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Treponema denticola improves adhesive capacities of *Porphyromonas gingivalis*

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SUMMARY

Porphyromonas gingivalis*, an important etiological agent of periodontal disease, is frequently found associated with *Treponema denticola*, an anaerobic spirochete, in pathogenic biofilms. However, interactions between these two bacteria are not well understood at the molecular level. In this study, we seek to link the influence of *T. denticola* on the expression of *P. gingivalis* proteases with its capacities to adhere and to form biofilms. The *P. gingivalis* genes encoding Arg-gingipain A (*RgpA*), Lys-gingipain (*Kgp*), and hemagglutinin A (*HagA*) were more strongly expressed after incubation with *T. denticola* compared with *P. gingivalis* alone. The amounts of the three resulting proteins, all of which contain hemagglutinin adhesion domains, were increased in culture supernatants. Moreover, incubation of *P. gingivalis* with *T. denticola* promoted static and dynamic biofilm formation, primarily via a time-dependent enhancement of *P. gingivalis* adhesion capacities on bacterial partners such as *Streptococcus gordonii*. Adhesion of *P. gingivalis* to human cells was also increased. These results showed that interactions of *P. gingivalis* with other bacterial species, such as *T. denticola*, induce increased adhesive capacities on various substrata by hemagglutinin adhesion domain-containing proteins.

INTRODUCTION

Periodontal diseases are multifactorial infections initiated by multispecies bacterial communities organized in a complex and dynamic biofilm (Rosan & Lamont, 2000). Early colonizers of the salivary pellicle on the tooth surface, mainly commensal oral streptococci such as *Streptococcus gordonii*, initiate biofilm formation by favoring adhesion and colonization by late pathogenic colonizers, including *Porphyromonas gingivalis* and *Treponema denticola*. *Porphyromonas gingivalis*, a gram-negative, black-pigmented, anaerobic rod, is widely recognized as an important etiological agent of periodontal disease (Lamont & Jenkinson, 1998). *Porphyromonas gingivalis* possesses several virulence factors, including adherence factors, responsible for biofilm formation and human cell colo-

nization, and proteases responsible for the destruction of periodontal tissues. Among *P. gingivalis* proteases, gingipains are cysteine proteinases, either arginine-specific (Rgp) or lysine-specific (Kgp), which possess propeptide and catalytic domains (Potempa *et al.*, 2003). Two different Rgp gingipains are expressed by *P. gingivalis*, RgpA and gpB. RgpA differs from RgpB by the presence of four C-terminal hemagglutinin adhesion (HA) domains, also found in Kgp and hemagglutinin A (hagA) (Han *et al.*, 1996; Curtis, 1997; Shibata *et al.*, 1999; Li *et al.*, 2011). These domains are involved in hemagglutination, hemoglobin-binding activity and adhesion.

The influence of other bacteria species on *P. gingivalis* is poorly understood, apart from the coaggregation phenomenon with microbial partners. Recent publications investigated *in vitro* the role of oral partners in adhesion and invasion of human cells with contradictory results (Saito *et al.*, 2009; Kirschbaum *et al.*, 2010), whereas a mouse model of periodontitis showed that the presence of *T. denticola* decreased the concentration of *P. gingivalis* necessary to develop infection (Orth *et al.*, 2011). *Treponema denticola* and *P. gingivalis* are frequently associated in oral biofilms (Kuramitsu *et al.*, 2005) and nutritional interactions between them have been described (Grenier, 1992). Interactions with first and later colonizers, more specifically with *T. denticola*, seem to influence the virulence of *P. gingivalis.* The *P. gingivalis* can coaggregate via its major fimbriae (FimA) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of *Streptococcus* species (Maeda *et al.*, 2004a,b). Moreover, *P. gingivalis* fimbriae can bind to *T. denticola* dentilisin, allowing coaggregation of both bacteria (Hashimoto *et al.*, 2003) or adhesion of *T. denticola* to *P. gingivalis* in biofilms (Vesey & Kuramitsu, 2004; Cogoni *et al.*, 2012). The role of the hemagglutinin/adhesin domain 1 (HA1 or Hgp44) in coaggregation with *T. denticola* is also documented (Ito *et al.*, 2010). Interestingly, molecules responsible for coaggregation are also implicated in biofilm formation such as FimA (Kuboniwa *et al.*, 2009), and their maturation is controlled by gingipain enzymes (Potempa *et al.*, 2003).

In this study, we investigated the influence of *T. denticola* on *P. gingivalis* focusing on virulence factors, including fimbriae and proteases. We showed that incubation of *P. gingivalis* with *T. denticola* enhanced the expression of *P. gingivalis* genes encoding gingipains *kgp*, *rgpA* and *hagA*, all of which contain HA domains, compared with the absence of *T. denticola*. Consequences of this increased expression were further evaluated on adhesive properties of *P. gingivalis*, on bacteria species and eukaryotic cells.

METHODS

Bacterial strains and cell lines

Porphyromonas gingivalis ATCC 33277 or *P. gingivalis*-SNAP26 (25) and *S. gordonii* DL1 pCM18 (GFP-mut3*), a gift from S. Molin (Molecular Microbial Ecology Group, Technical University of Denmark), were grown on blood Columbia agar plates and/or in a brain–heart infusion broth (AES Chemunex, Combourg, France) supplemented with menadione (10 μg ml^{-1}) and hemin (5 μg ml^{-1}; Sigma, Saint Quentin Fallavier, France). *Treponema denticola* ATCC 35404 were grown in NOS Spirochete medium (ATCC medium 1494; ATCC, Manassas, VA). For each experiment, cultures were used in the middle of log-phase growth at 37°C in an anaerobic chamber (MAC 500; Don Whitley Scientific, Shipley, UK) with 10% H_2, 10% CO_2, and 80% N_2.

KB epithelial cells were obtained from ATCC (HeLa derivative, ATCC CCL 17) and grown in Eagles' modified essential medium enriched with 10% fetal calf serum and an antibiotic mixture (10,000 U ml^{-1} streptomycin and 10,000 μg ml^{-1} penicillin) at 37°C with 5% CO_2 in a humidified atmosphere. Unless specified, all cell culture reagents were from Lonza (Levallois-Perret, France).

