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Dentinal tubule invasion and adherence by *Enterococcus faecalis*

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Abstract

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Aim To investigate dentinal tubule invasion and the predilection of *Enterococcus faecalis* for dentinal tubule walls.

Methodology The invasion of dentinal tubules in extracted human teeth by *E. faecalis* was measured *ex vivo* after 8 weeks of incubation. The canal walls of 16 root sections were either intact or instrumented with or without smear layer present. Extent and maximum depth of tubule invasion were assessed histologically and compared between groups. In the adherence study, 44 vertically split root samples were prepared to expose longitudinally aligned dentinal tubules and fractured orthodentine (OD). Surfaces were exposed to *E. faecalis* (erythromycin resistant strain, JH2-2 carrying plasmid pGh9:IS*S1*) and incubated aerobically for 2 h. Samples were processed for analysis using scanning electron microscopy. Bacterial adhesion to tubule walls versus fractured OD was calculated as number of cells per 100 μm^2.

Results The strain of *E. faecalis* used in this study showed moderate to heavy tubule invasion after 8 weeks. In the adhesion studies, significantly more bacteria adhered to fractured OD than to dentinal tubule walls (ANOVA, $P < 0.001$). With respect to the tubule wall, adherence was greater in inner versus outer dentine ($P = 0.02$) and greater when bacterial adhesion was tested in chemically defined medium than in phosphate-buffered saline (ANOVA, $P < 0.001$).

Conclusions Although *E. faecalis* readily invaded tubules, it did not adhere preferentially to tubule walls. Initial colonization of dentinal tubules by *E. faecalis* may depend primarily on other factors.

Keywords: adherence, dentinal tubules, *Enterococcus faecalis*, invasion.

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Introduction

Because apical periodontitis is usually caused by bacteria, a major objective of root canal treatment is to eliminate bacteria from infected root canals. Although bacterial infection can be substantially reduced by standard intracanal procedures (Byström & Sundqvist 1981, Byström & Sundqvist 1983, Byström *et al.* 1985, Dalton *et al.* 1998), it is difficult to render the root canal free from bacteria. Bacteria are located in inaccessible areas such as complicated root canal anatomy and dentinal tubules, and it is difficult to deliver antibacterial agents to these locations (Haapasalo & Ørstavik 1987, Berutti *et al.* 1997, Wu *et al.* 2006). Bacteria may survive and recolonize the root canal space whenever there is opportunity, and this may become a focal source for persistent infection.

Bacteria are commonly found within dentinal tubules of clinically infected canals (Ando & Hoshino 1990, Love & Jenkinson 2002, Siqueira *et al.* 2002). Amongst these bacteria, *Enterococcus faecalis* is of interest because it is the most frequently detected species in root filled teeth with persistent lesions (Molander *et al.* 1998, Sundqvist *et al.* 1998, Peciuliene *et al.* 2000, Peciuliene *et al.* 2001, Rocas *et al.* 2004). Some possible factors facilitating its long-term survival

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in the root canal system are its ability to invade dentinal tubules (Ørstavik & Haapasalo 1990, Love 2001), where it can survive for a prolonged period under adverse conditions such as starvation (Hartke et al. 1998, Figdor et al. 2003, Sedgley et al. 2005) and the high pH of calcium hydroxide medication (Evans *et al.* 2002, Gomes *et al.* 2002).

Although the mechanism of bacterial invasion is not completely understood, bacterial adhesion to dentinal tubule walls (TWs) is a logical early step in the process. Collagen is widely considered to be the primary substrate for specific binding of *E. faecalis* to dentine, and the collagen binding protein of *E. faecalis* (Ace) and a serine protease (Spr) have been proposed to play significant roles in binding to the root canal wall (Hubble *et al.* 2003). Ace also promotes *E. faecalis* binding to collagen type I (Nallapareddy *et al.* 2000a,b) and *in vitro ace* gene expression at 37 °C was enhanced in the presence of collagen (Nallapareddy & Murray 2006). However, the interaction of *E. faecalis* specifically with dentinal tubules has never been investigated.

Understanding the pattern and mechanisms of initial colonization of dentinal tubules by *E. faecalis* may assist the development of more effective clinical procedures for bacterial management and elimination. The aim of this study was to investigate *ex vivo* tubule invasion and adhesion to TWs by *E. faecalis.* Dentinal tubules within intact dentine are normally inaccessible for systematic investigation. Thus, for the adherence study, human tooth roots were split longitudinally to expose tubule surfaces. It was hypothesized that the dentinal TW may be a selective site for bacterial adherence and that alteration of dentine components may influence initial colonization.

Materials and methods

Teeth

Root samples were prepared from extracted, intact human teeth from young subjects, with complete root formation, which had been stored in 1% (w/v) chloramine T. Ethical approval was obtained from the Health Sciences Human Ethics Subcommittee of the University of Melbourne (HERC Project No. 050100).

Bacterial strain and growth conditions

Enterococcus faecalis (erythromycin resistant strain, JH2-2 carrying plasmid pGh9:IS*S1*, derived from the parental strain JH2) (Jacob & Hobbs 1974) was grown overnight from glycerol stock cultures. Brain heart infusion broth (Oxoid Ltd, Hampshire, UK) diluted in deionized water (1 : 5) was used as growth medium throughout the invasion study whilst chemically defined medium (CDM) adapted from Pichereau *et al.* (1999) was used in the binding study. CDM lacks any added proteins or peptides, and consists of amino acids, vitamins and minerals plus glucose. Erythromycin (6.5 μg mL^{-1}, Sigma, St Louis, MO, USA) was added to media immediately before use. An overnight broth culture was diluted 1 : 10 into 200 mL of fresh medium and incubated at 37 °C.

