Oral Microbiology Immunology 2007: 22: 169–174
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Effects of prolonged exposure to alkaline pH on *Enterococcus faecalis* survival and specific gene transcripts

O. K. Appelbe, C. M. Sedgley
Department of Cariology, Restorative Sciences and Endodontics, The University of Michigan, School of Dentistry, MI, USA

Appelbe OK, Sedgley CM. Effects of prolonged exposure to alkaline pH on Enterococcus faecalis *survival and specific gene transcripts.*
Oral Microbiol Immunol 2007: 22: 169–174. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard.

Introduction: The persistence of *Enterococcus faecalis* in treated root canals has been attributed to its resistance to the high pH of antimicrobial agents used during treatment, but the specific mechanisms are not clear. We investigated the survival and gene expression of *E. faecalis* maintained in alkaline media.
Methods: *E. faecalis* JH2-2 was maintained in media at pH 7, 10, 11 and 12 at either 25°C or 37°C for 1 week (168 h). At 24, 48, 72, 120 and 168 h, cell viability was determined in parallel with real-time quantitative polymerase chain reaction analyses of stress response genes (*dnaK*, *fba*, *ftsZ*, *GroEL*, *napA*, *pbp5*, *tsf* and *tuf*).
Results: After 1 week the *E. faecalis* showed survival levels of 100% in pH 7, 1% in pH 10, 0.001% in pH 11 and 0.00001% in pH 12 media. At 37°C increased levels of gene transcripts occurred between 72 and 120 h in pH 7 media for *ftsZ* and *dnaK*, and in pH 10 media for *ftsZ*, *pbp5*, *dnaK*, *napA*, *tsf*, *fba* and *GroEL*. No increase in transcripts was observed at 37°C in media at pH 11 or pH 12, nor at 25°C in any media.
Conclusion: Transcripts of *ftsZ*, a gene involved in cell division, increased by 37-fold after 120 h at pH 10 at 37°C. Overall, the greatest increase in levels of gene transcripts occurred in cultures maintained in pH 10 media at 37°C. These data may assist in understanding the survival strategies of *E. faecalis* following prolonged exposure to alkaline pH levels.

Key words: alkaline pH; *Enterococcus faecalis*; gene transcripts; prolonged exposure; survival

Christine Sedgley, Department of Cariology, Restorative Sciences and Endodontics, The University of Michigan, School of Dentistry, 1011 North University Avenue, Ann Arbor, MI 48109-1078, USA
Tel.: +1 734 615 3210;
fax: +1 734 936 1597;
e-mail: csedgley@umich.edu
Accepted for publication August 23, 2006

Enterococcus faecalis is the species most frequently cultured from the root canals of teeth with failed root canal treatment (11, 33). Complete debridement of infected root canals may be unachievable because of the inaccessibility of sites of infection in the root, for example accessory canals that instruments and irrigants cannot reach (22). *E. faecalis* cells can reside within dentinal tubules (24, 29), and remain viable in root-filled teeth for at least 12 months (29). Various 'virulence' factors have been identified in *E. faecalis* strains from root canals (31).

The persistence of *E. faecalis* in treated root canals has been attributed to its ability to resist the high pH of the antimicrobial agents commonly used during treatment (26, 35), especially calcium hydroxide paste [$Ca(OH)_2$; pH > 11.5], for which placement in the root canal for 1 week has been recommended (32). The resistance of *E. faecalis* in root canals to intracanal $Ca(OH)_2$ treatment may be influenced by the buffering effects of dentine (9, 36) so that the high pH may not be achieved within the dentinal tubules.

The antimicrobial activity of $Ca(OH)_2$ relates to its release of hydroxide ions (8). An abundance of hydroxide ions can cause many problems in the aqueous environment of the cell. The destruction of phospholipids, structural components of the cellular membrane, can be caused by the induction of lipid peroxidation by hydroxide ions (10). Genes are destroyed when hydroxide ions react with bacterial DNA and cause the denaturation of the strands (13). When *E. faecalis* cells enter a 'starvation phase' they possess greater resistance to the antibacterial effect of

$Ca(OH)_2$ (27), the mechanism for which has not been elucidated. The identification of genes expressed by *E. faecalis* cells in a prolonged stationary phase in alkaline pH might allow the eventual development of treatment protocols that can eradicate *E. faecalis* from infected root canals. In this study we examined the survival and targeted gene expression of *E. faecalis* maintained in media at pH 7, 10, 11 and 12 for 1 week.

