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Kinetics of coaggregation of *Porphyromonas gingivalis* with *Fusobacterium nucleatum* using an automated microtiter plate assay

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Coaggregation between *Porphyromonas gingivalis* and *Fusobacterium nucleatum* strains was previously studied using either a semi-quantitative macroscopic assay or radioactive tracer assays. A new automated microtiter plate assay is introduced, in which the plate reader (Vmax) was adapted to allow quantitative evaluation of the kinetics of coaggregation. *F nucleatum* PK 1594 coaggregated with *P. gingivalis* HG 405 with a maximal coaggregation rate of 1.05 mOD/min, which occurred at a *P. gingivalis* to *F. nucleatum* cell ratio of 1 to 2. *F. nucleatum* PK 1594 failed to do so with *P. gingivalis* strains A 7436 or ATCC 33277. Galactose inhibition of this coaggregation could be quantitatively measured over a wide range of concentrations to demonstrate its dose-dependent manner. *P. gingivalis* HG 405 failed to coaggregate with *F. nucleatum* strains ATCC 25586 and ATCC 49256. The assay used in the present study is a sensitive and efficient quantitative automated tool to study coaggregation and may replace tedious radioactive tracer assays.

Key words: coaggregation; *Fusobacterium nucleatum*; *Porphyromonas gingivalis*

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Coaggregation between oral bacterial strains is dependent on a plethora of bacterial adhesins that mediate this phenomenon. The coaggregation between *Porphyromonas gingivalis* and *Fusobacterium nucleatum* has been extensively studied by Kolenbrander et al. (11, 12), by Kinder & Holt (9, 10) and recently by Shaniztki et al. (14). It has been attributed to proteinaceous adhesins on *F. nucleatum* that recognize galactose-containing saccharides on the porphyromonas. Most of these coaggregation studies were aimed at elucidating the role of these adhesins in the complex events of dental plaque formation. Many of them were directed at high-aggregating pairs of strains, which coaggregate and sediment fast.

Interactions between *P. gingivalis* and *F. nucleatum* may not be limited only to dental plaque formation. Synergistic pathogenicity of *P. gingivalis* and *F. nucleatum* has also been reported in subcutaneous abscess models (1, 5, 15). Therefore, the present study focused on *P. gingivalis* strains that were recently studied for their pathogenicity in the murine subcutaneous chamber model (6, 7).

Most previous coaggregation studies used a macroscopic evaluation scale for coaggregation (2). However, since this method allows only semi-quantitative evaluation, methods based on radioactive labeling of the bacteria have also been used to provide quantitative analysis of coaggregation and of its inhibition by specific sugars (5, 11, 16).

In the present study we introduce a new approach that allows quantitative evaluation, as well as determination of the kinetics of the coaggregation of the bacteria studied. The coaggregation properties of three *P. gingivalis* strains with three potential *F. nucleatum* partners were evaluated using a new spectrophotometric method.

We report here on an adaptation of the kinetic automated microtiter plate reader Vmax for an effective and quantitative measurement of the kinetics of coaggregation between bacterial strains and the kinetics and other characteristics of coaggregation of *F. nucleatum* PK 1594 with *P. gingivalis* HG 405 and its absence with *P. gingivalis* A 7436 or *P. gingivalis* ATCC 33277.

Material and methods

Bacterial strains and growth conditions

Three *P. gingivalis* strains, HG 405, A 7436 and ATCC 33277, were used in this study as well as *Prevotella intermedia* CB21. All of the above were tested for coaggregation with three *F. nucleatum* strains: PK 1594, ATCC 25586 and ATCC 49256.

P. gingivalis A 7436 has been shown to be highly pathogenic and disseminating in the mouse model (6, 7). Both *P. gingivalis* strains HG 405 and ATCC 33277 were capable of establishing a locally contained infection in the chamber model, which progressed to local suppurative lesions, but were incapable of dissemination even at high infectious dose (6, 7).

