Oral bacteria in biofilms exhibit slow reactivation from nutrient deprivation

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The ability of oral bacteria to enter a non-growing state is believed to be an important mechanism for survival in the starved micro-environments of the oral cavity. In this study, we examined the reactivation of nutrient-deprived cells of two oral bacteria in biofilms, *Streptococcus anginosus* and *Lactobacillus salivarius*. Non-growing cells were generated by incubation in 10 mM potassium phosphate buffer for 24 h and the results were compared to those of planktonic cultures. When both types of cells were shifted from a rich, peptone–yeast extract–glucose (PYG) medium to buffer for 24 h, dehydrogenase and esterase activity measured by the fluorescent dyes 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) and fluorescein diacetate (FDA), respectively, was absent in both species. However, the membranes of the vast majority of nutrient-deprived cells remained intact as assessed by LIVE/DEAD staining. Metabolic reactivation of the nutrient-deprived biofilm cells was not observed for at least 48 h following addition of fresh PYG medium, whereas the non-growing planktonic cultures of the same two strains were in rapid growth in less than 2 h. At 72 h, the *S. anginosus* biofilm cells had recovered 78 % of the dehydrogenase activity and 61 % of the esterase activity and the biomass mm⁻² had increased by 30–35 %. With *L. salivarius* at 72 h, the biofilms had recovered 56 % and 75 % of dehydrogenase and esterase activity, respectively. Reactivation of both species in biofilms was enhanced by removal of glucose from PYG, and *S. anginosus* cells were particularly responsive to yeast extract (YE) medium. The data suggest that the low reactivity of non-growing biofilm cells to the introduction of fresh nutrients may be a survival strategy employed by micro-organisms in the oral cavity.

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INTRODUCTION

Biofilms are accumulations of micro-organisms within a complex matrix that adhere to a surface; they are closely linked with the aetiology of diverse chronic and recurrent human infections (Davey & O'Toole, 2000), device-related infections and treatment failures (Gilbert *et al.*, 2002; Hall-Stoodley *et al.*, 2004). In the human mouth, there is an unrestricted formation of natural microbial biofilms on the various tooth surfaces, generally referred to as dental plaque, which are associated with the occurrence of oral diseases such as dental caries, different forms of periodontal disease and root canal infections (Bowden, 1991; Chávez de Paz, 2007; Marsh, 2005; Sansone *et al.*, 1993; Socransky & Haffajee, 2002).

The rate of growth of dental plaque on tooth surfaces is dependent on ecological determinants such as the available nutrients, the competing organisms in the community and

the environmental conditions in the biofilm at the time. Compared to many ecosystems, the nutrient supply available to bacteria in the oral cavity is more abundant and localized at times when the host consumes food (feast), although the period of time over which this occurs is much shorter than when no added nutrients are consumed (famine) (Carlsson & Johansson, 1973; De Jong & Van der Hoeven, 1987; Van der Hoeven & Camp, 1991). In other micro-environments of the oral cavity, such as root canals of teeth, nutrients are commonly scarce and the colonizing micro-organisms frequently need to survive under nutrient deprivation. Bacteria under the stress of starvation have developed efficient adaptive regulatory reactions to shift their metabolic balance away from biosynthesis and reproduction, toward the acquisition of energy for existing biological functions (Giard *et al.*, 1997; Kim *et al.*, 2001; Matin, 1990; Watson *et al.*, 1998). Cultures of *Escherichia coli*, *Streptococcus equisimilis*, *Bacillus subtilis* and *Streptococcus pyogenes* that are deprived of amino acids, carbon, glucose or iron induce a stringent response dependent on the synthesis of guanosine nucleotides [(p)ppGpp], which favour activities that facilitate adaptation to the nutritional stress (Chatterji & Ojha, 2001;

Abbreviations: CSLM, confocal scanning laser microscopy; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; EB, ethidium bromide; FDA, fluorescein diacetate; PBS, 10 mM potassium phosphate buffer; PY, peptone-yeast extract; PYG, peptone-yeast extract-glucose; YE, yeast extract.

Mechold & Malke, 1997; Miethke *et al.*, 2006; Steiner & Malke, 2000). Despite considerable speculation, information is scarce concerning starvation responses among oral bacteria, or whether such responses are stimulated in the oral environment (Bowden & Li, 1997; Bowden & Hamilton, 1998).

From the physiological perspective, nutrient deprivation causes bacteria to reversibly switch to a state of metabolic arrest (Amy & Morita, 1983; Stevenson, 1977). At these very low metabolic rates, bacteria will survive a wide range of environmental threats, in addition to deprivation of nutrients, such as temperature shifts and extreme pH changes, as well as exhibiting decreased sensitivity to antimicrobial agents (Oliver, 1995). When the nutrient supply is favourable again, the stress response is released and the bacteria resume metabolic activity and cell division. A large amount of RNA and protein appears to be degraded rapidly at the onset of starvation (Jenkins *et al.*, 1988), which is believed to be part of a general stress response (GSR) that is connected to survival responses in changing environments like the oral cavity (Brown & Smith, 2001; Foley *et al.*, 1999).

