
Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide

M. Evans¹, J. K. Davies², G. Sundqvist³ & D. Figdor^{1,2,3}

¹School of Dental Science, University of Melbourne, ²Department of Microbiology, Monash University, Melbourne, Australia, and ³Department of Endodontics, Umeå University, Umeå, Sweden

Abstract

Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *International Endodontic Journal*, **35**, 221–228, 2002.

Aim This study sought to clarify the mechanisms that enable *E. faecalis* to survive the high pH of calcium hydroxide.

Methodology *E. faecalis* strain JH2-2 was exposed to sublethal concentrations of calcium hydroxide, with and without various pretreatments. Blocking agents were added to determine the role of stress-induced protein synthesis and the cell wall-associated proton pump.

Results *E. faecalis* was resistant to calcium hydroxide at a pH of 11.1, but not pH 11.5. Pre-treatment with

calcium hydroxide pH 10.3 induced no tolerance to further exposure at pH 11.5. No difference in cell survival was observed when protein synthesis was blocked during stress induction, however, addition of a proton pump inhibitor resulted in a dramatic reduction of cell viability of *E. faecalis* in calcium hydroxide.

Conclusions Survival of *E. faecalis* in calcium hydroxide appears to be unrelated to stress induced protein synthesis, but a functioning proton pump is critical for survival of *E. faecalis* at high pH.

Keywords: *E. faecalis*, proton pump, sodium hypochlorite, stress response.

Received 8 May 2001; accepted 24 July 2001

Introduction

Only a few studies have investigated the aetiology of failures in which the endodontic therapy has been well performed. These studies have revealed five factors that may contribute to a persistent periapical radiolucency after treatment. The factors are: intraradicular infection (Nair *et al.* 1990a); extraradicular infection by bacteria of the species *Actinomyces israelii* and *Propionibacterium propionicum* (Nair & Schroeder 1984, Sjögren *et al.* 1988); foreign body reaction (Koppang *et al.* 1989, Nair *et al.* 1990b); cysts, especially those containing cholesterol crystals (Nair *et al.* 1993); and fibrous scar tissue healing following conventional treatment (Nair *et al.* 1999). Of all these factors, it is generally believed that the major cause of root canal treatment failure is the persistence of microorganisms in the apical part of root-filled teeth.

Although information on the type of microorganisms that persist in the root-filled canal is limited, several studies have reported that root-filled teeth with persisting periapical lesions usually harbour one or a few bacterial species and that Gram-positive bacteria dominate the bacterial flora (Möller 1966, Molander *et al.* 1998, Sundqvist *et al.* 1998, Noda *et al.* 2000, Hancock *et al.* 2001). The species *Enterococcus faecalis* has been recovered in a high proportion of endodontic failures, approximately one third of the canals of root-filled teeth with persistent periapical lesions (Möller 1966, Molander *et al.* 1998, Sundqvist *et al.* 1998, Hancock *et al.* 2001).

The common recovery of *E. faecalis* from canals where the treatment has failed suggests it is an opportunistic pathogen whose persistence in the root canal presents a significant therapeutic problem. When *E. faecalis* has been identified in cases of failed endodontic treatment, it has proven to be difficult to eradicate during retreatment (Sundqvist *et al.* 1998), and the failure rate of retreatment is higher when *E. faecalis* has been recovered from the root canal at the time of root filling (Sundqvist *et al.* 1998). Once established in the root canal, *E. faecalis* faces

Correspondence: Dr David Figdor, 517 St Kilda Road, Melbourne, VIC 3004 Australia (tel: + 61 39866 4528; fax: + 61 39820 3102; e-mail: David.Figdor@med.monash.edu.au)

several challenges for survival, including an ability to withstand antimicrobial agents used during treatment and endure potential starvation in the cleaned and obturated canal. *E. faecalis* appears to be highly resistant to the medicaments used during treatment and is known to resist the antibacterial effect of dressing with calcium hydroxide (Byström *et al.* 1985, Haapasalo & Ørstavik 1987, Safavi *et al.* 1990, Ørstavik & Haapasalo 1990, Sato *et al.* 1993, Weiger *et al.* 1995, Siqueira & de Uzeda 1996, Tanriverdi *et al.* 1997, Waltimo *et al.* 1999). Interestingly, resistance to killing by calcium hydroxide is shared with the few other microorganisms that have been associated with endodontic failure, such as *Candida* species (Nair *et al.* 1990a, Waltimo *et al.* 1997, Waltimo *et al.* 1999) and *Actinomyces radidentis* (Kalfas *et al.* 2001).

