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Short communication

Aggregatibacter actinomycetemcomitans adhesion inhibited in a flow cell

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Introduction: Microbial interactions are considered important in the adhesion process of pathogenic bacteria in the oral cavity. This study addressed the hypothesis that a streptococcal biofilm influences the hard tissue colonization by the periodontopathogen *Aggregatibacter actinomycetemcomitans* under hydrodynamic conditions.
Methods: The colonization of a green-fluorescent-protein-labelled *A. actinomycetemcomitans* strain on surfaces coated with a streptococcal biofilm, was monitored in real time using a confocal laser scanning microscope-mounted flow cell. Culture and quantitative polymerase chain reaction data were obtained in parallel from a Modified Robbins Device.
Results: Colonization of *A. actinomycetemcomitans* was inhibited by the four tested streptococci (*Streptococcus sanguinis*, *Streptococcus cristatus*, *Streptococcus salivarius*, and *Streptococcus mitis*). The most inhibiting species was *S. sanguinis*.
Conclusion: These results confirmed the hypothesis that some bacterial species influence *A. actinomycetemcomitans* colonization of hard surfaces *in vitro* under hydrodynamic conditions.

Key words: adhesion; *Aggregatibacter actinomycetemcomitans;* flow cell; microbial interactions; real time

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Periodontitis causes severe damage in the oral cavity by affecting the tooth-supporting tissues (16) and increases the risk for certain systemic disorders (12). Although periodontitis is a multifactorial disease, a clear association exists between localized aggressive (or juvenile) periodontitis and colonization of *Aggregatibacter* (formerly known as *Actinobacillus*) *actinomycetemcomitans* (9). *A. actinomycetemcomitans* colonizes the oral cavity by its ability to adhere to soft and hard tissue surfaces (5). The biological factors that contribute to successful *A. actinomycetemcomitans* colonization are not well known. Epithelial colonization is influenced by environmental (21), viral (20), and bacterial (19) interactions. Some of these might also influence the colonization of hard tissues. Knowledge on the conditions that interfere with the emergence of pathogens is critical to understand the disease process and to develop new treatment strategies. The present study addressed the hypothesis that colonization of hard surfaces by streptococci interferes with the subsequent colonization of *A. actinomycetemcomitans*, under hydrodynamic conditions, using two *in vitro* models.

Materials and methods

Bacterial strains and culture conditions

A. actinomycetemcomitans ATCC 29522, containing pNP3M, a mutated version of the green fluorescent protein (GFP) expression vector pNP3 (14), *A. actinomycetemcomitans* ATCC 43718, *Streptococcus sanguinis* KTH-4, *Streptococcus cristatus* CC5A, *Streptococcus salivarius* TOVE, and *Streptococcus mitis* BMS were grown in brain–heart infusion broth (BHI, Oxoid, Basingstoke, UK). After harvesting by centrifugation (7970 *g* for 10 min), the bacterial pellets were resuspended in fresh medium at a concentration of 1×10^8 colony-forming units (CFU)/ml.

To obtain streptococcal biofilms, glass coverslips (22 × 22 mm) and discs (diameter 7 mm) were precolonized with streptococci in BHI in polystyrene well plates (Iwaki microplate; Scitech, Diu, Japan), 48 h before the experiment. The medium was refreshed after 24 h. All cultures were incubated at 37°C in a 5% CO_2

environment. The presence of a streptococcal biofilm was verified using gram staining.

Real-time exclusion assay

The flow cell system (Warner Instruments, Hamden, CT), mounted on an Olympus IX 70 confocal laser scanning microscope (CLSM; Olympus, Aartselaar, Belgium), contained a diamond-shaped imaging chamber with glass coverslips as the top and bottom plates (with 2.5 mm distance between). A clean (control) or a precolonized coverslip (test) was placed on the bottom of the chamber and a droplet of phosphate-buffered saline (PBS) was added before closing. Tygon perfusion lines were attached to the inlet and exit ports of the chamber and connected with a heated syringe (37°C) containing *A. actinomycetemcomitans* in colorless Dulbecco's modified Eagle's medium (Gibco, Life Technologies Ltd., Paisley, UK). The chamber and tubes were perfused with PBS to remove air bubbles (6). Subsequently, the bacterial suspension was pumped through the system for 3 h at a velocity of 200 μl/min (7).