Conditions of co-incubation of *Porphyromonas gingivalis* with *Treponema denticola*

All studies were performed in buffer containing 10 mM Tris–HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 8.0 (Rosen *et al.*, 2008). The *P. gingivalis* and *T. denticola* bacterial suspensions were harvested, washed three times, adjusted to the same optical density at 600 nm (OD_{600}), and mixed in equal volumes for different times of incubation. When specified, *S. gordonii* cells were also prepared in the same way. Controls of *P. gingivalis* alone were prepared with the same OD_{600} adjusted in the same final volume as for two bacterial species. These bacterial coincubation mixtures were used for all experiments: coaggregation activity, gene expression, protein

analysis, enzymatic or hemagglutination activity and adhesion to epithelial cells (chosen OD_{600} was 0.9–1.0) and biofilm experiments (chosen OD_{600} was 0.2). Ratios of *P. gingivalis* : *T. denticola* were therefore always 1 : 1 according to OD measurements.

Coaggregation activity

Autoaggregation or coaggregation activity of different coincubation bacterial mixtures was monitored by optical density at 600 nm using the turbidimetric assay as previously described (McIntire *et al.*, 1978).

Gene expression

RNA synthesis in bacterial *P. gingivalis* or *P. gingivalis–T. denticola* suspensions was stopped with two volumes of ice-cold methanol (methanol 60%, HEPES 62.5 mM, pH 6.5) after 1, 2, 10, 40 and 120 min of incubation as described above. Total RNA was extracted and purified using the RNeasy kit (Qiagen, Courtaboeuf, France) with a DNase treatment using the RQ1 RNase-free DNase 1 (Promega, Charbonnières, France) according to the manufacturer's instructions. Quality-checked RNA (1 μg) was used for reverse transcription using M-MLV-RT (Promega) following the manufacturer's protocol. Real time-PCR was performed on an ABI PRISM 7000 sequence detection system (95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min; Applied Biosystems, Villebon sur Yvette, France) in triplicate to evaluate hemolysin (*hem*), hemagglutinins (*hagA* and *C*), both fimbriae (*fimA* and *mfa1*), and gingipains (*kgp*, *rgpA* and *rgpB*) using qPCRMasterMixPlus for SYBR®GreenI according to the manufacturer's instructions (Eurogentec, Seraing, Belgium). Primer sequences used for messenger RNA amplification were designed and checked to amplify specifically messenger RNA from *P. gingivalis* (Table 1). Expression ($2^{\Delta\Delta C_t}$) was adjusted using internal reference (16S ribosomal RNA) and compared with *P. gingivalis* alone at the corresponding time during all experiments.

Protein analysis

After incubation times ranging from 0 to 60 min, *P. gingivalis*, *T. denticola* and *P. gingivalis/T. denticola* coincubation mixtures were harvested and pelleted. Supernatants were collected and concentrated using Centriplus YM-10 (Millipore, Billerica, MA) centrifugal filter devices. Pellets were used to prepare crude cell

Table 1 List of specific primers used for expression studies

Locus[1]	Gene	Description	Sequences 5′–3′	Length (bp)[2]
	16S rRNA	Ribosomal RNA	F: CAATCGGAGTTCCTCGTGAT R: TGGGTTTAAAGGGTGCGTAG	161
PGN_1733	*hagA*	Hemagglutinin A	F: ACAGCATCAGCCGATATTCC R: CGAATTCATTGCCACCTTCT	208
PGN_1906	*hagC*	Hemagglutinin C	F: ATTCAGAGCCAAATCCTCCA R: GTTTTCCCGGCTCCAAAA	142
PGN_1970	*rgpA*	Arginine-gingipain A	F: GCCGAGATTGTTCTTGAAGC R: AGGAGCAGCAATTGCAAAGT	276
PGN_1466	*rgpB*	Arginine-gingipain B	F: CGCTGATGAAACGAACTTGA R: CTTCGAATACCATGCGGTTT	230
PGN_1728	*kgp*	Lysine-gingipain	F: GTGTCGGCTTTGCCAACTAT R: AGGCTGAACACCAAATACGG	290
PGN_1802	*hem*	Hemolysin	F: ACGAAGCCTTGTTCTCCTCA R: CAATGAATATGCCGGTTTCC	189
PGN_0287	*mfa1*	Minor fimbriae	F: GAAAGTGCTGCTGGTAG R: CAGATGGGTTGTTGCTCA	118
PGN_0180	*fimA*	Major fimbriae	F: AATTCGAATCGTGCTTTTGG R: GTCTTGCCAACCAGTTCCAT	200

F, forward; R, reverse.

[1]Locus number and identification according to the NCBI *Porphyromonas gingivalis* genome database.

[2]Polymerase chain reaction product length (base pairs).

extracts. Briefly, pellets were suspended in 10 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma), 1 mM leupeptin (Sigma) and 5 mM EDTA (Sigma) before disruption by sonication. Supernatants and crude cell extracts were diluted in Laemmli buffer, boiled at 95° C, and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Amounts of 25 and 5 μg crude cell extract and supernatant, respectively, were loaded onto sodium dodecyl sulfate (10%) polyacrylamide gels. Proteins were transferred onto a nitrocellulose membrane before saturation with 5% low-fat milk in phosphate-buffered saline (PBS) for 2 h at room temperature. Incubations with a monoclonal primary antibody against HA domains at a 1 : 200 dilution (61BG13, a gift from R. Gmür, Institute of Oral Biology, University of Zurich, Switzerland) were performed in 2.5% low-fat milk in PBS containing 0.1% Tween-20 (PBST) at 4°C overnight. After three washes, membranes blotted with cell extracts or with supernatants were incubated for 1 h at room temperature in 2.5% low-fat milk PBST containing either IR-Dye 680LT Goat Anti-Mouse Secondary Antibody (Li-Cor Biosciences, Lincoln, NE) or horseradish peroxidase-conjugated anti-mouse IgG antibody (1 : 10,000). Immune detection was performed using the Odyssey infrared imaging system (Li-Cor Biosciences) for cell extracts or by chemiluminescence using the Super Signal kit (Pierce, Rockford, IL) for supernatants.

Adhesion to epithelial cells

KB cells were seeded into 24-well tissue culture plates at a density of approximately 2×10^5 cells per well. Cells were grown to confluent monolayers over a period of 48 h and then washed three times in PBS (pH 7.4). After incubation times of 0 or 120 min, 200 μl *P. gingivalis* alone or 200 μl *P. gingivalis*/*T. denticola* coincubation mixtures was incubated for 30 min with KB monolayers in antibiotic-free Eagles' modified essential medium (multiplicity of infection 1 : 100 for *P. gingivalis*). Unattached bacteria were removed following three washes of the monolayers with PBS. Percentages of *P. gingivalis* bacteria associated to KB cells were calculated by counting colony-forming units on blood Columbia agar plates after 7 days of anaerobic culture, normalized to bacteria added to cells.