Although *E. faecalis* has been reported to withstand the high pH of calcium hydroxide, relatively little is known about the survival mechanisms of *E. faecalis* that enable it to tolerate exposure to calcium hydroxide. In general, when bacteria face an adverse or potentially lethal challenge, a stress response is mounted that allows them to endure the threat, survive and recover (Parsell & Lindquist 1993, Welch 1993). This response has been described for a diverse range of stresses and in some bacterial species the adaptive response may contribute to pathogenesis (Foster & Hall 1990, Bearson *et al.* 1998, Castanie-Cornet *et al.* 1999). When a stress response is induced, this state may confer a general protection against a range of other stresses, for example a stress response induced by nutrient starvation provides protection against heat stress (Jouper-Jaan *et al.* 1992). *E. faecalis* has been shown to synthesize a variety of stress proteins when exposed to adverse environmental conditions (Hartke *et al.* 1998) including high salt concentration (Flahaut *et al.* 1996a), bile salts, acid, heat (Flahaut *et al.* 1996b), glucose starvation (Giard *et al.* 1997), high pH (Flahaut *et al.* 1997), sodium hypochlorite (Laplace *et al.* 1997) and starvation in tap water (Hartke *et al.* 1998).

If starvation or exposure to sodium hypochlorite solution or diffusing calcium hydroxide induces *E. faecalis* to enter a stress response then this might confer cross protection to *E. faecalis* when it is subsequently exposed to a calcium hydroxide dressing. This study sought to clarify the strategies employed by *E. faecalis* that may result in resistance to treatment. The aim was to investigate the stress response of *E. faecalis* to antimicrobial agents by exposing cells to sublethal concentrations of sodium hypochlorite and calcium hydroxide with or without a range of pretreatments. We also examined

alternative cell mechanisms that may be important for survival of *E. faecalis* at high pH.

Materials and methods

Bacterial strain and culture conditions

The bacterial strain used for all experiments was *E. faecalis* JH2-2, which is derived from the parental strain JH2 (Jacob & Hobbs 1974) and is representative of the species. Cultures were grown overnight in 10 mL Brain Heart Infusion broth (BHI, Oxoid Ltd, Basingstoke, UK) at 37°C, in air without shaking. A 2% inoculum in 50 mL BHI was then grown at 37°C to an optical density at 600 nm (OD_{600}) of 1.1. Preliminary experiments showed that cells stopped growing and entered stationary phase at this OD. All experiments described below were repeated in triplicate.

Antimicrobial solutions

Sodium hypochlorite solutions were prepared fresh daily by dilution of a 1% sodium hypochlorite solution (10 000 p.p.m. available chlorine, pH11; Milton®, Procter & Gamble, Parramatta, NSW, Australia) to 0.0001% or 0.005% solution (1 and 50 p.p.m. available chlorine, respectively). Available chlorine was confirmed by colorimetry (Lovibond 1000 Comparator, Salisbury, UK).

A saturated solution of calcium hydroxide was prepared by dissolving calcium hydroxide powder (Sigma Chemical Co., St Louis, MO, USA) overnight in de-ionized water. The saturated solution was centrifuged ($3000 \times g$, 10 min), the supernatant was removed and filtered using a Millex®-GP 0.22 µm Filter Unit (Millipore, Bedford, MA, USA) resulting in a stock calcium hydroxide solution, pH 11.5. Additional calcium hydroxide solutions of pH 11.1 and 10.3 were prepared by dilution of stock with de-ionized water (1 in 4 and 1 in 40, respectively).

Pre-treatment and challenge conditions

After cultures were grown to stationary phase, 10 mL aliquots were removed and cells were harvested by centrifugation ($3000 \times g$, 10 min). Cells were pretreated by resuspending cell pellets in 10 mL solutions of either 0.0001% sodium hypochlorite solution or calcium hydroxide solution pH 10.3 (or de-ionized water for controls) for 30 min. One mL aliquots were placed in Eppendorf tubes and cells recovered by centrifugation ($16\,000 \times g$, 3 min) and washed before challenge.

Table 1 Effect of pretreatment on survival of *E. faecalis* JH2-2 challenged with sodium hypochlorite and calcium hydroxide

Pre-treatment	Challenge					
	NaOCl, 0.005%		Ca(OH) ₂ , pH 11.1		Ca(OH) ₂ , pH 11.5	
	15 min	30 min	15 min	30 min	15 min	30 min
No pretreatment	1.2×10^{-4}	4.4×10^{-6}	4.8×10^{-1}	4.1×10^{-1}	2.6×10^{-4}	1.3×10^{-6}
Ca(OH) ₂ , pH 10.3	1.4×10^{-4}	1.6×10^{-5}	1.0×10^0	7.5×10^{-1}	5.7×10^{-4}	6.7×10^{-6}
NaOCl, 0.0001%	8.1×10^{-5}	4.3×10^{-6}	2.3×10^{-1}	1.3×10^{-1}	1.8×10^{-4}	1.7×10^{-6}

Data expressed as percentage survival, average of 3 experiments.

