STREPTOCOCCUS INTERMEDIUS INTERACTIONS

CHARACTERIZING COOPERATIVE AND COMPETITIVE INTERACTIONS INVOLVING *STREPTOCOCCUS INTERMEDIUS*

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ABSTRACT

The *Streptococcus* Anginosus/Milleri group (SMG) colonize mucosal surfaces in humans but are also associated with numerous respiratory and invasive infections. These infections are often polymicrobial in nature, with obligate anaerobes often being isolated. The group consists of three species, *S. anginosus*, *S. constellatus* and *S. intermedius*. SMG are considered to be lactic acid bacteria, producing acids such as lactate, formate and acetate as byproducts of their metabolism. Their genomes have been recently sequenced but little is known about their metabolism. Understanding the basis of their metabolism is beneficial in determining optimal growth conditions and mechanisms associated with their pathogenicity. The isolation of obligate anaerobes from SMG polymicrobial infections suggests that they have anoxic microenvironments. There is also some evidence for synergy between SMG species and anaerobes. While cooperation might be occurring with certain anaerobes, streptococci also produce inhibitors such as hydrogen peroxide and short peptides called bacteriocins. These give streptococci a competitive advantage in polymicrobial commensal communities such as the oral cavity. The *Streptococcus* invasion locus controls bacteriocin production in Group A streptococci and has been identified in SMG species as well. It is unknown if SMG have mechanisms to compete with closely related streptococci. The goal of my thesis is to characterize the cooperative and competitive interactions of *S. intermedius* with other species.

In chapter 2, we characterized the *in vitro* metabolism of *S. intermedius* under aerobic (5% CO_2) and anaerobic conditions. Using a transcriptomic and metabolomic approach, we mapped the pathways involved in *S. intermedius* B196 metabolism. We

found that there was a minimal upregulation of core pathways including carbohydrate metabolism under anaerobic conditions. Under aerobic conditions, oxidative stress genes were induced. An increased growth rate was also observed anaerobically.

In chapter 3, I demonstrated that *Streptococcus* strains, including *S. intermedius*, can deplete oxygen and create an anaerobic environment. Certain strains could support the viability of the obligate anaerobe *Prevotella melaninogenica* in broth cultures under hypoxic conditions, while others inhibited *Prevotella* by producing hydrogen peroxide. *S. intermedius* B196 has an alkylhydroperoxidase system (*ahpCF*), which is thought to endogenously detoxify peroxides. An *S. intermedius ahpCF* mutant produced hydrogen peroxide and inhibited *P. melaninogenica* in coculture. Complementation in *S. intermedius* restored *P. melaninogenica* viability in coculture. I demonstrated that the *ahpCF* peroxide detoxification system directly protects *S. intermedius* from peroxides and indirectly affects a polymicrobial community.

In chapter 4, we used a subcutaneous abscess model in BALB/c mice to demonstrate that *S. intermedius* promotes *P. melaninogenica* survival during co-infection in comparison to a *P. melaninogenica* mono-infection. *S. intermedius* induced abscesses appeared to induce apoptosis, necrosis and NETosis in neutrophils that infiltrated the site of infection. Our results demonstrate the complexity of SMG infections.

In chapter 5, I demonstrated that *S. intermedius* B196 produces inhibitors of other SMG in response to stimulation with the pheromone peptide SilCR. This is the first case of *S. intermedius* inhibiting a closely related SMG strain. A bioinformatic analysis was done on the *sil* system in SMG. The system is associated with a genetically

heterogeneous bacteriocin cluster which can carry any combination of sixteen putative open reading frames, six of which are putative bacteriocins.

