Competition Between Novel *Streptococcus* spp. from the Human Oral Cavity

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Abstract of Thesis

Genomics studies have shown that the oral microbiome harbors a complex bacterial community
and that many of its members have not been cultured. The bacteria of the oral microbiome are
known to form complex multispecies assemblages where species both compete and synergize to
colonize each niche in the mouth. Understanding how the dynamics of this microbial ecosystem
work could reveal how oral biofilm develops, how to control pathogenic bacteria or how
bacterial communities transition from commensal to disease causing. Co-culturing multiple
species together is a promising tool in microbial ecology as it may provide information missed
by –omics studies or studies of monocultures. An earlier study (Sizova et al. 2012) used a
combination of new and conventional culture techniques to generate a collection of oral bacteria
that had not previously been cultured. This collection includes numerous strains of novel
*Streptococcus*. This group of oral bacteria is of particular interest and importance. Many of the
oral streptococci are potential pathogens that are implicated in oral, respiratory and cardiac
disease (Kreth et al., 2009). They also interact and compete with each other as they colonize the
dental surface (Donoghue & Tyler, 1975; Kreth, Zhang, & Herzberg, 2008). These interactions
not only determine which strains are able to establish themselves within the niche, but also
whether or not a community containing a pathogenic species becomes infectious or remains
benign (Sbordone & Bortolaia, 2003). Our results show how phylogenetic distance, hydrogen
peroxide and carbon dioxide are involved in the interspecies interactions of our novel
*Streptococcus spp.* The combination of these factors in competition between *Streptococcus*
provides insights into the mechanisms involved in bacterial interactions, and highlights the
importance of volatile compounds in competition within microbial communities.
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Specific Aims

Genomics studies have shown that the oral microbiome harbors a complex bacterial community and that many of its members have not been cultured. The bacteria of the oral microbiome are known to form complex multispecies assemblages where species both compete and synergize to colonize each niche in the mouth. Understanding how the dynamics of this microbial ecosystem work could reveal how oral biofilm develops, how to control pathogenic bacteria or how bacterial communities transition from commensal to disease causing. Co-culturing multiple species together is a promising tool in microbial ecology as it may provide information missed by –omics studies or studies of monocultures. An earlier study (Sizova et al. 2012) used a combination of new and conventional culture techniques to generate a collection of oral bacteria that had not previously been cultured. This collection includes numerous strains of novel Streptococcus. This group of oral bacteria is of particular interest and importance. Many of the oral streptococci are potential pathogens that are implicated in oral, respiratory and cardiac disease (Kreth et al., 2009). They also interact and compete with each other as they colonize the dental surface (Donoghue & Tyler, 1975; Kreth, Zhang, & Herzberg, 2008). These interactions not only determine which strains are able to establish themselves within the niche, but also whether or not a community containing a pathogenic species becomes infectious or remains benign (Sbordone & Bortolaia, 2003). Our aims focus on using coculture to identify and examine interactions between novel and reference Streptococcus spp. to gain insights into the mechanisms involved, and analyze their possible implications for the oral ecosystem.

Aim 1: Use coculture to identify interactions in oral Streptococcus spp. and determine if a phylogenetic basis for competition exists. We will design a coculture assay to screen fourteen oral Streptococcus spp. strains recently isolated in our Laboratory and four isolates purchased
from public collections for pairwise interactions under anaerobic and microaerobic conditions. The design of the competition assay required us to address several key points. The assay must allow for cocultured cells to compete by using diffusible compounds or even through cell-cell contact, while each culture still must remain visibly distinct.

**Aim 2: Identify the mechanisms of interaction.** Once we have identified interspecies interactions we will attempt to identify the compound(s) that mediate these interactions. Extracts of solid media in which interactions were observed, spent media, and cell culture cups with filter bottoms will allow us to characterize the nature of the activity. We will manipulate culture conditions to determine how and why the interaction compounds might be produced. Given the number of observations between many pairs we were curious as to the possible mechanisms. We assumed interactions are mediated by a chemical compound(s) which we decided to “chase.” The simplest possibility we face is that the compound is abundant, stable, and extractable lending it to standard analysis techniques. This scenario allows us to explore specific mechanisms of interactions. However, there are potential complications if the compound does not exhibit the previously mentioned qualities and proves difficult to extract (ie: volatile compounds). If so we may only be able to gain some insights into the mechanisms. Our understanding of the nature of these compounds may be challenging, with the ease of analysis determining our ability to understand the mechanisms or not. With these thoughts under consideration we will conduct experiments to explore the inhibitory compounds.

**Aim 3: Genetic analysis of genes responsible for peroxide production.** All of the novel Streptococcus spp. used in this study have been submitted for whole genome sequencing to J. Craig Venter Institute (JCVI) as part of the Human Microbiome Project (NIH/HMP: http://commonfund.nih.gov/hmp/index). We can use these full genome sequences, along with
those from the reference strains and other available *Streptococcus* sequence to determine if there are differences in genes for peroxide production or tolerance of oxidative stress that explain the behavior of our strains, or could be predictive of inhibitory hydrogen peroxide production in as yet untested strains.

**Significance**

The oral microbiome contains up to 800 species of bacteria that comprise one of the best studied microbial biofilms (Paster et al., 2001; Dewhirst et al. 2010). The Human Oral Microbiome Database (HOMD, website: http://www.homd.org/index.php) contains comprehensive information on prokaryote “oral taxa” as well as links to available sequencing data including 355 annotated genomes (Chen et al. 2010). Of the taxa listed on the HOMD there are 42 *Streptococcus*. The high diversity and ease of access of the oral cavity provides an excellent means of studying microbial ecology, or how microbes interact with one another and with their environment (Konopka, 2006). The complexity of the oral environment leads to interesting inter- and intraspecies interactions and competition between members of the microbial community and successional or colonizing events following changes in the oral environment (Kolenbrander et al., 2006). These interactions are important because shifts in community structure that favor certain species, or species assemblages are known to result in dental disease. For example, *Streptococcus mutans* causes cavities in some individuals, but not when it is growing in complex multispecies biofilms with certain other *Streptococcus* species (Sbordone & Bortolaia, 2003).

We intend to use coculture as a means to discover and describe interactions between oral streptococci.
The oral environment provides an excellent system for studying microbial ecology, host-microbe and microbe-microbe interactions. However, there are challenges to working in the oral cavity. For example, of the numerous species of bacteria in the oral cavity, fewer than half have been cultured in the lab (Preza et al., 2009). It is believed that a sizable proportion of these uncultivated oral microbes are potentially pathogenic (Paster et al., 2001). Bringing the “uncultivables” into culture is therefore of principle importance. One method that increases recovery of oral microorganisms, and thus allows the study of community structure, is the use of an enamel chip incubated in situ to simulate the surface of the tooth. This method led to a successful isolation of previously uncultured organisms (Chalmers, Palmer, Cisar, & Kolenbrander, 2008). Other methods that have been used to drastically increase diversity of bacteria recovered from the oral cavity are the mini-trap to perform in vivo culture, anaerobic enrichments with specifically designed media, and single cell culture to recover slow growing strains. (Sizova et al., 2012; Sizova et al. 2013).