Materials and methods

Culture conditions and pH exposure

E. faecalis JH2-2 (14) was used in all experiments and grown aerobically at 37°C unless specified otherwise. JH2-2 was taken from storage at −80°C and grown on brain–heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD) supplemented with 1.5% agar incubated for 24–48 h. A single colony was transferred to BHI broth and the culture was grown overnight. A 1 : 10 dilution in fresh BHI broth was grown to an optical density at 560 nm of 1.0, corresponding to mid/late log phase of growth. Then, 35-ml aliquots (triplicates for each pH) were pelleted, resuspended in 35 ml buffered BHI broth at pH 7, 10, 11 or 12 (23), and incubated at either 25°C or 37°C. [Buffered BHI broth was prepared using buffer solutions made by mixing 0.2 M KH_2PO_4 and 0.2 M KOH for pH 7, 0.2 M $NaHCO_3$ and 0.2 M K_2CO_3 for pH 10.0, 0.2 M Na_2HPO_4 and 0.2 M KOH for pH 11.0 and pH 12.0, respectively (23).] Absorbance and pH readings were taken and serial dilutions were plated in triplicate onto BHI agar plates at 0 (baseline), 24, 48, 72, 120 (for 37°C only) and 168 h and incubated at 37°C for 48 h. Colony-forming units per ml were enumerated after 48 h.

RNA extraction and preparation

Extraction of RNA was performed for the pH 7, 10 and 11 cultures at each time interval; there was insufficient RNA available from the pH 12 samples. Total RNA was isolated as follows. Five milliliters of *E. faecalis* cells in suspension were placed in a 15-ml test tube and centrifuged (2500 ***g*** for 5 min at 4°C). The supernatant was carefully aspirated and the bacterial pellet was resuspended in 1.0 ml RNA *later* (Ambion, Austin, TX) and transferred to a 1.5-ml nuclease-free microfuge tube for overnight incubation at 4°C. RNA extraction was performed using the RiboPure-Bacteria Kit (Ambion) with the minor modifications that follow. The cells were collected by centrifugation (2500 ***g***, 10 min, 4°C). The supernatant was removed and cells were resuspended in 500 µl RNAwiz (Ambion). Cells were lysed by homogenization in a Mini Beadbeater-8 (Biospec Products, Bartlesville, OK) for 5 min followed by 5 min on ice. The RiboPure-Bacteria Kit protocol was followed thereafter, including the optional DNase I treatment, according to the manufacturer's protocol. A 1 : 50 dilution of RNA in 10 mM Tris–HCl (pH 8.0) was quantified using a Beckman Coulter DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). Substocks of 6 ng/µl were prepared and used for subsequent reverse transcription-polymerase chain reaction (RT-PCR) procedures.

Primers

Genes and primers are listed in Table 1; genes generally believed to be involved in the stress response were selected for evaluation (6, 12, 19, 20, 37). The nucleic acid sequences for the genes were obtained by searching the NCBI gene database (http://www.ncbi.nlm.nih.gov/). Unless otherwise reported, primers for this study were created using the PrimerQuest program on the Integrated DNA Technologies website (http://scitools.idtdna.com/Primerquest/). Each primer set returned by the program was tested for homology to other genes using the BLAST nucleotide algorithm (1).

Reverse transcription-polymerase chain reactions

RT-PCR was performed using an Eppendorf Mastercycler (Brinkmann Instruments, Inc., Westbury, NY). Approximately 30 ng RNA was placed in a 0.2-ml PCR tube and the volume was brought to 5 µl with diethylpyrocarbonate-treated water. The tubes were incubated at 70°C for 5 min to eliminate the secondary structure from the RNA. After incubation, 5 pmol/primer of gene-specific primers, 1X RT buffer (Invitrogen, Carlsbad, CA), and diethylpyrocarbonate-treated water were added for a volume of 17 µl. Ramp-down cycles were run for 1 min each to cover the annealing temperatures of all the gene-specific primers. Ten millimoles dithiothreitol (Invitrogen), 200 µM deoxynucleotide triphosphates (dNTPs; Invitrogen) and 100 U Superscript III reverse transcriptase (Invitrogen) were added to the tubes for a final

