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Response to alkaline stress by root canal bacteria in biofilms

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Abstract

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Aim To determine whether bacteria isolated from infected root canals survive alkaline shifts better in biofilms than in planktonic cultures.

Methodology Clinical isolates of *Enterococcus faecalis*, *Lactobacillus paracasei*, *Olsenella uli*, *Streptococcus anginosus*, *S. gordonii*, *S. oralis* and *Fusobacterium nucleatum* in biofilm and planktonic cultures were stressed at pH 10.5 for 4 h, and cell viability determined using the fluorescent staining LIVE/DEAD *Bac*Light bacterial viability kit. In addition, proteins released into extracellular culture fluids were identified by Western blotting.

Results *Enterococcus faecalis*, *L. paracasei*, *O. uli* and *S. gordonii* survived in high numbers in both planktonic cultures and in biofilms after alkaline challenge. *S. anginosus*, *S. oralis* and *F. nucleatum* showed increased viability in biofilms compared with planktonic cultures. Alkaline exposure caused all planktonic cultures to aggregate into clusters and resulted in a greater extrusion of cellular proteins compared with cells in biofilms. Increased levels of DnaK, HPr and fructose-1,6-bisphosphate aldolase were observed in culture fluids, especially amongst streptococci.

Conclusions In general, bacteria isolated from infected roots canals resisted alkaline stress better in biofilms than in planktonic cultures, however, planktonic cells appeared to use aggregation and the extracellular transport of specific proteins as survival mechanisms.

Keywords: apical periodontitis, bacterial stress, DnaK, HPr, root canal infection.

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Introduction

Inflammatory lesions in the periapical tissues usually occur as a result of root canal infection following partial or total breakdown of the pulp (Happonen & Bergenholtz 2003). It is well known that infected root canals can harbour an assortment of organisms that will provoke such lesions (Bergenholtz 1974, Sundqvist 1976). To resolve apical periodontitis, root canal treatment aims to eliminate the infectious elements residing within the root canal system. Such a treatment usually includes thorough mechanical instrumentation combined with the use of disinfectants as irrigating solutions and as intra-canal dressings between appointments (Byström 1986). Although bacteria are not expected to survive these procedures, clinical trials using bacterial culture tests have shown that complete bacterial elimination is not always feasible (Haapasalo *et al.* 2003).

Gram-positive facultative anaerobes, such as, *Streptococcus*, *Lactobacillus* and *Enterococcus* seem able to survive root canal treatment more readily than species of the anaerobic segment of the original microbiota (Chávez de Paz *et al.* 2003). This observation may reflect an increased capacity of these organisms to

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overcome changes in their environment induced by the treatment measures, including reduced nutritional supply, changed redox potential and the influence of antimicrobials (Sundqvist & Figdor 2003). Recently, considerable interest has been devoted to the ability of certain organisms, especially *Enterococcus faecalis*, to withstand extreme pH changes produced by the release of hydroxyl ions from intra-canal calcium hydroxide dressings (Byström *et al.* 1985, Gomes *et al.* 2001, Sukawat & Srisuwan 2002, Lynne *et al.* 2003). Although a direct physical contact with this caustic chemical at high concentrations will be lethal for any bacterial cell (Gordon *et al.* 1985), survival at sub-lethal concentrations may be a function of protective stress mechanisms, which are triggered by the environmental conditions. Knowledge of the mechanisms which allow microorganisms to remain viable in such alkaline environments is scant (Saito & Kobayashi 2003). The presence of acid-tolerant organisms in the microflora prevailing after calcium hydroxide medication suggests that the survival mechanism to alkaline stress is part of a general adaptive survival response. This may include the activation of ion-transport systems to balance intracellular and external pH levels. Indeed, it has been observed that proton-motive force is activated in *E. faecalis* in response to high pH (Booth & Kroll 1983, Booth 1985, Hall *et al.* 1995, Evans *et al.* 2002), and in *Bacillus* species responding to both alkaline and acid stress (Krulwich & Guffanti 1992, Cotter & Hill 2003).

In addition, pH changes in the environment may trigger the release of common cytosolic proteins outside the cell. Here, proteins will be expressed on the outer surface of the cell (Lewthwaite *et al.* 1998) and/or released into the environment (Nelson *et al.* 2001). For example, in *S. gordonii* the cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was primarily surface-associated when the organism was growing at pH 6.5, and shifted to >90% secreted in the culture supernatants at growth pH 7.5 (Nelson *et al.* 2001). Wilkins *et al.* (2003) reported that in *S. oralis* a shift from a neutral media (pH 7.0) to acidic conditions (pH 5.2) triggered the differential expression of 11 different surface-associated proteins. Amongst these proteins, GAPDH was downregulated after the acidic stress. Indeed, protein secretion is not only linked with pH changes, but also occurs under other environmental shifts. For example, the human pathogen *S. pyogenes* has been found to secrete ADP-ribosylating protein and GAPDH in response to iron starvation (Eichenbaum *et al.* 1996). Whilst the virulent potentials of these secreted proteins are not discarded, the mechanism behind these secretions *per se* is a consistent marker that uncovers a highly coordinated physiological regulation in response to alterations in the milieu.