Low metabolism of bacteria in biofilms has also been suggested for survival of oral bacteria. In a mixed continuous culture of *Streptococcus mutans* and *Lactobacillus casei* receiving high levels of fluoride (20 mM), the growth of *S. mutans* was inhibited below the level of detection and yet the organism survived for 5 days in the presence of fluoride, presumably in a biofilm on the surfaces of the chemostat (Bowden & Hamilton, 1989). In the present study, we investigated two clinical isolates from the human mouth, *Streptococcus anginosus*, a predominant streptococcus in subgingival plaque (Frandsen *et al.*, 1991), and *Lactobacillus salivarius*, a common commensal organism in dental plaque (Chávez de Paz *et al.*, 2004). Both species are persistent survivors in infected root canals of teeth even after several treatment sessions (Chávez de Paz *et al.*, 2004, 2005). The aim was to examine reactivation from non-growing cells in biofilm and planktonic cultures. For this, non-growing cultures of *L. salivarius* and *S. anginosus* were generated by nutrient deprivation in buffer and then provided with fresh nutrients in order to assess growth and metabolic reactivation. Membrane integrity and metabolic fluorescent staining analyses revealed significant differences in the physiological response of the biofilm and planktonic cultures of the same organism.

METHODS

Bacterial strains and growth conditions. *Streptococcus anginosus* was isolated from infected root canals of teeth (Chávez de Paz *et al.*, 2003) and identified using the primary tests and criteria described in a previous study (Chávez de Paz *et al.*, 2005). This strain was negative for β -N-acetylglucosaminidase, negative for α -arabinosidase, positive for β -glucosidase, negative for acid production from inulin and positive to arginine hydrolysis. *Lactobacillus salivarius* was isolated

from supra-gingival dental plaque (Chávez de Paz *et al.*, 2004) and identified to species level as described in a previous study (Chávez de Paz *et al.*, 2004). Briefly, the strain was selectively grown in Rogosa medium, tested for the fermentation of rhamnose and its metabolic products were analysed by gas-liquid chromatography. SDS-PAGE patterns of whole-cell proteins were matched with those of type strains used as positive controls. Strains were stored at -70°C using skim milk powder (Oxoid) diluted in double distilled water. Strains were recovered on blood agar in an atmosphere of 5% CO_2 in hydrogen at 37°C for 24 h.

To prepare the inoculum, colonies were transferred into liquid growth medium peptone–yeast extract–glucose (PYG) (Holdeman *et al.*, 1977), supplemented with 10 mM potassium phosphate buffer (PBS), pH 7.5, and incubated in an atmosphere of 5% CO_2 in hydrogen at 37°C overnight. Aliquots (500 μl) were transferred to 10 ml fresh PYG (pH 7.5) and incubated at 37°C ; the OD_{600} was monitored until late exponential growth was reached. Normal OD_{600} values at late exponential phase were 0.8 ± 0.1 for *S. anginosus* and 0.5 ± 0.1 for *L. salivarius*. Cells were harvested by centrifugation (3000 g, 5 min at 4°C), washed in 10 mM PBS and resuspended in PYG to a cell concentration of $1 \times 10^8 \text{ ml}^{-1}$.

Biofilm formation. The flow-chamber system μ -Slide VI for Live Cell Analysis (Integrated BioDiagnostics) (Fig. 1a), with working volumes of 150 μl , was used to create the biofilms used in the study. Each flow-chamber was inoculated with 30 μl of a washed suspension of cells grown to the late exponential phase in PYG, followed by addition of 100 μl of fresh PYG to give a final volume of 130 μl per flow-chamber. Flow chambers were incubated in an atmosphere of 5% CO_2 in hydrogen at 37°C and biofilm formation was monitored every hour by direct microscopic observation. After 24 h of biofilm growth, chambers were rinsed with PBS to remove non-adherent cells.

LIVE/DEAD, CTC and FDA/EB staining. Cell samples were stained separately for membrane integrity, dehydrogenase activity and esterase activity with fluorescent dyes. Hence, three triplicate sets of biofilms and planktonic samples were cultured under the same conditions, each set having its own unique total cell count. The LIVE/DEAD BacLight Bacterial Viability kit for microscopy (product no. L7007, Molecular Probes), was used. The LIVE/DEAD mixture was prepared by mixing components A + B in the kit, consisting of SYTO9 and propidium iodide, at a ratio of 1 : 100 with the inoculum medium (PYG or PBS). For planktonic cultures, 10 μl aliquots of the harvested–washed cells were incubated with 10 μl LIVE/DEAD mixture in the dark for 15 min at room temperature (25°C). For biofilms, 30 μl of the LIVE/DEAD mixture was added after removing the culture fluid from the flow-chamber and washing with PBS. Chambers were incubated at room temperature for 15 min. The fluorescence from stained cells was viewed using confocal scanning laser microscopy (CSLM) (see below).

The redox fluorescence dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Molecular Probes), which targets dehydrogenase activity, was used to assess the physiological status of the test bacteria. For planktonic cultures, 30 μl harvested bacterial culture was mixed with 3 μl CTC (50 mM) to obtain a final CTC concentration of 5 mM. CTC mixed cultures were left in the dark at room temperature for 50 min. For the biofilms, 30 μl CTC (5 mM) was added to the chamber after removing the culture fluid and washing with PBS, and incubated in the conditions described above. Fluorescence of the stained cells was examined by CSLM. Preliminary experiments with planktonic cultures showed that for *S. anginosus* at mid-exponential phase, $90\% \pm 3\%$ of the population was CTC active; while for *L. salivarius* $78\% \pm 5\%$ of the population was CTC active. Preliminary data have also shown that CTC staining yielded similar results in aerobic and anaerobic conditions.