Table 1. Genes and primers

Gene	Gene function	Primer	Primer sequence	Product size (bp)	Reference
E. faecalis 16S	Identification (ribosomal RNA)	Ef16SF	5′-ccgagtgcttgcactcaattgg-3′	138	(30)
		Ef16SR	5′-ctcttatgccatgcggcataaac-3′		
dnaK	Protein refolding and degradation	dnaKF	5′-ggaacaacaaactcagc-3′	1022	(19)
		dnaKR	5′-ccttggattgcagcaccc-3′		
fba	Glycolysis, gluconeogenesis	fbaF 588	5′-aaacatcaatgggcgctg-3′	150	This study*
		fbaR 738	5′-agaaccgacagcttcagc-3′		
ftsZ	Cell division	ftsZF203	5′-tggtttaggtgccggttcac-3′	788	This study*
		ftsZR 991	5′-tgtcttgtttgacggtgcgg-3′		
GroEL	Protein refolding and degradation	GroELF 288	5′-gccattgttcgtgaaggc-3′	158	(19)
		GroELR 446	5′-cggccttcagcttctagcgc-3′		
napA	Na^+/H^+ antiporter	napAF 492	5′-tgtcggcatgattggtgc-3′	347	This study*
		napAR 839	5′-agtctggcaaagaagacg-3′		
pbp5	Peptidoglycan synthesis	pbp5F	5′-catgcgcaattaatcgg-3′	444	(20)
		pbp5R	5′-catagcctgtcgcaaaac-3′		
tsf	Protein synthesis	tsfF 446	5′-aagatgttgctatgcacatcgcgg-3′	114	This study*
		tsfR 560	5′-cgaatgatttcacttcgccgcc-3′		
tuf	Protein synthesis	tufF 698	5′-aacgtggtgaagttcgcg-3′	379	This study*
		tufR 1077	5′-agcaacgttatcaccagg-3′		

Primer sequences were obtained from the TIGR V583 genome sequence (http://www.ncbi.nlm.nih.gov/).
*Primers created for this study used the PrimerQuest program on the Integrated DNA Technologies website (http://scitools.idtdna.com/Primerquest/).

reaction volume of 20 μl. For negative RT controls, Superscript III reverse transcriptase was replaced with diethylpyrocarbonate-treated water. Reactions were incubated at 42°C for 75 min followed by 65°C for 15 min.

For second-strand synthesis to check for DNA contamination, 4 μl RT product was placed in a 0.2-ml PCR tube; then 1X PCR buffer (Qiagen, Valencia, CA), 100 μM dNTPs (Invitrogen), 1.5 U *Taq* polymerase (Qiagen), 6 pmol of each primer and nuclease-free water were added to a volume of 30 μl per reaction. The reactions were incubated at 95°C for 5 min, followed by 35 cycles of 94°C for 20 s, a primer-dependent annealing temperature (range 51–66°C) for 45 s, and 72°C for 10 s to 1 min (duration dependent on length of PCR product, 30 s/500 base pairs, Table 1). Twenty microliters of PCR product was mixed with 2 μl loading buffer (1 g sodium dodecyl sulfate, 100 mg bromophenol blue, 40 ml 0.25 M disodium ethylenediaminetetraacetic acid, 10 ml distilled H_2O, 50 ml glycerol) and electrophoresed on a 1.0% agarose gel containing ethidium bromide and visualized under ultraviolet light.

Real-time quantitative PCR

The real-time quantitative PCR (qPCR) assays were performed with an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The reactions were conducted in triplicate for each gene and consisted of the following components: 2 μl of RT product; 3 μl 10X PCR buffer (Qiagen); 2 Units *Taq* DNA polymerase (Qiagen); 100 μM dNTPs (Invitrogen); 0.5X SYBR Green I dye (Applied Biosystems) (TE buffer, pH 8.0); 6 pmol of each primer; 2.5 mM $MgCl_2$ (Qiagen); 3% dimethylsulfoxide; and nuclease-free H_2O for a final volume of 30 μl. The efficiency of cDNA synthesis was standardized by using the same amount of cDNA template in the assays. Optimization of concentrations of $MgCl_2$, dimethylsulfoxide and primers has been previously described (30). The reaction cycle conditions were as described above. Negative controls (no DNA template) and negative amplification controls were prepared in triplicate alongside the experimental total DNA samples to normalize for any background signal obtained following amplification. At the completion of each assay, DNA melting curves were used to check for primer dimers or any nonspecific PCR products. To verify the sequences of the amplified products, sequencing and nucleotide sequence alignments were performed on a random selection of three to four products per gene.