Flow system for assays of biofilm formation and *Porphyromonas gingivalis* adhesion

Exponential bacterial cultures of *S. gordonii*-GFP-mut3*, *P. gingivalis*-SNAP26 and *T. denticola* were harvested. The *P. gingivalis* was stained as previously described by Nicolle *et al.* (2010), with 3 μM SNAP-Cell TMR Star (New England Biolabs, Ipswich, MA).

Analysis of biofilm formation was performed in sterile Ludin chambers (Life Imaging Services, Basel, Switzerland) or in VI 0.4 hydrophobic uncoate μ-slides (Ibidi, Planegg, Germany) connected to a peristaltic pump (Minipuls 3; Gilson, Middleton, WI) allowing a flow rate of 7 ml h^{-1} through silicone tubing in anaerobic conditions. Flow cells were coated with 0.22-μm filtered sterile human saliva (collected from at least six healthy volunteers, treated with 2.5 mM dithiothreitol and diluted in distilled water to obtain a 25% volume/volume solution) for 30 min before bacteria inoculation.

1. For assays of *P. gingivalis* adhesion: After saliva coating with or without *S. gordonii* inoculation (OD_{600} 0.04) for 30 min in the flowing system, *P. gingivalis* (OD_{600} 0.2) mixed with or without *T. denticola* (OD_{600} 0.2) were inoculated in flow cells for 15 min, either immediately or after a 120-min incubation.
2. For assays of mixed biofilm formation: *S. gordonii* (OD_{600} 0.04) and *P. gingivalis* (OD_{600} 0.2) with or without *T. denticola* (OD_{600} 0.2) were coincubated for 30 min before inoculation of the mixture for 3 h in the flowing system.

After washing with brain–heart infusion broth 1 : 50 for 30 min, flow cells were observed *in situ* with a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Wezlar, Germany). An HC PL Apo 63×, 1.4 NA, oil immersion objective lens was used for image capture and a numerical zoom of 2 was applied. The 488-nm argon laser and a 495–565-nm band-pass emission filter were used to detect *S. gordonii* bacteria expressing GFP-mut3*. The 561-nm diode laser and a 575–620-nm band pass emission filter were used to detect *P. gingivalis* TMR-Star positive bacteria. Control experiments did not show any fluorescence associated with *T. denticola* in these conditions. Biofilm stacks (123 × 123 μm) acquired at 0.5-μm intervals were scanned with a line average of 2. Leica software

(LAS AF V.2.2.1) was used for microscope piloting and image acquisition before image analysis by IMAGEJ V1.43m (National Institutes of Health, Bethesda, MD) and MATLAB with COMSTAT.

Evaluation of static biofilm formation by the Biofilm Control Ring Test

The ability of *P. gingivalis* to form a biofilm alone or in the presence of *T. denticola* was tested after different incubation times, as described above. Bacteria were harvested by centrifugation at 7000 ***g*** for 10 min and re-suspended in sterile brain–heart infusion medium. The Biofilm Control Technology (Saint Beauzire, France) was used to assess biofilm formation. The kit was used according to the manufacturer's instructions. Briefly, a solution of magnetic beads (toner, 10 μl ml^{-1}) was added to each bacterial suspension (200 μl) in wells of polystyrene strips. One well with toner and without bacteria was used as control for bead efficiency. After homogenization, cultures were incubated at 37°C in anaerobic conditions for various times ranging from 0 to 7 h to allow biofilm formation. At any time point, wells were scanned by a dedicated scanner before and after magnetization on the magnet support (block test). Before and after magnetization, each well was compared using the BIOFILM CONTROL Software, which obtains a BioFilm Index (BFI) (Chavant *et al.*, 2007). A high BFI (>7) value corresponds to a high mobility of beads under magnet action (no biofilm) whereas a low value (BFI < 2) reflects full immobilization of beads and formation of biofilm.

Statistical analysis

Results were subjected to one-way analysis of variance comparison (using the program EZANOVA). A *P*-value < 0.05 was considered statistically significant.

RESULTS

Porphyromonas gingivalis coaggregated with *Treponema denticola*

As shown in Table 2, *T. denticola* alone naturally begins to sediment only 1 h after shaking, with an aggregation factor of 50%. After 2 h, approximately 65% of *T. denticola* bacteria are settled at the bottom of the tube, compared with only 10 and 18% autoaggregation of *S. gordonii* and *P. gingivalis*, respectively.

Table 2 Autoaggregation and coaggregation with *Treponema denticola* as evaluated by turbidimetric assay

	Aggregation (%)[1]			
	10 min	30 min	60 min	120 min
Streptococcus gordonii	–[2]	–	–	10
Porphyromonas gingivalis	–	–	12	18
T. denticola	–	–	51	64
P. gingivalis/*S. gordonii*	–	22	27	31
P. gingivalis/*T. denticola*	–	51	45	70
S. gordonii/*T. denticola*	–	–	–	–

[1]Percentages of autoaggregation or coaggregation were calculated from values of optical densities monitored at 600 nm, as described by McIntire *et al.* (1978).
[2]Not significant.

No coaggregation between bacterial species was observed 10 min after mixing. Addition of *P. gingivalis* to *T. denticola* induced 51% coaggregation only 30 min after mixing, whereas no autoaggregation of either *P. gingivalis* or *T. denticola* was observed at the same time (Table 2). Moderate coaggregation between *P. gingivalis* and *S. gordonii* was also observed after 30 min, with a coaggregation factor of 22%. These coaggregations observed with *P. gingivalis* were not greatly enhanced by longer times of contact between bacteria, with coaggregation factors slightly increasing from 60 to 120 min. No specific coaggregation between *T. denticola* and *S. gordonii* was observed.