Fig. 1. (a) Flow-chamber system used for formation of biofilms. (b) Biofilm growth at 1, 6 and 24 h for *S. anginosus* (i–iii) and *L. salivarius* (iv–vi), using the BacLight LIVE/DEAD technique. The values below each image are the mean total cell counts for 10 images covering a total area of 0.5 mm² (means ± SEM of triplicate experiments). Bar, 50 μm.

Fluorescein diacetate (FDA), which reacts with non-specific esterases in metabolically active micro-organisms, was used as an alternative metabolic marker in biofilm cultures. A stock solution of FDA was prepared by mixing 5 mg FDA (Molecular Probes) ml⁻¹ in pure acetone. The working solution was prepared by mixing 5 μl FDA/acetone ml⁻¹ in the inoculum medium. After removing the culture fluid from the biofilm chamber and washing with PBS, 30 μl FDA working solution was added. Ethidium bromide (EB) was used as a counter-stain to visualize non-active cells. The EB stock solution was prepared by mixing 0.5 mg EB (Roth) ml⁻¹ NaCl, while the working solution was a 1:200 dilution of this in the inoculum medium. EB was added in 20 μl aliquots to the biofilm chambers already containing FDA. The biofilm chambers were incubated in the conditions described above. Fluorescence of the stained cells was examined by CSLM.

CSLM and total cell counts. All microscopy work was performed using an Eclipse TE2000 inverted confocal scanning laser microscope (Nikon). Ten randomly selected areas of each sample were imaged using a ×60 magnification oil immersion objective with a numerical aperture of 1.4, and the confocal pinhole set to a diameter of 30 μm. For the LIVE/DEAD and FDA/EB techniques, confocal illumination was provided by an Ar laser (488 nm laser excitation) fitted with a long-pass 515/30 filter for the green fluorescence signal (emitted by SYTO9 and FDA), and a long-pass 605/75 filter for the red fluorescence signal (propidium iodide and EB). Simultaneous dual-channel imaging was used to display green and red fluorescence. For detection of the red fluorescence by CTC staining, a G-HeNe laser was used (543 nm laser excitation), together with a long-pass 605/75 emission filter. A transmission emission channel using the same laser excitation was obtained from the same microscopic field. Simultaneous imaging was used to display red CTC fluorescence and the transmission emission channel, in order to determine the numbers of cells not reacting with the CTC stain.

In all cases, CSLM images were acquired by the software EZ-C1 v.3.40 build 691 (Nikon) at a resolution of 512 × 512 pixels and with a zoom factor of 1.0, giving a final resolution of 0.41 μm per pixel. Individual biofilm images covered an area of 0.05 mm² per field of view. Total cell counting was performed by the analysis software Cell C (Tampere University of Technology) which separated clusters of bacteria by erosion-dilation algorithms and gave results in binarized black and white images in Excel-ready CSV files. Individual cells were enumerated and their lengths, widths and areas calculated.

Image analyses. Confocal LIVE/DEAD and FDA/EB images were analysed by using a colour segmentation algorithm written in MATLAB 7.0 for Windows (MathWorks) which, by colour thresholding, separated the red and green fluorescence signals and measured the total areas (pixels²) covered by each segmented colour. To calculate the number of cells per segmented colour, colour areas were divided by the mean cell area, which was calculated from the total cell counts described above; the mean cell area of *L. salivarius* was 12 μm² = 76 pixels²; the mean cell area of *S. anginosus* was 1.1 μm² = 7 pixels².

The numbers of cells that reacted to CTC were calculated by separating the confocal images in two, one with the transmission emission channel, which registered the total cell numbers, and the other with the red fluorescence from CTC-stained cells. The image analysis software program NIS-Elements BR v.2.30 (Nikon) was used for the channel segmentation. Each image was analysed separately with the software CELL C, and the proportion of cells stained red by CTC based on the total cell count was calculated.

Nutrient deprivation and reactivation of biofilms. Once the biofilms had been established for 24 h, the PYG was removed and the chamber was washed with PBS; 130 μl 10 mM PBS (pH 7.5) was added and the mini-flow chambers were incubated in an atmosphere

of 5 % CO₂ in hydrogen at 37 °C. Slides were removed at 24 h and subjected to LIVE/DEAD and CTC staining and analysis. After the 24 h buffer incubation period, unstained mini flow-chambers were washed with PBS and inoculated with PYG (130 µl). Other media used to test reactivation of biofilm cells were prepared using the salt mixtures and water volumes in PYG: PYG devoid of glucose (PY), yeast extract (Difco) 10 mg ml⁻¹ (YE), Trypticase Peptone (BBL) 5 mg ml⁻¹ (peptone mix 1), Bacto Peptone (Difco) 5 mg ml⁻¹ (peptone mix 2), and 6 mM glucose. Additionally, the semi-defined medium (MADM) described by Bowden (1999), was used with and without glucose. The flow cells were then incubated in an atmosphere of 5 % CO₂ in hydrogen at 37 °C. Preliminary experiments indicated that reactivation of biofilms cells was more delayed compared to planktonic cells. Accordingly, the reactivation of biofilm cells was analysed with individual slides removed at 2, 4, 6, 24, 48, 72 and 96 h and subjected to LIVE/DEAD, CTC and FDA/EB staining and microscopic analysis as described above.

