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Role of hydrogen peroxidein competition and cooperation between *Streptococcus gordonii* and *Actinomyces naeslundii*

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Abstract

In dental plaque α-haemolytic streptococci, including *Streptococcus gordonii*, are considered beneficial for oral health. These organisms produce hydrogen peroxide (H_2O_2) at concentrations sufficient to kill many oral bacteria. Streptococci do not produce catalase yet tolerate H_2O_2. We recently demonstrated that coaggregation with *Actinomyces naeslundii* stabilizes arginine biosynthesis in *S. gordonii*. Protein arginine residues are sensitive to oxidation by H_2O_2. Here, the ability of *A. naeslundii* to protect *S. gordonii* against self-produced H_2O_2 was investigated. Coaggregation with *A. naeslundii* enabled *S. gordonii* to grow in the absence of arginine, and promoted survival of *S. gordonii* following growth with or without added arginine. Arginine-replete *S. gordonii* monocultures contained 20–30 μM H_2O_2 throughout exponential growth. *Actinomyces naeslundii* did not produce H_2O_2 but synthesized catalase, removed H_2O_2 from coaggregate cultures and decreased protein oxidation in *S. gordonii*. On solid medium, *S. gordonii* inhibited growth of *A. naeslundii*; exogenous catalase overcame this inhibition. In coaggregate cultures, *A. naeslundii* cell numbers were >90% lower than in monocultures after 24 h. These results indicate that coaggregation with *A. naeslundii* protects *S. gordonii* from oxidative damage. However, high cell densities of *S. gordonii* inhibit *A. naeslundii*. Therefore, H_2O_2 may drive these organisms towards an ecologically balanced community in natural dental plaque.

Keywords

oral streptococci; hydrogen peroxide; *Actinomyces naeslundii*; metal-catalyzed oxidation; *Streptococcus gordonii*; catalase

Introduction

Oral viridans streptococci, including *Streptococcus gordonii*, *Streptococcus sanguinis*, *Streptococcus oralis* and *Streptococcus mitis*, are primary colonizers of human dental plaque. In the first few hours after tooth brushing, viridans streptococci may constitute 60–80% of dental plaque bacteria (Nyvad & Kilian, 1990; Diaz *et al*., 2006), and these organisms remain present in high numbers for at least 24 h (Nyvad & Kilian, 1987). Non-mutans viridans

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streptococci do not appear to contribute to oral diseases and are considered commensal organisms in the oral cavity. Evidence suggests that colonization with viridans streptococci may exclude more pathogenic bacteria and protect against caries or periodontitis. For example, a negative correlation between *S. sanguinis* and mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) has been demonstrated in caries: in two independent studies individuals with extensive caries had significantly higher levels of mutans streptococci and significantly lower levels of *S. sanguinis* than those with no carious lesions (Nyvad & Kilian, 1990; Becker *et al*., 2002). High levels of viridans streptococci have been associated with low numbers of *Tannerella for sythensis* (formerly *Bacteroides for-sythus*) or *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* and the absence of periodontal disease (Hillman *et al*., 1985).

Viridans streptococci derive their name from the production of a greenish tinge (α-haemolysis) on blood agar. The α-haemolysin from *S. gordonii* has been identified as hydrogen peroxide (H_2O_2) (Barnard & Stinson, 1996), formed primarily by the action of pyruvate oxidase, SpxB (Hillman & Shivers, 1988; Spellerberg *et al*., 1996). Under laboratory conditions, many viridans streptococci produce sufficient quantities of H_2O_2 to kill *S. mutans* or periodontal pathogens (Holmberg & Hallander, 1973; Miyasaki *et al*., 1985; Kreth *et al*., 2005), and therefore, H_2O_2 may be an important mediator of competition in oral biofilms.

The deleterious effects of H_2O_2 on bacterial cells arise from the generation of hydroxyl radicals ($^{\bullet}OH$) in the presence of Fe(II), and the subsequent reaction of $^{\bullet}OH$ with molecules in the vicinity (Imlay, 2003). This can result in cellular damage by degradation of [4Fe–4S] clusters in enzymes and enzyme inactivation (Jang & Imlay, 2007), or by oxidation of macromolecules including DNA and proteins (Imlay, 2003). Peroxidogenic streptococci avoid autotoxicity by using Mn(II) in place of Fe(II) (Jakubovics *et al*., 2002; Tseng *et al*., 2002) and by limiting their reliance on [4Fe–4S] cluster proteins, for example by having no respiratory chain. Nevertheless, accumulation of H_2O_2 in batch cultures reduces stationary phase survival of at least some viridans streptococci (Eisenberg, 1973; Regev-Yochay *et al*., 2007). In bacteria exposed to oxidative stress, loss of viability in stationary phase follows the accumulation of oxidized proteins (Dukan & Nystrom, 1999). Several different amino acid side chains in polypeptides are susceptible to oxidation, and the introduction of carbonyl groups into the side chains of arginine, proline, lysine or occasionally threonine, is perhaps the most significant outcome (Nystrom, 2005). This process is irreversible and carbonylated proteins are targeted for degradation (Grune *et al*., 2003; Stadtman & Levine, 2003). The oxidized amino acids cannot be recovered and must be replaced by import from the extracellular milieu or by *de novo* synthesis. Not all polypeptides are equally affected by carbonyl modification and different stress conditions result in oxidation of different subsets of proteins (Noda *et al*., 2007).

