Bacterial colonization of enamel *in situ* investigated using fluorescence *in situ* hybridization

Ali Al-Ahmad,¹ Marie Follo,² Ann-Carina Selzer,¹ Elmar Hellwig,¹ Matthias Hannig³ and Christian Hannig¹

Correspondence

Christian Hannig
christian.hannig@
uniklinik-freiburg.de

¹Department of Operative Dentistry and Periodontology, University of Freiburg, Hugstetter Strasse 55, D-79106 Freiburg, Germany

²Department of Hematology and Oncology, Core Facility, Albert-Ludwig University, D-79085 Freiburg, Germany

³Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, University Hospital, Saarland University, Building 73, D-66421 Homburg/Saar, Germany

Oral biofilms are one of the greatest challenges in dental research. The present study aimed to investigate initial bacterial colonization of enamel surfaces *in situ* using fluorescence *in situ* hybridization (FISH) over a 12 h period. For this purpose, bovine enamel slabs were fixed on buccal sites of individual splints worn by six subjects for 2, 6 and 12 h to allow biofilm formation. Specimens were processed for FISH and evaluated with confocal laser-scanning microscopy, using probes for eubacteria, *Streptococcus* species, *Veillonella* species, *Fusobacterium nucleatum* and *Actinomyces naeslundii*. The number of adherent bacteria increased with time and all tested bacterial species were detected in the biofilm formed *in situ*. The general percentage composition of the eubacteria did not change over the investigated period, but the number of streptococci, the most frequently detected species, increased significantly with time (2 h: $17.7 \pm 13.8\%$; 6 h: $20.0 \pm 16.6\%$; 12 h: $24.7 \pm 16.1\%$). However, $\leq 1\%$ of the surface was covered with bacteria after 12 h of biofilm formation *in situ*. In conclusion, FISH is an appropriate method for quantifying initial biofilm formation *in situ*, and the proportion of streptococci increases during the first 12 h of bacterial adherence.

Received 17 March 2009

Accepted 5 June 2009

INTRODUCTION

Bacterial biofilms remain one of the greatest challenges in dentistry (Marsh & Bradshaw, 1995). Despite extensive research, there is still a strong demand for fundamental investigation of bacterial adherence to dental surfaces *in vivo* and *in situ* (Hannig *et al.*, 2007a, b, 2008a). Recent methods such as 4',6-diamidino-2-phenylindole staining and fluorescence *in situ* hybridization (FISH) allow the visualization and quantification of initial bacterial biofilms formed *in situ* on enamel specimens without any desorption procedure (Amann, 1995; Paster *et al.*, 1998; Schwartz *et al.*, 2003). Additionally, FISH offers the opportunity to differentiate several bacterial species (Amann, 1995; Paster *et al.*, 1998; Al-Ahmad *et al.*, 2007). About 50% of oral bacterial strains are not culturable using conventional culture plate methods (Aas *et al.*, 2005). Thus, many bacteria could be overlooked with this method. Furthermore, viable plate count techniques are known to select for certain bacterial species (Amann

et al., 1995). Another option is electron microscopic techniques, but these require extensive preparation of the sample, and quantification of adherent bacteria is difficult, despite transmission electron microscopy (TEM) and scanning electron microscopy (SEM) being the gold standards for ultrastructural exploration of the pellicle and of bacterial biofilms, respectively (Nyvad & Fejerskov, 1987; Hannig, 1999). During the first 12 h of bacterial colonization, microbial adherence is governed mainly by the pellicle (Marsh & Bradshaw, 1995; Hannig & Hannig, 2009). This proteinaceous layer is formed almost immediately on all solid substrates present in the oral cavity and provides several antibacterial mechanisms such as lysozyme and secretory immunoglobulin A (Pruitt *et al.*, 1969; Deimling *et al.*, 2007; Hannig *et al.*, 2009). However, many pellicle components such as amylase, mucins and proline-rich proteins serve as specific receptors for bacterial adherence (Hannig & Joiner, 2006). Furthermore, bacterial glucosyltransferases are immobilized at the tooth surface in an active conformation, forming glucans and mediating bacterial colonization (Vacca-Smith & Bowen, 2000). Thus, the role of the pellicle in bacterial biofilm formation is ambivalent (Hannig & Hannig, 2007). It is of great interest