While it is difficult to recreate multispecies communities ex situ, it is possible to reduce this complexity to pairs of cocultured species. Coculture of interacting organisms has been used successfully to induce production of novel secondary metabolites such as antimicrobial compounds or signaling molecules (Oh et al., 2007). It is our goal to use coculture to reduce the complexity of the oral microbial community to a manageable level while still retaining the ability to observe interspecies interactions and production of secondary metabolites that might not be produced in monoculture. Streptococcal species have long been known to produce peroxide in levels that can inhibit neighboring bacteria (Donoghue & Tyler, 1975; Pericone et al., 2000). Peroxide is produced by streptococcal species that have the pyruvate oxidase (spxB) and/or lactate oxidase (lox) gene as a result of aerobic metabolism (Chen et al., 2011; Garcia-Mendoza
et al., 1993; Kreth et al., 2008; Pericone et al., 2003; Tong et al., 2007; L. Y. Zheng et al., 2011). In *S. pneumoniae* pyruvate oxidase is responsible for 99% of H$_2$O$_2$ produced (Spellerberg et al. 1996). In addition to relying on oxygen metabolism SpxB production of H$_2$O$_2$ is regulated in two primary ways in most *Streptococcus*: upregulated by the SpxR DNA binding protein in response to metabolic state (Chen et al., 2011; Ramos-Montanez et al., 2008) and catabolite control protein A (CcpA), which represses SpxB in the presence of certain substrates (usually sugars) that vary between species of *Streptococcus* (Barnard & Stinson, 1999; Kreth et al., 2008; Zheng et al., 2011).

Peroxide plays an important role in virulence of streptococci. The H$_2$O$_2$ produced by *Streptococcus* has been shown to be toxic to alveolar epithelium in lung infections (Duane et al., 1993), kill endothelial cells in endocarditis (Stinson et al. 2003) and cause brain cell apoptosis in meningitis (Braun et al., 2000). Peroxide is also involved in colonization and competition in the oral ecosystem. Hydrogen peroxide can cause the release of extracellular DNA, which stimulates competence in *Streptococcus* (Kreth et al., 2008) and also encourages aggregation and adhesion to surfaces leading to biofilm formation (Das et al., 2011). Competition among oral streptococci is complex and involves many antimicrobial compounds in addition to H$_2$O$_2$, such as bacteriocins (Kreth et al., 2008). The lactic acid produced by *Streptococcus mutans* is inhibitory to many oral bacteria, but *Streptococcus oligofermentans* can utilize the lactic acid to produce H$_2$O$_2$ that in turn inhibits *S. mutans* (Tong et al., 2007). Some *Streptococcus* strains have also been shown to switch the substrates of H$_2$O$_2$ production to allow continued competition even at starvation levels of nutrients (L. Liu et al., 2012). Although H$_2$O$_2$ is only produced in aerobic environments the SpxB enzyme is present in low levels during anaerobic growth allowing for increased competition if the environment changes (L. Zheng et al., 2011). The high levels of
H$_2$O$_2$ production aerobically but not anaerobically also suggests that H$_2$O$_2$ is produced as a means to compete early in colonization before a mature, largely microaerobic to anaerobic biofilm has formed (L. Y. Zheng et al., 2011). To a degree, peroxide does negatively affect the growth of those species that produce it (Pericone et al., 2003) but the effects are stronger on competing species that lack catalase or peroxidase enzymes. For example: peroxide damages the machinery of glycolysis and protein synthesis in S. mutans (Baldeck & Marquis, 2008). The effects of H$_2$O$_2$ have been found to be similar between cells in suspension and those in biofilm, probably due to the small and diffusible nature of the molecule (Baldeck & Marquis, 2008). However, the effects are probably limited to very short range interactions in situ (100 µm) due to the quick decrease in concentration of H$_2$O$_2$ at a distance from biofilm and scavenging salivary peroxidases (X. Liu et al., 2011). Hydrogen peroxide not only works as an inhibitor of competitors in the environment, but the enzyme responsible (SpxB) also produces 85% of the acetyl phosphate (ATP precursor) generated in aerobic growth of S. pneumoniae, conferring a metabolic advantage to H$_2$O$_2$ producers (Pericone et al., 2003). In addition to hydrogen peroxide production, tolerance of reactive oxygen species may also play a role in competition between oral strains. Interestingly, SpxB, the enzyme responsible for peroxidogenisis in oral streptococci, also plays a role in tolerance to H$_2$O$_2$ by an unknown mechanism (Pericone et al., 2003). Various other detoxification enzymes include NADH oxidase, superoxide dismutase, thiol peroxidase, alkyl hydroperoxidase and glutathione transporters (Yamamoto et al., 2006; Yesilkaya, 2000; Hajaj et al., 2012; Paterson, 2006; Tsou et al., 2008).

The composition of the oral microbial community, mediated by competition among oral bacteria, including streptococci, can lead to a transition from a healthy (dental caries free) to a disease state (carious lesions) in the mouth (Becker et al., 2002). Therefore, it is important to
understand the complexities of competition between oral species. It is also important to find larger patterns in how oral species interact. Previous research by Cordero et al. (2012) has suggested that patterns in inhibitory interactions exist within bacterial populations. Their study found that there was a threshold of genetic distance above which the negative interactions increased sharply. Understanding if similar patterns exist within populations of oral bacteria can aid in understanding dynamics of dental disease.

Our overall goal is to determine if novel, previously uncultivated streptococci interact among themselves and with known species, and get insights into the mechanisms behind such interactions. We aim to provide at least a partial explanation for the complicated microbial interactions we and others observed in vivo and in vitro. We are especially interested to find out whether there is a connection between the degree of phylogenetic relatedness of two strains and the strength of their interactions. Hydrogen peroxide plays a critical role in competition between oral bacteria, and it would be interesting to learn if cells inhibit distantly related strains more than their kin. We are also interested in discussing how our findings inform the future research in microbial ecology of the oral microbiota.

**Materials and Methods**

*Bacterial strains.* The 14 streptococci strains used in this study were designated as *Streptococcus spp.* strains AS14, BS35b, AC15, CM7, OBCR6, ACC21, BS29a, AS20, BS21, CM6, ACS2, SR1, SR4, SR5. These strains were originally isolated in Epstein Laboratory by Sizova M.V. from subgingival dental plaque under strict anaerobic conditions. Samples were collected from 6 systemically and dentally healthy individuals (Sizova et al., 2012). To culture a wide variety of previously uncultured strains researchers used the mini-trap method for cultivation *in vivo* and
conventional anaerobic enrichment techniques, using media that did not contain glucose or other sugars.

Isolated strains were purified, and identified by 16S rRNA gene sequencing and submitted to JCVI for the whole genome sequencing via the Human Microbiome Project. All strains have been stored at the Laboratory collection and also deposited in BEI Resources under deposition numbers HMS-777, HMS-776, HMS-883, HMS-884, HMS-885, HMS-886, HMS-887, HMS-888, HMS-889, HMS-890, HMS-891, HMS-892, HMS-893, HMS-894, respectively. The GenBank accession numbers for 16S rRNA gene sequences of isolated strains are HQ616363, JN091082, HQ616356, JQ612581, HQ616373, HQ616352, JQ612583, HQ616366, JQ612582, HQ616372, HQ616360, JQ612587, JQ612588, JQ612579. The GenBank assembled genome sequences of strains BS35b and AS14 are ALKH00000000, ALKI00000000 respectively. Whole genome sequencing of 12 other strains is in progress; preliminary accession numbers are: PRJNA165927, PRJNA165925, PRJNA165929, PRJNA165931, PRJNA165933, PRJNA165935, PRJNA165937, PRJNA165939, PRJNA165941, PRJNA165943, PRJNA165945, PRJNA165947.

