Insights into passive ultrasonic irrigation

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Influence of *Streptococcus mutans* on *Enterococcus faecalis* biofilm formation

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Abstract

Introduction An important virulence factor of *Enterococcus faecalis* is its ability to form biofilms. Most studies on biofilm formation have been carried out using *E. faecalis* monocultures. Given the polymicrobial nature of root canal infections, it is important to understand biofilm formation of *E. faecalis* in the presence of other microorganisms. **Methods** Eight clinical strains of *E. faecalis* were tested for biofilm formation on hydroxyapatite discs in the presence and absence of a *Streptococcus mutans* biofilm. **Results** Significantly more *E. faecalis* viable cells were found in biofilms in the presence of *S. mutans*. This phenomenon was, however, strain dependent. Out of the eight strains tested, biofilm formation of strains AA-OR34, ER5/1 and V583 was not influenced by *S. mutans* biofilms. **Conclusions** The results from this study, especially the strain difference, underline the importance of studying this virulence factor in a more realistic multispecies setting.

Introduction

Enterococcus faecalis is a versatile pathogenic micro-organism that plays a major role in the etiology of persistent endodontic infections after initial treatment. It is commonly found in high numbers of root canal failures (1). Several traits of *E. faecalis* are related to its persistence in root canal infections. These include (i) extensive genetic polymorphisms (2), (ii) invasion into dentinal tubules (3), (iii) rapid adaptation to adverse conditions (4, 5), (iv) resistance to antimicrobial treatments (6), and (v) most importantly biofilm formation (7, 8).

Biofilms, by definition, are matrix-enclosed microbial communities in which cells adhere to each other and/or to surfaces or interfaces (9). It has been generally accepted that the physiology of a bacterium in planktonic culture is profoundly different from that of the same organism growing in a biofilm. Several studies have shown that *E. faecalis* cells in biofilms have increased adaptive capabilities to adverse conditions and display an increased resistance to antimicrobial treatments (10, 11). Moreover, cell surface proteins such as adhesins, which are essential for biofilm formation, are also related to the invasion of dentinal tubules (12, 13). The virulence of *E. faecalis* is thus significantly enhanced when the pathogen grows in a biofilm. Therefore, the factors affecting biofilm formation have been investigated extensively. Growth conditions, starvation and saliva coating, all influence biofilm formation (14). A number of virulence genes, including those encoding enterococcal surface protein (*esp*), gelatinase (*gelE*) and enterococcal polysaccharide antigen (*epa*), were also found to be involved in biofilm formation (7). In addition, variations in biofilm forming properties were observed for different *E. faecalis* isolates (15).

So far most studies on *E. faecalis* biofilm characteristics have been performed on monoculture biofilm. Although *E. faecalis* has sometimes been isolated as the sole infectious organism in root canals, the notion that endodontic infections are typically polymicrobial has become more and more favorable. A recent review (16) argues that the observation of *E. faecalis* mono-infections might be the result of sampling and culturing techniques that fail to detect low abundance, physiologically inactive and dormant microorganisms. By using more advanced molecular techniques *E. faecalis* was reported to co-exist with several other taxa in root canal-treated teeth (17). The polymicrobial nature of the infected root-canal was also reported in another study where 16S rDNA sequencing was used (18). Since communication between bacterial species has already been shown in other ecological systems (19), it is of interest to understand how the presence of other bacteria influences *E. faecalis* biofilm formation.

The aim of the current study is to investigate the effect of a *Streptococcus mutans* biofilm on hydroxyapatite on biofilm formation of eight *E. faecalis* clinical isolates. In a recent clinical study, with 16s rRNA probes (17), *Streptococcus* species were detected in 47% of root-canal treated teeth. *S. mutans* has been detected in root canal infections before (18, 20) and it is a strong biofilm former, therefore we chose to investigate its influence on *E. faecalis* biofilm formation. Since *S. mutans* biofilm formation is most potent in the presence of sucrose, we used this carbohydrate as the energy source in our biofilm experiments.

