PK947 and C104 which interacted only by lactose-reversible adherence became unbound.

**Sequential coaggregation of oral bacteria.** On the basis of the pattern specificity exhibited by pairs of oral bacteria (Fig. 2), a sequence of coaggregation partners was designed so that any given strain was a partner only of the strain added to the mixture of cells before and after it. In the example investigated here (Table 3), S. sanguis 34 coaggregated only with A. naeslundii PK25, which coaggregated only with 34 and C. ochracea ATCC 33596, which coaggregated only with PK25 and A. israelii PK16, which coaggregated only with ATCC 33596 and C. gingivalis DR2001. Accretion of any radioactively labeled strain in multicellular aggregates required the inclusion of its preceding partner. A diagrammatic representation of these results is shown in Fig. 7, which illustrates the potential for aggregate formation by accretion of cells belonging to diverse genera.

**DISCUSSION**

Coaggregations among oral bacteria appear to be the norm rather than the exception. They involve specific partnerships, the characteristics of which have been outlined in several surveys of the coaggregation properties of stock culture strains (4) and fresh isolates (16, 18–22). These pairwise coaggregations provided the information on which the multigeneric aggregation experiments were designed. In fact, the multigeneric aggregates are predictable composite replicas of the known independent pairwise interactions.

The participation of a given cell type in the multigeneric aggregates was monitored here by using radioactively labeled cells. Two essential elements of the radioactivity-based assay were the demonstrations that the coaggregations were stable (Fig. 3) and that nonreactive cell types were not trapped in the large flocculent multigeneric aggregates during the gentle centrifugation (Table 1). Thus, the formation of a coaggregation pair involved a specific recognition between

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**TABLE 2. Independent nature of lactose-reversible and -nonreversible coaggregations in multigeneric aggregates**

<table>
<thead>
<tr>
<th>Radioactive strain*</th>
<th>% Input cpm in coaggregates Before lactose</th>
<th>After lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. dentocariosa PK44</td>
<td>95</td>
<td>96</td>
</tr>
<tr>
<td>S. sanguis J22</td>
<td>69</td>
<td>64</td>
</tr>
<tr>
<td>A. naeslundii PK984</td>
<td>93</td>
<td>85</td>
</tr>
<tr>
<td>S. sanguis C104</td>
<td>42</td>
<td>5</td>
</tr>
<tr>
<td>A. naeslundii PK947</td>
<td>86</td>
<td>6</td>
</tr>
</tbody>
</table>

* In addition to these radioactively labeled strains, unlabeled A. israelii ATCC 10048 and S. sanguis H1 were added. Each radioactive strain was tested with the other six unlabeled strains with and without the addition of lactose (100 mM, final concentration). The percentage of input cpm in coaggregates was determined as described in the text.

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**TABLE 3. Sequential coaggregation of oral bacteria to form a multigeneric aggregate**

<table>
<thead>
<tr>
<th>Initial organism*</th>
<th>Radioactive partner</th>
<th>% Input cpm in coaggregated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>PK25</td>
<td>91</td>
</tr>
<tr>
<td>34 + PK25</td>
<td>ATCC 33596</td>
<td>47</td>
</tr>
<tr>
<td>PK25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PK16</td>
<td>ATCC 33596 + PK16</td>
<td>70</td>
</tr>
<tr>
<td>PK16</td>
<td>ATCC 33596 + PK16</td>
<td>59</td>
</tr>
<tr>
<td>PK16</td>
<td>PK25 + ATCC 33596 + PK16</td>
<td>14</td>
</tr>
<tr>
<td>PK16</td>
<td>PK25 + ATCC 33596 + PK16</td>
<td>18</td>
</tr>
</tbody>
</table>

* Bacterial strains used were S. sanguis 34, A. naeslundii PK25, C. ochracea ATCC 33596, A. israelii PK16, and C. gingivalis DR2001. The latter four strains were radioactively labeled and tested individually as coaggregation partners with the other unlabeled strains in various combinations of strains. The requirement for the immediately preceding strain (bridge organism) for inclusion of a radioactive strain in the multigeneric aggregate is demonstrated (e.g., requirement for C. ochracea ATCC 33596 before A. israelii PK16 could coaggregate). The percentage of input cpm in coaggregated cells was determined as described in the text.

