Association between Extracellular Material and Biofilm Formation in Response to Sodium Hypochlorite by Clinical Isolates of *Enterococcus faecalis*

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Abstract

Introduction: Extracellular material (ECM) surrounding *Enterococcus faecalis* may play a role in increasing resistance to environmental stresses. Our aim was to determine ECM levels in response to sub-minimal inhibitory concentrations of sodium hypochlorite (sub-MIC/NaOCl) or anaerobic growth and determine the impact on biofilm development. **Methods:** From 37 *E. faecalis* clinical strains, 19 were selected according to their biofilm-producing ability by using a crystal violet biofilm assay: 10 strong, 4 intermediate, and 5 non-biofilm producers. Biofilm assays were subsequently performed on all strains when subjected to sub-MIC/NaOCl. All strains were evaluated for ECM production under aerobic and anaerobic conditions and with sub-MIC/NaOCl. ECM production was assessed by using scanning electron microscopy. Double-blinded independent assessors were used to score levels of ECM production. The *esp* gene was detected by using polymerase chain reaction. Gelatinase activity was determined by using Todd-Hewitt and gelatin agar. **Results:** In aerobic conditions, ECM was expressed in all strains. In the presence of sub-MIC/NaOCl, of the 10 strong biofilm producers, 5 increased their ECM production, and 4 showed increased biofilm growth. Two strains had less ECM production and showed decreased biofilm growth. One isolate demonstrated no observable changes. Most non-biofilm producers demonstrated no observable differences in ECM production, although 1 strain increased biofilm growth. ECM production in anaerobic conditions was highly variable. The *esp* gene (n = 15) and gelatinase activity (n = 7) were evident among the isolates. **Conclusions:** Clonal diversity among strains of *E. faecalis* suggests that some strong biofilm producers can upregulate ECM production and increase biofilm growth in response to sub-MIC/NaOCl. *(J Endod 2017;■:1–5)*

Key Words

Biofilm, clonal diversity, *Enterococcus faecalis*, extracellular material, stress response

Significance

The present study assessed the association between biofilm and extracellular material levels in different environmental conditions for *E. faecalis*. Only biofilm producers were able to regulate extracellular material production in response to suboptimal growth conditions. Clonal diversity was marked.

The response to environmental stress is an important factor in understanding how bacteria survive chemical and physiological insults. In fact, certain *Enterococcus faecalis* strains are able to stimulate a response (eg, biofilm formation) to the presence of commonly used biocides, including sodium hypochlorite (NaOCl) (1). Biofilm formation could be considered a stress response to adverse environmental conditions and is associated with extracellular polysaccharides production (1).

The biosynthesis of extracellular polysaccharides in many bacteria is highly regulated and may play a role in many infections (2). These can form a loosely attached extracellular layer surrounding bacteria, which has been referred to as slime or extracellular matrix (ECM). The ECM may be related to pathogenesis by being involved in antibiotic resistance (3), adherence (4), and biofilm formation (5), plus it is thought to provide protection against host defenses (6, 7) and desiccation (4). Little is known regarding the role of ECM in *E. faecalis* biofilms to survive the physicochemical stresses associated with endodontic treatment. The ECM surrounding *E. faecalis* may play a role in upregulating biofilm growth and increasing resistance in the presence of environmental stresses occurring during root canal treatment.

E. faecalis can be found in previously filled root canals in the presence or absence of apical pathosis (8). This has been explained by differences in virulence abilities between isolates (8). In fact, *E. faecalis* isolates are able to synthesize proteins that can constitute virulence determinants, with the enterococcal surface protein and gelatinase production

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having particular relevance in endodontics. The enterococcal surface protein is a large chromosome-encoded surface adhesin that may play a role in evading the host immune system. The *esp* gene, which encodes this specific protein, is associated with biofilm attachment on abiotic surfaces and biofilm formation *per se* (9). Remarkably, this gene is widely present in clinical isolates of *E. faecalis* (10–12). On the other hand, gelatinase is an extracellular metalloproteinase that can hydrolyze the breakdown products of collagen and is also involved in biofilm development (13). Gelatinase's activity appears to be involved in the pathogenicity of endodontic disease (8) by contributing toward the microorganism's ability for long-term survival in obturated root canals (11).

Following on from our recent publication by Wilson et al (1), the main aim of the present study was to investigate the role of the ECM on biofilm growth by clinical isolates of *E. faecalis* when subjected to suboptimal growth conditions. The ECM layer may be an important virulence factor that enables certain (ie, strong ECM producers) isolates of *E. faecalis* to survive root canal treatment by increasing ECM and/or promoting biofilm growth. Similarly, the relationship of biofilm and ECM formation with other virulence determinants is not well-understood.

