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# *Aggregatibacter actinomycetemcomitans* serotype f O-polysaccharide mediates coaggregation with *Fusobacterium nucleatum*

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*Rupani D, Izano EA, Schreiner HC, Fine DH, Kaplan JB.* Aggregatibacter actinomycetemcomitans *serotype f O-polysaccharide mediates coaggregation with* Fusobacterium nucleatum.
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**Background/aims:** Intergeneric bacterial coaggregation may play an important role in plaque development.
**Methods:** In this study we investigated the coaggregation reaction between two periodontal pathogens, *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*.
**Results:** Previous studies showed that *A. actinomycetemcomitans* serotype b strains coaggregate with *F. nucleatum* strain PK1594, and that *A. actinomycetemcomitans* serotype b O-polysaccharide (O-PS) is the receptor responsible for coaggregation between *A. actinomycetemcomitans* and *F. nucleatum*. *A. actinomycetemcomitans* serotype f O-PS has been shown to be structurally and antigenically related to serotype b O-PS. In the present study we show that *A. actinomycetemcomitans* strain CU1060N, a serotype f strain, also coaggregated with *F. nucleatum* PK1594. Like coaggregation between serotype b strains and *F. nucleatum*, coaggregation between CU1060N and *F. nucleatum* was inhibited by galactose. An O-PS mutant of CU1060N failed to coaggregate with *F. nucleatum*.
**Conclusion:** We concluded that *A. actinomycetemcomitans* serotype f O-PS, like serotype b O-PS, mediates coaggregation between *A. actinomycetemcomitans* and fusobacteria.

Key words: *Actinobacillus actinomycetemcomitans*; ethidium bromide; rough colony; smooth colony

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Attachment of bacterial cells to host tissue is a prerequisite for colonization of the oral cavity. Oral bacteria can attach to dental or mucosal surfaces, or to cells of other bacterial species that have already colonized an oral surface. This last process, termed intergeneric coaggregation, is thought to play an important role in the development of dental plaque (10). Coaggregation may influence the species composition and metabolic activity of healthy and pathogenic dental plaque and other oral biofilms. Many prevalent oral bacteria exhibit intergeneric coaggregation phenotypes *in vitro* (9).

Coaggregation between the two gram-negative periodontopathogens *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) and *Fusobacterium nucleatum* has been studied in detail. Kolenbrander et al. (8) showed that *F. nucleatum* strain PK1594 coaggregated with *A. actinomycetemcomitans* strains Y4 and N27, two serotype b strains. Rosen et al. (14) showed that coaggregation between *A. actinomycetemcomitans* serotype b strains and fusobacteria was inhibited by *N*-acetylgalactosamine (GalNAc) or galactose, or by purified serotype b lipopolysaccharide (LPS), but

not by purified serotype a LPS. These findings indicate that the galactose-containing O-polysaccharide (O-PS) region of serotype b LPS mediates coaggregation with fusobacteria. The *F. nucleatum* adhesin that binds to *A. actinomycetemcomitans* serotype b O-PS is a 30-kDa outer membrane protein that also mediates other galactose-sensitive interactions of *F. nucleatum* including coaggregation with *Porphyromonas gingivalis*, hemagglutination, and attachment to mammalian cells (15, 18).

*A. actinomycetemcomitans* strains are classified into six serotypes (a, b, c, d, e, and f) corresponding to six structurally and antigenically distinct O-PS components of their respective LPS molecules (7). No studies on the coaggregation reactions between *F. nucleatum* and *A. actinomycetemcomitans* serotype c, d, e, or f strains have been reported. Previous studies showed that serotype f O-PS is structurally and antigenically related to serotype b O-PS (7). Both molecules contain a β-D-Gal*p*NAc single non-reducing end group linked to a linear polysaccharide backbone consisting of a repeating disaccharide unit. The observed structural similarity between *A. actinomycetemcomitans* serotype b and serotype f O-PS raises the possibility that serotype f strains may also coaggregate with fusobacteria. The purpose of the present study was to investigate the coaggregation reaction between *F. nucleatum* and all six known *A. actinomycetemcomitans* serotypes. In this report we present evidence that *A. actinomycetemcomitans* serotype b and f strains, but not serotype a, c, d, or e strains, coaggregate with fusobacteria, and that the O-PS molecule of *A. actinomycetemcomitans* serotype f strains is responsible for coaggregation with fusobacteria.