Data normalization

A primer efficiency-corrected comparative ΔC_t method was used for relative quantification. Raw threshold cycle (C_t) values were normalized based on primer efficiency determinations as follows. A standard curve was generated for each primer set using C_t values obtained from amplification of 10-fold serial dilutions of *E. faecalis* DNA ranging from 1 ng/μl to 100 fg/μl using the same components and quantitative PCR conditions as previously described. The lowest line estimate value for the eight primer sets was used to calculate normalization values. For each primer set the normalization value was then applied to the raw C_t values for that gene. Thereafter, primer efficiency-normalized C_t values were then calibrator normalized to the designated endogenous reference (in this case, the reference was time zero). Fold changes in relative levels of transcripts were determined at each incubation interval for each pH at each temperature, using the formula $2^{-\Delta\Delta C_t}$, where the first Δ was based on the primer efficiency and the second was based on the difference between the target at a specified time-point and time zero. A fold change of <-2 and >2 was considered to represent a real difference in levels of transcripts.

Results

Cell viability in alkaline environments

Viable cell counts were obtained from culturing on BHI agar after exposure at 25°C or 37°C at various pH values and time-points (Fig. 1). Viable counts at time zero corresponded to $\sim 1.0 \times 10^9$ colony-forming units/ml. After 1 week in medium at pH 7, viable counts remained at or above 1.0×10^9 colony-forming units/ml. The approximate percentage survival of cells compared to time zero after 1 week in alkaline media was 1% (pH 10), 0.001% (pH 11) and 0.00001% (pH 12). The pH of the buffered BHI media remained stable over the duration of the incubation periods (data not shown).

Gene expression

Table 2 shows the fold changes in levels of transcripts for experiments at 25°C and 37°C. At 25°C all genes showed a decrease in transcripts at all time intervals. In contrast, at 37°C when *E. faecalis* was grown in media at pH 7 and pH 10, all genes except *tuf* showed an increased level of transcripts at one or more time intervals but never at 168 h. In the pH 7 media, increased transcripts occurred with *ftsZ* at 24 and 48 h, and with *dnaK* at 24 h. In pH 10 media, increased transcripts occurred with *ftsZ* at 24, 48, 72 and 120 h, *pbp5* at 24, 72 and 120 h, *dnaK* at 24 and 120 h, *napA* at 72 and 120 h, *tsf* at 72 and 120 h, *fba* at 72 h and *GroEL* at 120 h. In pH 11 media at 37°C no increase in levels of transcripts was observed.

Discussion

When *E. faecalis* was grown at 37°C in media at pH 7 or pH 10, all the genes except *tuf* showed an increase in transcription level at one or more time intervals (but never at 168 h). The greatest increase in transcription level involved *ftsZ* at pH 10 at 37°C, which increased by 37-fold after 120 h. FtsZ is important in cellular division and may function as the universal prokaryotic division protein (21). It is interesting that despite a decrease in viability of two-orders of magnitude at pH 10 at 72 and 120 h (Fig. 1), there were corresponding 20-fold and 37-fold increases in levels of transcripts (Table 2). This surprising result appears counterintuitive and therefore remains the subject of further investigation. However, based on the proposed cytoskeleton functional homology between FtsZ and the eukaryotic protein tubulin (38), it could be speculated that the observed increase in *ftsZ* gene transcripts at pH 10 was associated with an increased cytoskeletal function in the remaining viable cells.

Overall, the greatest increase in levels of gene transcripts occurred in cultures maintained at 37°C in pH 10 medium. The observed increase of *ftsZ*, *pbp5*, *dnaK*, *GroEL*, *napA* and *tsf* gene transcripts in pH 10 cultures between the 72 and 120 h time-points at 37°C appeared to be associated with a slight increase in cultured cell growth during that time. In similar temperature conditions, levels of *fba*, *ftsZ*, *pbp5* and *tsf* transcripts increased in the period between the 48 and 72 h time-points at pH 10. Based on these data, these genes may be involved in growth recovery at these time intervals. Fba is known to play a role in central carbohydrate metabolic pathways (12) while DnaK and GroEL are known to be chaperone proteins that function in protein (re)folding and degradation (19). The penicillin-binding protein, Pbp5, which is involved in the terminal stages of peptidoglycan

