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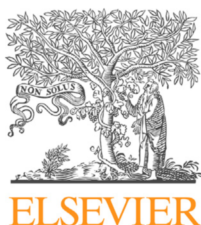

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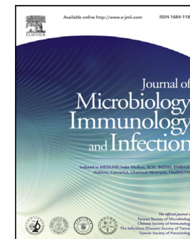

ORIGINAL ARTICLE

Coaggregation between *Prevotella oris* and *Porphyromonas gingivalis*

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Background/Purpose: The coaggregation of bacteria has been defined as one of the most important processes in the oral infection such as periodontitis. *Prevotella oris* and *Porphyromonas gingivalis*, which are two of the periodontopathogens, are frequently detected in severe forms of periodontal diseases. However, the interaction between *P. oris* and *P. gingivalis* is still unknown. In this study, the coaggregation of *P. oris* with nine oral bacterial species including *P. gingivalis* was examined.
Methods: All bacteria used in this study were cultured anaerobically and suspended in coaggregation buffer. Each cell suspension was mixed in a test tube and subjected to shaking at room temperature for 1 hour. Subsequently, the coaggregation values were scored. Furthermore, the effects of various chemical reagents, and heat, proteinase K, and serum treatment were examined.
Results: In this study, *P. oris* coaggregated only with *P. gingivalis*. A heat-stable, nonproteinous component of *P. oris* and a heat-labile, proteinous component of *P. gingivalis* play important roles in this coaggregation. In addition, this coaggregation was inhibited by L-arginine, L-lysine, and *Nα-p*-tosyl-L-lysine. Therefore, it was considered that a cell surface protein on *P. gingivalis*, such as gingipain, may be involved in the coaggregation. Furthermore, the coaggregation was not inhibited by serum treatment.
Conclusion: This is the first report to describe the coaggregation of *P. oris* and *P. gingivalis*. Our study proposes the possibility that *P. oris* may promote the colonization of *P. gingivalis* in an early stage of biofilm formation. Furthermore, this coaggregation may contribute to the initiation and progression of periodontitis.
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Introduction

Chronic periodontitis is a destructive inflammatory disease of the periodontal tissues, caused by various periodontopathogens.[1,2] To cause infection, it is necessary for periodontopathogens to coaggregate with early colonizing bacterial species to form an oral biofilm on the tooth surface or on the gingival epithelium. As such, the coaggregation of bacteria has been defined as one of the most important processes in the formation of periodontopathic biofilms.[3]

Prevotella oris is a nonpigmented, anaerobic, Gram-negative, rod-shaped bacterium frequently isolated from infectious oral lesions, such as those found in periodontal disease,[4] endodontic infection,[5] dentoalveolar abscess,[6] and spreading odontogenic infection,[7] and also from systemic infectious lesions, such as those found in empyema,[8] cervical spinal epidural abscesses,[8] and meningitis.[9] In previous studies, it has been reported that *P. oris* produces immunoglobulin A protease,[10] hyaluronidase,[11] and beta-lactamase [12] as pathogenic factors. However, there are no reports regarding the coaggregation of *P. oris* and other bacteria, except in the case of *Actinomyces israelii* and *Actinomyces odontlyticus*.[13]

Porphyromonas gingivalis is a member of the red complex group of bacteria frequently detected in severe forms of periodontal diseases.[14] Previously, it has been reported that *P. gingivalis* coaggregates with *Actinomyces naeslundii*,[15] *Actinomyces viscosus*,[16] *Streptococcus gordonii*,[17] *Streptococcus mutans*,[18] *Streptococcus oralis*,[19] *Prevotella intermedia*,[20] *Treponema denticola*,[21] *Treponema medium*,[22] and *Fusobacterium nucleatum*.[23] It is considered that these coaggregations may play important roles in the colonization of *P. gingivalis* with early colonizing bacteria.

In this study, the coaggregation of *P. oris* with nine oral bacterial species with potentially important roles in the formation of oral biofilms and in the progression of periodontitis was studied. To the best of our knowledge, this is the first report to describe that *P. oris* coaggregates with *P. gingivalis* in an *in vitro* setting. In addition, we sought to reveal the mechanism behind the coaggregation of *P. oris* and *P. gingivalis*, along with the effect of heat and proteinase K treatments, and treatment with various other reagents (including serum), on the coaggregation observed in the present study.

