

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, OS25	(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*, cytolytic 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 (OD₅₇₀) 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 OD₅₇₀ readings, the 12 optical density readings per strain were averaged to obtain the mean OD₅₇₀ reading for each strain as previously described (7).

Statistical Analysis

Because the data were not normally distributed, nonparametric tests were used. Mann-Whitney tests and Kruskal-Wallis tests were used to compare median OD₅₇₀ 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*), cytolytic 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.

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

Strain	Virulence genes†						
	<i>gelE</i>	<i>esp</i>	<i>asa</i>	<i>asa373</i>	<i>ace</i>	<i>cylA</i>	<i>efaA</i>
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*, cytolytic activator; *efaA*, endocarditis antigen.

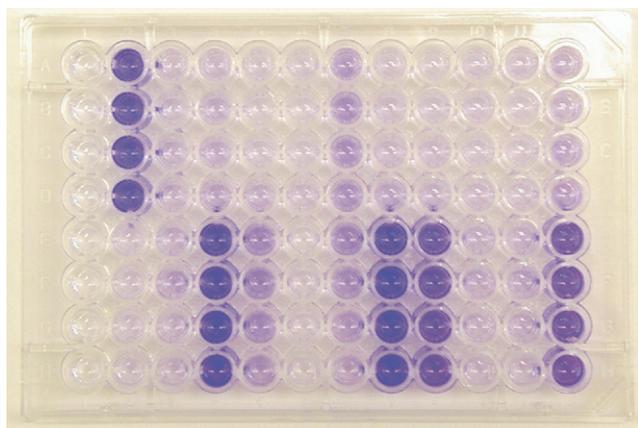


Figure 1. Representative microtiter plate with 24-hour *E. faecalis* biofilms after resolubilization of crystal violet used to stain-fixed biofilms. More intense coloration represents thicker biofilm formation. The five sets of more intensely stained quadruplicate samples are classified as “moderate” biofilm formers based on OD₅₇₀ readings (7). The left column of eight wells are negative controls where no biofilms were detected.

Results

Mean, median and range of OD₅₇₀ values and results of statistical tests are presented in Table 3 and Figure 2. Overall, mean OD₅₇₀ readings ranged from 0.012 to 1.801 (mean 0.201, median 0.084). Within specific groups, mean OD₅₇₀ readings ranged from 0.012 to 1.801 (mean 0.311, median 0.078) for endodontic strains, from 0.045 to 0.313 (mean 0.127, median 0.108) for oral strains, and from 0.030 to 0.157 (mean 0.073, median 0.067) for non-oral, non-endodontic strains. No biofilms were detected in negative control wells.

There was no significant relationship between the ability of *E. faecalis* to form biofilms and the source of isolates ($p = 0.066$). Within the root canal and oral isolates, there were no significant associations between biofilm OD₅₇₀ readings and the presence of the virulence determinants *asa*, *cylA*, *esp*, and *gelE* (all $p > 0.05$) (Table 3).

Discussion

Currently available information on the relationship between endodontics and microbial biofilms consists chiefly of observations of bacterial condensations in the root canal system (13) and the efficacy of

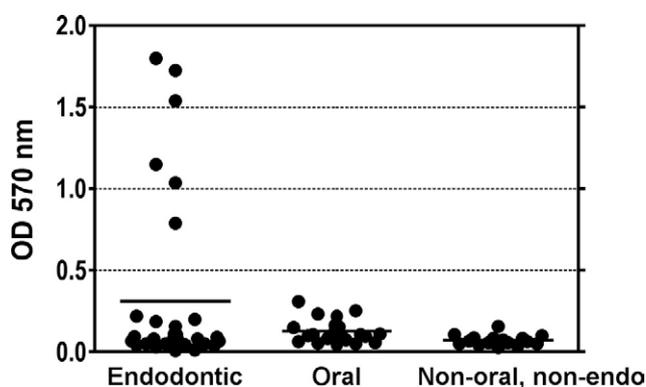


Figure 2. Optical density readings at 570 nm for each isolate. Each point represents the average of 12 readings for each strain.

selected irrigants and medicaments to disrupt them (14, 16, 20). The ability of *E. faecalis* to form biofilms on the walls of root canals has been shown in vitro (16–18). Under nutrient-rich conditions, *E. faecalis* ATCC 29212 biofilm penetration into dentinal tubules in vitro increases (17). Kishen et al. (18) suggested that the persistence of *E. faecalis* after root canal treatment may be associated with its capacity to induce apatite reprecipitation in mature biofilms. However, there is little information on the ability of clinical isolates to form biofilms. Therefore, the aim of this in vitro study was to investigate the biofilm-forming characteristics of *E. faecalis* isolates from root canals and the oral cavity. There was no statistically significant difference in biofilm formation between groups of endodontic, oral strains, and a “control” group of non-endodontic/non-oral *E. faecalis* strains.

