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# Intergeneric Bacterial Coaggregations Involving Mutans Streptococci and Oral Actinomyces

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**Mutans streptococci (MS) representing eight different serotypes were tested for their ability to coaggregate in vitro with oral actinomyces and other streptococcal species. Of the mutans streptococci tested, only strains of *S. cricetus* (formerly *S. mutans* serotype a) displayed pronounced coaggregations and only with certain strains of actinomyces. *S. cricetus* coaggregated, by lactose nonreversible mechanisms, with serotype 4 *Actinomyces naeslundii* WVU963 and WVU924 and with serotype 2 *Actinomyces odontolyticus* WVU758. The first pair was disaggregated by protein denaturants (e.g., sodium dodecyl sulfate and urea) and EDTA. This coaggregation was inhibited when the streptococcal, but not the actinomyces, partner was pretreated with either heat or protease, suggesting the presence of a protein mediator on only the streptococcal cell surface. The *S. cricetus-A. odontolyticus* coaggregation appeared to involve protein components on each cell, as shown by the lack of coaggregation after pretreatment of either cell type with heat or proteases. This coaggregation was also reversed by sodium dodecyl sulfate and urea, as well as by sodium deoxycholate, but not by EDTA. The data indicate that different mechanisms may be involved in each of these coaggregations.**

In the dynamic environment of the human oral cavity the ability of an organism to colonize necessarily depends upon a complex interplay of factors involving specific ion concentrations, pH, carbohydrate availability, and the ability of the organism to attach to tooth pellicle, soft tissue, or other established microorganisms. Clinical studies have demonstrated that in the presence of dietary sucrose certain mutans streptococci (MS) (*S. mutans* and *S. sobrinus*) are consistently found in elevated numbers, and they have been directly associated with dental caries (24, 32); however, the mechanisms by which MS are able to colonize the oral cavity are not completely defined. The prevailing theory is that MS attach to tooth pellicle by a sucrose-independent process and once attached, and in the presence of sucrose, accumulate by a dextran-mediated process. There are several reported examples of sucrose (dextran)-independent adherence of MS to plaque or saliva-coated hydroxyapatite (7, 8, 23, 35, 37, 38). Staat et al. (35) provided evidence that *S. mutans* possesses a protein adhesin that is involved in sucrose-independent attachment to pellicle and that glucosyltransferase (a dextran-synthesizing enzyme) plays a role only in cellular accumulation and not in adherence to pellicle. Other theories of sucrose-independent adherence mechanisms involve bridging of MS teichoic acid to acidic salivary glycoproteins in pellicle by calcium ions (33) or interaction of cellular components with blood group-reactive salivary mucins (14).

A theory first postulated by Gibbons and Nygaard (13) involves a role for intergeneric bacterial coaggregations in the formation of dental plaque. Many in vitro studies have demonstrated specific molecular interactions between different bacterial species that result in the formation of dextran-independent intergeneric coaggregates of bacteria (4–6, 10, 11, 13, 19–22, 27, 29, 31, 39–41). One study in particular found a pronounced coaggregation between *S. mutans* M-7 and *Veillonella alcalescens* V1 (a lactate-metabolizing organism) in gnotobiotic rats (27). In many of the studies, however, MS were included in the battery of coaggregation test organisms but either no intergeneric coaggregations were observed with MS (5, 27, 29, 39) or the reactions were weak and not studied further (11, 13, 31).

Since MS, along with *Streptococcus sanguis* and *Streptococcus mitis*, are considered to be early colonizers of tooth surfaces, they may play a potentially important role in plaque formation, once they are bound to pellicle, by coaggregating other bacterial species. In this study we reinvestigated non-dextran-mediated intergeneric coaggregations involving MS because of our interest in the biological significance of MS cell wall proteins and report preliminary data on coaggregations between one species of MS, *Streptococcus cricetus* (formerly *S. mutans* serotype a), and *Actinomyces naeslundii* serotype 4 and *Actinomyces odontolyticus* serotype 2.