F. nucleatum PK 1594 has been studied previously for its coaggregation with several other *P. gingivalis* strains and has been defined as having a galactose-inhibitable adhesin that mediates its coaggregation with *P. gingivalis* PK 1924 (11, 14), as well as its attachment to mammalian cells (16). *F. nucleatum* strains ATCC 25586 (subsp. *F. nucleatum*) and ATCC 49256 (subsp. *Prevotella vincentii*) are two of the type strains for sub-species groups of *F. nucleatum* proposed by Dzink et al. (3). *P. intermedia* CB 21 has been studied by Baumgartner et al. for its synergistic pathogenicity with *F. nucleatum* VPI 10197 (1) and was used in the present study as a non–*P. gingivalis* positive control that presented high coaggregation with *F. nucleatum* PK 1594.

All of these strains were grown anaerobically in Wilkins-Chalgren anaerobic broth (Oxoid), 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 passed three times in this growth medium before being used in the experiments. The cells were harvested from an overnight culture at a late exponential stage of growth.

Macroscopic coaggregation assay

The coaggregation assay originally described by Cisar et al. (2) and modified by Kolenbrander was used (11, 12). Briefly, bacteria of an overnight culture, which reached a late exponential or early stationary phase, were washed three times in an aggregation buffer consisting of 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ 0.15 M NaCl and 0.02% NaN_3, dissolved in 1.0 mM Tris and adjusted to pH 8.0. The microorganisms were then resuspended in this buffer at an absorbance at 660 nm (A_{660})=1.0 and kept at 4°C until used in the experiments.

To determine coaggregation, 150 µl of an A_{660}=1.0 suspension of each bacterium of the pairs of strains tested were mixed at room temperature. Coaggregation was then followed visually, using the scale described by Cisar et al. (2). In this scale, 4+ corresponded to formation of large, fast settling aggregates, with the supernatant remaining water-clear; 3+ was defined by the formation of large settling aggregates, but with slightly turbid supernatant; 2+ represented the presence of definite aggregates which did not settle immediately; 1+ was characterized by finely dispersed aggregates in a turbid background, and 0 had no visible aggregates with no reduction in turbidity. In experiments in which bacterial ratios other than 1:1 were tested, the total A_{660} of the mixture was also kept at 1.0.

Microscopic examination

Each mixed bacterial suspension that was macroscopically tested for coaggregation was also examined microscopically (phase contrast at ×1000 magnification). The observation was done on a "wet mount" as described by Kolenbrander & Andersen (11).

Vmax automated coaggregation assay

To quantitatively evaluate the coaggregation rate of weak coaggregating pairs and to quantitatively evaluate the influence of competitors with this coaggregation, the Vmax kinetic microtiter plate spectrophotometric reader (Molecular Devices Corp., Sunnyvale, CA) was utilized. It was adapted for a quantitative kinetic coaggregation assay of high sensitivity and reproducibility.

Flat-bottom 96-well microtiter plates (Maxisorp-immunoplates, Nunc) were pretreated by filling the wells with a solution of 0.05% Tween-20 in PBS, adjusted to pH 6.8, for 30 min. The buffer was then discarded and the plates were allowed to dry. Bacterial suspensions of each of the test strains in aggregation buffer (as described above) were adjusted to A_{660}=0.5 and added to the wells, fusobacteria first, followed by the porphyromonads. The total volume in each well was 110 µl, and the total A_{660} was kept at 0.5, unless otherwise specified.

Different proportions between the coaggregating partners have been previously reported to dramatically influence the outcome, even to the extent that the same pair of bacteria seemed to lack coaggregating capacity when mixed at other than optimal ratios (11). Therefore, each pair of bacteria was tested with a range of *P. gingivalis*: *F. nucleatum* ratios, including 10:1, 3:1, 2:1, 1:1, 1:2, 1:3 and 1:10, and controls were included that consisted of each of the strains alone. The various bacterial proportions were achieved by dispensing the proper volumes of each of the suspensions to maintain an A_{660} of 0.5. The assays were carried out in quadruplicate.

