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# New *Actinomyces* and *Streptococcus* Coaggregation Groups Among Human Oral Isolates from the Same Site

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The coaggregation properties of recent human oral streptococcal and actinomyces isolates from the same site were determined and compared with the coaggregation properties of well-characterized stock strains of these two kinds of bacteria. *Streptococcus sanguis*, *Actinomyces viscosus*, *Actinomyces naeslundii*, and phenotypically similar strains of actinomyces were isolated from subgingival samples from periodontally healthy older individuals, from persons participating in an experimental gingivitis study, and from young persons with localized (juvenile) and generalized (severe) periodontitis. All 34 of the actinomyces isolates coaggregated with reagent strains of *S. sanguis* that represented the four streptococcal coaggregation groups. Most of these actinomyces exhibited coaggregations identical to those of actinomyces stock strains. However, five isolates of an *Actinomyces* WVa-963 serovar exhibited a coaggregation pattern different from any previously described, which was used to define coaggregation group F. All coaggregations with members of this group were lactose inhibitable. Only 57% (8 of 14) of the recent *S. sanguis* isolates coaggregated with actinomyces reagent strains. But when the nonreactive streptococcal isolates were tested for their ability to coaggregate with actinomyces from the same patient, a new, highly specific coaggregation pattern (group 6) for *S. sanguis* was discovered. Coaggregation of these streptococci was observed only with certain isolates of *A. naeslundii* (members of coaggregation group D) from the same site, and none of these coaggregations were inhibited by lactose. Subsequent testing revealed that streptococci of group 6 coaggregated with group D actinomyces from other sources but not with actinomyces of other coaggregation groups. Only two strains of *S. sanguis* failed to coaggregate with any strain of actinomyces tested. These results indicate that nearly all fresh isolates of these species obtained from both diseased and healthy sites exhibit specific, nonrandom patterns of coaggregation and suggest the widespread occurrence of in vivo cell-to-cell recognition between oral actinomyces and streptococci.

Gibbons and Nygaard (3) reported that certain kinds of oral bacteria specifically interact with each other. Although most of the 23 strains they studied exhibited little or no coaggregation, a few coaggregated strongly. Subsequent studies, reviewed by Cisar (1), have delineated additional coaggregation groups and partially defined some of the mechanisms of coaggregation. Bacterial cells exhibit specific cell-to-cell recognition of isolates belonging to certain species, but not necessarily to all strains of a single species or all species of a genus. Examples of such specificity among human oral bacteria are: (i) *Bacterionema matruchotii* interacts with *Streptococcus* strains CC5A and CC6 to produce "corn cob configurations," but not with other streptococci (13); (ii) *Actinomyces naeslundii* ATCC 12104 coaggregates with *Streptococcus sanguis* 34 but not with *S. sanguis* DL1 (NCTC 7868), *Streptococcus mutans* (seven different strains), or *Streptococcus salivarius* (four different strains) (2); (iii) *S. salivarius* HB coaggregates with *Veillonella alcalescens* V1 but not with *Veillonella parvula* ATCC 10790 (14); (iv) *Actinomyces israelii* PK16 coaggregates with oral *Cytophaga* DR2001 but not with *Cytophaga* DR2002 (6); and (v) *Streptococcus morbillorum* PK509 coaggregates with *A. naeslundii* PK606 but not with *Actinomyces viscosus* T14V (8).

Such specificity provides a framework for a broader organization of bacterial strains into groups with distinct coaggregation patterns. Among the human oral streptococci and actinomyces, three basic kinds of coaggregations serve

to identify such patterns (2). They are distinguished on the basis of the effect of protease treatment or the heating of cells (85°C for 30 min) before mixing with the partner strain and are designated unimodal and bimodal coaggregations (5). The first kind of coaggregation is unimodal in that heat treatment of the streptococcal cells blocks their ability to coaggregate, but identical treatment of the actinomyces has no effect on coaggregation. The second kind is the opposite unimodal coaggregation in which heat treatment of the actinomyces cells renders them unable to coaggregate with streptococci, but the same treatment of the streptococci has no effect. Finally, bimodal coaggregation is a combination of the first two kinds where heat treatment of both cell types is required to prevent coaggregation. The second type of unimodal coaggregation is inhibited by addition of lactose, whereas most bimodal coaggregations are lactose inhibitable only if the streptococci are heated before coaggregation (2).