Nutrient deprivation and reactivation of planktonic cells. Cells were harvested in the late exponential phase of growth (described above), resuspended in 10 ml 10 mM PBS (pH 7.5) and incubated in an atmosphere of 5 % CO₂ in hydrogen at 37 °C. Samples were taken at 24 h to determine the OD₆₀₀, the number of cells that were stained fluorescent-green according to LIVE/DEAD, and the percentage of cells reacting to CTC. For reactivation, cells were collected by centrifugation (3000 g for 5 min), washed with PBS and resuspended in 10 ml fresh PYG. Cultures were incubated in an atmosphere of 5 % CO₂ in hydrogen at 37 °C. Since preliminary results had demonstrated that the reactivation of planktonic cells occurred within short time periods, samples were retrieved at 2, 4 and 6 h to check the cell parameters outlined above.

Statistical analysis. Each experiment was performed in triplicate to confirm that the patterns observed were reproducible. Two-way analysis of variance (ANOVA) was used to detect any significant variation in terms of time, variations among the three different biofilm or planktonic populations, interaction between time and bacterial populations, and random variation (error). The software package SPSS v.14 was used. The standard errors for the cell counts at each time point were calculated.

RESULTS

Reproducibility of results

The statistical analysis by the two-way ANOVA was used to determine whether there was any significant source of variation in the data resulting from the image analyses of the biofilm and planktonic populations. For most variables tested (see Methods), the two main sources of variation ($P < 0.001$) were time and random variation (error). No significant variations were observed between the three independent observations in terms of the cell counts resulting from the analyses of membrane integrity with the LIVE/DEAD technique and the reaction of cells to the CTC and FDA metabolic markers at each time point. The data reported are based on the mean of the three experimental cell counts each containing the total results of 10 images (biofilm area is 0.5 mm²).

Biofilm growth

Following inoculation of late-exponential phase cells to the flow chambers, growth in biofilms was monitored by direct microscopic observation and total cells counts. Fig. 1(b) shows LIVE/DEAD confocal images which demonstrate biofilm growth of *S. anginosus* (i–iii) and *L. salivarius* (iv–vi), at 1, 6 and 24 h (see also Fig. 2a and Fig. 5a). Normally, the biofilms formed at 24 h were mono-layered. There were significant differences between the cell counts on the biofilms at differing time points, with the exception of one time point with *L. salivarius*. Biofilms of *S. anginosus* after 1 h of incubation had a total of $3\,662 \pm 460$ cells in an area of 0.5 mm², while at 6 h the number of cells had increased twofold to $6\,794 \pm 503$ cells, and fivefold at 24 h to a total of $16\,658 \pm 1\,543$ cells. Biofilms of *L. salivarius* at 1 h of incubation had $3\,532 \pm 406$ cells, and despite a non-

Fig. 2. Effect of 24 h nutrient deprivation in buffer and subsequent reactivation in fresh PYG medium on the membrane integrity of *S. anginosus* in (a) biofilms and (b) planktonic cultures as assessed by (LIVE/DEAD) fluorescent staining. In (a), dark grey bars represent green, undamaged cells; white bars denote membrane-damaged cells. Total biofilm cell counts (per mm²) were calculated from a total area of 0.5 mm². In (b), grey bars represent the percentage of green, undamaged cells stained by LIVE/DEAD analysis and white bars indicate the percentage of membrane-damaged cells. ●, Cell growth (OD₆₀₀). All values are means $\pm$ SEM of triplicate experiments.

significant increase to 4873 ± 307 cells at 6 h, a significant threefold increase in growth to 9576 ± 1692 cells was registered at 24 h.

Nutrient deprivation and reactivation of *S. anginosus*

To ascertain whether oral bacteria enter a state of metabolic arrest as a survival mechanism for conditions of nutrient limitation in the mouth, we carried out experiments to mimic 'famine' and 'feast' conditions. This was done by creating nutrient deprivation in buffer, followed by the introduction of fresh PYG medium, with membrane integrity and metabolic activity of biofilms of the individual bacteria assessed throughout these conditions. A further objective was to compare the response of planktonic cultures of the same organisms under the same conditions. The hypothesis was that a delay in reactivation of the cells under either condition would constitute evidence for a slow physiological response.

Fig. 2 shows the effect of 24 h of incubation in buffer and subsequent reactivation with fresh PYG medium on the membrane integrity of *S. anginosus*, employing the LIVE/DEAD technique with biofilms (Fig. 2a) and planktonic cultures (Fig. 2b). Clearly, green cells, indicating undamaged cell membranes, dominated the populations of both culture types throughout the nutrient deprivation period and subsequent reactivation with fresh medium. As seen in Fig. 2(a), there were 16658 ± 1543 biofilm cells in 0.5 mm^2 at the onset of nutrient deprivation, 99 % (16059/16119) of which stained green (undamaged), and after 24 h of nutrient deprivation almost 100 % of the cells (18451/18488) were still undamaged. The addition of fresh medium did not alter the proportion of cells with sound membranes at 24 h, with 99 % (18695/18977) and 98 %

(24332/24569) at 48 h. At 72 h, a 37 % increase in the total cells (39168 ± 9276) was observed, which was associated with a slight decrease (5 %) in percentage of intact cells, while 86 % (40118/46716) of the cells were undamaged at 96 h.

