Characterization of Coaggregation of *Fusobacterium nucleatum* PK1594 with Six *Porphyromonas gingivalis* Strains

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Abstract

Coaggregation is a key mechanism in biofilm formation. The aim of this study was to characterize the coaggregation of *Fusobacterium nucleatum* PK1594 with six *Porphyromonas gingivalis* strains in terms of kinetics and sugars inhibition. This coaggregation was quantitatively characterized by using a kinetic coaggregation assay. Sugar inhibition profiles were also quantitatively defined. Four types of interactions among these coaggregation partners were found: (1) fast coaggregation that was substantially inhibited by galactose, lactose, and fucose (strain PK1924); (2) fast coaggregation that was not inhibited by any of the sugars tested (strain 274); (3) slow coaggregation that was either substantially or partially inhibited by the sugars mentioned (strains HG405 and W50, respectively); and (4) strains that did not coaggregate with the fusobacteria (ATCC33277 and A7436). These results suggest that adhesin(s) other than the well-known galactose-mediated ones may be involved in coaggregation between *F. nucleatum* PK1594 and *P. gingivalis* strains. *(J Endod 2009;35:50–54)*

Key Words

Biofilm, coaggregation, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*

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Bacterial biofilms have been observed in both infected root canals and periradicular tissues (1–5). They represent a common mechanism by which bacteria colonize and survive in their econiches (6).

Nair et al. (2) have recently shown that untreated recesses of root canals harbor bacteria in an organized biofilm, resembling to great extent the structure of dental plaque (2). Abundant intercellular matrix with a mixed bacterial population was observed including many bacteria with a fusiform morphology, resembling that of *Fusobacterium nucleatum* (2). These biofilms seemed to be unaffected by the irrigation of 5.25% sodium hypochlorite used during nonsurgical endodontic treatment.

Biofilms of endodontic pathogens have recently been studied for their susceptibility to root canal bactericidal agents (7–10). It became apparent that the high sensitivity of planktonic bacteria to substances such as sodium hypochlorite is greatly reduced when the same bacterium grows as a biofilm (7–9). These studies with single-species biofilms were recently extended to include dual-species biofilms of *F. nucleatum* and *Peptostreptococcus micros* (11). It was established that bacteria in these dual-species biofilms were even more resistant to the effect of sodium hypochlorite than their respective single-species biofilms. Furthermore, even bacterial susceptibility to antibiotics may dramatically change when they grow as a biofilm (12).

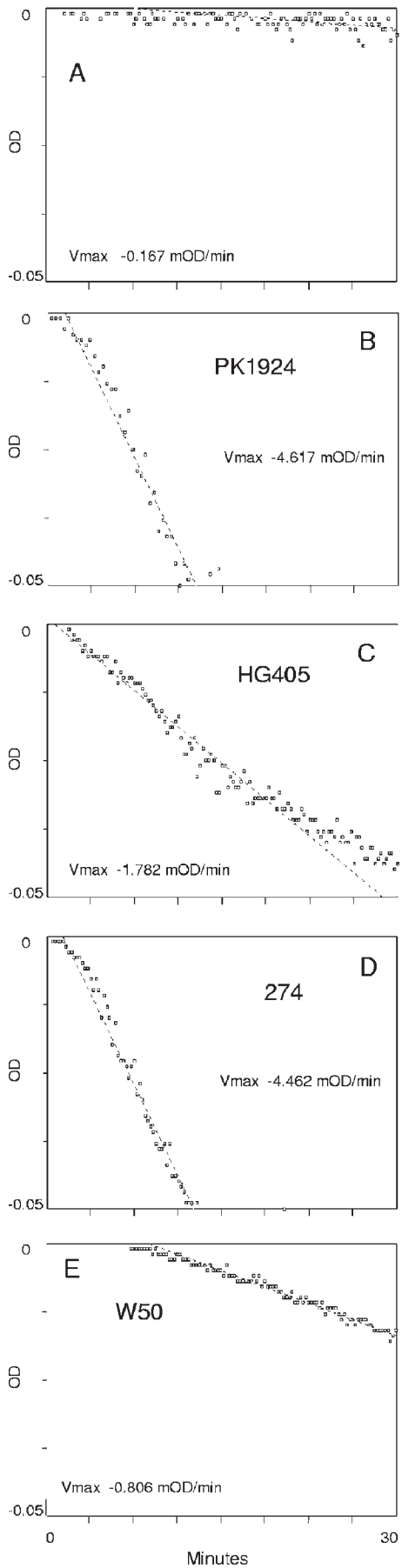
Some of the previously mentioned items may result from simple protection by the multilayer structure of the biofilm and its intercellular matrix; however, "quorum-sensing" mechanisms in this bacterial community may be of even greater significance (13, 14). Gene expression may be affected by such mechanisms, allowing the bacteria to become less susceptible to the antimicrobial agents.