In addition to strains available at the lab we used four reference Streptococcus sp. strains with well known characteristics and available whole genome sequences: S. mutans UA159 (accession AJ243965, oral taxon 686), S. oralis DSM 20627 (accession AF003932, oral taxon 707), S. anginosus DSM 20563 (accession AF104678, oral taxon 543), S. vestibularis DSM 5636 (accession AY188353, oral taxon 021).

Media for cultivation and assays. All cultures were maintained by growth at 37°C in liquid TY medium containing 28 g/L trypticase peptone (BD™, Sparks, MD), 20 g/L yeast extract (BD™, Sparks, MD) in anaerobic Hungate test tubes. Liquid medium was placed in Hungate tubes and sealed under anaerobic atmosphere (2-3% H2, 2-5% CO2, and 90-95% N2) prior to autoclaving.
(121°C), due to the anaerobic growth requirements of these *Streptococcus* strains. Pair wise interactions between strains were performed on ISO-Sensitest CM0437 (Oxoid Ltd. Hampshire, England), 23.4 g/L, a semi-defined medium, and standardized minimal glucose media (MG).

ISO-Sensitest medium contained in g/L: Casein, 11.0; Peptones, 3.0; Glucose, 2.0; NaCl, 3.0; Starch, 1.0; Na₂HPO₄, 2.0; C₂H₃NaO₂, 1.0; Magnesium glycerophosphate, 0.2; Calcium gluconate, 0.1; CoSO₄ • H₂O, 0.001; CuSO₄ • 5H₂O, 0.001; ZnSO₄, 0.001; FeSO₄, 0.001; MnCl₂ • 4H₂O, 0.002; Menadione, 0.001; Cyanocobalamin, 0.001; L-Cysteine hydrochloride 0.02; L-Tryptophan, 0.02; Pyridoxine, 0.003; Pantothenate, 0.003; Nicotinamide, 0.003; Biotin, 0.0003; Thiamine, 0.00004; Adenine, 0.01; Guanine, 0.01; Xanthine 0.01; Uracil, 0.01; pH 7.4 ± 0.2 @ 25°C. MG medium contained in g/L: Yeast extract, 0.5; K₂HPO₄, 3.2; KH₂PO₄, 7.0; NH₄Cl, 0.4; MgCl₂, 0.1; CaCl₂, 0.05; Glucose, 2.0. Hemolytic properties of streptococci strains were assessed on TY agar medium supplemented with sheep blood (40 ml/L).

For plating and pour plating 15 g/L Bacto agar (BD™, Sparks, MD) was used as a solidifying agent. Agar plates were incubated either inside anaerobic glove box (Coy Lab Grass Lake, MI) under anaerobic atmosphere (2-3% H₂, 2-5% CO₂, and 90-95% N₂) or in pack-rectangular 2.5 liter jars (Mitsubushi Gas Chemical Co., Inc., Japan) under micro aerobic conditions (flushed with N₂ and sealed).

**Competition assay design.** To assess interactions between different strains under microaerobic and anaerobic conditions we compared spots and streak techniques. Spot inoculations of competing strains were placed on solid medium surface adjacent to one another. Dense streak inoculations were used on the surface of plates (one loopful in one line) with competing strains adjacent to one another. We also used pour plating one strain, and adding competing strains to the surface. The pour plating was done by adding 100 μl of overnight liquid culture to 15 mL
portions of molten TY agar at 45°C prior to pouring each plate. The pour plating technique immobilized the strain in the agar and kept it in an environment of lower oxygen tension than surface plating. Once this plate solidified a loop (10 µl) of liquid overnight cultures of the each competing strain was streaked as a single dense line onto the surface (3 strains per plate) and the plates were incubated microaerobically or anaerobically at 37°C for 24-36 hours.

To extract potentially inhibitory compounds from zones of inhibition, following incubation the entire zone of inhibition was excised with a sterile spatula and placed into a 50 mL Falcon™ tube along with an equal volume of n-butanol. Samples in butanol were frozen at -80°C, thawed and pipetted to homogeneity for three freeze-thaw cycles. The homogenized samples were sent to Novobiotic Pharmaceuticals, where each sample was lyophilized and then reconstituted in 100% DMSO.

To test effect of spent medium we collected supernatant of overnight cultures by centrifuging at 10,000 for 10 minutes, followed by syringe-filtration (Millipore™ 0.22 µm). Reconstituted butanol extracts and spent media were tested on lawns of E. coli and S. aureus at Novobiotic and at the Lab on the entire strain collection by adding 10 µl to pour plated cultures.

*Cultivation and growth experiments.* To measure the range of typical hydrogen peroxide levels produced by different streptococci we inoculated all eighteen strains into Hungate test tubes with 10 ml of liquid MG medium. To obtain 1% and 10% concentrations (v/v) of oxygen in the headspace we sealed tubes under anaerobic conditions and then injected 0.07 ml and 0.7 ml of 99%- pure oxygen. H₂O₂ were measured in samples collected after 18 hours incubation at 37°C.

The growth dynamics of *Streptococcus* spp. strains CM6, SR5, CM7 was followed by OD600 and hydrogen peroxide formation. 100 µl of overnight culture adjusted to 0.1 OD 600 was
inoculated into liquid medium. Incubations were carried out in serum bottles with a total volume of 240 ml filled with 100 ml of MG medium. To obtain different initial concentrations of oxygen in the headspace we sealed flasks under anaerobic conditions and then injected 1.4 ml to 14 ml of 99% oxygen. Headspace oxygen level was adjusted to 1, 2.5, 5 and 10% v/v. Flasks were incubated at 37°C with no shaking and, samples were removed from each flask at hourly time points with a syringe and needle to minimize gas exchange to measure OD600 and H2O2 concentration. All experiments were completed in monocultures as well as in co-cultures when we inoculated two different strains simultaneously.

We also followed the dynamics of hydrogen peroxide production in solid medium. We pour-plated strains OBCR6 and AS20 into TY solid medium and then streaked strain CM6 onto the surface and incubated at 37°C. Plates were incubated in triplicate in separate anaerobic boxes for each time point. Small agar cores were taken every 4 hours by using wide-bore 250 µl pipette tips. Cores were collected at the inoculation site, and 0.5 cm and 1.0 cm away from the inoculation site of the surface strain. Samples were centrifuged at 16,000 x g for 10 minutes to separate liquid from the agar. 1µl was used for H2O2 analysis. Following sampling each plate was photographed to document growth and inhibition.

Hydrogen peroxide’s effect on bacterial growth was tested when 0.5 ml of 0.001M, 0.01M, 0.1M and 1M of H2O2 was added to cell culture dishes (Nunc™ 0.22 µm pore size, diameter 1cm) and placed directly on the surface of pour plated S. anginosus. Catalase effect on growth of pour plated S. anginosus, S. oralis and strains CM6, BS29, BS35b, AS14, ACC21 and AS20 was tested in TY agar medium supplemented with 200 U/ml of catalase enzyme (Catalase powder from A. niger, Fisher Hanover Park, IL). Catalase was added to molten and cooled down to 45 °C
medium immediately prior to pour plating. Zones of inhibition were measured after 18 hours of incubation at 37°C under micro aerobic conditions.

To determine if volatile, gaseous compounds influenced growth between competing strains coculture experiments were conducted with competing strains separated by a physical barrier but sharing the same headspace. This was achieved using 4-24x67mm well, rectangular, sterile, multidish plates with lids (Nunc International Rochester, NY) filled with 8ml TY agar in each well. Ten microliters overnight cultures of CM6 and OBCR6, undiluted and diluted to approximately 10^3 cells/ml by serial dilution in sterile H2O, were spread onto the surface of adjacent wells of the rectangular plates. Plates were incubated individually in pack-rectangular 2.5 liter jars (Figure 1) flushed with N2 or a 80/20% N2/CO2 mix where specified.

