Porphyromonas gingivalis and Treponema denticola
Synergistic Polymicrobial Biofilm Development

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Abstract
Chronic periodontitis has a polymicrobial biofilm aetiology and interactions between key bacterial species are strongly implicated as contributing to disease progression. Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia have all been implicated as playing roles in disease progression. P. gingivalis cell-surface-located protease/adhesins, the gingipains, have been suggested to be involved in its interactions with several other bacterial species. The aims of this study were to determine polymicrobial biofilm formation by P. gingivalis, T. denticola and T. forsythia, as well as the role of P. gingivalis gingipains in biofilm formation by using a gingipain null triple mutant. To determine homotypic and polymicrobial biofilm formation a flow cell system was employed and the biofilms imaged and quantified by fluorescent in situ hybridization using DNA species-specific probes and confocal scanning laser microscopy imagining. Of the three species, only P. gingivalis and T. denticola formed mature, homotypic biofilms, and a strong synergy was observed between P. gingivalis and T. denticola in polymicrobial biofilm formation. This synergy was demonstrated by significant increases in biovolume, average biofilm thickness and maximum biofilm thickness of both species. In addition there was a morphological change of T. denticola in polymicrobial biofilms when compared with homotypic biofilms, suggesting reduced motility in homotypic biofilms. P. gingivalis gingipains were shown to play an essential role in synergistic polymicrobial biofilm formation with T. denticola.

Introduction
Polymicrobial biofilms are dynamic structures that can alter form or composition based not only on environmental conditions including nutrient supply, shear forces and temperature but also on the synergies and antagonisms between the species that comprise the biofilm and the emergent properties that result from these interactions [1–3]. Microbial biofilms are the predominant bacterial lifestyle in habitats where shear forces and bulk phase movement result in removal of unattached bacteria. The oral cavity is a classic example where extensive bacterial biofilms (plaque) can develop on the non-shedding surfaces of teeth and result in the development of polymicrobial plaque-related diseases such as periodontitis [4].

Anaerobic, proteolytic bacterial species including Porphyromonas gingivalis, Treponema denticola and/or Tannerella forsythia are consistently found in elevated numbers in subgingival plaque samples taken from periodontally diseased subjects [5–7]. Of the three species, P. gingivalis and T. denticola are frequently found together in diseased sites [8,9], while T. forsythia may not co-localize spatially with the other two species [10,11]. Among the three species that are frequently found associated with the clinical measures of chronic periodontitis, P. gingivalis has been shown to be a major pathogen, with well-defined virulence factors including cell surface located proteolytic enzymes and adhesins (gingipains) [12,13]. Recent research suggests that a synergistic microbial community is more relevant to disease progression than individual species and it has been suggested that the abilities of micro-organisms to interact with one another are crucial for disease progression [14,15]. P. gingivalis has been shown to exert a community-wide pathogenic influence on the microbiota in an animal model and it has been suggested that the communication of P. gingivalis with other inhabitants of subgingival biofilm is crucial for the elevation of pathogenicity and disruption of host immune surveillance [16]. P. gingivalis and T. denticola displayed synergy in biofilm formation using a static biofilm model, but this was not observed with T. forsythia [17,18]. In dual-species biofilm experiments, T. denticola did not form homotypic biofilms, while P. gingivalis acted as an initial colonizer of the substratum, enabling subsequent incorporation of T. denticola [17,19]. P. gingivalis gingipains and T. denticola dentilisin have been shown to be involved in the coaggregation between the two species [19,20]. All of these studies on P. gingivalis and T. denticola synergistic biofilm formation used P. gingivalis strain 381 or ATCC 33277 in a static biofilm model as P. gingivalis W50 is considered to be poor biofilm former [21,22]. The simplicity of static biofilm system made it a widely used tool in biofilm research, especially for examining early events in biofilm formation [23]. However, its ability to generate mature biofilms is limited due to possible limitations of nutrient supply and lack of bulk phase movement. These limitations in the production of mature biofilms can be addressed using flow chambers and
continuous culture systems such as a chemostat [24]. A good example of the application of the later systems is the use of a chemostat to generate *T. denticola* homotypic mature biofilms [25]. We have previously shown that, using a flow cell model, *P. gingivalis* W50 was able to participate in polymicrobial mature biofilm formation with *T. denticola* and *T. forsythia* [26]. Although *P. gingivalis* W50 adhered poorly to the glass substratum, with only a few cells attached at the commencement of a constant flow, it managed to proliferate and became the dominant species in the mature polymicrobial biofilm. However, it remains to be elucidated how *P. gingivalis* W50 interacts with the other two species, especially *T. denticola*, and the mechanisms involved in polymicrobial biofilm formation.