By using the ability to coaggregate, the effect of heat, and the effect of lactose as the parameters measured in a survey of a stock culture collection of streptococci and actinomyces for coaggregation patterns, two coaggregation groups of actinomyces (groups A and B) and four groups of streptococci (groups 1 to 4) have been delineated (2). Each streptococcal group was characterized by a distinctive coaggregation pattern with the actinomyces groups and vice versa. Representative strains of each of these six groups were then used as reagent strains in an ensuing investigation of fresh oral actinomyces (7) and streptococci (8). Interestingly, the results of those studies showed that if the fresh isolates coaggregated with reagent strains, most exhibited coaggregation patterns identical to those initially found with stock cultures (2). A small percentage (about 10%) of the fresh streptococcal and actinomyces isolates exhibited slightly different coaggregation patterns which characterized three new actinomyces groups (groups C, D, and E) and one streptococcal group (group 5).

The current study was initiated during the investigation of fresh oral isolates (7, 8). Of special interest was the observation that only 60% of the fresh streptococcal isolates coaggregated with reagent actinomyces (8), whereas nearly all of the fresh actinomyces coaggregated with reagent streptococci (7). The focus of the current study was to investigate coaggregations between streptococci and actinomyces isolated from the same site. Three points were examined: (i) the hypothesis that the 40% of streptococcal isolates exhibiting no coaggregation could, in fact, participate in highly specific coaggregations with actinomyces present only at the same site on the tooth; (ii) the possibility that isolates from different disease states may exhibit new kinds of coaggregation patterns; and (iii) the possibility that same-site isolates may exhibit additional kinds of coaggregation not observed in isolate-reagent strain pairs.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** All strains were from humans and were grown in complex broth medium containing 0.2% glucose (9). The reagent strains used to test coaggregation properties of fresh isolates were those characterized in previous studies and are listed in footnotes *a* in Tables 2 and 3.

The fresh isolates examined in this study were obtained by procedures previously described (11, 12). Briefly, the isolates were from a periodontally healthy person, from individuals participating in an experimental gingivitis study (11), or from adolescents with localized periodontitis (periodontosis) or young adults with generalized periodontitis. Samples were from subgingival sites (11, 12) or from the tooth surface coronal to the gingival margin and adjacent to the subgingival site sampled (12). Thirty bacterial colonies were picked at random from cultures of diluted samples from each site; isolates were characterized and identified by morphological, biochemical, chromatographic, and electrophoretic methods outlined previously (4, 10). Isolates of actinomyces also were tested by fluorescent antibody reactions to identify *A. israelii* serovars I and II; *A. naeslundii* serovars I, II, and III; *A. viscosus* serovars I and II; and *Actinomyces* WVa-963. Isolates of actinomyces and *S. sanguis* from the same site or from different sites in the same individual were tested for coaggregation. Only one isolate of an actinomyces serovar or an *S. sanguis* biovar was tested from one site.

**Coaggregation assay.** A visual assay described previously (2) was used to determine coaggregation with reagent strains or between the fresh isolates. A reagent strain is one whose coaggregation properties have been thoroughly investigated by procedures outlined in an earlier investigation (2). The assay involves a scoring system of 0 for no visible coaggregation to 4 for maximum coaggregation. A score of 4 is given when large coaggregates are formed immediately upon mixing dense cell suspensions of the two partner strains. The coaggregates settle 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 was determined by heating a cell suspension at 85°C for 30 min before mixing with heated or unheated cells of a partner strain.

## RESULTS

**Coaggregation properties of fresh isolate-reagent strain pairs and fresh isolate-fresh isolate pairs.** The fresh streptococcal isolates were tested initially for their ability to coaggregate with reagent strains representing actinomyces coaggregation groups A and B, and the fresh actinomyces isolates were examined for coaggregation

with reagent strains of streptococcal coaggregation groups 1 to 4. With the exception of *Actinomyces* serovar WVa-963, all actinomyces coaggregated in patterns established in an earlier (2) or a concurrent (7) study. All members of *Actinomyces* serovar WVa-963, a previously untested serovar, exhibited a new coaggregation pattern designated group F (discussed below). In contrast, only 8 of 14 (57%) streptococcal isolates coaggregated with the reagent actinomyces. To test the possibility that the six nonreactive isolates may coaggregate with actinomyces isolates from the same person (including same-site isolates), these streptococci were paired with the fresh actinomyces isolates. Two streptococcal isolates did not coaggregate with any actinomyces tested. The other four coaggregated only with actinomyces isolates belonging to coaggregation group D, a group discovered in a parallel study (7). Members exhibiting this limited coaggregation pattern represent streptococcal group 6 (discussed below). Thus, by testing same-site isolates for coaggregation, a highly specific coaggregation pattern was found that would have been undetected by screening streptococci for their ability to coaggregate with the standard reagent strains of actinomyces groups A and B used formerly.