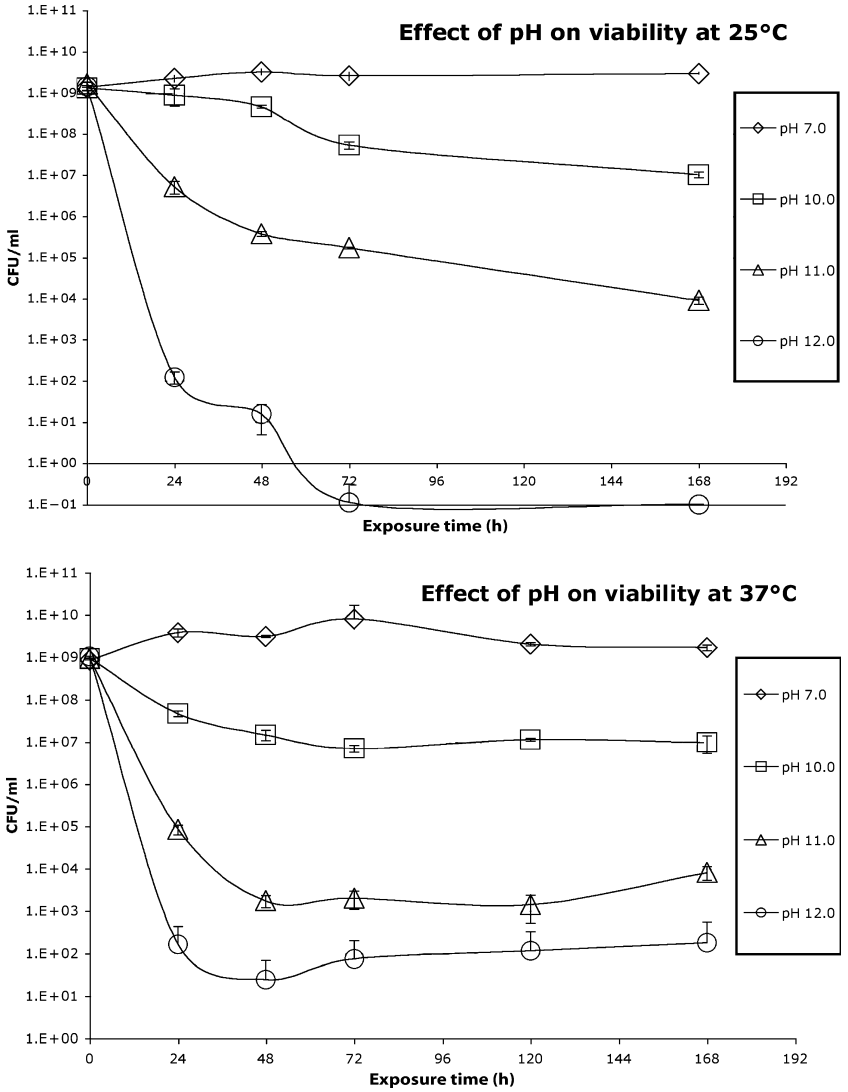

Fig. 1. Viable cell counts in colony-forming units (CFU) per ml of *Enterococcus faecalis* after incubation at 25°C (top) and 37°C (bottom) over various time periods at pH 7, 10, 11 and 12.

synthesis, may aid in survival under alkaline conditions (20).

Increased levels of transcripts of *tuf* were not evident under any experimental condition. Similarly, Heim *et al.* (2002) reported that *tuf* mRNA was initially detected in the viable but non-culturable state, but not detected in samples taken later. The *tuf* gene product, EF-TU-GDP, promotes the binding of aminoacyl-tRNA to the ribosome and may interact with proteins in the transcriptional machinery to act as a positive regulator of RNA synthesis (37). The *tsf* gene transcripts increased only moderately at 72 and 120 h at pH 10 at 37°C. When analysing *tsf* and *tuf* gene expression, it would be expected that their expression might be linked because *tsf* mediates the reformation of the EF-Tu-GDP complex into its active form (12). A possible explanation for the different levels of *tsf* and *tuf* gene transcripts observed may be that *tsf* recycling of EF-Tu-GDP is sufficient under stressful conditions, such as alkaline pH, and no new *tuf* need be transcribed.