Z scans, comprising six slices with distance in between of 1 μm, were made every 30 min. The focus was maintained in the centre of the chamber during the entire experiment. GFP was excited at a wavelength of 488 nm. The average fluorescence intensity of the Z stacks was determined using Fluoview 500® (Olympus). Data were expressed as proportions of the average fluorescence intensities of the Z stacks taken after 1, 1.5, 2, 2.5, and 3 h relative to the average fluorescence intensity of that Z stack after the first 30 min (baseline).

Microbial interference in the modified Robbins device

The Modified Robbins Device (MRD; Dentaid, Barcelona, Spain) (13) comprised four separate stainless-steel flow chambers, which were connected by tubes to a bioreactor. This bioreactor contained a continuous culture of *A. actinomycetemcomitans* in BHI. Peristaltic pumps brought the bacterial culture from the bioreactor to the flow chambers at a rate of 200 μl/min. Clean (control) or precolonized (test) glass discs were taken out of the MRD at specific time-points after the start of the experiment. The discs were vortexed and sonicated for 15 min at 100 W in 1 ml PBS. Serial dilutions were plated on blood agar and incubated for 3 days at 37°C in a 5% CO_2 environment. Colony counts were used to calculate the number of CFU/ml of *A. actinomycetemcomitans*. In addition, DNA was extracted (Instagene Matrix, Bio-Rad, Hercules, CA) from 100 μl of the undiluted bacterial suspension and used for a quantitative polymerase chain reaction (QPCR) assay (2). Bacterial quantification was based on a plasmid standard curve, based on known quantities of plasmid DNA with the target DNA sequence.

Statistical analysis

For each experimental design, eight independent experiments were performed. Log transformations were carried out on the original data. A linear mixed model was fitted and pairwise comparisons were calculated, which have been corrected for simultaneous hypothesis testing. The level of significance was set at $P < 0.05$.

Results

Bacterial colonization over time in flow cell

The colonization of glass by *A. actinomycetemcomitans* under flow conditions was monitored over 3 h in a parallel plate flow cell by fluorescence intensity measurements and in a MRD by bacterial culturing. Both flow cell systems showed a time-dependent linear increase in *A. actinomycetemcomitans* colonization (Fig. 1). The increase in fluorescence intensities matched very closely the increase in CFU ($R^2 = 0.96$). A 6.77-fold increase in *A. actinomycetemcomitans* was observed between 0.5 and 3 h by measurements of fluorescence, whereas a 6.85-fold increase was measured by bacterial culturing. These results indicated that both techniques can be used in parallel.

The CLSM images showed an increase in the amount of fluorescent *A. actinomycetemcomitans* attached to the bottom glass plate of the parallel plate flow chamber (Fig. 2A). Bacteria aggregated and formed microcolonies with a diameter of a few micrometers. No dense, tightly packed clusters of bacteria were observed. The microbial community had a more open biofilm architecture. The *A. actinomycetemcomitans* strain tested here demonstrated spreading across the glass rather than growth in the Z dimension. The majority of the biomass was located at or near the coverslip.

Effect of a streptococcal coating on *A. actinomycetemcomitans* adhesion

Real-time exclusion assay

For the exclusion experiments, glass slides were colonized by streptococcal strains 2 days before *A. actinomycetemcomitans* adhesion and clean glass surfaces served as controls. At the start of the experiments, a community of streptococci was attached to the coverslips (Fig. 2B and C). The amount was comparable for all strains used. Confocal images were taken at one spot in the parallel plate flow chamber. Figure 2 shows the Z stacks of images taken during adhesion of GFP-labelled *A. actinomycetemcomitans* to different surfaces. *A. actinomycetemcomitans* adhered to all of them, but there was large variability in the amount of fluorescent bacteria, depending on the precolonization (Fig. 2D). The streptococcal strain with