Number of cells at 0 min was $2.3 \pm 0.2 \times 10^9$ (= 100%).

Bacteria were challenged by exposure to sodium hypochlorite (0.005%), or calcium hydroxide solution (pH 11.1 or 11.5). At 0, 15 and 30 min, 1 mL samples were removed, cells recovered by centrifugation ($16\,000 \times g$, 3 min), washed, and resuspended in 0.9% sodium chloride solution. Viable counts were determined by serial dilution and plating onto BHI agar. The number of colony forming units (CFU) was determined after incubation for 48 h at 37°C in air.

Mechanisms of survival-response to lethal treatment

To check if protein synthesis was necessary for cell survival, some experiments were repeated in the presence of a protein synthesis inhibitor, chloramphenicol ($100 \mu\text{g mL}^{-1}$), which was added to the pretreatment and challenge solutions in relevant experiments. Chloramphenicol at a concentration of $100 \mu\text{g mL}^{-1}$ has been shown to block protein synthesis (Giard *et al.* 1996, Giard *et al.* 1997).

To determine if a proton pump was involved in resistance to high pH, nonadapted cell pellets were exposed to 1 mL calcium hydroxide (pH 11.1) in the presence of a proton pump inhibitor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, $100 \mu\text{M}$) (ICN Bio-medicals Inc., Aurora, OH, USA), for 15 and 30 min. CCCP has been shown to block the normal function of the proton pump at this concentration (Mugikura *et al.* 1990). Control experiments were performed under identical conditions, but with the calcium hydroxide solution adjusted to pH 7.0 by dilution. Cells were recovered and viability determined as above.

Results

Response to sodium hypochlorite

Preliminary experiments revealed that sodium hypochlorite solution at a 0.5 and 1% (v/v) concentration was

rapidly lethal, but *E. faecalis* survived a 0.0001% sodium hypochlorite solution (data not shown). Therefore, a sublethal concentration of 0.005% (v/v) sodium hypochlorite was used, which resulted in just $1.2 \times 10^{-4}\%$ of cells surviving after 15 min and $4.4 \times 10^{-6}\%$ cell survival after 30 min exposure (Table 1). Pre-treatment with 0.0001% sodium hypochlorite or calcium hydroxide pH 10.3 for 30 min did not induce tolerance or increase survival of *E. faecalis* in 0.005% sodium hypochlorite (Table 1).

Response to calcium hydroxide

When cells were exposed to calcium hydroxide pH 11.1 for 30 min, 0.4% cells survived, but a relatively small increase in alkalinity to pH 11.5 resulted in a dramatic drop in survival to just $1.3 \times 10^{-6}\%$ (Table 1). Pre-treatment with 0.0001% sodium hypochlorite had no effect on survival of cells challenged with calcium hydroxide pH 11.1 or 11.5 (Table 1). Pre-treatment with calcium hydroxide pH 10.3 for 30 min conferred no resistance by *E. faecalis* to further exposure to calcium hydroxide.

Effect of blocking protein synthesis

The protein synthesis inhibitor, chloramphenicol, was used to block protein production during antimicrobial challenge to determine the role of stress-induced proteins in cell survival. In the presence of $100 \mu\text{g mL}^{-1}$ chloramphenicol, there was no discernible difference in cell survival when cells were exposed to 0.005% sodium hypochlorite, with or without calcium hydroxide pH 10.3 pretreatment (Table 2). Similarly, there was no difference in cell survival when cells were exposed to calcium hydroxide (pH 11.1 and 11.5) in the presence of chloramphenicol, whether or not they were pretreated with calcium hydroxide pH 10.3 (Table 2).

Table 2 Effect of blocking protein synthesis with Chloramphenicol (Cn) on survival of *E. faecalis* JH2-2 when challenged with sodium hypochlorite and calcium hydroxide, with and without pretreatment

Pre-treatment	Challenge					
	NaOCl, 0.005%		Ca(OH) ₂ , pH 11.1		Ca(OH) ₂ , pH 11.5	
	15 min	30 min	15 min	30 min	15 min	30 min
No pretreatment*	1.2×10^{-4}	4.4×10^{-6}	4.8×10^{-1}	4.1×10^{-1}	2.6×10^{-4}	1.3×10^{-6}
No pretreatment + Cn	1.1×10^{-4}	7.8×10^{-6}	4.9×10^{-1}	3.7×10^{-1}	2.6×10^{-4}	1.6×10^{-6}
Ca(OH) ₂ , pH 10.3*	1.4×10^{-4}	1.6×10^{-5}	1.0×10^0	7.5×10^{-1}	5.7×10^{-4}	6.7×10^{-6}
Ca(OH) ₂ , pH 10.3 + Cn	1.1×10^{-4}	9.0×10^{-6}	9.7×10^{-1}	7.5×10^{-1}	7.4×10^{-4}	6.8×10^{-6}

Data expressed as percentage survival, average of three experiments.