Treponema denticola increased expression of *Porphyromonas gingivalis* genes implicated in virulence

Expression of *P. gingivalis* genes involved in virulence was analysed, after various coincubation times with *T. denticola* and compared with *P. gingivalis* alone. Genes encoding for HA-domain-containing proteins exhibited increased expression after *T. denticola* exposure for 1 min, with relative expression ratios of two-fold to eight-fold for *hagA* (2.23 ± 0.32), *kgp* (7.65 ± 1.24) and *rgpA* (6.37 ± 2.65) (Fig. 1A). The expression of these three genes then decreased, but was still up to two-fold greater for *rgpA* and *kgp* after 10 min of incubation with *T. denticola*; expression ultimately returned to control values (Fig. 1B). The

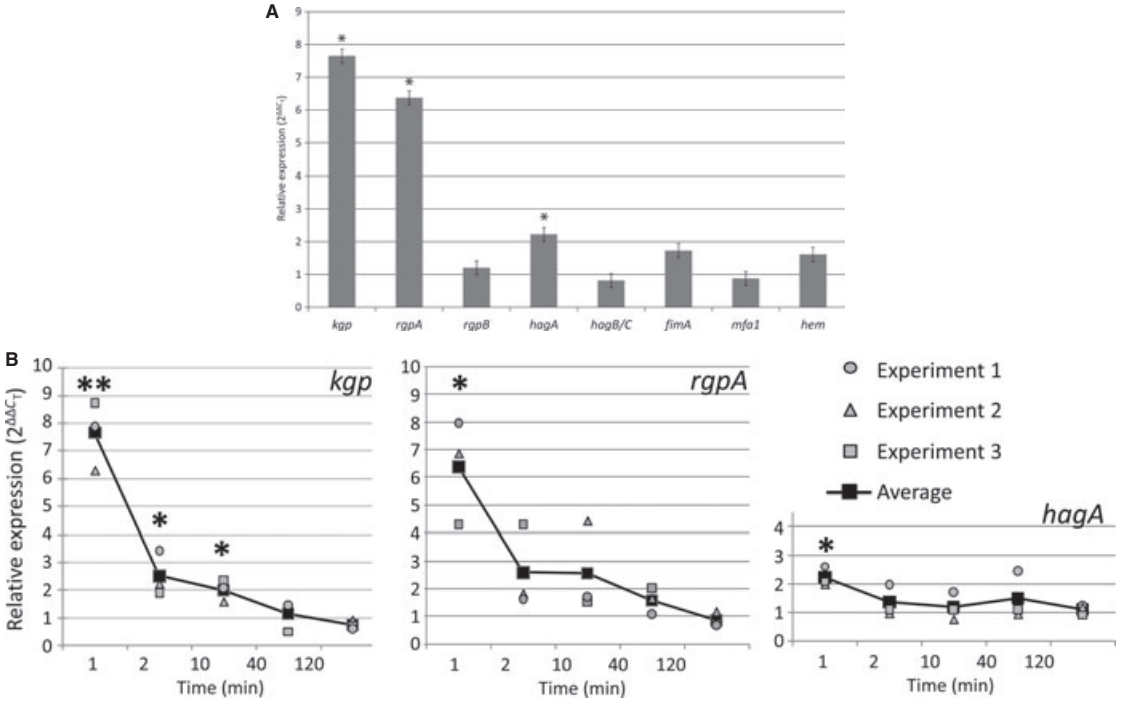

Figure 1 Effect of coincubation with *Treponema denticola* on the expression of *Porphyromonas gingivalis* virulence genes. (A) Relative expression of *P. gingivalis* genes after 1 min of coincubation of both bacteria species. (B) Effect of various incubation times with *T. denticola* on the expression of *P. gingivalis* genes. Relative gene expression as measured by reverse transcription–polymerase chain reaction ($2^{\Delta\Delta C_t}$) was adjusted using internal reference (16S ribosomal RNA) to *P. gingivalis* alone at corresponding times during all experiments. Results of three different experiments are presented, with their corresponding average. Asterisks (* and **) indicate significant differences between values of control bacteria (*P. gingivalis* alone) and bacteria after coincubation with *T. denticola* ($P < 0.05$ and $P < 0.01$, respectively).

expression of genes encoding fimbriae (*mfa1* and *fimA*), hemolysin (*hem*) and gingipain without HA domain (*rgpB*) were not significantly altered (Fig. 1A).

Levels of *P. gingivalis* proteins containing HA domains (RgpA, Kgp and HagA) were evaluated in bacteria pellets and their respective supernatants (Fig. 2) using an antibody raised against HA domains of RgpA. This antibody did not recognize any protein in the *T. denticola* protein extracts nor in the supernatant. In *P. gingivalis* protein extracts (Fig. 2, lane *P. gingivalis*), several forms of the proteins were detected, with main forms of approximately 39 and 44 kDa, as previously described (Booth & Lehner, 1997). Three other major bands were detected between 27 and 32 kDa, and a minor band at 14 kDa. After 0–60 min of coincubation with *T. denticola*, protein profiles of HA-domain-containing proteins in *P. gingivalis*/*T. denticola* mixtures were not modified compared with *P. gingivalis* alone, except for the minor 14-kDa band, which was less abundant.

In the corresponding *P. gingivalis*/*T. denticola* supernatants, main forms of approximately 39 and 44 kDa were also found, together with the 27-kDa form already detected in the pellet. Amounts of the major 39–44-kDa forms of the proteins were increased after 10 min compared with 0 min of coincubation. Moreover, the 27-kDa form, hardly visible in the supernatant at 0 min, appeared after 10 min. Amounts of these protein forms were maximal after 20 min of incubation with *T. denticola*, and then decreased.

Supernatants and cell pellets of bacteria were used to evaluate the enzymatic activity of *P. gingivalis* gingipains after coincubation with *T. denticola*. As *T. denticola* pellets and supernatants exhibited a significant enzymatic activity, comparison was made between 0 and 120 min of coincubation between *P. gingivalis* and *T. denticola* coincubation between *P. gingivalis* and *T. denticola* for 2 h induced increased Kgp-related and Rgp-related enzymatic activities in supernatants (see Fig. S1) compared with

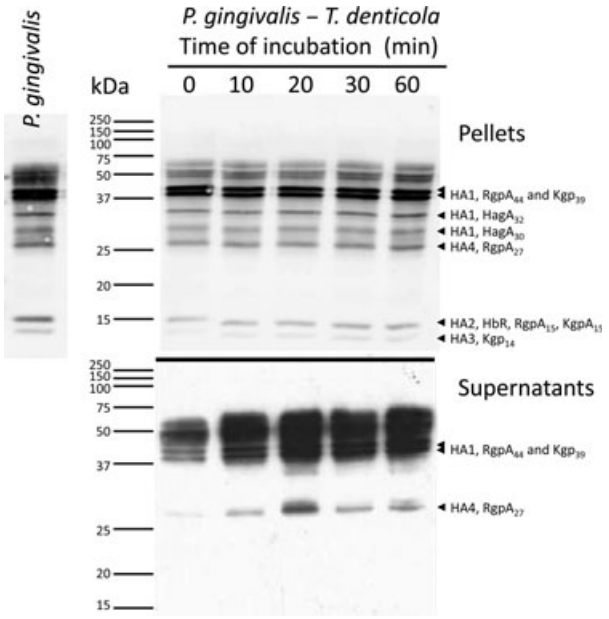

Figure 2 Expression of hemagglutinin/adhesin (HA) -containing proteins of *Porphyromonas gingivalis* after incubation with *Treponema denticola*. After various incubation times ranging from 0 to 60 min, supernatants and cell extracts of *P. gingivalis* and *T. denticola* mixtures were used for protein analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Detection of HA-domain-containing proteins was performed using antibody against RgpA (61BG13) combined with an infra-red imaging system for cells extracts or with chemiluminescence for supernatants. Equal amounts of proteins were loaded in each well. HbR, hemoglobin receptor.