The second reason for examining coaggregations between same-site isolates was to test the possibility that new kinds of interactions may occur that were not observed when fresh isolates were paired with reagent strains. For example, the coaggregation pattern exhibited by mixing a fresh streptococcal group 3 isolate with a fresh actinomyces group A isolate was compared to the pattern observed with (i) a fresh streptococcal group 3 isolate and a reagent actinomyces group A, (ii) a fresh actinomyces group A isolate and a reagent streptococcal group 3, and (iii) reagent strains of the two coaggregation groups. We found that after an isolate's coaggregation group status was identified, all combinations of coaggregations between fresh isolates and reagent strains were identical. In other words, fresh isolates and reagent strains representing the same coaggregation group exhibit indistinguishable coaggregation properties. This included the highly specific coaggregation between streptococcal group 6 and group D actinomyces.

**Distribution of fresh isolates among coaggregation groups.** Each of the 45 isolates was tested for its ability to coaggregate with representatives of actinomyces coaggregation groups A through F or streptococcal groups 1 through 6. The coaggregation patterns of actinomyces species and *S. sanguis* biovars I and II found in samples from eight persons are shown in Table 1. *A. naeslundii* serovars I, II, and III were distributed among coaggregation groups A, C, and D. Besides the two *Actinomyces* serovar WVa-963 isolates included in Table 1, three additional strains of serovar WVa-963 from three other patients also were members of coaggregation group F. The two actinomyces strains designated as "crosses" possessed antigens reacting with two antisera. *Actinomyces* NV were strains that reacted with antisera to *A. viscosus* serovar II and *A. naeslundii* serovars (usually serovar I). All but one of these *Actinomyces* NV strains were group A, which may reflect a dominant effect of the *A. viscosus* serovar II antigens. All *A. viscosus* serovar II strains tested to date, including those examined here, exhibit the coaggregation group A pattern.

TABLE 1. Coaggregation groups of *Actinomyces* serovars and *S. sanguis* biovars

| Patient no.[a] | Coaggregation group | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *A. naeslundii* serovar: | | | *Actinomyces* serovar: | | | *A. viscosus* serovar II | *S. sanguis* biovar: | |
| | I | II | III | 963 | Crosses | NV | | I | II |
| E1 (Ex) | D | | A | F | A | A | A | 6 | 6 |
| E2 (Ex) | D | | D | | C | A | A | 3 | 3 |
| E3 (Ex) | C | | D | | | A | A | 1 | 3 |
| D36 (SP) | C | A | | F | | A | A | —[b] | 3 |
| D39 (JP) | A | | A | | | A | A | 3 | 3 |
| D41 (OH) | C | | D | | | | | 6 | — |
| D42 (JP) | | | | | | A | A | | 3 |
| D43 (JP) | C | | | | | C | A | 6 | |

[a] Abbreviations in parentheses: Ex, person in adult experimental gingivitis study (11); SP. severe periodontitis, persons 18 to 30 years of age with generalized gingival inflammation and 5 mm or more loss of attachment on one or more surfaces of eight or more teeth in the presence of pockets 6 mm or deeper not limited to first molars and incisors; JP, juvenile periodontitis (periodontosis), adolescents or young adults (11 to 25 years of age) with initiation of severe destruction limited to first molars and incisors; OH, periodontally healthy adults at least 70 years of age.

[b] —, No coaggregation.

TABLE 2. Coaggregation pattern of actinomyces coaggregation group F (*Actinomyces* WVa-963 strain VPI D33C-25)

| Treatment | | Reaction (coaggregation score) with streptococcal group[a]: | | | | | |
|---|---|---|---|---|---|---|---|
| Streptococcus | Actinomyces | 1 | 2 | 3 | 4 | 5 | 6 |
| Heated[b] | Heated | 0 | 0 | 0 | 0 | 0 | 0 |
| Heated | None | 0 | 0 | 3[d] | 3[d] | 0 | 0 |
| None | Heated | 0 | 0 | 0 | 0 | 0 | 0 |
| None | None | 0 | 0 | 3[d] | 3[d] | 0 | 0 |

[a] The representatives of streptococcal groups 1 through 6 used were *S. sanguis* DL1 (NCTC 7868), H1, 34, and J22; *S. morbillorum* PK509; and strain VPI E1A-1A, respectively. Reactions were scored as 0 for no coaggregation and 3 for strong coaggregation.

