

## Research Article



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### Conflict of Interest

No potential conflicts of interest relevant to this article were reported.

# Surface interactions between two of the main periodontal pathogens: *Porphyromonas gingivalis* and *Tannerella forsythia*

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## ABSTRACT

**Purpose:** *Porphyromonas gingivalis* and *Tannerella forsythia* have been implicated as the major etiologic agents of periodontal disease. These two bacteria are frequently isolated together from the periodontal lesion, and it has been suggested that their interaction may increase each one's virulence potential. The purpose of this study was to identify proteins on the surface of these organisms that are involved in interbacterial binding.

**Methods:** Biotin labeling of surface proteins of *P. gingivalis* and *T. forsythia* and liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was performed to identify surface proteins involved in the coaggregating activity between *P. gingivalis* and *T. forsythia*.

**Results:** It was found that three major *T. forsythia* proteins sized 161, 100, and 62 kDa were involved in binding to *P. gingivalis*, and *P. gingivalis* proteins sized 35, 32, and 26 kDa were involved in binding to *T. forsythia* cells.

**Conclusions:** LC-MS/MS analysis identified one *T. forsythia* surface protein (TonB-linked outer membrane protein) involved in interbacterial binding to *P. gingivalis*. However, the nature of other *T. forsythia* and *P. gingivalis* surface proteins identified by biotin labeling could not be determined. Further analysis of these proteins will help elucidate the molecular mechanisms that mediate coaggregation between *P. gingivalis* and *T. forsythia*.

**Keywords:** Bacterial outer membrane proteins; Biotinylation; Periodontitis; *Porphyromonas gingivalis*; *Tannerella forsythia*

## INTRODUCTION

Bacterial pathogens have evolved sophisticated mechanisms to ensure survival within their hosts by colonizing host tissue, overcoming host resistance, and establishing specific niches [1]. In human periodontal disease, the microorganisms in dental plaque form a unique niche on the tooth surface and in periodontal pockets and mediate destruction of the periodontium

[2,3]. Dental plaque is a biofilm that consists of more than 700 different bacterial species [4-7], and it is believed to interact with the host as a dynamic and complex microbial community. Microbial interaction or cell-cell communication in dental plaque affects the structure and ultimately the behavior of the biofilm. The results of numerous studies strongly suggest that a population shift toward gram-negative anaerobic species in the plaque biofilm is responsible for the initiation and progression of periodontal disease [8-11]. Therefore, it is important to characterize interactions among dental plaque microorganisms, especially periodontopathic ones, in order to understand the microbial pathogenesis of periodontal disease.

The most prominent example of microbial interaction in dental plaque biofilm is coaggregation, or interbacterial binding. This phenomenon results from cell-to-cell recognition between specific microbial partners in dental plaque. It has been established that most bacteria in the oral cavity can physically interact with one or more specific partners, and this activity may be responsible for the development and formation of the biofilm [12,13]. Coaggregation can occur between early colonizers (i.e., *Streptococcus sanguis* and *Actinomyces viscosus*), between early and late colonizers (i.e., *Streptococcus gordonii* and *Fusobacterium nucleatum*), and between late colonizers (i.e., *Treponema denticola* and *Porphyromonas gingivalis*) [12,13]. In addition, it may facilitate physiological and metabolic interactions between microorganisms, which may lead to synergistic activity between dental plaque microorganisms and modify their potential virulence [14-16].

Two gram-negative strict oral anaerobes, *Porphyromonas gingivalis* and *Tannerella forsythia*, have been implicated, along with *Aggregatibacter actinomycetemcomitans*, as the major etiologic agents in periodontal disease [17]. Together with *T. forsythia*, *P. gingivalis* is frequently isolated from periodontal lesions [9,18]. In animal models, a mixed infection with these two organisms has significantly enhanced the virulence potential in abscess formation [19,20], suggesting a possible synergistic effect of *P. gingivalis* and *T. forsythia* in periodontal infection. Sonicated protein extracts of *T. forsythia* have been found to stimulate the growth of *P. gingivalis* in nutrition-depleted medium in a dose-dependent manner [21]. Collectively, these findings suggest a synergistic effect of *P. gingivalis* and *T. forsythia* in the pathogenesis of periodontal disease.