Immediately after the suspension of the second partner was added, the plates were inserted into the Vmax plate reader and the kinetics of the coaggregation followed for 30 min by monitoring the reduction in optical density. The reader was set to read each well every 14 s with repeated shaking and the limit set to optical density −0.05. The optical density diminished gradually with the aggregation, and the maximal slope of the resulting curve was expressed as Vmax value (expressed as change in (Δ) mOD/min), which was used to estimate different rates of aggregation. The correlation coefficient of the resulting curves was usually >93%, unless otherwise noted. For each group consisting of four wells, a mean Vmax value was calculated with a standard error of the mean usually within 5%.

Fusobacteria were always added first, while the second partner was added after a 2-min pause. This proved to be important, as it eliminated from the assay the slight background spontaneous sedimentation encountered with some fusobacterial strains. This slight sedimentation occurred before the plates were read and did not interfere with the assay.

Statistical analysis

For each of the coaggregation curves, a correlation coefficient was calculated by the Vmax software. Coaggregation rates (Vmax values) were expressed as means recorded in quadruplet wells. These values were compared to each other using Student's *t*-test.

Results

Macroscopic and microscopic evaluation of coaggregation

Coaggregation of *P. intermedia* CB 21 with *F. nucleatum* PK 1594 was macroscopically graded as 4+; microscopic examination revealed large coaggregates with no free unassociated bacteria in the background (Table 1). Coaggregation of *P. gingivalis* HG 405 with *F. nucleatum* PK 1594 was macroscopically graded as 2+. Microscopic examination revealed large coaggregates that were smaller than those of the previous pair; nevertheless, very few free bacteria could be found in the background. *P. gingivalis* A 7436 or ATCC 33277 failed to coaggregate with *F. nucleatum* PK 1594 (Table 1), which was verified microscopically.

Testing *F. nucleatum* strains ATCC 25586 and ATCC 49256 presented a problem: their homologous suspensions rapidly and spontaneously auto-aggregated, precluding true determination of their coaggregating relationships with the test *P. gingivalis* strains. Using the macroscopic assay alone, they could have been erroneously evaluated as "3+", as they presented with large aggregates with a turbid background. Nevertheless, microscopic examination revealed that the aggregates consisted of fusobacteria only, with no involvement of porphyromonads. Many porphyromonads could be seen in the background between the aggregates, with no apparent relation to the fusobacteria.

The size of the coaggregates of *P. gingivalis* HG 405 with *F. nucleatum* PK 1594 was affected by cell ratios, with smaller coaggregates formed at ratios higher or lower than 1:1. When only small coaggregates (containing few cells) were seen microscopically, no coaggregation could be observed in the macroscopic assay.

Table 1. Macroscopic and microscopic evaluation of coaggregation of *F. nucleatum* and *P. gingivalis* strain pairs

			Microscopic observation[b]	
		Macroscopic assay[a]	Aggregate	Bacteria in background
F. nucleatum PK 1594	*P. gingivalis* HG 405	2+	Large	Few
	P. gingivalis A 7436	0	None	All
	P. gingivalis ATCC 33277	0	None	All
	P. intermedia CB 21	4+	Large	None
P. gingivalis HG 405	*F. nucleatum* PK 1594	2+	Large	Few
	F. nucleatum ATCC 25568	"3+"*	*F. nucleatum* only	*P. gingivalis*
	F. nucleatum ATCC 49256	"3+"*	*F. nucleatum* only	*P. gingivalis*

[a] Macroscopic scale: 0 through 4+, as detailed in the text.
[b] Microscopic observation: presence of bacteria and aggregates.
* Aggregates of *F. nucleatum* alone with a turbid background of *P. gingivalis* alone could be erroneously interpreted as 3+ on the macroscopic scale. The microscopic observation revealed that the aggregates were made of *F. nucleatum* only and that there were no interrelations between the two strains.

