

# Influence of a model human defensive peroxidase system on oral streptococcal antagonism

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*Streptococcus* is a dominant genus in the human oral cavity, making up about 20% of the more than 800 species of bacteria that have been identified, and about 80% of the early biofilm colonizers. Oral streptococci include both health-compatible (e.g. *Streptococcus gordonii* and *Streptococcus sanguinis*) and pathogenic strains (e.g. the cariogenic *Streptococcus mutans*). Because the streptococci have similar metabolic requirements, they have developed defence strategies that lead to antagonism (also known as bacterial interference). *S. mutans* expresses bacteriocins that are cytotoxic toward *S. gordonii* and *S. sanguinis*, whereas *S. gordonii* and *S. sanguinis* differentially produce H<sub>2</sub>O<sub>2</sub> (under aerobic growth conditions), which is relatively toxic toward *S. mutans*. Superimposed on the inter-bacterial combat are the effects of the host defensive mechanisms. We report here on the multifarious effects of bovine lactoperoxidase (bLPO) on the antagonism between *S. gordonii* and *S. sanguinis* versus *S. mutans*. Some of the effects are apparently counterproductive with respect to maintaining a health-compatible population of streptococci. For example, the bLPO system (comprised of bLPO + SCN<sup>-</sup> + H<sub>2</sub>O<sub>2</sub>) destroys H<sub>2</sub>O<sub>2</sub>, thereby abolishing the ability of *S. gordonii* and *S. sanguinis* to inhibit the growth of *S. mutans*. Furthermore, bLPO protein (with or without its substrate) inhibits bacterial growth in a biofilm assay, but sucrose negates the inhibitory effects of the bLPO protein, thereby facilitating adherence of *S. mutans* in lieu of *S. gordonii* and *S. sanguinis*. Our findings may be relevant to environmental pressures that select early supragingival colonizers.

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## INTRODUCTION

The term 'microbial ecosystem' has been defined as an organization of micro-organisms that live in a particular niche, while subject to the environmental pressures of the niche (Raes & Bork, 2008). These pressures may be biotic (e.g. self, other microbial species, plants and animals) and abiotic (e.g. temperature, pH, nutrients, etc.) in origin. Dental plaques are examples of dynamic and exceedingly complex microbial ecosystems that are thought to comprise at least 800 bacterial species (Kroes *et al.*, 1999; Paster *et al.*, 2006; Aas *et al.*, 2005, 2008; Preza *et al.*, 2008; Becker *et al.*, 2002; Paster *et al.*, 2001; Dethlefsen *et al.*, 2007). Many of the environmental factors that influence the speciation of oral biofilms have been reviewed in other articles (Marsh, 2005; Overman, 2000; Rosan & Lamont, 2000; Sissons, 1997; Socransky & Haffajee, 2005; ten Cate, 2006), including the effects of pH (Burne & Marquis,

2000), nutrients (Sissons *et al.*, 2007), fluoride (Bowden, 1990) and various antimicrobials (Dashper *et al.*, 2007; Fine *et al.*, 2001; Leung *et al.*, 2005; Yang *et al.*, 2006; Zaura-Arite *et al.*, 2001). The effects of the host immune system on oral biofilms are less well understood. This paper focuses on the influence of defensive peroxidases on inter-streptococcal interactions.

There are two host-derived defensive peroxidases in the human oral cavity, salivary peroxidase (hSPO) and myeloperoxidase (hMPO) (Ashby, 2008; Ihalin *et al.*, 2006). hSPO is a normal non-inducible component of the saliva of the parotid and submandibular glands (Riva *et al.*, 1978), whereas hMPO is an offensive mechanism of neutrophilic polymorphonuclear leukocytes (PMNs). Employing hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as an oxidant, the defensive peroxidases use inorganic ions to produce antimicrobials that are generally believed to be more effective than H<sub>2</sub>O<sub>2</sub> itself. hMPO is capable of producing hypochlorous acid (HOCl), a powerful and comparatively indiscriminate biocide. While the production of HOCl by the hMPO system is possible in other environments (e.g. the gingival sulcus), it is disfavoured in the part of the oral

**Abbreviations:** bLPO, bovine milk lactoperoxidase; CLSM, confocal laser scanning microscopy; CV, crystal violet; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); hMPO, human myeloperoxidase; HRP, horseradish peroxidase; hSPO, human salivary peroxidase; LPO, lactoperoxidase.

cavity that is controlled by saliva (Ashby, 2008). Instead, hSPO and hMPO work in unison in the supragingival regime to exclusively oxidize thiocyanate ( $\text{SCN}^-$ ) to produce hypothiocyanite ( $\text{OSCN}^-$ , a reactive inorganic species), which constantly bathes early oral plaques (Ashby, 2008). We have employed bovine milk lactoperoxidase (bLPO), a readily available enzyme, as a surrogate for hSPO. The two enzymes are structurally and catalytically similar (Mansson-Rahemtulla *et al.*, 1988).

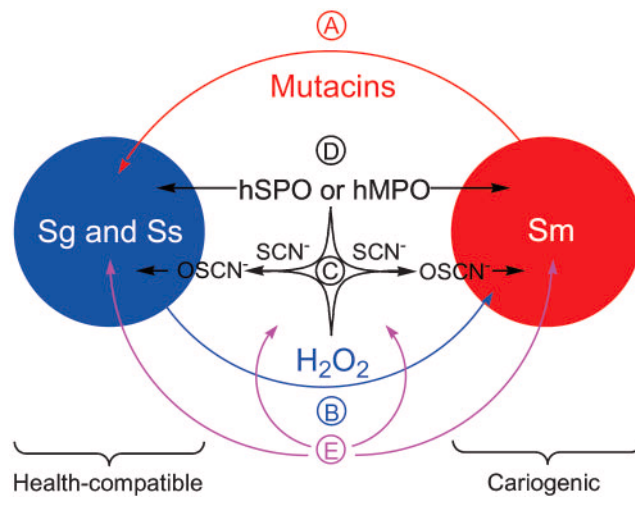
*Streptococcus* is a dominant genus in the human oral cavity, making up about 20% of the more than 800 species of bacteria that have been identified (Marsh, 1999), and about 80% of the early biofilm colonizers (Rosan & Lamont, 2000). Oral streptococci include both health-compatible (e.g. *Streptococcus gordonii* and *Streptococcus sanguinis*) and pathogenic species (e.g. the cariogenic *Streptococcus mutans*). Because the streptococci have similar metabolic requirements, they have developed defence strategies that lead to antagonism (also known as bacterial interference). *S. mutans* expresses bacteriocins that are cytotoxic toward *S. gordonii* and *S. sanguinis* (Hamada & Ooshima, 1975; Kreth *et al.*, 2008a), whereas under aerobic growth conditions *S. gordonii* and *S. sanguinis* differentially produce  $\text{H}_2\text{O}_2$ , which is relatively toxic toward *S. mutans* (Kreth *et al.*, 2008a).