[b] Cell suspension was heated at 85°C for 30 min.

[c] Coaggregation was reversed by addition of 0.06 M (final concentration) lactose.

The *S. sanguis* strains were distributed among coaggregation groups 1, 3, and 6, and, as discussed above, two strains failed to interact with any of the actinomyces tested. Also of interest was the random distribution among coaggregation groups of the fresh isolates from patients with various periodontal disease conditions. Thus, it appears that streptococci and actinomyces exhibiting these coaggregation characteristics are members of a resident population associated with several different states of oral health.

**Properties of new actinomyces coaggregation group F.** Only strains of *Actinomyces* serovar WVa-963 exhibited the coaggregation group F pattern, and the properties of this group are shown in Table 2. Members of group F coaggregate only with strains of streptococcal coaggregation groups 3 and 4, and in every case the coaggregations are inhibited by lactose. These coaggregations also are inhibited by heating the actinomyces cells but are unaffected by similar treatment of the streptococci.

**Properties of new streptococcal coaggregation group 6.** Four strains of *S. sanguis* showed the most specific coaggregation pattern yet reported (Table 3). These strains coaggregated only with members of actinomyces group D. Unlike the coaggregation properties of group F actinomyces, coaggregations with group 6 streptococci are prevented by heat treatment of the streptococci and are not inhibited by lactose.

**Coaggregation between actinomyces and streptococci isolated from the same patient or from the same site.** Coaggregation between streptococci and actinomyces isolated from the same person or from the same site was determined to estimate the potential for in vivo coaggregations.

Actinomyces-streptococcus pairs from seven of the eight persons coaggregated. The single *S. sanguis* isolate tested from this sample from the eighth patient (D41) was a member of the highly specific group 6 (Table 1). Neither of the two isolates of actinomyces tested from this person exhibited coaggregation group D properties.

*S. sanguis* and an actinomyces were isolated from the 10 tooth sites sampled from the eight patients. Actinomyces-streptococcus pairs from seven of these 10 sites coaggregated (Table 4). Of the three samples from which no coaggregating pairs were identified among the strains tested, the group 6 streptococcus was isolated from two samples (E1, 6F, and D43, 3M). The streptococcus from the subgingival sample of the mesial area of tooth 12, patient D36, did not coaggregate with any strain of actinomyces tested.

## DISCUSSION

Results reported here add to the growing body of evidence that establishes the existence of a nonrandom network of surface interactions between cells of certain human oral actinomyces and viridans streptococci. Only 2 of the 45 fresh isolates examined here failed to coaggregate. Of all the possible coaggregation patterns that could have taken place between these bacteria, only a few were observed. In fact, most were well characterized in other studies (2, 7, 8), but two (streptococcal group 6 and actinomyces group F) were new and highly specific in that the coaggregation partners were limited to members of only one or at most two coaggregation groups, respectively.

In this study, *A. viscosus*, *A. naeslundii*, and *S. sanguis* biovars I and II were of particular interest because the coaggregation properties of many of these strains were known (2, 7, 8). Two of the *Actinomyces* serovars, *Actinomyces*

TABLE 3. Coaggregation pattern of streptococcal coaggregation group 6 (*S. sanguis* biovar I, VPI strain E1A-1A)

| Treatment | | Reaction (coaggregation score) with actinomyces group[a]: | | | | | |
|---|---|---|---|---|---|---|---|
| Streptococcus | Actinomyces | A | B | C | D | E | F |
| Heated[b] | Heated | 0 | 0 | 0 | 0 | 0 | 0 |
| Heated | None | 0 | 0 | 0 | 0 | 0 | 0 |
| None | Heated | 0 | 0 | 0 | 3 | 0 | 0 |
| None | None | 0 | 0 | 0 | 3 | 0 | 0 |

[a] The representatives of actinomyces groups A through F used were: A, *A. viscosus* T14V; B, *A. naeslundii* ATCC 12104; C, *A. naeslundii* PK947; D, *A. naeslundii* PK606; E, *A. viscosus* T14AV; and F, *Actinomyces* WVa-963 strain VPI D33C-25. Reactions were scored as 0 for no coaggregation and 3 for strong coaggregation.