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## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The streptococcal strains used in this study were obtained from our culture collection, except for AHT, OMZ175, and the serotype h MS strain, which were obtained from W. Little, National Institute of Dental Research, Bethesda, Md. (AHT and OMZ175) and the American Type Culture Collection, Rockville, Md. (serotype h, ATCC 33748). All actinomycete strains except PK19 and PK29 were generously provided by S. Bragg, Centers for Disease Control, Atlanta, Ga. Strains PK19 and PK29 were provided by W. Clark, University of Florida, Gainesville.

All organisms were initially grown overnight (16 h) or until turbid at 37°C in autoclaved brain heart infusion broth supplemented with 0.3% yeast extract and then transferred to medium containing 0.5% each of tryptone, yeast extract, $KH_2PO_4$, and Tween 80 (26) and supplemented with 0.1%

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TABLE 1. List of organisms studied

| MS (serotype) | Test organism(s) (serotype) | |
|---|---|---|
| *S. cricetus* (a) | *S. salivarius* | *A. naeslundii* |
| AHT | ATCC 13419 | W569, X600, W869, |
| E49[a] | ATCC 27945 | PK19 (1) |
| OMZ61 | ATCC 25975 | W752, W1544 (2) |
| HS-6[a] | HHT | N16, WVU1468, |
| HS-1 | PC-1 (fresh | WVU1527 (3) |
| | isolate)[a] | WVU924, WVU963[b] (4) |
| *S. rattus* (b) | CDC 3549 | WVU1520, W1611, |
| BHT | | W1250 (NT[c]) |
| FA-1 | *S. mitis* | W1516, PK29 (NT) |
| | ATCC 6249 | |
| *S. mutans* | ATCC 903 | *A. viscosus* |
| GS-5 (c) | S-3 | T14V, T14AV, W859, |
| NCTC 10449 (c) | | W1053, M100, W1557 |
| Ingbritt 175 (c) | *S. sanguis* | (2) |
| Ingbritt 162 (c) | Challis c | |
| NG8 (c) | Challis e | *A. israelii* |
| NG7 (c) | Challis d | X372, A601, W726 (1) |
| NG5 (c) | Challis SR | W838, W1125, W748 (2) |
| B2 (e) | Blackburn | |
| V100 (e) | 71 × 43 | *A. odontolyticus* |
| B14 (e) | 71 × 35 | X363 (1) |
| OMZ175 (f) | 71 × 48 | WVU758 (2) |
| | 72 × 42 | |
| *S. sobrinus* | 72 × 33 | *A. propionica* |
| SL-1 (d) | 72 × 39 | W903, W973, W1054 (1) |
| 6715 (g) | 72 × 41 | W810, W852 (2) |
| K1-R (g) | 71 × 23 | |
| ATCC 33748 (h) | ATCC 15909 | *Bacterionema matruchotii* |
| | | W2596 and W1460 |
| | *Streptococcus* | |
| | *pyogenes* | *Rothia dentocariosa* |
| | Manfredo | X346, W876 (1) |
| | F365 | X348, W808 (2) |
| | T11 | WVU936 (3) |
| | *Lactobacillus* | *Nocardia asteroides* |
| | *casei* | W2967 |
| | ATCC 7469 | |

[a] Self-aggregating strain. E49 was subsequently cultured in brain heart infusion broth; no self-aggregation was observed in this medium.
[b] Also referred to as *Actinomyces* sp. strain WVa963.
[c] NT, Not typed.

glucose for actinomycetes and 0.5% glucose for streptococci. Growth was continued overnight or until the medium was turbid. All organisms were grown aerobically at 37°C except *Actinomyces israelii*, *Arachnia propionica*, and serotype 3 *A. naeslundii*, which were grown anaerobically under an 85% $N_2$–10% $H_2$–5% $CO_2$ atmosphere. After being harvested, cells were washed three times in sterile-filtered coaggregation buffer consisting of 20 mM Tris hydrochloride (pH 7.8), 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.15 M NaCl, and 0.02% $NaN_3$ (5). The final suspension was in coaggregation buffer to an $A_{600}$ of 1.5 (220 Klett units). The cell suspensions then were stored at 4°C.