The aims of the study were the following:

1. Determine whether ECM is upregulated or downregulated by *E. faecalis* in response to suboptimal growth conditions (ie, NaOCl sub-minimal inhibitory concentrations [sub-MIC] or anaerobiosis)
2. Determine whether ECM is associated with biofilm growth in response to sub-MIC levels of NaOCl
3. Understand the role of *esp* gene in *E. faecalis* in regard to biofilm and ECM formation
4. Understand the role of gelatinase production in regard to biofilm and ECM formation

Materials and Methods

Bacterial Strain Selection and Biofilm Determination

Thirty-seven *E. faecalis* clinical and reference strains were obtained as previously described (14). Initially, their ability to produce biofilms during a period of 24 hours was assessed by using a biofilm assay protocol previously described (9) and optimized for *E. faecalis* (15). Subsequently, the strains were ranked from the highest to the lowest biofilm producers on the basis of their mean optical density (OD) by using the crystal violet colorimetric assay. Finally, they were categorized according to their average OD at 570 nm as follows: strong ($0.55 \leq OD_{570nm}$), intermediate ($0.29 \leq OD_{570nm} < 0.55$), weak ($0.16 < OD_{570nm} < 0.29$), or non- ($0 \leq OD_{570nm} \leq 0.16$) biofilm producers.

Cultures were maintained on Todd-Hewitt agar (THA) plates (Oxoid, Basingstoke, UK), Todd-Hewitt broth (THB), or heart-infusion broth (HIB) (Oxoid). Purity was periodically checked by plating onto bile aesculin agar (Oxoid).

The 10 strongest biofilm producers were selected from the clinical isolates. Of the remaining strains, 3 intermediate producers and 5 non-biofilm producers (based on their OD ranking) were included. Finally, the type strain V583, which is deficient in the *esp* gene (16), was included to compare the relationship of *esp* gene with biofilm growth and ECM formation.

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) for NaOCl was obtained to assess biofilm formation by different isolates under environmental stress (ie, NaOCl sub-MIC). A 1% w/v dilution of a commercially available preparation was used as the highest concentration (Sigma-Aldrich, St Louis, MO). The MIC was determined by performing serial dilutions by using a 96-well microtiter tray in duplicate. One hundred microliters THB was added to each well, and then 100 μL 1% w/v NaOCl was dispensed into the first well of the row. Aliquots of 100 μL were used to make serial dilutions. To minimize errors in concentration associated with dehydration, a further aliquot of 100 μL THB was added to each well. Ten microliters of an O/N inoculum (normalized [OD_{570nm}] to 1.0), containing each isolate, was then added to each well of the experimental group. The positive control group contained 10 μL inoculum grown in THB without the addition of NaOCl, and the negative control group did not contain the inoculum but contained the biocide. After incubation (24 hours at 37°C), planktonic growth (OD_{570nm}) was confirmed by using a microplate reader (BioTek, Winooski, VT). The first experimental well that demonstrated bacterial growth (OD_{570nm}, comparable to the positive control group) before the MIC was reached was determined to be the sub-MIC to be used for the biofilm assays. For all the strains the sub-MIC values were 0.031% for NaOCl.

Biofilm Assays in the Presence of NaOCl at Sub-MIC Concentration

Biofilm assays were performed during 24 hours while subjected to sub-MIC levels of NaOCl by using the crystal violet colorimetric assay (9, 15). Plates were read before crystal violet staining to confirm growth in the experimental wells and the positive controls. The inoculum was grown overnight in THB at 37°C and was normalized ($OD_{570\ nm}$) to 1.0. Groups consisted of experimental group, NaOCl sub-MIC in THB + 10 μL inoculum (n = 36); control group 1, 200 μL THB + 10 μL inoculum (n = 36); control group 2, NaOCl sub-MIC in THB (n = 12 wells); and control group 3, 200 μL THB (n = 12 wells). The plates were read at OD_{570nm}. The average OD readings of control group 1 were subtracted from the average values of the experimental group (NaOCl sub-MIC). The OD readings were recorded as increased/decreased/no changes, and a percent relative to the strain control group was calculated.

Assessment of ECM

Congo red dye was purchased from Thermo-Fisher Scientific (Waltham, MA). All strains were grown on HIB agar containing 0.8 g/L Congo red dye and 36 g/L saccharose for 72 hours at 37°C under the following conditions: aerobic, aerobic in presence of sub-MIC NaOCl, and anaerobic (atmosphere containing, 5% CO_2, 5% H_2, 90% N_2). To prepare for scanning electron microscopy (SEM) imaging, square frustum sections (base, 8 mm wide × 5 mm high) were prepared by dissecting CRA sections containing colonies. Samples were fixed for 30 minutes in 4% paraformaldehyde/1.25% glutaraldehyde in phosphate-buffered saline (PBS) and 4% sucrose, pH 7.2. Fixative was removed, and samples were washed in PBS + 4% sucrose and soaked for 5 minutes. Samples were then placed in 2% osmium tetroxide in water for 30 minutes and dehydrated with 70% ethanol for 10 minutes, 90% ethanol for 10 minutes, and 100% ethanol for 10 minutes. The latter step (100% ethanol) was repeated 3 times. Hexamethyldisilazane (HMDS) 1:1 with ethanol was then added to the sample and removed before adding 100% HMDS for 10 minutes. HMDS was then removed, and the sample was allowed to dry in a fume hood. Samples were then attached to stubs and processed for SEM, as previously described (17). A Philips XL30 FEG (Eindhoven, Netherlands) microscope was used, with settings of 10 mm working distance and 10.0 kV beam. Sample preparation and imaging were carried out by a research assistant who was blinded to their group allocation.

Blinded (in regard to the strains and the environmental condition) assessments were conducted by 2 independent assessors. ECM