Examinations of extracted teeth with periapical lesions have confirmed the presence of microbial biofilm structures on root canal walls (see review by Svensäter & Bergenholtz 2004). For example, when sections were viewed by transmission electron microscopy, dense aggregates of cocci and rods embedded in an extracellular matrix were observed along the walls (Nair 1987). The use of scanning electron microscopy to localize root canal bacteria demonstrated micro-colonies of cocci, rods and filaments on the root canal walls (Molven *et al.* 1991, Sen *et al.* 1995). Furthermore, biofilms in root canals have been experimentally produced by use of monocultures of *E. faecalis* as well as mixtures of anaerobes. Thus, there is good reason to believe that biofilm formation in the root canal system is the rule rather than the exception.

There is a growing body of evidence that microorganisms grown in biofilms are phenotypically different from the same microorganism grown in liquid culture (Goodman & Marshall 1985, Costerton *et al.* 1987, Fletcher 1991). Most importantly, biofilm bacteria exhibit increased resistance to antimicrobial agents with some reports showing that biofilm bacteria can be up to 1000-fold more resistant (Gilbert *et al.* 1997, Johnson *et al.* 2002). Biofilms of oral bacteria have been found to be more resistant to chlorhexidine, amine fluoride, amoxycillin, doxycycline and metronidazole (Shani *et al.* 2000, Larsen 2002), although the mechanisms associated with such resistance have not yet been elucidated. With respect to endodontic treatment procedures involving calcium hydroxide medication, the susceptibility of biofilm bacteria to alkaline stress is of prime interest. As a consequence, the purpose of this study was to explore stress responses that may allow common clinical isolates from persisting root canal infections to survive alkaline shifts. The research was designed to observe whether differences existed in cell viability and extracellular protein transport between planktonic and biofilm cultures of the same organism grown *ex vivo*.

Material and methods

Bacterial isolates and growth media

The clinical strains employed in this study were *E. faecalis*, *Lactobacillus paracasei*, *Olsenella uli*, *Streptococcus anginosus*, *S. gordonii*, *S. oralis* and *Fusobacterium*

nucleatum, isolated in a previous study from samples of root canals that had been exposed to calcium hydroxide medication for at least 1 week (Chávez de Paz *et al.* 2003). The isolates had been identified to the species level as previously described (Chávez de Paz *et al.* 2004, 2005) and stored at −70 °C employing the CRYOBANK™ system (Copan Diagnostics Inc., Corona, CA, USA). The strains were recovered on Brucella agar medium (BBL Microbiological Systems, Cockeysville, MD, USA), with cultures of *E. faecalis* and streptococci incubated in an atmosphere of 5% CO_2 in nitrogen, whilst *L. paracasei*, *O. uli* and *F. nucleatum* were incubated anaerobically in an atmosphere of 10% H_2, 10% CO_2 in nitrogen at 37 °C for 24–48 h.

The liquid growth medium used throughout this study was the peptone-yeast extract-glucose medium (PYG) (Holdeman *et al.* 1977) supplemented with 10 mM potassium phosphate buffer (pH 7.0). In the experiments assessing bacterial survival after alkaline shock, the pH values of PYG were adjusted to pH 7, 8, 9, 10, 10.5, 11 and 12 using 8 N KOH. For all cultures, the pH was tested after incubation to ensure that pH was maintained at ±0.2 unit of the noninoculated medium. The incubation conditions for growth were those described above.

Determination of sub-lethal pH-values

For each strain, 100 μL aliquots of an overnight culture were transferred to 10 mL fresh PYG (pH 7.0) and incubated at 37 °C until the mid-exponential phase of growth was reached (OD ≈0.6–1.0), as measured by monitoring the optical density at 600 nm in a spectrophotometer for 6–12 h. The cells were collected at the middle of the exponential phase by centrifugation at 3000 × *g* (10 °C, 15 min), washed and re-suspended in potassium phosphate buffer (pH 7.0). Aliquots of 100 μL (≈5×10^8 cells) were inoculated into 10 mL of fresh medium buffered at pH 7, 8, 9, 10, 11 and 12 and incubated at 37 °C for 2, 6 and 24 h followed by diluting and plating on Brucella agar. After 2–5 day, the number of colony forming units (CFU) was semi-quantified according to the scale described by Dahlén *et al.* (1982) where 1, 1–10 CFU; 2, 11–100 CFU; 3, 101–1000 CFU; 4, 1001–10 000 CFU; and 5, >10 000 CFU. Each growth experiment was performed in triplicate for each strain and the viable cell counts collected at each time period statistically analysed using one-sample *t*-test to calculate mean ranges with 95% confidence intervals using the software SPSS for Windows (SPSS Inc., Chicago, IL, USA).

Surface-associated culture conditions

Biofilm cultures were generated on flat-bottomed wells in polystyrene micro-titre plates (6 × 15 mL; Becton Dickinson Labware, Lincoln Park, NJ, USA). Each well was inoculated with 10 mL of an exponential-phase cell suspension (≈ × 10^6 cells mL^{-1}) prepared as described above and the plates incubated for 20 h at 37 °C under the atmospheric conditions described above. The wells were carefully rinsed three times with distilled water to remove nonadherent cells and then divided into two lots: those receiving 10 mL of fresh growth medium buffered at pH 7.0 (control) and those receiving medium buffered at pH 10.5 (test wells). The micro-titre plates were then incubated at 37 °C for 4 h.