A maximum incubation period of 1 week was used, based on established clinical protocols that recommend placement of intracanal $Ca(OH)_2$ dressings for 1 week to eliminate bacteria surviving biomechanical instrumentation of the root canal (32). At least 99% of *E. faecalis* cells could not be cultured at either temperature after 1 week in an alkaline environment (Fig. 1). However, the number of living organisms could be underrepresented. For instance, in some situations, sublethally damaged organisms are undetected (2). Conversely, genes encoding virulence determinants are expressed in cells that have lost the ability to be cultured (5). Interestingly, at 25°C, or room temperature, all the genes showed a decrease in level of transcripts at every time interval (Table 2). Other data for this temperature are not available for comparison. Highly reduced levels probably relate, at least in some cases, to cell death.

While the intracellular pH values of bacteria grown in alkaline environments are generally more acidic than their extracellular environment (3), at a pH above 8.0, *Enterococcus hirae* does not appear to possess a system that maintains a constant intracellular pH (17). Many factors could potentially contribute to the tolerance of alkaline pH found in enterococci, including activation of specific proton pumps, and specific enzymatic systems and/or buffering devices that help to keep the internal pH constant. For example, a functioning proton pump is critical to the survival of *E. faecalis* at high pH (6). Secondary Na^+/H^+ antiporters are widely distributed among bacteria and some species have been found to produce a primary sodium pump (15, 18). Enzymes capable of remaining active in alkaline conditions, for example the Na^+-ATPase in *E. hirae* (16), could be required for bacteria to remain viable (7). In this study, levels of the Na^+/H^+ antiporter encoding NapA only increased at 120 h in pH 10 medium at 37°C, suggesting that the internal pH of the cells might have stabilized, but once the cells adapted, the increased expression of *napA* may have no longer been necessary.

In addition to transcription levels, the half-life of the RNA messages, the translation rates and the additional post-translational processing all contribute to gene expression. Since RNA is hydrolysed at alkaline pH (39), the data could reflect different degrees of 'protection' from hydrolysis at different pHs. Further, nucleases or other endogenous enzymes sensitive to pH, time, temperature or other uncontrolled variables may have been involved in modulating gene expression. Further studies are required to determine whether increased levels represent more synthesis or higher stability of transcripts, and whether there are variations in gene expression among *E. faecalis* strains.

When samples have been obtained over different time–courses, misinterpretation of the derived expression profile of the target genes is possible unless normalization of target gene expression levels is performed to compensate for sample-to-sample and run-to-run variations (25). The

Table 2. Fold-change in level of gene transcripts compared to time zero

	Incubation period (h)	Genes							
		dnaK	*fba*	*ftsZ*	*GroEL*	*napA*	*pbp5*	*tsf*	*tuf*
Temperature 25°C									
pH 7	24	−13	−693	−39	−394	−90	−492	−49	−249
	48	−8	−517	−17	−284	−106	−208	−2145	−197
	72	−7	−209	−22	−133	−65	−177	−333	−99
	168	−9	−516	−11	−380	−25	−156	−18	−90
pH 10	24	−7	−94	−17	−70	−12	−77	−48	−17
	48	−5	−13	−8	−20	−16	−20	−24	−20
	72	−12	−19	−16	−31	−8	−25	−27	−15
	168	−16	−68	−9	−62	−2	−8	−20	−4
pH 11	24	−17	−341	−77	−260	−125	−194	−57	−288
	48	−177	−553	−343	−1394	−435	−1087	−17,805	−1230
	72	−625	−356	−526	−578	−206	−422	−13,170	−548
	168	−625	−630	−1894	−3204	−442	−555	−30,048	−798
Temperature 37°C									
pH 7	24	**2**	−1	**29**	−3	−2	−2	−11	−4
	48	−1	−3	**3**	−4	−7	−9	−11	−11
	72	−2	−3	−6	−9	−17	−3	−16	−10
	120	−3	−2	−1	−7	−2	−18	−9	−15
	168	−4	−2	−16	−25	−6	−6	−61	−20
pH 10	24	**4**	−1	**6**	1	−1	**2**	−1	−2
	48	−2	−2	**2**	−14	−5	−2	−6	−3
	72	1	**3**	**20**	−2	1	**9**	**2**	1
	120	**4**	1	**37**	**3**	**9**	**10**	**3**	−1
	168	−4	−3	1	−18	−4	1	−29	−8
pH 11	24	1	−3	−7	−6	−5	−10	−43	−9
	48	−10	−4	−11	−22	−5	−15	−50	−24
	72	−7	−2	−27	−116	−5	−6	−163	−14
	120	−9	−3	−19	−58	−11	−26	−50	−22
	168	−7	−1	1	−27	−9	−37	−176	−25

A fold change of <−2 or >2 (bold) is considered to represent a real difference in levels of transcripts.