In a recent investigation into communication between *S. gordonii* and *Actinomyces naeslundii*, we observed that the *S. gordonii spxB* gene, encoding pyruvate oxidase, was upregulated in coaggregate cultures containing *A. naeslundii* compared with *S. gordonii* monocultures (Jakubovics *et al*., 2008). In addition, the expression of arginine biosynthesis genes was stabilized by coaggregation, and *S. gordonii* was able to grow in coaggregates under arginine-restricted conditions that did not support monoculture growth (Jakubovics *et al*., 2008). Considering that H_2O_2 in *S. gordonii* will oxidize arginine residues in proteins and place a burden on arginine biosynthesis, these observations suggested a possible role of H_2O_2 in the interaction between *S. gordonii* and *A. naeslundii*. Here, we investigated the ability of *A. naeslundii* to protect *S. gordonii* from H_2O_2-induced cell damage. In addition, the role of H_2O_2 as a mediator of intergeneric competition was evaluated.

Materials and methods

Bacterial strains and culture conditions

Streptococcus gordonii DL1 (Challis) and *A. naeslundii* MG1 (ATCC43146) were maintained by subculturing anaerobically (90% N_2/5% H_2/5% CO_2) at 37 °C in Todd–Hewitt broth (THB; Becton Dickenson, Sparks, MD) or on THB solidified with 1.5% Bacto agar. In some experiments THB medium was supplemented with 0.5% yeast extract (THBYE). Chemically defined medium (CDM) was based on Terleckyj's FMC medium for growth of streptococci (Terleckyj *et al*., 1975) with modifications as follows: L-leucine and L-isoleucine were added to a final concentration of 40 mg L^{-1} rather than 100 mg L^{-1} in FMC; L-arginine and L-histidine were at 100 mg L^{-1} rather than 200 mg L^{-1} in FMC; 0.1 mM $CaCl_2$ was included (not present in FMC) and the medium was adjusted to pH 7.3. For experiments involving coaggregate cultures, inocula were prepared by growing bacteria in an anaerobic environment for 16 h in TYEG consisting of 1% Bacto tryptone, 0.5% yeast extract, 0.3% K_2HPO_4 and 0.2% glucose, adjusted to pH 7.5 before autoclaving. Cells were harvested, washed twice in CDM or an identical medium lacking arginine (CDMΔarg) and resuspended in CDM or CDMΔarg. Cultures were adjusted to *c*. 5×10^9 CFUmL^{-1}. For coaggregate cultures, 300 μL of each strain were combined and vortex mixed for 10 s to form robust macroscopic coaggregates. Cultures were diluted to a final volume of 15 mL (*c*. 1×10^8 CFUmL^{-1} of each strain) in CDM or CDMΔarg and incubated aerobically without shaking. Equivalent monocultures containing *c*. 1×10^8 CFUmL^{-1} of *S. gordonii* or *A. naeslundii* were also prepared. Culture turbidity was measured using a Klett–Summerson spectrophotometer (Klett Manufacturing Co., New York) fitted with a 660 nm filter.

Enumeration of *S. gordonii* and *A. naeslundii* in mixed cultures

For enumeration of bacteria, 0.5-mL samples were removed from the culture and chains, clumps or coaggregates were disrupted by sonication in a Sonopuls ultrasonic homogenizer (Bandelin Electric, Berlin) equipped with a BR30 cup booster for 1 min on 50% maximum power. Samples were serially diluted 10-fold and 20 μL aliquots were dropped onto solidified THB medium or, to enumerate *A. naeslundii* in mixed cultures, onto THB supplemented with 128 mg L^{-1} mupirocin and 2.5 mg L^{-1} metronidazole (Lewis *et al*., 1995). Plates were incubated aerobically in 5% CO_2 atmosphere at 37 °C for 24 h (*S. gordonii*) or 48 h (*A. naeslundii*). Control experiments demonstrated that *A. naeslundii* colonies were not visible after 24 h and therefore only *S. gordonii* CFU from mixed cultures were counted at this point. Addition of mupirocin and metronidazole inhibited growth of *S. gordonii*, but had no effect on *A. naeslundii* colony formation (Jakubovics *et al*., 2008).