Bacterial coaggregation is a key mechanism in biofilm formation. As such, it was extensively studied in the context of dental plaque formation and periodontal disease (15–20). Kolebrander et al. (18–20) and Rickard et al. (21) greatly advanced our understanding of dental biofilm formation and concluded that *F. nucleatum* is a key component in this process, serving both as an early colonizer and a "bridge organism" that facilitates colonization of other bacteria (18–21).

Consequently, adhesin-mediated coaggregation between *F. nucleatum* and pathogenic strains of *Porphyromonas gingivalis* has been extensively studied by this group as well as others (16, 17, 22). It was established that galactose-mediated adhesin(s) were involved in these interactions (18, 19, 22). Furthermore, it has been shown that the same adhesin is also involved in the attachment of *F. nucleatum* PK1594 to mammalian cells, thus enabling it to be an early colonizer on host soft tissues as well (23).

A recent extensive study has extended these observations to the endopathogenic flora (24). Sixty-two strains were isolated from 10 cases of acute endodontic infection and studied for coaggregation. Of the 183 pairs tested, 81% coaggregated; one of the strongest coaggregations was observed with *F. nucleatum* (24). *F. nucleatum* is also the most frequently isolated bacterium from infected root canals with high positive correlation with *Porphyromonas endodontalis* (25). Similarly, *F. nucleatum* and *P. gingivalis* are the most common bacteria encountered in extraradicular biofilms covering the apices of roots with refractory apical periodontitis (5).

The elimination of bacterial biofilms in mechanically inaccessible recesses of the root canal or the extraradicular environment is a challenging task. One potential way to



explore this avenue is to find or develop bactericidal agents that will be effective on biofilm-residing bacteria (26). Another may be to study the mechanisms by which bacterial cells bind to each other in order to find ways to prevent this binding or design strategies to disperse them. Exploring coaggregation mechanisms is a potential way to address this issue.

The present study was designed to explore the interactions of *F. nucleatum* PK1594 with a group of six pathogenic *P. gingivalis* strains and test them for sugar-inhibition profiles.

Materials and Methods

Bacterial Strains and Growth Conditions

F. nucleatum PK1594 *and P. gingivalis* strains PK-1924, 274, HG405, W50, A7436, and ATCC 33277 were used in the present study. *P. gingivalis* PK-1924 was used as a coaggregating partner of *F. nucleatum* PK1594 in several studies (18, 19, 23) and was used in the present study as a reference strain (23). The pathogenicity of *P. gingivalis* 274 was studied by Sundqvist et al. (27) and that of *P. gingivalis* strains HG405, W50, A7436, and ATCC 33277 by Genco et al. (28) using the subcutaneous mouse chamber model.

All strains were grown anaerobically in Wilkins-Chalgren anaerobic broth (Oxoid Ltd, Basingstoke, Hampshire, England) in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 at 37°C (Coy anaerobic chamber). Bacterial strains were kept as frozen stocks and were started and transferred twice in this growth medium before being used in the experiments. Cells at late exponential to early stationary stage of growth were harvested and washed three times in a coaggregation buffer consisting of 0.1 mmol/L $CaCl_2$, 0.1 mmol/L $MgCl_2$, and 0.15 mol/L NaCl in 1.0 mmol/L Tris adjusted to pH 8.0 (15, 22). Bacteria were then resuspended in this buffer to an absorbance of $A_{660} = 1.0$ and kept at 4°C until used in the experiments.