Number of cells at 0 min was $2.3 \pm 0.2 \times 10^9$ (= 100%).

*Data from Table 1 included for comparison.

Effect of blocking proton pump

When the environmental pH rises, bacteria attempt to preserve the intracellular pH by pumping protons across the cytoplasmic membrane to maintain the cytoplasmic pH (Booth 1985, Booth 1999). The proton pump inhibitor, CCCP, was used to determine whether this mechanism might play a role in tolerance of *E. faecalis* to alkaline conditions.

When cells were exposed to calcium hydroxide at pH 11.1 for 30 min, there was a 20-fold reduction in cell survival in the presence of CCCP compared to calcium hydroxide exposure in the absence of the proton pump inhibitor (Fig. 1). The effect of CCCP was apparently time dependent because the effect was even more pronounced after 60 min of calcium hydroxide exposure (one experiment only; without CCCP 6.4×10^{-3} % of cells survived, but in the presence of CCCP, survival was 9.0×10^{-5} %). Control experiments showed that CCCP was not lethal to *E. faecalis* at pH 7.0 (Fig. 1).

Discussion

The common recovery of *E. faecalis* in root canals of teeth where endodontic treatment has failed implies that this species is intimately involved in the pathogenesis and maintenance of persisting apical periodontitis. One feature that may enable *E. faecalis* to persist in the root canal is an ability for it to survive conventional antimicrobial agents used during endodontic treatment, such as the alkaline pH of calcium hydroxide. *E. faecalis* is known to withstand a high pH; indeed this is an identifying characteristic of *E. faecalis* (Mundt 1986, Devriese *et al.* 1992). At pH 11.5 or greater, *E. faecalis* does not survive, yet it can survive at a pH below 11.5 as shown in this and earlier studies (Byström *et al.* 1985). Because of

Survival (%)

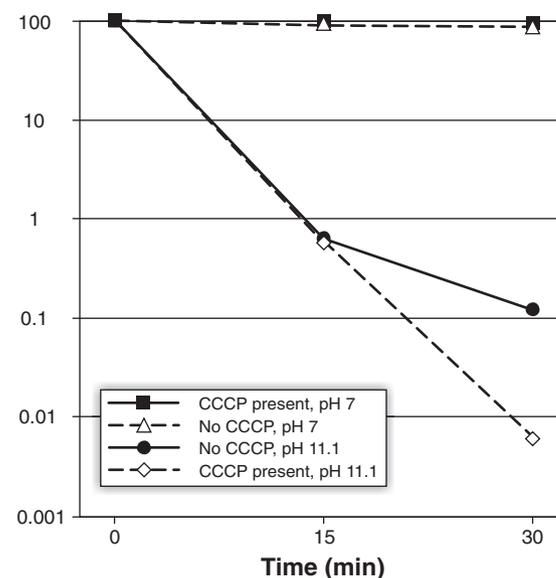


Figure 1 Survival of *E. faecalis* JH2-2 in calcium hydroxide pH 11.1 (and controls, pH 7.0) in the presence or absence of CCCP. Each data point is the average of 3 experiments.

the buffering effect of dentine (Wang & Hume 1988, Nerwich *et al.* 1993, Haapasalo *et al.* 2000), it is unlikely that the high pH of calcium hydroxide (>11.5) is attained within dentinal tubules where *E. faecalis* has the capacity, at least *in vitro*, to penetrate deeply (Haapasalo & Ørstavik 1987, Ørstavik & Haapasalo 1990, Safavi *et al.* 1990, Vahdaty *et al.* 1993, Peters *et al.* 2000). In radicular dentine, alkalinity may only reach pH 10.3 after dressing the canal with calcium hydroxide (Nerwich *et al.* 1993, Miñana *et al.* 2001).