Together, my thesis outlines that *S. intermedius* has specific mechanisms of cooperation and competition. These allow it to cooperate with obligate anaerobes such as *P. melaninogenica* and inhibit other SMG species. Oxygen depletion, hydrogen peroxide production and bacteriocin production are only three factors addressed in this thesis. However, there are many factors involved in shaping a polymicrobial environment with SMG species. More research in SMG polymicrobial interactions is required to fully understand SMG pathogenicity.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACN	Acetonitrile
ADI	Arginine deiminase pathway
ADP	Adenosine diphosphate
APC	Allophycocyanin
ATP	Adenosine triphosphate
BHI	Brain Heart Infusion
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony forming units
CHP	Conserved hypothetical protein
CO_2	Carbon dioxide
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
Cy5	Cyanine dye
Cy7	Cyanine dye
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dUTP	Deoxyuridine triphosphate
ESI	Electrospray ionization
EtOH	Ethanol
FITC	Fluorescein isothiocyanate
FMN	Flavin mononucleotide
FPKM	Fragments per kilobasepairs per million reads
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
GGS	Group G *Streptococcus*
GlcNAc	N-acetylglucosamine
Gly	Glycine
H_2	Hydrogen
H_2O	Water
H_2O_2	Hydrogen peroxide
H&E	Hematoxylin and eosin
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
IS	Internal standards
k_{app}’	Apparent retention factor
KVLB	Kanamycin vancomycin laked blood agar
LC-MS	Liquid chromatography-mass spectrometry

MeOH	Methanol
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
Min	Minutes
mL	Millilitres
mm	Millimetres
mM	Millimolar
MS	Mass spectrometry
MSI	Metabolomics standard initiative
N_2	Nitrogen
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced Nicotinamide adenine dinucleotide
NET	Neutrophil extracellular traps
ng	Nanograms
Nm	Nanometres
O_2	Oxygen
OD	Optical density
OPLS-DA	Orthogonal partial least-squares discriminative analysis
PB	Prussian Blue
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCA	Principal component analysis
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidyl-ethanolamine
Per	Photosynthetic apparatus found in *Glenodinium*
PG	Phosphatidylglycerol
Phe	Phenylalanine
PL	Phospholipids
PMN	Polymorphonuclear leukocytes
PPP	Pentose phosphate pathway
PTS	Phosphotransferase system
Q^2	Prediction statistic for OPLS-DA
QC	Quality control
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid

RS	Reference standard
SAM	S-adenosyl methionine
SMG	*Streptococcus* Milleri Group
TCA	Tricarboxylic acid cycle
THY	Todd Hewitt Broth with 5% Yeast extract
TOF	Time of flight
TSY	Tryptic soy broth with 5% yeast extract
U	Units of enzyme
UDP	Uridine diphosphate
v/v	Volume/volume
°C	Degrees celsius
µL	Microlitres
µM	Micromolar
µg	Micrograms
Δ	Deletion mutant

CHAPTER 1

INTRODUCTION

1.1 The *Streptococcus* genus

Streptococci were first visualized by Antonie van Leeuwenhoek in 1683 by sampling material from between teeth on a microscope. Almost 200 years later, in 1879, streptococci were isolated and proposed to be the etiological agent in puerperal sepsis by Louis Pasteur (Larson 1989). They were described by him as being small chain-forming microbes. In the last four centuries, streptococci have been isolated from numerous human diseases and attempts have been made to classify streptococci genetically and phenotypically to correlate strains with disease.

Classification of streptococci genetically is confounded by extensive horizontal gene transfer in these organisms as some are naturally competent (i.e. able to take up extracellular DNA). In addition to the 16S rRNA gene based approach, phenotypic and genetic delineation using multiple genes have been used as classification methods. An example of phenotypic classification includes Lancefield groupings based on cell wall carbohydrate composition in streptococci (Lancefield 1933). One limitation of Lancefield groupings is that some antigen groups include several species, making it hard to identify species based solely on this classification. Another limitation is that some species do not have a carbohydrate antigen assigned to them. Currently, a multiple gene based classification method provides the best option; but consideration of additional attributes is necessary with the increasing number of unclassified strains (Lal et al. 2011).

