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Role of *Fusobacterium nucleatum* and Coaggregation in Anaerobe Survival in Planktonic and Biofilm Oral Microbial Communities during Aeration

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Coaggregation is a well-characterized phenomenon by which specific pairs of oral bacteria interact physically. The aim of this study was to examine the patterns of coaggregation between obligately anaerobic and oxygen-tolerant species that coexist in a model oral microbial community. Obligate anaerobes other than *Fusobacterium nucleatum* coaggregated only poorly with oxygen-tolerant species. In contrast, *F. nucleatum* was able to coaggregate not only with both oxygen-tolerant and other obligately anaerobic species but also with otherwise-noncoaggregating obligate anaerobe–oxygen-tolerant species pairs. The effects of the presence or absence of *F. nucleatum* on anaerobe survival in both the biofilm and planktonic phases of a complex community of oral bacteria grown in an aerated (gas phase, 200 ml of 5% CO_2 in air · min^{-1}) chemostat system were then investigated. In the presence of *F. nucleatum*, anaerobes persisted in high numbers (>10^7 · ml^{-1} in the planktonic phase and >10^7 · cm^{-2} in 4-day biofilms). In an equivalent culture in the absence of *F. nucleatum*, the numbers of black-pigmented anaerobes (*Porphyromonas gingivalis* and *Prevotella nigrescens*) were significantly reduced ($P \leq 0.001$) in both the planktonic phase and in 4-day biofilms, while the numbers of facultatively anaerobic bacteria increased in these communities. Coaggregation-mediated interactions between *F. nucleatum* and other species facilitated the survival of obligate anaerobes in aerated environments.

The microbial composition of dental plaque is highly diverse, with as many as 300 genera of bacteria commonly isolated. This microflora contains bacteria with widely different requirements in terms of nutrition, environmental parameters, and reaction to the presence or absence of oxygen and other gases. For example, although the oral cavity is overtly aerobic, a large proportion of the oral microflora bacteria are obligately anaerobic (19). The mechanisms by which these anaerobes survive may depend on oxygen consumption by aerotolerant species, thereby generating gradients within plaque biofilms.

Experiments using a two-stage chemostat model system, in which the second-stage chemostat vessel was aerated, showed that anaerobes were able to persist and grow in both the planktonic and biofilm phases of growth (2). A subsequent experiment showed that they were unable to persist in such a system in the absence of facultative species, even in biofilms (4). These studies suggested that intimate interspecies interactions mediated anaerobe survival under these conditions.

Coaggregation has been shown to be a highly specific mechanism by which dental plaque bacteria may interact physically. Most authors have described the potential role of coaggregation in terms of the formation of dental plaque biofilms and, in particular, in accretion of secondary colonizers to the pioneer species in plaque, and thus the development of a spatially organized community (14). It is, however, also possible that coaggregation may provide some metabolic advantage (e.g., cross-feeding, enzyme complementation) to neighboring cells by facilitating close physical juxtaposition of partner cells, as has been shown for glucose metabolism of mixtures of *Actinomyces* and streptococci (10, 11).

The aim of the present study was initially to examine the pair-wise coaggregation patterns of the 10 bacterial species included in the chemostat system and, in particular, the interactions between obligately anaerobic and oxygen-tolerant species. Following on from this, the ability of *Fusobacterium nucleatum* and other coaggregating strains to act as bridges between otherwise-noncoaggregating obligately anaerobic–oxygen-tolerant species pairs was examined. Finally, the survival of anaerobes in planktonic and biofilm communities in which *F. nucleatum* was omitted was assessed to determine whether a relationship existed between coaggregation mediated by this organism and survival in hostile conditions.