## Materials and methods

### Bacterial strains and culture conditions

The *A. actinomycetemcomitans* strains used in the coaggregation assays are listed in Table 1. All strains exhibited a smooth-colony morphology on agar and were deficient in autoaggregation in broth (3). Strain IDH781S is a smooth-colony variant of clinical isolate IDH781 (6). Strain CU1060N is a spontaneous nalidixic acid-resistant variant of smooth-colony strain CU1060 (3). *A. actinomycetemcomitans* strains were maintained on tryptic soy agar containing 6 g/l yeast extract and 8 g/l glucose. *A. actinomycetemcomitans* cultures were incubated at 37°C in 10% $CO_2$ and were passaged twice weekly. *F. nucleatum* strains PK1594 and ATCC 10953 were grown in a MACS MG 250 anaerobic chamber (Microbiology International, Frederick, MD) at 37°C in 10% $CO_2$, 10% $H_2$ and 80% $N_2$. *F. nucleatum* cells were cultured on blood agar plates and passaged twice weekly.

*Table 1.* Coaggregation of *Aggregatibacter actinomycetemcomitans* smooth-colony strains with *Fusobacterium nucleatum* PK1594

| *A. actinomycetemcomitans* strain | Serotype | Source or reference[1] | Coaggregation phenotype[2] | |
|---|---|---|---|---|
| | | | − Gal | + Gal |
| SUNYab75 | a | ATCC | − | ND |
| ATCC 29523 | a | ATCC | − | ND |
| ATCC 29524 | b | ATCC | + | − |
| HK1651 | b | ATCC | + | − |
| Y4 | b | ATCC | + | − |
| JP2 | b | E. Lally | + | − |
| Aa307 | c | J. Zambon | − | ND |
| IDH781S | d | S. Kachlany | − | ND |
| IDH1705 | e | (6) | − | ND |
| CU1060N | f | D. Figurski | + | − |
| HS1035 (CU1060N *orfF1*::Tn*903*ϕ*kan*) | f | This study | − | ND |

[1]ATCC, American Type Culture Collection.
[2]Coaggregation phenotypes in the absence or presence of 10 mM galactose (Gal). ND, not done.

### Test tube coaggregation assay

For each test strain, a loopful of bacterial cells from an agar plate was transferred to a 1.5-ml microcentrifuge tube containing 500 µl coaggregation buffer [10 mM Tris (pH 8.0), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl]. The cells were dispersed with a disposable pellet pestle and then further dispersed by vortex agitation for 30 s. The absorbance of the cell suspension (at 595 nm) was adjusted to 0.6. Aliquots of each coaggregation partner (50 µl) were mixed in a 6 × 50-mm glass test tube (Fisher Scientific, Springfield, NJ). For controls, 100 µl of each test strain alone was transferred to a test tube. Tubes were incubated statically at room temperature and visually inspected after 15–30 min. A positive coaggregation phenotype was assigned when cells formed aggregates that settled to the bottom of the tube and the broth became clear (Fig. 1A). A negative coaggregation phenotype was assigned when the cell suspension remained turbid with few visible cell aggregates. Inhibition of coaggregation by galactose was determined by adding 0.1 volume of 100 mM galactose (in coaggregation buffer) to each cell suspension before mixing.

### Ethidium bromide coaggregation assay

Cell suspensions were prepared in coaggregation buffer as described above. Cells were stained by adding 0.1 volume of

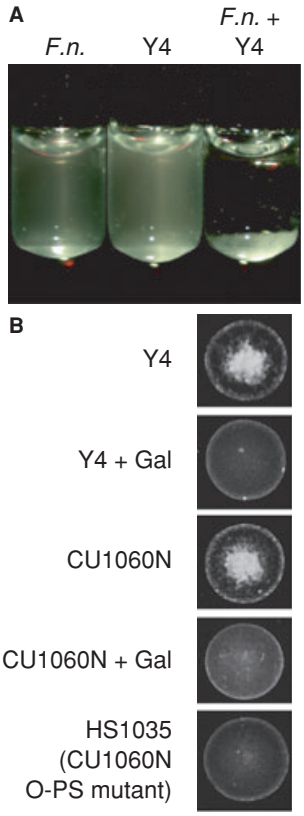


*Fig. 1.* Coaggregation reactions between *Aggregatibacter actinomycetemcomitans* strains and *Fusobacterium nucleatum* strain PK1594. (A) Test tube coaggregation assay. Tubes containing suspensions of *F. nucleatum* PK1594 (*F.n.*), *A. actinomycetemcomitans* strain Y4, or strains PK1594 plus Y4, were incubated statically for 30 min and photographed. (B) Ethidium bromide coaggregation assay. Ethidium bromide-stained cell suspensions of *F. nucleatum* PK1594 mixed with *A. actinomycetemcomitans* strains Y4, CU1060N or HS1035 were pipetted onto a cellophane sheet placed on a UV transilluminator. Cells were photographed under UV light using an orange filter after 10 min. Some assays were carried out in the presence of 10 mM galactose (Gal). Coaggregates appear as a bright spot on a dark background.