Abbreviations: CLSM, confocal laser-scanning microscopy; FISH, fluorescence *in situ* hybridization; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

to investigate the time point at which microbial adhesion overcomes the protective effects of the pellicle layer (Marsh & Bradshaw, 1995). Thus, the aim of the present *in situ* study, following on from our previous study (Hannig *et al.*, 2007a), was to monitor initial bacterial adherence to enamel *in situ* over a 12 h period on buccal sites using FISH, as previous studies have focused mainly on undisturbed plaque formation with special splints simulating proximal plaque formation (Dige *et al.*, 2007). It was hypothesized that the proportion of streptococci would increase within the investigated 12 h period, in accord with the general level of adherent bacteria.

METHODS

Subjects and specimens. Six healthy volunteers participated in the study. Visual oral examinations were carried out by an experienced dentist. The subjects showed no signs of gingivitis or caries. Informed written consent was given by the subjects about participation in the study. The study design was reviewed and approved by the Ethics Committee of the University of Göttingen (Proposal 16/6/05) and of the University of Freiburg (Proposal 222/08), Germany.

Cylindrical enamel slabs (diameter 5 mm, height 1.5 mm, surface area 19.63 mm²) were prepared from the labial surfaces of bovine incisors of 2-year-old cattle, which were all negative for bovine spongiform encephalopathy. The enamel surfaces were polished by wet grinding with abrasive paper (400–4000 grit). The smear layer on the slabs was removed by ultrasonication with NaOCl for 3 min (Hannig *et al.*, 2007a). The samples were then disinfected in ethanol (70 %) for another 3 min, washed in distilled water and stored in distilled water for 24 h before exposure in the oral cavity.

Pellicle and initial biofilm formation. For *in situ* exposure of enamel specimens, individual upper jaw splints were vacuum-formed from 1.5 mm thick methacrylate foils. Cavities were prepared in the buccal aspects of the splints at the sites of the premolars and the first molar. The slabs were fixed on the splints using polyvinylsiloxane impression material (Aquasil Light Body Dentsply DeTrey), exposing only the surfaces of the enamel slabs to the oral fluids. The splints were carried intra-orally for 2, 6 and 12 h, to allow pellicle formation and increasing bacterial adhesion and biofilm formation on the surfaces of the specimens. Each subject carried six samples per intra-oral exposure time on buccal sites. Volunteers refrained from eating and drinking 2 h before insertion of the splints and during intra-oral exposure.

After intra-oral exposure, the slabs were immediately removed from the splints and rinsed with running tap water for 5 s to remove non-adsorbed salivary remnants and bacteria.

FISH. FISH was conducted according to Amann *et al.* (1995) and was adapted to bovine enamel slabs as described previously (Amann *et al.*, 1995; Al-Ahmad *et al.*, 2007; Hannig *et al.*, 2007a). Briefly, initial biofilms formed on enamel slabs were fixed in 4 % paraformaldehyde in PBS (pH 7.2) for 12 h at 4 °C. After fixation, all specimens were washed with PBS and incubated again in a solution containing 50 % ethanol in PBS for 12 h. Subsequently, the specimens were washed twice with PBS, followed by incubation in a solution containing 7 mg lysozyme (105 000 U mg⁻¹; Fluka) ml⁻¹ in 0.1 M Tris/HCl (pH 7.2), 5 mM EDTA for 10 min at 37 °C to permeabilize the bacterial cells in the initial biofilm. The samples were dehydrated with a series of ethanol washes containing 50, 80 and 100 % ethanol for 3 min each. Specimens were incubated with the oligonucleotide probes at a concentration of 50 ng each per 20 ml hybridization buffer [0.9 M