Materials and Methods

Bacterial strains and growth conditions

The *E. faecalis* strains used in this study are E1, E2, E3, ER3/2s, ER5/1, AA-OR34, OS16, and V583 (all kind gifts from Dr. C.M. Sedgley) (15). The *S. mutans* UA159 strain used is also a clinical isolate (21). All bacterial strains were routinely grown anaerobically on brain-heart-infusion agar at 37 ℃. Biofilms were grown in modified semi-defined biofilm medium (BM) (22), which contains 76 mMol/L K_2HPO_4, 15 mMol/L KH_2PO_4, 10 mMol/L $(NH_4)_2SO_4$, 35 mMol/L NaCl, 2 mMol/L $MgSO_4$ $7H_2O$ and was supplemented with filter-sterilized vitamins (0.04 mMol/L nicotinic acid, 0.1 mMol/L pyridoxine HCl, 0.01 mMol/L pantothenic acid, 1 µMol/L riboflavin, 0.3 µMol/L thiamine HCl, and 0.05 µMol/L D-biotin), amino acids (4 mMol/L L-glutamic acid, 1 mMol/L L-arginine HCl, 1.3 mMol/L L-cysteine HCl, and 0.1 mMol/L L-tryptophan), and 0.3% (wt/vol) yeast extract, glucose (0.2%) or sucrose (0.2%) was added where indicated. The pH of this medium was 7.0.

Biofilm Assays

To avoid potential bacterial sedimentation in a flat-bottomed 96-well microtiter plate, the model which is commonly used for *E. faecalis* biofilm formation examination (15), we designed a new biofilm model where the substrata can be positioned vertically. In this model, we used a 24-well tissue culture

plate (Nunc^{TM}, Roskilde, Denmark). The top lid was replaced by a custom-made stainless-steel lid on which 24 clamps were fixed. Various substrata, such as hydroxyapatite discs or round glass cover slips can be inserted in the clamps. The clamps were positioned in such a way that the inserted substrata fit into the wells of the tissue culture plate without touching the wall (Figure 1). The stainless-steel lid together with the assembled substrata was sterilized by autoclaving before usage.

E. faecalis biofilms were grown anaerobically on HA discs in the absence or presence of a preformed 24 h *S. mutans* biofilm. In detail: one *S. mutans* UA159 colony was inoculated into BM medium with addition of 0.2% glucose and incubated overnight. The overnight culture was diluted to a final OD_{620} of 0.014 in fresh BM medium with addition of 0.2% sucrose and 1.5 mL of the cell suspension was dispensed into a sterile 24-well plate. The plate was then covered with a sterile stainless-steel lid containing standardized 9.5 mm diameter hydroxyapatite (HA) discs (HiMed INC, New York, USA). An equal number of HA discs were also inserted in sterile BM medium with 0.2% sucrose. After 24h incubation, HA discs with or without *S. mutans* biofilm were rinsed with Buffered Peptone Water (BPW, Oxoid, Hampshire, England) solution to remove non-adherent bacterial cells and were subsequently used as substrata for *E. faecalis* biofilm formation. For each *E. faecalis* strain, one colony was inoculated into BM with 0.2% sucrose and incubated overnight. This culture was then diluted to an OD_{620} of 0.05 in fresh BM medium with 0.2% sucrose and 1.5mL of the cell suspension was dispensed into a new sterile 24-well plate. The HA discs with or without 24h *S. mutans* biofilm were inserted in the cell suspension. After 8h incubation, the HA discs were rinsed with BPW and placed in fresh BM (with 0.2% sucrose). *E. faecalis* biofilm formation was evaluated after a further 16h incubation. Two to three experiments were carried out for each *E. faecalis* strain with triplicate samples in each experiment.