Bacterial viability

Viable counts of planktonic cells were obtained by plating aliquots on Brucella agar in triplicate as described above. The percentage live cells was determined using the LIVE/DEAD *Bac*Light bacterial viability kit (LIVE/DEAD) (Molecular Probes, Inc., Eugene, OR, USA) as outlined by the manufacturer. Briefly, aliquots of 10 μL cell culture were incubated with a mixture of SYTO 9 and propidium iodide in the dark at room temperature (25 °C) for 10 min. For each culture sample, the proportion of live cells (fluorescent green) and dead cells (fluorescent red) was estimated from 8 to 10 microscopic fields (at 1000×) using a Nikon microscope equipped with a halogen lamp and a 470–490 nm excitation filter. The fluorescent microscopic images were captured with a digital camera connected to the microscope. For averaging the proportion of total cell counts and viable bacteria (stained fluorescent green) in separate fields (viable bacteria/total amount of cells ×100), the software MATLAB, v.7.0 for Windows was used. The viability of biofilm cells was assessed by rinsing each micro-titre plate with distilled water followed by the addition of the two LIVE/DEAD fluorescent dyes. After incubation for 10 min, direct microscopic images of adherent cell cultures were documented and analysed in the same manner as planktonic cultures.

Analysis of extracellular proteins

The extracellular protein profiles generated by control and alkaline-stressed cells were assessed with both planktonic and biofilm cultures. The culture fluids were collected by centrifugation (3000 × *g*, 15 min) with

the resulting supernatant passed through a 0.2 μm filter to remove any remaining cells. The concentration of proteins in the cell-free culture fluids was determined with the Protein Determination Assay (Bio-Rad Laboratories, Hercules, CA, USA) based on Bradford's analysis (Bradford 1976). Concentrations were calculated by linear regression using the software provided by Fluostar Optima (BMG Labtechnologies, Offenburg, Germany). Triplicates of cell-free culture fluids were stored at −20 °C until subjected to one-dimensional electrophoresis [sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)] and two-dimensional electrophoresis (2-DE).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out as described previously (Chávez de Paz *et al.* 2005) with 10% polyacrylamide gels in 0.05 mol L^{-1} Tris, 0.4 mol L^{-1} glycine and 3.5 mmol L^{-1} SDS (pH 8.3) using the Mini Protean II electrophoresis system (Bio-Rad, Solna, Sweden). In all cases, the protein load in each well was 10 μg. Resolved proteins were visualized following staining with silver nitrate (Amersham Biosciences, Uppsala, Sweden) as described previously (Chávez de Paz *et al.* 2005). Dried gels were scanned (Canon N676U; Canon Inc., Tokyo, Japan) and band detection was carried out with the software package for imaging and analysing SDS-PAGE gels (Quantity One®; Bio-Rad Laboratories). The molecular weight of individual resolved proteins was estimated by comparison with broad-range molecular weight markers (Bio-Rad Laboratories). Three independent cultures were processed for each growth condition. For the comparison of the effect of elevated pH on the expression of extracellular protein, a more than twofold change of band intensity in each of three cultures was considered significant.

For 2-DE, the cell-free culture fluid was diluted in a sample buffer containing 8 mol L^{-1} urea, 2% Chaps (ICN Biomedicals Inc., Costamesa, CA, USA), 10 mmol L^{-1} dithiothreitol (DTT) (ICN Biomedicals Inc.) and 2% IPG-buffer (Amersham Biosciences) to give a protein concentration of 1 mg mL^{-1} and used to re-hydrate 7 cm linear immobilized pharmalyte gradient gel strips covering the pH range from 4 to 7 (Amersham Biosciences). The first dimensional isoelectric focusing was performed on the Multiphor II (Amersham Biosciences) and the second dimension in 14% polyacrylamide gels as previously described (Welin *et al.* 2004). Dried, silver-stained gels were scanned (Canon N676U) and spot detection was carried out with Delta2D image software (Decodon, Greifswald, Germany). Three independent cultures were processed for each growth condition and visual comparisons were made between protein spots of neutral and alkaline cultures. An increase or decrease in the relative amount of a particular protein was recorded if the result was observed in all three gels. To ensure that proteins in the cell-free culture fluids were not a result of cell lysis, whole-cell extracts of each culture were prepared (Welin *et al.* 2004) and analysed by SDS-PAGE and 2-DE.

Western blot analysis

The proteins DnaK and HPr were identified using Western blot analysis with polyclonal antibodies raised in rabbits, kindly provided by Dr A. Bleiweis and Dr R. Burne, University of Florida, USA. Following 2-DE, the proteins were transferred electrophoretically to Immobilon membrane (Millipore Intertech, Bedford, MA, USA) overnight at 30 V using a Bio-Rad trans-blot cell. Blots were incubated with the primary antibodies following blocking of nonspecific binding sites with 5% nonfat milk in Tris-buffered saline, pH 7.4 (TBS). After a wash with 0.1% nonfat milk in TBS, blots were incubated for 2 h with horseradish peroxidase-conjugated goat-anti-rabbit-IgG (Dako, Copenhagen, Denmark) in 1% nonfat milk and 0.05% Tween 20 in TBS. After washing in TBS, the blots were developed in the dark using 1% peroxidase substrate (3-amino-9-ethylcarazole in acetone; Sigma Chemical Co., St Louise, MO, USA) in 50 mmol L^{-1} sodium acetate buffer, pH 5.0, and 0.015% H_2O_2. Development was stopped by rinsing the blots in water.