**Coaggregation assay.** The assay we used for identifying coaggregating strains involved mixing equal volumes (0.2 ml each) of MS and the test organism in a test tube, vortexing, and incubating at room temperature for up to 24 h. The tubes were scored visually during this time for formation of coaggregates on a scale of 0 (no coaggregates) to +4 (large coaggregates that settled rapidly leaving a clear supernatant). The presence of each strain in the coaggregates was verified by light microscopy. Two volumes of each strain alone were observed for self-aggregation and scored in the same manner (5).

**Heat and protease treatment.** Each coaggregation partner was heated at 85°C for 30 to 60 min, washed once in coaggregation buffer, and resuspended to its original absorbance reading before being mixed with its similarly treated or untreated coaggregation partner. The coaggregates were scored as described above.

Proteases were purchased from Sigma Chemical Co., St. Louis, Mo. Proteinase K (protease type XI, P0390) and pronase E (protease type XIV, P5146) were used at 2 U/ml of cells. Trypsin (protease type III, T8253) was used at 100 to 10,000 U/ml of cells, and soybean trypsin inhibitor (type I-S, T9003) was used at 10 mg/ml of cells. For protease treatments each strain was incubated individually in coaggregation buffer at pH 7.5 with each protease for 1 h at 37°C at the final concentrations noted above. When soybean trypsin inhibitor was used, it was added to the cells at the same time as trypsin. The mixture was then incubated as indicated above. In all instances, cells in buffer were incubated at 37°C for 1 h with no added enzyme and scored as controls.

**Treatment of coaggregated pairs with protein denaturants, EDTA, and sugars.** To determine the nature of surface components responsible for cell-cell binding, coaggregated pairs were treated with a variety of potential disaggregating reagents. Portions (20 to 40 μl) of each stock test detergent were added to 400 μl of coaggregated cells to final concentrations of 0.01 to 1%; the detergents tested were sodium dodecyl sulfate (SDS), Tween 80, and Triton X-100. EDTA was tested in the same manner at final concentrations of 0.1 to 10 mM. For treatments with LiCl and urea, coaggregates were first centrifuged and then suspended in 1 and 2 M concentrations of each compound. Similarly, portions of lactose, galactose, rhamnose, glucose, melibiose, gentiobiose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic (sialic) acid stock solutions were added individually to the coaggregated pairs to final concentrations of 0.1 M. After treatments, pairs and similarly treated single-strain controls were scored.

## RESULTS

**Coaggregation of bacterial pairs.** Most of the test strains listed in Table 1 did not coaggregate with any of the MS under the conditions of the coaggregation assay. However, one species, *S. cricetus*, coaggregated strongly with two serotype 4 *A. naeslundii* strains, WVU963 and WVU924, as well as with the serotype 2 *A. odontolyticus* strain WVU758 (Table 2). All coaggregations were observed within seconds after vortexing. Figure 1 shows assay tubes containing either single strains (tubes a to d) or coaggregated pairs (tubes e to g) of these three species. Several weak scores (+1) were also noted with the same two *A. naeslundii* strains and certain strains of *S. mutans* and *S. sobrinus*. Coaggregation scores

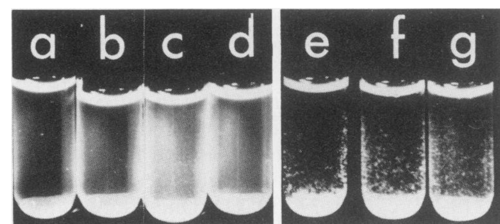


FIG. 1. Coaggregation assay tubes showing single-strain controls of *S. cricetus* AHT (a), *S. cricetus* E49 (b), *A. naeslundii* WVU963 (c), and *A. odontolyticus* WVU758 (d) and coaggregates AHT-WVU963 (e), E49-WVU963 (f), and AHT-WVU758 (g).