Figure 1. Anaerobic 2.5 liter jars with single multiwall plate for incubating vapor-competing strains.

*Peroxide assay and OD measurements.* Hydrogen peroxide concentration was measured with Pierce™ Aqueous Peroxide assay kits. For liquid cultures 0.5-1.0 ml aliquots were removed, centrifuged and tested for peroxide by adding 20 μl spent media to 200 μl of peroxide kit working reagent in a 96 well microtiter plate. For solid medium experiments 1 μl of extracted liquid was placed into the well of a 384 well microtiter plate along with 10 μl of peroxide kit...
working reagent. The kit’s reagents were mixed immediately prior to use. Samples were added, mixed and incubated for 20 minutes before analysis for color development. OD 595 nm was measured spectrophotometrically in a BioTek™ Synergy HT plate reader using Gen5 software.

Cell biomass was measured as OD 600 by Eppendorf BioPhotometer in 1 cm cuvette as well as in 96 well plates by BioTek™ Synergy HT plate reader.

Statistical calculations and data analysis. All calculations were performed with MS Excel. We used 2 to 5 replicas for all analytical measurements. The growth and competition experiments were completed at least twice.

We examined the probability of competitive interactions occurring given relatedness using Bayes’ theorem;

$$P(A \mid B) = \frac{P(A \mid B)P(A)}{P(B)}$$

This formula represents the probability of A (a negative interaction between strains) occurring given B (the genetic relatedness of the strains given 16S rRNA gene sequence similarity).

Phylogenetic trees were constructed using the ME algorithm via MEGA5 program package with bootstrap test with 1,000 replicates was used to evaluate robustness.

Strain relatedness was calculated as percent similarity between two 16S rRNA gene sequences based on the number of base differences per site. The analysis involved 18 16S RNA genes sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1186 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.
Statistical analysis of colony counts from CO\textsubscript{2} growth experiments was done using a 1-tailed t-test for unequal variances.

Venn diagrams were created by inputting lists of shared genes into the Venny website: http://bioinfogp.cnb.csic.es/tools/venny/

Jaccard similarity values were calculated using the number of similar genes provided by RAST and mGenome Subtractor data. The Jaccard coefficient was calculated using the following formula:

\[ J(A, B) = \frac{A \cap B}{A \cup B} = \frac{\text{Genes}_{AB}}{(\text{Genes}_A - \text{Genes}_{AB}) + (\text{Genes}_B - \text{Genes}_{AB}) + \text{Genes}_{AB}} \]

\( A \cup B \) represents the number of shared genes in addition to the number of each strain’s unique genes.

\( A \cap B \) represents the genes that are shared between one of the novel strains and the comparison strain. 

**Genome analysis**

The genomes were compared using DSMZ Genome-to-Genome Distance Calculator 2.0 (GGDC- http://ggdc.dsmz.de/distcalc2.php) to determine overall similarity between strains using the identities/High Scoring Pair length (HSP) formula. The genomes were analyzed in the mGenome Subtractor tool (http://bioinfo-mml.sjtu.edu.cn/mGS/) used to highlight the genes that are different between similar or competing strains. The differences highlighted by the mGenome Subtractor were sorted in Excel spreadsheets and genes related to peroxidogenesis, oxidative stress, CO\textsubscript{2} metabolism and streptococcal fratricide were identified.
Results

**Novel Streptococcus isolates:**

Our *Streptococcus* isolates represent 6 different oral taxa. The phylogenetic relationships of the strains used in this study are shown in Figure 2. The 14 oral strains from the Lab collection represent a wide genetic range of isolates, some of which share as little as 92.5% 16S rRNA gene sequence similarity. The novel isolates represent three streptococcal groups: Mitis (7 isolates), Salivarius (2 isolates) and Anginosus (5 isolates), and six different oral taxa, 071 (5 isolates), 758 (1 isolate), 721 (1 isolate), 755 (2 isolates), 644 (1 isolate), 543 (4) (http://www.homd.org) (Fig.1, Table 1). The list of novel isolates along with closest cultivable relative (GenBank) is shown in Table 1. Reference strains were selected as representatives of several oral *Streptococcus* groups: *S. mutans* UA159 (Mutans group, oral taxon 686), *S. oralis* DSM 20627 (Mitis group, oral taxon 707), *S. anginosus* DSM 20563 (Anginosus group, oral taxon 543), *S. vestibularis* DSM 5636 (Salivarius group, oral taxon 021).

This collection of strains provided a diverse sampling of *Streptococcus* that could potentially colonize the oral cavity and compete with one another.

**Competition assays:**

Initial attempts at using surface competition assays were unsuccessful for several reasons. Spot inoculations of competing strains adjacent to one another on the surface of plates did not produce enough growth to show consistent interactions, even under microaerobic conditions (flushed with N$_2$ to reduce oxygen stress). No interactions were observed when spot inoculations were plated anaerobically. More dense inoculations allowed for better growth but the interactions observed were not always reproducible. The best method we found to produce consistent growth of
competing strains was pour plating of one strain, and adding competing strains to the surface. This technique was used for all competition assays because it keeps cells localized and in close proximity to allow interactions to take place and make them easily observable (Figure 3). Multiple streaks could be done over the surface of the plate, and multiple species could be added to the embedded cells, increasing the throughput of the assay.

We screened our panel of Streptococci for pair-wise interactions using the pour plating procedure described above and observed strong inhibitory activity between many of the strains. Figure 3 shows typical zones of inhibition. Negative interactions ranged from complete inhibition to smaller, less dense microcolonies in the agar, occasionally bordered by rings of denser growth. Table 2 shows the results of three trials of pairwise interactions between the novel Streptococcus isolates and reference strains. The pairs that exhibited a negative interaction in at least one trial are indicated. Several of the novel streptococcal isolates exhibited negative effects on a wide range of competing strains (CM6, SR1, BS29, BS35b, S. oralis- all of which were in the Mitis group, hereafter referred to as inhibitory strains), while others showed very little inhibitory activity, (CM7, AC15, OBCR6, ACC21, S. anginosus- all in the Anginosus group, S. vestibularis, S. mutans- hereafter referred to as sensitive strains). Novel isolates from the Sanguinis and Salivarius clades exhibited intermediate sensitivity and inhibitory activity. The more inhibitory strains were less likely to be inhibited by another strain, while strains that did not negatively affect other strains were more likely to be inhibited. S. oralis was the most strongly inhibitory reference strain while CM6, SR1 and BS35b were the strongest novel strains.

Interestingly S. mutans, the strain implicated in dental caries and shown previously to inhibit other Streptococcus with bacteriocins (Kreth et al., 2008), did not exhibit inhibitory activity
against many other strains in our competition tests. All of the reference strains, besides *S. oralis*, were sensitive, in varying degrees, to the inhibitory strains from our collection.

Figure 2. Evolutionary relationships of 29 taxa. The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 0.16375033 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1163 positions in the final dataset.
<table>
<thead>
<tr>
<th>Oral strain (Lab Collection)</th>
<th>GB 16S rRNA sequence Accession</th>
<th>Closest relative from HOMD</th>
<th>Human Oral Taxon</th>
<th>16S% similarity to HOMD strain</th>
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</table>

Table 1. Novel *Streptococcus* isolates, GenBank 16S accession numbers, Genome ID and closest related oral taxon.
Table 2. Inhibitory activities within our Streptococcus strain collection and reference strains. Results of three trials of pairwise interaction combinations between all strains. An inhibitory effect of the streaked strain (blue, across top) on the species in the agar (orange, along side) in at least 1 of three trials is indicated by red (-). Absence of negative interactions is indicated by blue (+).