The present study focuses on the effects of components of the lactoperoxidase (LPO) system (comprising bLPO,  $\text{SCN}^-$  and  $\text{H}_2\text{O}_2$ ) on the pair-wise growth of *S. mutans*, *S. gordonii* and *S. sanguinis* and on the capacity of bLPO protein to influence the ability of individual bacterial species to form biofilm in an *in vitro* biofilm model. An effort has been made to simulate the aerobic growth conditions that probably exist during the initial colonization of tooth enamel (i.e. biofilm growth under low oxygen tension). The factors that we suggest are relevant to the subject of this study are summarized in Fig. 1. This is the first investigation of which we are aware that probes the influence of peroxidase systems on interspecies competition in a biofilm and on the biofilm-forming abilities of oral streptococci.

## METHODS

**Bacterial strains and media.** *S. mutans* UA140 (Qi *et al.*, 2001), *S. sanguinis* SK36 (Xu *et al.*, 2007) and *S. gordonii* DL1 (Pakula & Walczak, 1963) were grown aerobically (5%  $\text{CO}_2$ ) at 37 °C either in brain heart infusion broth (BHI; Difco) without shaking or on BHI agar plates.

**Competition assays on solid medium: effect of the peroxidase system.** To assess competitive growth, a protocol described previously was used with modifications (Kreth *et al.*, 2008a). Briefly, 8  $\mu\text{l}$  of an overnight culture of each species in BHI medium was inoculated as the pioneer colonizer onto a 90 mm BHI agar plate. The BHI medium and the agar plates were alternatively supplemented or not supplemented with 10 mM  $\text{SCN}^-$ . bLPO (TATUA Co-operative Dairy Company) was introduced using two different methods: 100  $\mu\text{l}$  PBS containing 250  $\mu\text{g}$  bLPO was spread as a lawn



**Fig. 1.** Competitive host peroxidase and streptococcal defence mechanisms in the supragingival region of the oral cavity. (A) Cariogenic *S. mutans* produces bacteriocins (mutacins) that are known to be cytotoxic to other oral streptococci. (B) Oral health-compatible *S. gordonii* and *S. sanguinis* differentially produce  $\text{H}_2\text{O}_2$  under aerobic growth conditions, thereby promoting an ecological advantage. (C) The human supragingival peroxidase system (consisting of the enzyme hSPO or hMPO, the substrate  $\text{SCN}^-$  and the oxidant  $\text{H}_2\text{O}_2$ ) produces the antimicrobial hypothiocyanite ( $\text{OSCN}^-$ ). (D) The peroxidase proteins themselves are known to be antimicrobial. (E) Environmental pressures, which may be biotic or abiotic in origin, could affect the above defensive mechanisms.

using a Spiral Biotech Autoplate 4000, or 20  $\mu\text{g}$  bLPO was added to the 8  $\mu\text{l}$  of inoculum of both the pioneer and the competing species just before plating. A reference culture (spatially separated from the pioneer and subsequent competing inocula) was inoculated at the same time. After incubation for 16 h, 8  $\mu\text{l}$  of the competing species was inoculated next to the pioneer colonizer such that the colonies almost touched each other. A reference of the competing colonizer was inoculated at the same time. The inoculation order was pioneer/competitor: *S. mutans/S. gordonii*, *S. gordonii/S. mutans*, *S. mutans/S. sanguinis*, *S. sanguinis/S. mutans*, *S. gordonii/S. gordonii*, and *S. sanguinis/S. gordonii*. The plates were prepared in triplicate. After incubation for an additional 16 h, the plates were photographed with an in-house-constructed, optical density-calibrated digital camera system. Growth inhibition was assessed by the presence of a proximal zone of inhibition at the intersection with the pioneer colony.

**Competition assays on solid medium: effect of the catalase.** A protocol analogous to the one used to investigate the effects of the bLPO system was followed, except that no  $\text{SCN}^-$  was added to the 8  $\mu\text{l}$  inocula of both the pioneer and the competing species just before plating. Alternatively, 8  $\mu\text{l}$  PBS containing 8  $\mu\text{g}$  catalase was added to the site of inoculation, and once absorbed, 8  $\mu\text{l}$  of the inocula followed. Both procedures for introducing catalase gave the same visual result. The inoculation order was pioneer/competitor: *S. mutans/S. sanguinis*, *S. sanguinis/S. mutans* and *S. sanguinis/S. sanguinis*.

**Biofilm quantification with 96-well microtitre dish assay.** Biofilm formation was measured using a modification of the

Christensen microtitre plate test (Christensen *et al.*, 1985). Microtitre wells (Falcon Microtest 96, Becton Dickinson) were inoculated from overnight BHI-grown cultures of *S. mutans*, *S. gordonii* or *S. sanguinis* diluted 1:60 in fresh BHI medium with or without the addition of bLPO and SCN<sup>-</sup>, as indicated (final volume 150 µl with 10<sup>7</sup> cells ml<sup>-1</sup>). The cells were grown for 16 h, and the medium was removed by inverting the dish with shaking. The remaining cells were stained with 150 µl per well of crystal violet (CV) (2.3% w/v, Accustain Crystal Violet Solution, Sigma Diagnostics) for 15 min, and the microtitre dish was subsequently washed twice with water and air-dried. Biofilm formation was quantified by solubilization of the CV staining in 150 µl of 95% ethanol per well. The absorbance of the resulting solution (100 µl) was measured at 570 nm with a microplate reader (model 680, Bio-Rad). Experiments were repeated three times with multiple replicates (at least eight) per microtitre dish.

