Research in Microbiology 167 (2016) 393–402

www.elsevier.com/locate/resmic

Original article

A dual-species microbial model for studying the dynamics between oral streptococci and periodontal pathogens during biofilm development on titanium surfaces by flow cytometry

Anita Manti [a,1], Eleonora Ciandrini [a,1], Raffaella Campana [a], Sabrina Dominici [a], Caterina Ciacci [a], Sara Federici [b], Davide Sisti [a], Marco B.L. Rocchi [a], Stefano Papa [a], Wally Baffone [a,*]

[a] *Department of Biomolecular Science, Via Saffi 2, University of Urbino "Carlo Bo", 61029 Urbino, Italy*
[b] *CRB – Centro di Ricerche Biotecnologiche, Università del Sacro Cuore, via Milano 24, 26100 Cremona, Italy*

Received 25 November 2015; accepted 17 March 2016
Available online 28 March 2016

Abstract

The association of the pioneer organisms *Streptococcus mutans* ATCC 25175 or *Streptococcus oralis* ATCC 9811 with secondary colonizers *Fusobacterium nucleatum* ATCC 25586 or *Porphyromonas gingivalis* ATCC 33277 during biofilm development on titanium surfaces was evaluated by flow cytometry (FCM) using specific polyclonal antibodies.

ELISA and FCM were employed, revealing high antibody sensitivity and specificity. Biofilm formation of four dual-species combinations was analyzed by crystal violet staining, while the association between streptococci and periodontal pathogens was assessed using FCM. Dual-species association between *S. oralis* and *P. gingivalis* or *F. nucleatum* showed a proportional decrease in *S. oralis* during biofilm development, with a concomitant increase in *P. gingivalis* or *F. nucleatum*. This trend was not observed in either of the dual-species associations of *S. mutans* with the periodontal pathogens.

Our dual-species microbial model, which employed FCM, proved to be useful in the study of partnerships between bacteria in oral associations, showing that the presence of primary colonizers is required for the establishment of secondary colonizers in biofilms. The proposed experimental approach is technically simple to prepare and analyze, and also proved to be reproducible; hence, it is well-suited for investigating the development and dynamics of oral communities.

Keywords: Oral streptococci; Periodontal pathogens; Biofilm; Titanium; Flow cytometry

1. Introduction

Oral surfaces provide favorable habitats for microorganisms and allow bacterial growth to high densities, which results in biofilm development [1]. The growth of microorganisms on the tooth surface results in two patterns of biofilm, the supra-gingival biofilm prevalently characterized by Gram-positive bacteria, comprehending *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus mitis* and lactobacilli; and the sub-gingival biofilm dominated by Gram-negative anaerobic bacteria, such as *Aggregatibacter*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* [2,3].

The main cause of dental caries is usually supra-gingival biofilm, while the sub-gingival microbiome is associated with gingivitis and periodontal disease [4], two of the most common oral diseases in humans. In the oral cavity, bacteria may attach to different types of surfaces, including prosthetic devices and dental implants. In the latter case, biofilms have not been well characterized, although the formation and

* Corresponding author. Department of Biomolecular Science, Via S. Chiara 27, 61029 Urbino, Italy. Tel.: +39 0722 303543; fax: +39 0722 303541.
E-mail address: wally.baffone@uniurb.it (W. Baffone).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.resmic.2016.03.004
0923-2508/

maturation of biofilms on dental implants may have pathogenic implications in the development of peri-implant mucositis or peri-implantitis [5,6]. In fact, immediately after dental implant placement, the abutment surface is colonized by bacterial species such as *Streptococcus* spp. and *Actinomyces* spp. Which, over the course of biofilm maturation, develop a polymicrobial community [7,8] that includes other members belonging to the red, orange and green complexes [5,7,9]. Microorganisms in dental biofilm are identified in "complexes" based on the frequency with which microorganisms are recovered together. These microbial complexes have been associated with various stages of disease initiation and progression: members of the yellow complexes (*Streptococcus* spp.) and the purple complexes (*Actinomyces odontolyticus* and *Veillonella parvula*) are the early colonizers and members of the green (*Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens* and *Capnocytophaga* spp.), orange (*Fusobacterium*, *Prevotella* and *Campylobacter* spp.) and red (*P. gingivalis*, *Bacteroides forsythus* and *Treponema denticola*) complexes are the secondary colonizers [10,11].

