

Biofilm Formation in Medicated Root Canals

John W. Distel, DMD, MS, John F. Hatton, DMD, and M. Jane Gillespie, PhD

The hypothesis that *Enterococcus faecalis* resists common intracanal medications by forming biofilms was tested. *E. faecalis* colonization of 46 extracted, medicated roots was observed with scanning electron microscopy (SEM) and scanning confocal laser microscopy. SEM detected colonization of root canals medicated with calcium hydroxide points and the positive control within 2 days. SEM detected biofilms in canals medicated with calcium hydroxide paste in an average of 77 days. Scanning confocal laser microscopy analysis of two calcium hydroxide paste medicated roots showed viable colonies forming in a root canal infected for 86 days, whereas in a canal infected for 160 days, a mushroom-shape typical of a biofilm was observed.

Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis showed no differences between the protein profiles of bacteria in free-floating (planktonic) and inoculum cultures. Analysis of biofilm bacteria was inconclusive.

These observations support potential *E. faecalis* biofilm formation in vivo in medicated root canals.

The most common reasons for failures in conservative root canal therapy are related to problems in instrumentation. However, occasionally, bacteria resistant to conservative therapy may also be involved (1). "Bacteria-associated endodontic failures together with pulp-periapical infections refractory to conventional treatment represent the unresolved bacteriological problems in endodontics" (2). Numerous studies have shown that persistent endodontic infections are often caused by *Enterococcus faecalis* (1, 3).

Virulence factors of *E. faecalis*, such as hemolysin, gelatinase, and enterococcal aggregation substance (EAS) play an important role in the bacterium's pathogenesis (4). However, the mechanism through which *E. faecalis* persists in the root canal is not well understood. *E. faecalis* seems to be highly resistant to the medications used during treatment and is one of the few organisms that has been shown to resist the antibacterial effect of calcium hydroxide (5, 6). There is little research to explain why *E. faecalis* is resistant to root canal therapy. It is easily destroyed when grown in vitro, but it becomes resistant when present in the environment of the root canal system (7). Therefore, *E. faecalis* must undergo

some type of change while in the root canal system, possibly activating some virulence factor that makes it more resistant. Alternatively, it may form a biofilm.

Biofilms, also known as plaque, are complex communities of bacteria embedded in a polysaccharide matrix (8). Suspended, i.e. planktonic, bacteria that are either leaving or joining the biofilm surround the biofilm. The growth conditions vary between biofilm and planktonic environments. For this reason, proteins expressed by biofilm bacteria may differ from those expressed by their planktonic counterparts, and both the biofilm bacteria and the planktonic bacteria may differ from bacteria maintained in the laboratory.

The purpose of this study was to test the hypothesis that *E. faecalis* forms a biofilm that allows it to resist common intracanal medications and to chronically infect the root canal system. *E. faecalis* colonization of extracted roots medicated with calcium hydroxide was observed over time by using scanning electron microscopy (SEM) and scanning confocal laser microscopy (SCLM). Also, protein profiles of planktonic and biofilm cultures of *E. faecalis* were compared by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Forty-six human maxillary anterior teeth were prepared and placed in a test model as described in a previous publication (7). Briefly, the clinical crown was removed at or near the CEJ to obtain a standard root length of 15 mm. The canals were instrumented to #50, 1-mm short of the apex, maintaining patency with a #25 and the coronal portion prepared with Gates Glidden drills (#2-4). A 3-mm reservoir was prepared with a #4 round bur.

The test model (7) was composed of 5-ml Wheaton serum vials with fitted rubber stoppers (Wheaton, Millville, NJ). A hole was made through the center of every rubber stopper, and each instrumented root was inserted through the stopper to the CEJ. This positioned the reservoir portion of the root outside of the vial and the remaining portion of the root within the vial. Cylinders prepared from polyvinyl tubing were affixed to the external surface of the vial to create an additional reservoir and barrier to prevent leakage of the bacteria or the culture medium over the sides of the apparatus. The vials were filled with brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) so that approximately 2 mm of the root apex was immersed in the broth.