In the current study we used a flow cell biofilm model to investigate the ability of *P. gingivalis* and *T. denticola* to form homotypic and polymicrobial biofilms and the role of *P. gingivalis* gingipains in biofilm formation. We demonstrate that both *P. gingivalis* W50 and *T. denticola* ATCC 35405 form extensive mature homotypic biofilms and that there is a strong synergy between the two species in biofilm formation and development. In addition *P. gingivalis* gingipains are essential for biofilm formation and the interactions of *P. gingivalis* with *T. denticola*.

**Methods**

**Bacterial Strains and Growth**

Bacterial strains used for this study were *Porphyromonas gingivalis* W50, *Trentepohlia denticola* ATCC 35405 and *Tannarella forsythia* ATCC 43037. A gingipain-null mutant of *P. gingivalis* W50 lacking RgpA, RgpB and Kgp (*P. gingivalis* W50ABK) was obtained from the culture collection of the Oral Health Cooperative Research Centre, The University of Melbourne, Australia and was created as described previously [27]. All of the cultures were grown anaerobically at 37°C in a MACS MG500 anaerobic workstation (Don Whitley Scientific, U.K.) containing a gaseous mix of 5% hydrogen, 5% carbon dioxide and 90% nitrogen. *P. gingivalis* was grown in brain heart infusion (BHI), *T. denticola* was grown in oral bacteria growth medium (OBGM), and *T. forsythia* was grown in tryptic soy broth supplemented with 0.3% yeast extract (TSBYK), vitamin K (0.4 μg/mL) and N-acetylglucosaminic acid (NAM) (10 μg/mL) (Sigma Aldrich, MO, USA) as described previously [26,28]. Each species was transferred into fresh OBGM to obtain exponential growth. The optical density of the bacterial cultures was adjusted with fresh OBGM to give an absorbance of 2.0 at a wavelength of 650 nm prior to snap freezing in liquid nitrogen and storage at −70°C. OBGM contained brain heart infusion (12.5 g/L), tryptone soya broth (10 g/L), yeast extract (7.5 g/L), sodium thiosulphate (0.5 g/L), asparagine (0.25 g/L), D-glucose (2 g/L), ascorbic acid (2 g/L), sodium pyruvate (1 g/L) and sodium bicarbonate (2 g/L), L-cysteine (1 g/L), ammonium sulfate (2 g/L), thiamine pyrophosphate (6 mg/L), heat inactivated rabbit serum (5% vol/vol), haemin (5 mg/L), menadione (1 mg/L), N-acetylglucosaminic acid (10 mg/mL) and a volatile fatty acid mix (0.5% vol/vol). All species, including the *P. gingivalis* W50ABK mutant grew well as planktonic cultures in OBGM.

**Flow Cell Preparation**

A single track (40 mm long, 16 mm wide and 2 mm deep) was milled into a high-density polyethylene block, serving as the incubation chamber for the flow cell. A standard-sized, uncoated glass microscope coverslip (ProSciTech, QLD, Australia), which served as the attachment substratum for the biofilm, was secured to the flow cell with a silicone adhesive (GE Silicones, General Electric Company, Waterford, NY). Sodium hypochlorite with 0.5% available chlorine was pumped through the flow cell system for 2 h to ensure sterility. This was followed by overnight rinsing with sterile ultrapure water to flush out the bleach. The flow cell system was then treated with pre-reduced 20% OBGM for 2 h at 37°C in an MG500 anaerobic workstation to condition the glass surface with medium prior to inoculation.