Quantitive determination of H_2O_2 concentrations

The concentration of H_2O_2 in culture supernatants was determined using horseradish peroxidise (HRP) and Amplex UltraRed reagent (Invitrogen, Carlsbad, CA). Cells were harvested by centrifugation in a swing-out rotor at 3200 ***g*** at 25 °C for 7 min. The supernatant was collected, filtered through a 0.22-μm pore membrane and diluted 10-fold in 50 mM sodium phosphate buffer, pH 7.4. Triplicate 50 μL samples were mixed with 50 μL sodium phosphate buffer containing 1 U HRP and 0.1 mM Amplex UltraRed and incubated at 25 °C for 30 min. Fluorescence (525 nm excitation/590 nm emission) was measured using a VICTOR^3 microplate reader (Perkin Elmer, Waltham, MA). For each assay, a standard curve was prepared by diluting a 30% H_2O_2 solution (Sigma, St. Louis, MO).

Assessment of protein oxidation

Proteins were extracted from *S. gordonii* or *A. naeslundii* cells by enzymatic digestion of the cell wall and bead beating (Jakubovics *et al*., 2000). Following protein extraction, samples

were divided and the protein concentration was determined in one portion using a BCA assay kit (Pierce, Rockford, IL). To the other portion, dithiothreitol (50 mM final concentration) was added and the samples were frozen immediately at − 20 °C and analyzed together when all samples were collected. Proteins containing carbonyl groups were visualized by Western blotting using the Oxyblot kit (Chemicon International, Temecula, CA) as outlined by the manufacturer. Briefly, protein extracts were denatured by mixing with an equal volume of 12% sodium dodecyl sulphate and carbonyl groups were derivatized to 2,4-dinitrophenylhydrazone (DNP) by addition of 2,4-dinitrophenylhydrazine and incubation for 15 min at 25 °C. The reaction was neutralized and proteins were separated on a 12% polyacrylamide gel. Proteins were blotted to a nitrocellulose membrane, and DNP was detected by immunostaining with a specific antibody.

H_2O_2 detection in bacterial colonies and agar plate competition assay

Bacterial monocultures in CDM were prepared as described above, and 10 μL portions were spotted onto solidified THB medium. In some cases, 10 U catalase (Sigma) were included in the CDM used to resuspend *S. gordonii* cells. For competition assays, a drop of *S. gordonii* culture was placed on the plate and allowed to dry. Within 5 min, *A. naeslundii* was spotted in close proximity but not overlapping with the *S. gordonii* drop. The plates were incubated aerobically in 5% CO_2 atmosphere for 48 h at 37 °C. Where appropriate, H_2O_2 was detected by flooding the plate with 3 mL detection reagent [100 mM potassium phosphate, pH 6.0 containing 20 U mL^{-1} HRP and 1 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)]. Excess detection reagent was removed immediately and plates were incubated at 25 °C for 15 min before imaging. The presence of H_2O_2 was indicated by a purple colour.

Results

Coaggregation with *A. naeslundii* enhances growth and survival of *S. gordonii*

Recently, we have shown that coaggregation with *A. naeslundii* enables *S. gordonii* to initiate aerobic growth in low (0.025 mM) arginine, equivalent to the concentration of free arginine in saliva (Jakubovics *et al*., 2008). Under these conditions, there was a small (*c*. 1 log) decrease in the number of viable *S. gordonii* cells in monoculture after 24 h incubation (Jakubovics *et al*., 2008). To determine whether the extracellular arginine concentration influences the ability of *S. gordonii* to survive in stationary phase or to grow in coaggregation with *A. naeslundii*, monocultures and coaggregate cultures were set up using a CDM or a modified medium lacking arginine (CDMΔarg). Cultures were incubated aerobically for up to 24 h and viable counts of streptococci were determined. In CDMΔarg medium, *S. gordonii* monocultures rapidly lost viability, and viable counts decreased by *c*. 1 log in 6 h and *c*. 3 log after 24 h (Fig. 1). Coaggregation enabled *S. gordonii* to initiate growth in the absence of arginine and to remain viable for ≥24 h after inoculation. The onset of streptococcal growth did not occur for ≥6 h and viable counts decreased at a similar rate to monocultures during this lag phase. In CDM (0.5 mM arginine), *S. gordonii* grew to high cell densities within 6 h in monoculture, but rapidly lost viability in stationary phase (Fig. 1). Viable counts decreased approximately two orders of magnitude between 6 and 24 h. By contrast, in coaggregates *S. gordonii* CFU remained stable or even increased slightly between 6 and 24 h after inoculation.