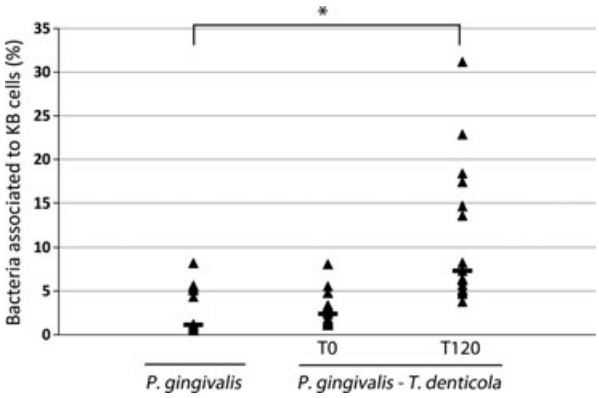

Figure 3 Adhesion of *Porphyromonas gingivalis* on epithelial KB cells in the presence of *Treponema denticola*. Either *P. gingivalis* alone or *P. gingivalis*/*T. denticola* coincubation mixtures after 0 (T0) or 120 min (T120) of incubation were used in adhesion assays. Percentages of *P. gingivalis* associated with KB cells were evaluated by colony-forming unit (CFU) counts and normalized to the number of bacteria added to cells. Values represented by dots were obtained from three different experiments with at least four different values for each condition. A median was calculated (black bold line) and the asterisk (*) indicates significant differences between assays with *P. gingivalis* alone and assays with a mixture of *T. denticola* and *P. gingivalis* after 120 min of incubation ($P < 0.05$).

0 min of coincubation between both species. The activity of both proteases in cell pellets was not modified by between 0 and 2 h coincubation (see Fig. S1).

The *P. gingivalis* hemagglutination activity was also evaluated after various times of coincubation with *T. denticola* (see Fig. S2). Hemagglutination activity of *P. gingivalis* was enhanced with coincubation time with *T. denticola*, from 15 min to 2 h.

Treponema denticola increased *Porphyromonas gingivalis* adhesion on epithelial cells

The presence of *T. denticola* did not significantly modify levels of *P. gingivalis* bacteria associated with KB cells compared with *P. gingivalis* alone when the *T. denticola*/*P. gingivalis* mixture was immediately added on cells (T0, Fig. 3). However, a prolonged coincubation of 2 h between *P. gingivalis* and *T. denticola* increased the percentage of *P. gingivalis* attached to KB cells with a 7.3 median percentage compared with 1.4% bacteria attached when *P. gingivalis* was added alone (T120, Fig. 3).

Treponema denticola increased *Porphyromonas gingivalis* adhesion for biofilm formation

Adhesion of *P. gingivalis* on slides coated with saliva with or without *S. gordonii* was equivalent, with *P. gingivalis* biomasses of 0.32 and 0.20 $\mu m^3\ \mu m^{-2}$ respectively. The *P. gingivalis* was uniformly scattered on the slides (Fig. 4A,B, first columns and Table 3).

The addition of *T. denticola* to *P. gingivalis* just before inoculation of bacteria in the flow increased the adhesion of *P. gingivalis* on slides coated with saliva with or without *S. gordonii* from two-fold to three-fold (Table 3). The distribution of *P. gingivalis* was also modified, with bacteria being mainly organized into clusters and only a few single bacteria scattered throughout (Fig. 4A,B, second columns).

A 2-h period of coincubation with *T. denticola* did not enhance *P. gingivalis* adhesion on slides coated with saliva alone, compared with *P. gingivalis* alone or with *T. denticola* at 0 min of coincubation (Fig. 4A,