Because endodontic treatment involves repeated or alternate use of sodium hypochlorite solution and calcium hydroxide at various stages of treatment, *E. faecalis* is likely to be exposed to an alkaline pH, which is sublethal. These conditions could adapt or 'pretreat' cells by eliciting a stress response that enhances their survival. For example, adaptation of *E. faecalis* by incubation at low temperatures confers an increased ability for cells to withstand freezing (Thammavongs *et al.* 1996). It therefore seems reasonable to suspect that repeated exposure of *E. faecalis* to sodium hypochlorite solution and calcium hydroxide might induce resistance to subsequent exposure at levels that might normally be lethal.

The initial experiments confirmed the survival of *E. faecalis* in an alkaline high pH. After 30 min exposure in calcium hydroxide pH 11.1, some cells (0.4%) survived, however, at pH 11.5 >99.99% were killed. Next, *E. faecalis* was pretreated with calcium hydroxide pH 10.3 before challenging cells in calcium hydroxide (pH 11.1 and 11.5) and these experiments revealed a minimal increase in survival by *E. faecalis*, much less than reported in another study (Flahaut *et al.* 1997). Pre-treatment with sodium hypochlorite solution had no effect on survival by *E. faecalis* in calcium hydroxide. Thus, these results show that pretreatment by calcium hydroxide and sodium hypochlorite solution is of minor importance in the ability of *E. faecalis* to survive the high alkalinity of calcium hydroxide.

The capacity to mount a stress response with synthesis of general or specific stress-induced proteins is an important survival mechanism (Foster & Spector 1995). The production of these proteins is a fundamental biological response in many bacterial species and protects cells from adverse conditions, facilitating cell recovery when more favourable conditions are re-established (Parsell & Lindquist 1993, Welch 1993). *E. faecalis* has been shown to produce a number of such proteins in response to other environmental stresses, including glucose starvation (Giard *et al.* 1997), elevated temperatures (Boutibonnes *et al.* 1993) and bile salts (Flahaut *et al.* 1996b). To determine the role of stress-induced proteins in surviving the alkaline pH of calcium hydroxide, protein production was blocked with chloramphenicol during pretreatment and challenge with calcium hydroxide. Stopping protein synthesis had no effect on cell survival, which implies that stress-induced protein production is not important for survival of *E. faecalis* at high pH, a finding consistent with Flahaut *et al.* (1997). The induction of tolerance to high pH was independent of protein synthesis because pretreatment in the presence of chloramphenicol did not alter cell survival.

In acid or alkaline environments, most bacterial cells maintain pH homeostasis in which the internal pH is kept within a narrow range so that enzymes and proteins maintain normal function (Booth 1985). The pH homeostasis is based on two principal components: a passive function consisting of a low cell membrane permeability to ions and a buffering ability of the cytoplasm; and an active mechanism that functions mainly through controlled transport of cations (potassium, sodium and protons) across the cell membrane (Booth 1985, Kakinuma 1998, Booth 1999). In acidic environments, a cation antiport system is thought to raise the internal pH by expelling protons across the cell wall. In an alkaline medium, cations/protons are pumped into the cell to lower the internal pH (Booth 1985, Hall *et al.* 1995, Booth 1999). Extensive work with *Enterococcus hirae* (formerly *Streptococcus faecalis*) has confirmed a fundamental role for a potassium/proton antiport system in maintaining cytoplasmic pH in an alkaline environment (Kakinuma 1987, Kakinuma & Igarashi 1990, Kakinuma & Igarashi 1995, Kakinuma & Igarashi 1999).

In order to determine the role of the proton pump in maintaining survival of *E. faecalis* in a high pH environment, CCCP was used to shut down the pump. CCCP is known to block proton movement across cell membranes (Kinoshita *et al.* 1984). In the presence of CCCP, there was a 20-fold reduction in cell survival after 30 min exposure to high pH compared to cells that were not exposed to CCCP. The effect of CCCP was apparently time-dependent because the effect was more pronounced – a 70-fold reduction – after 60 min of calcium hydroxide exposure (one experiment only). These results show that a functioning proton pump, which drives protons into the cell to acidify the cytoplasm, is critical for the survival of *E. faecalis* in a highly alkaline environment. Presumably, when the environmental alkalinity reaches pH 11.5 or above, this life-saving mechanism is overwhelmed.

During clinical endodontic treatment, sodium hypochlorite solution is usually used in a concentration range of 0.5–5% and preliminary experiments confirmed that exposure to 0.5 and 1% sodium hypochlorite solution for 5 min was lethal to *E. faecalis* (data not shown). Indeed, exposure to 0.005% sodium hypochlorite resulted in >99.99% killing of *E. faecalis* at 30 min. It should be noted that these concentrations are considerably below those used in conventional clinical treatment, yet even at this low concentration sodium hypochlorite solution was highly bactericidal. Pre-treatment with 0.0001% sodium hypochlorite did not induce tolerance against further exposure to sodium hypochlorite, which

is consistent with another study (Laplace *et al.* 1997) showing that pretreatment of *E. faecalis* with sodium hypochlorite had no effect on cell survival to further sodium hypochlorite challenge. In all probability, any stress or adaptive response mounted by *E. faecalis* is completely overwhelmed by the lethal effect of sodium hypochlorite.