[b] Cell suspension was heated at 85°C for 30 min.

TABLE 4. Coaggregation groups found in individual tooth sites

| Patient no. | Tooth (area) sampled[a] | Coaggregation group detected | | Coaggregation |
|---|---|---|---|---|
| | | Streptococcus | Actinomyces | |
| E1 | 6F (S) | 6 | A | No[b] |
| | 6D (S) | 6 | D | Yes |
| E2 | 4M (S) | 3 | D | Yes |
| E3 | 3M (S) | 1 | D | Yes |
| D36 | 12M (RS, S) | —[c] | A, A, C | No |
| | 2M (RS) | 3 | A, F | Yes |
| D39 | 29M (RS) | 3 | A, A | Yes |
| | 29M (RS) | 3 | A, A | Yes |
| D42 | 30M (RS) | 3 | A, A | Yes |
| D43 | 3M (RS) | 6 | A, C, C | No |

[a] Military tooth numbering system 1 through 32. Abbreviations: D, distal; F, facial; M, mesial; (S), subgingival; (RS), supragingival surface cornonal to the gingival margin after the area had been cleaned with a sterile toothpick.

[b] Group D actinomyces were isolated from the same tooth, distal surface.

[c] —, No coaggregation.

WVa-963 and *Actinomyces* NV, had not been tested before.

Earlier surveys used only reagent strains of known coaggregation groups, whereas pairs of fresh isolates as well as fresh isolates with reagent strains were examined here. The coaggregating pairs found among fresh isolates in seven of eight persons and 7 of 10 sites indicated that coaggregation is frequent if not universal and may play an important role in the development of tooth surface plaque. Some *Actinomyces* or *S. sanguis* strains of interest might easily have been missed because only 30 total bacterial isolates, picked at random, were examined from each sample. Discovery of the highly specific coaggregation between the new group 6 streptococci and group D actinomyces suggests that the two coaggregation-negative streptococcal isolates found in this study may be coaggregation partners in equally specific interactions with as yet untested strains of actinomyces. One of the practical results of this discovery is that coaggregation of a group 6 streptococcus with an unknown actinomyces isolate provides a rapid and positive identification of the actinomyces as a member of coaggregation group D. It is significant that once an isolate was established as a member of a certain coaggregation group, no new interactions between fresh isolates were observed when compared to reagent strains. This strongly suggests that the surface components that mediate coaggregation are stable structures that continue to be expressed upon subculturing a strain in stock culture.

The *Actinomyces* WVa-963 strains exhibited identical coaggregation patterns characterizing a new coaggregation group F. Similarly, all *A. viscosus* serovar type II strains tested are members of coaggregation group A. These results suggest a relationship between serovar and coaggregation pattern since both reflect the existence of specific cell surface components. Evidence to confirm such speculation may come from analyses of the serological properties of coaggregation-defective mutants like those recently described for *A. viscosus* (5).

The results of this study confirm and extend previous observations on the coaggregation properties of oral streptococci and actinomyces (2, 7, 8). First, each of about 60 strains of *A. viscosus* tested to date coaggregates with *S. sanguis* reagent strains in a specific pattern designated group A. Second, most *A. naeslundii* strains coaggregate with *S. sanguis*, but in a variety of coaggregation patterns. Third, only about 60% of the *S. sanguis* isolates coaggregate with reagent strains of actinomyces groups A and B. We observed a similar low percentage (57%) of coaggregation among the streptococcal isolates examined here when actinomyces reagent strains of groups A and B were tested as partners. However, when it was discovered that one of these coaggregation-negative isolates coaggregated strongly with a certain actinomyces (member of coaggregation group D) isolated from the same site, further testing revealed that nearly 90% of the *S. sanguis* isolates coaggregated when a representative strain for group D actinomyces was included in the screening procedure. Thus, most strains of *A. viscosus* and *A. naeslundii* coaggregate with *S. sanguis* and certain related streptococci. Our results suggest that frequent coaggregations among same-site isolates obtained from various periodontal disease conditions are likely. The in vivo significance of these coaggregations remains to be established. However, we recently found that these coaggregations also occur in saliva (Kolen-

brander and Phucas, unpublished data). Thus, it is probable that cells of these two kinds of oral bacteria do interact in their natural ecological niche.

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