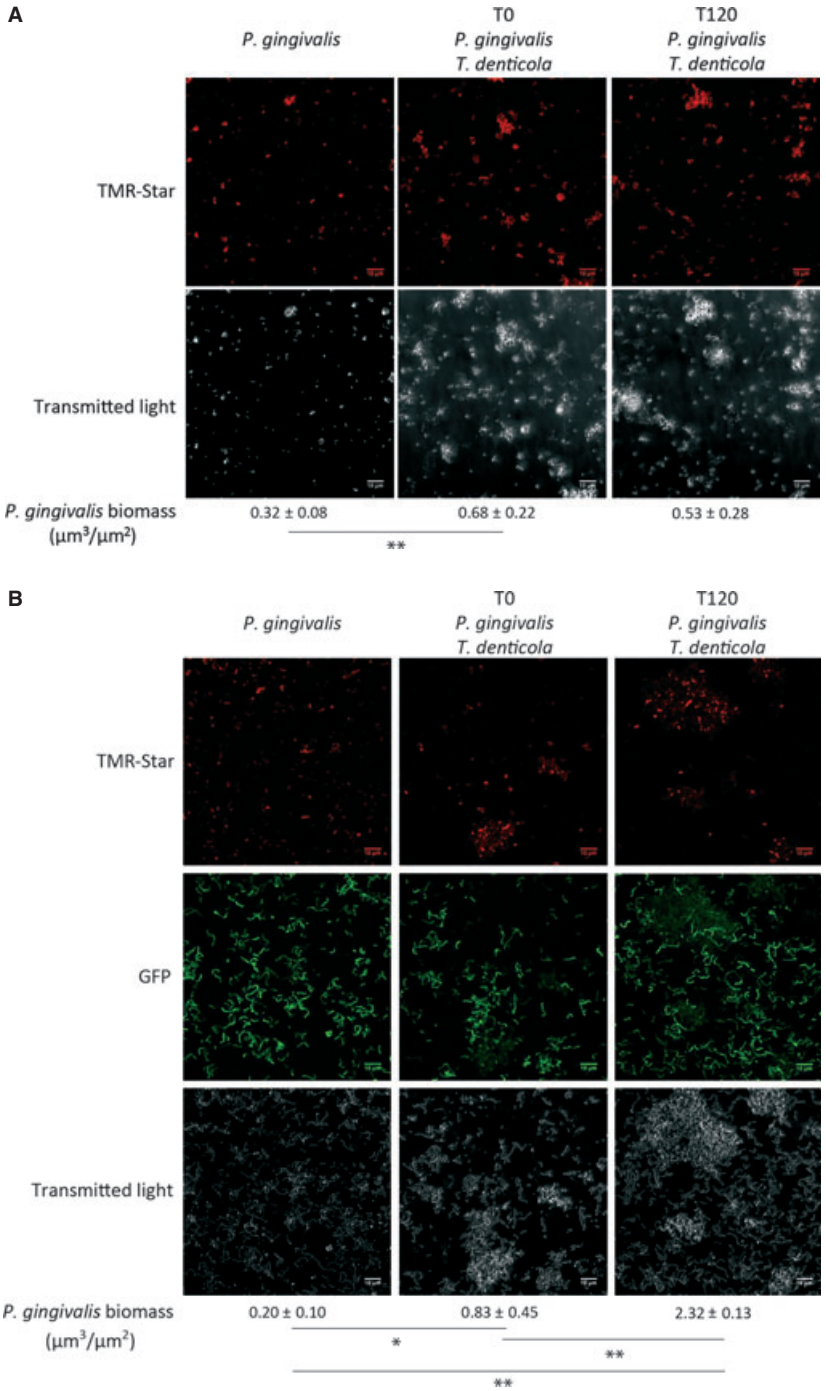

Figure 4 Adhesive properties of *Porphyromonas gingivalis* in flowing conditions. After saliva coating without (A) or with (B) *Streptococcus gordonii* inoculation for 30 min in the flowing system, *P. gingivalis* mixed with or without *Treponema denticola* were inoculated in flow cells for 15 min, either immediately (T0) or after a 120-min period of incubation (T120). Flow cells were observed by confocal laser scanning microscopy and image analysis and biomass quantification of *P. gingivalis* were performed using ImageJ and Matlab-Comstat software. The *P. gingivalis* biomasses of three different experiments were reported, together with images of a representative experiment showing the maximum projection of acquired stacks of TMR-Star fluorescence (staining for *P. gingivalis*), GFP fluorescence (staining for *S. gordonii*), or of transmitted light (showing all bacterial species). Asterisks (* and **) indicate significant differences between *P. gingivalis* biomasses of bacteria under the different conditions used ($P < 0.05$ and $P < 0.01$, respectively).

Table 3 Influence of *Treponema denticola* on adhesion capacities of *Porphyromonas gingivalis* to salivary pellicle and *Streptococcus gordonii*

	P. gingivalis biomass ($\mu m^3\ \mu m^{-2}$)	
Bacterial partners	Saliva	Saliva + *S. gordonii*
P. gingivalis	0.32 ± 0.08	0.20 ± 0.10
P. gingivalis/*T. denticola:* (T0)[1]	0.68 ± 0.22	0.83 ± 0.45
P. gingivalis/*T. denticola:* (T120)[1]	0.53 ± 0.28	2.32 ± 0.13

[1]The *P. gingivalis* and *T. denticola*, were coincubated for 0 min (T0) or 120 min (T120) before inoculation in the flowing system.

T120 third column and Table 3). The distribution of *P. gingivalis* was also comparable. In contrast, adhesion of *P. gingivalis* on *S. gordonii* was increased 12-fold (7.3–24-fold) after 2 h of coincubation with *T. denticola* compared with *P. gingivalis* alone and 2.8-fold (1.7–6.4-fold) compared with the presence of *T. denticola* without any incubation time (Table 3). The size of *P. gingivalis* clusters was also enhanced (Fig. 4B, third column).

Treponema denticola promoted biofilm formation by *Porphyromonas gingivalis*

Study of the development of a static biofilm showed that a complete biofilm was formed by *P. gingivalis* alone within 7 h, with a calculated BFI of 1.7 ± 0.2 (Fig. 5A,B). *Treponema denticola* alone did not form any biofilm by 7 h (BFI > 8, Fig. 5A,B). A mixture of *P. gingivalis* and *T. denticola* with a coincubation period <30 min did not increase the biofilm formation rate, as shown in Fig. 6. However, after a coincubation of 120 min, a complete mixed biofilm was already observed at 3 h and was stable up to 7 h (BFI < 2).

The effect of *T. denticola* on formation of a 3-h dynamic multi-bacterial biofilm was secondarily assessed. As shown in Fig. 6, the biomasses associated with *P. gingivalis* after coincubation for 30 min with *T. denticola* were increased 40-fold compared

