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Quantitation of biofilm and planktonic life forms of coexisting periodontal species

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Background: Complexity of oral polymicrobial communities has prompted a need for developing *in vitro* models to study behavior of coexisting bacteria. Little knowledge is available of *in vitro* co-growth of several periodontitis-associated species without early colonizers of dental plaque.
The aim: was to determine temporal changes in the quantities of six periodontal species in an *in vitro* biofilm model in comparison with parallel planktonic cultures.
Material and methods: Porphyromonas gingivalis, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Parvimonas micra*, *Campylobacter rectus* and *Fusobacterium nucleatum* were anaerobically grown as multispecies and monospecies biofilms and parallel planktonic cultures using cell culture plates and microfuge tubes, respectively. After incubating 2, 4, 6, 8 days, biofilms and planktonic cultures were harvested, DNA extracted and the target species quantified using qPCR with species-specific 16S rDNA primers. Biofilm growth as monocultures was visualized at day 2 and 8 with confocal microscopy and crystal violet staining.
Results: The six species were found throughout the test period in all culture conditions, except that *P. gingivalis* and *F. nucleatum* were not detected in multispecies planktonic cultures at day 8. In multispecies biofilm, *P. gingivalis* qPCR counts (cells/ml) increased ($P<0.05$) from day 2–8 and were then higher ($P<0.05$) than those of *A. actinomycetemcomitans* and *C. rectus*, whereas in monospecies biofilm, *P. gingivalis* counts were lower ($P<0.05$) than those of the other species, except *A. actinomycetemcomitans*. When multi- and monospecies biofilm cultures were compared, *P. gingivalis* counts were higher ($P<0.05$) but those of the other species, except *P. intermedia*, lower ($P<0.05$) in multispecies biofilm. Comparison between planktonic and biofilm cultures showed that *A. actinomycetemcomitans*, *P. micra* and *C. rectus* had higher ($P<0.05$) counts in planktonic cultures no matter whether grown in mono- or multispecies environment.
Conclusions: Six periodontal species were able to form multispecies biofilm up to 8 days *in vitro* without pioneer plaque bacteria. *P. gingivalis* seemed to prefer multispecies biofilm environment whereas *P. micra* and *A. actinomycetemcomitans* planktonic culture.

1. Introduction

Dental plaque is a site-specific and unique bacterial community in the human body. The ancient biofilm lifestyle provides plaque bacteria with powerful tools for survival and persistence that still are only partly understood. Although biofilm research started among environmental microbiologists [1], biofilms formed by human indigenous microbiota are currently recognized as causative agents of various chronic, treatment-resistant infections, including periodontitis [2–4].

Periodontitis-associated bacteria grow in dental plaque biofilms, but they can also be found on oral mucosae and in saliva. Plaque formation follows a regimented pattern through specific adhesin-receptor interactions mediating adherence of certain oral bacteria, including streptococci, actinomycetes and veillonellae to pellicle, a salivary glycoprotein coat on tooth surfaces [5]. Thereafter, other bacteria successively join the developing dental plaque that gradually evolves towards its climax community [6]. Most periodontitis-associated species are obligate anaerobes e.g., *Porphyromonas gingivalis*, *Prevotella intermedia*, *Parvimonas micra*

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and *Fusobacterium nucleatum*, or facultative anaerobes such as *Campylobacter rectus* and *Aggregatibacter actinomycetemcomitans* [7,8] that are usually found in mature dental plaque community. Despite the fact that these species are more common and occur at higher levels in periodontitis than healthy subjects there are individual differences in species combinations and quantities in plaque [9].

Due to the vast diversity and complexity of dental plaque [9] *in vitro* models for subgingival or supragingival plaque biofilm [10,11] attempt to advance understanding of the inter- and intraspecies behavior of indigenous oral bacteria in oral biofilm communities. So far available knowledge to a great extent derives from studies using monospecies biofilm design [12–14], whereas few investigators have reported data on the characteristics of multispecies biofilms of periodontitis-associated bacteria. In the latter studies early plaque colonizers, such as various oral streptococci, have been used in adjunction to some Gram-negative periodontal species [15,16]. Contrary to previous studies we chose to test biofilm and planktonic co-growth of six periodontitis-associated species, all late colonizing species in dental plaque [17] that grow in the top to intermediate zones in natural subgingival biofilms [18]. *F. nucleatum* has an exceptional ability to coaggregate with a variety of plaque bacteria [19], which suggests that this long rod-shaped bacterium can function as a bridge organism between early and late colonizing species in dental plaque [20].

Saliva continuously washes mucosal and dental surfaces and collects detached bacteria. This leads to the constant salivary presence of bacterial cells and their aggregates that can be regarded as representatives of planktonic bacteria floating free in saliva. Saliva with live bacteria [21] is the likely vehicle for intraoral but also person-to-person transmission of periodontitis-associated bacteria, with their levels being a determinant for successful transmission [22]. Loose bacteria have no time to multiply in saliva before getting swallowed, but after each swallowing incident part of whole saliva remains on oral surfaces [23], which may result in reattachment of bacteria. However, behavior of periodontitis-associated bacteria grown as planktonic co-cultures *in vitro* is largely unknown, but could be of interest due to the common intraoral and extraoral spread of bacteria in saliva.