Loss of viability in stationary phase has been linked to H_2O_2 production by viridans streptococci, and can be averted in *Streptococcus pneumoniae* by disruption of *spxB*, the gene encoding the major H_2O_2-producing enzyme, pyruvate oxidase (Regev-Yochay *et al*., 2007). We tested survival of an *spxB* mutant of *S. gordonii* (kindly donated by R. J. Lamont) in aerobic CDM. In monoculture, this strain grew at a similar rate to wild type, reaching 2×10^9 CFU mL^{-1} after 6 h. However, unlike wild-type *S. gordonii*, the *spxB* mutant remained viable in monoculture and $>1 \times 10^9$ CFU mL^{-1} were recovered after 24 h (data not shown).

Actinomyces naeslundii depletes H_2O_2 from *S. gordonii* cultures and protects *S. gordonii* from protein oxidation

To examine the role of H_2O_2 in *S. gordonii* and *A. naeslundii* cultures, H_2O_2 concentrations were measured in bacterial supernatants throughout aerobic growth of *S. gordonii* and *A. naeslundii* in monoculture and in coaggregate CDM (0.5 mM arginine) cultures. H_2O_2 was barely detectable in *A. naeslundii* cultures during incubation for 6 h. On the other hand, *S. gordonii* monocultures maintained a stable H_2O_2 concentration of *c*. 25 μM (Fig. 2a and b). In coaggregate cultures, H_2O_2 was initially low (*c*. 7 μM) but increased after 2 h as the streptococci outgrew the *Actinomyces* cells. By 4 h, the concentration of H_2O_2 exceeded 15 μM and it remained stable at this level until ≥6 h after inoculation.

Oxidative damage to bacterial proteins was assessed using the Oxyblot kit. This procedure derivatizes carbonyl groups, caused by oxidation of arginine, proline or lysine residues in proteins, to 2,4-dinitrophenylhydrazone and detects the modified proteins with a specific antibody. Protein oxidation was measured after 3 h growth, when the streptococci had reached late exponential phase. By this time streptococci had outgrown *Actinomyces* and constituted 85–90% of cells in coaggregate cultures (Fig. 2a). From measurements of proteins recovered per cell in monoculture, the efficiency of protein extraction was found to be equivalent for *S. gordonii* and *A. naeslundii* (data not shown). Therefore, it was predicted that 85–90% of proteins extracted from coaggregate cultures incubated for 3 h originated from *S. gordonii*. Examination of Coomassie-stained gels (not shown) confirmed that the one-dimensional protein profiles of coaggregate cultures looked very similar to those of *S. gordonii* monocultures. Extensive protein oxidation was observed in preparations from *S. gordonii* monocultures, with a particularly strong band corresponding to *c*. 55 kDa (Fig. 2c). In contrast, only a small number of bands from *A. naeslundii* protein extracts cross-reacted with the Oxyblot antibody. In coaggregate cultures, protein oxidation was markedly reduced compared with *S. gordonii* monocultures. A band at *c*. 55 kDa was apparent, but several others that were present in monoculture extracts, including a band at 48 kDa and five bands at >100 kDa, were absent from coaggregate culture extracts (Fig. 2c).

Antibacterial effects of *S. gordonii* -produced H_2O_2 against *A. naeslundii*

Several studies have documented the bactericidal properties of H_2O_2 produced by viridians streptococci against oral and extraoral bacteria (Holmberg & Hallander, 1973; LeBien & Bromel, 1975; Miyasaki *et al*., 1985; Kreth *et al*., 2005). A simple assay, based on growth on solidified THB medium, was used to determine whether *A. naeslundii* growth is inhibited by *S. gordonii*. Initially, production of H_2O_2 by *S. gordonii* and *A. naeslundii* on THB agar was assessed by a colorimetric reaction that produces purple staining in the presence of H_2O_2. This assay clearly demonstrated production of H_2O_2 by *S. gordonii* but not by *A. naeslundii* (Fig. 3a). Next, 10 μL drops containing 10^6 CFU of an exponential phase culture of each strain were spotted in close proximity on solidified THB medium and incubated aerobically under 5% CO_2 for 48 h. *Actinomyces naeslundii* did not grow within *c*. 10 mm of *S. gordonii* (Fig. 3b). However, mixing *S. gordonii* cells with 10 U catalase prior to spotting on the agar reduced the zone of inhibition to < 1 mm.