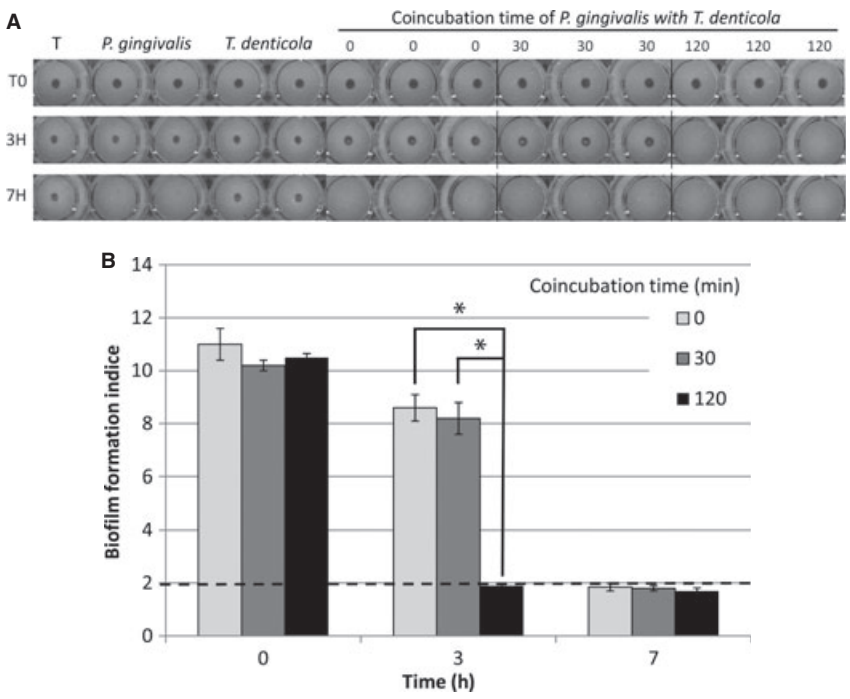

Figure 5 Effect of *Treponema denticola* on formation of static biofilm by *Porphyromonas gingivalis*. The capacity of *P. gingivalis* to form a biofilm was tested after 0, 30 or 120 min of coincubation with *T. denticola* by the Biofilm Ring Test. After harvesting, bacteria suspensions were incubated in the presence of magnetic beads (toner) for 0, 3 and 7 h in anaerobic conditions. (A) Biofilm visualization after magnetization of beads on a magnet support. A ring or a central point is indicative of the absence of biofilm formation. (B) Numerically evaluated biofilm using the 'biofilm formation indice' (BFI). Low BFI (≈2) reflects full immobilization of beads and formation of a biofilm. T (toner) corresponds to the internal control of each experiment, which consists of magnetic beads without bacteria. Asterisks (*) indicate significant differences of calculated BFI between 30 or 120 min of coincubation and absence of coincubation ($P < 0.05$).

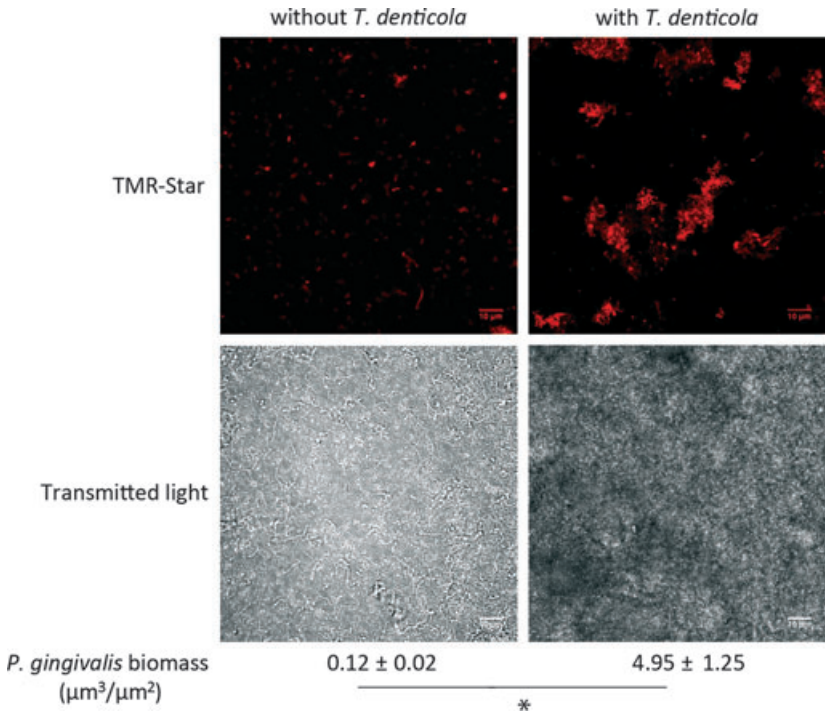

Figure 6 Effect of *Treponema denticola* on formation of a mixed dynamic biofilm by *Porphyromonas gingivalis* and *Streptococcus gordonii*. The *P. gingivalis*-SNAP26 marked with TMR-Star and *S. gordonii*-GFPmut3*, with or without *T. denticola*, were inoculated for 3 h in a flow cell before biofilm observation by confocal laser scanning microscopy. Image analysis and biomass quantification of *P. gingivalis* were performed using ImageJ and Matlab-Comstat software. The *P. gingivalis* biomasses of three different experiments were reported, together with images of a representative experiment showing the maximum projection of acquired stacks of TMR-Star fluorescence (staining for *P. gingivalis*) or of transmitted light (showing all bacterial species). Asterisks (*) indicate significant differences between *P. gingivalis* biomasses of control bacteria without *T. denticola* and bacteria with *T. denticola* ($P < 0.05$).

with *P. gingivalis* in the absence of *T. denticola*. In addition, *T. denticola* modified *P. gingivalis* distribution and induced the formation of large clusters.

DISCUSSION

Bacterial inter-relation in the promotion and development of *P. gingivalis* biofilms is under investigation by many researchers (Yamada *et al.*, 2005; Rosen *et al.*, 2008; Ito *et al.*, 2010; Kirschbaum *et al.*, 2010; Verma *et al.*, 2010; Daep *et al.*, 2011; Orth *et al.*, 2011). Synergy between *P. gingivalis* and *T. denticola* has been demonstrated in a periodontitis mouse model using a consortium of bacteria (Kesavalu *et al.*, 2007). Orth *et al.* (2011) demonstrated that the presence of *T. denticola* reduced the concentration of *P. gingivalis* necessary to develop infection. However, virulence determinants of *T. denticola* and *P. gingivalis* that promote synergy in infectious capacities and in the development of *P. gingivalis* biofilm are still unknown. In this study, we explored the influence of *T. denticola* coincubation with *P. gingivalis* on *P. gingivalis* gene expression, coaggregation and adhesion capacities. A short direct contact between *P. gingivalis* and *T. denticola* has been demonstrated as essential for interaction and cooperation (Yamada *et al.*, 2005; Saito *et al.*, 2009). In our model, a short incubation was followed by the overexpression of genes implicated in virulence, specifically in adhesion (*hagA*) and also in enzymatic reactions (*rgpA* and *kgp).* Only genes encoding proteins with HA domains were increased. Rapidly (10–20 min), proteins containing HA domains were enhanced in the bacterial supernatant. Gingipains and hemagglutinins must be exported and processed by proteolysis to exhibit functional activity. Different forms of the proteins coexist, with some attaching to the cell membrane as protein complexes and others being fully secreted (Potempa *et al.*, 1995, 2003). In our hands, the increase of HA-domain-containing proteins was detected in the

bacteria supernatant, especially HA1 domains (proteins with a molecular weight of 30–44 kDa) and HA4 domains (27-kDa form in RgpA). These proteins may correspond to fully secreted proteins, or to proteins detached from cell membrane by the centrifugation step performed to prepare cell extracts.