The mechanisms involved in coaggregation have been described for interactions between several of the early colonizers of dental plaque biofilm, including *S. gordonii*, *S. sanguis*, and *A. viscosus* [13]; however, similar information is not yet available for most of the periodontopathic pathogens, including *P. gingivalis* and *T. forsythia*. Elucidating these mechanisms may help us better understand the formation and development of periodontitis-associated dental plaque and, thereby, the pathogenesis of periodontal infection.

So far, the exact mechanisms by which *P. gingivalis* and *T. forsythia* interact have not been adequately studied, although it has been suggested that these mechanisms are mediated through protein-protein interactions [11,22]. In our previous study [23], it was observed that cell-cell contact between *P. gingivalis* and *T. forsythia* was confirmed by electron and epifluorescence microscopic studies. In addition, it was suggested that the coaggregation is a protein-protein interaction, as protease treatment of *P. gingivalis* and *T. forsythia* completely blocked the coaggregating activity. The purpose of this study was to identify the surface proteins involved in interbacterial binding between *P. gingivalis* and *T. forsythia*.

## MATERIALS AND METHODS

### Bacterial strains

*T. forsythia* ATCC 43037 cells were grown on agar plates containing 3.8% heart infusion broth (Becton Dickinson, Sparks, MD, USA), 0.005% N-acetylmuramic acid (Fisher Scientific, Pittsburgh, PA, USA), 5% fetal bovine serum (FBS) (Fisher Scientific), 5 µg/mL of hemin (Fisher Scientific), 0.5 µg/mL of menadione (Fisher Scientific), and 5% yeast extract (Becton Dickinson). Cells of *P. gingivalis* strain 381 were grown on blood agar plates containing 3% tryptic soy agar (Becton Dickinson), 0.5% yeast extract, 5 µg/mL of hemin, 1 µg/mL of menadione, and 5% sheep's blood. These bacteria were cultivated under an anaerobic atmosphere (10% H<sub>2</sub>, 5% CO<sub>2</sub>, and 85% N<sub>2</sub>) at 37°C in a Forma anaerobic chamber (Thermo Scientific, Waltham, MA, USA), as described elsewhere.

### Immunofluorescence microscopy

The bonds between the bacterial cells and the biotin-labeled surface proteins of the counterpart cells were visualized as follows. Surface proteins from *T. forsythia* or *P. gingivalis* cells were labeled with biotin, as described below, and were mixed with the counterpart *P. gingivalis* or *T. forsythia* cells, respectively, for 30 minutes. Bound cells were placed on glass slides, heat-fixed, and washed with methanol. After being blocked with 1% BSA/TBS for 30 minutes, the bacterial cells were incubated with a 1:100 dilution of streptavidin–fluorescein isothiocyanate (FITC) (Thermo Scientific). All the procedures were performed at room temperature. The bacterial cells were observed using a Zeiss LSM 510 confocal microscope (Zeiss, Oberkochen, Germany).

### Biotinylation and identification of surface proteins bound to counterpart bacterial cells

The surface proteins of *T. forsythia* and *P. gingivalis* were labeled with biotin as follows. Bacterial cells were cultivated, harvested, and washed three times with PBS. Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) was added to the individual bacterial cells at a final concentration of 1 mg/mL, and the cells were incubated at RT for 30 minutes by rotation. Unbound biotin was removed by washing the cells three times with 0.1 M of glycine. Biotinylated surface proteins from *T. forsythia* were extracted with 2% deoxycholic acid (Thermo Fisher Scientific), dialyzed in 10 mM of PBS, and then mixed with whole *P. gingivalis* cells for 2 hours at RT. The resulting *P. gingivalis* cells labeled with biotinylated *T. forsythia* surface proteins were then separated by SDS-PAGE and were transferred to a nitrocellulose membrane, as described elsewhere. Subsequently, biotinylated *T. forsythia* surface proteins were detected with the use of streptavidin–horseradish peroxidase (HRP) (Thermo Fisher Scientific) and 4-chloro-1-naphthol (4-CN) (Thermo Fisher Scientific), according to the manufacturer's instructions. The same procedures were performed for identifying the biotinylated surface proteins of *P. gingivalis* that are involved in the interaction with *T. forsythia* cells.