NaCl, 20 mM Tris/HCl (pH 7.2), 25 % formamide, 0.01 % (w/v) SDS]. Hybridization was conducted in 96-well plates (Greiner Bio-One) at 46 °C for 2 h. Following probe hybridization, samples were incubated for 15 min at 48 °C in wash buffer containing 20 mM Tris/HCl (pH 7.5), 5 mM EDTA, 159 mM NaCl and 0.01 % (w/v) SDS. After washing, the labelled samples were analysed in a chambered coverglass (μ-Slide 8 well; ibidi) by confocal laser-scanning microscopy (CLSM; TCS SP2 AOBs, Leica) using a ×63 water-immersion objective (HCX PL APO/bd.BL 63.0 × 1.2 W; Leica). A total of three sites (1024 × 1024 pixels, 0.196 mm²) was evaluated per enamel slab. HPLC-purified oligonucleotide probes for *Streptococcus* species, *Fusobacterium nucleatum*, *Veillonella* species, *Actinomyces naeslundii* and eubacteria were synthesized commercially and 5'-end-labelled with different fluorochromes (Thermo Electron). The 5' modification was chosen after testing the different fluorochromes in a multiplex FISH assay using the bacterial strains above, which were used to test the specificity of the different probes (Al-Ahmad *et al.*, 2007). The eubacterial probe EUB 338 (5'-GCTGCTCCCGTAGGAGT-3'; Amann *et al.*, 1990) was 5'-labelled with fluorescein and used to visualize the entire bacterial population within the plaque specimens. STR 405 (5'-TAGCCGTCCTTTCTGGT-3'; Paster *et al.*, 1998) was 5'-labelled with Cy3 and used to stain oral streptococci. E79 (5'-AATCCCTCCTTCAGTGA-3'; Paster *et al.*, 1998) was used to detect *Veillonella* species and was 5'-labelled with Texas Red. *F. nucleatum* was detected using FUS 664 [5'-CTTGAGTTCCGC(C/T)TACCTC-3'; Thurnheer *et al.*, 2004], which was 5'-labelled with Cy5. IF 201 (5'-GCTACCGTCAACCCACCC-3'; Foster & Kolenbrander, 2004) was labelled with Pacific Blue and used to visualize *A. naeslundii*. The specificities of the oligonucleotide probes were tested using different strains as described previously (Al-Ahmad *et al.*, 2007).

Excitation of the FISH probes was carried out at the following wavelengths: Pacific Blue, 405 nm; fluorescein, 488 nm; Cy3, 546 nm; Texas Red, 594 nm; and Cy5, 633 nm. Fluorescence emission of the probes was measured at the following wavelengths: Pacific Blue, 406–473 nm; fluorescein, 495–565 nm; Cy3, 493–538 nm; Texas Red, 599–670 nm; and Cy5, 550–592 nm. To minimize spectral overlap between the probes, CLSM was carried out sequentially for each image. Image analysis was conducted as described elsewhere in detail (Al-Ahmad *et al.*, 2007). In brief, within each area, the thickest point was measured by determination of the upper and lower boundaries of the biofilm. This procedure was repeated twice more and the mean thickness of the biofilm was determined from the three measurements. Biofilms were scanned from these three starting points, generating sections of approximately 0.5 μm thickness each at 2 μm intervals throughout the biofilm layers (to avoid overlaps). Standard images were made with a zoom setting of 1.7 corresponding to physical dimensions of 140 × 140 μm for each image. The area of each section was transformed into a digital image containing 1024 × 1024 pixels.

To quantify the biomass of the different targets within the oral biofilm, total fluorescent staining of the CLSM micrographs was analysed using the image analysis program MetaMorph version 6.3r7 (Molecular Devices). The program was used to calculate the biofilm composition from stacks of five-channel images by measuring voxel intensities. Fluorescence intensity thresholds were set manually for each of the fluorescent colours. The EUB 338 probe corresponding fluorescent volume was set as 100 % of bacterial biomass in the biofilm. All other targets were calculated as a percentage of the biomass calculated with EUB 338.

Electron microscopy. Selected samples were analysed by electron microscopy.

To prepare samples for TEM, they were rinsed gently in phosphate buffer solution (pH 7.4) to remove any non-adherent bacteria or proteins and dropped immediately into 3 % glutaraldehyde fixing

solution. Post-fixation was carried out in 2 % osmium tetroxide. After dehydration and embedding in Araldite M, the enamel slabs were decalcified in 4 % EDTA (pH 7.2) and re-embedded. Ultrathin sections (~50 nm) were prepared in an ultramicrotome (Ultracut E; Reichert) with a diamond knife. These sections were contrast stained with uranyl acetate followed by lead citrate and examined under a Philips 201 transmission electron microscope at a magnification of $\times 30\,000$.