Biofilm evaluation

Since our biofilms only consist of *S. mutans* and *E. faecalis* we used colony forming units (CFUs) to evaluate the composition of biofilms. Each HA disc with biofilms was carefully removed from the lid with forceps and placed in a sterile vial containing 1mL Cystein Peptone Water (CPW). One mL liquid phase of each biofilm-well was also transferred to a sterile vial. All samples were then kept on ice. Both biofilm and liquid phase samples were dispersed by sonication on ice for 30 s at an amplitude of 40W (Vibra cell^{TM}, Sonics & Materials INC, USA) and vortex-mixing for 30s. After serial dilution, aliquots were plated out on BHI agar plates. The plates were incubated anaerobically at 37 ℃ for 2 days. The number of bacterial colonies was counted and expressed as colony forming units (CFUs). *S. mutans* and *E. faecalis* were characterized based on their specific colony morphology.

Statistics

Data were analyzed with the Statistical Package for Social Science (Version 15.0, SPSS, Chicago, IL, USA). The independent samples *t* test was used to compare biofilm cell CFU counts between two groups (with/without pre-formed *S. mutans* biofilms). One-way ANOVA was used to evaluate CFU differences within a group. The CFU counts were log transformed before the statistical tests. $p < 0.05$ was considered as significant.

Results

In this study, HA discs with or without *S. mutans* biofilms were used to grow *E. faecalis* biofilms. Within 24 hours *S. mutans* UA159 was able to form biofilms on the HA discs with an average of 6.6 ($\pm$0.3) $\log_{10}$ CFU/HA disc. In parallel, HA discs without *S. mutans* biofilms were incubated in sterile medium for 24h to exclude a potential medium effect on *E. faecalis* biofilm formation on the discs.

After 24h incubation with the various *E. faecalis* strains, biofilm formation of the two groups was evaluated. Five strains displayed significantly higher *E. faecalis* CFU counts on the HA discs covered with *S. mutans* biofilms than on the clean HA discs ($p < 0.05$). In other words, for these strains *E. faecalis* biofilm formation was enhanced by the pre-formed *S. mutans* biofilm. For strains ER5-1, AA-OR34 and V583, no significant difference in biofilm formation was observed between with/without *S. mutans* biofilm groups (Figure 2).

To evaluate whether the increased biofilm formation is related to growth differences between the *E. faecalis* strains, we also measured CFU counts in the liquid phase of each biofilm-well, so that the total bacterial CFU counts could be calculated. The total CFU number per well was calculated as follows: CFU-total = 1.5x *E. faecalis* liquid phase CFU (per ml) + *E. faecalis* biofilm CFU (per disc) + *S. mutans* biofilm CFU (per disc).

S. mutans liquid phase CFU counts were neglected because the bacterial cell numbers were below the detection limit (10^2 CFUs per disc). For all tested *E. faecalis* strains, the CFU-total reached

around 10^9 CFU (except for strain E3) after 24 hours of growth (data not shown). This number was not affected by the presence of the *S. mutans* biofilms. Strain E3 scored a significantly lower CFU-total number than the other clinical strains (in both groups). Figure 3 shows the relative contribution of *E. faecalis* biofilm CFUs and liquid phase CFUs to the total cell amount. In all cases most *E. faecalis* cells grow in the liquid. Again, the presence of *S. mutans* biofilms on the HA discs in most cases increases the percentage of *E. faecalis* biofilm cells, except for strains ER5-1 and V583.

Biofilm formation of *S. mutans* was also evaluated. Without *E. faecalis* present the total CFUs of *S. mutans* increased to 8.0 (±0.2) $\log_{10}$CFU/HA disc (in this case after 48h). When the discs were incubated with *E. faecalis*, the total CFUs reached only 7.1(±0.6) $\log_{10}$CFU/HA disc.