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**FIG. 6.** Diagrammatic representation of the independent nature of lactose-reversible and -nonreversible coaggregations. Illustrated here is the selective release of cells that coaggregate only by lactose-reversible mechanisms and the maintenance of aggregates consisting of lactose-nonreversible interactions following the addition of lactose to the multigeneric aggregate.
the two cell types which created a sufficiently strong bond that was not broken by simple mechanical procedures such aspipetting, vortexing, and centrifugation; nor was thisinteraction disrupted by the addition of a large excess ofunlabeled competitor cells (Fig. 3D), although mixing a largeexcess of homologous competitor cells before the addition ofthe partner strain did result in strong competition (Fig. 3B).The inability of unlabeled competitor cells to release homol-ogous labeled cells from preformed coaggregates indicates thatonce a cell participates in a coaggregation, it is likely toremain bound to its partner. This does not imply thatcoaggregation is irreversible or that coaggregates do notchange in size from large masses to smaller masses bymechanical stress, but rather that the cell-to-cell interactionequilibrium strongly favors coaggregation, and once it oc-curs, individual free cells are not likely to be released fromthe aggregates.

Release of free cells from multigeneric aggregates wasreadily shown, however, by the addition of a high concentra-tion (100 mM) of lactose but not sucrose. The fact thatlactose but not sucrose inhibited and reversed the lactosesensitive interactions supports the concept that only a fewsugars are responsible for the observed specificity of theinteractions. Such sugar specificity is in agreement with theextensive studies of McIntire and co-workers (26–28) whocompared the inhibition of streptococcal–actinomycetes coag-gregations by a variety of sugars. The specificity of coaggre-gation was further shown by the independent nature oflactose-reversible and -nonreversible interactions (Table 2and Fig. 5 and 6). Only those aggregates that were main-tained solely by lactose-reversible coaggregations were com-pletely disrupted by lactose (Fig. 5). But more importantly,in aggregates consisting primarily of lactose-nonreversibleinteractions (Fig. 6), bacteria that were capable of bothlactose-reversible and -nonreversible coaggregations were notreleased from the aggregate by lactose. The sugar specifi-city of lactose-nonreversible coaggregations is an area ofactive interest. Inhibition of lactose-insensitive coaggrega-tions by the following sugars has been shown: (i) N-acetylneuraminic acid inhibits S. sanguis PK1317 and A. naeslundii PK606 (17) as well as S. sanguis H1 and A. naeslundii PK1643 (37); (ii) the trisaccharide N-acetylace-ramin-lactose completely inhibits, and N-acetylneuraminicacid, N-acetylgalactosamine, and N-acetylgalactosamine eachpartially inhibit, coaggregation between Capnocytophaga gingivalis DR2001 and A. israelii PK16 (12, 13); and (iii)t-rhamnose and d-fucose inhibit A. naeslundii PK984 and C. ochracea ATCC 33596 coaggregations (E. Weiss, personalcommunication).

Although they are independent, both lactose-reversible and -nonreversible coaggregations appear to cooperate inestablishing the network of interactions observed in thecurrent study. It is likely that sugar-specificity and lectin-carbohydrate interactions are reflected in coaggregationpartnerships. Clearly, coaggregation is not random (Fig. 2),and this was also shown by the partner specificity in thesequential addition of different strains to a preexisting aggre-gate (Table 3 and Fig. 7). Some interactions were lactose reversible (S. sanguis with A. naeslundii PK25 and the latterstrain with C. ochracea ATCC 33596) and some were not (C. ochracea ATCC 33596 with A. israelii PK16 and PK16 with C. gingivalis DR2001). Adherence of each new strain re-quired the previous attachment of a specific partner. Thisresulted in a multigenericaggregate with a controlled butchanging bacterial population. The implication of this kind ofcell-to-cell recognition in vivo is obvious in that dentalplaque is thought to mature by accretion of oral bacteria.Accordingly, the oral econiche is known to change fromprimarily streptococci and actinomyces in newly formedplaque to include various gram-negative bacteria in maturingdental plaque (25, 36). Taken collectively, the results re-port here illustrate that the multigeneric aggregates are anintricate combination of pairwise coaggregations acting in-dependently. The independence is manifested as stability ofcoaggregating pairs, competition and bridging among celltypes, and partner specificity, the key principles for accre-tive multigeneric aggregations.

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LITERATURE CITED