In all experiments, the purity of all bacterial cultures was determined by both Gram staining and by culture analysis using Brain Heart Infusion (BHI) agar (Oxide Ltd., Hampshire, UK) under both aerobic and anaerobic conditions.

Tubule invasion study

Sample preparation

Sixteen premolar teeth with oval-shaped canals were decoronated at the cemento-enamel junction (CEJ), and 2 mm of the apical root was removed with a diamond saw. One side of the canal wall was prepared with Gates–Glidden drills up to two sizes larger than the original canal size, whilst the other side of the canal was left intact (Fig. 1), providing both natural canal wall and prepared canal wall in each root segment.

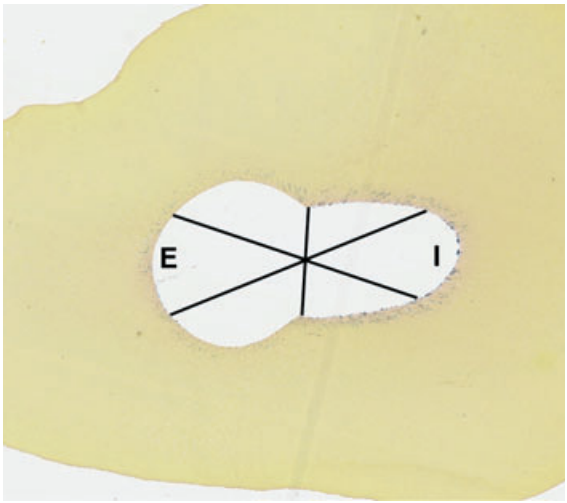

Figure 1 Histological sample prepared for *Enterococcus faecalis* invasion study. One side of the root canal wall was enlarged using Gates–Glidden drills (E), whilst the other side remained intact (I). Prepared and intact root canal walls were divided into three segments each for scoring purposes.

During canal preparation, root samples were divided into two groups. In one group (n = 8), root canals were irrigated with normal saline solution throughout the preparation, leaving the smear layer intact. Root canals in the other group were irrigated with 1% NaOCl and 17% ethylenediaminetetraacetic acid (EDTA) was left in the canal for 3 min before final rinsing with 3 mL of 1% NaOCl solution. Thus, prepared root segments had smear layer present or absent from the prepared canal wall. Then, two dentine blocks per root (3 and 5 mm in height) were obtained by transverse sectioning at levels of 3 and 8 mm from the CEJ, using a low speed diamond saw under copious coolant (Fig. 2). A bucco-lingual groove 1.5 mm in depth was prepared at the apical end of the 5 mm block to facilitate vertical splitting after incubation. Prepared samples from each tooth were individually stored in moist conditions and sterilized by gamma irradiation (25 kGy).

Dentinal tubule invasion by Enterococcus faecalis

Sterilized root canal specimens were inoculated with 5 mL *E. faecalis* culture grown in diluted BHI broth with added erythromycin (6.5 μg mL^{-1}) and incubated (37 °C) for 8 weeks. During the incubation period, 4.5 mL of the bacterial suspension was replaced with fresh medium twice a week. At the end of the incubation period, the 3-mm dentine blocks were removed from the bacterial suspension, washed with phosphate-buffered saline (PBS) and processed for histological examination using light microscopy. The 5-mm dentine blocks were initially fixed with 2.5% glutaraldehyde in PBS and longitudinally split with a sharp blade and prepared for scanning electron microscopy (SEM).

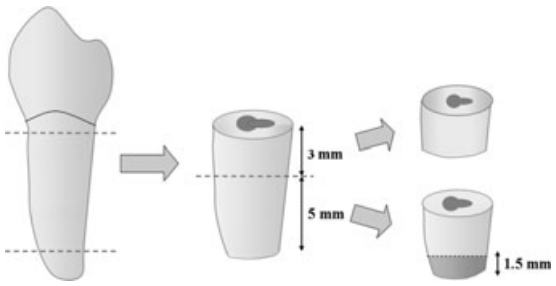

Figure 2 Diagram of sample preparation for invasion study. Teeth were decoronated and 2 mm of the apical root was removed. One side of the root canal wall was left intact, whilst the other side was enlarged using Gates–Glidden drills. After sectioning, 3 and 5-mm dentine blocks were obtained. A 1.5-mm bucco-lingual groove was made as the guide for root splitting.

Sample processing for light and scanning electron microscopy

The samples for light microscopy were processed according to standard histological procedures. Triplicate transverse 3-μm decalcified sections from the coronal third were obtained at the level of 2–3 mm from the CEJ and stained with a modified Brown and Brenn staining technique for observing any bacteria located in dentinal tubules (Love 2001). Slides were scanned using an automated scanner (ScanScope®, Aperio Technologies Inc., Vista, CA, USA), which provided a digital image for analysis using ImageScope™ software (Aperio Technologies Inc.).

Samples for SEM were fixed in 2.5% glutaraldehyde in PBS, immersed in serial dilutions of acetone, critical point dried and gold-sputter-coated for examination under field emission SEM (FESEM, model XL30 FEG, Philips, Eindhoven, the Netherlands).

Scoring of dentinal tubule invasion

The scoring system for tubule invasion was modified from the tubule invasion index (TI) (Love 2001). An imaginary line from the junction of the prepared and natural root canal wall was drawn from one side of the root canal wall to the opposite side (Fig. 1). The centre of this line was identified and from this position, the canal wall was divided into six segments including three equal lengths of prepared canal sites and three equal lengths of natural, unprepared canal wall. The extent of bacterial invasion within each of these lengths was determined by the number of tubules containing bacteria (400× magnification). The extent of tubule invasion by *E. faecalis* was scored 0, 1, 2 or 3 when 0, 1–20, 20–50 or >50 tubules were infected in the segment, respectively. The score 0 was interpreted as nil, 1 as mild, 2 as moderate and 3 as heavy invasion (Love 2001). The categorical data were then statistically analysed to compare the effect of smear layer on the extent of tubule invasion using Pearson's chi-squared test, adjusted for clustering within tooth samples, at 95% significance level.