The roots in the test model were randomly divided into four groups. Group 1: 15 roots medicated with a commercially prepared calcium hydroxide (Ca(OH)₂)/methylcellulose paste (Pulpdent,

Watertown, MA) placed in the canals to working length by means of a lentulo spiral (Brasseler, Savannah, GA). Group 2: 15 roots fitted with size #50 Ca(OH)₂ points (Roeko, Duarte, CA) with tug back as specified by the manufacturer. Group 3: eight roots served as a positive control by leaving the canals empty with no medication and a sterile cotton pledget placed in the reservoir. Group 4: eight roots served as a negative control by leaving the canals empty and a sterile cotton pledget placed in the reservoir with no bacterial inoculation. Groups 1, 2, and 3 were inoculated with bacteria as described below.

The cotton pledgets placed in the reservoir of the test roots in groups 1 to 3 were soaked with 50 μ l of a pure culture of *E. faecalis* [American Type Culture Collection (ATCC 4083), Rockville, MD]. The culture was grown in BHI broth to late exponential phase and adjusted against an uninoculated BHI control to an optical density at 540 nm of 1.534 using a CARY 1 BIO Spectrophotometer (Varian, Australia). Fresh, 50- μ l aliquots of the adjusted culture were added every 24 h to recontaminate the cotton pellet. At this OD₅₄₀, 50 μ l was sufficient to deliver approximately 1.5×10^8 bacteria as determined by a cell count in a Petroff-Hauser Chamber.

The test model was incubated at 37°C in an aerobic incubator and checked daily for turbidity in the broth below the root. The number of days it took for the appearance of bacterial growth was recorded as the time required for contamination of the root canal by *E. faecalis*. To confirm that contamination in samples showing turbidity was due to *E. faecalis*, the BHI broth culture was plated on tryptic soy agar with 5% blood (Gibson Laboratories, Lexington, KY) incubated as described above and examined for typical *E. faecalis* colonies.

SEM Analysis

Ten roots in groups 1 and 2 and five roots in groups 3 and 4 were prepared for SEM analysis. The infected groups, 1, 2, and 3, had become contaminated as indicated by growth in the broth under the tooth root. Longitudinal grooves were cut along the entire length of each root. The roots were then split with a hammer and chisel into two halves as described by Sen et al. (9). Each root half was then gently washed in 0.2 M potassium phosphate buffer (PBS), pH 7.2, at 4°C to 6°C. The roots were then fixed in 2% glutaraldehyde at 4°C to 6°C for 24 h, washed with PBS for 15 min, and postfixed for 12 h at 4°C to 6°C in 1% (wt/vol) osmium tetroxide. PBS was used as a final wash. Dehydration was performed with an ascending acetone series (30%, 60%, 100%) for 10 min each. The roots were dried by using a SAMDRI PVT-3 critical point dryer apparatus (Tousimis Research Corp., Rockville, MD) using liquid CO₂ replacement. Each root was mounted and coated with a 200 Å layer of gold palladium. Canal observations were performed by using a JEOL JSM-35CF scanning electron microscope at 25 kV. Photographs were recorded on Polaroid Type 55 film.