For SEM, selected samples were fixed in 2.5 % glutaraldehyde for 2 h at 4 °C and dehydrated in an increasing ethanol series. After critical point drying, the samples were sputtered with gold. Samples were examined under a Philips 501 scanning electron microscope at a magnification of $\times 2500$ –5000.

Statistics. Statistical evaluation was performed by analysis of variance followed by the Scheffé procedure ($P < 0.05$) using SPSS version 16.0.

RESULTS AND DISCUSSION

In the present study, adherent bacteria on enamel slabs were visualized successfully using FISH after intra-oral exposure for 2–12 h. All bacterial species tested were detectable on enamel samples exposed to the oral fluids of different subjects. Representative images of the initial bacterial colonization are given in Figs 1–3. A high level of inter-individual and intra-individual variability was recorded. Single bacteria as well as monolayered chains or multilayered and three-dimensional aggregates of bacteria were observed, confirming the semi-planktonic

nature of bacterial populations in saliva. Single bacteria were mainly detected after 120 min, whereas bacterial aggregates and micro-colonies dominated the 12 h samples. Most bacteria were identified as having a coccoid morphology, but fibrils and rod-shaped bacteria were also visible. Micro-colonies of bacteria were mainly found after 6 and 12 h. Aggregates of different species were found after just 120 min. Certain areas colonized with one species were also observed, but this was rare and was observed mainly with streptococci. Representative electron microscopic micrographs are given in Fig. 4. The TEM micrograph shows the integration of bacteria into the ultrastructure of the pellicle and the formation of a bacterial multilayer after 12 h. The SEM micrograph shows incomplete coating of the enamel surface by a bacterial monolayer with several multilayered micro-colonies. This corresponded well with the CLSM pictures, as did the observed micromorphology of the bacteria.

In this study, the covering grade of the substratum was calculated. Although it is of great relevance, this parameter is often neglected in studies concerning the oral biofilm. Only a small area of the enamel surface was coated with bacteria and the covering grade detected in the present *in situ* study demonstrated that the initial oral biofilm increased only after 12 h to cover $1.14 \pm 2.88\%$ of the surface with bacteria. This was significantly more than recorded after 2 h ($0.44 \pm 2.20\%$) and 6 h ($0.38 \pm 0.92\%$) ($P < 0.001$). However, high variability of the covered area

Fig. 1. FISH staining of samples exposed to oral fluids *in situ* for 2 h. Eubacteria are shown in green, streptococci in magenta, *Veillonella* species in red, *F. nucleatum* in yellow and *A. naeslundii* in blue. Note the different morphology of the bacterial aggregates. Panel (d) shows a detailed view of (c).

Fig. 2. FISH staining of samples exposed to oral fluids *in situ* for 6 h. Eubacteria are shown in green, streptococci in magenta, *Veillonella* species in red, *F. nucleatum* in yellow and *A. naeslundii* in blue. Note the different morphology of the bacterial aggregates. Panel (d) shows a detailed view of (c).

was observed. A previous *in vitro* study demonstrated the relevance of the covering grade for studying the impact of different substances on the growth of *Streptococcus mutans* biofilms (Al-Ahmad *et al.*, 2008).

The composition of the adherent bacteria was calculated as a percentage of the number of eubacteria. The proportion of the different species differed significantly, irrespective of the oral exposure time ($P < 0.001$; Fig. 5). The significantly highest proportion of the investigated species was made up of streptococci followed by *Veillonella* species, *F. nucleatum* and *A. naeslundii*. The individual frequency of all single species differed significantly from each other except for *F. nucleatum* and *A. naeslundii* ($P < 0.001$).