The maximum depth of tubule invasion in μm was also measured in each of the six compartments, as mentioned previously, using the imaging processing software. The maximum penetration depth of prepared and natural canal sites from all sections were added and averaged for the maximum penetration depth of the group. The difference in maximum penetration

depth amongst groups was statistically analysed using a generalized linear model approach at 95% significance level.

Adhesion to split dentine surface

Tooth preparation

Intact, noncarious premolar teeth were decoronated and split vertically into two halves to expose longitudinally aligned dentinal tubules and fractured OD (Fig. 3). Although fractured OD is not normally accessible to bacteria, the fractured surface of OD was used as a control for adhesion relative to the TW. The fractured surfaces were likely to have abundant exposed mineralized collagen. Paired samples were utilized to compare a range of treatment effects on bacterial adhesion, including sterilization methods (autoclaving and gamma irradiation vs. untreated freshly split surfaces) and nutrient availability. Eight split samples were assigned to each experimental group. Split samples were sterilized by either autoclaving (121 °C, 20 min) or gamma irradiation (25 kGy), whilst nonsterilized fresh samples were copiously washed with sterile de-ionized water.

Adherence conditions

Split dentine samples from each tooth were individually immersed in 5 mL *E. faecalis* culture grown in CDM to log phase ($0.5–0.6 \times 10^8$cells per mL), except where indicated, and incubated aerobically (37 °C, 2 h) without shaking. CDM was used throughout this part of the study to avoid any potential interference of exogenous proteins with binding. After incubation, samples were removed, washed three times (shaking at 120 rpm in PBS, 5 min) to remove nonadherent bacteria and processed for SEM.

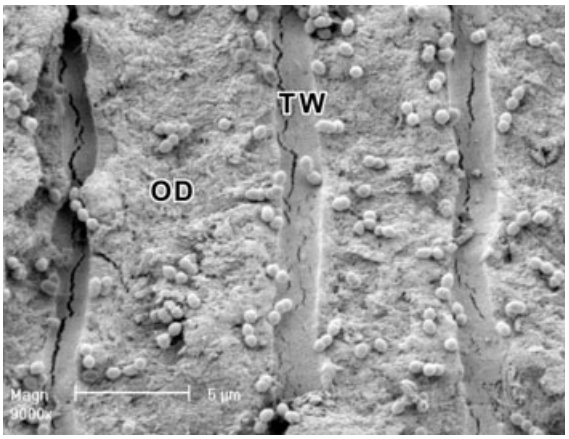

Figure 3 Scanning electron micrograph showing *Enterococcus faecalis* adherence to two exposed structures, orthodentine (OD) and tubule walls (TW) of split dentine surface (9000×).

To investigate the effect of culture medium on adhesion, paired dentine samples were exposed to bacterial suspensions in either CDM or PBS. Bacteria resuspended in PBS were grown in CDM to mid-exponential phase (optical density = 0.6, 570 nm) centrifuged (8000 *g*, 20 min, 4 °C), and resuspended in PBS to the same cell density as the bacteria in CDM. Cell density of bacteria in both groups was determined by optical density measurement at a wavelength of 570 nm before exposure to tooth samples.

Evaluation of adherence to dentinal tubules

At each location (e.g. coronal inner dentine), 10 randomly selected nonoverlapping fields within the overall area were imaged, beginning at a specific point (e.g. 100 μm from the pulpal surface). Scanning electron micrographs of inner and outer root dentine were captured at 100–300 μm from root canal walls and within 1 mm of the external root surface, respectively.

Bacterial adhesion was calculated as the average number of bacterial cells per unit area of dentine (100 μm^2), based on 10 separate fields. Bacteria attached to different dentine surfaces (TW vs. fractured OD) were counted, and the surface area of the two structures (TW and OD) in each section was measured, using a specific biological imaging program (Olysia® Imaging Software, Olympus Corporation, Tokyo, Japan) (Fig. 3).

The number of bacteria per 100 μm^2 and the ratio of TW/OD adherence at each location were calculated and average values from each sample were compared within and amongst groups with different variables. Finally, adhesion to different dentine structures, locations and depths and the effect of sterilization methods and growth activity on adhesion were statistically analysed using ANOVA (General Linear Model) at 95% significance level.

Results

Bacterial invasion of tubules

The strain of *E. faecalis* used was able to invade dentinal tubules to a heavy extent. Overall, both the extent of tubule invasion and the maximum tubule penetration into the natural root canal wall were significantly greater than into the prepared root canal surface ($P < 0.001$). There was a low extent of tubule invasion

Table 1 Extent of tubule invasion (median value) and average of the maximum penetration depths (μm) at different areas of the canal surface irrigated with either saline (smear layer present) or 1% NaOCl and 15% EDTA (smear layer removed) after 8 weeks incubation at 37 °C

	Extent of tubule invasion[a]		Maximum penetration depth (mean[b] ± SE[c]) (μm)	
	Unprepared canal	Prepared canal	Unprepared canal	Prepared canal
Saline	3	0*	202.4 ± 16.2	31.3 ± 12.3**
NaOCl and EDTA	3	3	193.9 ± 15.3	156.2 ± 25.3

[a]Modified from tubule invasion index (TI) of Love (2001), where 0 = no invasion, 3 = heavy invasion.
[b]Average of 81 values from eight roots irrigated with saline or 93 values from eight roots replicates irrigated with NaOCl and EDTA.
[c]Robust standard errors which adjust estimates for clustering of values from tooth samples.
*Significantly different from all other groups ($P < 0.001$).
**Significantly less than the average maximum penetration depth values of other groups ($P < 0.001$).