Scanning Confocal Laser Microscopy

After becoming contaminated, two roots from group 1 were examined by SCLM. One root became contaminated after it had been inoculated daily for 86 days; the other after it had been inoculated daily for 160 days. To prepare these for SCLM, roots were split as described above and gently washed in PBS. Cyanoacrylate was used to mount root halves on 24 \times 30-mm glass cover

slips glued to a customized chamber that used rubber hose washers to keep the root immersed in a BacLight bacterial viability stain (Molecular Probes, Eugene, Oregon). The BacLight stain is comprised of two fluorescent dyes, SYTO9 and propidium iodide, with excitation/emission maxima of 480 nm/500 nm and 490 nm/635 nm, respectively. Viable cells stain with SYTO9 and fluoresce green, whereas dead cells stain with propidium iodide and fluoresce red. Observations were performed on a Zeiss LSM 410 scanning confocal laser microscope (Zeiss, Oberkochen, Germany) by using a \times 63 water immersion lens and a fluorescein long pass filter. The excitation wavelength was 488 nm and light above 500 nm was collected. Photographs were produced on a Sony UP-5200MB video printer (Sony Corp., Tokyo, Japan).

Preparation of Cell Lysates

The bacteria that breached the CaOH₂ points and CaOH₂ paste moved down the root canal to the apex where they grew to create turbidity in the BHI broth. These *E. faecalis* cells were possibly coming off of the biofilms in the root, and therefore, likely to express the same genes and thus proteins as the planktonic cells most closely associated with the biofilm.

To investigate potential differences in protein expression, the protein profiles of cells in each inoculated group were compared to the inoculum culture by SDS-PAGE. Cell-free lysates were prepared from cells collected from planktonic cultures that grew in the BHI under the tooth roots and from the *E. faecalis* stock culture that was used to inoculate the roots. Bacterial cells were collected from roots of each contaminated group by brief sonication (five roots from group 2 and three roots from each of the other groups). All collected cells were washed in PBS twice, centrifuged, and stored at -20°C.

Two methods were used to extract proteins from the cells, trichloroacetic acid (TCA)/SDS and lysozyme treatment. Before treatment, the cells were suspended to an OD₅₄₀ of 1.0 to 1.15. For TCA/SDS treatment, the cells were pelleted in a clinical centrifuge at maximum speed. They were suspended in 0.5 ml of 10% TCA and incubated overnight at 4°C. PBS was used to wash the suspension three times at maximum speed using an Eppendorf microcentrifuge. The resulting cell pellet was suspended in 100 μ l of distilled water to which 100 μ l of \times 2 SDS-PAGE sample buffer (0.5 M Tris, pH 6.8) was added. This was heated for 10 min at 95°C and cooled on ice; it was reheated for 5 min at 95°C, just before being loaded on the gel.

For lysozyme treatment, the cell suspension adjusted to the desirable OD₅₄₀ was centrifuged in a clinical centrifuge at maximum speed, and the resulting pellet was suspended in 1 ml of 0.5 M Tris buffer, pH 6.8 containing 1 mg/ml lysozyme, 2 mM tetrasodium EDTA, and 1 mM phenyl-methyl sulfonyl fluoride (PMSF). These were incubated on a rotator overnight at 37°C. The resulting lysates were assayed for total protein, and if necessary, concentrated by using a Centricon centrifugal filter unit (Millipore, Bedford, MA) with a 10 kDa cutoff.

Analytical Techniques

SDS-PAGE was performed on cell lysates by using 1.5 mm vertical slab gels with either a 5% to 20% (wt/vol) acrylamide gradient or 12% (wt/vol) acrylamide, containing 0.2% (wt/vol) SDS, with a 4.5% (wt/vol) acrylamide stacking gel. SDS-PAGE