MATERIALS AND METHODS

Bacterial strains. The following bacteria were used in the study: *Streptococcus mutans* R9, *Streptococcus sanguis* 209, *Streptococcus oralis* EF186, *Lactobacillus rhamnosus* AC413, *Actinomyces naeslundii* WVU627, *Neisseria subflava* A1078, *Veillonella dispar* ATCC 17745, *Porphyromonas gingivalis* W50, *Prevotella nigrescens* T588, and *F. nucleatum* ATCC 10953. The strains were maintained in 10% glycerol in BM broth in the gas phase above liquid nitrogen (at approximately −130°C).

Coaggregation assay. Each species was inoculated into 1 liter of BM medium (20) and grown at 37°C. *N. subflava* was grown in a static culture in air for 3 days; *S. sanguis*, *S. oralis*, *S. mutans*, *A. naeslundii*, and *L. rhamnosus* were grown in static cultures in an anaerobic atmosphere (80% N_2, 10% CO_2, 10% H_2) for 4 days; and *V. dispar*, *F. nucleatum*, *P. nigrescens*, and *P. gingivalis* were grown in static cultures under an anaerobic atmosphere for 7 days. Bacteria were harvested by centrifugation at 5,000 × *g* for 20 min and resuspended in coaggregation buffer (7). This buffer contains 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.15 M NaCl, and 3.1 mM NaN_3, dissolved in 1 mM Tris adjusted to pH 8. The strains were washed twice in coaggregation buffer and finally resuspended to give an optical density of 2.0 at 660 nm. Equal volumes of 2 ml of each bacterial suspension were mixed together by vortex mixing, with each possible pair of species, in test tubes. Individual bacterial suspensions were dispensed alone as controls. Coaggregation was scored on the following scale after 90 min, as described by Cisar et al. (7): 0, no coaggregation visible; 1+, small coaggregates, remaining in solution; 2+, larger coaggregates, not immediately falling out of solution; 3+, large coaggregates, immediately clearing, slightly turbid suspension; 4+, large coaggregates, settling immediately.

Coaggregation experiments involving three organisms were also carried out. All pairs of noncoaggregating anaerobe–oxygen-tolerant species were allowed to

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TABLE 1. Coaggregation scores of pairs of the 10 oral bacterial species

Species	Coaggregation score with[a]:									
	A. naeslundii	*F. nucleatum*	*L. rhamnosus*	*N. subflava*	*P. gingivalis*	*P. nigrescens*	*S. mutans*	*S. oralis*	*S. sanguis*	*V. dispar*
A. naeslundii	1+									
F. nucleatum	4+	4+								
L. rhamnosus	2–3+	3+	2–3+							
N. subflava	2+	4+	3+	0–1+						
P. gingivalis	0	3+	2–3+	0–1+	0					
P. nigrescens	1–2+	2–3+	2–3+	0–1+	0–1+	1+				
S. mutans	1–2+	4+	3+	1+	0–1+	0–1+	0–1+			
S. oralis	0	4+	3+	0–1+	0	0–1+	2+	2+		
S. sanguis	3–4+	3–4+	2–3+	2–3+	0	0	1–2+	1–2+	2–3+	
V. dispar	1+	3+	2+	0–1+	0	0–1+	1+	1–2+	1+	0–1+

[a] Coaggregation scored as described in Materials and Methods, read at 90 min following vortex mixing.

coaggregate for 90 min. After this time, coaggregation was recorded, 2 ml of *F. nucleatum* was added, and coaggregation was scored again 90 min later.

Continuous-culture growth conditions and inoculation. The continuous-culture system consisted of a two-stage system, set up as described previously (2). Briefly, the first-stage vessel was operated anaerobically (gas phase, 5% CO_2 in N_2) and was linked to a second-stage vessel, which was supplied with a gas phase of 200 ml of 5% CO_2 in air $\cdot$ min^{-1}. The first-stage vessel was a conventional fermentor vessel, but the second-stage vessel consisted of a wide-top vessel with a top plate modified to allow the aseptic insertion and removal of hydroxyapatite discs. The growth medium was BMHGM medium (a diluted basal peptone medium supplemented with porcine gastric mucin) (5), supplied to the first-stage culture at 50 ml $\cdot$ h^{-1}, to give a dilution rate of 0.1 h^{-1} (corresponding to a mean generation time of 6.9 h). The overflow weir of the first-stage vessel was connected to the second-stage vessel, thus giving a 50-ml $\cdot$ h^{-1} flow rate of culture into the second-stage vessel. In addition, fresh medium was supplied to the second-stage vessel at a rate of 75 ml $\cdot$ h^{-1}. Culture pH was maintained in each vessel at 7.0 $\pm$ 0.1 (set point $\pm$ range) by the automatic addition of 2 M NaOH, and the temperature was maintained at 37 $\pm$ 0.1°C.