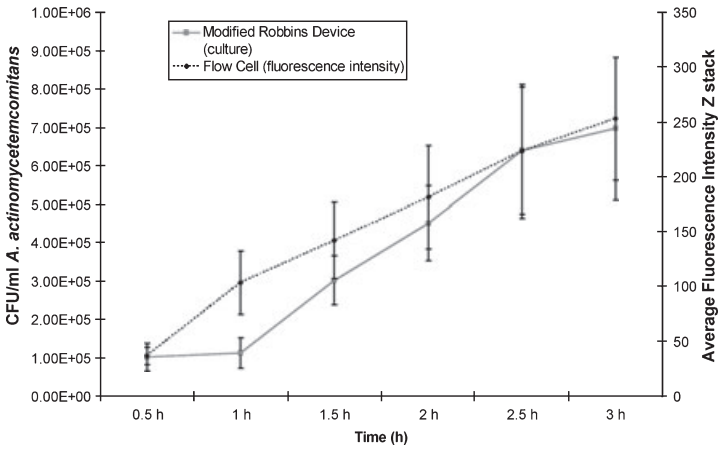

Fig. 1. Aggregatibacter actinomycetemcomitans adhesion followed in time. Comparison of the time-dependent increase in average fluorescence intensity, determined with the visual model, with the number of *A. actinomycetemcomitans* detected by culture of the samples in the Modified Robbins Device (MRD). Error bars represent standard deviations.