### In-gel enzymatic digestion of proteins

Detected protein bands were excised from the gel, transferred to 100 µL of 0.01 M of dithiothreitol/0.1 M of Tris (pH 8.5), and incubated at 55°C for 1 to 2 hours. After cooling, the gel bands were incubated with 100 µL of 0.03 M of iodoacetamide/0.1 M of Tris (pH 8.5) for 30 minutes in the dark, were washed twice with 200 µL of 0.05 M of Tris (pH 8.5)/30% acetonitrile for 20 minutes with shaking, and were then washed once with 100 µL of acetonitrile for 2 to 3 minutes until the gel turned an opaque white. After removing the acetonitrile, the gel pieces were dried for 20 to 30 minutes in a Speed-Vac Concentrator

(Thermo Scientific). Gels were digested by adding 0.08 to 0.10  $\mu\text{g}$  of modified trypsin (sequencing grade, Roche Molecular Biochemicals, Indianapolis, IN, USA) in 15 to 20  $\mu\text{L}$  of 0.025 M of Tris (pH 8.5) or enough volume to completely hydrate the gel. The tubes were placed in a heating block at 32°C and left overnight. Peptides were extracted with 50  $\mu\text{L}$  of 50% acetonitrile/2% trifluoroacetic acid, and the combined extracts were either dried or reduced in volume in a Speed-Vac Concentrator to about 10  $\mu\text{L}$  pending further analysis.

### Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis

LC-MS/MS analysis was performed on a Micromass hybrid quadrupole/time-of-flight mass spectrometer with a nanoelectrospray source (Waters, Milford, MA, USA). Capillary voltage was set at 1.8 kV and cone voltage at 32 V; the collision energy was set according to the mass and charge of the ion, from 14 eV to 50 eV. Chromatography was performed on an LC Packings HPLC with a C18 PepMap column using a linear acetonitrile gradient with a flow rate of 200 nL/min. Raw data files were processed using MassLynx ProteinLynx software (Boston, MA, USA), and .pkl files were submitted for a database search at <http://www.matrixscience.com> using the Mascot scoring algorithm. The peptide sequences of the identified proteins were determined from a database containing whole genome sequences of *T. forsythia* (available at <http://www.ncbi.nlm.nih.gov/genome>).

## RESULTS

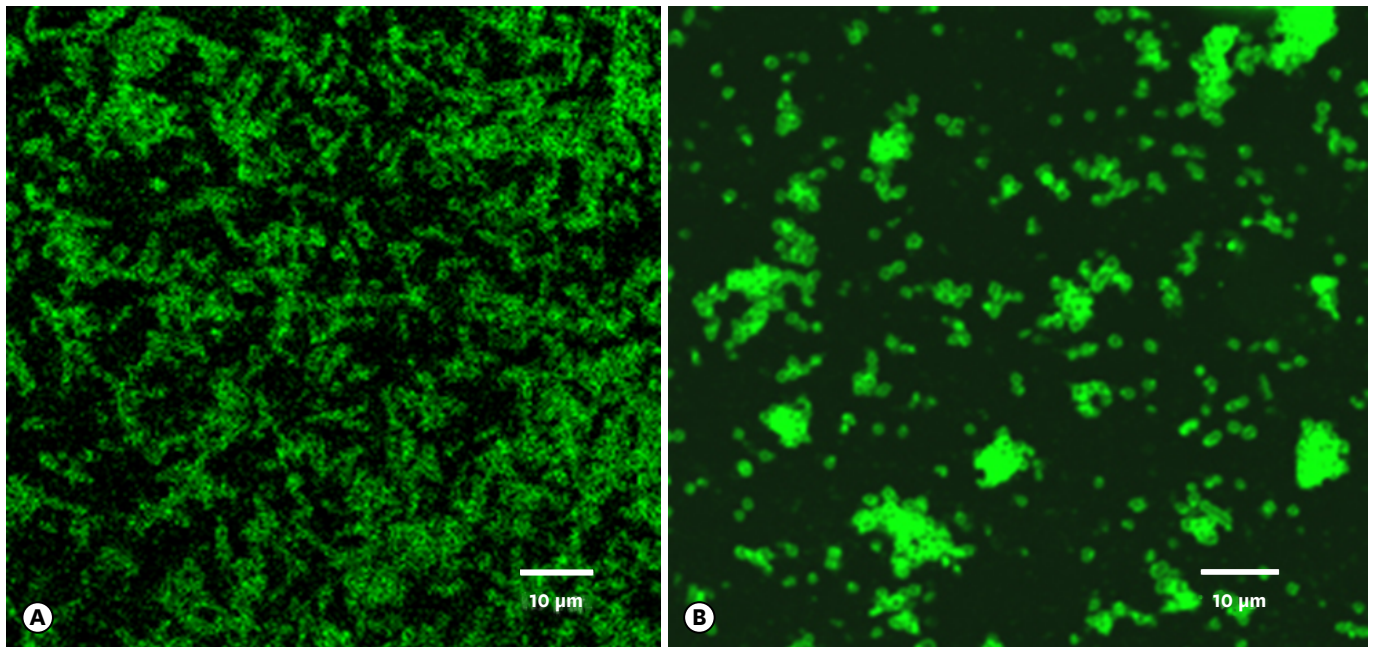
### Confirmation of biotin-labeled surface proteins bound to *P. gingivalis* and *T. forsythia* cells