Streptococci, the most dominant bacterial fraction, were found to make up about one-quarter of the initial biofilm. In other *in vivo* and *in situ* studies evaluating the initial bacterial colonization over periods of 4–48 h, 50–85 % of the detected bacteria were identified as streptococci (Nyvad & Kilian, 1987, 1990). A general dominance of streptococci was observed in a recent morphological FISH study without quantification of the micro-organisms (Dige *et al.*, 2007), whereas in another investigation of early microbial colonization occurring during the first 2 h based on a chequerboard DNA–DNA assay, only 25 % of the bacteria were identified as streptococci (Li *et al.*, 2004). Several aspects have to be considered in this context. Culture methods usually select for certain species. Thus,

streptococci in particular might be overestimated whereas other species such as *A. naeslundii* might be underestimated (Nyvad & Kilian, 1987, 1990; Al-Ahmad *et al.*, 2007). Several FISH studies have been conducted with comparable protocols and identical rRNA gene oligonucleotide probes (Al-Ahmad *et al.*, 2007; Dige *et al.*, 2007; Hannig *et al.*, 2007a). A recent morphological CLSM study found streptococci to be the predominant colonizers of early dental biofilms monitored over 6–48 h (Dige *et al.*, 2007). In contrast, our previous studies yielded a lower proportion of streptococci within the first 120 min of biofilm formation, as well as after 2 days or more of plaque formation (Al-Ahmad *et al.*, 2007; Hannig *et al.*, 2007a). None the less, the present data showed a significant increase in the proportion of streptococci with oral exposure time of the samples ($P < 0.001$). After 12 h, a higher frequency of streptococci was recorded compared with at 2 and 6 h (2 h vs 12 h, $P < 0.001$; 6 h vs 12 h, $P = 0.005$). This is in good accordance with a previous investigation based on FISH analysing bacterial adherence during the first 120 min, indicating a significant increase in streptococci (Hannig *et al.*, 2007a). Also, after 1 day of bacterial adhesion in the oral cavity, a high proportion of streptococci (40 %) was detected (Al-Ahmad *et al.*, 2007). A characteristic feature of streptococci is their short doubling time compared with other bacterial species. The doubling time of *S. mutans* is 1.4 h, whereas that of *Actinomyces viscosus* has been determined as 2.7 h (Beckers

Fig. 3. FISH staining of samples exposed to oral fluids *in situ* for 12 h. Eubacteria are shown in green, streptococci in magenta, *Veillonella* species in red, *F. nucleatum* in yellow and *A. naeslundii* in blue. Note the different morphology of the bacterial aggregates. Panels (b), (d) and (f) show detailed views of (a), (c) and (e), respectively.

& van der Hoeven, 1984). Furthermore, many components of the pellicle layer serve as specific binding sites for the adherence of streptococci (Douglas, 1994; Scannapieco, 1994). In addition, the presence of enzymically active glucosyltransferases in the pellicle promotes preferential adherence of streptococci (Scheie *et al.*, 1987; Schilling & Bowen, 1992; Hannig *et al.*, 2008b).

The proportion of *F. nucleatum* was also influenced by oral exposure time of the enamel slabs ($P=0.034$). After 6 h, there was a significantly higher proportion of *F. nucleatum* than after 12 h ($P=0.036$; Fig. 5). For *Veillonella* species and *A. naeslundii*, no significant impact of the formation time on the proportion of these species was found. *Veillonella* species are a relevant component of early oral

biofilms and have been found to make up 5 % of the initial plaque biomass, corresponding well with the present data (Nyvad & Kilian, 1990; Palmer *et al.*, 2006). These species metabolize organic acids such as lactate to shorter organic acids, hydrogen and carbon dioxide (Delwiche *et al.*, 1985). Some *Veillonella* species co-aggregate with streptococci (Palmer *et al.*, 2006).

F. nucleatum together with *A. naeslundii* represented only a minor component of the initially adhering bacteria. *F. nucleatum* is regarded as a relevant initiator of periodontal and gingival inflammation caused by matured supra- and subgingival plaque (Christersson *et al.*, 1991), whereas *Actinomyces* species are important for caries lesions of the root surface (Preza *et al.*, 2008). However, they were

Fig. 4. Electron micrographs. (a) TEM of a pellicle with adherent bacteria on an enamel sample at the buccal site of the maxillary first molar after a period of 12 h. The pellicle shows a rather homogeneous globular ultrastructure. Note the electron-dense basal layer (arrows) directly above the enamel surface. The enamel is not visible due to the decalcification process. The bacteria have a coccoid morphology. Original magnification: $\times 10\,000$. (b) SEM of a bacterial monolayer and bacterial aggregates on an enamel sample exposed to oral fluids for 12 h (first molar, buccal site). Some parts of the enamel are still free of bacteria. The adherent micro-organisms are coccoid or rod-shaped. The multilayered areas indicate some kind of co-aggregation. Compare with Figs 2 and 3. Original magnification: $\times 2500$.