Initially, all 10 bacterial species described above were inoculated into the first-stage vessel from a pooled inoculum, which had been stored in the gas phase above liquid nitrogen, as described previously (6). Subsequently, the experiment described above was repeated but with *F. nucleatum* omitted from the inoculum, so that a nine-species culture was developed. In both experiments, growth in the first-stage vessel was allowed to reach a steady state for at least 10 times the mean generation time (i.e., 3 to 4 days) before this vessel was connected to the second-stage vessel; and growth in the second-stage vessel, in turn, was allowed to reach a steady state before experiments were begun. Following establishment of a steady state, hydroxyapatite discs were inserted in the culture for 4 days to allow biofilms to develop (3). Bacteria from biofilms and planktonic culture were enumerated by serial decimal dilution and plating onto a range of selective and nonselective agar media, followed by counting of colonies, as described previously (5).

Statistical analysis. Log_{10}-transformed counts of total numbers of bacteria and of individual species in the two aerated cultures (i.e., with and without *F. nucleatum*) were compared by using Student's *t* test, with significance assumed at a P of <0.05.

RESULTS

Pair-wise coaggregation. The aerobe *N. subflava* coaggregated only poorly with the obligate anaerobes *P. gingivalis*, *P. nigrescens*, and *V. dispar* (Table 1). All strains coaggregated with *F. nucleatum*, and this species also auto-aggregated strongly (Table 1). Coaggregations between *F. nucleatum* and *A. naeslundii*, *F. nucleatum* and *S. mutans*, and *F. nucleatum* and *S. oralis* gave unequivocal 4+ results. *S. sanguis* coaggregated strongly with *A. naeslundii*, *L. rhamnosus*, and *N. subflava*; *L. rhamnosus* also coaggregated strongly with *S. mutans*, *S. oralis*, and *N. subflava* and, to some extent, with *S. sanguis*, *P. gingivalis*, and *P. nigrescens*.

Three-species coaggregation. *F. nucleatum* was able to act as a bridge between all of the otherwise-noncoaggregating (or weakly coaggregating) pairs of aerobe-anaerobe species, in all cases tested (Table 2). Despite the strong auto-aggregation of *F. nucleatum*, the 3+ scores for the three-species coaggregates (large coaggregates, immediately clearing, leaving a slightly turbid suspension), in comparison with generally very low scores for the pairs (0 to 1+, corresponding either to no coaggregation at all or to small coaggregates, remaining in solution), indicate that the majority of the noncoaggregating bacteria were precipitated by the addition of this species.

Chemostat community composition in the first-stage (anaerobic) chemostat. The compositions of the steady-state communities that developed in the first-stage chemostat, in the presence and absence of *F. nucleatum*, are shown in detail in Table 3. The numbers of the majority of species in the two communities were not markedly different, although the viable counts of streptococci and *N. subflava* were increased significantly in the absence of *F. nucleatum*.

Community development in the aerated second-stage chemostat. (i) Environmental conditions. The environmental conditions in the aerated second-stage chemostats were broadly similar in the parallel experiments with and without *F. nucleatum*. The redox potential (E_h) was -259 ± 14 mV in the presence of *F. nucleatum* and -280 ± 65 mV in the absence of *F. nucleatum*. These values were not significantly different (*t* test, $P = 0.55$). Dissolved oxygen (dO_2) was only intermittently detectable in the culture at 0 to 20% saturation with the same gas mixture (5% CO_2 in air).