Results

Sub-lethal response zone

Initially, the viability of the test strains was determined in nutrient-rich media over a range of alkaline pH and times of exposure to determine the pH at which the bacteria were stressed, but not killed. The resulting CFU counts were organized in plot boxes (Fig. 1) where each plot box represents a time of exposure (2, 6 or 24 h) and includes the mean CFU values for all of the seven test strains. Within the plot boxes, the first in viability ranking was normally *E. faecalis*, followed by *L. paracasei*, *O. uli* and *S. gordonii* as the most tolerant strains, whilst *F. nucleatum* was always the least tolerant. The strains showed similar CFU counts up to pH 10 following a 2 h exposure, however, beyond this level viability began to decrease. At 6 h exposure, no

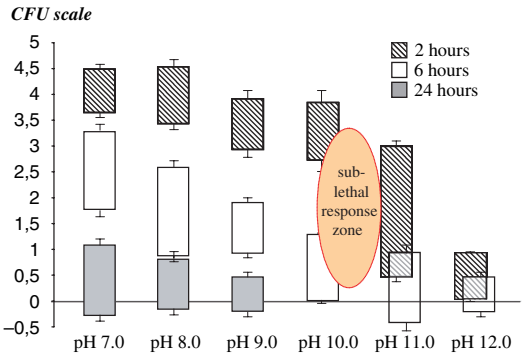

Figure 1 Box plots show the analyses of viable bacterial counts (CFU) at different pH exposures and time intervals. Figures are based on one-way *t*-tests with standard deviation margins. The sub-lethal response zone was determined to be between pH 10 and 11 (10.5) and between 2 and 6 h (4 h) (encircled zone).

growth was observed for *F. nucleatum* beyond pH 9. At pH 10, no growth was registered for *O. uli*, *S. anginosus*, *S. gordonii* and *S. oralis*. After 24 h, none of the strains grew after exposure above pH 8, with the exception of *E. faecalis* which grew up to pH 9. On the basis of these findings, it was determined that the 'sub-lethal response zone' for all strains was between 0 and 6 h, and at a pH level between 10 and 11 (delineated in Fig. 1). Future tests involved incubation at pH 10.5 for 4 h.

Effects of alkaline challenge on cell viability

The effect of alkaline pH on the viability of planktonic and biofilm cells was assessed using the LIVE/DEAD fluorescent staining method to determine the percentage of live and dead cells after 4 h incubations at pH 7.0 and 10.5. As seen in Table 1, planktonic cells of *E. faecalis*, *L. paracasei*, *O. uli* and *S. gordonii* were the least affected by alkaline exposure with a total number of viable green-stained cells of 81% (±3), 98% ± (2), 85% (±4) and 66% ± (7), respectively. On the other hand, *F. nucleatum* (1%), *S. anginosus* (3%) and *S. oralis* (13%) were significantly more vulnerable corresponding to approximately one on the CFU scale. The corresponding photomicrographs of the staining patterns for the planktonic cells can be seen in Fig. 2 for pH 7 (column A) and pH 10.5 (column B). The formation of cell clusters was a general phenomenon after alkaline exposure (see column B) with *S. anginosus* forming clusters at neutral pH.

The data in Table 1 and Fig. 2 (column C) indicate that, in general, biofilm cells have a greater capacity for survival at alkaline pH than planktonic cells. This can be seen by observing that all three of the most susceptible cells in planktonic culture, e.g. *F. nucleatum*, *S. anginosus* and *S. oralis*, survived to higher levels in biofilms with the latter two increasing from 3% and 13% to 84% and 48%, respectively. Whilst *F. nucleatum*, increased from 1% to 14% in biofilms, observation of the LIVE/DEAD staining (Fig. 2, column C) indicates that the culture was significantly stressed even in a biofilm. Two strains, *L. paracasei* and *O. uli*, were somewhat less resistant in biofilm culture falling from 98% and 85% survival in planktonic culture to 71% and 46% in biofilm culture, respectively.

Protein release at alkaline stress

To ascertain the extent of the protein released from cells during the alkaline shock with the test strains, the proteins in the extracellular fluid were collected and subjected to SDS-PAGE followed by band detection and quantification by image analysis. Since *F. nucleatum*

Table 1 Mean averages (%) of viable (green) cells, calculated from 8 to 10 micro-photographic images using the LIVE/DEAD (BacLight) fluorescent stain

	Planktonic cells		Biofilm cells	
	pH 7.0	pH 10.5	pH 7.0	pH 10.5
E. faecalis	100	81 ± 3	99 ± 1	96 ± 3
F. nucleatum	99 ± 1	1 ± 1	100	14 ± 2
L. paracasei	100	98 ± 2	98 ± 2	71 ± 5
O. uli	100	85 ± 4	87 ± 7	46 ± 2
S. anginosus	92 ± 2	3 ± 1	100	84 ± 6
S. gordonii	93 ± 3	66 ± 7	93 ± 5	64 ± 10
S. oralis	100	13 ± 4	84 ± 5	48 ± 5

Images were taken from planktonic cell cultures and cells growing in biofilms, at neutral pH (7.0) and after alkaline challenge (pH 10.5) (see Fig. 2). The standard deviation for the averages is shown after the ±sign.