already present among the initially adhering bacteria. The proportion of *F. nucleatum* was found to increase over 7 days of undisturbed plaque formation, but seems to be of less relevance at buccal sites during the first 12 h (Al-Ahmad *et al.*, 2007). This could be caused by higher oxygen concentrations in these sites and in the first 12 h, as *F. nucleatum* has been reported to be a late colonizer, depending on the decrease in oxygen, in a nascent five-species *in vitro* biofilm (Guggenheim *et al.*, 2001; Al-Ahmad *et al.*, 2007).

Overall, there was high intra-individual as well as inter-individual variability of bacterial adhesion and surface colonization, indicating that not only biofilm formation but also initial bacterial adhesion occurs in a subject-dependent manner (Diaz *et al.*, 2006; Al-Ahmad *et al.*, 2007; Dige *et al.*, 2007). None the less, in general, the number of detected adherent bacteria on buccal sites was

still quite low after 12 h. Contact with the oral soft tissues as well as salivary flow are assumed to limit glucan formation and thus adherence of streptococci on buccal sites. More extensive plaque formation has been observed previously at undisturbed proximal sites (Diaz *et al.*, 2006; Dige *et al.*, 2007). In addition to the occurrence of shear forces, antibacterial components of the pellicle layer such as lysozyme as well as the turnover processes of this proteinaceous layer will impair bacterial adhesion in a healthy oral cavity (Hannig & Joiner, 2006; Hannig & Hannig, 2007).

About 70 % of the detected bacteria could not be assigned to the tested species. It is possible that members of the genera *Gemella*, *Granulicatella*, *Neisseria*, *Prevotella*, *Campylobacter* and *Rothia*, as well as *Eikenella corrodens*, may contribute to the initial adherence, although they were not probed in the present study (Li *et al.*, 2004; Diaz *et al.*,

Fig. 5. Composition of the adherent bacteria. Each species was calculated as a percentage of the total eubacteria. Results are shown as means $\pm$ SD for six subjects, with six samples per subject and exposure time.

2006). Some of these non-streptococcal species have a more-or-less coccoid morphology (Li *et al.*, 2004). In general, more than 700 different bacterial phenotypes may occur in oral fluids (Aas *et al.*, 2005).

This study showed the semi-planktonic nature of bacteria in the oral fluid. This is seldom mentioned in studies concerning salivary bacteria, as microbiologists tend only to differentiate sessile from planktonic bacteria. This aspect, which has been discussed previously in our studies combining different methods to detect adherent oral bacteria *in situ* (Hannig *et al.*, 2007a), should again be highlighted by the results presented in this study.

Recently, Henssge *et al.* (2009) analysed different house-keeping genes in *A. naeslundii* isolates from various sources. The authors paid attention to the difficulties in separation of the different *A. naeslundii* genotypes using 16S rRNA gene sequences and, according to their detailed gene analysis, they proposed the name *Actinomyces oris* sp. nov. for *A. naeslundii* genospecies 2 and *Actinomyces johnsonii* sp. nov. for *A. naeslundii* genospecies WVA 963 and separated these two species from *A. naeslundii* genospecies 1. As a result, we cannot exclude the possibility that the *A. naeslundii*-specific oligonucleotide probe used in this study bound to all three *Actinomyces* strains mentioned above. This should be taken into consideration in future studies on the prevalence of *Actinomyces* strains in the oral cavity (Henssge *et al.*, 2009). None the less, the present data may serve as a reference for future studies testing the effect of oral healthcare products or antibacterial surface coatings on initial biofilm formation in the oral cavity. Further studies are necessary to investigate other oligonucleotide probes, as well as the site-specific mode of bacterial adhesion.

ACKNOWLEDGEMENTS

This study was supported in part by the German Research Foundation (DFG proposals HA 2718/3-3 and HA 5192/1-2). We would like to thank Bettina Spitzmüller and Gabriele Braun for their excellent technical support.