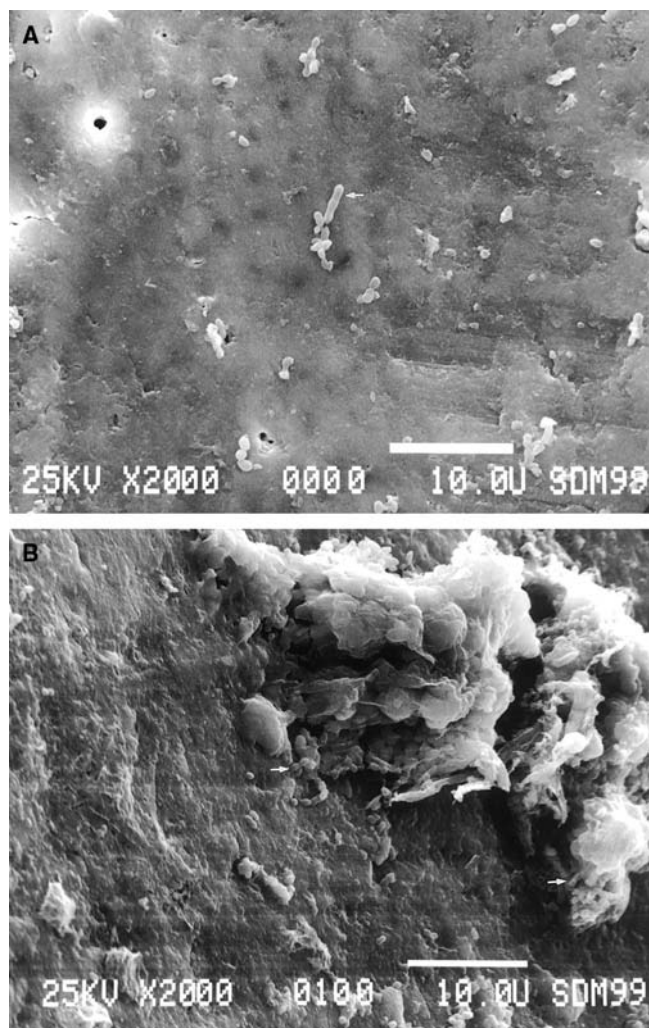


FIG 1. SEM of *E. faecalis* colonizing root canals medicated with (A) calcium hydroxide points at 2 days and (B) calcium hydroxide paste at 77 days. Bar = 10 μ m. Arrows indicate examples of *E. faecalis* cells. The cells are coated with gold palladium.

used the Laemmli buffer system (10). Protein bands in the SDS-PAGE gels were visualized by the Bio-Rad silver stain kit (Bio-Rad Laboratories, Hercules, CA). Protein assays were conducted by using a Pierce Commassie Plus assay kit (Pierce Chemical Co., Rockford, IL).

RESULTS

SEM Analysis

Roots from each group were observed under SEM. Canals were compared in each group for colony formation by *E. faecalis*. Group 4 (negative control) showed the root canal with no bacterial colonization after 60 days of incubation. Group 3 (positive control) showed total colonization of root canals by *E. faecalis* in all specimens after 2 days of incubation. Separate colonies were observed adhering to the root canal walls with short filaments growing out from the cells. Similar to group 3, group 2 (calcium hydroxide point medication; Fig. 1A) showed colonization of all samples after 2 days of incubation. In some samples of group 2, fewer colonies were observed than in group 3.