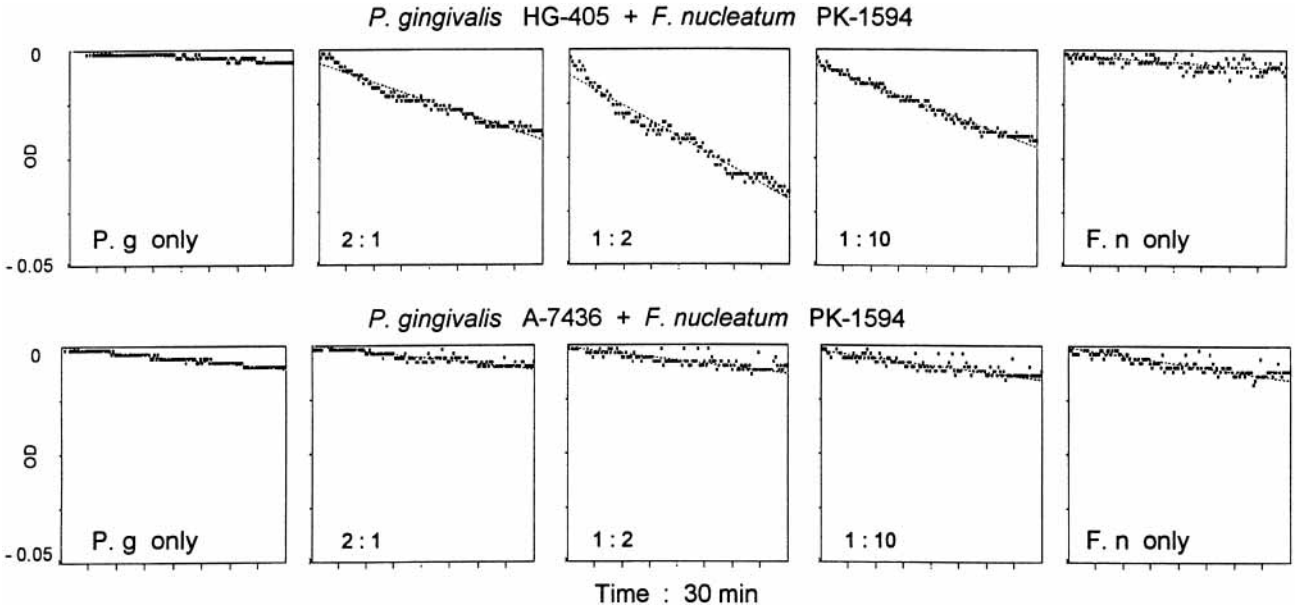

Fig. 1. Vmax coaggregation assay: coaggregation patterns. Coaggregation patterns of *P. gingivalis* HG 405 with *F. nucleatum* PK 1594 and absence of coaggregation between *P. gingivalis* A 7436 and *F. nucleatum* PK 1594. *P. gingivalis* to *F. nucleatum* ratios of 2:1, 1:2 and 1:10, compared to *P. gingivalis* only and *F. nucleatum* only controls. Each curve is representative of the Vmax recording of the coaggregation in each of the 4 wells of a group. Correlation coefficients in the three coaggregating combinations (ratios 2:1, 1:2 and 1:10) were >95. A 30-min assay, limit set at OD −0.05.