Several *in vitro* studies have shown sodium hypochlorite solution to be lethal to *E. faecalis*, yet when *E. faecalis*-infected dentine is exposed to sodium hypochlorite solution, some cells of *E. faecalis* may survive (Ørstavik & Haapasalo 1990, Vahdaty *et al.* 1993, Siqueira *et al.* 1997, Heling & Chandler 1998). The recovery of *E. faecalis* in the root canals of failed cases after endodontic retreatment (Sundqvist *et al.* 1998) also implies an ability of *E. faecalis* to survive, albeit rarely, the antimicrobial action of sodium hypochlorite in the root canal. Our findings show that an adaptive stress response to sodium hypochlorite solution does not account for survival of *E. faecalis*. Another possible reason could be strain variation – some strains of *E. faecalis* have been reported to survive low concentrations of sodium hypochlorite (Kearns *et al.* 1995). However, more likely explanations include the possibility that sodium hypochlorite solution occasionally may not reach places where *E. faecalis* may hide, such as in the dentinal tubules or the more inaccessible areas of the root canal system; or, that sodium hypochlorite is buffered by dentine (Haapasalo *et al.* 2000).

In summary, this study has confirmed that *E. faecalis* is resistant to killing by calcium hydroxide at or below pH 11.1. An adaptive response in alkaline pH and stress-induced protein synthesis appear to play minor roles in cell survival, however, a functioning proton pump with the capacity to acidify the cytoplasm is critical for survival of *E. faecalis* at high pH. *E. faecalis* is efficiently killed by sodium hypochlorite; in fact, the concentrations used during clinical treatment are many orders of magnitude higher than that required for lethal action. These findings contribute to our understanding of the response by *E. faecalis* to these antimicrobial agents, yet eradication of *E. faecalis* from an infected root canal remains a significant clinical challenge for modern endodontic treatment.

Acknowledgements

This study was supported by grants from the Australian Society of Endodontology Inc, the Australian Society of Endodontology (Vic Branch), the Australian Dental Research Foundation Inc and Melbourne University.

References

- Bearson B, Wilson L, Foster J (1998) A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. *Journal of Bacteriology* **180**, 2409–17.
- Booth I (1985) Regulation of cytoplasmic pH in bacteria. *Microbiological Reviews* **49**, 359–78.
- Booth I (1999) The regulation of intracellular pH in bacteria. *Novartis Foundation Symposium* **221**, 19–37.
- Boutibonnes P, Giard J, Hartke A, Thammavongs B, Auffray Y (1993) Characterization of the heat shock response in *Enterococcus faecalis*. *Antonie Van Leeuwenhoek* **64**, 47–55.
- 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.
- Castanie-Cornet M-P, Penfound T, Smith D, Elliott J, Foster J (1999) Control of acid resistance in *Escherichia coli*. *Journal of Bacteriology* **181**, 3525–35.
- Devriese L, Collins M, Wirth R (1992) The Genus *Enterococcus*. In: *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn, pp. 1465–81. New York, USA: Springer-Verlag.
- Flahaut S, Benachour A, Giard J-C, Boutibonnes P, Auffray Y (1996a) Defence against lethal treatments and de novo protein synthesis induced by NaCl in *Enterococcus faecalis* ATCC 19433. *Archives of Microbiology* **165**, 317–24.
- Flahaut S, Hartke A, Giard J-C, Auffray Y (1997) Alkaline stress response in *Enterococcus faecalis*: Adaptation, cross-protection, and changes in protein synthesis. *Applied and Environmental Microbiology* **63**, 812–4.
- Flahaut S, Hartke A, Giard J-C, Benachour A, Boutibonnes P, Auffray Y (1996b) Relationship between stress response towards bile salts, acid and heat treatment in *Enterococcus faecalis*. *FEMS Microbiology Letters* **138**, 49–54.
- Foster J, Hall H (1990) Adaptive acidification tolerance response of *Salmonella typhimurium*. *Journal of Bacteriology* **172**, 771–8.
- Foster J, Spector M (1995) How *Salmonella* survive against the odds. *Annual Review of Microbiology* **49**, 145–74.
- Giard J-C, Hartke A, Flahaut S, Benachour A, Boutibonnes P (1996) Starvation-induced multiresistance in *Enterococcus faecalis* JH2-2. *Current Microbiology* **32**, 264–71.
- Giard J-C, Hartke A, Flahaut S, Boutibonnes P, Auffray Y (1997) Glucose starvation response in *Enterococcus faecalis* JH2-2: survival and protein analysis. *Research in Microbiology* **148**, 27–35.
- Haapasalo M, Ørstavik D (1987) *In vitro* infection and disinfection of dentinal tubules. *Journal of Dental Research* **66**, 1375–9.
- Haapasalo H, Sirén E, Waltimo T, Ørstavik D, Haapasalo M (2000) Inactivation of local root canal medicaments by dentine: an *in vitro* study. *International Endodontic Journal* **33**, 126–31.