Table 3. The percent similarity as calculated from the number of base differences per site from between 16s rRNA gene sequences are shown. The analysis involved 18 nucleotide sequences.
Figure 3. Zone of inhibition with bands of dense/less dense growth of pour-plated bacteria imbedded within agar medium. Left: Picture of *S. anginosus* (pour-plated in agar) inhibited by *S. oralis* (top-streak) with multiple bands of microcolonies grown within the agar medium. Center: Image of this banded zone of inhibition through a dissecting scope (120 ×). Right: SR1 and CM6 produce zones of inhibition on pour plated SR4.

*Phylogenetic patterns of interactions:*

We used the competition assays of pairwise interactions within our pool of strains to search for such patterns. Data from three replicates of microaerobic (purged with N2) competition trials in TY media were analyzed with respect to the 16s rRNA gene sequences similarities (Table 3) of the strains to examine the patterns of interactions in our strain collection on a phylogenetic basis. Some strains showed a pattern of increased inhibition, measured as the zone diameter in mm, against more distantly related strains, while others were more strongly inhibited by more similar strains (Figure 4). For example, CM6 (Mitis group) was most active against the distantly related Anginosus group strains, while AS14 (Mitis group) was inhibited by other members of the same group. There was no clear trend of increased competition, as measured by size of zone of inhibition, as a function of genetic distance among all strains. We also examined the probability of competitive interactions occurring given relatedness using Bayes’ theorem, P (A|B). This analysis did show that there is a general increase in competitive interactions with increase an in
genetic distance (Figure 5). The probability of negative interactions occurring begins to rise between strains that are less than 95% related by 16s rRNA sequence. The 95% similarity corresponds with the value suggested by Yarza et al., 2008 to delineate bacterial genera. This pattern could suggest that oral bacterial strains compete more with distantly related neighbors (those not within the same genus) than closely related ones. In the oral ecosystem this may serve as an indication that closely related strains compete less with one another, treating them as “self”, in a highly competitive environment for space and resources.

Figure 4. Left: *Streptococcus* sp. strain AS14 (Mitis group) was inhibited more by closer relatives (Mitis group). Right: Strain CM6 (Mitis group) inhibited distantly related strains more (Anginosus group)
Figure 5. Probability of a negative interaction occurring (A) between strains given their relatedness (R) by 16s rRNA gene sequence. Strains’ 16s rRNA gene sequences similarities ranged from 92% to 99.9% similar.

**Identifying inhibitory compounds:**

Our first step in determining the nature of the inhibitory interactions between our strains was to ascertain if the inhibitory activities were extractable. Extracts reconstituted in DMSO and spent media were tested for activity, but did not produce the same zones of inhibition that were achieved with fresh, competing cultures. The lack of activity found in the extracts and spent media, even on strains that were sensitive to live cultures, indicated that the negative interactions were caused by a compound that was either not extractable, was volatile or has a short half-life.

When extracts and spent media showed no inhibitory activity we considered a possibility that growth inhibition was caused a compound that was either too unstable to survive the extraction
process or volatile and did not persist after the bacteria stopped growing. We next attempted to perform competition assays in other media compositions to search for patterns that might elucidate the mechanisms of inhibition. We used ISO-sensitest, which contains glucose (2.0 g/L), and observed larger zone inhibition sizes than in glucose-free TY medium. Anaerobic competition assays were conducted in TY and ISO-sensitest and produced no observable inhibitory interactions. We knew that many Streptococcus are capable of producing hydrogen peroxide and suspected that this may be the case for our strains. Available information indicated that sugar concentration plays a role in peroxide production, and that many pathogenic and commensal Streptococcus are known to produce hydrogen peroxide as a byproduct of aerobic metabolism (Chen et al., 2011; Garcia-Mendoza et al., 1993; Kreth et al., 2008; Pericone et al., 2003; Tong et al., 2007; L. Y. Zheng et al., 2011). The lack of inhibition under anaerobic conditions is thus consistent with the idea that hydrogen peroxide is causing the inhibition we observed under microaerobic conditions.

In an additional set of experiments all of our reference and Lab strains were streaked onto TY blood as peroxidogenic Streptococcus cause alpha-hemolysis on blood agar (Barnard & Stinson, 1996). Therefore, strains that produced hydrogen peroxide would be distinguishable by a green area of ‘hemolysis’ around the bacterial growth. All of the inhibitory novel isolates (Mitis group: CM6, BS35b, BS29, SR1, BS21, AS14 and Salivarius group: ACS2) produced alpha hemolysis when grown microaerobically. Anaerobically we observed no alpha-hemolysis on blood agar for any strains, presumably because peroxide levels were too low due to the lack of oxygen needed for hydrogen peroxide production. To determine if H₂O₂ was the primary means of inhibition we added catalase to competition assays between strains that had previously proved to be antagonistic in order to neutralize any peroxide that was produced. The competition pairs we
used were strains CM6, BS29, SR1, BS35b and S. oralis as competitors from the Mitis group streaked on to the surface and strains AS14, ACC21 or AS20 and S. anginosus as sensitive strains in the agar. All of these strain pairs were tested in duplicate. The catalase competition assays were performed microaerobically in TY and showed no inhibition indicating that hydrogen peroxide was, indeed, responsible for our strains competitive interactions.

Hydrogen peroxide production was measured with all 18 Streptococci strains grown in MG medium with initial oxygen level of 1% or 10%. Two oxygen concentrations were chosen to determine if higher levels of oxygen resulted in more peroxide or inhibited the growth of the Streptococci and resulted in less peroxide. High producing Mitis strains (CM6, SR1, BS35b, BS21, BS29) produced 130-160 µM H₂O₂ in 10% oxygen (Figure 6). More H₂O₂ was produced in 10% O₂ than 1%. Interestingly, a small amount peroxide was detected from sensitive strains and strains that were minimally inhibitory: SR5 and AS14. To test if hydrogen peroxide was inhibitory to our strains at these concentrations we placed H₂O₂ placed in cell culture dishes on top of a sensitive strain (S. anginosus). This produced similar size zones of inhibition to those that were observed in pair-wise competition assays (Figure 7). 0.01M H₂O₂ concentration of hydrogen peroxide produced zones of inhibition (5mm) that most resembled those from competition assays. These zones also had bands of dense growth at the perimeter of the zone of inhibition similar to those observed in some of the competition assays. Interestingly, the amount of hydrogen peroxide needed to show this pattern (10 mM) was larger than the range of peroxide concentrations measured from the inhibitory novel strains (0.15 mM), which could be due to the method of delivery: diffusion out of a culture dish compared to the metabolic byproduct from growing cells. This evidence further supports the hypothesis that hydrogen peroxide is responsible for the antagonism between our Streptococcal isolates.
Figure 6. Overnight peroxide concentration from *Streptococcus* strains, grown in MG media with 1% or 10% O₂ in headspace. Single replicate is shown.