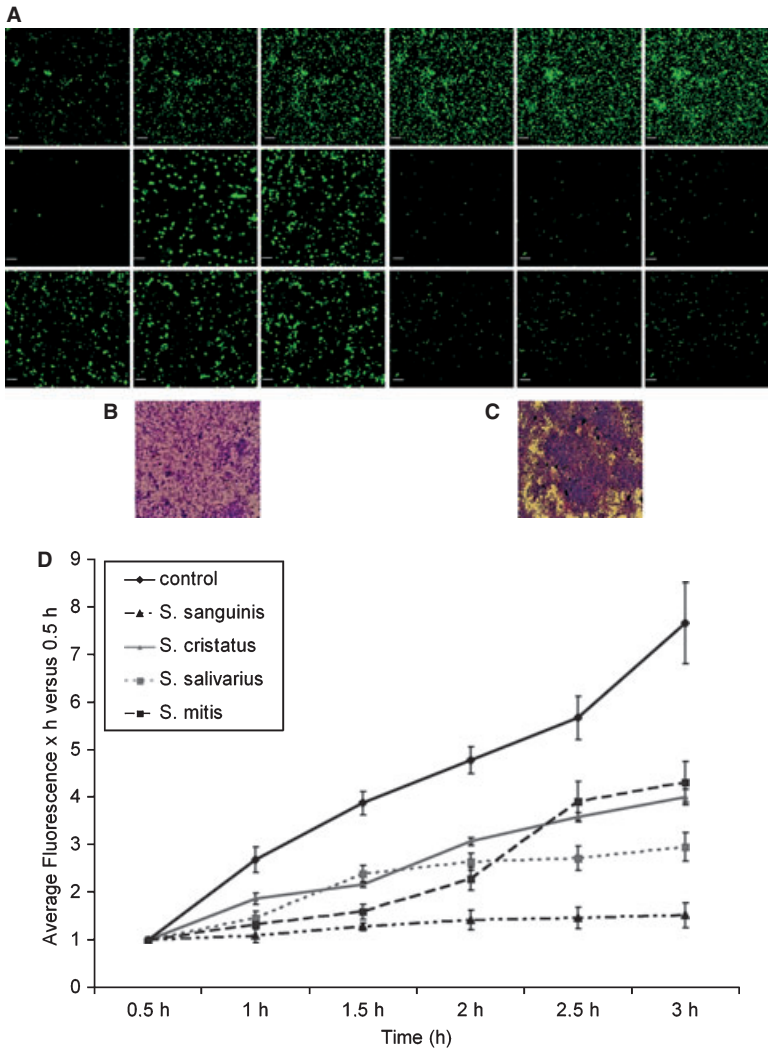

Fig. 2. Adhesion and exclusion of green fluorescent protein (GFP)-labelled *Aggregatibacter actinomycetemcomitans* in real time. Z stacks of confocal images during an adhesion experiment with GFP-labelled *A. actinomycetemcomitans* on a clean surface (A), a surface preconditioned with *Streptococcus cristatus* (B), and a surface preconditioned with *Streptococcus sanguinis* (C). Bars = 10 μm. The time interval between successive images is 0.5 h. The first Z stack was made at 0.5 h after the start of the experiment (0.5, 1, 1.5, 2, 2.5, and 3 h). The streptococcal preconditioning of the glass surfaces at the start of the experiment is shown after gram staining (1000×). Exclusion of *A. actinomycetemcomitans* adhesion by streptococcal strains based on fluorescence intensity measurements (D). Average fluorescence intensity measurements were performed on Z stacks of confocal images taken during exclusion experiments with GFP-labelled *A. actinomycetemcomitans* in a parallel plate flow chamber. Bars represent standard deviations.

the most inhibiting effect towards *A. actinomycetemcomitans* adhesion was *S. sanguinis*. A statistically significant difference was seen throughout the experiment between the adhesion of *A. actinomycetemcomitans* to a clean surface vs. a *S. sanguinis*-precolonized coverslip. At the 3-h time-point *S. sanguinis* caused a reduction of 80.2% compared with the control. *S. cristatus*, *S. mitis*, and *S. salivarius* also had reducing effects. A significant difference ($P < 0.05$) from the control situation was found from the 1.5-h time-point. The reduction at 3 h was 47.7% for *S. cristatus*, 61.5% for *S. salivarius*, and 43.6% for *S. mitis*. After 2.5 h, the differences between the three groups were also statistically significant ($P < 0.05$).

Microbial interference in a MRD

Analysis of variance tests for both assays (microbial culture and QPCR) showed no interaction between time and strain. This means that differences between strains were equal for each time-point. The effect of preconditioning on the subsequent adhesion of *A. actinomycetemcomitans* could therefore be analyzed by using the average over the different time-points (Fig. 3). The amount of *A. actinomycetemcomitans* quantified by both the microbial culturing technique and the QPCR assay was significantly different on a *S. sanguinis*-precoated surface compared with on a clean surface ($P < 0.05$) with a decrease in the number of pathogens of respectively 59.4 and 72.6%. *S. cristatus* diminished *A. actinomycetemcomitans* adhesion by 22.8% for culture and 25.2% for QPCR.

Combination of the culture and QPCR data allowed an estimation of the percentage viability in the biomass. There were no statistically significant differences between the percentage vitality of the biomass on the different pretreated surfaces (87.5% on the *S. sanguinis*-precoated surfaces, 81.9% on *S. cristatus*-precoated surfaces, and 84.7% for the control).

Discussion

Alternative therapies in periodontology are more than welcome because (re)colonization by pathogens after treatment is a problem (15) and because of the growing concern of antibiotic resistance. Colonization by periodontopathogens starts with their adhesion to host surfaces and beneficial bacteria could be applied in a 'replacement therapy' to inhibit this process by forming a protective biofilm in the pockets (19). The inhibitory effect of some streptococcal strains on the adhesion of *A. actinomycetemcomitans* to a glass surface under flow conditions was evaluated in this study using two experimental models.

A parallel plate flow system for real-time observation of bacterial adhesion under hydrodynamic conditions has been described (3, 17). These experiments were based on phase contrast microscopy, and their usability was limited for species with similar morphologies (4) or when complex communities were analyzed. In the visual model presented here, fluorescently tagged bacteria combined with CLSM, enabled real-time monitoring of bacterial adhesion in an environment with other bacteria with similar morphology, even when they extended above one layer (6).