Historically, Grenier (1992) was the first to demonstrate coaggregation between *P. gingivalis* and *T. denticola* with bimodal interaction. These bacteria were frequently identified together at *in vivo* disease sites (Kigure *et al.*, 1995; Socransky *et al.*, 1998). In the present study, we confirmed that these two bacteria of the red complex coaggregated. The *P. gingivalis* surface HA1 domains from HagA, Kgp and RgpA proteins (Potempa *et al.*, 2003) were recently identified as key adhesion factors for coaggregation with *T. denticola* (Ito *et al.*, 2010). Our study confirmed the importance of these three genes for coaggregation of *P. gingivalis* and *T. denticola*. Indeed, coaggregation between bacteria increased from 30 min until 120 min when it was maximal, with a parallel increase of protein levels and probably of enzymatic activity of RgpA and Kgp. The time-course of events linked to HA-domain proteins is therefore in accordance with coaggregation levels observed at the same times between bacteria. The HA-domain proteins not only promote but also maintain coaggregation. Moreover, increased hemagglutination capacities of *P. gingivalis* after 2 h of incubation with *T. denticola* were observed, also due to HA1 domains, which play a role in hemagglutination (Sakai *et al.*, 2007). Previous work showed that Kgp plays a role in decreasing autoaggregation capacities of *P. gingivalis* (Kuboniwa *et al.*, 2009). As observed in our model, an increase in Kgp levels may also favor coaggregation with *T. denticola* over autoaggregation.

Porphyromonas gingivalis adhered significantly more strongly to KB cells after incubation with *T. denticola* in a time-dependent manner, as also shown by Kirschbaum *et al.* (2010) with *F. nucleatum.* Moreover, an increase in *P. gingivalis* capacity of attachment on a first colonizer such as *S. gordonii* was induced by time-dependant exposure to *T. denticola*. Cell-to-cell interactions, either between bacteria species or with eukaryotic cells, may be involved in these modifications. As *T. denticola* and *S. gordonii* are unable to interact as shown in coaggregation experiments, incubation with *T. denticola* induced subsequent changes in *P. gingivalis* adhesion capacity to *S. gordonii* and to epithelial cells. Selective adhesion of *P. gingivalis* to specific oral streptococcal species has been shown to be driven by protein–protein interactions between the minor fimbrial antigen (Mfa) of *P. gingivalis* and the streptococcal antigen I/II (Brooks *et al.*, 1997; Demuth *et al.*, 2001; Park *et al.*, 2005). In the same way, even if HA1 domains can bind to host proteins of the epithelium such as fibronectin, fibrinogen and collagen type V (Sakai *et al.*, 2007), adhesion and invasion of oral epithelial cells are driven by the major fimbriae FimA (Njoroge *et al.*, 1997). In our experiments, expression of *fimA* and *mfa1* was not increased. The mechanism of enhanced adhesion (to epithelial cells and to a first bacterial colonizer) could however be a result of gingipain overexpression. Prefimbrilin (FimA) and the 75-kDa protein precursor (Mfa1) are matured by Rgp, which is the major processing enzyme in the virulence pathways of *P. gingivalis* (Kadowaki *et al.*, 1998). Rgp are also suggested to facilitate colonization/adhesion through the proteolytic exposure of cryptitopes in host tissue (Gibbons *et al.*, 1990). Facilitated processing of fimbriae and proteolysis of host proteins by increased levels of gingipains can therefore be responsible for improved adhesion of *P. gingivalis* to such substrata. Analysis of fimbrial processing by gingipains after incubation with *T. denticola* could provide clues to the molecular mechanisms involved in *P. gingivalis*/*T. denticola* interaction.

In vivo study of *P. gingivalis* colonization by introduction of bacteria into the mouth of human volunteers resulted in almost exclusive *P. gingivalis* localization in areas of streptococci-rich plaque (Slots & Gibbons, 1978). However, in a model of mixed dynamic biofilm, we showed that *P. gingivalis* had difficulties in forming an important biofilm with only *S. gordonii* in flowing conditions. Association with *T. denticola* induced a 40-fold increase of *P. gingivalis* biomasses in the biofilm. The increased formation of dynamic biofilm in flowing conditions with *T. denticola* was probably in part a consequence of the increased adhesion of *P. gingivalis* observed in adhesion assays. In all assays, the presence of *T. denticola* in the inocula was sufficient to induce the formation of *P. gingivalis* clusters, easily found on various substrata such as saliva, *S. gordonii*, and epithelial cells. Previous results obtained on *P. gingivalis* homotypic biofilms showed that Rgp are involved in bacteria

microcolony morphology and allow an increase in colony size (Kuboniwa *et al*., 2009). The present study also demonstrated that the presence of *T. denticola* induced an increase in Rgp that could be responsible for the observed increase in clusters.

In static biofilms, long incubation times between *P. gingivalis* and *T. denticola* also lead to accelerated kinetics of biofilm formation. As described by Yamada *et al.* (2005), equivalent numbers of *T. denticola* and *P. gingivalis* are required for maximum synergistic mixed biofilm formation. As no substratum. such as saliva. or bacteria species like *S. gordonii* was used in these conditions, HA-domain-containing proteins are likely to be involved only in promoting *P. gingivalis*/*T. denticola* interactions. However, other molecular processes not yet evaluated, such as the secretion of matrix, may also be increased.

In conclusion, an increase in the adhesion capacities of *P. gingivalis* after incubation with *T. denticola* was observed, which at least partially resulted in improved biofilm formation. *Treponema denticola*, usually associated with *P. gingivalis* in the red complex, was therefore confirmed as an enhancer of *P. gingivalis* virulence, as *in vivo* studies also suggest (Orth *et al*., 2011). Moreover, *T. denticola* may also take advantage of *P. gingivalis* by enhancing its own recruitment in the biofilm. Our data highlight the essential role of incubation time and substratum used to study biofilm formation in flow conditions. The importance of HA domains in molecular interactions between *P. gingivalis* and *T. denticola* have been underlined, together with their influence in adhesion to other bacterial partners or eukaryotic cells. Other roles of HA-domain-containing proteins need further study to elucidate their mechanism of action on adhesion capacities and therefore on biofilm formation. Exopolysaccharide production of *P. gingivalis* or *T. denticola* could also be investigated. The dynamic biofilm model described in this study, with *S. gordonii* as a first colonizer, followed by incubated *P. gingivalis* with various partners such as *T. denticola*, will be used to further investigate bacterial interactions in the oral biofilm.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Enzymatic activities of *Porphyromonas gingivalis*/*Treponema denticola* coincubation mixtures.

Figure S2. Hemagglutination activity of *Porphyromonas gingivalis*.

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