Figure 7. Cell culture cups on pour plated culture of *S. anginosus*. Hydrogen peroxide solutions: 1 M (top right), 0.1M (top left), 0.01M – note rings in zone of inhibition (bottom right), 0.001M(bottom left)
*Liquid culture peroxide time course:*

Dynamics of hydrogen peroxide production by different strains were followed in experiments with controlled initial glucose and oxygen concentration. Three strains were selected as representatives of the inhibitor (strain CM6), intermediate (strain SR5), and sensitive (strain CM7) groups of strains. Figure 8 shows the difference in growth and peroxide production from each of the three groups over a 10 hour time period. CM6 shows hydrogen peroxide concentration beginning to rise after only 3 hours while the level spikes, then drops for SR5 and no level is detected for CM7. A self-inflicted bactericidal effect from H$_2$O$_2$ can be seen from the decreasing optical density at the end of the growth curve of CM6. We also used a coculture method that combined two competing strains in the same culture flask. We expected to see increased peroxide production from the inhibitory strains when paired with a sensitive strain, indicating an antagonistic reaction. However, we did not observe any patterns in peroxide production when strains were grown together that indicated they were responding competitively. The patterns of hydrogen peroxide concentration and OD600 seem to be a blend of the two strains individual time courses. These coculture patterns seem to suggest that this type of mixed culture experiment does not have the ability to resolve competitive interactions caused by hydrogen peroxide. Additionally, many *Streptococcus* exhibit regulation/repression of peroxide production in the presence of various metabolites in a concentration dependent manner, such as carbon catabolite repression, so it is possible that the concentration of glucose we used may not be optimal for peroxide production for each of our strains.
Figure 8 continued on pg. 28
Concentric rings of inhibition:

One of the most puzzling observations in our interaction tests was that of concentric rings of growth within some of the zones of inhibition (Figure 3). The rings consisted of areas with denser microcolonies in the agar, alternating with areas of less dense growth. In these rings the microcolonies of the inhibited strain are immobilized in the agar and *Streptococcus* are non-motile, preventing any form of motility contributing the pattern. It seems unlikely that nutrient availability next to the zone of inhibition is the cause of denser growth, as this would result in a single dense band, not multiple bands. Diffusion of the inhibitory compound through the agar seems unlikely to be the cause of the pattern because the decreasing concentration of the inhibitory compound away from the inhibitory strain should not permit increased growth of an inner ring, only to inhibit the next rank of microcolonies. No inhibitory compounds besides hydrogen peroxide have been identified in our experiments, but we were unable to replicate the
multiple ringed patterns by using peroxide alone (Figure 7). We, therefore, decided investigate peroxide concentrations on a fine scale within the ringed zones of inhibition of the competition trials.

*Solid medium peroxide time course:*

To investigate the role of hydrogen peroxide and ring formation, we designed an experiment to test peroxide concentrations directly from solid media competition assays. Due to the volatile, reactive nature of hydrogen peroxide, these experiments were conducted as a time course in order to more accurately track the concentration of peroxide. Competition trials in TY between strain CM6 (inhibitor) and strains OBCR6 and AS20 (both sensitive) were conducted microaerobically. Samples of the agar surrounding the CM6 inoculation were taken at 4 hour intervals and growth inhibition was photographed over a 24 hours. High concentrations of hydrogen peroxide did not diffuse a great distance from the inoculation site of CM6 (Figure 9). Concentrations at 0.5 cm distance from the streak were only 37% and 17% of that at the streak itself (for OBCR6 and AS20 respectively) at 24 hours. These lower concentrations are unlikely to cause complete inhibition of the sensitive strain, but may cause metabolic or physiological changes that result in the altered appearance of growth in the form of rings. The solid media time course also revealed that the H₂O₂ concentrations were not consistent for the duration of the 24 hour time period. There was significant undulation of the concentrations of H₂O₂. The concentration rose from zero to a maximum of over 200 µM at 8 hours, and then dropped to a local minimum of under 100 µM at 16 hours before rising to the high concentration of 200 µM again by 24 hours. The decrease in H₂O₂ concentration was also present when CM6 was grown alone, but to a much lesser degree. The decrease suggests transcriptional bursting may be a possible explanation for the movement of H₂O₂ concentration; if pyruvate oxidase, the enzyme
primarily responsible for H\textsubscript{2}O\textsubscript{2} production, undergoes series of fluctuations in transcription, the
levels of H\textsubscript{2}O\textsubscript{2} would exhibit similar fluctuations. The decreases in hydrogen peroxide for the
intermediate time period may also be due to depletion of a certain nutrient and subsequent
substrate switching. However, because the fluctuation is greater in the coculture condition, it is
likely that the sensitive strain may be reacting to the H\textsubscript{2}O\textsubscript{2} produced by its competitor. The
explanation for the sharper drop in peroxide concentration during time course experiments of
cocultured strains than in axenic trials could be as simple as H\textsubscript{2}O\textsubscript{2} becoming reduced as it reacts
with sensitive cells, or that there is an adaptive response to peroxide by the sensitive strains.
Although the mechanisms involved remain unclear, the variations in hydrogen peroxide
concentration over time and distance could certainly help to explain the concentric rings of
growth observed in the competition trials.

Figure 9 continued on pg. 31-32
Figure 9 continued on pg. 32
Figure 9. Peroxide concentration measured from agar cores during 24 hour competition trial. Pictures of growth (A: AS20 w/ CM6 streak) and time points were taken every 4 hours. Distance of sample from is indicated by line color. AS20 in agar, CM6 streak (B), OBCR6 in agar, CM6 streak (C), Unstreaked pour plates of AS20, OBCR6 and uninoculated TY (D), Uninoculated TY CM6 streak (E) (control). Error bars represent standard deviation (3 biological replicates each containing 3 technical replicates).

Effects of CO2/anaplerotic reactions

Many bacteria, including *Streptococcus sp.*, have long been known to have a CO2 requirement (Valley and Rettger, 1927). However, Streptococci are also able to generate enough CO2 endogenously to grow in the absence of supplementary CO2, unless it is continuously purged from their environment (Repaske et al., 1974). In *Streptococcus* CO2 is converted to carbonate by streptococcal carbonic anhydrase, used in anaplerotic reactions as a substrate for enzymes such as phosphoenolpyruvate carboxylase and carbamoyl-phosphate synthase, and produced by other enzymes in the cell (e.g., pyruvate oxidase). Under normal conditions, CO2 produced by biological processes within the culture is enough to meet the demand for CO2, but when the environment is constantly purged with N2 even the more robust strains (CM6) show greatly reduced growth. Although streptococcal interactions and streptococcal dependence on CO2 have
been explored separately in the past, the role of CO₂ in interspecies interactions has not been
closely studied. Our experiments sought to elucidate any volatile/gaseous compounds involved in
competition. For this purpose, we cocultured the inhibitor strain CM6 and sensitive strain
OBCR6 in separate wells of a petri dish, such that they would be physically separated yet shared
the same headspace. OBCR6 showed significantly (p = 0.011) more CFU’s when grown with
CM6 than when grown alone (Figure 10). The apparent growth stimulation of OBCR6 by CM6
was replicated by incubation with 80/20% - N₂/CO₂ gas mixture in the absence of CM6,
indicating the observed growth stimulation was likely due to increased CO₂ availability.