(ii) Planktonic communities. Data on the composition of steady-state, aerated planktonic cultures in the presence and absence of *F. nucleatum* are presented in Table 4. The total

TABLE 2. Coaggregation pattern of noncoaggregating anaerobe-aerobe species pairs at 90 min after *F. nucleatum* addition

Anaerobe-aerobe pair	Coaggregation score
P. gingivalis-A. naeslundii	3+
P. gingivalis-N. subflava	3+
P. gingivalis-S. mutans	3+
P. gingivalis-S. oralis	3+
P. gingivalis-S. sanguis	3+
P. nigrescens-A. naeslundii	3+
P. nigrescens-N. subflava	3+
P. nigrescens-S. mutans	3+
P. nigrescens-S. oralis	3+
P. nigrescens-S. sanguis	3+
V. dispar-A. naeslundii	3+
V. dispar-L. rhamnosus	3+
V. dispar-N. subflava	3+
V. dispar-S. mutans	3+
V. dispar-S. oralis	3+
V. dispar-S. sanguis	3+

TABLE 3. Compositions of planktonic communities in first-stage (anaerobic) chemostat in the presence and absence of *F. nucleatum*

Species	Steady-state $\log_{10}$ CFU ml^{-1}[a] (%)		P[b]
	With *F. nucleatum* ($n = 3$)	Without *F. nucleatum* ($n = 4$)	
S. mutans	5.02 ± 0.41 (0.1)	6.95 ± 0.17 (10.8)	0.0003
S. oralis	6.53 ± 0.47 (4.6)	7.24 ± 0.06 (21.1)	0.02
S. sanguis	6.26 ± 0.38 (2.5)	7.23 ± 0.19 (20.6)	0.007
L. rhamnosus	4.94 ± 0.08 (0.1)	6.02 ± 0.52 (1.3)	0.07
A. naeslundii	3.85 ± 1.21 (0.01)	5.34 ± 1.28 (0.3)	0.17
P. gingivalis	7.28 ± 0.16 (25.8)	7.26 ± 0.60 (22.1)	0.96
P. nigrescens	7.22 ± 0.31 (22.4)	6.42 ± 0.72 (3.2)	0.13
F. nucleatum	7.25 ± 0.31 (24.0)		
V. dispar	7.18 ± 0.28 (20.5)	7.23 ± 0.29 (20.6)	0.80
N. subflava	2.03 ± 0.34 (0.0001)	4.87 ± 0.12 (0.08)	<0.0001
Total	7.869 ± 0.20	7.917 ± 0.20	0.50

[a] Values are means ± standard deviations.
[b] Determined by Student's *t* test. *P* values of < 0.05 are considered significant.

TABLE 5. Compositions of 4-day biofilm communities grown in aerated conditions in the presence and absence of *F. nucleatum*

Bacterium	Biofilm $\log_{10}$ CFU · cm^{-2}[a] (%)		P[b]
	With *F. nucleatum* ($n = 3$)	Without *F. nucleatum* ($n = 4$)	
S. mutans	4.28 ± 0.13 (0.1)	6.81 ± 0.35 (9.8)	<0.001
S. oralis	5.42 ± 0.10 (1.4)	7.13 ± 0.45 (20.5)	0.002
S. sanguis	5.24 ± 0.15 (0.9)	7.02 ± 0.39 (15.9)	<0.001
L. rhamnosus	3.15 ± 0.09 (0.01)	6.86 ± 0.36 (11.1)	<0.001
A. naeslundii	3.83 ± 0.53 (0.03)	6.21 ± 0.70 (2.5)	0.005
P. gingivalis	6.45 ± 0.34 (14.9)	4.20 ± 0.86 (0.02)	0.001
P. nigrescens	6.04 ± 0.47 (5.8)	3.09 ± 0.64 (0.002)	<0.001
F. nucleatum	6.59 ± 0.32 (20.5)		
V. dispar	5.82 ± 0.10 (3.5)	6.47 ± 0.14 (4.5)	0.001
N. subflava	7.00 ± 0.22 (52.8)	7.37 ± 0.25 (35.7)	0.09
Total	7.278 ± 0.252	7.817 ± 0.24	0.02