Recent papers have classified streptococci based on multiple genes (Olson et al. 2013; Gao et al. 2014; Póntigo et al. 2015). One study analyzed 138 *Streptococcus* strains and generated a *Streptococcus* delineation tree based on 278 orthologous proteins (Figure

1.1, Gao et al., 2014). Another study used the 16S rRNA gene, DNA dependent RNA polymerase β subunit (*rpoB)*, superoxide dismutase (*sodA)*, elongation factor Tu (*tuf)*, RNase P component class B (*rnpB)*, DNA gyrase subunit B (*gyrB)*, molecular chaperone (*dnaJ*) and DNA repair protein (*recN)* to generate a similar tree (Pontigo et al., 2015). The genus *Streptococcus* is divided into several major groups. The pyogenic group, so termed based on its disease manifestation in humans, includes *S. pyogenes*, *S. agalactiae*, *S. uberis*, *S. iniae*, *S. equi* and *S. dysgalactiae*. *S. pyogenes* is also referred to as Group A streptococci (GAS) based on its Lancefield Grouping. *S. agalactiae* and *S. uberis* are referred to as Group B streptococci (GBS). GAS and GBS are associated with many disease manifestations in humans (Schuchat 1999; Farley and Strasbaugh 2001; Walker et al. 2014). The Mitis group species predominantly colonize the oral cavity and upper respiratory tract of humans. These species include *S. mitis*, *S. gordonii*, *S. pneumoniae* and *S. oralis*. *S. pneumoniae* is a well known pathogen in this group (Bogaert et al. 2004). It is carried as an asymptomatic commensal in humans but causes infection in young and elderly humans. The Anginosus group includes *S. anginosus*, *S. constellatus* and *S. intermedius*. Species in this group are also human colonizers and are often overlooked as pathogens despite causing more than half of all pyogenic *Streptococcus* infections (Laupland et al. 2006). The Salivarius group (*S. salivarius*, *S. thermophiuls* and *S. vestibularis*) include streptococci that often colonize the oral cavity (Frandsen et al. 1991). The Bovis group (*S. bovis*, *S. gallolyticus*, *S. pasteurianus, S. macedonicus*, *S. equinus* and *S. infantarius*) has been associated with colonic cancer (Abdulamir et al. 2011) while the Mutans group is associated with dental caries (Nicolas and Lavoie 2011).