16S rRNA and glyceraldehyde-3-phosphate dehydrogenase genes have traditionally been used as 'housekeeping genes' for real-time qPCR quantification of gene expression, based on the assumption that these genes will be constitutively expressed. However, under many conditions, they are not constitutively expressed (4, 28, 34). For example, Richardson *et al.* (28) showed that 16S rRNA and glyceraldehyde-3-phosphate dehydrogenase expression in *Actinobacillus actinomycetemcomitans* is a dynamic process and adapts to different growth conditions. In the present investigations, the use of housekeeping genes was considered an unreliable option because of the wide range of experimental conditions. Therefore, the reliability of real-time qPCR was optimized by generating calibration curves for the primers, using identical amplification efficiencies for both the baseline target and the higher pH targets, and by using a standardized amount of cDNA template in the qPCR assays. Thereafter, relative expression levels of gene transcripts at pH 7, 10 and 11 relative to baseline at time zero were calculated using a primer efficiency-corrected comparative ΔC_t method.

The greatest increase in overall levels of gene transcripts, in particular *ftsZ*, was seen at 120 h in cultures maintained in medium at pH 10 at 37°C. These data provide new information on the effect of prolonged exposure to highly alkaline pH on viability of *E. faecalis* in broth, and represent a first step in the process to identify proteins that might be important to the survival of *E. faecalis* in high pH environments. It is hoped that future studies can incorporate the rapid advances underway in DNA microarray technology and proteomics. Extrapolation of the present data to the clinical situation can only be speculative, in part because *E. faecalis* might exist in the root canal system as part of a polymicrobial biofilm as opposed to pure culture in prolonged stationary phase in broth in these studies. However, the present data may contribute towards better understanding the survival strategies of *E. faecalis* following prolonged exposure to pH levels similar to those encountered in root canals receiving intracanal $Ca(OH)_2$ treatment.

References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990: **215**: 403–410.
2. Blackburn CW, McCarthy JD. Modifications to methods for the enumeration and detection of injured *Escherichia coli* O157:H7 in foods. Int J Food Microbiol 2000: **55**: 285–290.
3. Booth IR. The regulation of intracellular pH in bacteria. Novartis Found Symp 1999: **221**: 19–28.
4. Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR – a perspective. J Mol Endocrinol 2005: **34**: 597–601.
5. Chaiyanan S, Chaiyanan S, Huq A, Maugel T, Colwell RR. Viability of the nonculturable *Vibrio cholerae* O1 and O139. Syst Appl Microbiol 2001: **24**: 331–341.
6. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. Int Endod J 2002: **35**: 221–228.
7. Flahaut S, Hartke A, Giard JC, Auffray Y. Alkaline stress response in *Enterococcus faecalis*: adaptation, cross-protection, and changes in protein synthesis. Appl Environ Microbiol 1997: **63**: 812–814.
8. Freeman BA, Crapo JD. Biology of disease: free radicals and tissue injury. Lab Invest 1982: **47**: 412–426.
9. Haapasalo HK, Sirén EK, Waltimo TM, Ørstavik D, Haapasalo MP. Inactivation of local root canal medicaments by dentine: an *in vitro* study. Int Endod J 2000: **33**: 126–131.
10. Halliwell B. Oxidants and human disease: some new concepts. FASEB J 1987: **1**: 358–364.
11. Hancock HH, Jr, Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001: **91**: 579–586.
12. Heim S, Lleo MM, Bonato B, Guzman CA, Canepari P. The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determined by proteome analysis. J Bacteriol 2002: **184**: 6739–6745.
13. Imlay JA, Linn S. DNA damage and oxygen radical toxicity. Science 1988: **240**: 1302–1309.
14. Jacob AE, Hobbs SJ. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J Bacteriol 1974: **117**: 360–372.
15. Kakinuma Y, Igarashi K. Amplification of the Na^+-ATPase of *Streptococcus faecalis* at alkaline pH. FEBS Lett 1990: **261**: 135–138.
16. Kakinuma Y, Igarashi K. Mutants of *Streptococcus faecalis* sensitive to alkaline pH lack $Na^{(+)}$-ATPase. J Bacteriol 1990: **172**: 1732–1735.
17. Kobayashi H, Saito H, Futatsugi L, Kakegawa T. Cation movements at alkaline pH in bacteria growing without respiration. Novartis Found Symp 1999: **221**: 235–242.
18. Krulwich TA. Na^+/H^+ antiporters. Biochim Biophys Acta 1983: **726**: 245–264.
19. Laport MS, Lemos JA, Bastos Md Mdo C, Burne RA, Giambiagi-De Marval M. Transcriptional analysis of the groE and dnaK heat-shock operons of *Enterococcus faecalis*. Res Microbiol 2004: **155**: 252–258.
20. Lleo MM, Pierobon S, Tafi MC, Signoretto C, Canepari P. mRNA detection by reverse transcription-PCR for monitoring viability over time in an *Enterococcus faecalis* viable