2 mg/ml ethidium bromide (in water) to each cell suspension. Cells were incubated at room temperature for 10 min, harvested by centrifugation, and then washed twice with coaggregation buffer. Cells were resuspended in fresh coaggregation buffer at an absorbance of 595 nm ($A_{595}$) of 0.6 and then mixed in equal volumes with their appropriate coaggregation partner in a polypropylene microcentrifuge tube. A volume of 25 μl of each cell mixture was pipetted onto a cellophane sheet placed on a UV transilluminator. For controls, 25 μl of each test strain alone was pipetted onto the cellophane sheet. Cells were visually inspected under UV light after 10 min. A positive coaggregation phenotype resulted in the appearance of a bright fluorescent spot on a dark background (Fig. 1B).

#### Isolation of a serotype f O-PS mutant

*A. actinomycetemcomitans* strain CU1060N was randomly mutagenized with transposon IS*903*ϕ*kan* as previously described (7). Approximately 5000 independent mutants were selected on tryptic soy agar plates containing 20 μg/ml kanamycin. Three mutants that produced rougher-textured colonies on agar, which is characteristic of O-PS mutants in gram-negative bacteria, were selected. DNA sequence analysis of polymerase chain reaction (PCR) products amplified using inverse PCR primers *kan*Start and *kan*Stop (7) indicated that the transposon in one of the rough-colony mutants (designated HS1035) inserted into *orfF1* of the serotype f-specific O-PS gene cluster (after base-pair 5678 in GenBank accession number AF213680). PCR analysis of genomic DNA from strains CU1060N and HS1035 using primer sets *kan*Start/P2, *kan*Stop/P8 and P2/P8 (7) confirmed that strain HS1035 contained a single IS*903*ϕ*kan* insertion in *orfF1*.

#### Enzyme-linked immunosorbent assay (ELISA)

Strains CU1060N and HS1035 were grown in tryptic Soy broth containing 6 g/l yeast extract and 8 g/l glucose. LPS was purified from each strain by phenol extraction as previously described (7). LPS was quantified using the *Limulus* amebocyte lysate (LAL) assay (catalog no. C0031, Associates of Cape Cod, East Falmouth, MA) using *Escherichia coli* endotoxin as a standard. The LAL assay was carried out according to the instructions provided by the manufacturer. Equal amounts of LPS from each strain were coated onto a Nunc-Immuno microtiter plate, reacted with rabbit antiserum raised against CU1000 whole cells, and then quantified by ELISA using a goat antirabbit immunoglobulin G alkaline phosphatase-conjugated secondary antibody as previously described (7).

## Results

#### *F. nucleatum* coaggregates with *A. actinomycetemcomitans* serotype b and f strains

Coaggregation between *A. actinomycetemcomitans* and *F. nucleatum* PK1594 was detected by using a visual test tube coaggregation assay (Fig. 1A). *A. actinomycetemcomitans* strains ATCC 29524, HK1651, Y4, and JP2 (all serotype b) and strain CU1060N (serotype f) exhibited a strong coaggregation phenotype (Table 1). Serotype a, c, d, and e strains did not coaggregate with *F. nucleatum* PK1594. Coaggregation of *A. actinomycetemcomitans* serotype b and serotype f strains with *F. nucleatum* PK1594 was completely inhibited by 10 mM galactose. None of the *A. actinomycetemcomitans* strains coaggregated with *F. nucleatum* strain ATCC 10953.

Coaggregation phenotypes were confirmed by using a visual ethidium bromide coaggregation assay (Fig. 1B). All coaggregation partners exhibited the same coaggregation phenotype in the ethidium bromide assay as they did in the test tube assay. In addition, all coaggregation reactions were inhibited by 10 mM galactose in the ethidium bromide assay.

#### *A. actinomycetemcomitans* serotype f O-PS mediates coaggregation with *F. nucleatum*

To determine whether O-PS plays a role in coaggregation of *A. actinomycetemcomitans* CU1060N with *F. nucleatum* PK1594, we constructed a CU1060N O-PS mutant strain. The mutant (designated HS1035) contained a transposon insertion in *orfF1*, which encodes a putative glycoside transferase required for the synthesis of serotype f O-PS (Fig. 2A). In an ELISA, LPS from HS1035 reacted weakly with anti-CU1000 rabbit antiserum compared to the reactivity exhibited by LPS from the parental strain CU1060N (Fig. 2B). These findings confirm that strain HS1035 was deficient in O-PS production. Strain HS1035 failed to coaggregate with *F. nucleatum* PK1594 in both the test tube coaggregation assay (Table 1) and the ethidium bromide coaggregation assay (Fig. 1B). These findings are consistent with the hypothesis that serotype f O-PS mediates the binding of *A. actinomycetemcomitans* CU1060N to *F. nucleatum* PK1594.