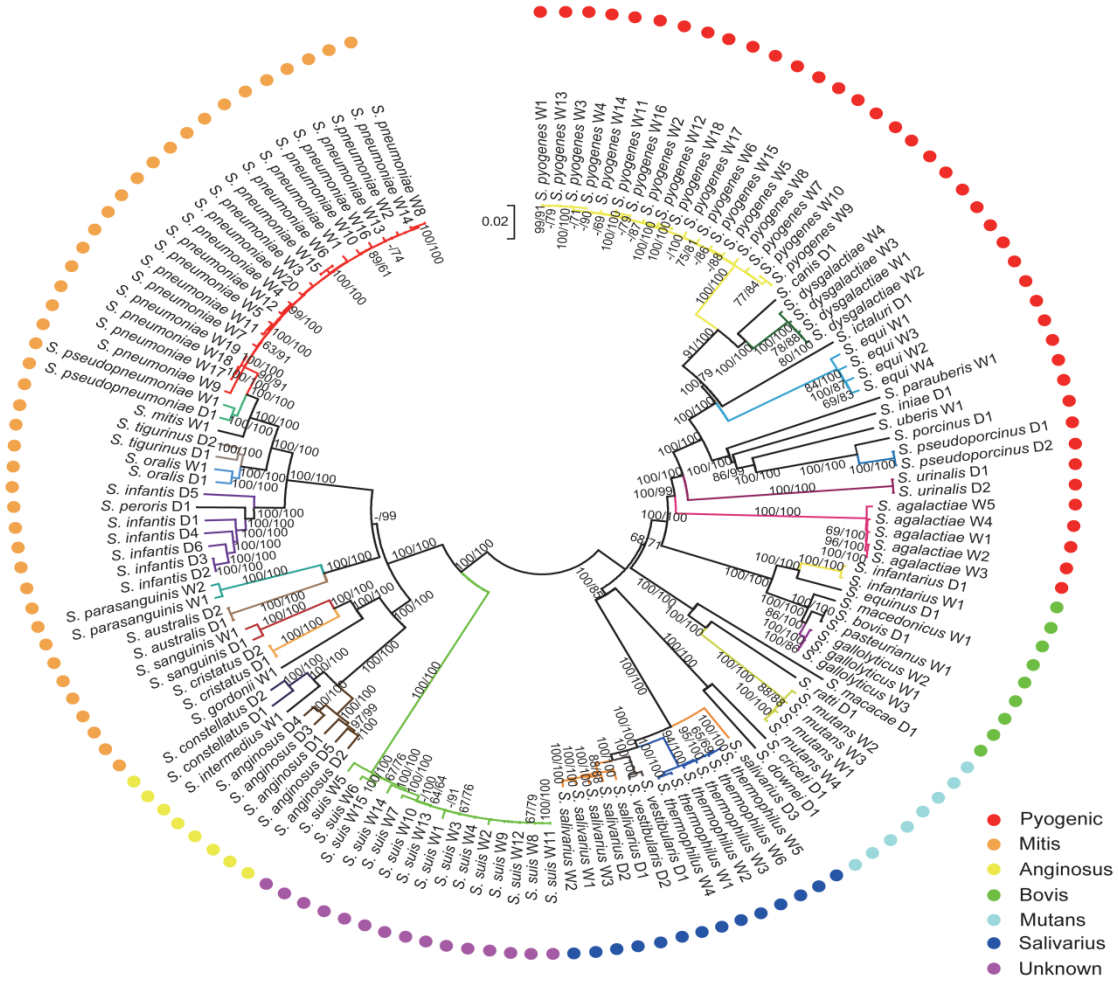

Figure 1.1: Phylogenomic tree of the *Streptococcus* genus (taken from Gao et al., 2014). Analysis of 278 orthologous groups was used to delineate 138 strains of streptococci using maximum likelihood (bootstrap value indicated as numerator) and neighbor joining (bootstrap value indicated as denominator) algorithms.

Aside from these, new groups have also been proposed (Póntigo et al. 2015). With the advantage of high-throuput sequencing becoming more accessible and less expensive, *Streptococcus* species delineation will gain more clarity in the next coming years.

1.2 *Streptococcus* associations with humans

In humans, streptococci are found as commensals in the oral cavity, the gastrointestinal tract, the upper respiratory tract, skin and the urogenital tract (Human Microbiome Project Consortium 2012). When host barriers are compromised, streptococci can cause invasive and sometimes systemic infections. Streptococci are associated with a broad spectrum of infections, ranging from localized abscesses to systemic bacteremia and even endocarditis (Li et al. 2000b; Farley and Strasbaugh 2001; Mitchell 2003; Tran et al. 2008; Abdulamir et al. 2011; Walker et al. 2014). As commensals, *Streptococcus* colonization depends on the bacterial strain, host age and health status and environmental factors (Obaro and Adegbola 2002; Zaura et al. 2009; Dewhirst et al. 2010; Zarco et al. 2012; Human Microbiome Project Consortium 2012; Welch et al. 2016). They compete with microbial residents and can even overthrow the dynamics of a niche (Krone et al. 2014; Thevaranjan et al. 2016). There is a need to further explore the commensal and pathogenic role of these overlooked bacteria.

1.3 *Streptococcus* Anginosus/Milleri group

The *Streptococcus* Anginosus/Milleri group (SMG) strains were first isolated in the early 20th century (Andrewes and Horder 1906; Prevot 1924; Prevot 1925). SMG consists of three distinct yet closely related species namely, *S. anginosus*, *S. constellatus* and *S. intermedius. S. anginosus* is further divided into *S. anginosus* subsp. *whileyi* and *S.*