GFP was often used as a species-specific marker for living bacteria and GFP has also already been applied in the field of adhesion research (1). The pNP3-mutated GFP-containing plasmid is a high copy number, segregationally stable vector

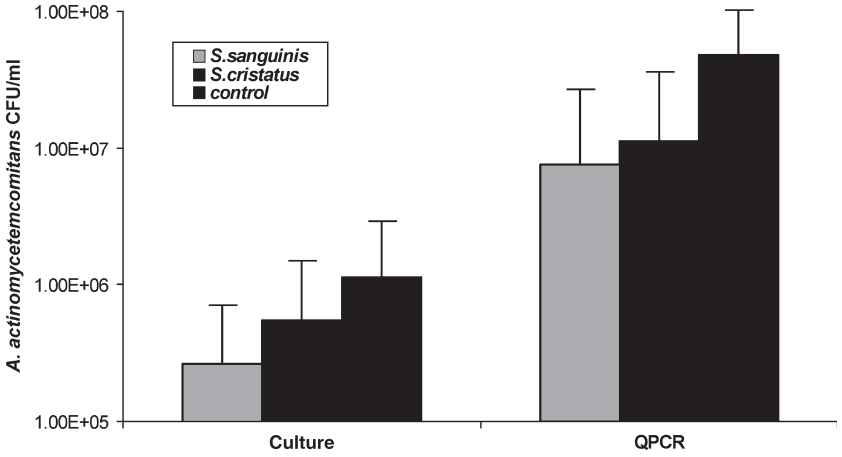

Fig. 3. Effect of a streptococcal biofilm on subsequent *Aggregatibacter actinomycetemcomitans* adhesion determined with a Modified Robbins Device. The number of *A. actinomycetemcomitans* adhering to different precolonized surfaces was determined with microbial culture and quantitative polymerase chain reaction. Bars represent standard deviations.

(14). Prior testing has shown that GFP-transformed bacteria have the same adhesion potentials as the wild-types and that no plasmid transfer occurred by natural competence between *A. actinomycetemcomitans* and the streptococci.

Hydrodynamic conditions, present in the *in vivo* ecological niche of the bacteria, were taken into account in this project because these conditions produce a clearance effect and shear forces, which might have an impact on the results. As such this new model better mimics the *in vivo* situation for studying bacterial adhesion and interference in real time.

All four streptococci had an antagonistic effect on subsequent *A. actinomycetemcomitans* adhesion. *S. cristatus*, *S. mitis*, and *S. salivarius* had minor effects, whereas *S. sanguinis* showed strong inhibition. The differences in the ability of *A. actinomycetemcomitans* to adhere to the precolonized surfaces were not linearly correlated with the amount of streptococci attached, so occupational inhibition was obviously not the (only) interaction mode present.

The visual flow cell data confirmed the results from earlier static experiments on epithelial cells (19). Again, *S. sanguinis* was the best inhibitor of subsequent *A. actinomycetemcomitans* adhesion and *S. salivarius* and *S. mitis* scored in between no inhibition and that of *S. sanguinis*. The rather unexpected inhibitory effect of *S. cristatus* in this study might be explained by an increased sterical hindrance as the result of the flow, and shows the importance of introducing flow to the study design.

The *A. actinomycetemcomitans* strain used in present study was a smooth, non-fimbriated strain. The random accumulation of the bacteria seen on the microscope images was described earlier for other smooth *A. actinomycetemcomitans* strains (7). Bacterial colonization shows an open architecture and reduced height. Rough strains would present towers of microcolonies, anchored by a small contact area with the surface.