REFERENCES

- Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I. & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol* **43**, 5721–5732.
- Al-Ahmad, A., Wunder, A., Auschill, T. M., Follo, M., Braun, G., Hellwig, E. & Arweiler, N. B. (2007). The *in vivo* dynamics of *Streptococcus* spp., *Actinomyces naeslundii*, *Fusobacterium nucleatum* and *Veillonella* spp. in dental plaque biofilm as analysed by five-colour multiplex fluorescence *in situ* hybridization. *J Med Microbiol* **56**, 681–687.
- Al-Ahmad, A., Wiedmann-Al-Ahmad, M., Auschill, T. M., Follo, M., Braun, G., Hellwig, E. & Arweiler, N. B. (2008). Effects of commonly used food preservatives on biofilm formation of *Streptococcus mutans* *in vitro*. *Arch Oral Biol* **53**, 765–772.
- Amann, R. I. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In *Molecular Microbial Ecology Manual*, vol. 3.3.6, pp. 1–15. Edited by A. D. L. Akkermans, J. D. van Elsas & F. J. de Bruijn. Dordrecht: Kluwer.
- Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. & Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**, 1919–1925.
- Amann, R. I., Ludwig, W. & Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59**, 143–169.
- Beckers, H. J. & van der Hoeven, J. S. (1984). The effects of mutual interaction and host diet on the growth rates of the bacteria *Actinomyces viscosus* and *Streptococcus mutans* during colonization of tooth surfaces in di-associated gnotobiotic rats. *Arch Oral Biol* **29**, 231–236.
- Christersson, L. A., Zambon, J. J. & Genco, R. J. (1991). Dental bacterial plaques. Nature and role in periodontal disease. *J Clin Periodontol* **18**, 441–446.
- Deimling, D., Hannig, C., Hoth-Hannig, W., Schmitz, P., Schulte-Mönting, J. & Hannig, M. (2007). Non-destructive visualisation of protective proteins in the in situ pellicle. *Clin Oral Investig* **11**, 211–216.
- Delwiche, E. A., Pestka, J. J. & Tortorello, M. L. (1985). The Veillonellae: Gram-negative cocci with a unique physiology. *Annu Rev Microbiol* **39**, 175–193.
- Diaz, P. I., Chalmers, N. I., Rickard, A. H., Kong, C., Milburn, C. L., Palmer, R. J., Jr & Kolenbrander, P. E. (2006). Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Appl Environ Microbiol* **72**, 2837–2848.
- Dige, I., Nilsson, H., Kilian, M. & Nyvad, B. (2007). *In situ* identification of streptococci and other bacteria in initial dental biofilm by confocal laser scanning microscopy and fluorescence *in situ* hybridization. *Eur J Oral Sci* **115**, 459–467.
- Douglas, C. W. (1994). Bacterial–protein interactions in the oral cavity. *Adv Dent Res* **8**, 254–262.
- Foster, J. S. & Kolenbrander, P. E. (2004). Development of a multispecies oral bacterial community in a saliva-conditioned flow cell. *Appl Environ Microbiol* **70**, 4340–4348.
- Guggenheim, M., Shapiro, S., Gmür, R. & Guggenheim, B. (2001). Spatial arrangements and associative behavior of species in an in vitro oral biofilm model. *Appl Environ Microbiol* **67**, 1343–1350.
- Hannig, M. (1999). Transmission electron microscopy of early plaque formation on dental materials in vivo. *Eur J Oral Sci* **107**, 55–64.
- Hannig, M. & Hannig, C. (2007). Does a dental biofilm, free of bacteria, exist in situ? *J Parodontol Implantol Orale* **26**, 187–200.
- Hannig, C. & Hannig, M. (2009). The oral cavity – a key system to understand substratum-dependent bioadhesion on solid surfaces in man. *Clin Oral Investig* **13**, 123–139.
- Hannig, M. & Joiner, A. (2006). The structure, function and properties of the acquired pellicle. *Monogr Oral Sci* **19**, 29–64.
- Hannig, C., Hannig, M., Rehmer, O., Braun, G., Hellwig, E. & Al-Ahmad, A. (2007a). Fluorescence microscopic visualization and quantification of initial bacterial colonization on enamel *in situ*. *Arch Oral Biol* **52**, 1048–1056.
- Hannig, M., Kriener, L., Hoth-Hannig, W. & Schmidt, H. (2007b). Influence of nano-composite surface coating on biofilm formation in situ. *J Nanosci Nanotechnol* **7**, 4642–4648.
- Hannig, C., Spitzmüller, B., Al-Ahmad, A. & Hannig, M. (2008a). Effects of Cistus-tea on bacterial colonization and enzyme activities of the *in situ* pellicle. *J Dent* **36**, 540–545.
- Hannig, C., Ruggeri, A., Al-Khayer, B., Schmitz, P., Spitzmüller, B., Deimling, D., Huber, K., Hoth-Hannig, W., Bowen, W. H. & Hannig, M. (2008b). Electron microscopic detection and activity of glucosyl-