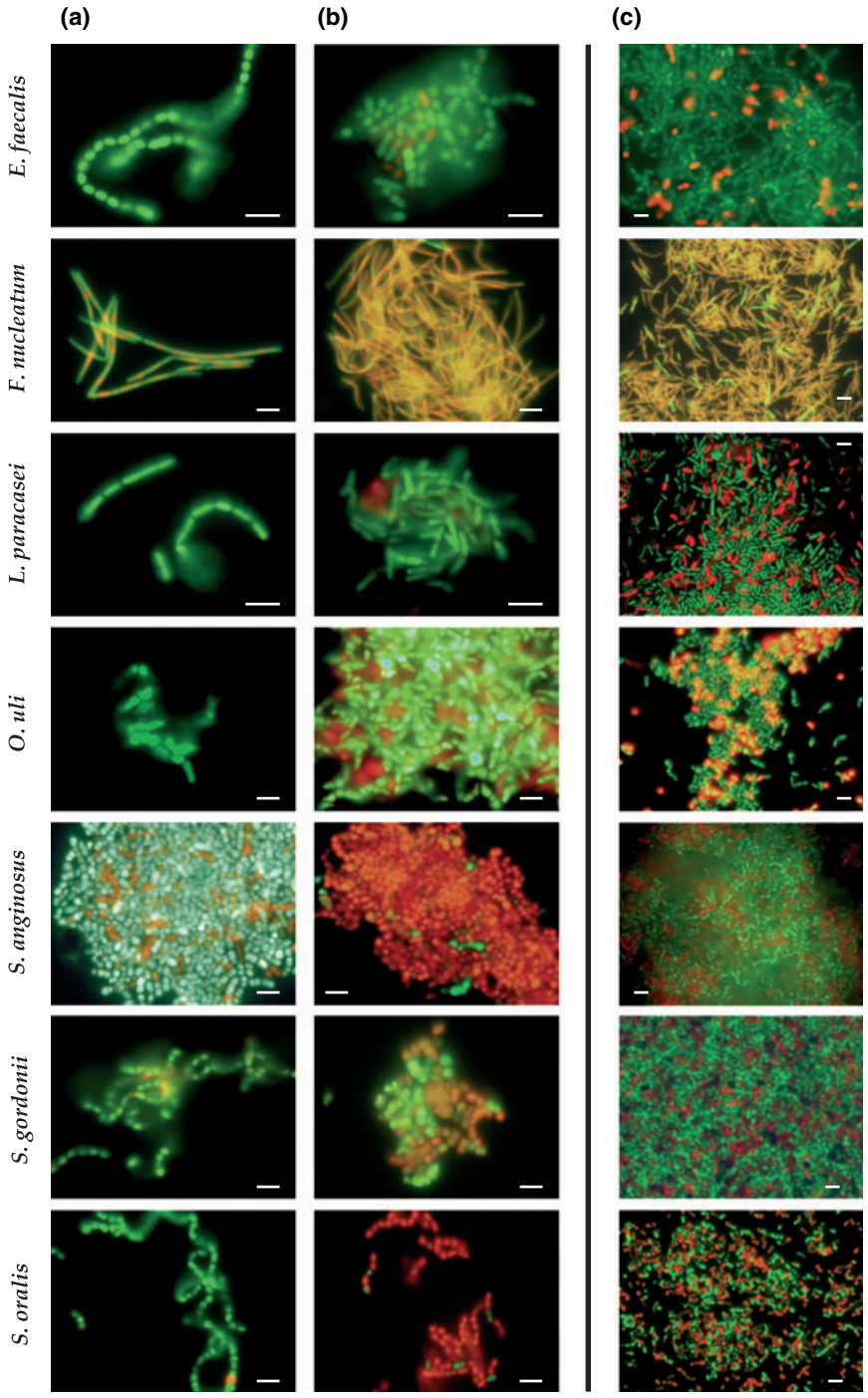

Figure 2 Fluorescence micrographs using LIVE/DEAD staining (magnification ×1000). Column (a) shows planktonic cells of the seven strains tested at neutral media (pH 7) (zoom×10). Column (b) shows planktonic cells after exposure to pH 10.5 for 4 h (zoom ×10), and column (c) show biofilm cells exposed to alkaline challenge (pH 10.5) for 4 h. Bars, 2 μm.

was a poor survivor in both cultures, it was excluded from the analysis. Fig. 3 compares the protein profiles from the SDS-PAGE gels of the pH 10.5 cultures with those of the same strain at the control pH of 7.0 and the results indicate great variation between the strains in the total number of proteins released from the cells.