To test whether H_2O_2 from *S. gordonii* was bactericidal against *A. naeslundii*, monocultures and coaggregate cultures in CDM were incubated aerobically for 24 h and viable *A. naeslundii* cells were enumerated on selective agar. Unlike *S. gordonii*, *A. naeslundii* cells in monoculture did not lose viability within this time frame (Fig. 4a), even though the medium supported only weak growth of *A. naeslundii*. In coaggregate cultures, *A. naeslundii* CFU increased slightly over the first 6 h. Between 6 and 24 h, *A. naeslundii* CFU dropped by almost three orders of magnitude, indicating that these cells were efficiently killed by *S. gordonii* during this period. Because the growth of *A. naeslundii* in CDM was poor, we assessed the

inhibitory properties of *S. gordonii* towards *A. naeslundii* in a rich medium (THBYE) that supported growth of *A. naeslundii* to high cell densities (Fig. 4b). By 6 h after inoculation, *A. naeslundii* cell numbers had increased greater than threefold in both monoculture and coaggregate cultures. In monoculture, *A. naeslundii* reached $>10^9$ CFU mL^{-1} by 24 h and cell numbers did not increase after prolonged (48 h) incubation (data not shown). In coaggregate culture, there was a marked (*c*. 2.5-fold) decrease in viable cell numbers between 6 and 24 h, indicating that *S. gordonii* inhibited the growth of *A. naeslundii* cells in this medium.

Discussion

Dental plaque is a complex microbial ecosystem in which viridans streptococci, including *S. gordonii*, and *A. naeslundii* are often found in high numbers from the earliest stages. Several factors may enhance the coexistence of these species. For example, efficient degradation of host carbohydrates often requires combinations of enzymes from more than one bacterial species (Bradshaw *et al*., 1994). *Streptococcus gordonii* can utilize free sialic acids, but does not produce sialidase and therefore cannot release sialic acids from host glycoproteins (Bradshaw *et al*., 1994; Byers *et al*., 1996). *Actinomyces naeslundii* has sialidase activity (Costello *et al*., 1979; Bradshaw *et al*., 1994) and could potentially supply nutrients for *S. gordonii*. On the other hand, *A. naeslundii* lacks several glycolytic and proteolytic activities that are produced by *S. gordonii*, including *N*-acetyl-glucosaminidase, α-fucosidase, gly-pro diamino peptidase and trypsin-like protease (Bradshaw *et al*., 1994). In addition, there is evidence that the ability of *A. naeslundii* to bind surface receptors on *S. gordonii* contributes to its retention in biofilms under flowing saliva (Palmer *et al*., 2001). Here, we demonstrate that H_2O_2 production results in *S. gordonii* obtaining a benefit from *A. naeslundii* in the form of protection against oxidative stress. When *S. gordonii* cells significantly outnumber *A. naeslundii*, H_2O_2 production overwhelms the *Actinomyces* cells and kills them as well as itself. Therefore, H_2O_2 may be an additional factor that drives coexistence and modulates the *A. naeslundii* population while selecting against overgrowth of *S. gordonii*.

In an attempt to identify the key gene functions of *S. gordonii* that are involved in interactions with *A. naeslundii*, we recently applied DNA microarray analysis to *S. gordonii* monocultures and coaggregate cultures containing *A. naeslundii* (Jakubovics *et al*., 2008). These experiments demonstrated that *S. gordonii* arginine biosynthesis is a major pathway affected by coaggregation. Further investigation demonstrated that aerobic arginine biosynthesis is inefficient in *S. gordonii* and only occurs when ≥0.1 mM arginine is supplied initially (Jakubovics *et al*., 2008). However, coaggregation with *A. naeslundii* can overcome this arginine requirement and enable growth of *S. gordonii* in the absence of arginine. Arginine is a well-recognized nutritional antioxidant (Fang *et al*., 2002) and can react directly with free radicals to neutralize them (Lass *et al*., 2002). Furthermore, arginine residues in proteins are sensitive to an irreversible oxidation reaction in the presence of free radical generating systems such as H_2O_2/Fe^{2+} (Stadtman & Levine, 2003). Therefore, H_2O_2 produced by *S. gordonii* is likely to deplete the intracellular arginine pool and increase the requirement for arginine. Bacterial cell membranes are largely permeable to H_2O_2 (Seaver & Imlay, 2001), and consequently removal of H_2O_2 from the bulk medium by *A. naeslundii* will reduce the H_2O_2 concentration inside *S. gordonii* cells. In control experiments, we found that addition of exogenous catalase (10 U mL^{-1}) produced higher growth yields of *S. gordonii* in low arginine concentrations (0.016 or 0.032 mM arginine) compared with cultures that did not contain catalase (data not shown). Coaggregation may also provide a local microenvironment that protects *S. gordonii*, for example by producing anaerobic pockets.