transferase B, C, and D in the *in situ* formed pellicle. *Arch Oral Biol* **53**, 1003–1010.

Hannig, C., Spitzmüller, B. & Hannig, M. (2009). Characterization of lysozyme activity in the *in situ* pellicle using a fluorimetric assay. *Clin Oral Invest* **13**, 15–21.

Henssge, U., Do, T., Radford, D. R., Gilbert, S. C., Clark, D. & Beighton, D. (2009). Emended description of *Actinomyces naeslundii* and descriptions of *Actinomyces oris* sp. nov. and *Actinomyces johnsonii* sp. nov., previously identified as *Actinomyces naeslundii* genospecies 1, 2 and WVA 963. *Int J Syst Evol Microbiol* **59**, 509–516.

Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., Socransky, S. S. & Oppenheim, F. G. (2004). Identification of early microbial colonizers in human dental biofilm. *J Appl Microbiol* **97**, 1311–1318.

Marsh, P. D. & Bradshaw, D. J. (1995). Dental plaque as a biofilm. *J Ind Microbiol* **15**, 169–175.

Nyvad, B. & Fejerskov, O. (1987). Transmission electron microscopy of early microbial colonization of human enamel and root surfaces *in vivo*. *Scand J Dent Res* **95**, 297–307.

Nyvad, B. & Kilian, M. (1987). Microbiology of the early colonization of human enamel and root surfaces *in vivo*. *Scand J Dent Res* **95**, 369–380.

Nyvad, B. & Kilian, M. (1990). Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. *Caries Res* **24**, 267–272.

Palmer, R. J., Jr, Diaz, P. I. & Kolenbrander, P. E. (2006). Rapid succession within the *Veillonella* population of a developing human oral biofilm *in situ*. *J Bacteriol* **188**, 4117–4124.

Paster, B. J., Bartoszyk, I. & Dewhirst, F. E. (1998). Identification of oral streptococci using PCR-based, reverse-capture, checkerboard hybridization. *Methods Cell Sci* **20**, 223–231.

Preza, D., Olsen, I., Aas, J. A., Willumsen, T., Grinde, B. & Paster, B. J. (2008). Bacterial profiles of root caries in elderly patients. *J Clin Microbiol* **46**, 2015–2021.

Pruitt, K. M., Caldwell, R. C., Jamieson, A. D. & Taylor, R. E. (1969). The interaction of salivary proteins with tooth surface. *J Dent Res* **48**, 818–823.

Scannapieco, F. A. (1994). Saliva–bacterium interactions in oral microbial ecology. *Crit Rev Oral Biol Med* **5**, 203–248.

Scheie, A. A., Eggen, K. H. & Rolla, G. (1987). Glucosyltransferase activity in human *in vivo* formed enamel pellicle and in whole saliva. *Scand J Dent Res* **95**, 212–215.

Schilling, K. M. & Bowen, W. H. (1992). Glucans synthesized *in situ* in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. *Infect Immun* **60**, 284–295.

Schwartz, T., Hoffmann, S. & Obst, U. (2003). Formation of natural biofilms during chlorine dioxide and u.v. disinfection in a public drinking water distribution system. *J Appl Microbiol* **95**, 591–601.

Thurnheer, T., Gmur, R. & Guggenheim, B. (2004). Multiplex FISH analysis of a six-species bacterial biofilm. *J Microbiol Methods* **56**, 37–47.

Vacca-Smith, A. M. & Bowen, W. H. (2000). *In situ* studies of pellicle formation on hydroxyapatite discs. *Arch Oral Biol* **45**, 277–291.