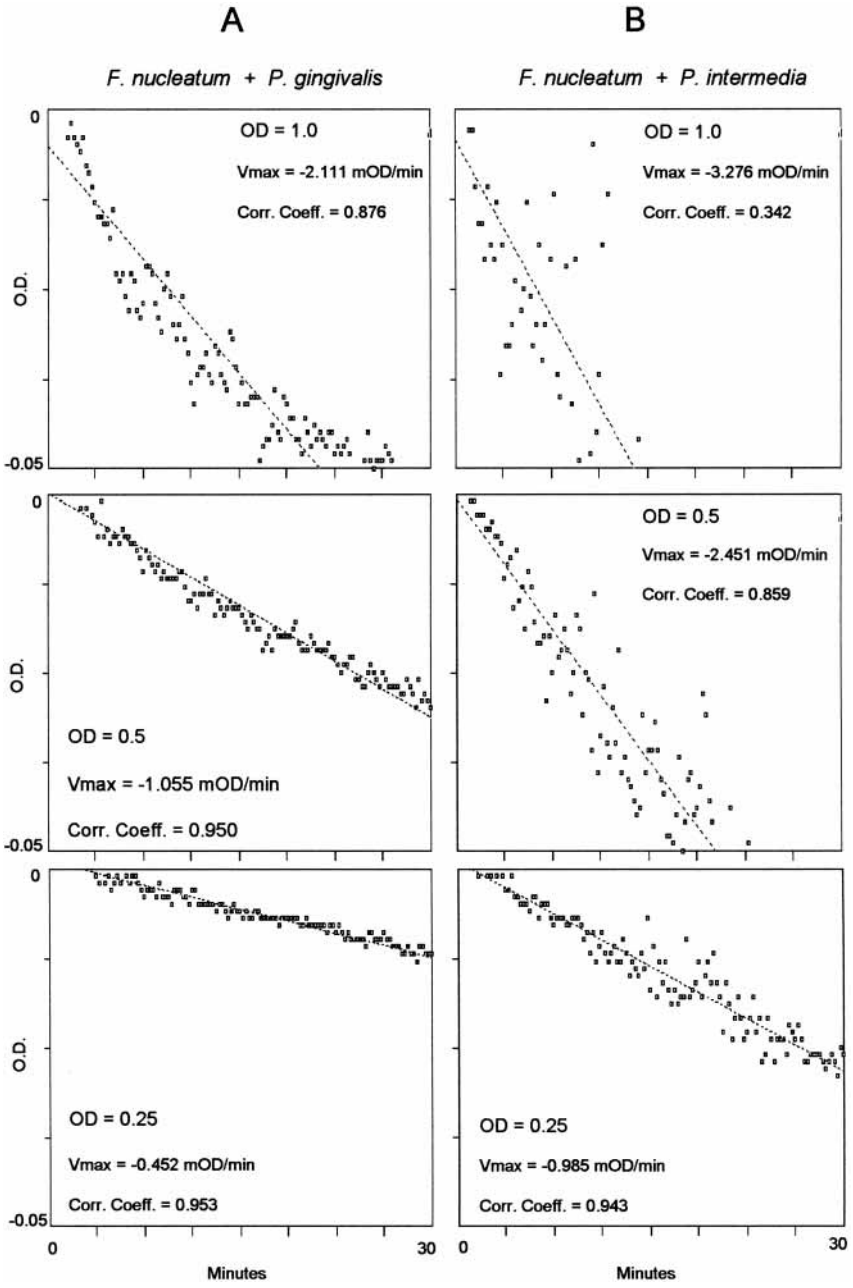

Fig. 2. Effect of bacterial concentrations on the coaggregation patterns. **A.** Coaggregation of *P. gingivalis* HG 405 with *F. nucleatum* PK 1594 at OD 1.0, 0.5 and 0.25. **B.** Coaggregation of *P. intermedia* CB 21 with *F. nucleatum* PK 1594 at OD 1.0, 0.5 and 0.25. Correlation coefficients as well as the rates of coaggregation are influenced by bacterial concentrations. Each curve is representative of the Vmax recording of the coaggregation in each of the four wells of a group.

Vmax coaggregation assay: technical aspects and considerations

The Vmax assay was initially attempted with bacterial suspensions of A_{660}=1.0, which resulted in a rather scattered line (Fig. 2A). Only when diluting the suspensions to A_{660}=0.5 could relatively smooth curves be obtained (Fig. 1, 2A). When the porphyromonads or the fusobacteria were tested as pure suspensions, almost no change in the optical density could be observed over a 30-min period (Fig. 1).

Assays lasting for 30 min or longer yielded reproducible results; however, shorter periods, such as 10 min, gave misleading results due to the influence of slight variability in handling times. We therefore selected the period of 30 min for routine use. The uniformity of the curves generated from the quadruplicate wells was usually high. The correlation coefficient of the estimated Vmax rates of each curve was generally greater than 93%. The standard error of the mean of the Vmax estimates for the replicates was usually within 5%.

