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Stimulation of growth of *Porphyromonas gingivalis* by cell extracts from *Tannerella forsythia*

Masahiro Yoneda[1], Toru Yoshikane[1], Noriko Motooka[2], Kazuhiko Yamada[1], Kazuhiro Hisama[1], Toru Naito[1], Ichizo Okada[1], Masaharu Yoshinaga[1], Keitaro Hidaka[1], Koichi Imaizumi[1], Katsumasa Maeda[2], Takao Hirofuji[1]
[1]Section of General Dentistry, Department of General Dentistry, Fukuoka Dental College and [2]Section of Periodontology, Department of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, Japan

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Objective: In order to examine if *Tannerella forsythia* stimulates the growth of *Porphyromonas gingivalis*, an *in vitro* study was performed.

Background: *P. gingivalis and T. forsythia* are often isolated simultaneously from active periodontitis sites, indicating that these bacteria somewhat interact in the periodontal environment. We reported previously that mixed infection of *P. gingivalis* and *T. forsythia* synergistically induced lesion formation in a murine abscess model, and gingipains of *P. gingivalis* played an important role in this synergism. One of the possible mechanisms of this synergism is growth promotion by coinfection of the two bacteria.

Methods: Cell extracts of *T. forsythia* were added to the nutrition-decreased medium and the promotion of growth of *P. gingivalis* was examined.

Results: Sonicated extract of *T. forsythia* stimulated growth of *P. gingivalis* in nutrition-decreased medium in a dose-dependent manner. Proteins appeared to be the nature of growth-promoting factor, and the cell extract of *T. forsythia* had no stimulating effect on the growth of *P. gingivalis* strain devoid of gingipain activities.

Conclusion: A product or a component of *T. forsythia* seemed to stimulate growth of *P. gingivalis* under nutrition-limited conditions. Gingipains are considered to play an important role in digestion or uptake of this growth-promoting factor. The interaction between *T. forsythia* and *P. gingivalis* in growth may be in part related with the synergistic virulence in a murine model.

Masahiro Yoneda, Section of General Dentistry, Department of General Dentistry, Fukuoka Dental College, 2-15-1, Tamura, Sawara-ku, Fukuoka 814–0193, Japan
Tel: +81 92 801 0411 ext. 125
Fax: +81 92 801 4909
e-mail: yoneda@college.fdcnet.ac.jp

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Subgingival plaque samples contain more than 300 cultivable bacterial species (1). A number of studies have indicated that only some of these microorganisms may be responsible for initiation/progression of periodontal diseases. Among them, pathogenic subgingival microorganisms include *Porphyromonas gingivalis*, *Tannerella forsythia*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Treponema denticola*. In addition, some other bacteria are implicated as possible periodontopathogens. These bacteria are usually found in combination in periodontal pockets rather than alone, suggesting that some of the bacteria may cause destruction of the periodontal tissue in a cooperative manner (2). In a murine abscess model, combinations of *P. gingivalis–Fusobacterium nucleatum*, *P. gingivalis–T. denticola*, and *P. gingivalis–A. actinomycetmcomitans* exhibited enhanced virulence compared to monoinfections (3–5). We have previously reported that *P. gingivalis and T. forsythia* also show synergistic virulence in a murine abscess model (6).

The importance of mixed infections in periodontal environments is

generally accepted, but the mechanisms underlying these phenomena have not yet been elucidated.

There may be several types of bacterial interactions *in vivo*, but one of the important interactions is considered to be nutritional relationship, i.e. production of growth-stimulating factors for other bacteria. Interbacterial interactions have been reported among *P. gingivalis*, *T. forsythia*, and other bacteria (7–10). In this study, we examined the effect of cell extracts from *T. forsythia* on the growth of *P. gingivalis*, and confirmed their growth-promoting effect.

Material and methods

Bacterial strains and culture conditions

P. gingivalis ATCC 33277 and KDP128 (*rgpA rgpB kgp*) (11) were maintained on CDC anaerobic blood agar (Becton Dickinson, Cockeysville, MD, USA) in an anaerobic atmosphere (80% N_2, 10% H_2, 10% CO_2), and inoculated in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) supplemented with hemin (5 μg/ml) and menadione (1 μg/ml). Erythromycin (10 μg/ml) was used to maintain the KDP128 strain. *T. forsythia* ATCC 43037 was maintained on CDC-anaerobic blood agar cocultured with *F. nucleatum* ATCC 23726 under anaerobic conditions. *T. forsythia* was also grown in brain heart infusion broth containing yeast extract (0.5%), hemin (5 μg/ml), menadione (1 μg/ml), *N*-acetylmuramic acid (0.001%, Sigma Chemical Co., St. Louis, MO, USA), and fetal bovine serum (5%, Gibco-BRL, Grand Island, NY, USA). *F. nucleatum* ATCC 23726 and *Bacteroides fragilis* RIMD 023001 were grown as previously reported (12).