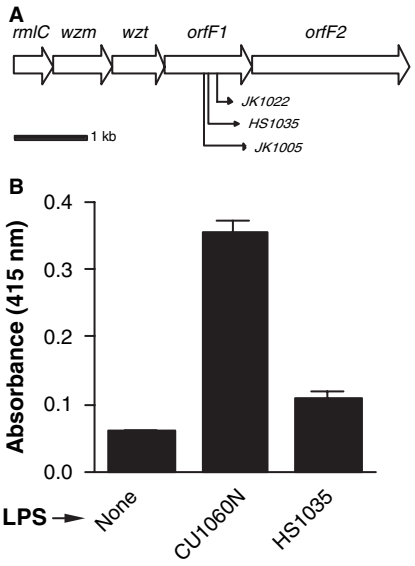


*Fig. 2.* Characterization of *Aggregatibacter actinomycetemcomitans* serotype f O-PS mutants. (A) Partial genetic map of the serotype f O-PS gene cluster (7). Open arrows indicate open reading frames and direction of transcription. Arrows below *orfF1* indicate the location and direction of transcription of transposon IS*903*ϕ*kan* insertions in three O-PS mutant strains. Mutants JK1022 and JK1005 were described in (7). (B) Cross-reactivity of *A. actinomycetemcomitans* LPS with anti-CU1000 rabbit antiserum as measured by ELISA. Values show mean and range for duplicate wells. Control wells contained no LPS.

## Discussion

In the present study we showed that *A. actinomycetemcomitans* serotype b and f strains, but not serotype a, c, d, or e strains, coaggregated with *F. nucleatum* PK1594. As shown for serotype b (14), our results suggest that the β-D-Gal*p*NAc epitope of *A. actinomycetemcomitans* sero type f O-PS mediates coaggregation with *F. nucleatum*. These findings are consistent with those of previous studies demonstrating structural and antigenic similarity between the serotype b and f O-PS molecules, and homology between the serotype b-specific and f-specific O-PS gene clusters (7).

Several studies suggest that different *A. actinomycetemcomitans* serotypes are associated with periodontal health, periodontitis, and non-oral infections. Although serotype f strains are rare in the general

population (Sirkka Asikainen, personal communication), two studies reported that serotype f strains accounted for 15–30% of strains isolated from localized aggressive periodontitis patients (7, 11). Serotype f strains are rarely isolated from healthy subjects. It is possible that serotype f strains exhibit increased pathogenic potential because of their ability to coaggregate with *F. nucleatum*, which is one of the most abundant oral bacteria and the species most often associated with destructive periodontal disease (14). *A. actinomycetemcomitans* serotype b, which also coaggregates with fusobacteria, has also been shown to be a dominant serotype in localized aggressive periodontitis patients (5).

Several studies have examined the effect of coaggregation on the species composition and metabolic activity of complex mixed-species oral biofilms grown *in vitro* or *in vivo*. Coaggregation has been shown to facilitate biofilm formation by certain oral bacteria *in vitro* (4, 13, 16, 19). One study reported that weakly-coaggregating strains of *A. actinomycetemcomitans* and *F. nucleatum* still formed mixed-species biofilms *in vitro* (2), although only one *A. actinomycetemcomitans* strain of unspecified serotype was tested. Coaggregation has also been shown to influence synergistic biochemical interactions in multispecies biofilms grown *in vitro* (1). Palmer et al. (12) showed that coaggregation plays a role in the initial colonization of retrievable intraoral enamel chips worn by human volunteers, suggesting that coaggregation may play a role in early plaque development. Serotype-specific coaggregation between *A. actinomycetemcomitans* and *F. nucleatum* could be a useful tool for studying the formation of *A. actinomycetemcomitans*/*F. nucleatum* mixed-species biofilms *in vitro*, and for studying the potential clinical significance of coaggregation between these two periodontal pathogens *in vivo*.

Suzuki et al. (17) recently showed that *A. actinomycetemcomitans* serotype c O-PS, but not serotype a, b, d, or e O-PS, mediates coaggregation between *A. actinomycetemcomitans* and *P. gingivalis* strain ATCC 33277. Serotype f strains were not tested in this study. If the complex *in vitro* serotype-specific coaggregation reactions between *A. actinomycetemcomitans* and *F. nucleatum*, and between *A. actinomycetemcomitans* and *P. gingivalis*, are relevant *in vivo*, then they probably play an important role in the ecology of *A. actinomycetemcomitans* and in the evolution of *A. actinomycetemcomitans* O-PS genes.

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