Kinetics of coaggregation: *P. gingivalis* HG 405 with *F. nucleatum* PK 1594

At the optimal suspension concentration (A_{660}=0.5), this coaggregation proceeded at a constant rate of 1.05 mOD/min for at least 30 min (Fig. 2A). The bacterial cell proportions of *P. gingivalis* HG 405 mixed with *F. nucleatum* PK 1594 substantially influenced the rate of the resulting coaggregation, as detected by the Vmax assay. The highest rate was recorded when the *P. gingivalis* to *F. nucleatum* ratios were 1:2 and 1:3 (Fig. 1, 3). Altering the ratios outside these optima, in favor of either the porphyromonads or the fusobacteria, consistently lowered the Vmax rates of coaggregation. There was no coaggregation recorded with homologous suspensions of either of these bacteria and the Vmax values remained very low.

P. gingivalis A 7436, on the other hand, did not coaggregate with *F. nucleatum* PK 1594 as measured by this assay (Fig. 1). *P. gingivalis* ATCC 33277 also failed to coaggregate with *F. nucleatum* PK 1594 (data not presented).

Kinetics of coaggregation: *P. intermedia* CB 21 with *F. nucleatum* PK 1594

The coaggregation between *P. intermedia* CB 21 and and *F. nucleatum* PK 1594 that was evaluated as 4+ in the macroscopic assay was also tested by the Vmax method. The above conditions did not provide a "smooth" curve for this highly coaggregating pair. At A_{660}=1.0, the coaggregation occurred too quickly, compromising the quality of the curves generated (correlation coefficient 0.34) (Fig. 2B). This was in accordance with the observations in the macroscopic assay in which occasional sedimenting aggregates formed fast while most of the bacteria were still in the suspension (data not presented). Adjusting the bacterial suspension to A_{660}=0.5 resulted in a slower but more orderly reaction with a correlation coefficient of 0.86. Further reduction to A_{660}=0.25 resulted in a

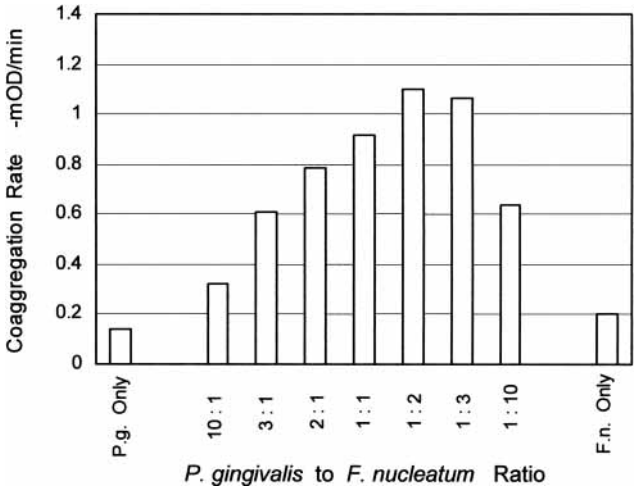

Fig. 3. Vmax coaggregation assay: coaggregation rates of *P. gingivalis* HG 405 with *F. nucleatum* PK 1594. Mean coaggregation rates of *P. gingivalis* HG 405 with *F. nucleatum* PK 1594 expressed as a time-dependent reduction in optical density (660 nm). Each bar represents the mean rate of coaggregation in mOD/min of a group of four wells (SEM <5%).