Discussion

The data presented in this paper demonstrate that the presence of a streptococcal-biofilm on hydroxyapatite significantly increases the biofilm formation of five *E. faecalis* clinical strains. The reason for this increased biofilm formation is not yet clear. Since the total bacterial cell number remains the same irrespective of the presence of a *S. mutans* biofilm on the substrata, this effect was not due to changes in the growth-rate of *E. faecalis*. Coaggregation, defined as cell-to-cell adhesion in which cells of one microbial species adhere more or less specifically to those of a different species (23), may play a role. So far coaggregation (or maybe better specific cell-to-cell adhesion) has not yet been reported for *E. faecalis* and *S. mutans*. Still it is clear from our data that *E. faecalis* biofilm formation can be strongly influenced by other species that are present in the root canal.

From the eight strains tested, three strains exhibited different dual species biofilm characteristics. Strains ER5/1, AA-OR34 and V583 did not display increased biofilm formation in the presence of pre-formed *S. mutans* biofilms, which is different from other tested strains. However, their biofilm formation characteristics were similar to the other strains when grown as monoculture. Strains E1, E2, E3, AA-OR34 and OS16, used in this study, were isolated from a rinse sample of an endodontic patient. Strains ER3/2s and ER5/1 were isolated from a retreated root canal and strain V583 was a non-oral strain (15). Although clinical strain diversity might be related to the origin of the strains, in this case it does not explain the difference in biofilm characteristics.

As mentioned, enterococcal surface proteins are also involved in *E. faecalis* biofilm formation (24). The presence of the *esp* gene in these eight clinical strains has been studied previously (15, 25, 26), and it was shown that only strain E2 and ER3/2s possess *esp*. Therefore, the biofilm characteristics reported in the current study can not be related to the presence/absence of the *esp*-gene.

The biofilm model chosen in this study has several advantages over the often used comparable Calgary biofilm model (27). The Calgary biofilm model is a combination of a standard 96-well microtiter plate and a lid with an identical number of polystyrene pegs that fit into the wells. It has been successfully used to monitor e.g. the antibiotic susceptibility of microbial biofilms (28). Advantages of the Calgary model are that biofilm formation is not obscured by sedimentation, possible contamination can be eliminated and it is easy to use. Unfortunately the substratum of the model is limited to polystyrene. The model used in our study combines the advantages of the Calgary biofilm model with a flexible choice of substrata. HA discs, dentin discs or glass cover slips can be easily inserted into and removed from the clamps fixed on the custom-made lid. Since biofilm characteristics are affected by the substratum (29), this can now be easily evaluated (or avoided). In the current study, we were able to use standardized HA discs to mimic the root canal wall. An additional advantage of the stainless-steel lid is that the entire model can be autoclaved after assembly, allowing for an even wider use of substrates.

Increased resistance to antimicrobial treatment in biofilms has been reported for many bacterial species, including *E. faecalis*. New endodontic treatments have therefore been evaluated on *E. faecalis* biofilm instead of planktonic cells. Our study shows that the presence of other microorganisms has a marked effect on *E. faecalis* biofilm mode of growth. Hence, evaluation of these new treatments should be performed in a polymicrobial setting. Moreover, our results showed that not all tested clinical isolates behave in the same way. Strains that show similar biofilm formation characteristics as mono-species diverge in their interaction in dual species biofilms. This emphasizes the need for studying this important virulence factor in a multispecies setting.

Figure 1. Pictures of the biofilm model used in this study. (*A*) Custom-made stainless-steel lid on which 24 clamps are fixed. Substrata glass coverslips or HA disks are shown. (*B*) Position of the substrata (HA disks) in the 24-well plate, at the time of biofilm growth.