Coaggregation Assay

The Thermomax-automated microtiter plate reader (Molecular Devices Corp, Sunnyvale, CA) was used to quantitatively study the kinetics of coaggregation using the method described by Metzger et al. (29). Briefly, flat bottom 96-well microtiter plates (Maxisorp-immunoplates, Nunc, Rochester, NY) were pretreated with 0.05% Tween-20 in phosphate-buffered saline (pH 6.8) for 30 minutes, the buffer was then discarded, and the plates were allowed to dry. Bacterial suspensions of each of the test strains, in the coaggregation buffer, were adjusted to A_{660} 0.5 or 1.0 and added to the wells, fusobacteria first, followed by the porphyromonads. Cell ratios of coaggregating partners have been previously reported to greatly affect the reaction's outcome (18, 19, 29). Therefore, each coaggregating pair was tested with the following *P. gingivalis:F. nucleatum* cell ratios: 9:1, 3:1, 2:1, 1:1, 1:2, 1:3, and 1:9. Controls consisting of each of the strains alone were also included in each experiment. The final volume in each well was kept at 110 μL. Each variable was studied in quadruplicate, and each experiment was designed so that it could be performed in a single 96-well microtiter

Figure 1. Coaggregation patterns of *F. nucleatum* PK1524 with four *P. gingivalis* strains. (*A*) *F. nucleatum* PK1594 alone (control). Coaggregation between (*B*) *F. nucleatum* PK1594 and *P. gingivalis* PK1924, (*C*) *P. gingivalis* HG405, (*D*) *P. gingivalis* 274, and (*E*) *P. gingivalis* W50. Each panel presents the up to 129 readings performed in a single representative well using the Vmax-automated coaggregation assay. Each well contained 110 μL of bacterial suspension ($A_{660} = 1.0$), with the *F. nucleatum* to *P. gingivalis* cell ratio of 1:1. The oblique dotted line represents the maximal coaggregation rate as defined by the software. *P. gingivalis* cell suspension alone gave results similar to that shown in panel *A*.

TABLE 1. Interaction of *F. nucleatum* PK1594 with Six *P. gingivalis* Strains

P. gingivalis Strain	Coaggregation with *F. nucleatum* PK1594	Optimal *P.g.:F.n.* Ratio	Maximal Coaggregation Rate −mO/min
PK1924	+	1:1	4.60 (±0.24)
274	+	1:1	4.37 (±0.03)
HG405	+	2:1	1.99 (±0.0.28)
W50	+	1:1	0.78 (±0.03)
ATCC 33277	0	—	—
A7436	0	—	—

Bacterial suspensions (A_{660} = 1.0) of *F. nucleatum* PK1594 and each of the *P. gingivalis* strains were mixed at *F. nucleatum: P. gingivalis* cell ratios ranging from 1:9 to 9:1 and the ratio resulting in a maximal rate of coaggregation determined.
P.g., P. gingivalis; F.n., F. nucleatum.

plate. The results of each plate were analyzed separately. Each experiment was performed at least twice.

The fusobacteria were placed first in the wells, immediately followed by the second partner, and the plates were inserted into the reader. Kinetics of the coaggregation was followed for 30 minutes by monitoring the decline in optical density (OD). The plate reader was set to read each well every 14 seconds, with repeated mixing, and the reading limit was set to −0.05 OD. The OD diminished gradually with the progress of coaggregation. The maximal slope of the resulting curve was designated as the "Vmax value," expressed in −mOD/min, which was used here as a value expressing the rate of coaggregation (29). For each group consisting of four wells, a mean Vmax value was calculated, and the standard error of the mean usually did not exceed 5% to 10%.

Sugar Inhibitors

When testing the effect of the sugars, this assay allowed a quantitative comparison of the degree of inhibition provided by each of them. Galactose, lactose (Galβ1-4Glc), or fucose (6-deoxy galactose) was used, at 60 mmol/L, to inhibit the coaggregation, whereas glucose or mannose, at the same concentration, was used as the control.