**Growth of Biofilm in Flow Cells**

Snap frozen stocks of each strain were thawed and used as the inoculum. Inocula for polymicrobial biofilms were first coaggregated by mixing 0.5 mL portions of *P. gingivalis*, *T. denticola* and *T. forsythia* prior to inoculation. After inoculation, the system was incubated for 1 h prior to a constant flow (3 mL/h) of OBGM diluted 4:1 with water to 20% full strength.

Biofilms adhering to the glass coverslips were harvested 90 h after the commencement of medium flow. They were first rinsed *in situ* with phosphate buffered saline (PBS) to remove culture medium and unattached bacterial cells prior to fixation with 4% paraformaldehyde for 1 h at room temperature. After fixation, residual paraformaldehyde was flushed out with PBS. For subsequent *in situ* analyses by scanning electron microscopy (SEM), the coverslip was removed from the flow cell using a diamond pen. For fluorescence staining, the biofilm was embedded in acrylamide and subjected to fluorescent *in situ* hybridisation (FISH) using *T. denticola* Snap frozen stocks of each strain were thawed and used as the inoculum. Isolates for polymicrobial biofilms were first coaggregated by mixing 0.5 mL portions of *P. gingivalis*, *T. denticola* and *T. forsythia* prior to inoculation. After inoculation, the system was incubated for 1 h prior to a constant flow (3 mL/h) of OBGM diluted 4:1 with water to 20% full strength.

**Fluorescent Staining of Biofilms**

Biofilms were fluorescently stained essentially as described [26]. Single-species biofilms were stained with Syto 9 DNA dye (6 μM, Life Technologies, Grand Island, NY), and polymicrobial biofilms subjected to fluorescent *in situ* hybridisation (FISH) using species-specific probes.

**Confocal Laser Scanning Microscopy (CLSM) and Image Analysis**

Fluorescently labelled biofilms were visualised on a confocal laser scanning microscope as described previously [26]. The confocal datasets, 3 image stacks in random positions from each of two biological replicates, were analysed with COMSTAT software to determine biometric parameters of the biofilm [29]. The biometric data were statistically analysed using independent t-test and a P value of <0.05 was considered to be statistically significant. Three-dimensional reconstructed images were produced using Zeiss LSM image browser (Carl Zeiss, Germany).

**Scanning Electron Microscopy (SEM)**

Biofilm samples for SEM were prepared as described [26] and imaged with a Philips XL30 field-emission scanning electron microscope (Philips, Eindhoven, Netherlands) at a voltage of 2 kV.

**Results**

**Synergistic Biofilm Formation by *P. gingivalis* and *T. denticola***

Homotypic biofilms of *P. gingivalis* W50 and *T. denticola* were harvested from the flow cell 90 h after the commencement of constant medium flow. Both *P. gingivalis* W50 and *T. denticola* ATCC 35405 formed mature biofilms, characterised by a heterogeneous architecture consisting of microcolonies surrounded by open areas with more scattered colonisation, as revealed by
biovolume and substratum coverage of the single microcolony from two biological replicates (Table 1). The images taken at five randomly chosen positions each containing biofilms were more than three times greater than T. denticola biofilms (Table 1), suggesting T. denticola has a greater potential for forming homotypic biofilms. The maximum thickness of P. gingivalis biofilms was comparable to that of T. denticola, however, the average thickness of P. gingivalis W50 biofilms was more than double, indicating that P. gingivalis biofilms have a greater cell density (Table 1).