Methods

Bacterial strains and culture conditions

P. oris JCM 8540^T, the sole strain available from bacterial culture collections, and WK 1 isolated from periodontal pocket of a patient with chronic periodontitis were used in the present study. *P. gingivalis* ATCC 33277^T, *F. nucleatum* JCM 8532, *P. intermedia* ATCC 25611^T, *Prevotella denticola* JCM 8528, *Prevotella loescheii* JCM 8530^T, *Prevotella oralis* JCM 12251^T, *A. viscosus* ATCC 19246, *S. mutans* ingbritt, and *Streptococcus sanguinis* ATCC 10556 were also used in this study. These strains were cultured anaerobically in tryptic soy broth supplemented with 0.5% yeast extract, 5 μg/mL hemin, and 1 μg/mL menadione at 37°C in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. The cells were collected in the late exponential growth phase by centrifugation at 10,000×*g* for 15 minutes and washed three times with coaggregation buffer solution (1 mM Tris–HCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.15 M NaCl, and 0.02% NaN_3, pH 7.5).[24]

Coaggregation assay

The cell suspensions were adjusted to 1.8 (OD 600 nm) for *P. oris*, *P. loescheii*, *P. intermedia*, *A. viscosus*, *S. mutans*, and *S. sanguinis*, and 0.65 for *P. gingivalis* and *F. nucleatum*, using a UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). The coaggregation assay was performed according to Cisar et al.[24] The *P. oris* cell suspension (200 μL), additional cell suspensions (200 μL), and chemical reagents or coaggregation buffer (100 μL) were mixed in a test tube and subjected to shaking at room temperature on a shaker (150 rpm) for 1 hour. Subsequently, the coaggregation values were scored from negative to 4+ described in Cisar et al.[24] Each experiment was performed at least twice on separate days.

Effect of heat and proteinase K treatments on coaggregation

P. oris and *P. gingivalis* cells were treated at 100°C for 10 minutes or treated individually with 2 mg/mL proteinase K at 37°C for 1 hour. After washing three times with the coaggregation buffer, the coaggregation values of the treated cells were measured.

Effects of various chemical reagents on coaggregation

The addition of 12.5 to 100 mmol/L amino acids (L-arginine, L-lysine, L-cysteine, L-proline, L-threonine, L-glycine, L-serine, L-alanine, L-glutamine, L-valine, L-isoleucine, L-methionine, L-histidine, L-lysine, and L-phenylalanine) and 100 mmol/L sugars (D-mannitol, xylitol, maltose, xylose, L-arabinose, D-fructose, D-galactose, D-glucose, L-ramnose, D-mannose, D-sorbitol), in addition to 0.6 mmol/L EDTA and 10–20 mmol/L *N*α-*p*-tosyl-L-lysine (TLCK), was examined with respect to their effects on cell coaggregation. Following the mixing of bacterial cells together with these reagents, the coaggregation of *P. oris* and *P. gingivalis was* measured.

Effect of serum on coaggregation

After collection of horse blood, an equal volume of Alsever's solution was added immediately, and the mixture was refrigerated at 4°C until use. To obtain the serum, the blood was centrifuged at 1500×*g* for 10 minutes. The *P. oris* and *P. gingivalis* cells were pretreated with serum for 10 minutes at room temperature and washed with coaggregation buffer three times, after which coaggregation values were obtained.

Results

Coaggregation of *P. oris* with other bacterial species

In this study, coaggregation of two *P. oris* strains, including JCM 8540^T and WK 1, with nine additional bacterial species was examined (Table 1). In these bacterial species, self-aggregations were not observed except for *A. viscosus* and *F. nucleatum*. For all combinations of bacterial cells used as partner species, the two strains of *P. oris* formed aggregation clumps with *A. viscosus*, *F. nucleatum*, or *P. gingivalis*. However, because self-aggregations were observed for *A. viscosus* and *F. nucleatum* in the coaggregation buffer solution, *P. oris* could coaggregate strongly with *P. gingivalis* only. Following this result, the coaggregation of *P. oris* and *P. gingivalis* was studied further.

Effects of the heat and proteinase K treatments on coaggregation

After the individual treatment of *P. oris* JCM 8540^T, WK 1, and *P. gingivalis* bacterial cells with heat or proteinase K, the degree of coaggregation between the treated cells was measured (Table 2). The results show that both heated strains of *P. oris* coaggregated with the nonheated *P. gingivalis*, although the coaggregation value fell from 3+ to 2+. In contrast, when heated, *P. gingivalis* did not coaggregate with either the heated or nonheated *P. oris* strains.