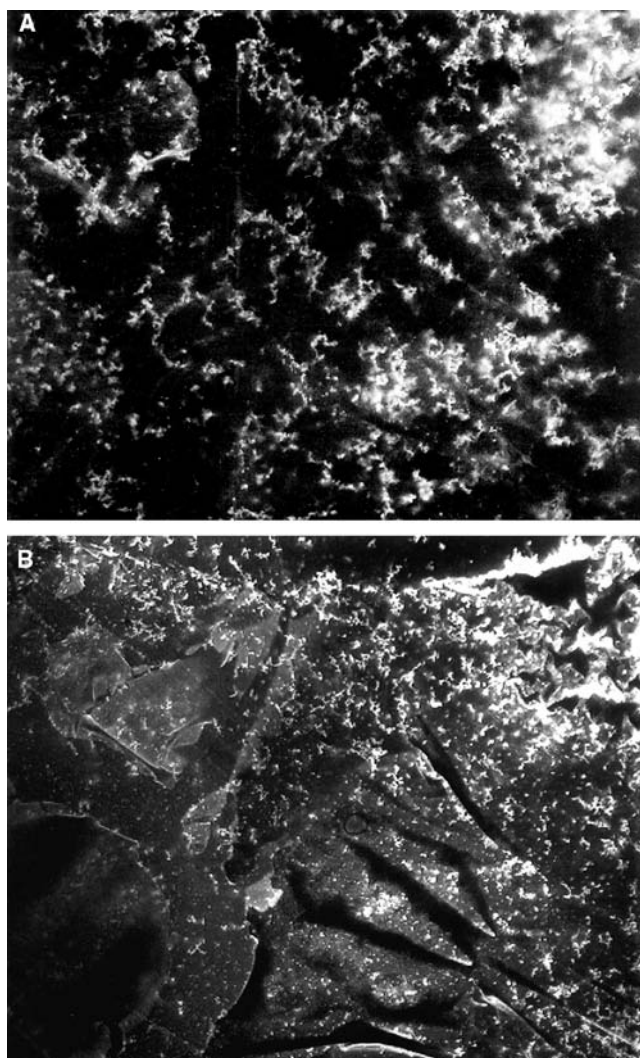


FIG 2. SCLM of fluorescing *E. faecalis* colonizing the surface of (A) a calcium hydroxide medicated root canal at 86 days and (B) calcium hydroxide itself at 86 days (magnification $\times 630$). The cells are stained with the BacLight viability stain containing SYTO9 and propidium iodide.

Group 1 (calcium hydroxide paste medication; Fig. 1B) showed total colonization of all samples after an average of 77 days of incubation. Bacteria were observed to form branching networks of filamentous material, which encompassed the bacteria in a dense heavy matrix. These palisade-like structures adhered to the root canal wall and protruded out in a mushroom-shaped colony (Fig. 1B). A notable difference in colonization patterns was observed between groups, such as group 1, that were inoculated over a long-term (Fig. 1B) and groups, such as groups 2 and 3, that were inoculated over a short-term (Fig. 1A). That is, a well-developed biofilm was observed in most specimens of group 1 (Fig. 1B).

SCLM Analysis

Two roots from group 1 were observed under SCLM. One root, inoculated for 86 days [Fig. 2 (A and B)], showed colonization of both the dentin surface (Fig. 2A) and the surface of calcium hydroxide (Fig. 2B). The other root, inoculated for 160 days (Fig. 3), showed colonies forming a mushroom-shape, a form that is

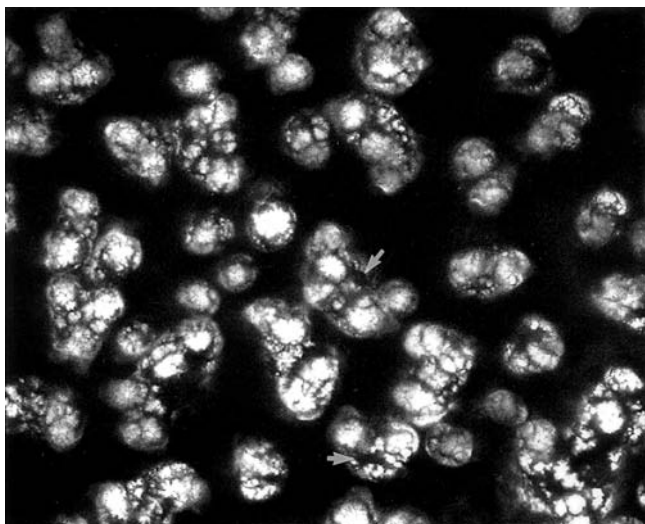


FIG 3. SCLM of fluorescing *E. faecalis* colonizing the surface of a calcium hydroxide medicated root canal at 160 days (magnification $\times 630$). The cells are stained as described in Fig. 2. Arrows indicate examples of possible water channels.

typical of a well-developed biofilm. In Fig. 3, vacant areas in individual colonies were observed that were possible water channels. The depth of the apparent biofilm was determined by SCLM scanning of 1- μm sections from top to bottom. The 160-day sample measured 28 to 30 μm , the 86-day sample measured 21 μm . The BacLight viability stain showed that approximately 90% of *E. faecalis* cells were viable at the time of observation. This was true even of cells growing directly on calcium hydroxide paste (Fig. 2B).