The fact that CM6 can support its own growth with endogenously produced CO₂, and produces
more H₂O₂ than OBCR6, is not a coincidence. The enzyme pyruvate oxidase converts pyruvate
to acetyl phosphate generating both H₂O₂ and CO₂ as byproducts. Higher activity of this one
enzyme in CM6 would explain both the higher levels of H₂O₂ as well as the growth inducing
levels of CO₂. The combination of H₂O₂ inhibition and CO₂ growth stimulation, as well as the
different rates these two chemicals diffuse through the medium, coupled with their apparently
pulsed production, could lend explanations as to why there were bands of inhibition and denser
growth in our competition assays. However, the fact that this endogenously produced gaseous
compound clearly affects interactions between two species has implications that extend beyond
this one interaction: almost all existing screens for biologically active compounds involve
bioassay-guided fractionation, leading to losses of all volatile/gaseous/reactive compounds. Our
competition assays between *Streptococcus* strains have shown that such “lost” compounds may
have marked effects on bacterial interactions.
Figure 10. Top plate: Outer wells contain 10 µl undiluted overnight culture of CM6, inner wells contain 10 µl of 10^5 dilution of OBCR6. Bottom plate: Outer wells contain 10 µl undiluted overnight culture of OBCR6, inner wells contain 10 µl of 10^5 dilution of OBCR6. Outer wells of both plates show growth (CM6/OBCR6 as a lawn), but only the plate containing both CM6 and OBCR6 has growth of diluted OBCR6 in inner wells.

**Genome analysis**

We compared the genomes of the selected type strains to either AS14 or BS35b (two of our novel isolates with full genome sequences available). We used mGenome Subtractor to identify which genes were shared between strains and mapped these genes to RAST annotations of the query genomes. This mapping allowed us to match shared genes, RAST annotated genes and hypothetical functional proteins, giving us the ability to create a Venn diagram of the metabolic
functions shared between each strain (Figure 11). Of BS35b’s 1780 genes, 1542 were shared with at least one other selected strain while AS14 shared 1343 genes out of 2066 with at least one other strain in the group. We also used a Jaccard similarity index to compare our strains. As our Jaccard index compares numbers of shared genes, it is an indication of metabolic similarity. If two strains possess only genes coding for the same functional proteins then the ratio of genes shared between two strains to those shared genes plus unique genes will be higher and the coefficient will be close to 1. High coefficient values indicate the two strains have a large number of genes with the same functional role, and likely correlates to metabolic similarity while a low value indicates few genes with the same function as a proportion of total genes between the two strains. Table 4 shows the Jaccard similarity indexes for the two novel strains AS14 and BS35b compared to the type strains used in the mGenome subtractor Venn diagram comparison. Similarity values were low for most strains, which is surprising given that most of these organisms are thought to exist in similar niches within the oral cavity. Strain BS35b had a higher Jaccard similarity, at 0.658, to S. oralis than between any other two strains. The high degree of similarity suggests that, not only are the two closely related, but they are similar metabolically and probably engage in competition with other Streptococcus using the same mechanisms.

These trends in the number of shared genes corresponded with the relatedness of the strains by 16s and GGDC (Table 6). We identified genes from the RAST annotation of each strain that could potentially be involved in competition: pyruvate oxidase, oxidative stress genes including superoxide dismutase, genes involved in CO₂ metabolism and anaplerotic reactions such as PEP oxidase and carbonic anhydrase, and genes involved in bacteriocin production and streptococcal fratricide (Table 5). Inhibitory and sensitive strains shared similar numbers of genes related to H₂O₂ production, oxidative stress, and anaplerotic reactions. This finding indicates that the
differences in inhibitory H$_2$O$_2$ production and growth patterns under limited CO$_2$ among our strains are likely under the control of transcription factors not identified by our genomic analysis. Interestingly, genes for streptococcal fratricide and bacteriocins were enriched in the inhibitory strains. Although no inhibitory or stimulatory compounds, other than CO$_2$ and H$_2$O$_2$, were identified from our coculture interactions, it is possible that there are synergistic effects between the bacteriocin or streptococcal fratricide genes and the high levels of H$_2$O$_2$ produced by the inhibitory strains.

The genome-genome distance calculator (GGDC) provides a more robust analysis of full genome similarity than 16S rRNA genes alone. The GGDC generates simulated DNA-DNA hybridization (DDH) values that can be used to infer the relatedness of compared strains (a DDH value <70% is accepted to indicate two strains are a separate species). The GGDC results from our strains as well as other type strains, found in table 6, provide useful insight into the phylogenetics of our streptococci. For example, strains that appeared to be closely related by 16s were actually distinct species by genome comparison. Strain BS35b was 97.79% similar to S. oralis by 16S rRNA gene sequence, but the value of DDH is only 50, clearly demonstrating that the two strains are different species. Also, there were much greater differences between some of the strains than would normally be expected from two members of the same genus: S. mutans DDH value was less than 30 for both of the novel strains. It was also surprising to find such low full genome similarity values between strains that shared high numbers of similar genes: AS14 shared 1050 genes with S. anginosus but the DDH value for these strains was only 25.5, a number that might be low enough to group the two strains into different genera. Our calculations from the GGDC not only serve to highlight the enormous genetic diversity of the Streptococcus genus, but clearly identify the novel strains AS14 and BS35b as distinct novel species.
Figure 11. Venn diagram of genes from AS14 (A) and BS35b (B) in common with *S. mutans* (blue), *S. oralis* (green), *S. anginosus* (red), and either AS14 or BS35b (yellow). The number in each area represents genes shared with the background strain and overlapping strains.
Table 4. Jaccard similarity index of functional genes. This index indicates the number of genes shared as a proportion of the total genes in AS14 or BS35b.

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<th>Strain</th>
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<th>BS35b</th>
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<tr>
<td>AS14</td>
<td>-</td>
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<tr>
<td>BS35b</td>
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Table 5 continued on pg. 39

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<td>Produces H2O2 as a byproduct of ATP synthesis, responsible for 99% of H2O2 production well as tolerance</td>
<td>Spellerberg, B. 1996</td>
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<td>Lactate oxidase</td>
<td>Produces H2O2 as a byproduct of ATP synthesis</td>
<td>Tong, 2007; Liu 2012</td>
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<tr>
<td>Thiamine pyrophosphokinase</td>
<td>Produces substrate of SpxB; H2O2 production</td>
<td>Chen, 2011</td>
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<td>Alkylhydroperoxide reductase D</td>
<td>Involved in oxidative stress</td>
<td>Paterson, G. K. et al 2006</td>
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<td>Glutathione peroxidase</td>
<td></td>
<td>Tsou, C. C. 2008, sources 10, 11</td>
</tr>
<tr>
<td>NADH oxidase,</td>
<td>Resistance to H2O2 stress</td>
<td>Yamamoto et al 2006</td>
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<tr>
<td>Superoxide dismutase</td>
<td>protects against oxidative stress by reducing superoxide into H2O2</td>
<td>Liochev and Fridovich 2007, Yesilkaya 2000</td>
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<td>Thiol peroxidase</td>
<td>Reduces H2O2, plays critical role in H2O2 control</td>
<td>Hajaj 2012</td>
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<td>Mn 2+ ABC membrane transporter</td>
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<td>Dintilhac, 1997</td>
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<td>Mn 2+ efflux</td>
<td>MntE mutant has increased H2O2 and resistance to ROS</td>
<td>Rosch, 2009</td>
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<tr>
<td>SpxR</td>
<td>Positively regulates SpxB in response to energy and metabolic state (may bind ATP or AMP and CoA compounds)</td>
<td>Ramos et al 2008</td>
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<tr>
<td>Two component regulator of iron uptake</td>
<td>Regulates Mn 2+ uptake, decreases H2O2</td>
<td>Ong 2013</td>
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Table 5 continued on pg. 39
### Anaplerotic genes