In clinical situations associated with failing implants or peri-implant diseases, biofilms showed a predominance of orange and red complex species [8,12,13]. In addition, the presence of oral pathogens has been associated with serious systemic conditions such as bacterial endocarditis [14], chronic obstructive pulmonary disease and other diseases [15].

Biofilm formation and development depend on the interactions between different species of bacteria and is a very ordered and coordinated process involving a series of sequential steps, namely, the formation of the acquired pellicle, followed by colonization by primary and then secondary colonizers. The acquired pellicle, composed of a variety of host-derived molecules, coats oral surfaces and represents a source of receptors for primary colonizers such as *Streptococcus* spp., *Actinomyces* spp., *Capnocytophaga* spp., *Eikenella* spp., *Haemophilus* spp. and *Veillonella* spp. [16]. The pioneer bacteria, attached to the pellicle, excrete an extracellular matrix (ECM), composed of polysaccharides and other macromolecules such as nucleic acids [17] and structural and functional proteins [18], which help the bacteria to remain bound together [19].

The diversity and complexity of oral biofilm have been extensively reviewed [17], but not fully characterized. Techniques such as fluorescence in situ hybridization (FISH), also used in combination with confocal laser scanning microscopy (CLSM) [19,20], are useful methods for the study of oral biofilm. Flow cytometry (FCM) may be a helpful alternative to the above-mentioned techniques [21]. FCM detection of specific microorganisms requires monoclonal or polyclonal antibodies, which have the advantage of not requiring serious pretreatment processes. FCM can therefore be used in combination with conventional total cell count or viability dyes [22,23].

According to what was reported above, a biofilm appears as a systematic, complex and dynamic unit, in which interspecies communication plays a central role in formation, growth and maturation. Therefore, it is essential to consider the biofilm as a community in continuous transformation and with great variability in microbial species. To increase the understanding of interactions among oral species, we used an in vitro simplified model system based on flow cytometry to evaluate the association between two oral streptococci and two periodontal pathogens during biofilm formation. For this purpose, first, four dual-species biofilms were developed on titanium surfaces, one of the most common materials used in oral implants. *S. mutans* ATCC 25175 and *Streptococcus oralis* ATCC 9811 were used as pioneer microorganisms, to provide specific binding sites for subsequent incorporation of the secondary colonizers, i.e. *F. nucleatum* ATCC 25586 and *P. gingivalis* ATCC 33277. In order to obtain more information on the proportion of each secondary colonizer grown in association with the different primary colonizers during biofilm development, each species was quantified by flow cytometry in combination with specific polyclonal antibodies, developed against the tested oral bacteria in our laboratory.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Four oral pathogen reference strains, *S. mutans* ATCC 25175, *S. oralis* ATCC 9811, *F. nucleatum* ATCC 25586 and *P. gingivalis* ATCC 33277, purchased from the American Type Culture Collection, were utilized in this study. *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 were maintained on Columbia agar base (Oxoid, Milan, Italy) supplemented with 5% sheep blood (Oxoid) at 37 °C for 24 h, while *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25586 were cultivated in sheep blood agar base (Oxoid) with 5% sheep blood (Oxoid) at 37 °C under an anaerobic atmosphere for 48–72 h (Anaerogen System, Oxoid). All the strains were kept at −80 °C in nutrient broth (Oxoid) with 15% glycerol.

For optimization of anaerobic strain growth in liquid media, bacteria were seeded in brain heart infusion (BHI, Oxoid) supplemented with 5% fetal calf serum (FCS, Sigma–Aldrich, Milan, Italy), 1% yeast extract (Oxoid), hemin (5 mg/ml) (Sigma–Aldrich) and menadion (10 mg/ml) (Merck, Darmstadt, Germany). For all experiments, to obtain bacterial suspensions of the anaerobic strains, *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25586 were grown in this medium at 37 °C under anaerobic conditions for periods of up to 5–7 days under static conditions. *S. mutans* ATCC 25175 and *S. oralis* ATCC 9811 were grown in brain heart infusion (BHI, Oxoid) supplemented with 1% yeast extract (Oxoid) at 37 °C for 24 h.

2.2. Preparation of antibodies

Polyclonal antibodies were developed in rabbit against *S. mutans* ATCC 25175, *S. oralis* ATCC 9811, *F. nucleatum* ATCC 25586 and *P. gingivalis* ATCC 33277. Four New Zealand ra, with a bacterial suspension (10^{10} cells/ml), inactivated in glutaraldehyde 2.5% overnight at 4 °C and emulsified in incomplete Freund's adjuvant (Sigma–Aldrich). The