In the present study, the range of OD₅₇₀ readings was from 0.012 to 1.801. The method was selected to allow general comparisons of biofilm formation by *E. faecalis* strains recovered from human infections at other sites (7, 21, 22). The present OD₅₇₀ readings for the endodontic and oral strains were somewhat lower than those previously reported for strains associated with endocarditis (7), slightly lower than urinary tract infection strains (22) and similar to those associated with intravascular catheter-related bloodstream infections (21). The criteria used to categorize biofilm-formation capability in polystyrene wells have varied. For example, Sandoe et al. (21) defined any OD₅₉₀ readings greater than zero to indicate an ability to form biofilms. They reported that all *E. faecalis* strains recovered from intravascular catheter-related bloodstream infections formed biofilms when grown in Brain Heart Infusion (OD₅₉₀ range, 0–0.180; median, 0.084). By using these parameters, 100% of *E. faecalis* strains evaluated in the present study qualified as “biofilm formers.” However, based on the more stringent criteria adopted by Mohamed et al. (7) used to study endocarditis strains, only strains with OD₅₇₀ readings of >0.5 were classified as biofilm formers and then further classified into the categories “weak” (<1), “medium” (<2), or “strong” (>3). By using these parameters, only 6 of the 54 endodontic and oral strains (11%) could be considered “biofilm formers,” one endodontic strain qualifying as a “weak” biofilm former and the other five endodontic strains as “moderate” biofilm formers. These results for root canals and oral isolates contrast with those of Mohamed et al. (7) who reported that 100% of their 79 endocarditis strains were biofilm formers (9% weak, 52% moderate, and 39% strong) compared with 20% of 22 urine strains, 12% of 15 hospital fecal specimens, and 15% of 16 community fecal specimens.

Several investigations thus far on biofilms and root canals have focused on the species *E. faecalis* (14, 16–18). A recent study addressing *E. faecalis* biofilms grew their specimens under a flow system inducing a stress on the bacteria, which was considered to induce more resilient biofilms (14).

TABLE 3. OD₅₇₀ Readings of *E. faecalis* Strains

	n	Median	Min–max range	p
Overall (n = 70)	70	0.084	0.012–1.801	
Endodontic	33	0.078	0.012–1.801	0.066
Oral	21	0.108	0.045–0.313	
Non-endodontic, non-oral	16	0.067	0.030–0.157	
Clinical endodontic and oral isolates	54	0.092	0.012–1.801	
Country of origin				
Sweden	32	0.071	0.012–1.801	0.102
United States	22	0.108	0.052–0.313	
Virulence genes				
<i>asa</i> -positive	44	0.087	0.012–1.801	0.947
<i>asa</i> -negative	10	0.105	0.052–0.235	
<i>cyl</i> -positive	10	0.073	0.042–0.791	0.367
<i>cyl</i> -negative	44	0.097	0.012–1.801	
<i>esp</i> -positive	25	0.109	0.016–1.801	0.252
<i>esp</i> -negative	29	0.083	0.012–1.541	
<i>gelE</i> -positive	45	0.083	0.012–1.801	0.160
<i>gelE</i> -negative	9	0.152	0.054–0.313	

Sandoe et al. (21) reported that *E. faecalis* formed thicker biofilms when grown under low nutrient (saline) versus nutrient-rich (BHI) conditions (21). In the present study, polystyrene microtiter plates and nutrient-rich media were used, and biofilms were grown under stationary conditions because it was thought that the root canal space would likely have minimal, if any, fluid flow. However, without further studies, it is not reasonable to extrapolate these data to mean that a higher biofilm score correlates with enhanced survival in the root canal system. Bacteria colonizing dentin inhabit an extremely complex surface topography with rough surface irregularities and dentinal tubules awash with necrotic tissue, quite unlike the smooth, sterile conditions found in the polystyrene plates used in this study. In addition, endodontic infections are typically polymicrobial (5). Others have concluded that heterogenous colonies possess a distinct advantage over homogenous ones in production of surface biofilms (23).

Of interest was whether greater biofilm production capacity was associated with virulence traits identified in the same strains (10, 11). Overall, no relationship was found between the ability of *E. faecalis* isolates to form biofilms and possession of the virulence determinants *asa*, *cylA*, *esp*, and *gelE*. In contrast, a positive correlation between biofilm production and *gelE* in *E. faecalis* strains was initially reported for endocarditis strains (7) but later qualified to apply to the combination of *gelE* and *esp* (8). The six isolates showing a greater capability to form biofilms than other strains were all root canal isolates originally recovered from both primary and retreatment cases in Sweden (11). The five “medium” biofilm producers had phenotypic and genotypic similarities in terms of the presence of a cryptic 5.1-kb plasmid and a clumping response in the presence of pheromone as well as virulence genes for gelatinase (*gelE*), aggregation substance (*asa*), endocarditis antigen (*efaA*), and collagen-binding antigen (*ace*) (11). Recently, Kowalski et al. (19) observed that the expression of the collagen-binding protein, Ace, was associated with stronger adhesion to dentin when grown at 46°C. Because the virulence determinants *ace* and *efaA* were detected in almost all root canal and oral isolates (9–11) (Table 2), these factors could not be investigated here.

To our knowledge, this is the first report on biofilm formation by clinical endodontic and oral *E. faecalis* strains. The results indicate that, in contrast to strains from other clinical sources, in particular endocarditis strains (7, 21), *E. faecalis* strains from oral and endodontic sources have a lower inherent capacity to form biofilms. This is of interest because prophylactic antibiotic coverage is provided for patients at risk although its value and efficacy is difficult to confirm (24). Although more data are obviously required on the biofilm-forming capabilities when grown on biological surfaces including dentin and heart valves, the present data suggest that the biofilm-forming capacity of root canal and oral strains may not be an important contributory factor for their presence in these environments. The absence of this putative “virulence” characteristic further questions the significance of *E. faecalis* in endodontic infections (25). However, the variations observed in these clinical isolates suggest that biofilm formation might be a factor when considering the virulence phenotype of endodontic strains in general.

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