The results of the LIVE/DEAD staining for planktonic cells of *S. anginosus* (Fig. 2b) indicated that during incubation in buffer and reactivation, the majority of cells that were counted had intact membranes, although some red damaged cells were evident at each time point. At the onset of nutrient deprivation, 91 % of the initial population of cells (34247 ± 1861) was undamaged (green), while after 24 h of incubation in buffer, the membrane integrity dropped to 84 %. Two hours after the addition of fresh medium, the OD_{600} of the total cell population had increased from 0.71 ± 0.01 to 0.92 ± 0.08 , which was accompanied by an increase in the proportion of green cells to 88 %. The OD_{600} of the total cell population was maintained at 0.93 at 4 h and 6 h of reactivation; similarly, the percentage of cells with intact membranes was maintained at 89 % at 4 h and 6 h following reactivation.

Metabolic activity of *S. anginosus*

The redox fluorescence dyes CTC and FDA, which target dehydrogenase activity and esterase activity, respectively, were used to assess the metabolic activity of *S. anginosus* after 24 h incubation in buffer and subsequent reactivation in fresh PYG medium. The proportions of cells which were able to reduce CTC and produce a red fluorescence signal indicating dehydrogenase activity in biofilms and planktonic cultures are shown in Figs 3(a) and (b), respectively. As seen in Fig. 3(a), biofilms had 14777 ± 2456 cells at the onset of nutrient deprivation, 65 % (9624 ± 1852) of which showed red fluorescence indicating CTC reduction.

Fig. 3. Effect of 24 h nutrient deprivation in buffer and subsequent reactivation in fresh PYG medium on the metabolic activity of *S. anginosus* in (a) biofilms and (b) planktonic cultures. In (a), grey bars represent red, dehydrogenase-active CTC-reducing cells, hatched bars represent enzyme active FDA-reactive cells and white bars indicate the number of inactive cells. Total biofilm cell counts (per mm^2) were calculated from a total area of 0.5 mm^2 . In (b), grey bars represent the percentage of the population stained red by CTC and white bars indicate the percentage of inactive cells. ●, Cell growth (OD_{600}). All values are means $\pm$ SEM of triplicate experiments.

However, after 24 h of nutrient deprivation, none of the $15\,406 \pm 1\,158$ total cells showed metabolic activity (see representative image in Fig. 4a). As seen in Fig. 3(a) and Fig. 4, no CTC-reactive red cells were present at 2, 24 and 48 h following the addition of fresh nutrients, indicating no detectable dehydrogenase activity in cells. However, dehydrogenase reactivation increased with time: there were 78 % active red cells at 72 h ($16\,232/20\,928$ cells) (Fig. 4e) and 93 % at 96 h ($26\,665/28\,658$ cells).

The proportion of cells which were able to reduce FDA and produce a green fluorescent signal is also presented in Fig. 3(a). At the onset of nutrient deprivation, 68 % ($10\,242/15\,086$) of cells reacted to FDA, i.e. showed esterase activity; however, after 24 h of nutrient deprivation, less than 1 % of the $14\,281 \pm 720$ total cells showed esterase activity. Following the addition of fresh nutrients, no increase in FDA-reactive green cells was observed at 2 and 24 h. However, esterase activity was observed at 48 h with 10 % ($1\,752 \pm 545$) green active cells; this increased to 61 % ($16\,344 \pm 1\,821$ cells) at 72 h and 83 % ($25\,380 \pm 2\,016$ cells) at 96 h.

By way of comparison, planktonic cells also became metabolically inactive during the 24 h nutrient deprivation period (Fig. 3b). As seen at the onset of nutrient deprivation, 76 % of the initial total cells ($15\,151 \pm 517$) showed metabolic activity, while after 24 h nutrient deprivation, none of the cells in the total population were metabolically active (Fig. 4f). As in the LIVE/DEAD experiment (Fig. 2), the addition of fresh medium resulted in cell growth and rapid renewal of planktonic cell metabolic activity. After 2 h, the OD₆₀₀ of planktonic cells

increased from 0.71 to 0.92; CTC staining indicated that 96 % of the cells were red and active (Fig. 4g). At 4 h, 93 % of the cells were active at an OD of 0.93. The population remained constant at 6 h; however, metabolic activity decreased to 71 %.

Nutrient deprivation and reactivation of *L. salivarius*

As with *S. anginosus*, LIVE/DEAD fluorescent staining tests with biofilm and planktonic cultures of *L. salivarius* revealed a high proportion of green cells, indicating undamaged cell membranes throughout the 24 h incubation in buffer and reactivation in fresh medium. As seen in Fig. 5(a), 99 % of the biofilm cells were green from the onset of nutrient deprivation to the 48 h sample in the reactivation period. A slight decrease was observed 72 h after the addition of fresh PYG medium, where 95 % of the cells ($10\,246/10\,828$ cells) were undamaged, while the fluorescent green cell count dropped to 82 % ($11\,909/14\,451$ cells) at 96 h. Similarly, 99 % of the total cells in planktonic cultures of *L. salivarius* (Fig. 5b) showed green fluorescence at the end of the nutrient deprivation phase and through 4 h of the recovery period, while membrane integrity declined to 86 % at 6 h (Fig. 5b).

Metabolic activity of *L. salivarius*

As seen in Fig. 6(a) and Fig. 7, at the onset of nutrient deprivation, 56 % ($4\,595/8\,252$) of cells reduced CTC, while after 24 h nutrient deprivation, none of the cells in the

Fig. 4. Visual summary of the CTC-metabolic data for *S. anginosus*. Dual-channel confocal photomicrographs display cells by transmission emission and CTC staining of biofilms (a–e) and planktonic cultures (f–g). The values below each image are the numbers of CTC-stained (red) cells, indicating metabolic activity, and the total cell counts (all values are means $\pm$ SEM of triplicate experiments); the percentages given are for 10 images in an area of 0.5 mm^2 . Images cover a microscopic field of $20 \text{ } \mu\text{m}^2$. Bar, $5 \text{ } \mu\text{m}$.