anginosus subsp. *anginosus* (Jensen et al. 2013). *S. constellatus* is divided into *S. constellatus* subsp. *pharyngis, S. constellatus* subsp. *viborigensis* and S. *constellatus* subsp. *constellatus* (Jensen et al. 2013). Jensen and colleagues used seven housekeeping genes to divide SMG strains into seven clusters and attempted to phenotypically characterize the clusters with some success **(Figure 1.2**) (Jensen et al. 2013). Overall, SMG strains can be grouped under five Lancefield classifications (A, C, F, G, no antigen) and can be non-hemolytic, α-hemolytic or β-hemolytic (**Figure 1.2**) (Colman and Williams 1972; Jensen et al. 2013). In spite of serological and haemolytic differences, SMG species share a number of physiological characteristics. These include biochemical processes such as fermentation of various carbohydrates, production of acetoin from glucose and hydrolyzing esculin and arginine (Parker and Ball 1976; Facklam 1977; Ball et al. 1979). SMG colonies are typically described as being 0.5 – 1 mm pinpoint colonies and can have smooth or rough morphologies. Aerobic growth is enhanced in the presence of carbon dioxide, though the reason for this dependence is unknown (Ball et al. 1979).

As commensals, SMG species have been isolated from the oral cavity, the throat, feces and female urogenital tract (Long and Bliss 1934; Smith and Sherman 1938; Mejàre and Edwardsson 1975; Wort 1975; Bannatyne and Randall 1977; Unsworth et al. 1980). SMG is also associated with a number of diseases affecting various sites of the human body. They are often isolated from respiratory infections and conditions including lung abscesses, empyema, cystic fibrosis, chronic obstructive pulmonary disorder and asthma (Lee et al.; Hocken and Dussek 1985; Van der Auwera 1985; Gossling 1988; Ripley et al. 2006; Sibley et al. 2008; Navratilova et al. 2016). They are associated with pharyngitis