Statistical Analysis

The correlation coefficient of the resulting aggregation curves was automatically calculated by the Softmax software (Molecular Devices Corp) serving the plate reader and was usually >93%, unless otherwise noted. A Student *t* test was used to compare between the groups in the sugar blocking assays.

Results

Coaggregation Kinetics

When *F. nucleatum* PK1594 was mixed with *P. gingivalis*, coaggregation occurred, and its rate (expressed as −mOD/min) could be quantitatively followed (Fig. 1). *P. gingivalis* PK1924 and 274 aggregated with *F. nucleatum* PK 1594 at fast rates of 4.60 (±0.24) and 4.37 (±0.30) −mOD/min, respectively (Fig. 1). *P. gingivalis* HG405 coaggregated with *F. nucleatum* PK1594 at a slower rate of 1.81 (±0.20) −mOD/min, whereas strain W50 coaggregated at a low rate of 0.78 (±0.03) −mOD/min (Fig. 1). No coaggregation occurred between *F. nucleatum* PK1594 and *P. gingivalis* strains A7436 and ATCC33277 (Table 1).

Because coaggregation with *P. gingivalis* strains PK1924 and 274 was fast, the following experiments with these strains were performed at A_{660} = 0.5 to minimize the potential influence of variations in handling time on the results (29). Coaggregation rates were influenced by bacterial proportion in the mixture (Fig. 2). When *F. nucleatum* PK1594 was mixed with *P. gingivalis* PK1924 at A_{660} = 0.5, maximal coaggregation rate of 3.20 (±0.04) −mOD/min occurred at a ratio of 1:1. Maximal coaggregation rates with *P. gingivalis* strains W50 and 274 also occurred at a cell ratio of 1:1 (Table 1). With *P. gingivalis* HG405, a maximal coaggregation rate occurred with a *F. nucleatum*:*P. gingivalis* ratio of 2:1.

Sugar Inhibitors

Galactose, lactose, and fucose (6-deoxy galactose), at a concentration of 60 mmol/L, inhibited the coaggregation of *F. nucleatum* PK1594 with three of the tested *P. gingivalis* strains (Fig. 3). The coaggregation with strain PK1924 was inhibited by these sugars by 76%, 80%, and 82%, respectively ($p < 0.001$), Coaggregation with strain HG405 was inhibited by these sugars by 80%, 89%, and 59% ($p < 0.001$), whereas the coaggregation rate with strain W50 was reduced only by 62%, 43%, and 62%, respectively ($p < 0.01$). On the other hand, the coaggregation of *F. nucleatum* PK1594 with *P. gingivalis* 274 was unaffected by these sugars (Fig. 3). Glucose and mannose, which served as controls, had no effect on the coaggregation of any of the previously mentioned pairs (data not presented).

Discussion

Coaggregation between *F. nucleatum* and *P. gingivalis* strains was previously studied using either a macroscopic visual coaggregation assay (15, 18, 19), assays based on radioactive labeling of bacteria (23, 31), or fluorochromes and confocal microscopy (24). In the present study, the kinetic coaggregation assay, introduced by Metzger et al. (29) was used, which uses the coaggregation rate as a quantitative parameter rather than the end result used in all former assays. Koop et al. (30) defined two phases in bacterial coaggregation, the first in which small nonsedimenting coaggregates are formed and a second in which fast sedimentation of the heavy aggregates occurs. The coaggregation assay used in the present study kinetically followed this first initial phase in which aggregates were being formed. Many of the former assays evaluated mainly the end result, namely the presence of large sedimented aggregates. The assay required certain adaptations when fast coaggregating pairs were concerned. When the fast coaggregating strains were used, with bacterial suspensions of A_{660} = 1.0, the assay reached its −0.05 mOD limit within 13 minutes. This did not present a problem when running screening experiments such as presented in Figure 1. Nevertheless, when running more complex experiments, which re-