Fig. 5. Effect of 24 h nutrient deprivation in buffer and subsequent reactivation in fresh PYG medium on the membrane integrity of *L. salivarius* in (a) biofilms and (b) planktonic cultures as assessed by (LIVE/DEAD) fluorescent staining. In (a), dark grey bars represent green, undamaged cells, while white bars denote membrane-damaged cells. Total biofilm cell counts (per mm²) were calculated from a total area of 0.5 mm². In (b), grey bars represent the percentage of green, undamaged cells stained by LIVE/DEAD analysis and white bars indicate the percentage of membrane-damaged cells. ●, Cell growth (OD₆₀₀). All values are means ± SEM of triplicate experiments.

total population ($9\,546 \pm 4\,080$) showed dehydrogenase activity (Fig. 7a). Of particular interest was the fact that the addition of fresh medium to the non-growing biofilms did not result in CTC reduction (i.e. red cells) at 24 h and 48 h samples (Figs 7c and d, respectively). Reactivation started at 72 h, when 56 % ($5\,056/9\,102$) of the cells showed dehydrogenase activity (Fig. 7e); the fraction of these cells increased to 79 % ($7\,665/9\,654$ cells) at 96 h. Unlike in *S. anginosus*, no significant increase in the biomass mm⁻² was linked to the metabolic reactivation.

Fig. 6(a) shows the proportion of cells which were able to reduce FDA and produce a green fluorescent signal. At the onset of nutrient deprivation, 42 % ($3\,501/8\,340$) of cells

reduced FDA; however, after 24 h nutrient deprivation, less than 1 % of the total cells ($8\,547 \pm 1\,111$) showed esterase activity. Following the addition of fresh nutrients, no increase in FDA-reactive green cells was observed at 24 h. However, esterase activity was observed as active green cells in 15 % ($1\,082 \pm 253$) of cells at 48 h; this increased to 75 % ($6\,595 \pm 485$) at 72 h, and 90 % ($7\,509 \pm 696$ cells) at 96 h.

As seen in Fig. 6(b), the metabolic reactivation of planktonic cultures of *L. salivarius* was much faster than that seen with corresponding biofilm cultures. At the onset of nutrient deprivation, 46 % of the cells ($6\,090/13\,278$) were active; however, after 24 h in buffer, only 3 % of the cells reduced CTC (Fig. 7f). Within 2 h of the addition of

Fig. 6. Effect of 24 h nutrient deprivation in buffer and subsequent reactivation in fresh PYG medium on the metabolic activity of *L. salivarius* in (a) biofilms and (b) planktonic cultures. In (a), grey bars represent red, dehydrogenase active CTC-reducing cells, hatched bars represent enzyme active FDA-reactive cells and white bars indicate the number of inactive cells. Total biofilm cell counts (per mm²) were calculated from a total area of 0.5 mm². In (b), grey bars represent the percentage of the population stained red by CTC and white bars indicate the percentage of inactive cells. ●, Cell growth (OD₆₀₀). All values are means ± SEM of triplicate experiments.

Fig. 7. Visual summary of the CTC-metabolic data for *L. salivarius*. Dual-channel confocal microphotographs display cells by transmission emission and CTC staining of biofilms (a–e) and planktonic cultures (f–g). The values below each image are the numbers of CTC-stained (red) cells, indicating metabolic activity, and the total cell counts (all values are means ± SEM of triplicate experiments); the percentages given are for 10 images in an area of 0.5 mm². Images cover a microscopic field of 30 µm². Bar, 5 µm.

fresh medium, there was a significant increase to 78 % in the number of cells stained fluorescent red with CTC (Fig. 7g) and this metabolic activity was associated with an increase in OD₆₀₀ values from 0.46 ± 0.05 to 0.55 ± 0.03 , indicating cell growth. The planktonic population increased to OD₆₀₀ 0.61 ± 0.02 at 4 h, with 77 % of cells registered as metabolically active. At 6 h, the OD₆₀₀ measurements did not change (0.61), however, CTC reduction dropped to 62 %.

Effect of different medium in reactivation of biofilm cells

Seven additional media were tested for reactivation of nutrient-deprived biofilm cells, five containing isolated components of PYG and two semi-defined: MADM and MADM without glucose. As shown in Table 1, both organisms showed dehydrogenase and esterase activity with PY and YE. Reactivation of *S. anginosus* in PY started at 48 h, when 16 % and 30 % of cells were reactive with CTC and FDA, respectively, and an 11 % increase in biomass mm⁻² was seen. Reactivation of cells gradually increased, with 59 % and 61 % reactive cells and a 34 % increase in biomass mm⁻² at 72 h, and 85 % and 88 % reactive cells and 46 % increase in biomass mm⁻² at 96 h. As shown in Table 1, YE induced a faster reactivation of *S. anginosus*, with 4 % of cells displaying esterase activity at 24 h. At 48 h, 54 % and 41 % were metabolically active cells (CTC and FDA, respectively) with an increase in biomass mm⁻² of 26 %; at 72 h, 76 % and 77 % cells were reactivated, with a 45 % increase in biomass. For *L. salivarius*, PY was more effective than PYG for reactivation, since at 72 h reactivation, 50 % and 66 % of cells were active, with an increase in biomass of 36 %. Unlike with PYG, an increase in biomass (20 %) at 96 h was also seen by *L. salivarius*

reactivated with YE. Peptone mix 1, peptone mix 2, 6 mM glucose, MADM and MADM without glucose did not show any improvement in the reactivation of both species.