but nonculturable population maintained in a laboratory microcosm. Appl Environ Microbiol 2000: **66**: 4564–4567.
21. Lutkenhaus J, Addinall SG. Bacterial cell division and the Z ring. Annu Rev Biochem 1997: **66**: 93–116.
22. Nair PN, Henry S, Cano V, Vera J. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after 'one-visit' endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005: **99**: 231–252.
23. Nakajo K, Nakazawa F, Iwaku M, Hoshino E. Alkali-resistant bacteria in root canal systems. Oral Microbiol Immunol 2004: **19**: 390–394.
24. Peters LB, Wesselink PR, Moorer WR. Penetration of bacteria in bovine root dentine *in vitro*. Int Endod J 2000: **33**: 28–36.
25. Pfaffl MW, Hageleit M. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol Lett 2001: **23**: 275–282.
26. Portenier I, Waltimo TMT, Haapasalo M. *Enterococcus faecalis* – the root canal survivor and 'star' in post-treatment disease. Endod Topics 2003: **6**: 135–159.
27. Portenier I, Waltimo T, Ørstavik D, Haapasalo M. The susceptibility of starved, stationary phase, and growing cells of *Enterococcus faecalis* to endodontic medicaments. J Endod 2005: **31**: 380–386.
28. Richardson J, Craighead JC, Cao SL, Handfield M. Concurrence between the gene expression pattern of *Actinobacillus actinomycetemcomitans* in localized aggressive periodontitis and in human epithelial cells. J Med Microbiol 2005: **54**: 497–504.
29. Sedgley CM, Lennan SL, Appelbe OK. Survival of *Enterococcus faecalis* in root canals *ex vivo*. Int Endod J 2005: **38**: 735–742.
30. Sedgley CM, Nagel AC, Shelburne CE, Clewell DB, Appelbe O, Molander A. Quantitative real-time PCR detection of oral *Enterococcus faecalis* in humans. Arch Oral Biol 2005: **50**: 575–583.
31. Sedgley CM, Molander A, Flannagan SE et al. Virulence, phenotype and genotype characteristics of endodontic *Enterococcus* spp. Oral Microbiol Immunol 2005: **20**: 10–19.
32. Sjögren U, Figdor D, Spangberg L, Sundqvist G. The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. Int Endod J 1991: **24**: 119–125.
33. Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998: **85**: 86–93.
34. Vandecasteele SJ, Peetermans WE, Merckx R, Van Eldere J. Quantification of expression of *Staphylococcus epidermidis* housekeeping genes with Taqman quantitative PCR during *in vitro* growth and under different conditions. J Bacteriol 2001: **183**: 7094–7101.
35. Waltimo T, Trope M, Haapasalo M, Orstavik D. Clinical efficacy of treatment procedures in endodontic infection control and one year follow-up of periapical healing. J Endod 2005: **31**: 863–866.
36. Wang JD, Hume WR. Diffusion of hydrogen ion and hydroxyl ion from various sources through dentine. Int Endod J 1988: **21**: 17–26.
37. Young CC, Bernlohr RW. Elongation factor Tu is methylated in response to nutrient deprivation in *Escherichia coli*. J Bacteriol 1991: **173**: 3096–3100.
38. Yu XC, Margolin W, Gonzalez-Garay ML, Cabral F. Vinblastine induces an interaction between FtsZ and tubulin in mammalian cells. J Cell Sci 1999: **112**: 2301–2311.
39. Zhou DM, Taira K. The hydrolysis of RNA: from theoretical calculations to the hammerhead ribozyme-mediated cleavage of RNA. Chem Rev 1998: **98**: 991–1026.