- Hall H, Karem K, Foster J (1995) Molecular responses of microbes to environmental pH stress. *Advances in Microbial Physiology* **37**, 229–72.
- Hancock HH, Sigurdsson A, Trope M, Moiseiwitsch J (2001) Bacteria isolated after unsuccessful endodontic treatment in a North American population. *Oral Surgery Oral Medicine Oral Pathology, Oral Radiology and Endodontics* **91**, 579–86.
- Hartke A, Giard J-C, Laplace J-M, 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.
- Heling I, Chandler N (1998) Antimicrobial effect of irrigant combinations within dentinal tubules. *International Endodontic Journal* **31**, 8–14.
- Jacob A, Hobbs S (1974) Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. zymogenes. *Journal of Bacteriology* **117**, 360–72.
- Jouper-Jaan Å, Goodman A, Kjelleberg S (1992) Bacteria starved for prolonged periods develop increased protection against lethal temperatures. *FEMS Microbiology Ecology* **101**, 229–36.
- Kakinuma Y (1987) Lowering of cytoplasmic pH is essential for growth of *Streptococcus faecalis* at high pH. *Journal of Bacteriology* **169**, 4403–5.
- Kakinuma Y (1998) Inorganic cation transport and energy transduction in *Enterococcus hirae* and other streptococci. *Microbiology and Molecular Biology Reviews* **62**, 1021–45.
- Kakinuma Y, Igarashi K (1990) Mutants of *Streptococcus faecalis* sensitive to alkaline pH lack Na⁺-ATPase. *Journal of Bacteriology* **172**, 1732–5.
- Kakinuma Y, Igarashi K (1995) Potassium/proton antiport system of growing *Enterococcus hirae* at high pH. *Journal of Bacteriology* **177**, 2227–9.
- Kakinuma Y, Igarashi K (1999) Isolation and properties of *Enterococcus hirae* mutants defective in the potassium/proton antiport system. *Journal of Bacteriology* **181**, 4103–5.
- Kalfas S, Figdor D, Sundqvist G (2001) A new bacterial species associated with failed endodontic treatment: Identification and description of *Actinomyces radidentis*. *Oral Surgery Oral Medicine Oral Pathology, Oral Radiology and Endodontics* **92**, 208–14.
- Kearns A, Freeman R, Lightfoot N (1995) Nosocomial enterococci: resistance to heat and sodium hypochlorite. *Journal of Hospital Infection* **30**, 193–9.
- Kinoshita N, Unemoto T, Kobayashi H (1984) Proton motive force is not obligatory for growth of *Escherichia coli*. *Journal of Bacteriology* **160**, 1074–7.
- Koppang H, Koppang R, Solheim T, Aarnes H, Stølen S (1989) Cellulose fibers from endodontic paper points as an etiological factor in postendodontic periapical granulomas and cysts. *Journal of Endodontics* **15**, 369–72.
- Laplace J-M, Thuault M, Hartke A, Boutibonnes P, Auffray Y (1997) Sodium hypochlorite stress in *Enterococcus faecalis*: Influence of antecedent growth conditions and induced proteins. *Current Microbiology* **34**, 284–9.
- Miñana M, Carnes D, Walker W III (2001) pH changes at the surface of root dentin after intracanal dressing with calcium oxide and calcium hydroxide. *Journal of Endodontics* **27**, 43–5.
- Molander A, Reit C, Dahlén G, Kvist T (1998) Microbiological status of root-filled teeth with apical periodontitis. *International Endodontic Journal* **31**, 1–7.
- Möller Å (1966) Microbiological examination of root canals and periapical tissues of human teeth. *Odontologisk Tidskrift (special issue)* **74**, 1–380.
- Mugikura S, Nishikawa M, Igarashi K, Kobayashi H (1990) Maintenance of a neutral cytoplasmic pH is not obligatory for growth of *Escherichia coli* and *Streptococcus faecalis* at an alkaline pH. *Journal of Biochemistry* **108**, 86–91.
- Mundt J (1986) Enterococci. In: *Bergey's Manual of Systematic Bacteriology*, Vol. 2, pp. 1063–5. Baltimore, USA: Williams & Wilkins.
- Nair P, Schroeder H (1984) Periapical actinomycosis. *Journal of Endodontics* **10**, 567–70.
- Nair P, Sjögren U, Figdor D, Sundqvist G (1999) Persistent periapical radiolucencies of root-filled human teeth, failed endodontic treatments, and periapical scars. *Oral Surgery Oral Medicine Oral Pathology, Oral Radiology and Endodontics* **87**, 617–27.
- Nair P, Sjögren U, Krey G, Kahnberg K-E, Sundqvist G (1990a) Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. *Journal of Endodontics* **16**, 580–8.
- Nair P, Sjögren U, Krey G, Sundqvist G (1990b) Therapy-resistant foreign body giant cell granuloma at the periapex of a root-filled human tooth. *Journal of Endodontics* **16**, 589–95.
- Nair P, Sjögren U, Schumacher E, Sundqvist G (1993) Radicular cyst affecting a root-filled human tooth: a long-term post-treatment follow-up. *International Endodontic Journal* **26**, 225–33.
- Nerwich A, Figdor D, Messer H (1993) pH changes in root dentin over a 4-week period following root canal dressing with calcium hydroxide. *Journal of Endodontics* **19**, 302–6.
- Noda M, Komatsu H, Inoue S, Sano H (2000) Antibiotic susceptibility of bacteria detected from the root canal exudate of persistent apical periodontitis. *Journal of Endodontics* **26**, 221–4.
- Ørstavik D, Haapasalo M (1990) Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Endodontics and Dental Traumatology* **6**, 142–9.
- Parsell D, Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annual Review of Genetics* **27**, 437–96.
- Peters L, Wesselink P, Moorer W (2000) Penetration of bacteria in bovine root dentine *in vitro*. *International Endodontic Journal* **33**, 28–36.
- Safavi K, Spångberg L, Langeland K (1990) Root canal dentinal tubule disinfection. *Journal of Endodontics* **16**, 207–10.
- Sato T, Hoshino E, Uematsu H, Noda T (1993) Predominant obligate anaerobes in necrotic pulps of human deciduous teeth. *Microbial Ecology in Health and Disease* **6**, 269–75.
- Siqueira J, de Uzeda M (1996) Disinfection by calcium hydroxide

