Biofilm Formation of Oral and Endodontic *Enterococcus faecalis*

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Abstract

Biofilms are complex aggregations of microorganisms attached to a surface. The formation of biofilms might facilitate certain survival and virulence characteristics under some situations. This study tested the hypothesis that the ability of *Enterococcus faecalis* to form biofilms is related to the source of the strains. *E. faecalis* strains recovered from root canals ($n = 33$), the oral cavity ($n = 21$), and non-oral/non-endodontic sources ($n = 16$) were studied. Biofilms were grown in tryptic soy broth in 96-well plates for 24 hours at 37°C, fixed with Bouin's fixative, and stained with 1% crystal violet. Optical density at 570 nm (OD_{570}) was measured by using a microtiter plate reader. Experiments were performed in quadruplicate on three occasions and mean OD_{570} readings determined for each strain. There were no statistically significant differences between groups ($p = 0.066$, Kruskal-Wallis). Within the root canal and oral isolates there were no significant associations between biofilm formation and the presence of the virulence determinants *asa, cylA, esp*, and *gelE*. *(J Endod 2007;33:815–818)*

Key Words

Biofilm, endodontic, *Enterococcus faecalis*, in vitro, oral

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Biofilms are sessile microbial communities composed of cells irreversibly attached to a substratum and interface or to each other (1). Ultrastructurally biofilms form tower- or mushroom-shaped microcolonies with interspersed channels that are separate from the external environment and through which fluids move by convection (2). The cells within biofilms produce the matrix of extracellular polymeric substances (1). Cells located more deeply in the biofilm are exposed to environmental conditions that differ from those at the surface including decreased oxygen tension. This results in altered phenotypes in terms of growth rate and gene transcription that might facilitate certain survival and virulence characteristics (3). The slow metabolic rate of microorganisms in biofilms as well as the extracellular matrix of the biofilm can impede the effectiveness of many antimicrobials (1, 4). For example, the inhibition of *Enterococcus faecalis* biofilms require very high concentrations of antibiotics such as ampicillin, vancomycin, and linezolid (4).

E. faecalis is an opportunistic pathogen and one of the leading causes of nosocomial infections. *E. faecalis* is also frequently isolated from the failed root canals undergoing retreatment (5, 6), albeit in low numbers as a proportion of the overall bacterial load (6). The ability of *E. faecalis* to form biofilms may confer an ecological advantage in certain situations. For example, clinical strains of *E. faecalis* isolated from infective endocarditis patients were significantly associated with greater biofilm formation than nonendocarditis clinical isolates (7). This may be attributable in part to specific virulence traits such as gelatinase production and presence of the adherence determinant *esp*; this combination was shown to be associated with the formation of thicker biofilms (8). These virulence traits and others have also been identified in clinical isolates of *E. faecalis* from root canals and the oral cavity (9–11).

Conditions under which biofilms might occur in infected root canals in vivo are not well understood (12). Biofilms have been described as present in the undebrided parts of the root canal system of surgically resected root apices (13). In vitro studies have focused on the efficacy of selected irrigants and medicaments to remove biofilms grown in wells (14), on membrane filters (15), and on dentin samples (16–19) by using one or a few strains of selected species found in root canal infections including *E. faecalis* (14–19). However, apart from one study that included a root canal isolate (15), no information could be found on the biofilm-forming capabilities and characteristics of clinical isolates of *E. faecalis* recovered from root canals or the oral cavity nor on their relative capacity for biofilm formation compared with strains associated with other human infections.

The aim of this study was to evaluate quantitatively biofilm formation by *E. faecalis* isolates recovered from root canals ($n = 33$) and the oral cavity ($n = 21$); the phenotype and genotype of these strains have been previously reported (9–11). A group of nonoral, nonendodontic strains ($n = 16$) was included for comparison. The hypothesis tested was that *E faecalis* strains from different sources vary in their ability to form biofilms. Associations between biofilm formation and the presence of previously determined virulence traits (9–11) were also evaluated.

Materials and Methods

Microorganisms

All bacterial strains ($n = 70$) used in these investigations and their sources are listed in Table 1. Details on phenotypic and genotypic characteristics of the endodontic and oral strains are available elsewhere (9–11); details not already published are shown in Table 2 and were obtained by using methods described previously (11).

TABLE 1. *E. faecalis* Strains Studied and Their Source

Bacteria source	Strains	Reference
Oral (n = 21)		
Tongue swab-endodontic patient	AA-T4, AA-T26	(9)
	GS-34	(26)
Oral rinse-dental student	C1	(10)
Oral rinse-endodontic patient	E1, E2, E3, E4, E5, E6, E7, E8, E10, E11	(10)
	OS16,0S25	(27)
	AA-OR3,AA-OR4, AA-OR26, AA-OR34	(9)
Saliva	OG1	(28)
Endodontic (n = 33)		
Primary treatment	GS3, GS6, GS7, GS8, GS13, GS18, GS19, GS22, GS24, GS27, GS28, GS31, GS32	(11)
Orthograde retreatment	GS1, GS2, GS12, GS16, GS25, GS33	
	ER3/2s, ER5/1	(26)
"Endodontic treatment"	GS4, GS5, GS9, GS10, GS14, GS15, GS17, GS21, GS23, GS26, GS29, GS30	(11)
Other (non-oral, non-endodontic) (n = 16)		
Human nosocomial	368	(29)
	DS16	(30)
Human bacteremia	MMH594	(31)
	V583	(32)
Laboratory strains	JH2-2, FA2-2, OG1X/pAD1, OG1X/pAM373, DS16C1, DS16C2, DS16C3, OG1X, OG1RF, TX5128, FA2-2/pAT28, FA2-2/pESPF	*

*References available upon request.