Preparation of sonicated extracts of bacterial cells

Bacterial cells grown in liquid medium were harvested by centrifugation and washed with phosphate-buffered saline. Cells were disrupted by sonication on ice and unbroken cells were removed by centrifugation (13). Clear supernatants were sterilized by filtration through a 0.22 μm filter.

Growth stimulation experiments

Growth promotion was examined by using the modified method of Takahashi and Sato (14). Tryptic soy broth, which was prepared according to the manufacturer's recommendations, was diluted to 40% of original concentration with phosphate-buffered saline and supplemented with hemin and menadione. Then 100 μl of *P. gingivalis* suspension was inoculated into 5 ml of diluted tryptic soy broth, with or without bacterial cell extracts. Diluted tryptic soy broth supplemented with bacterial cell extracts was incubated at the same time and served as a blank. Bacterial growth was measured as optical densities at 600 nm.

Preliminary characterization of growth-promoting factor from *T. forsythia*

Cell extracts were heat-treated (80°C, 10 min) or dialyzed against phosphate-buffered saline using Spectra/Por 1 dialysis membrane (molecular weight cut-off 6000–8000, Spectrum Co. Laguna Hills, CA, USA) before growth-promotion assay.

Results

Tryptic soy broth concentration and growth of *P. gingivalis*

The stimulation of growth of *P. gingivalis* was observed under nutrition rich conditions (data not shown), but to make the growth promoting effect clearer, we prepared nutrition-decreased medium. Tryptic soy broth was diluted with phosphate-buffered saline, and overnight-cultured *P. gingivalis* was inoculated and incubated for 48 h (Fig. 1). When tryptic soy broth was diluted to 40% of original concentration, the growth rate was approximately half that of the original tryptic soy broth. Therefore, we used tryptic soy broth that was diluted to 40% (diluted tryptic soy broth) for the subsequent experiments.

Growth stimulation by cell extracts from *T. forsythia*

Sonicated extracts from *T. forsythia* stimulated the growth of *P. gingivalis* in a dose-dependent manner (Fig. 2). Addition of the same concentration of tryptic soy broth instead of cell extracts also promoted growth of *P. gingivalis*, but the effect was far smaller than the cell extracts (data not shown), indicating that this growth promotion was not due to a mere nutritional effect. As

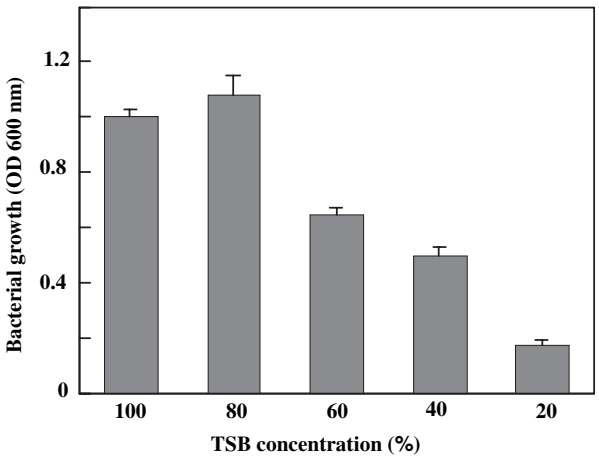

Fig. 1. Tryptic soy broth concentration and growth of *Porphyromonas gingivalis*. After tryptic soy broth was diluted with phosphate-buffered saline, hemin and menadione were added. Then, 100 μl of overnight-cultured *P. gingivalis* suspensions were inoculated into 5 ml of medium and incubated for 48 h. TSB, tryptic soy broth.

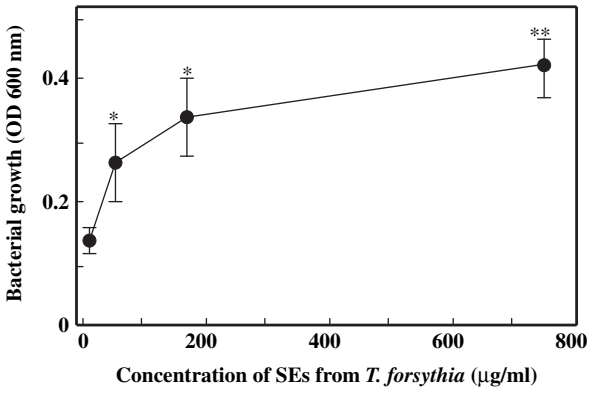

Fig. 2. Dose-dependent growth stimulation by sonicated extracts from *Tannerella forsythia*. Different amounts of sonicated extracts from *T. forsythia* (45, 180 or 720 μg/ml) were added to the diluted tryptic soy broth. Then, 100 μl of overnight-cultured *Porphyromonas gingivalis* suspensions were inoculated into 5 ml of diluted tryptic soy broth, and incubated for 14 h. $*p < 0.05$ and $**p < 0.001$, mean significant difference from growth without sonicated extracts from *T. forsythia*. SEs, sonicated extracts.