Polymicrobial biofilms formed by P. gingivalis W50, T. denticola and T. forsythia consisted of large microcolonies (Figure 1c). T. forsythia was present in extremely low numbers and was only forsythia T. forsythia and T. denticola formed the basal layer of the mature polymicrobial biofilms, while P. gingivalis was the dominant species of the microcolonies (Figure 1c). T. denticola was closely associated with P. gingivalis in microcolonies, but its abundance decreased towards the top of the microcolonies (further away from the substratum). P. gingivalis, T. denticola and T. forsythia polymicrobial biofilms showed significantly higher colonisation of the substratum compared with either P. gingivalis or T. denticola alone (Figure 2). There was an approximately three-fold increase in total biovolume for P. gingivalis and a six-fold increase for T. denticola in polymicrobial microcolonies compared with single-species biofilms (Figure 2).

The thickness of biofilms for each species in microcolonies also increased dramatically (Figure 2). The total biovolume and average thickness of the two species together in polymicrobial biofilms increased by approximately four and seven-fold, respectively, when compared with the sum of each species in homotypic biofilms, suggesting a strong synergy in biofilm formation between these species. Moreover, the morphology of T. denticola in single-species biofilms (Figure 1b) was distinctly different from that in the polymicrobial biofilm (Figure 1c). In single-species biofilms, the vast majority of T. denticola cells did not display the spiral morphology that is characteristic of spirochetes. In contrast, in the polymicrobial biofilms, T. denticola cells retained their typical coiled morphology.

T. forsythia did not form mature single-species biofilms in the flow cell system, although it grew well in the growth medium as a planktonic culture. Furthermore, there was no significant difference in biometric parameters of double-species biofilms formed by P. gingivalis and T. denticola when compared with polymicrobial biofilms formed by the three species (data not shown).

**Gingipains are Essential for Mature Biofilm Formation and the Interaction Between P. gingivalis and T. denticola**

The P. gingivalis W50ABK mutant, lacking functional cell surface-located RgpA, RgpB and Kgp gingipains, was used in this study to investigate the role of gingipains in polymicrobial biofilm formation. P. gingivalis W50ABK was not able to form mature biofilms after 90 h incubation, with only a few thin clumps of cells up to 12 μm in depth adhering to the glass substratum (Figure 3a). Few cells were detected using SEM, suggesting that the attachment of P. gingivalis W50ABK to the glass substratum was not strong enough to withstand the extensive sample preparation required for SEM. When P. gingivalis W50ABK was incubated with T. denticola and T. forsythia, W50ABK was present in low numbers, similar to what was observed in single-species biofilm of the W50ABK mutant. It was not possible to accurately enumerate P. gingivalis W50ABK in the polymicrobial biofilm due to the extremely low number of cells. The main component of the polymicrobial biofilm was T. denticola and the biofilm structure was similar to T. denticola single-species biofilm, except for a small amount of P. gingivalis W50ABK associated with T. denticola microcolonies (Figure 3b).

When compared with single-species T. denticola biofilms, there was no significant difference in biometric parameters of T. denticola in the polymicrobial biofilms with P. gingivalis W50ABK and T. forsythia, as determined from five CLSM images at random positions from two biological replicates (Table 1), thus indicating that there was no synergy in biofilm formation between P. gingivalis W50ABK and T. denticola.