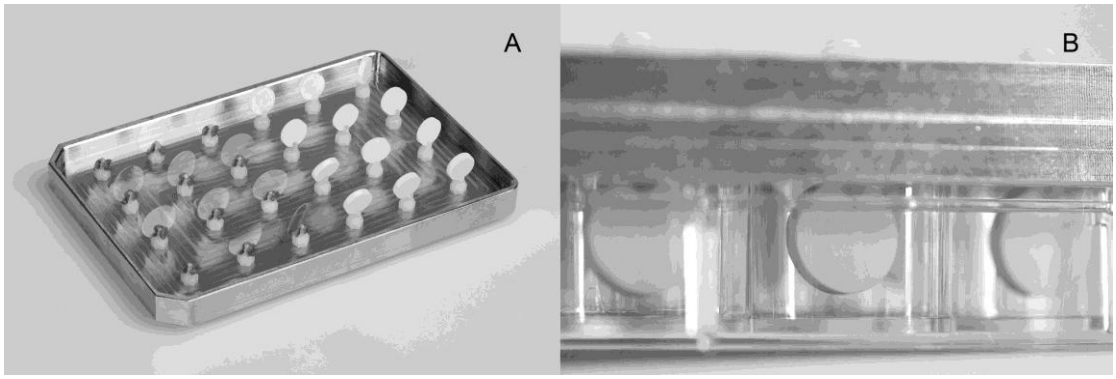


Figure 2. Viable counts (log_{10}CFU/disc) of *E. faecalis* in biofilms. *E. faecalis* biofilm cell counts are presented when it grew on HA disc only (white bar) or HA disc covered with *S. mutans* biofilms (grey bar). Eight *E. faecalis* clinical isolates were tested. * indicates significant differences in biofilm formation between two groups ($p < 0.05$).