**Confocal laser scanning microscopy (CLSM).** Biofilms for CLSM imaging were grown essentially as described above for the 96-well plate assay. The cell suspensions were inoculated into the Lab-TekII Chamber Slide System (Nalge Nunc International), where the objective slide was replaced with a thin cover slide to accommodate the working distance of the microscope objective. Biofilms were grown aerobically for 16 h at 37 °C. Cells were stained with a LIVE/DEAD Bacterial Viability kit (Molecular Probes) according to the manufacturer's recommendations. CLSM was performed using a LSM-510META laser scanning confocal microscope (Carl Zeiss) equipped with detectors and filter sets for visualizing green and red fluorescence. Images were obtained with a ×63 water (1.2 numerical aperture) objective.

**Rate of reaction of H<sub>2</sub>O<sub>2</sub> with BHI.** The rate of reaction of H<sub>2</sub>O<sub>2</sub> with BHI was determined by adding a 500 µM bolus of H<sub>2</sub>O<sub>2</sub> to BHI broth followed by periodic sampling. The concentration of H<sub>2</sub>O<sub>2</sub> was determined using a modification of a protocol described by Gilliland (1969). Briefly, 50 µl of 0.1% w/v *o*-dianisidine (ICN) in methanol was added to 750 µl of 100 mM acetate buffer (pH 4.5) with 2.0 µg horseradish peroxidase (HRP; Sigma) in methanol, followed by 200 µl of the sample of BHI which contained H<sub>2</sub>O<sub>2</sub>. The reaction mixture was incubated at 20 °C for 10 min, and the chromophore was stabilized by adding 50 µl of 4 M HCl. The absorbance at 400 nm was determined using an identical solution (*o*-dianisidine + HRP + BHI) without H<sub>2</sub>O<sub>2</sub> as a blank, and the concentration was calculated from a standard curve prepared using a 500 µM solution of H<sub>2</sub>O<sub>2</sub> [from a 100 mM stock solution in water that was prepared from a commercial 30% H<sub>2</sub>O<sub>2</sub> solution (Sigma)]. The concentration of H<sub>2</sub>O<sub>2</sub> in the 100 mM stock solution was determined spectrophotometrically ( $\epsilon_{240}=43.6 \text{ M}^{-1} \text{ cm}^{-1}$ ) using an HP 8452A diode array UV-visible spectrophotometer.

**Rate of reaction of OSCN<sup>-</sup> with BHI.** The rate of reaction of OSCN<sup>-</sup> with BHI was determined by a colorimetric assay using 5-thio-2-nitrobenzoic acid (TNB) (Aune & Thomas, 1977). As H<sub>2</sub>O<sub>2</sub> is known to interfere with the assay (Bulaj *et al.*, 1998), it was first established that the BHI contained no measurable H<sub>2</sub>O<sub>2</sub>, using the above HRP/*o*-dianisidine method. TNB was prepared *in situ* by reduction of a 520 µM solution of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; TCI) in 0.1 M phosphate buffer (pH 7.4) with a 0.5 M stock solution of 2-mercaptoethanol in water. Excess 2-mercaptoethanol was avoided by monitoring the absorption of DTNB ( $\lambda_{\text{max}}=324 \text{ nm}$ ,  $\epsilon_{324}=17780 \text{ M}^{-1} \text{ cm}^{-1}$ ) (Riddles *et al.*, 1979). The final concentration of TNB was determined spectrophotometrically ( $\lambda_{\text{max}}=412 \text{ nm}$ ,  $\epsilon_{412}=14150 \text{ M}^{-1} \text{ cm}^{-1}$ ) (Eyer *et al.*, 2003) as 860 µM (~95% reduction of the original DTNB). A 1 mM solution of OSCN<sup>-</sup> was prepared in 0.1 M phosphate buffer (pH 7.4) by reacting 1 mM H<sub>2</sub>O<sub>2</sub> with 10 mM SCN<sup>-</sup> in the presence of 10 µg bLPO ml<sup>-1</sup>. The resulting solution of OSCN<sup>-</sup> was mixed 1:1 with BHI broth to give a 500 µM solution of OSCN<sup>-</sup> in 50% BHI. The amount of

OSCN<sup>-</sup> in 25 µl BHI solution was determined periodically by adding it to a 50 µM solution of TNB (prepared by adding 58 µl TNB stock solution to 917 µM 0.1 M phosphate buffer at pH 7.4). Taking into account the 2:1 stoichiometry of the TNB to OSCN<sup>-</sup> reaction, the concentration of OSCN<sup>-</sup> was determined spectrophotometrically by monitoring the absorption of TNB at 412 nm.

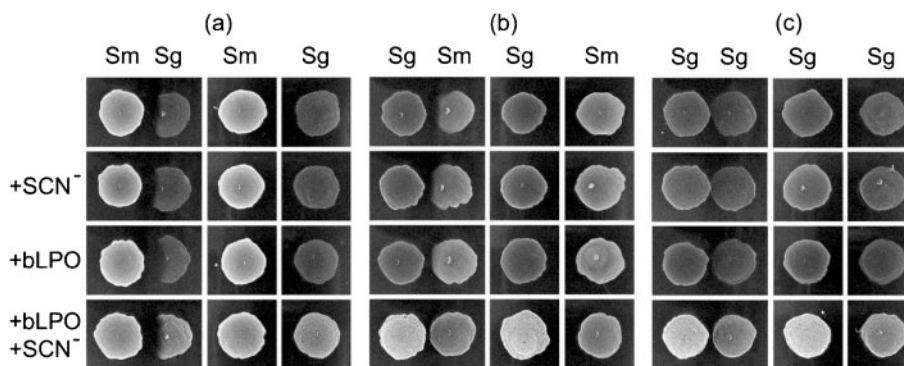
**Concentration of thiols in BHI.** Thiols in BHI were quantified with Ellman's reagent (DTNB) using a published procedure (Bulaj *et al.*, 1998; Ellman, 1959).