SDS-PAGE Gel Electrophoresis Analysis

SDS-PAGE gel electrophoresis compared *E. faecalis* planktonic cultures to stock cultures. No notable differences were observed between the protein profiles of these cultures (data not shown). This indicated that *E. faecalis* did not express different proteins in the presence of the medications, and it assured that the cultures were not contaminated during the experiment. We tried to compare the protein profiles of *E. faecalis* cells growing in planktonic cultures to the cells that were colonizing the root canal. However, few cells were recovered from the root canals and examination by phase microscopy indicated that these cells were embedded in a matrix. This matrix was only partially soluble with the TCA/SDS procedure. SDS-PAGE of this material from group 1 did not give well-separated protein bands; instead, a protein smear was observed with a single band at 32 kDa (data not shown). The observed protein smear suggests the presence of active proteases in the sample; however, more research is needed to ascertain the proteins in this putative biofilm.

DISCUSSION

This in vitro study focused on *E. faecalis* colonization of the medicated root canal. *E. faecalis*, a facultatively anaerobic group D streptococcus, is a saprophytic component of the enteric flora. *E. faecalis* is seldom found in primary endodontic infections; however, it is a species often isolated in retreatment cases of apical

periodontitis (1, 11). *E. faecalis* may be encountered either as a monoinfection or mixed with one or more species (1); therefore, it is one of the few bacteria to be isolated as a monoculture from the root canal. *E. faecalis* is not indigenous to the oral cavity, indicating that it is an exogenous infection that can enter the root canal, survive the intracanal medication treatment, and then persist after obturation (3). This study is in agreement with previous studies, which have shown that calcium hydroxide medication is not effective against *E. faecalis* infection of root canals (5, 6). This study also demonstrated that in both short-term and long-term incubation periods, *E. faecalis* colonized medicated root canals with possible biofilm formation in the long-term experiments.

Biofilms are defined as "polysaccharide matrix enclosed bacterial populations adherent to each other and/or to surfaces or interfaces" (8). Biofilms are highly organized structures consisting of mushroom-shaped clumps of bacteria bound together by a carbohydrate matrix and surrounded by water channels that deliver nutrients and remove wastes (12, 13). Bacteria sequestered in biofilms are shielded and are often much harder to kill than their free-floating or "planktonic" counterparts (12). Biofilms have been observed in a number of lesions of human bacterial diseases (8). Examples include infections of the oral soft tissues and teeth, as well as the middle ear, gastrointestinal, and urogenital tract. Biofilms have also been observed on invasive medical devices, such as indwelling catheters, cardiac implants, and tracheal and ventilator tubing (8).

In this study, *E. faecalis* was observed with SEM in root canals of all specimens inoculated with bacteria. Only 2 days were needed for *E. faecalis* to colonize all samples either medicated with calcium hydroxide points (group 2) or without medication (group 3). In these samples, which received short-term exposure to the bacteria, pairs or short chains of cells were seen adhering to the dentin surface.

In samples medicated with calcium hydroxide paste (group 1), SEM analysis revealed colonies of *E. faecalis*. These colonies were comprised of bacteria embedded in branching networks of filamentous material. This filamentous material, which likely represented an extracellular polysaccharide produced by the bacteria (12), made it difficult to distinguish individual cells. Thus, the SEM studies revealed a notable difference between *E. faecalis* colonization patterns when short-term (groups 2 and 3) was compared with long-term (group 1) bacterial exposure with the complex cellular organization characteristic of a well-developed biofilm (14) observed upon long-term exposure.