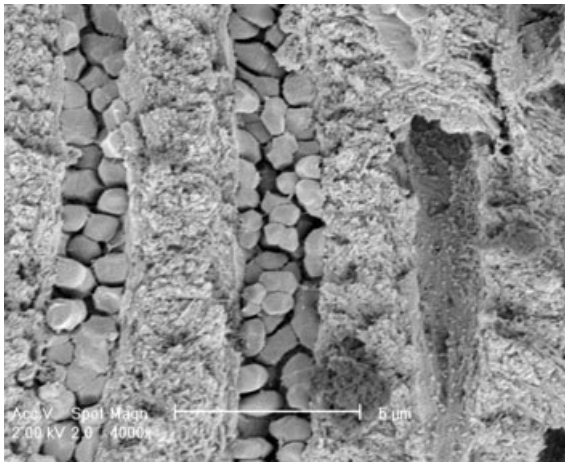

Figure 4 Scanning electron micrograph showing random tubule invasion by *Enterococcus faecalis*. Whilst some tubules were densely packed, the adjacent tubule was empty. Pleomorphic change was observed for bacteria densely packed in dentinal tubules.

(median value: 0) with shallow bacterial penetration in prepared canals irrigated with normal saline (i.e. with smear layer present) (Table 1). Depth of tubule penetration by *E. faecalis* in root canals with smear layer removed was not significantly different in both prepared (156.2 ± 25.3 μm) and unprepared areas of the canal (193.9 ± 15.3 μm) (ANOVA, $P = 0.07$).

Using SEM, bacterial invasion of tubules was irregular, with some tubules containing densely packed cells whilst adjacent tubules were empty (Figs 4 and 5). Morphological change was observed in densely packed bacteria that appeared to be pleomorphic (Fig. 4), whereas less densely packed bacteria (Fig. 5) retained a typical round or oval shape. The surface texture of tubules varied considerably with some tubules having numerous exposed collagen fibres, whilst adjacent tubules had a smooth surface (Fig. 5). Presence or absence of intratubular bacteria was not dependent on the tubule surface. When the smear layer was present, large numbers of bacteria attached to the smear layer with very few bacterial cells present in underlying tubules (Fig. 6, Table 1).

Bacterial adherence

Dentine surfaces

For freshly split or gamma-irradiated dentine exposed to bacterial cultures in CDM, mean values of bacterial cells adhering to TW and OD at the coronal inner location ranged from 7.4 to 8.9 and 29.9 to 30.5 cells per 100 μm^2, respectively (Table 2). Bacterial adhesion to TWs was significantly and consistently less than to fractured OD ($P < 0.001$) in all experimental conditions applied (Table 2). Even when collagen fibres lining the inner TW were clearly visible, little adherence occurred (Fig. 7). The average ratio of adherent cells to TW versus OD at coronal inner dentine from all experiments ranged from 0.3 to 0.5. The ratios were not affected by most test conditions applied, including sterilization method, location and media.

Sterilization method

Whilst the extent of *E. faecalis* adhesion was similar in freshly split and gamma-irradiated samples (ANOVA, $P = 0.6$), significantly less adhesion (approximately one-third) was found with autoclaved samples ($P < 0.001$) (Table 2).

Location

Significantly greater adherence of *E. faecalis* to dentinal TWs occurred at inner versus outer dentine ($P = 0.02$) (Table 2). Adherence to TWs of coronal dentine was not significantly greater than in apical dentine ($P = 0.4$) (Table 2).

Medium

Dentine specimens exposed to bacterial cultures grown in CDM had approximately double the bacterial adherence

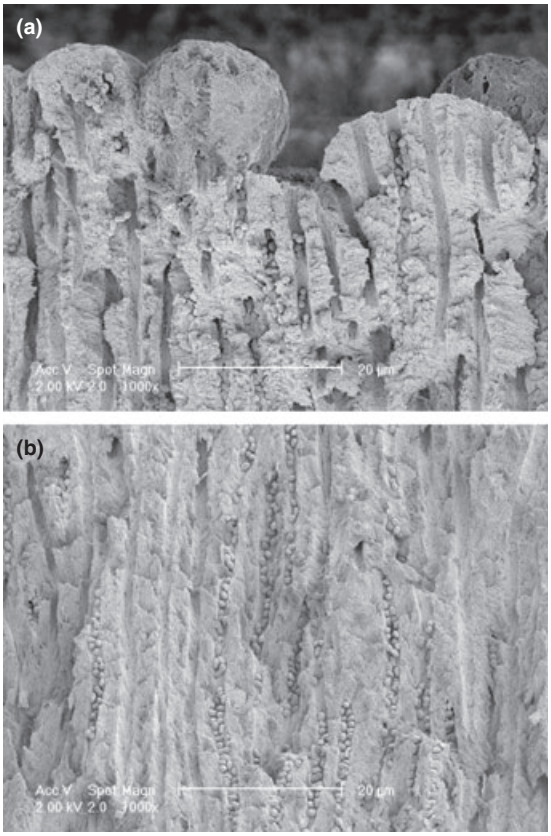

Figure 5 Scanning electron micrograph showing the pattern of tubule invasion. (a) Dentinal tubules at the intact pulpal surface were randomly invaded by *Enterococcus faecalis* cells. (b) Colonization of *E. faecalis* in dentinal tubules approximately 100 μm from the pulpal surface. Some tubules were occupied by bacteria, whilst adjacent tubules were empty.

compared to specimens exposed to bacteria resuspended in PBS (ANOVA, $P < 0.001$) (Table 2).