TABLE 2. Positive coaggregations involving MS[a]

| MS | Test strain(s) | Score[b] |
|---|---|---|
| *S. cricetus* | | |
| AHT | *A. naeslundii* WVU963 and WVU924[c] | +3–+4 |
| E49 | *A. naeslundii* WVU963 and WVU924 | +3 |
| OMZ61 | *A. naeslundii* WVU963 and WVU924 | +1–+2 |
| HS-6[d] | *A. naeslundii* WVU963 and WVU924 | +4 |
| HS-1 | *A. naeslundii* WVU963 and WVU924 | +2 |
| *S. cricetus* | | |
| AHT | *A. odontolyticus* WVU758 | +2 |
| E49 | *A. odontolyticus* WVU758 | +2 |
| HS-6[d] | *A. odontolyticus* WVU758 | +3 |
| HS-1 | *A. odontolyticus* WVU758 | +2 |
| *S. sobrinus* 6715 | *A. naeslundii* PK19 and PK29 | +1 |
| *S. mutans* | | |
| Ingl75 | *A. naeslundii* WVU963 and WVU924[c] | +1 |
| NG7 | *A. naeslundii* WVU963 and WVU924 | +1 |
| B2 | *A. naeslundii* WVU963 and WVU924 | +1 |
| V100 | *A. naeslundii* WVU963 and WVU924 | +1 |
| *S. sobrinus* | | |
| SL-1 | *A. naeslundii* WVU963 and WVU924 | +1 |
| 6715 | *A. naeslundii* WVU963 and WVU924 | +1 |

[a] Coaggregations were not reversed by 0.1 M lactose, galactose, rhamnose, glucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, melibiose, gentiobiose, or sialic acid.
[b] Scoring is explained in the text. All strains listed (MS and actinomyces) are from human sources, except for E49, HS-6, and HS-1 (from hamsters) and OMZ61 (from rats).
[c] Suspensions of these two strains occasionally produced minute self-aggregates with a score of +1 or less.
[d] HS-6 self-aggregated with a score of +2.

were consistently +3 to +4 with *S. cricetus* and *A. naeslundii* WVU963 and WVU924, except with strain OMZ61, while scores with *S. cricetus* and *A. odontolyticus* were normally +2. None of these coaggregations was reversed or lessened by the addition of 0.1 M lactose or any other sugar tested. The addition of whole, clarified saliva to centrifuged coaggregates or the suspension of each partner in saliva before mixing did not significantly alter the degree of coaggregation (data not shown). Of note was the discovery of a very pronounced sialic acid (0.15 M)-reversible coaggregation between several strains of *S. salivarius* and *A. odontolyticus* WVU758 (data not shown).

**Effects of heat and protease treatments on coaggregation.** Coaggregations involving *S. cricetus* and *A. naeslundii* were partially inhibited when the streptococci were heated, but no similar effect was seen when the actinomyces were heated (Table 3). On the other hand, heating either member of the *S. cricetus-A. odontolyticus* pair prevented visible coaggregation. Protease treatment had an equally profound effect on cell-cell binding. Treatment of the streptococci with proteinase K, as well as pronase E (2 U/ml) and trypsin (1,000 U/ml for *A. naeslundii* coaggregations and 10,000 U/ml for *A. odontolyticus* coaggregations), resulted in complete inability to coaggregate with either *A. naeslundii* or *A. odontolyticus*. Conversely, only *A. odontolyticus* was susceptible to these protease treatments; *A. naeslundii* appeared not to possess vulnerable surface proteins. In some instances, small aggregates were observed when *S. cricetus* HS-6 was treated. As noted, this strain self-aggregates to a certain extent, and after treatment with trypsin and pronase E it continued to do so. The addition of soybean trypsin inhibitor to the streptococcal samples to which trypsin was added prevented the loss of ability to coaggregate with the actinomyces.

**Effect of chemical agents on coaggregation.** Several of the chemicals tested elicited disaggregating effects (Table 4). SDS, an anionic detergent, caused complete visual disaggregation of *S. cricetus-A. naeslundii* and *S. cricetus-A. odontolyticus* coaggregated pairs. Sodium deoxycholate (SDe), an anionic cholesterol-derived bile salt with detergentlike properties similar to those of SDS, also had a disaggregating effect but only on the *S. cricetus-A. odontolyticus* pairs and at a concentration of 0.2% (concentrations up to 1% SDe did not disaggregate *A. naeslundii* pairs). Similar effects of SDS and SDe were noted by McIntire et al. (28) in disaggregating *Actinomyces viscosus* T14V and *S. sanguis* 34 and by Cisar et al. (5) with non-lactose-reversible coaggregations involving T14V and all four coaggregation groups of *S. sanguis*. Triton X-100 and Tween 80, both nonionic detergents, had no effect on coaggregation (data not shown).