Briefly, previous analyses included (1) phenotypic tests for antibiotic resistance, clumping response to pheromone, and production of gelatinase, hemolysin, and bacteriocin; (2) genotype analysis based on polymerase chain reaction amplification of virulence determinants encoding gelatinase *gelE*, cytolysin activator *cylA*, endocarditis antigen *efaA*, aggregation substances *asa* and *asa373*, and adherence factors *esp* and *ace*; and (3) physical DNA characterization using pulsed-field gel electrophoresis of genomic DNA and plasmid analysis (9–11).

E. faecalis strains were taken from −80°C stocks and plated onto Todd Hewitt Broth (THB; Becton, Dickinson and Co, Sparks, MD) supplemented with 1.5% agar and incubated aerobically at 37°C for 24 hours. For each strain expected colony and cell morphology and gram stain reaction were verified.

Biofilm Assays

Biofilm assays were conducted based on a previously described method (7). Briefly, for each strain, one colony was transferred to tryptic soy broth (TSB, Bacto Tryptic Soy Broth medium, contains 0.25% glucose, Becton, Dickinson and Co) and incubated overnight under stationary aerobic conditions at 37°C. The cultures were diluted 1:100 in medium and 200 μL of this cell suspension was dispensed into sterile flat-bottomed 96-well polystyrene microtiter plates (Falcon; Becton, Dickinson and Co, Franklin Lakes, NJ). Four wells per strain were inoculated. For negative controls, TSB alone was dispensed into eight wells per tray. After stationary aerobic incubation at 37°C for 24 hours, broth was carefully drawn off by using a multichannel pipettor. Wells were washed three times with 200 μL phosphate-buffered saline. Biofilms were fixed with 200 μL of Bouin's Fixative (Ricca Chemical Company, Arlington, TX) for 30 minutes, and wells were washed with distilled water. Biofilms were stained with 200 μL of 1% crystal violet solution in water for 30 minutes, and wells were washed with distilled water. Microtiter plates were inverted on a paper towel and air dried. To quantify biofilm production, 200 μL of ethanol-acetone (80:20, vol/vol) was added to each well to destain the biofilms (Fig. 1). Thereafter, the optical density of the resolubilized crystal violet was measured at 570 nm (OD_{570}) by using a microtiter plate reader (Bio-Tek ELx800, Winooski, VT). Each assay was performed in quadruplicate on three occasions for a total of 12 readings for each strain. Wells containing uninoculated medium served as negative controls and to determine background optical density. After subtraction of the mean background OD_{570} readings, the 12 optical density readings per strain were averaged to obtain the mean OD_{570} reading for each strain as previously described (7).

TABLE 2. Characteristics of *E. faecalis* Strains*

Strain	Virulence genes†						
	gelE	*esp*	*asa*	*asa373*	*ace*	*cylA*	*efaA*
E1	+	−	+	−	+	+	+
E2	−	+	+	−	+	−	+
E3	+	−	+	−	+	−	+
E4	+	−	+	−	+	−	+
E5	+	+	−	−	+	−	+
E6	+	−	−	−	+	−	+
E7	−	−	+	−	+	−	+
E8	+	−	−	−	+	−	+
E10	+	−	+	−	+	−	+
E11	−	+	+	−	+	−	+
C1	+	−	−	−	+	−	+
OS16	+	−	+	−	+	−	+
OS25	+	−	+	−	+	−	+
OG1	+	−	−	−	+	−	+
ER3/2s	−	+	−	−	+	−	+
ER5/1	+	−	−	−	+	−	+

*See references 9–11 for methods and results for other endodontic and oral strains listed in Table 1.
†*gelE,* gelatinase; *esp* and *ace,* surface adherence factors; *asa* and *asa373,* aggregation substance; *cylA,* cytolysin activator; *efaA,* endocarditis antigen.

Statistical Analysis

Because the data were not normally distributed, nonparametric tests were used. Mann-Whitney tests and Kruskall-Wallis tests were used to compare median OD_{570} readings according to source (root canal, oral, non-root canal/non-oral) and the presence of enterococcal virulence genes for aggregation substance (*asa*), surface adhesin (*esp*), cytolysin activator (*cylA*), and gelatinase (*gelE*) previously identified (9–11) (Table 2). GraphPad Prism for Macintosh (Version 4.0c; GraphPad Software, Inc, San Diego, CA) was used for all calculations. Significance was set at $p < 0.05$.