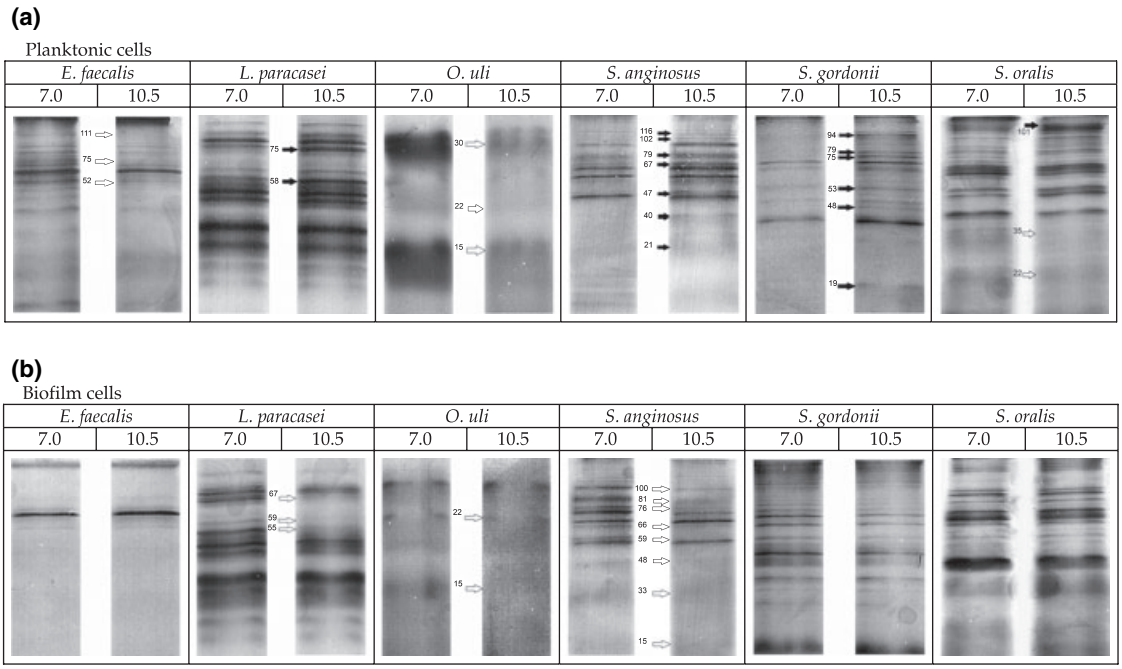

Figure 3 Silver stained SDS-PAGE patterns of extracellular proteins in cultures after 4 h exposure to pH 10.5 in planktonic (a) and biofilm cells (b). Black arrows indicate new proteins or proteins that were enhanced (≥2-fold) after alkaline exposure. White arrows indicate proteins which their intensity was diminished <2-fold. Numbers correspond to the molecular weight of the protein (kDa).

Analysis of the total protein released indicates that the amount in the extracellular fluid at pH 10.5 from all of the planktonic cells was greater (mean ratio = 3.28) than that released at pH 7.0 (Table 2). The biofilm cells, on the other hand, released less protein than the planktonic cells (mean ratio = 1.27) and in two cases, *S. gordonii* and *S. oralis*, the ratio of the proteins released under alkaline stress conditions at pH 10.5 was less than that a pH 7.0. As seen in Table 3, the analysis was simplified by identifying only those proteins whose release was increased or repressed by alkaline shock. Using this approach, the six planktonic cultures at pH 10.5 increased the release of a total of 16 proteins, the majority by *S. anginosus* (7) and *S. gordonii* (6), whilst three strains, *E. facealis*, *O. uli* and *S. oralis*, repressed the release of eight proteins. None of the biofilm cultures exhibited enhanced protein release at pH 10.5 and only three strains, *L. paracasei*, *O. uli*, and *S. anginosus*, showed repressed release of a total of 13 proteins with *S. anginosus* accounting for eight of such proteins. Clearly, the biofilm cells reacted differently to the pH 10.5 shock than the same cells in planktonic culture.

Table 2 Amount of proteins detected in supernatants of cell cultures in planktonic and biofilms growth

	Planktonic cells			Biofilm cells		
	pH 7.0	pH 10.5	Ratio 10.5/7.0	pH 7.0	pH 10.5	Ratio 10.5/7.0
E. faecalis	6.1*	28.8*	4.7	10.5*	30.4*	2.9
L. paracasei	4.1	15.3	3.7	7.2	8.1	1.1
O. uli	7.0	26.1	3.7	8.4	9.2	1.1
S. anginosus	5.2	18.2	3.5	7.0	8.6	1.2
S. gordonii	10.0	16.0	1.6	6.9	6.0	0.9
S. oralis	4.4	11.1	2.5	9.2	3.9	0.4

Readings were taken at neutral pH (7.0) and after alkaline stress (pH 10.5).
*$\mu g\ mL^{-1}$.

Table 3 Number of proteins identified in SDS-PAGE gels (see Fig. 3), which were enhanced or diminished at alkaline stress

	Planktonic cells (pH 10.5)		Biofilm cells (pH 10.5)	
	Enhanced	Diminished	Enhanced	Diminished
E. faecalis	0	3	0	0
L. paracasei	2	0	0	3
O. uli	0	3	0	2
S. anginosus	7	0	0	8
S. gordonii	6	0	0	0
S. oralis	1	2	0	0

Numbers are shown for planktonic and biofilm growth conditions.

Identity of altered proteins released

The identification of specific proteins released into the extracellular fluid by planktonic and biofilm cultures of *S. anginosus* following 4 h incubation at pH 7.0 and 10.5 was undertaken by 2-DE and Western blotting (Fig. 4). The question of whether proteins in the cell-free culture fluid were present due to cell lysis was resolved by comparing protein patterns of whole cell extracts and cell-free culture fluids. For each strain used in this study, such comparisons showed that common cytoplasmic proteins, such as lactate dehydrogenase and pyruvate oxidase, were not present in the corresponding culture fluid indicating that no significant cell lysis had occurred during the 4 h incubation periods. Similar experiments with other strains produced similar results.

For planktonic cells at pH 7.0, the proteins Dnak, Hpr, fructose-1,6-bisphosphate aldolase (FBA) and GAPDH were identified in culture fluids of the seven strains tested, predominantly amongst the streptococci (e.g. Fig. 4a). Under planktonic conditions, the proteins Dnak, Hpr and FBA increased ≥2-fold in the culture fluid after the increase of pH to 10.5 (Fig. 4b). No change was observed in the relative amount of GAPDH. For biofilm cells at neutral pH, the relative levels of Dnak, Hpr, FBA and GAPDH were the same as in planktonic cells exposed to alkaline conditions (Fig. 4c). Conversely, when biofilm cells were exposed to pH 10.5 the concentration of these proteins was reduced (Fig. 4d).