[a] Values are means ± standard deviations.
[b] Determined by Student's *t* test. *P* values of <0.05 are considered significant.

viable count of the community was slightly increased in the absence of *F. nucleatum*. *N. subflava* predominated in the planktonic phase of both of the aerated cultures, comprising 46 and 69% of the total CFU in the 9- and 10-species communities, respectively. Anaerobes were able to persist and grow in high numbers in the planktonic phase when *F. nucleatum* was present. However, in the absence of *F. nucleatum*, the proportions of all of the anaerobes were reduced, with the log-transformed numbers of the black-pigmented species (*P. nigrescens* and *P. gingivalis*) reduced significantly (*t* test, $P < 0.001$). In contrast, the numbers of other facultative species in the community (the three streptococci and two gram-positive rods) were significantly increased in the absence of *F. nucleatum*.

(iii) Biofilm communities. The data on the composition of 4-day biofilms are shown in Table 5, and these largely mirrored the composition of the planktonic phase. The total viable counts in the biofilms developed in the absence of *F. nucleatum* were increased, but the proportions of black-pigmented anaerobes were lower, and their $\log_{10}$ viable counts were significantly reduced (*t* test, $P \leq 0.001$). This decrease contrasted with a large and significant increase in the numbers of facultatively anaerobic species and also with an increase in the numbers of *V. dispar*.

TABLE 4. Compositions of planktonic communities in aerated second-stage chemostat in the presence and absence of *F. nucleatum*

Species	Steady-state $\log_{10}$ CFU ml^{-1}[a] (%)		P[b]
	With *F. nucleatum* ($n = 6$)	Without *F. nucleatum* ($n = 5$)	
S. mutans	5.05 ± 0.92 (0.08)	6.81 ± 0.09 (2.8)	0.002
S. oralis	6.56 ± 0.26 (2.7)	7.39 ± 0.09 (10.9)	≪0.001
S. sanguis	6.33 ± 0.67 (1.6)	7.21 ± 0.12 (7.2)	0.018
L. rhamnosus	4.53 ± 0.93 (0.03)	6.49 ± 0.36 (1.4)	<0.0017
A. naeslundii	4.20 ± 0.41 (0.01)	6.58 ± 0.70 (1.7)	≪0.001
P. gingivalis	7.17 ± 0.16 (11.1)	6.37 ± 0.24 (1.0)	<0.001
P. nigrescens	7.10 ± 0.26 (9.4)	4.62 ± 1.08[c] (0.02)	≪0.001
F. nucleatum	7.44 ± 0.11 (20.6)		
V. dispar	7.04 ± 0.20 (8.2)	7.16 ± 0.41 (6.4)	0.52
N. subflava	7.79 ± 0.90 (46.2)	8.19 ± 0.21 (68.6)	0.36
Total	8.125 ± 0.31	8.351 ± 0.10	>0.05

[a] Values are means ± standard deviations.
[b] Determined by Student's *t* test. *P* values of <0.05 are considered significant.
[c] Two nondetected counts were replaced by counts of 4.00.

DISCUSSION

Bacteria are found in many natural environments which, at least superficially, appear to be hostile. For example, obligately anaerobic bacteria may be recovered from a wide variety of habitats which are highly aerated (e.g., the oral cavity, the skin, and seawater). The bulk of natural bacterial populations exist in biofilms, and these may afford protection to the constituent organisms (8, 9). The majority of natural ecosystems support the growth of diverse mixed populations of bacteria, which often appear to have conflicting environmental requirements (e.g., aerobes and obligate anaerobes coexisting at a site) (15). A wide spectrum of physical, biochemical, and genetic interactions between different species enable bacteria in nature to exist as true communities, as suggested by Wimpenny (23). In multispecies biofilms, distinct activity domains may develop, depending on the spatial organization of bacteria and on their metabolic activities. As a result of these factors, spatial heterogeneity develops; this can allow sharp gradients of nutrients, pH, and oxygen, etc., to develop over relatively short distances. Thus, species with radically differing requirements can coexist in biofilms.