**Biofilm Surface Structure Revealed by Scanning Electron Microscopy**

SEM was used to examine topographies of polymicrobial biofilms with high magnification. On the surface of polymicrobial microcolonies formed by wild-type P. gingivalis, T. denticola and T. forsythia, P. gingivalis was the major component (Figure 4). T. denticola was closely associated with P. gingivalis and showed the typical spiral morphology characteristic of spirochetes (Figure 4). T. denticola cells on the surface of microcolonies connected distant P. gingivalis cells (Figure 4a). P. gingivalis cells were found attached to the ends of T. denticola cells that projected out from the surface of the microcolonies (Figure 4b, arrows). A large number of outer membrane vesicles (OMVs) were observed on the surface of wild-type P. gingivalis cells in the polymicrobial biofilm (Figure 4c). In P.
gingivalis homotypic biofilms, OMVs were also observed on the cell surface with a similar abundance. When *P. gingivalis* W50ABK was co-inoculated with *T. denticola* and *T. forsythia*, the majority of cells in biofilms were found to be *T. denticola*, with a few *P. gingivalis* cells either associated within the microcolony (Figure 5a), or at the outer edges of the microcolonies (Figure 5b). The surface of the *P. gingivalis* W50ABK mutant was rough and the shape of the cells less symmetrical (Figure 5a) when compared with wild-type (Figure 4c). Most of the *T. denticola* cells incubated with the *P. gingivalis* W50ABK mutant lost the typical spiral morphology (Figure 5a). Blebbing of the outer membrane of *P. gingivalis* W50ABK mutant cells was not as abundant as that found with wild type (Figure 4c). Furthermore, the blebbing of the mutant exhibited morphology that was distinct from the well-formed OMVs found on wild-type *P. gingivalis* cells (Figures 4c and 5a). Interestingly, filamentous structures were found on *P. gingivalis* W50ABK mutant cells in polymicrobial biofilms formed with *T. denticola* and *T. forsythia*, connecting individual *P. gingivalis* cells (Figure 5b) or between *P. gingivalis* and *T. denticola* (Figure 5a).

### Discussion

Due to the wide-spread use of static biofilm assays on polystyrene surface, *P. gingivalis* W50, which generally adheres poorly to substratum, has been considered as a poor biofilm former [21,22]. However, it has been suggested that *P. gingivalis* W50-like strains are more virulent in experimental infections and cause a more invasive type of infection when compared with *P. gingivalis* 381/ATCC 33277-like strains, which cause a more localised infection [30–32]. By using a dynamic system with a low flow rate designed to mimic the flow of gingival crevicular fluid within the periodontal pocket, we showed that *P. gingivalis* W50 was in fact able to form extensive, mature homotypic biofilms.

Table 1. Biometric parameters of *P. gingivalis* and *T. denticola* homotypic biofilm and polymicrobial biofilm formed by *P. gingivalis* W50 ABK, *T. denticola* and *T. forsythia* harvested at 90 h.

| Biometric parameters | Homotypic biofilms | Polymicrobial biofilm with *P. gingivalis* W50ABK |
|----------------------|--------------------|-----------------------------------------------|
|                      | *P. gingivalis* W50 | *T. denticola* | *T. denticola* |
| Biovolume (μm^3/μm^2) | 3.63±0.36* | 0.94±0.62 | 1.40±0.33 |
| Average thickness of biofilm (μm) | 4.93±0.51* | 2.25±1.27 | 4.48±2.94 |
| Maximum thickness of biofilm (μm) | 29.66±8.09* | 25.67±6.13 | 36.64±14.04 |
| Substratum coverage (%) | 28.42±1.71* | 6.06±6.57 | 6.48±0.94 |

Data are expressed as means ± standard deviations of two biological replicates, from five CLSM images at random positions from each biological replicate. All images were analysed using COMSTAT software.

*Significantly different to *T. denticola* homotypic biofilm values, as determined by Students’ T-test (p≤0.05).

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Figure 2. Synergistic *P. gingivalis* and *T. denticola* biofilm formation. Both polymicrobial and homotypic biofilms were produced using a flow cell under identical conditions. The two sets of bars above the species name refer to the biometric parameters measured using the species-specific fluorescent probe for the species grown either as part of the polymicrobial biofilm or as a homotypic biofilm. The primary vertical axis (left) is for maximum (grey bars) and average (black bars) biofilm thickness (μm) and the secondary vertical axis (right) is for biovolume (white bars) (μm^3/μm^2).

Data are expressed as means ± standard deviations of five CLSM images at random positions from biological replicates. All images were analysed using COMSTAT software.