When various concentrations of EDTA (up to 10 mM) were added to each of the coaggregated pairs, total disaggregation was observed at 1.0 mM in the *A. naeslundii* group but no effect was observed in the *A. odontolyticus* group. Of

TABLE 3. Heat and protease treatments

| Actinomyces | *S. cricetus* strain | Score after treatment[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Heat | | | | Proteinase K (2 U/ml)[b] | | |
| | | C | S | A | SA | C[c] | S | A |
| *A. naeslundii* WVU963 and WVU924[d] | AHT | +4 | +2 | +4 | +2 | +3–+4 | 0 | +3 |
| | E49 | +3 | +2 | +3 | +2 | +2 | 0 | +2 |
| | OMZ61 | +2 | 0 | +2 | 0 | +1 | 0 | +1 |
| | HS-6[e] | +4 | +2–+3 | +4 | +2–+3 | +3–+4 | 0 | +3 |
| *A. odontolyticus* WVU758 | AHT | +2 | 0 | 0 | 0 | +2 | 0 | 0 |
| | E49 | +2 | +1 | 0 | 0 | +1 | 0 | 0 |
| | HS-6[e] | +3 | +1 | +1 | +1 | +3 | 0 | +1 |

[a] Scoring is explained in the text. C, Control (neither strain was treated); S, only streptococcus was treated; A, only actinomyces was treated; SA, both streptococcus and actinomyces were treated.
[b] All single-strain, enzyme-treated controls were negative for self-aggregation. All single strain, non-enzyme-treated controls were negative for self-aggregation except HS-6 (+2).
[c] When either actinomyces or streptococcus was treated with protease, a 37°C no-enzyme control was also included. The 37°C control was mixed with its untreated coaggregation partner, and the resulting coaggregation score was used as the control value.
[d] Both WVU963 and WVU924 cells, when heated, displayed minute aggregates.
[e] HS-6 cells, after heating, had a score of +1. All other streptococcal heated controls had a score of 0.

TABLE 4. Treatment of coaggregating pairs with chemical agents

| Actinomyces | *S. cricetus* strain | Score after treatment[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Control (untreated) | SDS (0.1%) | SDe[b] (0.2%) | EDTA (1.0 mM) | LiCl (2.0 M) | Urea (2.0 M) |
| *A. naeslundii* WVU963 and WVU924 | AHT | +3–+4 | 0 | +3–+4 | 0 | +2 | +1 |
| | E49 | +2–+3 | 0 | +2–+3 | 0 | +2 | 0 |
| | OMZ61 | +1 | 0 | +1 | 0 | +1 | 0 |
| | HS-6 | +3–+4 | +1–+2 | +3–+4 | +1 | +3 | +2 |
| *A. odontolyticus* WVU758 | AHT | +2 | 0 | 0 | +2 | 0 | 0 |
| | E49 | +2 | 0 | 0 | +2 | 0 | 0 |
| | HS-6 | +3 | 0 | 0 | +3 | +1 | +1–+2 |

[a] When each strain alone, except HS-6, was treated with the chemicals, no self-aggregates were observed. HS-6, treated alone, was scored +1 with SDS, SDS and +2 with the rest. Final concentrations are shown in parentheses. Scoring is explained in the text.

[b] CHAPSO (3-[3-cholamidopropyl dimethylammonio]-1-[2-hydroxyl-1-propane sulfonate]; Calbiochem-Behring, La Jolla, Calif.) and digitonin, both cholesterol derivatives like SDe, had no effect on coaggregation.

note is the finding that EDTA-washed cells of the *A. naeslundii* group coaggregated upon the addition of 2 mM $CaCl_2$ or $MgCl_2$ (data not shown), indicating that $Mg^{2+}$ and $Ca^{2+}$ may be involved in the coaggregation process. Urea, thought to denature proteins in their hydrophobic regions and to generally disrupt noncovalent bonds, totally disaggregated pairs in both the *A. naeslundii* and *A. odontolyticus* groups. Again, strain HS-6 self-aggregated, so a residual score of +1 to +2 was observed. Finally, LiCl, a strong ionic protein-dissociating agent, caused some reduction in the ability of the *A. naeslundii* and *S. cricetus* strains to coaggregate and caused total disaggregation of *A. odontolyticus* and *S. cricetus*.