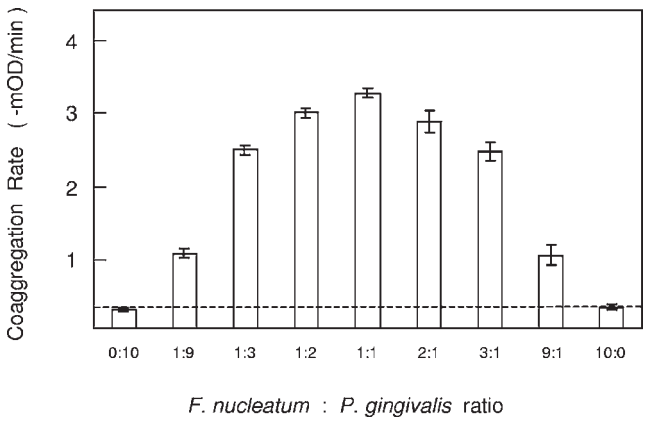


Figure 2. Bacterial cell ratios and coaggregation rates. Coaggregation rates between *F. nucleatum* PK1594 and *P. gingivalis* PK1924 as a function of bacterial cell ratios (bacterial suspensions of A_{660} = 0.5). Each bar represents the mean coaggregation rate, expressed in −mOD/min, in four wells (± standard error of the mean). The dotted line represents the higher of the background spontaneous sedimentation rates of either of the bacteria alone.