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The role of motile bacteria such as T. denticola in polymicrobial biofilms, in polymicrobial biofilms. SEM images showed that in mature biomass, as well as the restored spiral morphology of P. gingivalis. However the findings of this current study demonstrate that the resultant enhanced nutrient flow enables a higher biofilm biomass to be sustained. A T. denticola flagella hook figE mutant formed polymicrobial biofilms with P. gingivalis, but was only found in the basal layer of the polymicrobial biofilms and no synergy was observed (unpublished data). This suggests that the motility of T. denticola plays an important role in synergistic biofilm formation with P. gingivalis.

The mature polymicrobial biofilm consisted predominantly of P. gingivalis, which has previously been shown to be abundant in subgingival plaque from deep periodontal pockets and the level of which, once above a certain threshold, was predictive of disease progression [42]. In our previous study [26], it was shown that only a few cells of P. gingivalis W50 were attached to the glass substratum after the commencement of a constant flow, but the bacterium proliferated quickly after 24 h. P. gingivalis coaggregates with T. denticola [43,44] and it has been shown that a P. gingivalis gingipain-null mutant lost the ability to coaggregate with T. denticola [20]. Our current study shows that gingipains are essential for interaction between P. gingivalis and T. denticola since the P. gingivalis W50ABK mutant had no impact on T. denticola biomass and morphology.

Notably, in polymicrobial biofilms formed by wild-type P. gingivalis, T. denticola and T. forsythia, a large number of OMVs were found on the surface of P. gingivalis cells. It has been suggested that OMVs plays an important role in intercellular communication and are involved in biofilm formation and virulence of other bacterial species [45–47]. A recent study of Pseudomonas putida showed that the formation of OMVs leads to an increase in cell surface hydrophobicity, making cells attach more easily to each other as well as the substratum, thus enhancing their ability to form biofilms [48]. It has been suggested that P. gingivalis selectively sorts outer membrane proteins into OMVs, resulting in an enrichment of gingipains in OMVs [49]. Besides the catalytic domain, gingipains also encode non-catalytic adhesion domains and these adhesins have been shown to be responsible for the interaction of P. gingivalis with other bacteria, including T. denticola [20,50,51]. It is possible that this enrichment of adhesins in OMVs contributed to the synergistic biofilm formation by P. gingivalis and T. denticola. In polymicrobial biofilms formed by P. gingivalis W50ABK, T. denticola and T. forsythia, some blebbing of the outer membrane of the W50ABK mutant was observed. However, it appeared that it represented only the initial stages of vesiculation.
Figure 4. SEM micrographs of polymicrobial biofilms involving wild-type *P. gingivalis*, *T. denticola* and *T. forsythia*. *T. denticola*, the long thin spirochaete; *P. gingivalis*, the smaller grape-like coccobacillus and *T. forsythia* the larger fusiform rod. Panels B and C are higher magnifications of sections in Panel A. (a) *T. denticola* forming bridges. (b) *P. gingivalis* attached to the ends of *T. denticola* projecting out of the microcolonies. (c) Outer membrane vesicles on the surface of *P. gingivalis*.

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since not many well-formed OMVs were observed on the surface of the mutant compared with wild-type cells. This finding may suggest that gingipains facilitate the maturation of P. gingivalis OMVs. The inability of P. gingivalis W50ABK to produce OMVs and to form mature homotypic P. gingivalis biofilms suggests that gingipains facilitate the maturation of P. gingivalis biofilm formation or that biofilm formation is an important trigger for OMV production. Interestingly, in poly microbial biofilms with the W50ABK mutant, an enrichment of filamentous appendages was observed. The filamentous structures connected adjacent P. gingivalis cells or P. gingivalis with T. denticola cells. It is possible that in the absence of gingipains in the P. gingivalis W50ABK mutant, other pathways (e.g., minor fimbrine production) are upregulated to compensate for the loss of cell surface adhesins and OMVs.

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