The use of a rough, instead of a smooth, strain could have had an impact on the results of the present study. Clinical, rough *A. actinomycetemcomitans* strains have fimbriae that enhance the attachment potential of the bacteria (8). The exact interaction mechanism of the streptococci with *A. actinomycetemcomitans* is not yet revealed and no information is available about the direct influence of streptococci on *A. actinomycetemcomitans* fimbriae. For *Porphyromonas gingivalis* it is, for example, known that one of the surface proteins of *S. cristatus* directly causes repression of the *P. gingivalis* fimbrial gene (*fimA*) (23). For *A. actinomycetemcomitans* such information cannot be found in the literature. It is, however, known from previous studies that environmental factors, such as pH, NaCl, temperature, oxygen, and iron concentration, can influence the expression of *A. actinomycetemcomitans* fimbriae (7). When the streptococci lower the pH in the environment, this might for example indirectly lead to changes in the expression of *A. actinomycetemcomitans* fimbriae. Such changes would then decrease the adhesion capacity of the *A. actinomycetemcomitans*. This suppression of fimbrial gene expression would be an additional inhibiting mechanism, which cannot be present in the current experiments because they were executed with a non-fimbriated strain.

S. sanguinis was selected for this study because it produces a bacteriocine (sanguicine) and hydrogen peroxide (10). The reason for selecting *S. salivarius* was its *in vivo* inhibition of the emergence of *Streptococcus mutans* (18). The rather strong inhibiting effect of *S. mitis* at the start diminished with time. This species can release biosurfactants as an indirect defense mechanism against other colonizing strains on the same substratum (22). When some *A. actinomycetemcomitans* manage to adhere, the biosurfactants on the surface probably partly lose their effect and further adhesion happens more easily.

To determine whether changes in growth rate in the presence of the streptococci could have affected the accumulation of *A. actinomycetemcomitans*, the growth rate of planktonic *A. actinomycetemcomitans* was followed in time with and without the four streptococcal strains in BHI (unpublished data). The *A. actinomycetemcomitans* monoculture presented normal growth with an exponential phase starting after 2–4 h. The mixed cultures of *A. actinomycetemcomitans* and the streptococcal strains initially showed similar progress in *A. actinomycetemcomitans* growth. After 6 h however, the number of *A. actinomycetemcomitans* in the mixed solutions decreased compared to the control solution. Regarding the time frame of the current experiments, this effect had no influence on the *A. actinomycetemcomitans* colonization in the present study. Previously, tests have been performed to check if the streptococcal strains could cause an inhibition of growth on the agar plates during the postadhesion-experiment culturing period. No growth inhibition was seen (19).

The effects of *S. sanguinis* and *S. cristatus* under flow conditions were also examined by a second *in vitro* model, the MRD. An important advantage of this type of flow cell is the possibility of checking different strains at the same time, under identical conditions. Attached bacteria can be quantified upon removal. *S. sanguinis* caused a higher reduction in the number of total and cultivable adhered *A. actinomycetemcomitans* compared to *S. cristatus*. The streptococcal strains reduced adhesion and therefore influenced both the viable and total cell counts. The pretreatments had no effect on the percentage vitality of the biomass.

On the clean surfaces there was a good correlation between fluorescence intensity measurements and culture results obtained with both models. The results from the exclusion assays indicated, however, that the reduction by the same strains was more prominent in the parallel plate flow cell model than in the MRD set up. The visual experiments show, moreover, an increase of the effect in time, while in the MRD experiments the differences between the strains are equal for each time-point. These differences might be related to a decrease in pH in the flow chamber with time caused by the streptococci. A known characteristic of the fluorescent protein is its reduced fluorescence at low pH. For *Streptococcus gordonii* it was shown that the use of a flow chamber could resolve this problem, because such a system prevents the accumulation of lactic acid produced by *S. gordonii* (11). Flow velocity plays of course an important role. It might be that the flow is slow enough here for the streptococci to cause a reduction in pH, which leads to a decrease of the fluorescence signal. This probably causes a more pronounced exclusion effect than the realistic effect.

The exclusion assays showed that some streptococcal species can interfere with the adhesion of *A. actinomycetemcomitans* on a hard surface under hydrodynamic conditions. The different techniques used, with their own specific characteristics, identified *S. sanguinis* as the most effective inhibitor of *A. actinomycetemcomitans* adhesion. The exact nature of these interactions warrants further investigation. These data support the idea that bacterial interactions could possibly be used as a new treatment approach to periodontal disease.

Acknowledgments

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