SCLM analysis of group 1 also showed a relationship between the length of bacterial exposure and the maturity of the biofilm formed. Root canal surfaces that were inoculated daily for 86 days with *E. faecalis* were colonized by what has been described by other workers as either microcolonies or developing biofilms (8), whereas root canals inoculated daily for 160 days displayed mushroom-shaped structures characteristic of well-developed biofilms (14). In the latter, vacant areas in individual colonies were observed that were possible water channels, which are believed to be a primitive circulatory system responsible for the delivery of nutrients to and removal of waste from biofilm bacteria (12, 13).

In our study, the depths of the biofilms measured 28 to 30 μm after 160 days of inoculation and 21 μm after 86 days. The similarity in depth of the 86-day and 160-day biofilms supports our assumption that the 86-day colonies were developing into biofilms. A previous study of tooth enamel showed biofilms 75 to 220 μm in depth forming in 4 days (14). The biofilms reported here were much shorter and required longer to develop. The low compliance

environment and the small space available in the root canal may have caused the shorter biofilms observed in this study. Because a critical cell density is required to initiate biofilm formation (15), nutrient limitation or the antimicrobial activity of calcium hydroxide might also have contributed to the shorter biofilms and the extended time periods required for their formation in the medicated root canals.

If *E. faecalis* can form biofilms in root canals, this might explain its ability to persist in that environment. Compared with planktonic cells, biofilm bacteria are up to 1000-fold more resistant to phagocytosis, antibodies, and many antibiotics (8, 12, 15). Factors contributing to resistance include the impenetrable polysaccharide coating on the biofilm bacteria and the ability of biofilm bacteria to survive without dividing. In addition, the physical conditions available to support bacterial growth, such as pH, ion concentration, nutrient availability, and oxygen supply (8, 15), vary throughout the biofilm. Most antimicrobics aren't active in a variety of physical environments, and many can only act on dividing cells.

The proximity of individual bacteria in biofilms also increases the opportunity for gene transfer (15), making it possible to convert a previously avirulent organism into a highly virulent pathogen or a bacterium that is susceptible to antimicrobics into a resistant one. This potential for gene transfer within biofilms is particularly significant in the case of *E. faecalis*, because a number of *E. faecalis* virulence factors are encoded on transmissible plasmids. These include collagenase (16), gelatinase, and adhesins (4), all with the potential to contribute to survival in and colonization of the root canal.

In previous SEM studies, bacteria have been observed colonizing the periapical tissues (17, 18) and root canal (9, 19). In some of these studies, structures typical of bacterial colonization have been observed, including filaments that seem to hold the bacteria to each other or to the dentin surface (18) and an amorphous material, presumably polysaccharide, covering the bacteria (17). These are now known to be characteristics of biofilms (15), but the SCLM data presented here is the first to demonstrate bacteria forming the classic bacterial biofilm architecture in the root canal.

In summary, this study has presented evidence of *E. faecalis* colonization and biofilm formation in root canals of human teeth. To develop new treatments to eradicate *E. faecalis* from persistent root canal infections, the mechanisms through which the bacterium maintains these infections must be understood. Further research on *E. faecalis* biofilms may contribute to this understanding.

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Dr. Distel was a resident in the graduate endodontics program at the Saint Louis University Health Sciences Center, St. Louis, Missouri 63103. He is presently in private practice limited to endodontics in Chicago, Illinois. Dr. Hatton is director of graduate endodontics, Center for Advanced Dental Education, Saint Louis University Health Sciences Center, St. Louis, Missouri 63103. Dr. Gillespie is head, Section of Microbiology, Department of Applied Dental Medicine, Southern Illinois University School of Dental Medicine, Alton, Illinois 62002. Address requests for reprints to Dr. John F. Hatton, Director of Graduate Endodontics, Southern Illinois University School of Dental Medicine, 2800 College Avenue, Alton, IL 62002.

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