**Statistical methods.** Statistical analysis of two datasets was performed with QuickCalcs online calculators (<http://www.graphpad.com/quickcalcs/index.cfm>) using the *t* test software presenting mean and SD. Differences between group means (bLPO concentrations) were tested with ANOVA (<http://statpages.org/anova1sm.html>). Statistically significant differences were set at a *P* value of <0.05.

## RESULTS

### The bLPO system protects streptococci from the toxic effects of H<sub>2</sub>O<sub>2</sub>

To assess the influence of each component of the bLPO system (comprised of bLPO, SCN<sup>-</sup> and H<sub>2</sub>O<sub>2</sub>), the effects of BHI (control), BHI+SCN<sup>-</sup>, BHI+bLPO, and BHI+bLPO+SCN<sup>-</sup> were investigated using a competition assay on agar. For the agar with SCN<sup>-</sup>, BHI was supplemented with 10 mM NaSCN before autoclaving. For the agar with bLPO, the protein was filter-sterilized and either spread as a lawn on the agar before inoculation or bLPO was added to the inocula. The two methods of introducing bLPO gave visually indistinguishable results. Typical results are illustrated in Fig. 2. Visually similar results were obtained when *S. sanguinis* was substituted for *S. gordonii* for experiments analogous to those of Fig. 2(a, b) (data not shown). Proximal zones of inhibition around competing colonies of *S. gordonii* and *S. sanguinis* were observed under all growth/medium conditions when *S. mutans* was the pioneer (e.g. Fig. 2a). In the absence of bLPO, proximal zones of inhibition around competing colonies of *S. mutans* were observed when *S. gordonii* (or *S. sanguinis*) was the pioneer (e.g. Fig. 2b). In some cases, bLPO alone resulted in a reduction in the zone of inhibition for competing colonies of *S. mutans* when *S. gordonii* or *S. sanguinis* was the pioneer (e.g. Fig. 2b for bLPO + SCN<sup>-</sup>). Reduction of the zones of inhibition by the protein alone was never as notable as when the protein was added in conjunction with SCN<sup>-</sup>. Mature colonies of *S. gordonii* retarded the establishment of adjacent colonies of *S. gordonii* (Fig. 2c) and *S. sanguinis* (data not shown). For all colonies of *S. gordonii* and *S. sanguinis* (regardless of whether they were pioneers or competitors), the presence of bLPO + SCN<sup>-</sup> resulted in more dense colonies (e.g. *S. gordonii* in Fig. 2 for the control versus bLPO + SCN<sup>-</sup>).



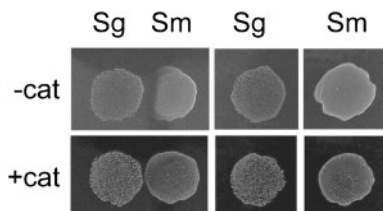
**Fig. 2.** Effect of the bLPO system on the growth of *S. mutans* and *S. gordonii*. (a) *S. mutans* was inoculated first and grown aerobically (5% CO<sub>2</sub>) for 16 h at 37 °C on BHI agar with (+SCN<sup>-</sup>) or without 10 mM SCN<sup>-</sup> and with (+bLPO) or without 20 µg bLPO per 8 µl of inoculum. *S. gordonii* was then inoculated next to the pioneer colonizer (with or without bLPO in the inoculum), and the plates were incubated for 16 h and then photographed. The columns labelled Sm and Sg are reference cultures grown in spatial isolation. (b) The same protocol was followed, except that *S. gordonii* was the pioneer colonizer and *S. mutans* was the competing colonizer. (c) The same protocol was followed as for (a) and (b), except that *S. gordonii* was both the pioneer colonizer and the competing colonizer.

### Catalase protects the streptococci from the toxic effects of H<sub>2</sub>O<sub>2</sub>

It has been previously suggested that catalase-negative oral bacteria that grow in the vicinity of catalase-positive bacteria are afforded protection from H<sub>2</sub>O<sub>2</sub> (Jakubovics *et al.*, 2008). Accordingly, we have observed that inhibition of competing colonies of *S. mutans* by pioneer colonies of *S. gordonii* and *S. sanguinis* was abolished by catalase (e.g. Fig. 3). Not surprisingly, the addition of catalase to the inocula had no effect on the bacteriocin-induced inhibition of competing colonies of *S. gordonii* and *S. sanguinis* by pioneer colonies of *S. mutans* (data not shown).

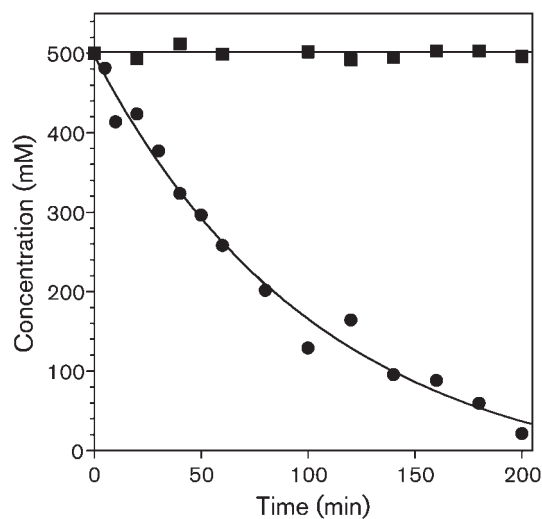
### Hypothiocyanite is not an obvious influencing factor

In contrast to H<sub>2</sub>O<sub>2</sub>, which is relatively inert towards thiol moieties, OSCN<sup>-</sup> is reactive, and in addition it spontaneously decomposes (via an unknown mechanism) in the absence of a reaction partner (Aune & Thomas, 1977; Christy & Egeberg, 2000; Tenovuo *et al.*, 1986; Thomas,



**Fig. 3.** Effect of catalase on the growth of *S. gordonii* and *S. mutans*. The same protocol was followed as for Fig. 2, except that catalase was employed instead of bLPO.