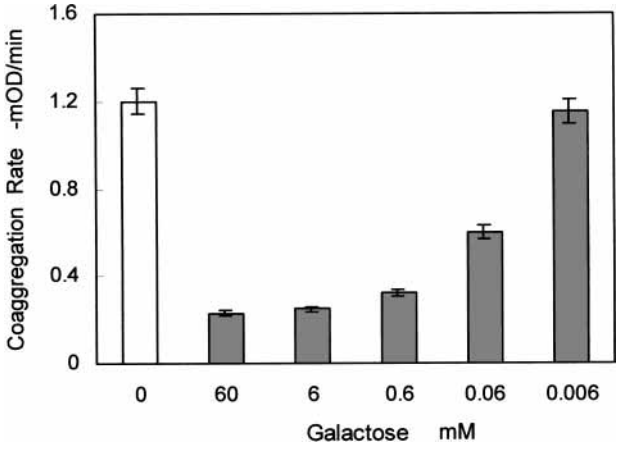

Fig. 4. Effect of galactose on the coaggregation of *P. gingivalis* HG 405 with *F. nucleatum* PK 1594. Each bar represents the mean rate of coaggregation in mOD/min of a group of four wells (±SEM).

slower rate of coaggregation, improving the correlation coefficient to 0.94.

Galactose-mediated inhibition of coaggregation

When galactose was added to the bacterial suspensions containing *P. gingivalis* HG 405 and *F. nucleatum* PK 1594, it blocked the coaggregation as detected in either the macroscopic or microscopic assay (data not presented) or in the Vmax assay (Fig. 4). Galactose concentrations of 6 to 60 mM fully inhibited the coaggregation as detected not only by the Vmax assay but also by both macroscopic and microscopic analyses. With lower galactose concentrations (between 0.06 and 0.0006 mM), an inhibitory dose response was observed that could be quantitatively measured by the Vmax assay (Fig. 4). This dose response compared favorably with the microscopic analysis but was difficult to detect by macroscopic assay. Glucose or mannose, which were used as a control, had no effect on the coaggregation (data not presented).

Discussion

Interactions between certain strains of *P. gingivalis* and *F. nucleatum*, which resulted in their coaggregation, have been previously studied and characterized (9–12, 14). A comprehensive study by Kolenbrander et al. has shown that, while *F. nucleatum* has many coaggregating partners, *F. nucleatum* was the only demonstrated coaggregating partner for *P. gingivalis* (12). Furthermore, it has been shown that this coaggregation was mediated by a lactose- or galactose-inhibitable adhesin (11).

In most of these coaggregation studies, a macroscopic assay (2) was effectively used. This simple method proved to be effective and easy to apply and was based on visual examination of a mixture of two bacterial cell suspensions. The degree of coaggregation was defined by the detection of aggregate formation and by their sedimentation, resulting in the clearing of the supernatant of the bacteria. The method allowed efficient processing of large numbers of samples, but it had two major drawbacks: (a) it was limited to a semi-quantitative evaluation of the aggregation and (b) when carried out with bacterial cell ratios other than the optimal, it may lead to erroneous negative results.

Quantitative spectrophotometric methods have been previously used by Gibbons et al., Ericson et al. and others to measure aggregation and coaggregation (4, 8, 13). In these methods, the reduction in the optical density of a bacterial suspension was used as a means to follow the formation and sedimentation of the aggregates. Most of these assays were conducted in vertical cuvettes or tubes, using a horizontal light path. The coaggregation and sedimentation of the aggregates in those assays removed the bacteria from the light path, thus reducing the suspension's optical density. Though allowing a quantitative continuous monitoring of the process, processing of large numbers of replicate samples was rather cumbersome and slow.

In some recent studies, methods based on radioactive labeling of the bacteria have been introduced, to permit quantitative evaluation (9, 11, 16). Radioactive nucleotides were used to label one strain of bacteria or both and coaggregation was measured by the amount of tracer in the sedimented coaggregate. This allowed quantitative evaluation of the effects of lactose and other related sugars on the coaggregation (11, 16) as well as that of saliva and serum (9). Though effective, this method had several drawbacks, such as the release of the tracer from the bacteria into the medium, complicating the interpretation of the results. When this method was applied to kinetic studies, it was tedious and required repeating of the samples for each time point studied.