Comparatively, when coaggregations of the cells treated with proteinase K were examined, the treated *P. oris* coaggregated strongly with the nontreated *P. gingivalis*. However, the treatment of *P. gingivalis* inhibited the coaggregation with the nontreated *P. oris*, demonstrated by an aggregation score reduction from 3+ to 1+.

Inhibitory effects of various chemical reagents and serum on coaggregation

The coaggregation between *P. oris* and *P. gingivalis* was effectively inhibited with L-arginie, L-lysine, and TLCK, and was strongly concentration dependent. However, there were no effects observed with any of the other amino acids (Table 3). The sugar, EDTA, and serum treatments had no affect on coaggregation (Table 3).

Table 1 Coaggregation of *P. oris* with other oral bacteria

Bacteria	Coaggregation with		
	Coaggregation buffer	*P. oris* JCM 8540^T	*P. oris* WK 1
S. mutans ingbritt	0	0	0
S. sanguinis ATCC 10556	0	0	0
A. viscosus ATCC 19246	1+	1+	1+
F. nucleatum JCM 8532	3+	3+	3+
P. gingivalis ATCC 33277^T	0	3+	3+
P. intermedia ATCC 25611^T	0	0	0
P. denticola JCM 8528	0	0	0
P. loescheii JCM 8530^T	0	0	0
P. oralis JCM 12251^T	0	0	0

Table 2 Effects of the heat and proteinase K treatments on the coaggregation of *P. oris* and *P. gingivalis*

Bacteria		Coaggregation
P. oris JCM 8540 ^{T}	*P. gingivalis* ATCC 33277 ^{T}	3+
P. oris JCM 8540 ^{T} (H)	*P. gingivalis* ATCC 33277 ^{T}	2+
P. oris JCM 8540 ^{T}	*P. gingivalis* ATCC 33277 ^{T} (H)	0
P. oris WK 1	*P. gingivalis* ATCC 33277 ^{T}	3+
P. oris WK 1 (H)	*P. gingivalis* ATCC 33277 ^{T}	2+
P. oris WK 1	*P. gingivalis* ATCC 33277 ^{T} (H)	0
P. oris JCM 8540 ^{T} (P)	*P. gingivalis* ATCC 33277 ^{T}	4+
P. oris JCM 8540 ^{T}	*P. gingivalis* ATCC 33277 ^{T} (P)	1+
P. oris WK 1 (P)	*P. gingivalis* ATCC 33277 ^{T}	4+
P. oris WK 1	*P. gingivalis* ATCC 33277 ^{T} (P)	1+

Discussion

There have been no reports describing the coaggregation of *P. oris* and periodontopathogens, such as *P. gingivalis* and

Table 3 Effects of various reagents and serum treatment on coaggregation of *P. oris* and *P. gingivalis*

Tested reagent (mmol/L)	Coaggregation	
	P. oris JCM 8540^T + *P. gingivalis* ATCC 33277^T	*P. oris* WK 1 + *P. gingivalis* ATCC 33277^T
Buffer	3+	3+
Sugars 100 mmol/L	3+	3+
EDTA 0.6 mmol/L	3+	3+
L-Arginine 100 mmol/L	0	0
50 mmol/L	0	0
25 mmol/L	0	0
12.5 mmol/L	0	2+
L-Lysine 100 mmol/L	0	2+
50 mmol/L	0	3+
25 mmol/L	1+	3+
12.5 mmol/L	2+	3+
Other amino acids 100 mmol/L	3+	3+
TLCK 10 mmol/L	1+	1+
20 mmol/L	0	0
Blood serum	3+	3+

Prevotella species. In the present study, we observed that *P. oris* coaggregates with *P. gingivalis* only (Table 1) when the coaggregation of two strains of *P. oris* with nine bacterial species, including five *Prevotella* species, was examined. Therefore, to the best of our knowledge, this is the first report to describe the coaggregation of *P. oris* and *P. gingivalis*. It is known that *P. gingivalis* is isolated from the infected gingival crevice and that it coaggregates with many bacterial species, including *A. viscosus*, *S. gordonii*, *S. mutans*, *F. nucleatum*, and *T. denticola*. This coaggregation is an important step in the formation of oral biofilms.