The surface proteins of *T. forsythia* and *P. gingivalis* cells were labeled with Sulfo-NHS-LC-Biotin. Unbound biotins were removed by washing three times with 100 mM of glycine. To check whether the surface proteins could bind to the counterpart cells, biotinylated *T. forsythia* and *P. gingivalis* cells were dissolved in 2% deoxycholic acid and underwent dialysis in 10 mM of phosphate buffers to remove the detergents in the solution. *T. forsythia* lysates and *P. gingivalis* lysates were incubated with *P. gingivalis* and *T. forsythia* cells, respectively, at 4°C overnight. The unbound lysates were removed by centrifugation and washed twice with PBS. *T. forsythia* cells and *P. gingivalis* cells that were bound with *P. gingivalis* and *T. forsythia* surface proteins, respectively, were visualized on confocal microscopy with streptavidin-FITC (Figure 1).

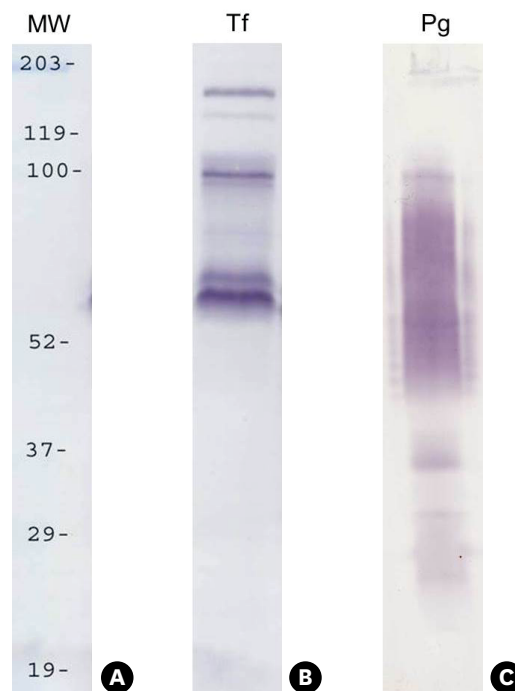
### Identification of surface proteins bound to *T. forsythia* and *P. gingivalis*

*T. forsythia* and *P. gingivalis* cells to which the surface proteins of *P. gingivalis* and *T. forsythia* cells, respectively, were attached were dissolved in 3% SDS buffer. The resultant total proteins were separated in 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. Biotinylated proteins were then detected with streptavidin-HRP and 4-CN. It was found that three major *T. forsythia* proteins having molecular masses of 161, 100, and 62 kDa were involved in binding to *P. gingivalis* cells, and that *P. gingivalis* proteins having molecular masses of 35, 32, and 26 kDa were involved in binding to *T. forsythia* cells (Figure 2).

LC-MS/MS analysis of the 100-kD *T. forsythia* protein that was bound to *P. gingivalis* revealed that this protein was actually a TonB-linked outer membrane protein (118 kD). However, the nature of the other *T. forsythia* and *P. gingivalis* surface proteins could not be determined by LC-MS/MS analysis.



**Figure 1.** Surface proteins of *P. gingivalis* and *T. forsythia* bound to *T. forsythia* (A) and *P. gingivalis* cells (B), respectively, as observed under epifluorescence microscopy using FITC-conjugated streptavidin antibody. Fluorescence-labeled rod-shaped *T. forsythia* cells are clearly evident (A). Fluorescence-labeled coccobacilli-shaped *P. gingivalis* cells can be seen (B). For details, refer to the main text.