Discussion

In this study, *E. faecalis* was chosen as the test organism because it is known to invade dentinal tubules *in vitro* (Haapasalo & Ørstavik 1987, Love 2002, Weiger *et al.* 2002) and appears to be associated with persistent periapical pathosis (Molander *et al.* 1998, Siqueira & Rocas 2004, Stuart *et al.* 2006). Examples of factors facilitating its presence in filled root canals with persistent lesions include the ability to invade dentinal tubules (Haapasalo & Ørstavik 1987, Love 2002, Weiger *et al.* 2002), ability to tolerate the high pH of calcium hydroxide (Evans *et al.* 2002) and ability to withstand long periods of starvation with subsequent

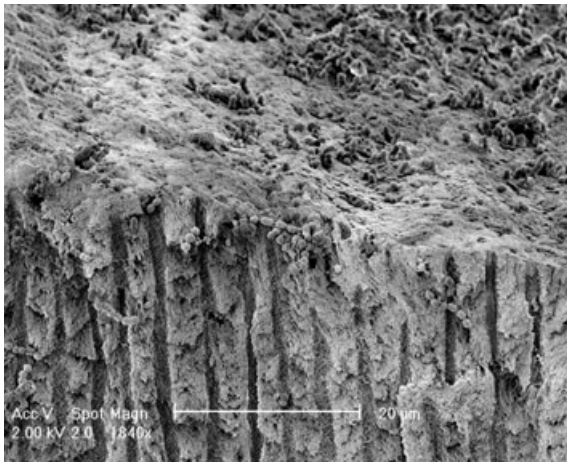

Figure 6 Scanning electron micrograph showing root canal wall covered with smear layer. Bacteria can be seen associated with the smeared surface and no tubule invasion was present.

recovery in the presence of serum (Figdor *et al.* 2003). It was also shown to survive long term in the root canal even without additional nutrients (Sedgley *et al.* 2005).

The *E. faecalis* strain used here has been widely used in other endodontic studies that investigated the effects of antimicrobial agents (Turner *et al* 2004, Rossi-Fedele & Roberts 2007), starvation (Appelbe & Sedgley 2007), resistance to high pH (Evans *et al* 2002) and most importantly invasion of dentinal tubules (Love 2001). This study has confirmed the invasion of dentinal tubules by this bacterium. The depth of tubule invasion in the present study is slightly higher than the reported depth of bacterial penetration in clinical samples with periapical lesions, which ranged between 10 and 150 μm (Sen *et al.* 1995). The heavy extent of tubule invasion is comparable to results from a previous experimental study of *E. faecalis* in which the TI was used as a scoring system (Love 2001). These results demonstrate that the strain is suitable as a representative of bacteria with the ability to invade dentinal tubules.

Significantly less tubule invasion was found in prepared canals irrigated with normal saline solution, compared with other groups. This was caused by the presence of smear layer and smear plugs that impede tubule invasion (Love 2002). Greater invasion was observed in prepared canals when the smear layer was removed by irrigation with NaOCl and EDTA. The similar extent of tubule invasion in natural canal wall and prepared canal with smear layer removed showed that *E. faecalis* was able to invade tubules in conditions of both primary infection and persistent infection. This

Table 2 Mean and standard deviation of bacterial adherence and tubule wall (TW)/orthodentine (OD) ratio values of samples in groups with different sterilization methods, locations or media

Factors	Subgroup	Bacterial adherence[a] (cells per 100 μm^2)		TW/OD ratio
		Tubule wall (mean ± SD)	Orthodentine (mean ± SD)	
Sterilization method[a]	Gamma irradiated (n = 16)	8.9 ± 5.7	29.9 ± 12.0	0.3 ± 0.1
	Autoclaved (n = 16)	3.2 ± 2.0	9.2 ± 6.4	0.4 ± 0.1
	Freshly split (n = 16)	7.4 ± 4.2	30.5 ± 27.2	0.3 ± 0.2
Location (n = 12)	Coronal inner	9.1 ± 3.6	28.7 ± 5.6	0.3 ± 0.1
	Coronal outer	5.0 ± 2.3	23.1 ± 3.4	0.2 ± 0.1
	Apical inner	6.1 ± 4.0	23.4 ± 11.8	0.3 ± 0.1
	Apical outer	2.9 ± 1.4	19.8 ± 2.7	0.1 ± 0.1
Media (n = 8)	CDM	8.7 ± 4.4	24.8 ± 8.9	0.36 ± 0.1
	PBS	3.6 ± 1.9	9.5 ± 5.1	0.5 ± 0.4

CDM, chemically defined medium; PBS, phosphate-buffered saline.
[a]Paired-split surface samples from the eight roots were used to compare two treatment methods (gamma irradiation vs. autoclaving, gamma irradiation vs. freshly split and autoclaving vs. freshly split group). Therefore 48 split root samples from 24 roots were included in this experiment.

might support the recent finding that equal amounts of *E. faecalis* were detected in both situations using PCR techniques (Gomes *et al.* 2006).

The geometry of dentinal tubules with a narrow lumen (1–2 μm) and considerable length (2–3 mm) complicates experimental studies of dentine binding. Therefore, vertically split root samples were utilized for the adherence study. TWs throughout their length were equally exposed to bacteria, making systematic investigation possible. Using split root samples also provided access to fractured OD, which is not normally exposed. Bacterial adherence to fractured OD could be used as a reference for the less mineralized OD surface compared with more highly mineralized peritubular dentine of the TW.