The kinetic coaggregation assay introduced here makes use of an automated microtiter plate reader and of the useful software that serves it. This assay is highly sensitive and reproducible and may be adapted for either high- or low-coaggregating bacterial pairs. Slight changes in coaggregation rate can easily be detected. The assay is quantitative and allows large numbers of samples

and variables to be effectively handled at one time. Furthermore, it permits an automated processing of the data for analysis. In addition, the assay allows the study of the kinetics of the coaggregation process, using repeated measures of the same samples, thus introducing a feature that until now required extensive and elaborate work.

The vertical light beam used by this type of instrument makes it essentially different from assays using a horizontal light beam passing above the settling aggregates in a non-mixed cuvette. Reading through the bottom of the plate with mixing eliminated the requirement for sedimentation as a measure of aggregation and at proper cell ratios served as a direct measure of aggregation.

The kinetic assay uses the coaggregation rate as a quantitative parameter rather than the end result (sedimentation) used in former assays. Koop et al. (13) defined two phases in coaggregation, the first in which non-sedimenting coaggregates are formed and a second in which fast sedimentation of the aggregates occurs. The coaggregation assay used in the present study kinetically follows this first initial phase, while many of the former assays were evaluating only the end result of sedimentation of the aggregates.

Using this assay, the kinetics and nature of the weak coaggregation of the pair *P. gingivalis* HG 405 and *F. nucleatum* PK 1594 were studied. The activity was weak and proceeded at a slower rate compared with the coaggregation of the same *F. nucleatum* strain with *P. intermedia* CB 21. When optimal bacterial concentrations and ratios were used, this coaggregation proceeded at a constant rate for the duration of the experiment and was typical to the specific pair. It was demonstrated that the coaggregation of *P. gingivalis* HG 405 differed from that of *P. gingivalis* strains A 7436 and ATCC 33277, which did not express this trait. This coaggregation was fully blocked by galactose and was most probably mediated through the same adhesin that was defined by Kolenbrander & Andersen as mediating the coaggregation of this strain of *F. nucleatum* with *P. gingivalis* strain PK 1924 (11).

Our results indicate and confirm that bacterial cell ratios greatly influence the rate of coaggregation, to the extent that the same pair of bacteria may apparently fail to coaggregate when out of the optimal range. It was at the unfavorable cell ratios that the Vmax assay was especially helpful: the macroscopic assay failed to detect any coaggregation of these ratios. On the other hand, microscopic examination of these mixtures verified that (i) the bacteria did adhere to each other and (ii) the small size of the aggregates most probably prevented a positive result in the macroscopic assay. The size of the coaggregates was in agreement with the Vmax data, as small aggregates could be seen (microscopically) in the unfavorable ratios that expressed lower rates of coaggregation in the Vmax assay.

During these studies, it became apparent that a microscopic evaluation of the coaggregation should be an essential part of such a study. While there is no real need for it when detecting a positive high coaggregation in the macroscopic observation, it becomes essential when marginal or negative results are evaluated. When such results are encountered by macroscopic evaluation, only the microscopic assay can clearly determine whether the bacteria fail to associate with each other, or alternatively that the attachment of the bacteria to each other does occur; however, no visible coaggregates formed due to an unfavorable numerical proportion between the bacteria. Furthermore, when spontaneous autoaggregation of the fusobacteria occurred, only microscopic examination could prevent misleading conclusions (Table 1).

Galactose inhibition of the coaggregation between *F. nucleatum* PK 1594 and *P. gingivalis* HG 405 was used in the present study to illustrate the potential of the assay to quantitatively study the effect of inhibitory saccharides. At the higher galactose concentrations, which fully blocked the coaggregation, the macroscopic assay proved effective. However, when lower galactose concentrations were used it became difficult to apply. The Vmax assay, on the other hand provides an objective, quantitative estimation of this dose-dependent physical interaction.

The Vmax coaggregation assay described here is a powerful tool that may permit a quantitative and kinetic evaluation of properties, competition and mechanisms involved in coaggregation. In this sense, it has a benefit over commonly used semi-quantitative assays and is a favorable replacement for the relatively tedious, radioactive quantitative assays.

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