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<td>PEP carboxylase</td>
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<td>carbamoyl phosphate synthase</td>
<td>Fix CO2 to glutamine</td>
<td>Arioli, 2007</td>
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<td>Carbonic Anhydrase</td>
<td>Convert CO2 to HCO3</td>
<td>Burghout, 2010</td>
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<td>Dihydrofolate synthase, folypolyglutamate synthase</td>
<td>Folate biosynthesis</td>
<td>Burghout, 2013</td>
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<tr>
<td>Acetyl-coA carboxyl transferase beta chain</td>
<td>RAST colicin/bacteriocin AND fatty acid biosynthesis)</td>
<td>RAST</td>
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### Bacteriocin/competition genes

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<tbody>
<tr>
<td>Dihydrofolate synthase, folypolyglutamate synthase</td>
<td>RAST colicin/bacteriocin indicates both folate synth AND part of bacteriocine production cluster</td>
<td>RAST</td>
</tr>
<tr>
<td>tRNA pseudorodine synthase A</td>
<td>RAST colicin/bacteriocin</td>
<td>RAST</td>
</tr>
<tr>
<td>Amidophosphoribosyltransferase</td>
<td>RAST colicin/bacteriocin</td>
<td>RAST</td>
</tr>
<tr>
<td>Acetyl-coenzyme A carboxyl transferase beta chain</td>
<td>RAST colicin/bacteriocin AND fatty acid biosynthesis</td>
<td>RAST</td>
</tr>
<tr>
<td>Two-peptide bacteriocin CibA</td>
<td>RAST bacteriocin/Streptococcal fratricide</td>
<td>RAST</td>
</tr>
<tr>
<td>Choline binding protein D</td>
<td>RAST bacteriocin/fratricide</td>
<td>RAST</td>
</tr>
<tr>
<td>Cell wall-associated murein hydrolase LytC</td>
<td>RAST bacteriocin/fratricide</td>
<td>RAST</td>
</tr>
<tr>
<td>Immunity factor ComM</td>
<td>RAST bacteriocin/fratricide</td>
<td>RAST</td>
</tr>
</tbody>
</table>

Table 5. Genes involved in hydrogen peroxide production and reactive oxygen species tolerance, anaplerotic enzymes and CO₂ metabolism, and streptococcal fratricide and bacteriocin production.
<table>
<thead>
<tr>
<th>Reference genome</th>
<th>DDH</th>
<th>Model C.I.</th>
<th>Distance</th>
<th>Prob. DDH &gt;= 70%</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. oralis</td>
<td>50.6</td>
<td>3.333892</td>
<td>0.0702</td>
<td>20.92</td>
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<td>S. mitis</td>
<td>37.7</td>
<td>3.009717</td>
<td>0.1075</td>
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<tr>
<td>S. pneumoniae TIGR4</td>
<td>37.5</td>
<td>3.016131</td>
<td>0.1084</td>
<td>1.38</td>
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<td>S. pneumoniae D39</td>
<td>37.4</td>
<td>3.017088</td>
<td>0.1087</td>
<td>1.35</td>
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<tr>
<td>S. gordonii</td>
<td>33.7</td>
<td>2.678629</td>
<td>0.1237</td>
<td>0.43</td>
</tr>
<tr>
<td>S. vestibularis</td>
<td>31.0</td>
<td>2.603793</td>
<td>0.137</td>
<td>0.16</td>
</tr>
<tr>
<td>S. parasanguinis</td>
<td>30.6</td>
<td>2.660304</td>
<td>0.1387</td>
<td>0.14</td>
</tr>
<tr>
<td>AS14</td>
<td>30.6</td>
<td>2.61092</td>
<td>0.1391</td>
<td>0.13</td>
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<tr>
<td>S. anginosus</td>
<td>29.9</td>
<td>2.595093</td>
<td>0.1429</td>
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<tr>
<td>S. sanguinis</td>
<td>29.3</td>
<td>2.625722</td>
<td>0.1462</td>
<td>0.08</td>
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<tr>
<td>S. constellatus</td>
<td>28.8</td>
<td>2.579438</td>
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<td>S. infantarius</td>
<td>28.4</td>
<td>2.571276</td>
<td>0.1511</td>
<td>0.05</td>
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<td>S. mutans</td>
<td>27.8</td>
<td>2.542404</td>
<td>0.1552</td>
<td>0.04</td>
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<td>S. intermedius</td>
<td>27.3</td>
<td>2.591186</td>
<td>0.158</td>
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<td>S. pyogenes</td>
<td>25.4</td>
<td>2.564713</td>
<td>0.171</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 6 continued on pg. 41
Table 6. Digital genome-genome hybridization distance calculations (GGDC); identities/HSP length. Strains BS35b (A) and AS14 (B) compared to type strains of other members of the *Streptococcus* genus. Columns represent DNA-DNA hybridization (DDH) value, confidence interval, phylogenetic distance and probability DDH is greater than 70%, indicating the strains compared represent the same species.

**Future directions**

This research has unearthed important Streptococcal interactions and elucidated some of the mechanisms involved. We are also interested in discovering more about the dynamics of microbial interactions. One conceptually simple way to do this is to visually observe the bacteria as they interact with each other. However, it is difficult to track individual species of bacteria in complex communities. Fluorescence *in situ* hybridization (FISH) can be utilized to visualize many different families or genera of bacteria simultaneously, but most FISH tags are not species specific (Valm et al., 2011). These techniques also require fixation of the bacterial community,
meaning a single interaction cannot be tracked over time. If, however, the strains of interest could be genetically manipulated to produce different fluorescent proteins it would be possible to observe their growth as it happens, using epifluorescence or confocal microscopy. Another option would be to perform studies on survivorship in competition between the novel isolates. This would provide useful information on the nature of the deleterious effects of H₂O₂, not only on the competing strain, but on the producers of H₂O₂ as well. We can accomplish this by introducing antibiotic markers into each strain. The antibiotic markers would allow us to recover cells of each strain from mixed cultures, select for each strain’s marker separately, and count CFUs. One potential pitfall is that some oral Streptococcus are easily transformable while others are more reluctant to take up foreign DNA and maintain those genes while not under selection to do so. If these difficulties can be overcome we could have a powerful tool for examining the dynamics of multispecies communities with competing Streptococcus strains.

Imaging mass spectroscopy (such as MALDI-IMS) provides a valuable tool to analyze and identify compounds or profiles from competing organisms and could aid in discovering the causes for multiple bands of inhibition. Our competition assays’ plate format lends itself well to analysis with this method. Imaging mass-spec could be used to produce profiles of all compounds present within each region of the inhibitory interactions, allowing us to spatially map what is occurring in each band of a zone of inhibition, analyze the profiles and potentially identify compounds that are present. This type of analysis could provide information as to what compounds are involved in the interactions between our novel isolates in addition to hydrogen peroxide.

Further studies of our novel streptococci should seek to explain the role of CO₂ metabolism in growth and interactions. Our studies indicated that H₂O₂ production involved in competition and
CO₂ metabolism are interconnected with the enzyme pyruvate oxidase likely at the center of this pathway. Pyruvate oxidase mutants should be constructed and growth and interaction experiments conducted in CO₂ rich and CO₂ poor conditions. Other genes central to CO₂ metabolism, such as carbonic anhydrase, should also be examined for the role they play in the competition, H₂O₂ and CO₂ pathways.

More importantly, the impact that this simple gaseous molecule has on interactions indicates the need to study a new dimension of bacterial interactions and biologically active molecules: volatile compounds. This enormous class of compounds is essentially ignored by current methods. The impact of volatile compounds should be examined for every system where interactions, competition or biologically active compounds are suspected using methods that focus on gas exchange or shared headspace.

Literature cited