1981). We determined that the half-life of OSCN<sup>-</sup> in BHI (Fig. 4) is similar to that in PBS under similar conditions (data not shown). The kinetics of decomposition of OSCN<sup>-</sup> in BHI are consistent with our observation that the BHI does not contain thiol groups (data not shown). At room temperature, 500 µM OSCN<sup>-</sup> decomposed in BHI in a first-order process with a half-life of about 1 h, with no detectable OSCN<sup>-</sup> remaining after about 4 h (Fig. 4). Under similar conditions at 37 °C, no OSCN<sup>-</sup> was detected after 1 h (data not shown). In contrast, no loss



**Fig. 4.** Rate of decomposition of an initial concentration of OSCN<sup>-</sup> of 500 µM at pH 7.4 and 19 °C in the presence of 50% BHI (●). A first-order fit is illustrated ( $k=9.5 \pm 1.4 \times 10^{-3} \text{ min}^{-1}$ ;  $t_{1/2}=73 \text{ min}$ ). H<sub>2</sub>O<sub>2</sub> (initial concentration=500 µM) is stable under the same conditions (■).

of  $\text{H}_2\text{O}_2$  was observed in BHI over a period of 5 h at 20 °C (Fig. 4).

Our observations suggest that under the conditions of our competitive assays on solid media, the  $\text{OSCN}^-$  that is produced decomposes before it has a marked effect on adjacent colonies. However, there is also no inhibitory effect of  $\text{OSCN}^-$  on the pioneer colonies of *S. gordonii* and *S. sanguinis* (when grown in the presence of  $\text{bLPO} + \text{SCN}^-$ ). Indeed, denser colonies of these  $\text{H}_2\text{O}_2$ -producing bacteria were observed in the presence of  $\text{bLPO} + \text{SCN}^-$ . This observation suggests that  $\text{H}_2\text{O}_2$  is more cytotoxic to *S. gordonii* and *S. sanguinis* than the  $\text{OSCN}^-$  that is presumably produced. This issue will be addressed further in the Discussion (see Fig. 8). We note that because  $\text{OSCN}^-$  decomposes faster than  $\text{H}_2\text{O}_2$ , the competition assay on solid media does not address the issue of whether  $\text{OSCN}^-$  or  $\text{H}_2\text{O}_2$  is more cytotoxic to *S. mutans*.

### LPO inhibits biofilm formation by streptococci, but sucrose overcomes the effect for *S. mutans* only

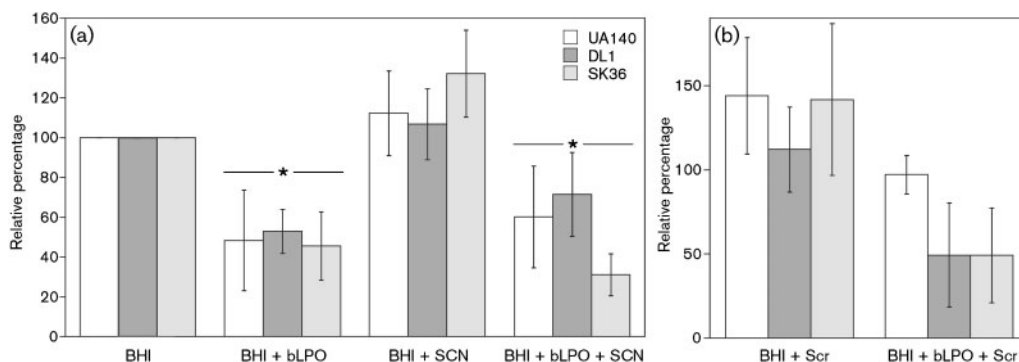
Oral streptococci must attach to the tooth surface to avoid eradication by saliva flow. After attachment, the cells develop into stratified biofilm structures. This process is accompanied by the presence of saliva and salivary components, including the defensive peroxidase systems. To learn whether the LPO system influences the biofilm formation of *S. sanguinis*, *S. gordonii* and *S. mutans*, the relative biomass of attached cells after overnight biofilm development was assessed with a microtitre plate biofilm assay. Compared with the BHI control, cells grown in the presence of 1 mg  $\text{bLPO ml}^{-1}$  showed a reduced ability to form biofilm on the polystyrene surface. The biomass decreased to about 50 % (Fig. 5a). A similar decrease was

observed when  $\text{SCN}^-$ -containing medium was supplemented with  $\text{bLPO}$ . Both reductions were significant for all strains as determined by Student's *t* test ( $P < 0.05$ ). In contrast, BHI supplemented with  $\text{SCN}^-$  did not decrease in biomass, indicating that  $\text{bLPO}$  alone is sufficient to exert an effect on the biofilm-forming abilities of oral streptococci. The terminal cell density of all species (determined spectrophotometrically) was not influenced by  $\text{bLPO}$ ,  $\text{SCN}^-$  or  $\text{bLPO} + \text{SCN}^-$  (versus the BHI control) under the tested conditions, suggesting that the reduced biomass was not a consequence of decreased growth (data not shown).

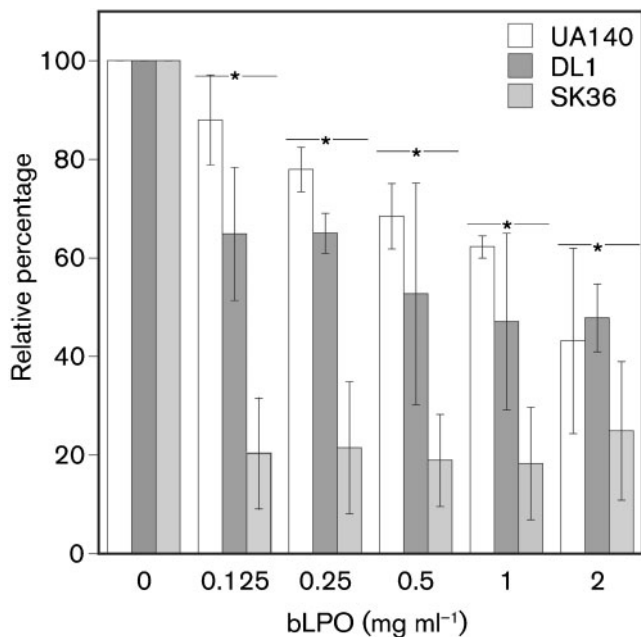
Earlier reports have shown that *S. mutans* is better able to withstand shear stress (Yang *et al.*, 2006) and antimicrobials (Kreth *et al.*, 2008b) when grown in the presence of sucrose. This has been attributed to the production of extracellular glucan polymers. Addition of 0.5 % sucrose increased the attached biomass of *S. mutans* and *S. sanguinis* slightly, but seemed to have no effect on *S. gordonii*. Addition of 0.5 % sucrose to cultures with  $\text{bLPO}$ , however, increased the attached biomass of *S. mutans* to a value that was similar to that for the BHI control. Accordingly, the difference between  $\text{bLPO}$  and  $\text{bLPO}$  with sucrose was statistically significant for the attached biomass of *S. mutans* ( $P < 0.05$ ). Sucrose addition did not influence the biomass of *S. sanguinis* and *S. gordonii* when grown in the presence of  $\text{bLPO}$  (Fig. 5b). This suggests that sucrose-dependent glucan formation can overcome the inhibitory effect of  $\text{bLPO}$  during *S. mutans* biofilm formation.