When all disaggregated pairs (from the treatments discussed above) were centrifuged, washed, and resuspended in coaggregation buffer, no permanent effects of the denaturants were seen. All coaggregations were restored to untreated control levels.

## DISCUSSION

Based on the preliminary data presented here, it is apparent that specific mechanisms exist for coaggregations involving *S. cricetus* and *A. naeslundii* or *A. odontolyticus*. It is likely that a protein-protein interaction is involved in coaggregation of *S. cricetus* and *A. odontolyticus*, since coaggregation was prevented when either species was treated with protease or heat or when protein denaturants were added to the coaggregates. Less clear is the requirement for divalent cations, since the addition of EDTA to coaggregates did not disrupt them. Disaggregation produced by the anionic detergents SDS and SDe could indicate displacement of required cations by these negatively charged molecules and/or temporary denaturation of proteins involved in the coaggregations. The coaggregations involving *S. cricetus* and *A. naeslundii* appear to involve a protein(s) on the streptococcal surface only since the *A. naeslundii* strains were insensitive to heat and protease treatment. These coaggregations were also affected by the protein denaturants (LiCl, urea, and SDS) but not by SDe. Here, however, EDTA was effective in causing total disaggregation of all pairs of cells. The possibility exists that there is a lectinlike interaction between these two species. Several such interactions have been discovered involving streptococci and actinomyces which also are dependent upon the presence of divalent cations, particularly calcium (3, 5, 29). These interactions normally are lactose reversible, but the coaggregations involving *S. cricetus* and *A. naeslundii* were not, nor were they reversed by any of the other saccharides or amino sugars tested. Also, periodate (0.01 M) oxidation of *A. naeslundii* WVU963 whole cells (overnight at 4°C) did not prevent coaggregation with *S. cricetus* AHT (data not shown), indicating that reducing sugars on the actinomycete are not involved. It is not likely that lipoteichoic acid, a calcium-binding surface amphiphile on MS, is involved in any of the coaggregations since it is not proteinaceous and is found in the walls of all MS, most of which did not coaggregate in this study. However, it may be that lipoteichoic acid plays a secondary role by adding extra cohesiveness to the coaggregation. DiRienzo et al. (10) have shown evidence that *S. sanguis* lipoteichoic acid binds to one type of receptor on *Fusobacterium nucleatum* in the formation of "corncobs."

The clinical relevance of these findings is not clear because *S. cricetus*, although cariogenic in animals (16, 30), is not a species commonly isolated from humans. This may be attributed in part to decreased viability of *S. cricetus* once it is removed from the oral cavity and/or culture techniques used for isolating MS. One widely used medium incorporates bacitracin as a selective agent for MS, but this antibiotic inhibits the growth of *S. cricetus* (9, 15). It is also possible that the low recovery of *S. cricetus* may be due to decreased viability in the oral cavity. For example, lysozyme, a prominent salivary enzyme, is reported to be inhibitory to the growth of *S. cricetus* and *Streptococcus rattus* (18). Iacono et al. (18) found that the growth of these two species was inhibited by 1% of the dosage required to inhibit *S. mutans* and *S. sobrinus*. However, in two studies significant numbers of *S. cricetus* were isolated from plaque samples from predominantly non-Western populations (2, 34). Of most note was an Egyptian population in which *S. cricetus* (as well as *S. rattus*) was detected in 50% of those sampled, whereas *S. mutans* was found in only 10%. Other possible explanations for the scarcity of *S. cricetus* isolates in supragingival plaque may be that (i) this species, in forming coaggregates with actinomyces, expedites removal of cellular flocs from the oral cavity, a theory proposed by Mandel (25) and others for saliva-mediated aggregation and clearance of organisms, and (ii) *S. cricetus* favors a more sequestered niche among the subgingival flora. MS have been detected in subgingival plaque and have also been associated with root caries (12, 36); however, which species of MS are present has not been elucidated. Two reports also cite the

prevalence of actinomyces in carious lesions of the root surface (17, 36), and another identifies *A. odontolyticus* as being significantly associated with progressive carious lesions (1).

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