Discussion

Increasing scientific information indicates that survival of bacteria against antimicrobial agents and toxic environments is enhanced significantly by association with a surface in a biofilm compared with that of a

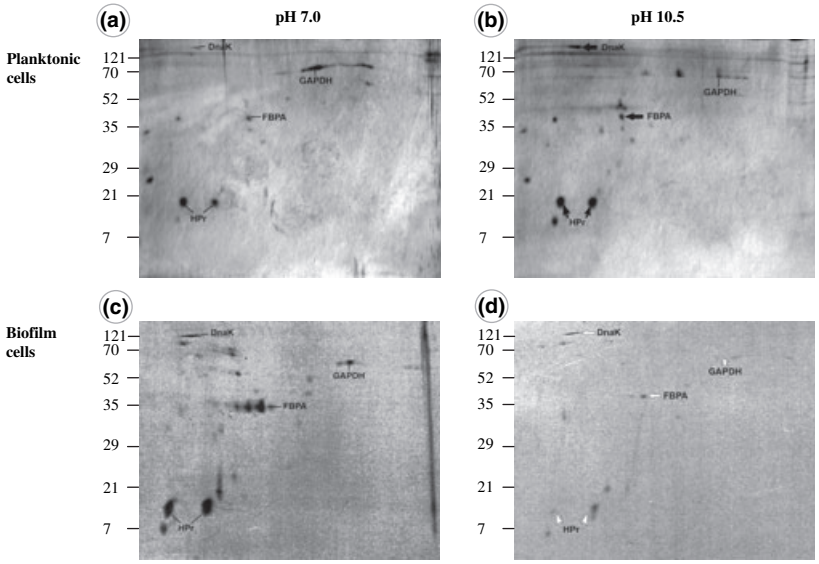

Figure 4 Two-dimensional electrophoresis (2-DE) images of proteins exported by *Streptococcus anginosus* in (a) planktonic conditions at (pH 7), (b) planktonic cells after alkaline exposure pH 10.5 for 4 h, (c) biofilms in neutral pH, and (d) biofilms after alkaline stress. The vertical axis represents molecular weight (Mw) in kDa. The proteins DnaK, GAPDH, FBA and HPr are showed. After alkaline exposure, black arrows indicate proteins that were enhanced ≥2-fold and white arrows indicate proteins diminished <2-fold.

liquid phase or planktonic culture (Costerton *et al.* 1999, Fux *et al.* 2005, Szomolay *et al.* 2005). This finding is particularly relevant to bacteria in the mouth as illustrated by recent research that has demonstrated that oral streptococci exhibit enhanced survival in low pH environments in biofilms (Svensäter *et al.* 2001). There is clear evidence that the physiology of bacterial cell in a biofilm differs from that of the same cell in planktonic phase (McNeill & Hamilton 2003) and that alkaline and acidic environments can signal cellular stress responses that can enhance survival (Stancik *et al.* 2002). Thus, it is realistic to imagine that the exposure of root canal bacteria to alkaline pH might trigger differential survival mechanisms between organisms in biofilms and those not associated with a surface. Therefore, the objective of the present study was to examine the survival of a selected group of oral bacteria in biofilms under alkaline stress and to observe the release of proteins during stress, a known response of bacteria to environmental change.

As seen in Table 1, the survival response of the test organisms in planktonic and biofilm culture to a shift in pH from 7.0 to 10.5 for 4 h varied, in some cases, considerably. Clearly, *E. faecalis* is an alkaline pH survivor whose viability was somewhat enhanced in biofilm culture, whereas, *F. nucleatum* was a poor survivor even in a biofilm. The most striking change was observed with *S. anginosus*, whose viability increased from 3% in planktonic culture to 84% in a biofilm. Survival of *S. oralis* was also impressive, increasing from 13% to 48% in the biofilm. On the other hand, *S. gordonii*, a relatively good survivor, was not influenced by the culture status, whilst *L. paracasei* and *O. uli* exhibited reduce survival in a biofilm. This evidence suggests that *S. anginosus* and *S. oralis* have a more flexible physiology that responds positively to biofilm growth with regard to shifts to alkaline pH, whilst *L. paracasei* and *O. uli* did not.

Visualization of the cultures under alkaline conditions by the LIVE/DEAD staining method was informative since it indicated that microbial aggregation appeared to be a natural response to alkaline stress by planktonic cells (Fig. 2, column B). However, the killing rate of *F. nucleatum*, *S. anginosus* and *S. oralis* indicates that such cell–cell aggregation was not always successful in enhancing survival. Microbial aggregation has been proposed to be a natural protective mechanism exercised by bacteria on exposure to harsh environmental conditions (Trueba *et al.* 2004). In a mechanism termed 'surface-sensing', a bacterial population exposed to an environmental change will sense and process the chemical information from their milieu and thereby adjust their phenotypic properties (Prigent-Combaret *et al.* 1999, Otto & Silhavy 2002). It is also possible that microbe-microbe adherence may have been triggered by signalling pathways involved in the quorum-sensing system (Parsek & Greenberg 2005, Suntharalingam & Cvitkovitch 2005).