### Biofilm inhibition by LPO is dose-dependent

Addition of varying concentrations of  $\text{bLPO}$  from 0.125 to 2 mg  $\text{ml}^{-1}$  resulted in a significant dose-dependent inhibition of biofilm growth for *S. mutans* ( $P = 0.0065$ ) and *S. gordonii* ( $P = 0.04$ ) (Fig. 6), as determined by a one-



**Fig. 5.** Quantification of attached biomass using a microtitre plate biofilm assay. Biofilm formation of *S. mutans* (UA140, white), *S. gordonii* (DL1, dark grey) and *S. sanguinis* (SK36, light grey) on microtitre plates was quantified with CV staining. Values were normalized to the BHI control, which was set to 100 %. (a) Biofilm biomass of cultures in BHI medium, BHI plus  $\text{bLPO}$  (1 mg  $\text{ml}^{-1}$ ), BHI plus  $\text{SCN}^-$  (10 mM) and BHI plus  $\text{bLPO}$  (1 mg  $\text{ml}^{-1}$ ) and  $\text{SCN}^-$  (10 mM). (b) Biofilm biomass of cultures supplemented with 0.5 % sucrose and 0.5 % sucrose plus  $\text{bLPO}$  (1 mg  $\text{ml}^{-1}$ ). Data presented are the means  $\pm$  SD from three independent experiments. Asterisks indicate statistical significance.

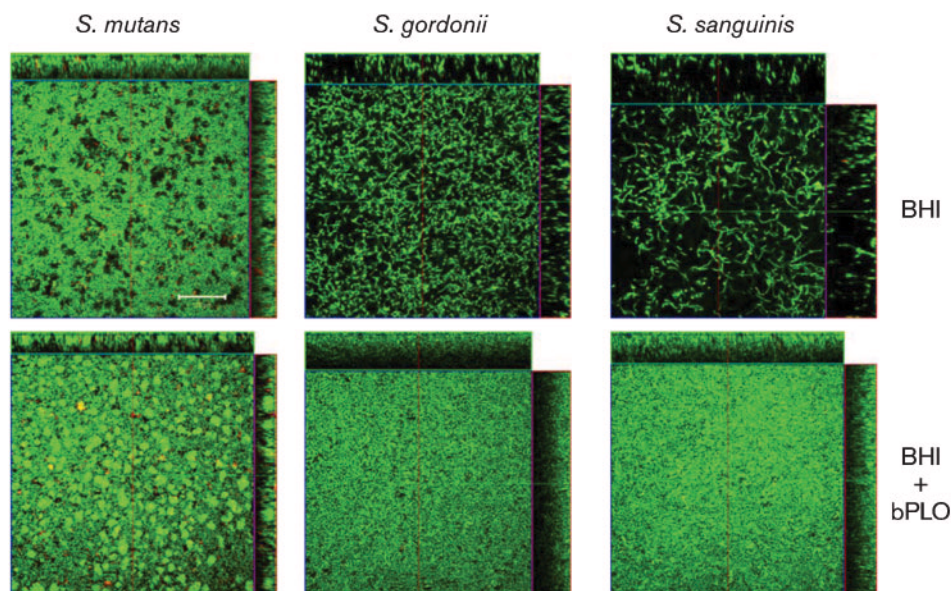


**Fig. 6.** Dose-dependent effect of bLPO protein (no SCN<sup>-</sup>) upon biofilm growth of *S. mutans* (UA140, white), *S. gordonii* (DL1, dark grey) and *S. sanguinis* (SK36, light grey) using the 96-well plate assay. Cultures were grown in BHI medium containing different concentrations of LPO (ranging from 0.125 to 2 mg ml<sup>-1</sup>). Values were normalized to the BHI control, which was set to 100%. Data presented are the means  $\pm$  SD from three independent experiments. Asterisks indicate statistical significance.

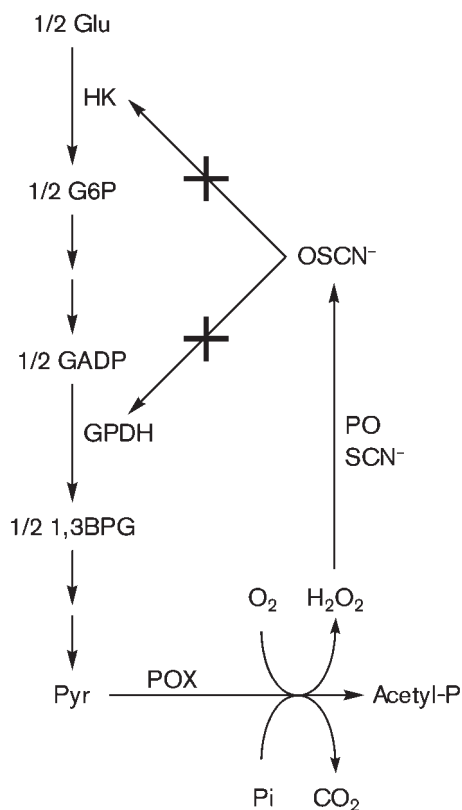
way ANOVA statistical test. Interestingly, for *S. sanguinis* the inhibition under the tested conditions was similar for all tested concentrations of bLPO. This suggests a differential response of oral streptococci to the presence of bLPO during biofilm formation.