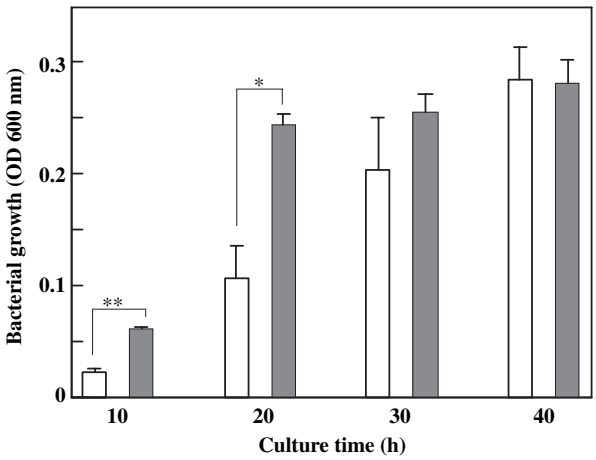

Fig. 3. Growth stimulation at different culture stages. A total of 100 μl of overnight-cultured *Porphyromonas gingivalis* suspensions were inoculated into 5 ml of diluted tryptic soy broth with (■) or without (□) sonicated extracts from *Tannerella forsythia* (180 μg/ml) and incubated for the indicated periods. $*p < 0.005$ and $**p < 0.001$, mean significant difference between growth with or without sonicated extracts from *T. forsythia*.

Table 1. Effect of treatment of sonicated extracts from *Tannerella forsythia* on growth promotion

Additive	Treatment	OD 600 nm (mean ± SD)
None	–	0.149 ± 0.018
T. forsythia SEs[a]	none	0.314 ± 0.020**
T. forsythia SEs[a]	heat[b]	0.266 ± 0.016*
T. forsythia SEs	dialysis[c]	0.317 ± 0.021**

[a]180 μg/ml.
[b]*T. forsythia* SEs were heated at 80°C for 10 min.
[c]*T. forsythia* SEs were dialyzed against phosphate-buffered saline.
$*p < 0.05$ and $**p < 0.0005$, mean significant difference from growth without sonicated extracts from *T. forsythia*.
SEs, sonicated extracts.

shown in Fig. 3, the growth-promoting effect was evident at the early stages of growth, and after prolonged culture, the growth rates were almost the same in the presence or absence of sonicated extracts from *T. forsythia*. Therefore, the following experiments were performed at 14 h incubation. The growth curve also showed that cell extracts from *T. forsythia* seemed to have a greater effect on the doubling time than the final yield of *P. gingivalis* (data not shown).

Characterization of growth-promoting factor

To characterize the nature of the growth-promoting factor of sonicated extracts from *T. forsythia*, it was pretreated before the assay (Table 1). Heat treatment (80°C for 10 min) of sonicated extracts from *T. forsythia* partially abolished the growth-promoting effect. On the other hand, dialysis had no effect on growth promotion. In our previous report, we showed that gingipains of *P. gingivalis* played an important role in the synergistic virulence (6). Therefore, we examined the effect of gingipains on the growth interaction between *P. gingivalis* and *T. forsythia*. As shown in Table 2, sonicated extracts from *T. forsythia* did not stimulate growth of *P. gingivalis* KDP128, which is devoid of gingipain activities.

Effect of various bacterial cell extracts on growth of *P. gingivalis*

To exclude the possibility that the growth promotion found above was

Table 2. Effect of sonicated extracts from *Tannerella forsythia* on the growth of gingipain mutant[a]

Additive	OD 600 nm (mean ± SD)
none	0.193 ± 0.009
T. forsythia SEs[b]	0.194 ± 0.030

[a]*Porphyromonas gingivalis* KDP128 (*rgpA rgpB kgp*) was used.
[b]180 μg/ml.
SEs, sonicated extracts.

due to non-specific nutrition, cell extracts from various bacteria were examined for their growth-stimulating effects (Fig. 4). Cell extracts from *P. intermedia and B. fragilis* had no effect on the growth of *P. gingivalis* under the conditions used in this experiment. Sonicated extracts from *T. forsythia* showed the greatest stimulating effect among the bacterial cell extracts. Sonicated extracts from *F. nucleatum* also promoted growth of *P. gingivalis*. Bovine serum albumin slightly stimulated growth of *P. gingivalis*, but not significantly.