Not all bacterial proteins are equally sensitive to oxidation and it is thought that proteins that bind metal ions or interact with metals transiently are especially prone to metal-catalyzed oxidation (Nyström, 2005). In *Escherichia coli*, arginine biosynthesis enzymes are highly

represented within the carbonylation-sensitive proteins that have been identified to date. Thus, in a recent study of proteins that were oxidized in response to carbon, nitrogen or phosphate starvation, 62 carbonyl-containing proteins were identified of which three were arginine biosynthesis enzymes (Noda *et al*., 2007). These were acetylornithinase (ArgJ) and arginosuccinate synthase (ArgG), oxidized during nitrogen limitation, and carbamoyl phosphate synthase large subunit (CarA) that was oxidized upon carbon starvation (Noda *et al*., 2007). Carbonyl modification of proteins can result in loss of enzyme activity, a phenomenon that was first demonstrated with glutamine synthetase (Levine *et al*., 1981). Further studies will be required to determine whether arginine biosynthesis enzymes in *S. gordonii* are specific targets of protein oxidation in the presence of H_2O_2.

Removal of high concentrations of H_2O_2 by bacteria is associated with catalase activity. Streptococci do not make catalase; production of catalase within the genus *Actinomyces* is strain dependent (Collins *et al*., 2000). The strain used here, *A. naeslundii* MG1, generated bubbles on immersion in 3% H_2O_2, indicating that catalase was produced. This is consistent with the annotated genome sequence of *A. naeslundii* MG1, which includes a single catalase, encoded by *hktE* (http://cmr.tigr.org/cgi-bin/CMR/GenomePage.cgi?org=gan). In coaggregate cultures in CDM, *A. naeslundii* initially removed H_2O_2 from the medium, presumably due to catalase activity. However, after prolonged incubation (>6 h), this activity was insufficient to protect *A. naeslundii* cells from being killed. Similarly, on solidified medium and in nutrient-rich planktonic cultures, *S. gordonii* H_2O_2 inhibited growth of *A. naeslundii*. These findings are in line with studies on several oral and extra-oral bacteria, which have demonstrated that production of catalase is not sufficient for resistance to H_2O_2 from viridans streptococci (Miyasaki *et al*., 1985; Uehara *et al*., 2001; Regev-Yochay *et al*., 2006).

In summary, the data presented here support the hypothesis that H_2O_2 drives the changes in *S. gordonii* arginine homeostasis that occur upon coaggregation with *A. naeslundii* (Jakubovics *et al*., 2008). In addition, H_2O_2 secreted by *S. gordonii* is a key factor influencing the dynamics of interactions between *S. gordonii* and *A. naeslundii in vitro*. It will be important to determine how H_2O_2 influences the ecology of mixed species communities in open systems where nutrients are constantly replenished and waste products removed, as occurs in the oral cavity. Further, the role of saliva in interbacterial interactions needs to be addressed, because salivary components including secretory IgA and lactoperoxidase are known to affect the antibacterial properties of H_2O_2 (Carlsson, 1980; Adamson & Carlsson, 1982; Carlsson *et al*., 1983; Uehara *et al*., 2006). Such interactions are fundamental to the formation of stable dental plaque communities.

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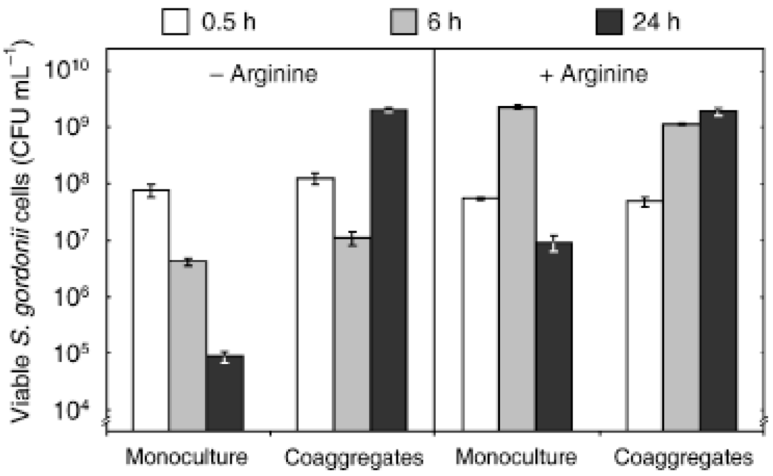

Fig. 1.

Enhanced survival of *Streptococcus gordonii* by coaggregation with *Actinomyces naeslundii*. Monocultures or coaggregate cultures in CDMΔarg (left panel) or CDM (right panel) were incubated aerobically and viable counts of *S. gordonii* cells were determined at the times indicated. Data shown are mean values of triplicate measurements and SDs from triplicate measurements. Data represent one of two experiments that gave similar results.