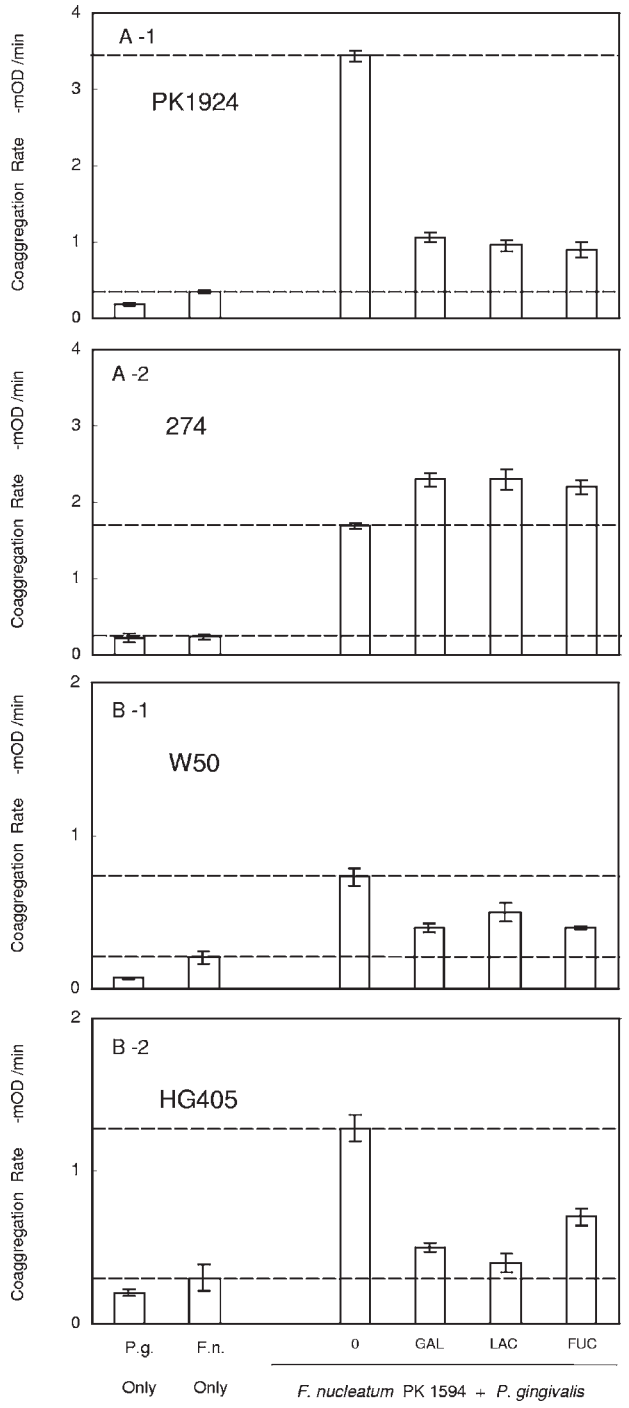


Figure 3. Sugar inhibition of coaggregation between *F. nucleatum* PK1594 and four *P. gingivalis* strains. Coaggregation rates in wells containing bacterial combination in proportions that resulted in maximal coaggregation rates. *F. nucleatum* and *P. gingivalis* alone serve as controls. Sugars were used at a final concentration of 60 mmol/L to test for their inhibition of coaggregation. The upper dotted line represents the coaggregation rate of the bacteria with no sugar added. The lower dotted line represents the higher of the background spontaneous sedimenteation rates of each of the bacteria alone. A-1 and A-2 were high coaggregating pairs and were used at A_{660} = 0.5. B1 and B2 were low coaggregating pairs and were used at A_{660} = 1.0 (note also the change in scale). Each bar represents the mean coaggregation rate, expressed in −mOD/min, in four wells (± standard error of the mean). P.g., *P. gingivalis* alone; F.n., *F. nucleatum* alone; GAL, galactose; LAC, lactose; FUC, fucose. Glucose and mannose had no inhibitory effect (data not presented).

quired the simultaneous use of many of the wells of the microtiter plate (such as the sugar inhibition assays), handling time became a critical issue. The elapsed time between adding the second coaggregating partner to the first and last wells may greatly influence the results in case of an extremely fast coaggregation (29). To overcome this problem, two approaches were taken: (1) the bacterial suspensions were used at A_{660} = 0.5 to slow down the coaggregation rate and (2) two positive control groups were included in each plate, one that was handled first and another that was handled last, when adding the second coaggregating partner. Only by verifying that these two controls gave identical results could one be sure that the handling-time problem did not affect the results. Lowering the concentration of the bacterial suspensions of the fast coaggregating pairs to A_{660}= 0.5 proved to be essential in order to reproducibly achieve this goal with the dual controls.

When variation of kinetics of the interaction between the strains was taken together with the pattern of sugar inhibition, four patterns of *P. gingivalis* interaction with *F. nucleatum* PK1594 could be defined: (1) fast coaggregation (with a rate higher than 4.0 −mOD/min) that was substantially inhibited by the sugars (strain PK1924), (2) fast coaggregation that was not affected by the sugars tested (strain 274), (3) slow coaggregation (with a rate lower than 2.0 −mOD/min) that was either substantially or partially inhibited by the sugars (strains HG405 and W50, respectively), and (c) no coaggregation with *F. nucleatum* PK1594 (strains A7436 and ATCC 33277).

Based on the sugar-inhibition profiles, the coaggregation *of P. gingivalis* PK1924, HG405, and W50 with *F. nucleatum* PK1594 most probably involved the galactose- and lactose-mediated adhesin of the latter (18, 19, 22, 23). Nevertheless, the effective but not total inhibition in the case of PK1924 and HG405 and its partial nature in the case of strain W50 may indicate potential involvement of additional adhesins. The fourth coaggregating strain, *P. gingivalis* 274, most probably interacted with *F. nucleatum* PK1594 by a different adhesin, which was not inhibited by any of the sugars tested.

Shaniztki et al. (31) defined at least three different coaggregating mechanisms of *F. nucleatum* PK1594: one involving a galactose-mediated adhesin, which mediates the coaggregation with *P. gingivalis* PK1924, another that involves a N-acetylneuraminic acid–mediated adhesin that was responsible for its coaggregation with *Actinomyces israelii* PK16, and a third coaggregation mechanism with *Actinomyces naeslundii* T14V for which no inhibitor was found. It is possible that the coaggregation with strain 274 was mediated by one of the other adhesins, which could have also potentially contributed to the coaggregation with strains HG405 and W50. Further studies will be required to elucidate in full these interactions and their potential in vivo implications.

A better understanding of coaggregation mechanisms among endodontic pathogens may allow a better understanding of biofilm formation, potentially leading to the development of new strategies to disperse root canal biofilms, which are otherwise unaffected by current endodontic irrigating solutions.

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