DISCUSSION

A state of metabolic arrest, often referred to as dormancy, caused by nutrient deprivation has been intensely studied in cultures of *E. coli* and *Micrococcus luteus* grown in liquid or planktonic suspensions (Jenkins *et al.*, 1988; Kaprelyants & Kell, 1993), but little is known about this process for oral bacteria or organisms in biofilms. In oral micro-environments such as endodontically treated root canals of teeth, it is generally assumed that persisting bacteria survive nutrient deprivation and other stresses by entering non-growing physiological states (Chávez de Paz, 2007). In this study, we were able to distinguish non-growing states of *L. salivarius* and *S. anginosus* in biofilm cultures while incubated in fresh medium after a period of nutrient deprivation in buffer. Importantly, after 24 h of incubation in buffer, essentially all cells showed intact membranes (fluorescent green according to LIVE/DEAD analysis) (Figs 2a, 5a), with no measurable metabolic activity detected using the CTC and/or FDA fluorescent markers (Figs 3a, 4a, 6a, 7a). The slow physiological reactive phenotype in biofilm cultures was recognized when fresh PYG was added to the flow chambers and the undamaged populations of *S. anginosus* (Fig. 4b–d) and *L. salivarius* (Fig. 7b–d) showed no significant metabolic activity at 48 h. It was apparent that the slow physiological response of *L. salivarius* biofilm cells was longer lasting than that of *S. anginosus* since no increase in the biomass mm⁻² was evident after the addition of fresh medium. Thus, while both organisms survived the 24 h nutrient deprivation period with intact

Table 1. Percentage of *S. anginosus* and *L. salivarius* cells reactive with CTC and FDA, and the biomass increase during reactivation in PYG, PY and YE medium

Time (h)	PYG*			PY†			YE‡		
	CTC	FDA	Biomass mm ⁻²	CTC	FDA	Biomass mm ⁻²	CTC	FDA	Biomass mm ⁻²
<i>S. anginosus</i>									
0	–	–	–	–	–	–	–	–	–
24	–	–	–	–	–	–	–	4 ± 1	–
48	–	10 ± 3	–	16 ± 2	30 ± 8	11 ± 8	54 ± 6	41 ± 4	26 ± 6
72	78 ± 5	61 ± 7	28 ± 3	59 ± 4	61 ± 4	34 ± 8	76 ± 3	77 ± 4	45 ± 2
96	93 ± 3	83 ± 7	43 ± 2	85 ± 2	88 ± 9	46 ± 7	90 ± 1	93 ± 5	51 ± 5
<i>L. salivarius</i>									
0	–	–	–	–	–	–	–	–	–
24	–	–	–	–	–	–	–	–	–
48	–	15 ± 3	–	14 ± 2	29 ± 5	–	4 ± 1	7 ± 1	–
72	56 ± 4	75 ± 6	–	50 ± 2	66 ± 5	36 ± 1	41 ± 9	45 ± 9	–
96	79 ± 4	90 ± 8	–	88 ± 4	84 ± 7	51 ± 3	53 ± 10	67 ± 6	20 ± 4

*Peptone-yeast-glucose.

†PYG without glucose.

‡10 mg yeast extract ml⁻¹.

–, No cells stained or no measurable biomass.

cell populations, their recovery from nutritional stress was different, suggesting that the response to other stresses might be similar.

The above hypothesis on the slow physiological response of biofilm cells is supported by the data generated by planktonic cultures incubated in buffer. As in the biofilm cultures, the number of metabolically active cells decreased dramatically to zero in both strains following 24 h in buffer (Figs 3b, 4f, 6b, 7f); however, the number of green undamaged cells did not change significantly, such that, at the beginning of the reactivation period with fresh PYG, the cultures were dominated (84–99%) by undamaged cells that were metabolically inactive. However, unlike in the biofilms, the addition of fresh nutrients resulted in significant levels of CTC reduction at 2 h, indicating an immediate increase in metabolism and rapid reactivation of the planktonic cultures (Figs 4g, 7g). This observation highlights the difference in physiology between the biofilm and planktonic cultures, and also confirms the slower physiological response of biofilm cells (Mah & O'Toole, 2001). The data produced by this study show that biofilm cells exhibit a slow physiological response, and unlike cells in planktonic culture, do not reactivate in short time periods even under optimal conditions.