It has been assumed that *P. oris* is one of the early colonizers in periodontal pockets, as *P. oris* has been isolated from the periodontal pocket before and after periodontal therapy.[7] Thus, our findings also suggest that *P. oris* may be actively involved in the formation of periodontopathic biofilms by acting as a target organism for *P. gingivalis* together with other early colonizer bacteria.

The coaggregation was inhibited by the heat or proteinase K treatment of *P. gingivalis*, but not completely inhibited by the heat treatment of *P. oris* (Table 2). These results suggest that a heat-stable, nonproteinous component of *P. oris* together with a heat-labile proteinous component of *P. gingivalis*, is involved in the coaggregation. It is interesting that a strong coaggregation was observed when using *P. oris* cells treated with proteinase K (Table 2). This result may indicate that a proteinous component of *P. oris*, such as fimbria, was decomposed by proteinase K, and as a result, the coaggregation factor of *P. oris* was exposed on the cell surface. Thus, it was assumed that the proteinous components of *P. oris* partially inhibit coaggregation with *P. gingivalis*, although the significance of this phenomenon remains unknown.

The coaggregation of *P. oris* and *P. gingivalis* was not inhibited by either sugars or EDTA (Table 3). These results suggest that lectin-like binding, such as that observed with the coaggregation of *F. nucleatun* and *P. gingivalis*,[25] and bicationic metal ions are not involved in the coaggregation.

This coaggregation was inhibited by TLCK (Table 3), indicating the involvement of gingipain, a major proteinase in or secreted from *P. gingivalis*, because TLCK is an inhibitor of gingipain.[22,26] The coaggregation of *P. intermedia*, one of the black-pigmented *Prevotella* bacterial species, and *P. gingivalis* was also inhibited by TLCK.[20] It is already clear that the gingipain—adhesin complex is involved in the coaggregation between *P. intermedia* and *P. gingivalis*. Although the Arg-gingipain genes are encoded from *rgpA* and *rgpB* and Lys-gingipain gene is encoded from *kgp*, Kamaguchi et al[20] demonstrated that the gingipain—adhesin complex on *P. gingivalis* interacts with *P. intermedia* when using the *rgpA rgpB*, *rgpA kgp*, *rgpA rgpB kgp*, and *rgpA kgp hagA* mutants of *P. gingivalis*. Furthermore, Kamaguchi et al[27] revealed that the *P. gingivalis* adhesion molecules involved in the coaggregation with *P. intermedia* were encoded by the gingipain genes. The coaggregation of *P. oris* and *P. gingivalis* may be similar to that observed between *P. intermedia* and *P. gingivalis*.

In this study, the coaggregation of *P. oris* and *P. gingivalis* was inhibited by L-lysine and especially L-arginine. The coaggreation of *P. intermedia* and *P. gingivalis* is also inhibited by arginine. Similarly, the inhibition of coaggregation by L-arginine was also described for the coaggregation of *P. gingivalis* and *S. oralis*,[19] *A. viscosus*,[16] *T. denticola*,[21] and *T. medium*.[28]

Some studies have reported that proteinous components derived from saliva or serum inhibit the coaggregation of periodontal bacteria.[29—31] In fact, periodontopathogens in the gingival sulcus or periodontal pockets are bathed with saliva and gingival cervicular fluid containing many proteinous components. Therefore, it is important to understand whether or not bacterial species coaggregate with each other in the saliva or gingival cervicular fluid. Thus, we examined the effect of serum treatment on this coaggregation. As shown in Table 3, after the serum treatment, the coaggregation of *P. oris* and *P. gingivalis* was determined to have the same coaggregation values as with the nonserum treatment. This result suggests that the coaggregation of *P. oris* and *P. gingivalis* may actually occur *in vivo*.

In conclusion, the coaggregation of *P. oris* and *P. gingivalis* was observed in an *in vitro* setting for the first time, and that a heat-stable, nonproteinous component on *P. oris* and a heat-labile, proteinous component of *P. gingivalis* were involved in the coaggregation. Our study proposes the possibility that *P. oris* may promote the colonizing of *P. gingivalis* in an early stage of biofilm formation. Furthermore, this coaggregation may contribute to the initiation and progression of periodontitis. Further studies are needed to clarify the identity of the coaggregation factors and the mechanism underlying the coaggregation of *P. oris* and *P. gingivalis*.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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