Although moderate to heavy tubule invasion was found after 8 weeks of incubation, *E. faecalis* had only limited tendency to adhere to the surface of dentinal tubules. These observations suggest that *E. faecalis* has only a low predilection for tubules and that colonization may be influenced by other factors such as adhesin-related gene expression, nutrient conditions, amount of peritubular dentine deposition and ability of bacteria to proliferate as chains (Love *et al.* 1997). In this study, adherence was evaluated using bacteria grown in CDM rather than in BHI (as was used for tubule invasion). For adherence, it is essential to avoid the presence of any exogenous proteins or peptides that may interfere with binding. It is possible that the expression of cell surface receptors is affected by the growth medium used; however, cells grown in both BHI and CDM were found to bind readily to collagen-coated wells in a separate study (data not shown). Whilst the extent of bacterial adhesion to the dentine surface may be influenced by nutrient conditions, it cannot account for the low adherence of bacteria to TW relative to OD.

Adherence is considered to be the first step for bacterial colonization of host tissue, including tubule invasion, and is mediated by bacterial specific cell-surface components (adhesins) (Patti *et al.* 1994). It has been shown that Ace (collagen-binding protein of enterococci) promoted *E. faecalis* adhesion to some extracellular matrix proteins including collagen type I (Nallapareddy *et al.* 2000a). As collagen type I is the main organic component of dentine, it is widely considered to be a major substrate for *E. faecalis* binding to dentine. A study of mutant strains of *E. faecalis* also showed that Ace and serine protease (Spr) played significant roles in *E. faecalis* binding to root canal walls (Hubble *et al.* 2003). In that study, however, the canal walls had been subjected to instrumentation with endodontic files, and the resultant smear layer (Drake *et al.* 1994, Hülsmann *et al.* 2003, Teixeira *et al.* 2005) was not removed prior to the measurement of binding. Hence, their results may reflect bacterial adherence to smeared dentine rather than binding to natural root canal wall.

Most studies of *E. faecalis* Ace function were performed *in vitro* at 46 °C because of the low level of *ace* gene expression at 37 °C (Nallapareddy *et al.* 2000a, Nallapareddy & Murray 2006). However, it has been shown in both *in vivo* and in laboratory studies that

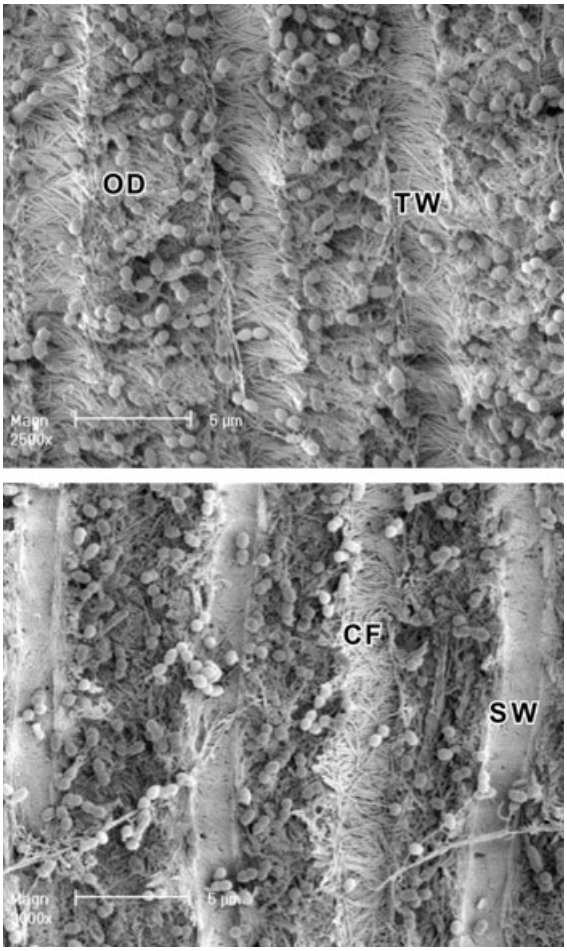

Figure 7 Two scanning electron micrographs showing more adherence of *Enterococcus faecalis* to fractured orthodentine (OD) compared to tubule wall surface (TW) (9000×). This characteristic was observed whether collagen fibres were clearly visible on the tubule wall (CF) or whether the tubule had a smooth wall (SW).

bacterial colonization of dentinal tubules does occur at 37 °C, including this study. Evidence of anti-Ace antibodies detected in human sera collected from patients with *E. faecalis* endocarditis has also been reported (Nallapareddy *et al.* 2000b). Moreover, *in vitro* expression of Ace was shown to be induced during some specific conditions such as early growth phase (Hall *et al.* 2007) and the presence of collagen type I or serum (Nallapareddy & Murray 2006) or elevated temperature (Xiao *et al.* 1998, Hubble *et al.* 2003). Thus, the regulation of *ace* gene expression *in vivo* may be enhanced by specific conditions or the presence of substrate and it may play a role in *E. faecalis* binding *in vivo*. Considerable tubule invasion may also be influenced by stress such as low nutrient conditions during the incubation period or the presence of unmineralized collagen. However, other receptors, substrates, not yet identified binding sites, bacterial hydrophobicity and electrostatic charges that may play roles in initial and nonspecific dentine binding must still be considered.

When dentine specimens were exposed to bacteria suspended in PBS (i.e. non-nutrient conditions), there was less bacterial adhesion to the dentine surface compared with bacterial cultures in CDM. One possible explanation for this difference is that metabolic activity may be required for the expression of specific proteins (receptors) on the cell surface that is expressed mainly in the presence of an inducer (i.e. collagen type I). Bacterial cells suspended in PBS would be unable to synthesize new protein because of lack of an energy source. However, other explanations cannot be excluded such as differences in other constituents of the two media that might influence adherence. In this regard, CDM rather than a protein containing medium (such as BHI) was used so that no exogenous proteins were present during dentine adhesion.