Discussion

We have previously reported synergism between *P. gingivalis* and *T. forsythia* in a murine abscess model (6).

In this *in vitro* study, we showed that sonicated extracts from *T. forsythia* stimulated growth of *P. gingivalis* in a dose-dependent manner. It is interesting to note that this growth promotion was evident only at the early stages of growth. Availability of nutrients seemed important especially at the early stage of bacterial growth. *T. forsythia* is known to be an important risk factor for the destructive periodontal disease (15), and virulence factors of this microorganism are extensively studied these days.

Decrease of the growth-promoting effect by heat treatment indicates that the proteins in the sonicated extracts from *T. forsythia* are in part responsible for the growth stimulation. On the other hand, no change of growth-stimulating effects by dialysis indicates that the low molecular weight materials may not be the major growth-promoting factor in this experiment. Purification and further characterization of the growth-promoting factor from the cell extracts are now in progress.

Several growth-promoting factors have been reported for some bacterial combinations, and these are fatty acids, amino acids or small peptides (16). We did not use spent culture medium because the culture medium contains fetal bovine serum and it was difficult to exclude the effect of this additive. There is a possibility that metabolic byproducts in the spent culture medium may be more effective in growth promotion of *P. gingivalis*. Further experiments using spent culture medium are necessary.

We also reported previously that the synergism of these two bacteria in a murine abscess model was found only with wild-type *P. gingivalis* (6, 17). When mutant strains devoid of gingipain activities were used, only the additive effect, not the synergistic effect, was observed for a mixed infection with *T. forsythia*. Gingipains have many biologic activities (18), and we have reported some of the virulence factors of these enzymes (19, 20). Grenier *et al.* reported that *P. gingivalis* digests proteins with gingipains and takes in the smaller molecules for its growth (9). In the present study, sonicated extracts from *T. forsythia* did not promote growth of a *P. gingivalis* strain devoid of gingipain activities, and gingipains seemed to play an important role in the digestion or uptake of the growth-promoting factor from sonicated extracts from *T. forsythia*.

Cell extracts from *F. nucleatum* also stimulated growth of *P. gingivalis*. It is known that *P. gingivalis and F. nucleatum* have a synergistic effect in a murine abscess model (21). The synergism of this combination may in part come from the growth-promoting effect of *F. nucleatum*. Bovine serum albumin also slightly promoted growth of *P. gingivalis* (not significant). We could not exclude the possibility that some of the growth promotion is due to a non-specific nutritional factor.

P. gingivalis is isolated from deep periodontal pockets. On the other hand, *T. forsythia* is often found from shallower pockets as well as deep pockets (15). *T. forsythia* may be changing the nutrition-limited environments such as shallow pockets to environments suitable for the growth of *P. gingivalis*, resulting in attachment loss.

The nutritional interaction cannot fully explain the bacterial synergism, but it may be playing some roles in the initiation and progression of periodontal disease.

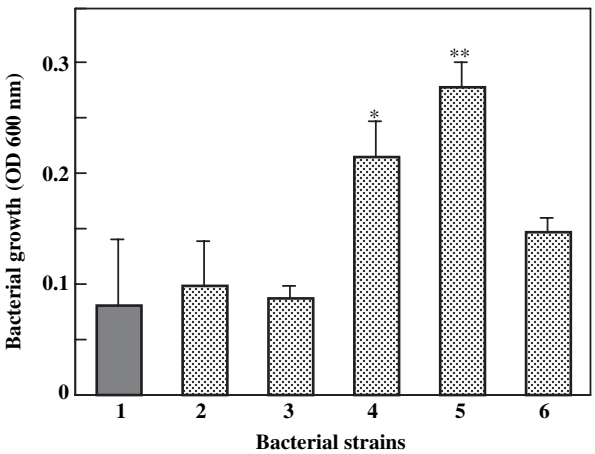

Fig. 4. Effect of various bacterial cell extracts on growth of *Porphyromonas gingivalis*. Sonicated extracts from various bacteria or bovine serum albumin (180 μg/ml) were added to diluted tryptic soy broth. Then, 100 μl of overnight-cultured *P. gingivalis* suspensions were inoculated into 5 ml of diluted tryptic soy broth with or without additives and incubated for 14 h. 1, no additive; 2, *Prevotella intermedia*; 3, *Bacteroides fragilis*; 4, *Fusobacterium nucleatum*; 5, *Tannerella forsythia*; 6, bovine serum albumin. *$p < 0.05$ and **$p < 0.001$, mean significantly different from growth without additives.

Acknowledgements

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