### LPO alters biofilm architecture

Addition of bLPO to the growth medium during biofilm formation has an obvious effect on the biofilm mass of all three tested oral streptococci. To further characterize the effect of bLPO on biofilms of *S. mutans*, *S. sanguinis* and *S. gordonii*, the biofilm architecture was investigated with CLSM. Static overnight biofilms were grown and stained with LIVE/DEAD viability stain for visualization. *S. mutans* grown in BHI shows a characteristic biofilm architecture (a confluent layer of cells punctuated by void areas) that has been described previously (Kreth *et al.*, 2008b). Cells grown with bLPO seemed to cluster in dense micro-colonies with an absence of void areas. Overall, the biofilm of *S. mutans* appeared denser when grown in the presence of bLPO (Fig. 7). The biofilm architectures of *S. sanguinis* and *S. gordonii* appeared similar to one another in BHI medium. Both species formed long chains that seemed to be loosely attached and tangled together, leaving larger void areas between chains. Growth in the presence of bLPO again changed the biofilm appearance dramatically. Both *S. sanguinis* and *S. gordonii* appeared densely packed, the void areas disappeared completely, and the height of the biofilm was reduced. In summary, the biofilms of *S. mutans*, *S. sanguinis* and *S. gordonii* grown in the presence of bLPO



**Fig. 7.** Biofilm architecture of cultures grown with and without LPO. CLSM images of *S. mutans*, *S. sanguinis* and *S. gordonii* biofilms grown in the Lab-TekII Chamber Slide system. Cells were stained with the LIVE/DEAD viability fluorescent stain. Bar, 100  $\mu$ m.



**Fig. 8.** Feedback limitation of H<sub>2</sub>O<sub>2</sub> production by pyruvate oxidase (POX)-catalysed oxidation of pyruvate (Pyr) through inhibition of the upstream glycolytic enzymes hexokinase (HK) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) by host peroxidase (PO=hSPO and hMPO)-catalysed production of OSCN<sup>-</sup>. G6P, glucose 6-phosphate; GADP, glyceraldehyde 3-phosphate; 1,3BPG, 1,3-bisphosphoglycerate.

seemed to be more compact than their counterparts grown in BHI alone. However, LIVE/DEAD viability stain did not reveal any differences between BHI and BHI+bLPO-grown cells (Fig. 7).

## DISCUSSION

The results we have presented illustrate the influences of the bLPO system on the interspecies competition among three oral bacteria. Fig. 1 summarizes the competitive host and bacterial defence mechanisms that are potentially relevant to the subject of our study. (A) Cariogenic *S. mutans* produces bacteriocins, including lantibiotic mutacin I and non-lantibiotic mutacin IV, that are known to be cytotoxic to other oral streptococci (Hamada & Ooshima, 1975; Kreth *et al.*, 2008a). (B) Commensal (oral health-compatible) *S. gordonii* and *S. sanguinis* differentially produce H<sub>2</sub>O<sub>2</sub> under aerobic growth conditions, thereby promoting an ecological advantage (Kreth *et al.*, 2008a). (C) The human supragingival peroxidase system (consisting of the enzyme hSPO or hMPO, the substrate SCN<sup>-</sup>,

and the oxidant H<sub>2</sub>O<sub>2</sub>) produce the antimicrobial hypothiocyanite (OSCN<sup>-</sup>). (D) The peroxidase proteins themselves are known to be antimicrobial (Tenovuo & Knuutila, 1977b). (E) Environmental pressures that may be biotic (e.g. other microbial species) or abiotic (e.g. nutrients) in origin could affect the above defensive mechanisms. The last category is intended to draw attention to the fact that the *in vitro* models we have employed in our investigation are incomplete. Thus, the intent of the present study has been to uncover parameters that may be relevant in the oral cavity, but, as a caveat, these data do not unequivocally demonstrate the dominance of (A–D) over (E) *in vivo*. Indeed, some of the factors that enhance or diminish the influence of (A–D) are discussed next.

## Inhibition of oral streptococci by hypothiocyanite

It has been previously demonstrated that *S. mutans* is inhibited by the LPO + SCN<sup>-</sup> + H<sub>2</sub>O<sub>2</sub> system when H<sub>2</sub>O<sub>2</sub> is added exogenously (Donoghue *et al.*, 1985; Hoogendoorn, 1976; Kersten *et al.*, 1981; Mansson-Rahemtulla *et al.*, 1987; Rupf *et al.*, 2001; Tenovuo & Knuutila, 1977a; Tenovuo & Knuutila, 1977b; Thomas *et al.*, 1983; Thomas *et al.*, 1994; van der Hoeven & Camp, 1993). Furthermore, Thomas *et al.* (1983) have shown that the amount of H<sub>2</sub>O<sub>2</sub> that is produced by some strains of *S. mutans* during aerobic growth is sufficient to activate the bLPO system. In contrast to the large number of studies of the effects of the LPO and related systems on *S. mutans*, there have been relatively few studies of the effect on *S. sanguinis* (formally *sanguis*) (Carlsson, 1980; Carlsson *et al.*, 1983; Courtois *et al.*, 1995; van der Hoeven & Camp, 1993), and we are not aware of any efforts to quantify the effects of the peroxidase systems on *S. gordonii*. Nonetheless, a number of other streptococcal species have been shown to be susceptible to inhibition by OSCN<sup>-</sup> in sufficient concentration, including *Streptococcus oralis* (van der Hoeven & Camp, 1993), *Streptococcus mitior* (Donoghue *et al.*, 1987), *Streptococcus rattus* (Donoghue *et al.*, 1987), *Streptococcus uberis* (Marshall *et al.*, 1986), *Streptococcus dysgalactiae* (Mickelson & Brown, 1985), *Streptococcus agalactiae* (Mickelson, 1979) and *Streptococcus pyogenes* (Mickelson, 1966). Importantly, many factors determine susceptibility to inhibition by OSCN<sup>-</sup>, including pH, the flux of the peroxidase system (availability of H<sub>2</sub>O<sub>2</sub>), cell thiol content, stored carbohydrate content, and the ability of the medium to quench OSCN<sup>-</sup>. Peroxidase systems produce OSCN<sup>-</sup> at the expense of H<sub>2</sub>O<sub>2</sub>, but both chemical species can be cytotoxic; apparently, H<sub>2</sub>O<sub>2</sub> can be more so under some circumstances (see below).