In biofilms, the proportion of cells unaffected by the alkaline exposure (stained green) (Fig. 2, column C) was high, in agreement with several reports that have indicated that bacteria in biofilms are better able to withstand environmental change than planktonic cells (Costerton *et al.* 1999, Fux *et al.* 2005). One of the common explanations for this increased resistance is that biofilm cells become phenotypically different by exhibiting new and more effective physiological properties in biofilms (Stewart & Costerton 2001). Coupled to this is the concept of persister cells having unique qualities for survival (Bigger 1944, Moyed & Bertrand 1983, Lewis 2001, Spoering & Lewis 2001). The possibility for such cells in the alkaline-treated cell populations can be seen in the pH 10.5-planktonic cells of *S. anginosus*, where the percentage of red (dead) cells accounted for >90% of the cells (Fig. 2, column B). Within the total population a few green (live) cells existed in the bulk of the dead cells and may, in fact, represent a small group of persister cells. Various explanations suggest that they represent cells (a) in some protected part of their cell cycle, (b) are capable of rapid adaptation, (c) are in a dormant state, or (d) are unable to initiate programmed cell death in response to the stimulus (Lewis 2005). Thus, persister cells represent a recalcitrant sub-population that will not die and are capable of initiating a new population with normal susceptibility once the antibacterial effect has been dissipated (Balaban *et al.* 2004, Keren *et al.* 2004, Harrison *et al.* 2005).

As shown by SDS-PAGE (Fig. 3), a highly variable number of proteins was released by the planktonic and biofilm cells exposed to pH 7.0 and 10.5 in the present study with more total protein released by the planktonic cells than by the biofilm cells. This latter finding suggests that, upon exposure to alkaline conditions, the planktonic cells required increased cell–cell communication via extracellular proteins to trigger cellular aggregation in an attempt to enhance survival. Biofilms would not require such communication being in a more defensible position on the substratum surface. Furthermore, by comparing the *S. anginosus* 2-DE profiles in Fig. 4, it is apparent that the adherence of

the organism to the biofilm surface triggered the release of proteins at pH 7.0 that were released by the alkalization of the planktonic cells.

The most frequently identified proteins released from the alkaline-stressed cells were the phosphocarrier HPr, the heat-shock chaperone DnaK, FBA and GAPDH. All these proteins are housekeeping molecules functioning in the cytoplasm under normal circumstances. HPr is a protein of the sugar phosphotransferase system and has surface-associated functions such as in regulating sugar transport and signal transduction (Vadeboncoeur 1995). HPr has been suggested to participate actively in maintaining a neutral cytoplasmic pH upon acid stress. For example, in *Lactococcus lactis*, the cytoplasmic levels of HPr were reported to increase after exposure to pH 4.5 (Frees *et al.* 2003), whilst Wilkins *et al.* (2003) reported that HPr was closely associated with the cell surface of *S. oralis* and was the most abundant protein to be found at both neutral pH and after acid challenge. As reported in the current study, there is no previous evidence describing the export of this protein following alkaline challenge. In fact HPr has been only identified extracellularly in cultures of *S. pyogenes* (Gerlach *et al.* 1992). How HPr is exported and its physiological role outside the cell is presently unknown but presumably relates to its highly effective phosphotransferase capacity required for adaptation in times of environmental changes.

The intracellular expression of DnaK has been reported to be enhanced in *S. mutans* upon acid stress, hence the increased production of this chaperone was correlated with the physiological adaptation of this microorganism to acidic environments (Svensäter *et al.* 2001). Although reports on the presence of this protein outside the cell are scant, it has been reported in a previous study that the presence of this molecular chaperone extracellularly in supernatant cultures of different streptococcal strains isolated from teeth undergoing root canal treatment (Chávez de Paz *et al.* 2005). In the current study, these results were confirmed and it was further observed that the levels of extracellular DnaK were enhanced following alkaline stress in planktonic cultures. This finding may suggest the importance of this chaperone in bacterial adaptation to a variety of environmental changes.

Fructose-1,6-bisphosphate aldolase represents a group of very active enzymes that participate in basic metabolic pathways (for a review see Hall *et al.* 1999). The presence and enhanced expression following alkaline stress has not been previously reported but as the intracellular levels of FBA were also enhanced in planktonic cultures of *S. mutans* exposed to acid stress (Welin *et al.* 2003), it is likely that this enzyme participates in the physiologic adaptation of bacteria exposed to environmental challenges, especially when cells are growing in planktonic conditions.

Of the four proteins identified by Western blotting in supernatant cultures, GAPDH is the only one that was previously reported to be released extracellularly upon acid stress in *S. gordonii* (Nelson *et al.* 2001). Wilkins *et al.* 2003 also reported that the levels of surface-associated GAPDH were downregulated after acidic stress. These findings have given important clues as to the function of this enzyme in bacterial adaptation and stress response. However, the results of the current study suggest that the efflux of the protein is not specifically linked to alkaline stress, since the levels registered for GAPDH in planktonic and biofilm culture fluids were similar under both neutral and alkaline conditions.

Conclusion

This study has demonstrated that the test strains, when established in biofilms, are more resistant to alkaline than the same cells in fluid (planktonic) culture. Also noteworthy was the observation that organisms known to thrive under acidic conditions, e.g. *Streptococcus*, *Lactobacillus* and *F. nucleatum*, were also capable of adapting and surviving alkaline stress at pH 10.5 for 4 h. The exact mechanisms behind the survival of bacteria exposed to alkaline challenge were not completely established in this study; however, the release of proteins to the extracellular fluid phase coupled with cell-to-cell aggregation may play a role in microbial survival adaptation of cells in the planktonic phase. Such phenotypic modifications may enhance the survival capacity of sub-populations of cells. Populations in the root canal that are capable of surviving imposed endodontic treatment measures would thus remain in the environment once the effects of noxious stimuli have worn off.

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