- pastures of dentinal tubules infected with two obligate and one facultative anaerobic bacteria. *Journal of Endodontics* **22**, 674–6.
- Siqueira J, Machado A, Silveira R, Lopes H, de Uzeda M (1997) Evaluation of the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of *Enterococcus faecalis* from the root canal, *in vitro*. *International Endodontic Journal* **30**, 279–82.
- Sjögren U, Happonen R, Kahnberg K, Sundqvist G (1988) Survival of *Arachnia propionica* in periapical tissue. *International Endodontic Journal* **21**, 277–82.
- Sundqvist G, Figdor D, Persson S, Sjögren 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.
- Tanriverdi F, Esener T, Erganiş, Belli S (1997) An *in vitro* test model for investigation of disinfection of dentinal tubules infected with *Enterococcus faecalis*. *Brazilian Dental Journal* **8**, 67–72.
- Thammavongs B, Corroler D, Panoff J-M, Auffray Y, Bouttibonnes P (1996) Physiological response of *Enterococcus faecalis* JH2-2 to cold shock: growth at low temperatures and freezing/thawing challenge. *Letters in Applied Microbiology* **23**, 398–402.
- Vahdaty A, Pitt Ford T, Wilson R (1993) Efficacy of chlorhexidine in disinfecting dentinal tubules *in vitro*. *Endodontics and Dental Traumatology* **9**, 243–8.
- Waltimo T, Sirén E, Ørstavik D, Haapasalo M (1999) Susceptibility of oral *Candida* species to calcium hydroxide *in vitro*. *International Endodontic Journal* **32**, 94–8.
- Waltimo T, Sirén E, Torkko HL, Olsen I, Haapasalo MP (1997) Fungi in therapy-resistant apical periodontitis. *International Endodontic Journal* **30**, 96–101.
- Wang J, Hume W (1988) Diffusion of hydrogen ion and hydroxyl ion from various sources through dentin. *International Endodontic Journal* **21**, 17–26.
- Weiger R, Manncke B, Werner H, Löst C (1995) Microbial flora of sinus tracts and root canals of non-vital teeth. *Endodontics and Dental Traumatology* **11**, 15–9.
- Welch W (1993) How cells respond to stress. *Scientific American* **268**, 56–64.