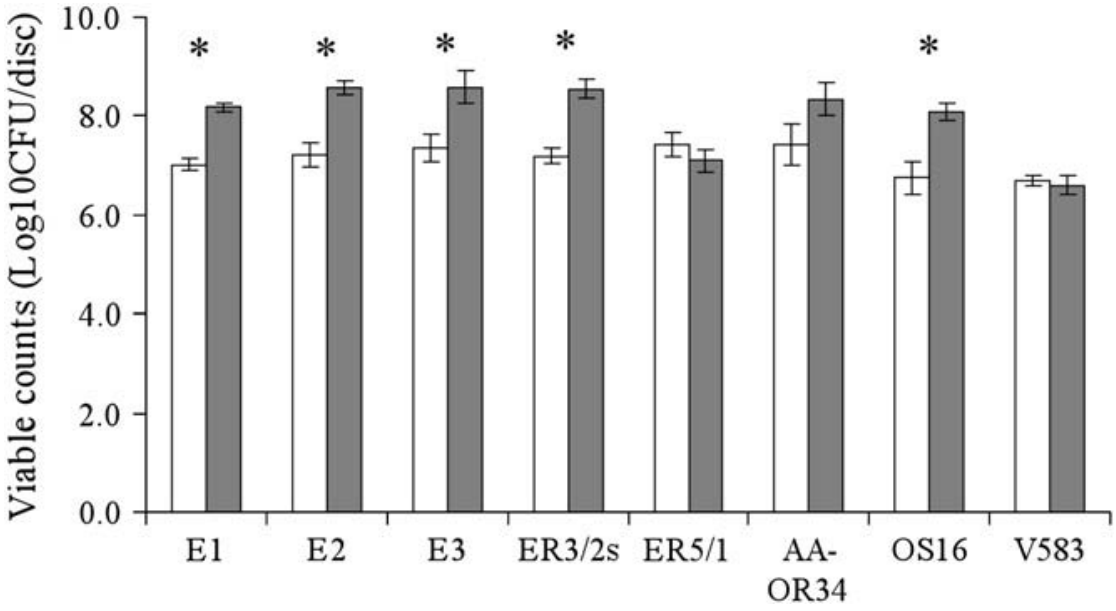
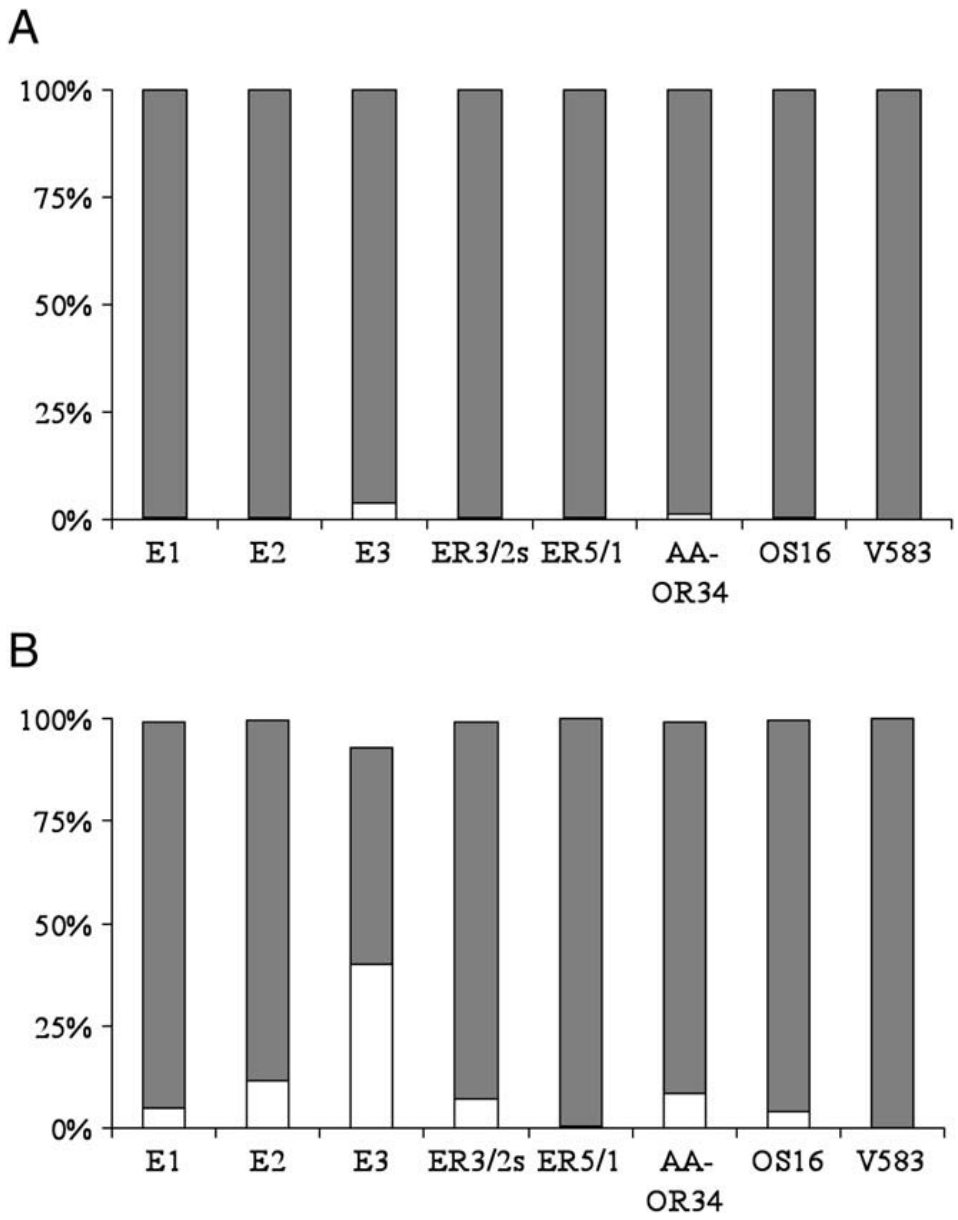


Figure 3. Percentage of *E. faecalis* biofilm CFUs (white bars) and liquid phase CFUs (grey bars) to the total cell amount. The percentage is calculated from the total cell number, which is determined as follows: total cell number= 1.5x *E. faecalis* suspension CFU (per mL) + *E. faecalis* biofilm CFU (per disc) + *S. mutans* biofilm CFU (per disc). Figure 3A represents data from those grown on clean HA discs and figure 3B represents data from those grown on HA discs covered by 24h *S. mutans* biofilm.



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