S. anginosus and *L. salivarius* have different core physiological traits which may explain the differences observed in the time to reactivate from non-growth while forming biofilms. The main physiological characteristics of *S. anginosus* are the production of acetoin from glucose, and the hydrolysis of arginine (Ruoff, 1988). As arginine

represents 8% of the amino acid content of PYG, it is possible that arginine is used as an alternative source to generate usable energy in the form of ATP during slow reactivation (Moat & Foster, 1995). However, independent peptone mixtures in PYG did not reactivate *S. anginosus*. Interestingly, YE reactivated *S. anginosus* faster than PYG and PY (Table 1). In an additional experiment in which we measured pH in a culture of *S. anginosus* in YE during the exponential phase (data not shown), a pH drop from 7.5 to 5.5 was recorded, indicating that the 17.5% of non-defined carbohydrates in YE may be the main source for reactivation of *S. anginosus*. On the other hand, *L. salivarius* has a homofermentative metabolism as it produces large amounts of lactic acid from glucose, levulose, melibiose, trehalose, lactose, mannitol, etc. (Rogosa *et al.*, 1953). However, the metabolic reactivation and biomass increase of *L. salivarius* with PY (Table 1) may indicate that the excess glucose in PYG was to some extent inhibitory to reactivation of *L. salivarius* cells from nutrient deprivation (Fig. 6a).

The methodology used in this study has allowed us to distinguish between membrane integrity and dehydrogenase and esterase activity of cells that had undergone nutrient deprivation and were reactivated by addition of fresh nutrients. The BacLight LIVE/DEAD assay system determined whether membrane damage had occurred during the period of nutrient deprivation. In both strains, neither the 24 h period of incubation in buffer nor reactivation with fresh medium significantly altered the proportion of green, undamaged cells. The CTC marker complemented the information given by the LIVE/DEAD

staining by indicating the dehydrogenase activity of the cells under the same conditions. CTC scavenges electrons from microbial oxidation/reduction reactions and is intracellularly reduced to bright red formazan precipitates by the electron transport system (ETS) components or dehydrogenases in metabolically active micro-organisms. Kaprelyants & Kell (1993) reported that in aerobic environments, CTC was reduced directly by ETS-associated dehydrogenase enzymes in *M. luteus*, and Lopez-Amoros *et al.* (1995) demonstrated that CTC could be reduced by NAD(P)H in fixed (formalin) *E. coli*. Smith & McPeters (1997) further clarified that CTC reduction in aerobic *E. coli* K-12 cultures is linked with dehydrogenase activity. In the present study, the relationship between growth and the ability to reduce CTC was optimal in the preliminary planktonic experiments with *S. anginosus* and *L. salivarius*, where exponential-phase growth was positively correlated with high levels of CTC reduction. This was also seen with the first reactivation sample at 2 h, where CTC measurements increased in accordance with the increase in the bacterial population. CTC is commonly referred to as a marker of respiration; thus its effectiveness in oxygen-limited environments is thought to be doubtful. In the present study, we worked with CTC in biofilm and planktonic cultures under strict anaerobic conditions, and the results obtained were similar to preliminary experiments in aerobic cultures (data not shown). These data suggest that the reduction of CTC by bacteria is independent of the availability of oxygen, thus confirming the results from previous studies showing CTC reduction by a variety of anaerobes during all phases of growth (Bhupathiraju *et al.*, 1999; Fukui & Takii 1989; Walsh *et al.*, 1995). Seemingly, intracellular enzymes (e.g. dehydrogenases) present during anaerobic processes (fermentation, nitrate reduction) are also capable of reducing CTC.

Since previous studies reported discrepancies with CTC and various cell-specific methods designed to distinguish active from inactive cells (Karner & Fuhrman 1997; Nielsen *et al.*, 2003), we included FDA as an extra marker of metabolic activity. FDA is hydrolysed in active bacteria by esterases, although other enzymes such as proteases and lipases can also hydrolyse FDA. The product of this enzymic conversion is fluorescein, which can be visualized within cells by fluorescence microscopy. FDA has previously been used to determine amounts of active bacteria in different environments including dental plaque (Brex *et al.*, 1990; Netuschil *et al.*, 1998). In the present study, no major discrepancies were observed between the CTC and FDA results (Figs 3a, 6a). Normally, FDA yielded a slightly higher value of active cells than CTC (Table 1), and on some occasions, FDA-reactive cells were seen when no CTC activity was registered, e.g. at 48 h reactivation with PYG by *S. anginosus* (10 %) and *L. salivarius* (15 %), and at 24 h reactivation with YE by *S. anginosus* (4 %). Similar differences between CTC and other vitality markers have been previously reported and indicated that CTC might detect cells which have greater metabolic activity compared

to most cells that are scored as active (Longnecker *et al.*, 2005; Sherr *et al.*, 1999). In conclusion, the combination of LIVE/DEAD, CTC and FDA staining techniques to detect cells in a non-growing state and to evaluate their slow physiological response in biofilms appears to be satisfactory. However, other methods are obviously required for biofilm studies and might involve the use of more specific markers for other key cell-specific activities, such as fluorescence *in situ* hybridization (Nielsen *et al.*, 2003).

The presence of bacteria in a state of non-growth could explain the survival of bacteria in persisting root canal infections, where micro-organisms must survive very low nutrient supplies, as well as exposure to calcium hydroxide when it is used as intracanal medication in root canal treatment (Chávez de Paz *et al.*, 2003, 2007). In this line of argument, disease could be prevented not only by targeting 'pathogenic' organisms, but also by interfering in the reactivation of non-growing organisms. This work demonstrates that a phenotype giving rise to a slow recovery response is a characteristic of biofilm cells not shown by planktonic cells. The methodology used in this work could also be applied to test the physiological response of cells towards stresses such as antiseptics or antibiotics. Furthermore, studies on the factor(s) that promote recovery of the cellular metabolic activity are important to obtain information leading to a greater understanding of oral microbial ecology. The greater understanding of bacterial physiology and non-growing states has significance in relation to the control of oral diseases.

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