In the present study, less bacterial adherence occurred to autoclaved dentine, compared with gamma-irradiated and freshly split dentine. As collagen is generally considered to be the substrate for binding to dentine (Love and Jenkinson 2002, Hubble *et al.* 2003), less *E. faecalis* adherence to autoclaved teeth may be caused by alteration of dentine components such as collagen by autoclaving (White *et al.* 1994).

The different levels of bacterial adherence at different locations may result from varying extents of peritubular dentine deposition, with its higher mineral and lower collagen content in outer root dentine compared with inner dentine. Love (1996) also reported low bacterial adherence (*Streptococcus gordonii*) to outer sclerotic dentine. This difference seems to support the significance of organic matrix components in bacterial binding. The deposition of more mineralized dentine with increasing age may explain the variations in adherence amongst tooth samples, which is in agreement with an earlier study (Siqueira *et al.* 2002).

The ability of *E. faecalis* to grow as chains has been suggested as another explanation for the moderate to high extent of tubule invasion (Love & Jenkinson 2002), in spite of its low affinity for dentinal tubules. It is possible that only adherence to the tubule orifice is required for invasion. After initial attachment to the poorly/nonmineralized pre-dentine at the tubule orifices, deeper penetration may not require specific binding as invasion may result from intratubular cell

growth (Love & Jenkinson 2002). This somewhat speculative suggestion has not been investigated systematically but deserves further study.

In this study, scanning electron micrographs showed densely packed bacteria within tubules, with irregular shape (Fig. 4), whilst less densely packed cells retained normal morphology (Fig. 5). It is possible that bacteria themselves may occupy dentinal tubules and impede access of medium to bacteria, leading to nutrient deprived conditions of bacteria in the deeper part of the tubule. SEM in a previous study showed that alteration in shape of some *E. faecalis* JH2-2 cells was observed within 3 to 7 weeks after incubation in oligotrophic conditions (Hartke *et al.* 1998). This extreme starved condition may be applicable to explain the pleomorphic shape of packed cells in dentinal tubules with possibly low nutrient condition in this study.

Conclusion

Under the experimental conditions, a strain of *E. faecalis* readily invaded dentinal tubules even though the organism seemed to have low affinity for TWs. Dentine components may include selective factors for bacterial adherence and invasion as implied by the effect of autoclave sterilization. Also, adherence was higher in the less mineralized inner dentine than outer dentine. However, low adherence was observed regardless of the appearance of the TW surface. Therefore, the initial colonization of dentinal tubules by *E. faecalis* may be primarily dependent on other factors such as the environmental conditions inducing surface receptor related gene expression. The further investigation of such factors that may play roles in tubule colonization and possible competitive agents is still necessary. Understanding these mechanisms may lead to development of novel clinical strategies to inhibit bacterial colonization and to manage these infections in the future.

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References

Ando N, Hoshino E (1990) Predominant obligate anaerobes invading the deep layers of root canal dentin. *International Endodontic Journal* **23**, 20–7.

Appelbe OK, Sedgley CM (2007) Effects of prolonged exposure to alkaline pH on *Enterococcus faecalis* survival and specific gene transcripts. *Oral Microbiology and Immunology* **22**, 169–74.

Berutti E, Marini R, Angeretti A (1997) Penetration ability of different irrigants into dentinal tubules. *Journal of Endodontics* **23**, 725–7.

Byström A, Sundqvist G (1981) Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. *Scandinavian Journal of Dental Research* **89**, 321–8.

Byström A, Sundqvist G (1983) Bacteriologic evaluation of the effect of 0.5 percent sodium hypochlorite in endodontic therapy. *Oral Surgery, Oral Medicine, Oral Pathology* **55**, 307–12.

Byström A, Claesson R, Sundqvist G (1985) The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. *Endodontics and Dental Traumatology* **1**, 170–5.

Dalton BC, Ørstavik D, Phillips C, Pettiette M, Trope M (1998) Bacterial reduction with nickel-titanium rotary instrumentation. *Journal of Endodontics* **24**, 763–7.

Drake DR, Wiemann AH, Rivera EM, Walton RE (1994) Bacterial retention in canal walls *in vitro*: effect of smear layer. *Journal of Endodontics* **20**, 78–82.

Evans M, Davies JK, Sundqvist G, Figdor D (2002) Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *International Endodontic Journal* **35**, 221–8.

Figdor D, Davies JK, Sundqvist G (2003) Starvation survival, growth and recovery of *Enterococcus faecalis* in human serum. *Oral Microbiology and Immunology* **18**, 234–9.

Gomes BP, Ferraz CC, Garrido FD *et al.* (2002) Microbial susceptibility to calcium hydroxide pastes and their vehicles. *Journal of Endodontics* **28**, 758–61.

Gomes BP, Pinheiro ET, Sousa EL *et al.* (2006) *Enterococcus faecalis* in dental root canals detected by culture and by polymerase chain reaction analysis. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **102**, 247–53.

Haapasalo M, Ørstavik D (1987) *In vitro* infection and disinfection of dentinal tubules. *Journal of Dental Research* **66**, 1375–9.

Hall AE, Gorovits EL, Syribeys PJ *et al.* (2007) Monoclonal antibodies recognizing the *Enterococcus faecalis* collagen-binding MSCRAMM Ace: conditional expression and binding analysis. *Microbial Pathogenesis* **43**, 55–66.

Hartke A, Giard JC, Laplace JM, Auffray Y (1998) Survival of *Enterococcus faecalis* in an oligotrophic microcosm: changes in morphology, development of general stress resistance, and analysis of protein synthesis. *Applied and Environmental Microbiology* **64**, 4238–45.