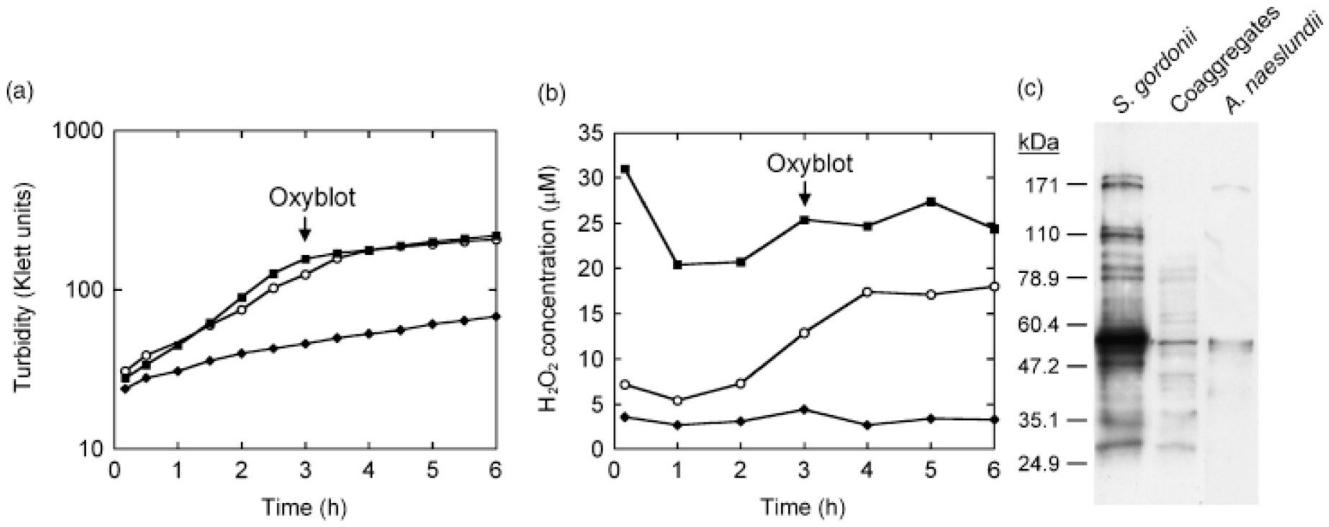

Fig. 2.

Effects of coaggregation on H_2O_2 concentration and protein oxidation in *Streptococcus gordonii*. Monocultures or coaggregate cultures in CDM (0.5 mM arginine) were incubated aerobically. Turbidity (a) and extracellular H_2O_2 concentrations (b) were determined at intervals. Symbols represent *S. gordonii* monocultures (■), coaggregate cultures containing *S. gordonii* and *Actinomyces naeslundii* (○) and *A. naeslundii* monocultures (◆). After 3 h incubation, samples were removed and proteins extracted. (c) Equal amounts (1 μg) of proteins were separated using SDS-PAGE and oxidized proteins containing carbonyl groups were detected by immunostaining with the Oxyblot kit. Approximately 85–90% of proteins in the sample from coaggregate cultures originated from *S. gordonii*. Experiments were repeated three times with similar results.