In the present study we applied a simple, cost-effective biofilm model that later on can be used for e.g. structural studies of periodontal species biofilms and to test their responses to antimicrobial agents. Methods previously used to quantify *in In the present study we applied a simplvitro*-grown biofilm biomass include crystal violet [24,25], safranin staining [26,27] and fluorescent stain Syto9 [28]. The mentioned methods do not separate different bacterial species in the biofilm biomass and are therefore not suitable for the present study. Instead, similar to some previous studies [29,30], we chose quantitative real-time PCR (qPCR) for species-specific quantification of the selected bacteria.

Recent molecular biological studies have revealed that the location of periodontitis-associated species in subgingival plaque is in its superficial layers [18]. Therefore, it seems likely that these species can grow together without species regarded as early plaque colonizers [19]. To test this hypothesis we designed a study where the aim was to determine temporal changes in the quantities of six periodontitis-associated bacteria grown together or separately as biofilms and respectively, as planktonic cultures.

2. Material and methods

2.1. Reference bacteria, culture conditions and 16S rDNA sequencing-based identity verification

Reference strains *F. nucleatum* ssp. *polymorphum* NCTC 10562, *P. gingivalis* ATCC 33277, *P. intermedia* ATCC 25611 and *P. micra* CCUG 46357 (Table 1) were purchased from culture collections. *C. rectus* strain Umea-12 was a gift from R. Claesson (Umea University, Sweden) and *A. actinomycetemcomitans* strain SA269 (a clinical, rough-colony strain) was from S. Asikainen's strain collection. Except *A. actinomycetemcomitans*, which was grown on tryptic soy agar, all other strains were cultured on brucella blood agar. All strains were incubated in anaerobic conditions (10% H_2, 5% CO_2, 85% N_2) at 37 °C using Anoxomat™ Mark II anaerobic gas filling system (Mart Microbiology, The Netherlands) or by using AnaeroGen™ bags (Oxoid, Hampshire, England) in jars; *C. rectus* for 6–7 days, *P. gingivalis* for 4–5 days and all others for 3 days.

As customary in our laboratory, the identities of test strains were confirmed by PCR amplification using GeneAmp® 9700 (Applied Biosystems) and sequencing of variable region V4 of 16S rRNA gene as described [31]. In brief, complete 16S rDNA sequence of ~1500 bp was amplified for each species using universal forward primer D88 (5′-GAGAGTTTGATYMTGGCTCAG) and reverse primer E94 (5′-GAAGGAGGTGWTCCARCCGCA). Ready-To-Go® PCR beads were reconstituted with 18 μl sterile water, each primer was added to a final concentration of 0.2 μM and 200–500 ng template DNA was added. The initial denaturation at 95 °C for 10 min, 30 cycles of 95 °C for 1 min, 50 °C for 30 s and 72 °C for 30 s was followed by a final elongation at 72 °C for 5 min. For verifying the amplicon sizes a 5-μl aliquot of each PCR product was first run on a 1% agarose gel and the remainder was purified using QiaQuick® PCR purification spin columns (Qiagen). Purified amplicons were sequenced by using primers B34 (ACGGGAGGCAGCAGY) and F17 (CCGTCWATTCMTTTGAGTTT) targeting variable region V4 in the 16S rRNA gene [31]. Sequencing was performed using a BigDye Terminator® kit on Beckman Coulter CEQ™ 8000 sequencer. Sequence data obtained was used for similarity search at NCBI BLAST.

2.2. Biofilm and planktonic cultures

Bacterial cultures were set up on different days prior to the biofilm and planktonic culture experiments, depending on the incubation time (described above) required for obtaining sufficient growth. Established methods [25] with modifications were used for biofilm culture. Briefly, the six test bacteria (Table 1), each separately (monospecies biofilm) and all mixed together (multispecies biofilm), were cultured in 1-ml total volume in 24-well cell culture plates [BD Bioscience (biofilm culture)] for 2, 4, 6, and 8 days in anaerobic conditions as detailed above. For inoculum preparation the bacterial cells were harvested from agar plates with sterile plastic loops and suspended in sterile PBS. The suspensions were washed two times in sterile PBS by centrifuging at 5000 ×g for 5 min. For OD_{600} measurements, bacterial suspensions were 10-fold diluted, resulting in nearly uncolored suspensions for pigmented species.

Monospecies biofilms and broth cultures for planktonic bacterial growth were initiated by inoculating 24-well plates and microfuge tubes, respectively, containing 900 μl brucella broth with a 100-μl aliquot from an $OD_{600} = 1$ suspension of each species.

For multispecies biofilms and planktonic cultures, a total of a 100-μl inoculum was prepared in a microfuge tube by combining equal volumes of $OD_{600} = 1$ suspensions from each species. The species mixture was transferred into 900 μl of brucella broth to be aliquoted into wells of 24-well plates or microfuge tubes. At the end of each incubation time point, supernatant broth was aspirated and the biofilms were washed once with sterile water to remove unattached bacteria. The biofilms were then thoroughly scraped off from the wells. Removal of biofilms was confirmed with stereomicroscopic examination. From planktonic cultures, only