Hubble TS, Hatton JF, Nallapareddy SR, Murray BE, Gillespie MJ (2003) Influence of *Enterococcus faecalis* proteases and the collagen-binding protein, Ace, on adhesion to dentin. *Oral Microbiology and Immunology* **18**, 121–6.

Hülsmann M, Heckendorff M, Lennon A (2003) Chelating agents in root canal treatment: mode of action and indications for their use. *International Endodontic Journal* **36**, 810–30.

Jacob AE, Hobbs SJ (1974) Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. zymogenes. *Journal of Bacteriology* **117**, 360–72.

Love RM (1996) Adherence of *Streptococcus gordonii* to smeared and nonsmeared dentine. *International Endodontic Journal* **29**, 108–12.

Love RM (2001) *Enterococcus faecalis* – a mechanism for its role in endodontic failure. *International Endodontic Journal* **34**, 399–405.

Love RM (2002) The effect of tissue molecules on bacterial invasion of dentine. *Oral Microbiology and Immunology* **17**, 32–7.

Love RM, Jenkinson HF (2002) Invasion of dentinal tubules by oral bacteria. *Critical Reviews in Oral Biology & Medicine* **13**, 171–83.

Love RM, McMillan MD, Jenkinson HF (1997) Invasion of dentinal tubules by oral streptococci is associated with collagen recognition mediated by the antigen I/II family of polypeptides. *Infection and Immunity* **65**, 5157–64.

Molander A, Reit C, Dahlen G, Kvist T (1998) Microbiological status of root-filled teeth with apical periodontitis. *International Endodontic Journal* **31**, 1–7.

Nallapareddy SR, Qin X, Weinstock GM, Hook M, Murray BE (2000a) *Enterococcus faecalis* adhesin, Ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infection and Immunity* **68**, 5218–24.

Nallapareddy SR, Singh KV, Duh RW, Weinstock GM, Murray BE (2000b) Diversity of *ace*, a gene encoding a microbial surface component recognizing adhesive matrix molecules, from different strains of *Enterococcus faecalis* and evidence for production of Ace during human infections. *Infection and Immunity* **68**, 5210–7.

Nallapareddy SR, Murray BE (2006) Ligand-signaled upregulation of *Enterococcus faecalis ace* transcription, a mechanism for modulating host-E. faecalis interaction. *Infection and Immunity* **74**, 4982–9.

Ørstavik D, Haapasalo M (1990) Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Endodontics and Dental Traumatology* **6**, 142–9.

Patti JM, Allen BL, McGavin MJ, Hook M (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. *Annual Review of Microbiology* **48**, 585–617.

Peciuliene V, Balciuniene I, Eriksen HM, Haapasalo M (2000) Isolation of *Enterococcus faecalis* in previously root-filled canals in a Lithuanian population. *Journal of Endodontics* **26**, 593–5.

Peciuliene V, Reynaud AH, Balciuniene I, Haapasalo M (2001) Isolation of yeasts and enteric bacteria in root-filled teeth with chronic apical periodontitis. *International Endodontic Journal* **34**, 429–34.

Pichereau V, Bourot S, Flahaut S, Blanco C, Auffray Y, Bernard T (1999) The osmoprotectant glycine betaine inhibits salt-induced cross-tolerance towards lethal treatment in *Enterococcus faecalis*. *Microbiology* **145**, 427–35.

Rocas IN, Siqueira JF Jr, Santos KR (2004) Association of *Enterococcus faecalis* with different forms of periradicular diseases. *Journal of Endodontics* **30**, 315–20.

Rossi-Fedele G, Roberts AP (2007) A preliminary study investigating the survival of tetracycline resistant Enterococcus faecalis after root canal irrigation with high concentrations of tetracycline. *International Endodontic Journal* **40**, 772–7.

Sedgley CM, Lennan SL, Appelbe OK (2005) Survival of *Enterococcus faecalis* in root canals ex vivo. *International Endodontic Journal* **38**, 735–42.

Sen BH, Piskin B, Demirci T (1995) Observation of bacteria and fungi in infected root canals and dentinal tubules by SEM. *Endodontics and Dental Traumatology* **11**, 6–9.

Siqueira JF Jr, Rocas IN, Lopes HP (2002) Patterns of microbial colonization in primary root canal infections. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **93**, 174–8.

Siqueira JF Jr, Rocas IN (2004) Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **97**, 85–94.

Stuart CH, Schwartz SA, Beeson TJ, Owatz CB (2006) *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment. *Journal of Endodontics* **32**, 93–8.

Sundqvist G, Figdor D, Persson S, Sjogren U (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **85**, 86–93.

Teixeira CS, Felippe MC, Felippe WT (2005) The effect of application time of EDTA and NaOCl on intracanal smear layer removal: a SEM analysis. *International Endodontic Journal* **38**, 285–90.

Turner SR, Love RM, Lyons KM (2004) An in-vitro investigation of the antibacterial effect of nisin in root canals and canal wall radicular dentine. *International Endodontic Journal* **37**, 664–71.

Weiger R, de Lucena J, Decker HE, Lost C (2002) Vitality status of microorganisms in infected human root dentine. *International Endodontic Journal* **35**, 166–71.

White JM, Goodis HE, Marshall SJ, Marshall GW (1994) Sterilization of teeth by gamma radiation. *Journal of Dental Research* **73**, 1560–7.

Wu MK, Dummer PM, Wesselink PR (2006) Consequences of and strategies to deal with residual post-treatment root canal infection. *International Endodontic Journal* **39**, 343–56.

Xiao J, Hook M, Weinstock GM, Murray BE (1998) Conditional adherence of *Enterococcus faecalis* to extracellular matrix proteins. *FEMS Immunology and Medical Microbiology* **21**, 287–95.