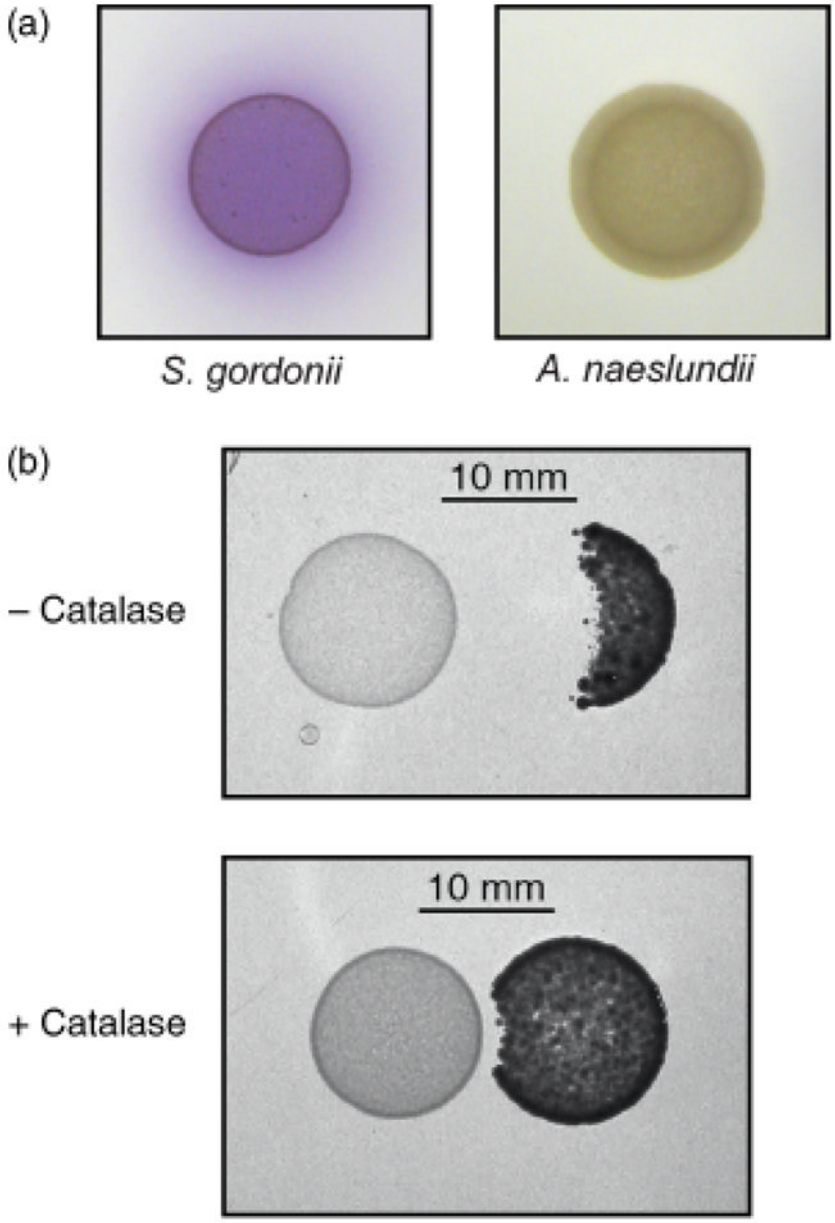

Fig. 3.

Inhibition of *Actinomyces naeslundii* growth by *Streptococcus gordonii*-produced H_2O_2. (a) A colorimetric assay, based on conversion of an indicator, ABTS, in the presence of HRP and H_2O_2 was used to detect H_2O_2 in cells grown on THB agar. The purple colour on and around *S. gordonii* cells indicates the presence of H_2O_2. (b) *Streptococcus gordonii* (left) and *A. naeslundii* (right) were spotted in close proximity on THB agar without (upper panel) or with (lower panel) prior addition of 10 U catalase to the *S. gordonii* cells.

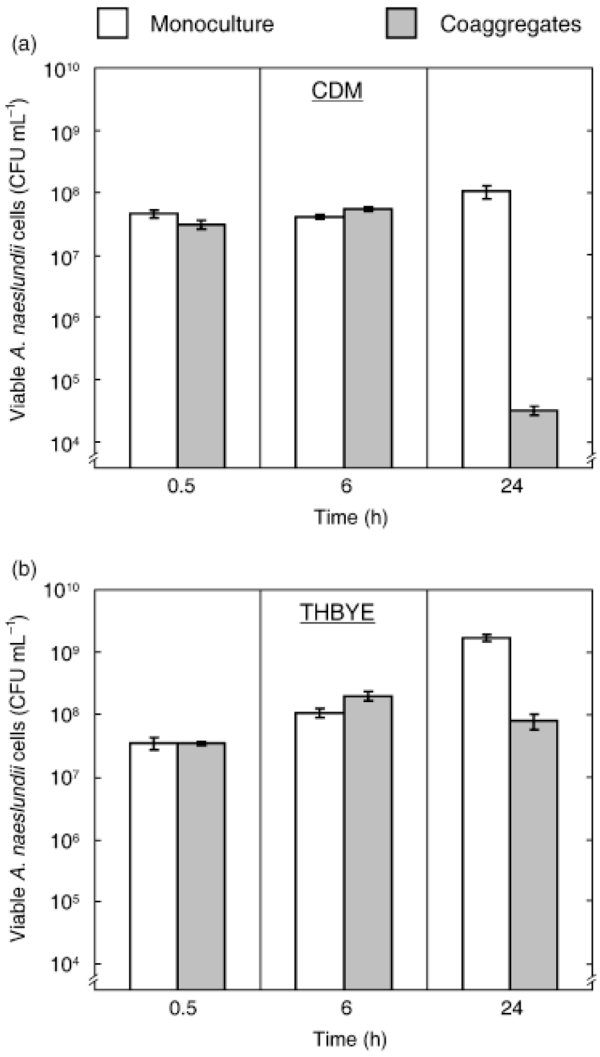

Fig. 4.

Inhibitory properties of *Streptococcus gordonii* towards *Actinomyces naeslundii* in CDM (0.5 mM arginine) (a) and THBYE medium (b). Viable *A. naeslundii* cells in aerobic monocultures or coaggregate cultures containing *S. gordonii* were enumerated on selective agar. Experiments were performed twice with similar results. Data are means and SDs from triplicate measurements of one representative experiment.