## The peroxidase system protects streptococci from H<sub>2</sub>O<sub>2</sub> toxicity

Although H<sub>2</sub>O<sub>2</sub> production is part of the lifestyle of streptococci, they lack conventional defence mechanisms against the toxicity of H<sub>2</sub>O<sub>2</sub> (e.g. catalase and glutathione). Resistance of streptococci to H<sub>2</sub>O<sub>2</sub> toxicity has been

attributed to the absence of haemoproteins and related metalloenzymes (Dolin, 1961). The relative importance of  $H_2O_2$  as a toxic agent in the oral cavity is difficult to assess because varying amounts act on microcosm plaques in diverse environments. Individual susceptibilities of oral streptococci to  $H_2O_2$  toxicity vary. Some mutants streptococci accumulate up to 2 mM  $H_2O_2$  in their media during growth on glucose (Thomas & Pera, 1983; Thomas, 1985), whereas other oral streptococci are inhibited by micromolar concentrations of  $H_2O_2$  (Adamson & Carlsson, 1982; Carlsson *et al.*, 1983; Donoghue *et al.*, 1987). Furthermore, there are likely to be synergisms in multi-species plaques. For example, while pure cultures of oral streptococci produce  $H_2O_2$ ,  $H_2O_2$  is not found in dental plaque or salivary sediment, despite streptococci being major components of their mixed bacterial populations. This could be due to the rapid consumption of  $H_2O_2$  by the hSPO and hMPO systems. Indeed, it has been suggested that the human peroxidases of the oral cavity protect host tissues (Carlsson, 1987; Tipton *et al.*, 1995) and  $H_2O_2$ -sensitive bacteria (Adamson & Carlsson, 1982) by consuming endogenous  $H_2O_2$  (with the production of the comparatively less toxic OSCN<sup>-</sup>). An alternative mechanism for the consumption of free  $H_2O_2$  is by catalase-positive species of bacteria (e.g. *Neisseria*, *Haemophilus*, *Actinomyces* and *Staphylococcus* species; see Fig. 3) (Ryan & Kleinberg, 1995).

*S. sanguinis* and *S. gordonii* produce  $H_2O_2$  under conditions of aerobic mixed-acid fermentation during the oxidation of pyruvate (Pyr) as catalysed by pyruvate oxidase (Pox) (Kreth *et al.*, 2008a). Under the conditions of our agar plate assay, bLPO + SCN<sup>-</sup> protects *S. mutans* from the cytotoxic effects of  $H_2O_2$  produced by *S. sanguinis* and *S. gordonii* (Fig. 2b). While this might be considered counterproductive for a host defence mechanism that presumably protects the oral cavity from cariogenic pathogens, we note that bLPO + SCN<sup>-</sup> also facilitates the growth of the health-compatible *S. sanguinis* and *S. gordonii* (see Fig. 2 for the control versus bLPO + SCN<sup>-</sup>). Thus, bLPO + SCN<sup>-</sup> is capable of protecting  $H_2O_2$ -producing bacteria from themselves. Of particular relevance are the results shown in Fig. 2(c), which suggest that regrowth adjacent to more mature,  $H_2O_2$ -producing colonies may be facilitated by the peroxidase system. The presence of bLPO + SCN<sup>-</sup> results in denser colonies of *S. gordonii* and *S. sanguinis*, which implies that the OSCN<sup>-</sup> that is presumably generated by the peroxidase system is less cytotoxic than the  $H_2O_2$  that is consumed in the process. However, compounding factors may be relevant: it is known that the bLPO + SCN<sup>-</sup> +  $H_2O_2$  system is capable of inhibiting enzymes in the glycolytic metabolic pathway, including hexokinase (HK) (Adamson & Pruitt, 1981; Pruitt & Adamson, 1977) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) (Shin *et al.*, 2001). It is therefore conceivable that a feedback mechanism exists by which the peroxidase system simultaneously destroys  $H_2O_2$  and inhibits key metabolic steps in the glycolytic pathway that

may disfavour the production of  $H_2O_2$  by Pox (Fig. 8). We note that the cell density that is achieved for *S. gordonii* (Fig. 3) and *S. sanguinis* (data not shown) in the presence of catalase is less than that achieved in the presence of bLPO + SCN<sup>-</sup> (e.g. Fig. 2 for *S. gordonii*), which is consistent with the hypothesis shown in Fig. 8.

### Which factors dominate in the oral cavity?

The presumed role of the innate components of the host defences in the oral cavity is to place selective pressures on pathogens while passively facilitating the growth of health-compatible bacterial species. Using *in vitro* models, we have illustrated herein two major effects of a (model) host peroxidase system on oral streptococcal speciation: (1) the peroxidase system protects all of the streptococci from  $H_2O_2$ ; and (2) the peroxidase protein inhibits biofilm formation. We note that our observations are consistent with previous studies (which have primarily employed planktonic cultures). While the overall effect of innate defence mechanisms such as the peroxidases benefits the host, there may be unintended consequences of the non-specific host defensive stratagems at the bacterial species level. For example, the peroxidase system suppresses the  $H_2O_2$ -induced antagonism of cariogenic *S. mutans* by health-compatible *S. gordonii* and *S. sanguinis*. Also, in the case of *S. mutans* (but not the health-compatible streptococci), sucrose overcomes the inhibition of biofilm growth by the bLPO protein, perhaps thereby giving the pathogen an ecological advantage. However, what are missing from this simple description are the differential effects of the innate defence mechanisms. While *in vitro* experiments such as those described here are capable of identifying relevant parameters that may affect speciation of multispecies biofilms, they do not necessarily indicate the dominant factors *in vitro*. Finally, we note that the present study has focused on conditions that are pertinent to the early colonization of teeth (i.e. aerobic growth conditions), and it is possible that the peroxidase systems afford host defence under different conditions, such as during the development of mature plaque biofilms. A challenge that remains for researchers is the transition from *in vitro* models such as the ones herein to *in vivo* experiments that assess the environmental factors that influence the microbial shifts associated with oral infectious diseases.

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