**Figure 2.** Cell-surface labeling with biotin. The surface proteins of *P. gingivalis* and *T. forsythia* were labeled with Sulfo-NHS-LC-Biotin and mixed with *T. forsythia* and *P. gingivalis* cells, respectively. Subsequently, the mixtures were subjected to SDS-PAGE, and then transferred onto nitrocellulose membranes. Biotinylated *P. gingivalis* and *T. forsythia* surface proteins were detected with streptavidin-HRP and 4-CN. Lanes: MW, molecular weight marker; Tf, biotinylated *T. forsythia* surface proteins bound to *P. gingivalis*; Pg, biotinylated *P. gingivalis* surface proteins bound to *T. forsythia*

## DISCUSSION

The purpose of this study was to identify surface proteins that are involved in interbacterial binding between *P. gingivalis* and *T. forsythia*. So far, the molecular mechanisms responsible for the interaction between *P. gingivalis* and *T. forsythia* have not been elucidated. Yoneda et al. [19] found that mixed infections with *P. gingivalis* and *T. forsythia* had a synergistic effect in causing abscess formation in mice and suggested a role for gingipains of *P. gingivalis* in this effect. Gingipains are cysteine proteinases known to produce usable peptides and amino acids through the digestion of host proteins. Their biological functions include disturbance of host immune systems, impairment of the bactericidal functions of polymorphonuclear leukocytes, acquisition of heme, and maturation of fimbriae [24]. Furthermore, Kamaguchi et al. [25] revealed the role of adhesion domains within gingipains in the coaggregating activity between *P. gingivalis* and *Prevotella intermedia*. Therefore, the gingipains of *P. gingivalis* may be involved in interactions with other microorganisms in dental plaque biofilms. On the other hand, the molecular components of *T. forsythia* involved in interbacterial binding have not yet been identified, except for a surface (S-) layer. Shimotahira et al. [26] showed that the S-layer is in fact involved in coaggregation with *Streptococcus sanguis*, *Streptococcus salivarius*, and *P. gingivalis*, because the S-layer-deficient mutants exhibited reduced coaggregation with these microorganisms.

In our previous study, we showed that *T. forsythia* possesses a unique surface (S-) layer that is involved in hemagglutination, in the attachment to and invasion of epithelial cells, and in abscess formation in mice [27]. We subsequently determined that the S-layer is composed of two large glycoprotein subunits [28]. Being an outermost surface structure and a hemagglutinin, the S-layer could presumably be involved in the coaggregation of *T. forsythia* with *P. gingivalis*. In addition, the S-layer-deficient mutant appeared to exhibit reduced coaggregating activity with *P. gingivalis*, as compared with that of the wild-type strain [26]. These findings suggest that the S-layer is, at least in part, involved in coaggregation with *P. gingivalis*. Considering that heat treatment of *T. forsythia* significantly increased its coaggregating activity [23], it is likely that the carbohydrate components of the S-layer are involved in the surface interaction with *P. gingivalis*. However, the results of the present study did not provide direct evidence that the S-layer of *T. forsythia* is involved in a surface-surface interaction with *P. gingivalis*, since biotin-labeling of the S-layer was not confirmed in SDS-PAGE. It can be postulated that the S-layer was not adequately labeled with biotin and was therefore not identified in the surface interaction with *P. gingivalis*.

One of the *T. forsythia* surface proteins bound to *P. gingivalis* cells was identified; however, other biotin-labeled *T. forsythia* and *P. gingivalis* surface proteins could not be determined by means of LC-MS/MS, since there were probably too many minor protein bands or non-specific backgrounds associated with the major proteins to allow a precise sequence determination. The single identified *T. forsythia* surface protein was TonB-linked outer membrane protein (Gene ID: TF1439). Although the function of this surface protein has not yet been determined, it has been shown to be produced only *in vivo* in patients with chronic periodontitis [29]. Therefore, along with its involvement in the coaggregation with *P. gingivalis* and *in vivo* expression inside the host, the TF1439 protein may be involved in chronic periodontitis. Based on our results, it can be postulated that *T. forsythia* binds to *P. gingivalis* in the actual disease process, enhancing the virulence potential of these two bacteria, but this possibility will require further testing.

In conclusion, the interbacterial binding between *P. gingivalis* and *T. forsythia* appears to be mediated by multiple protein–protein interactions. One *T. forsythia* outer membrane protein (TonB-linked outer membrane protein) was identified as a surface protein involved in this coaggregating activity. Other unidentified surface proteins should be determined from *T. forsythia* and *P. gingivalis* in order to elucidate the molecular mechanisms that mediate coaggregation between these two periodontal pathogens.

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