Chemostats have been used to model oral microbial communities (5, 18, 20), and surfaces have been introduced into these systems to allow biofilm development (3). Recently, we have examined the effects of aeration on the development of these oral bacterial communities. Initial experiments examined the importance of the "aerobe" *N. subflava* in the reaction of the community to oxygen. The *N. subflava* population was increased in aerated cultures, and the oxygen added was rapidly depleted. The numbers of anaerobes remained high ($>10^7$ ml^{-1}). In the absence of *N. subflava*, oxygen remained in higher concentrations in the culture and yet the anaerobes were still able to persist and grow in both the planktonic and biofilm phases of growth (2). A later study showed that anaerobes were able to survive only when facultative or aerobic species were present. In the absence of such species, the anaerobes died quickly, and even biofilms could not provide a haven for the anaerobes under these circumstances (4).

A number of previous studies have suggested an important role for *F. nucleatum* in the microbial ecology of the oral cavity. This species has been proposed as a key organism in bridging between early colonizers (such as streptococci) and bacteria (especially obligate anaerobes) more usually associated with mature dental plaque (14). This phenomenon has thus usually been considered in terms of facilitating an ordered microbial succession during the formation of dental plaque and in the development of a structured "climax plaque." In the present study, aerobes and facultative species were unable to coaggregate with any of the anaerobes except *F. nucleatum*. Thus, the protection afforded to these anaerobes by growth in a mixed culture, which was apparent in our previous studies (2, 4), could not be mediated by direct coaggregation. The coaggregation patterns of *F. nucleatum* suggested a possible role for this organism in this protective effect. Indeed, this study has shown that *F. nucleatum* could act as a bridge between otherwise-noncoaggregating pairs to form three-species aggregates.

A striking finding from these studies was the persistence of obligate anaerobes in aerated environments, not only in biofilms but also in planktonic cultures. When *F. nucleatum* was omitted from the inoculum, however, the composition of the aerated community was significantly perturbed. In particular, the viable counts of the black-pigmented anaerobes *P. gingivalis* and *P. nigrescens* were significantly reduced (1,000- and 100-fold, respectively). The fact that these effects were observed in both biofilm and planktonic phases of growth suggests that ordered, metabolically organized aggregates were responsible for the persistence of the anaerobes. The data suggest that gradients in oxygen must be generated within these aggregates over relatively short distances. Metabolism of aerobic and/or oxygen-tolerant species may reduce the concentration of oxygen to levels that the defenses of obligate anaerobes are able to detoxify (15). These microbial community effects result from intimate physical contact, spatial organization, and efficient metabolic coupling, as was reported with glucose metabolism by coaggregating *Streptococcus-Actinomyces* pairs (10, 11). This study indicates that microbial community effects may be as important as, if not more important than, the beneficial effects often ascribed to the biofilm mode of growth for oral bacteria.

The findings of this study have shown that coaggregation may provide additional benefits to the interacting species, beyond promoting adherence and facilitating bacterial succession. In addition to allowing close coupling of obligate anaerobes with oxygen-tolerant species, coaggregation may encourage other important interactions. Mixed consortia are required for the efficient degradation of complex host proteins and glycoproteins (1, 12, 13, 21, 22). It may be that coaggregation is also a strategy for increasing the probability of interactions between species with complementary metabolic capabilities. This helps to explain the powerful homeostatic properties of oral microbial communities, despite regular perturbations (16). It also follows that interfering with community interactions may be a promising route towards an ecological approach to plaque control (17).

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