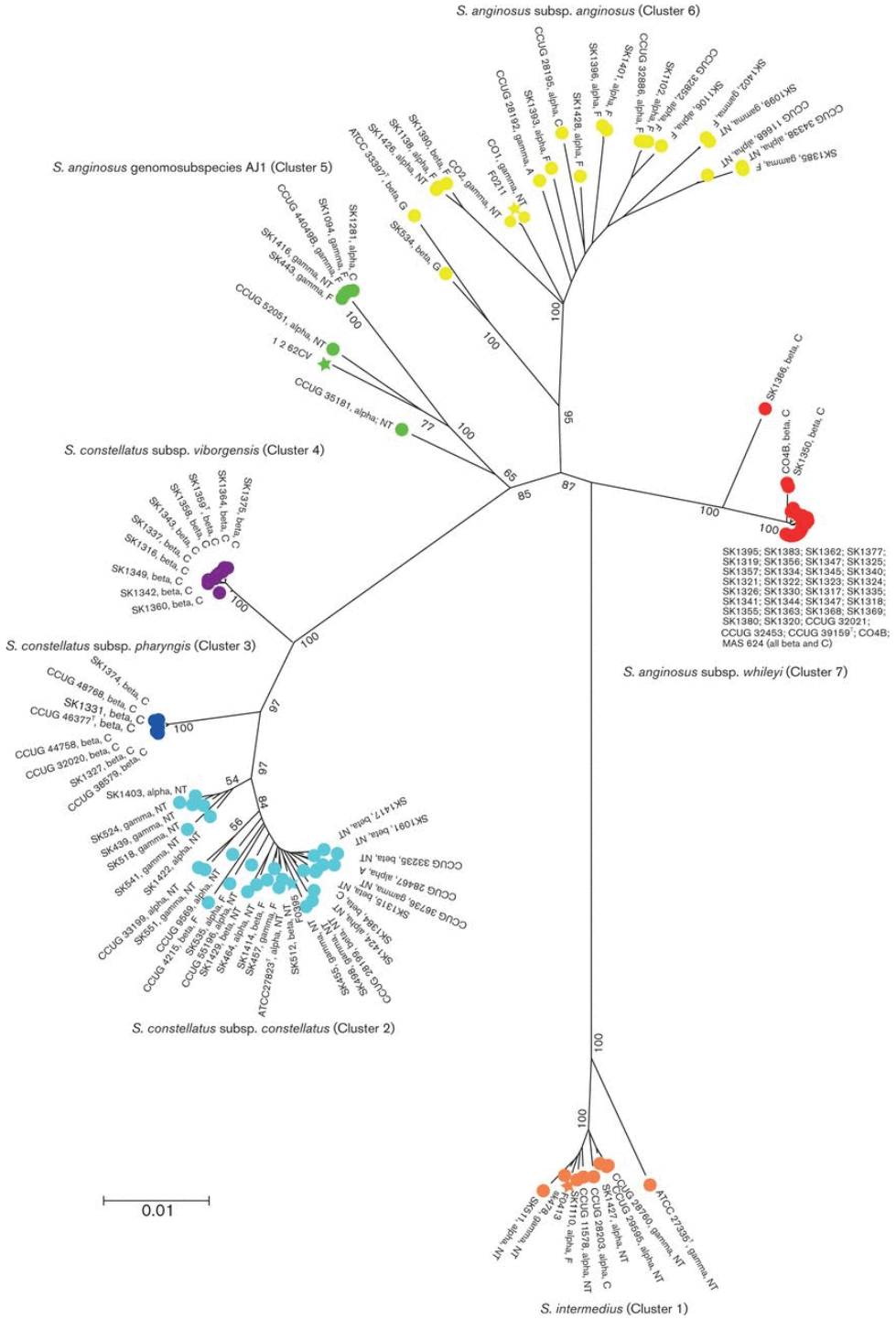

Figure 1.2: A minimum evolution tree based on seven housekeeping genes in 128 *Streptococcus* anginosus strains (Jensen et al. 2013). The tree divided strains into seven colour coded clusters as shown. Stars indicate sequences obtained from whole genomes. Bootstrap values are as shown. The hemolysis and Lancefield classifications are indicated for each strain.

(Poole and Wilson 1979), liver abscesses (Dorvilus and Edoo-Sowah; Clarridge et al. 2001; Rashid et al. 2007), and appendicitis (Poole and Wilson 1979; Madden and Hart 1985). Invasive SMG strains have been isolated from central nervous system infections such as head and neck abscesses (de Louvois 1980; Hirai et al. 2005) and meningitis (Koepke et al. 1965; Tecson-Tumang et al. 1982). Urogenital carriage in humans is thought to cause SMG associated neonatal sepsis (Cox et al. 1987). In addition, SMG has been isolated from bodily fluids including blood and cerebrospinal fluid (Parker and Ball 1976; Clarridge et al. 2001). SMG species are thought to disseminate through the bloodstream (Parkins et al. 2008; Giuliano et al. 2012). They have even been isolated from cases of endocarditis (Woo et al. 2004; Rashid et al. 2007).

There is some variation in characteristics observed within SMG species. *S. intermedius* and *S. constellatus* are more often isolated from abscesses in comparison to *S. anginosus* (Clarridge et al. 2001). Studies predominantly support *S. intermedius* association with central nervous infections and *S. anginosus* association with gastrointestinal disease (Whiley et al. 1992; Bantar et al. 1996). *S. constellatus* is associated with both. Identification of SMG species involved in invasive infection could have a prognostic benefit as infection with *S. intermedius* has been associated with a worse outcome in terms of length of hospital stay and mortality in comparison to *S. anginosus* and *S. constellatus* respectively (Junckerstorff et al. 2014). While these generalizations have been made, there are exceptions (Junckerstorff et al. 2014).

1.3.1 SMG Metabolism

Streptococci are considered to be lactic acid bacteria, a name given due to their production and secretion of acids such as lactate and acetate as an output of carbohydrate metabolism. Lactic acid bacteria include genera such as *Lactococcus*, *Lactobacillus* and *Streptococcus*. The carbohydrate metabolism of these bacteria can vary by species, but an overarching theme includes their ability to differentially use carbohydrates to produce fermentative acids such as lactate, formate and acetate. Not all lactate producers are considered lactic acid bacteria (eg. spore formers such as *Bacillus coagulans*).

SMG are facultative anaerobes. Their growth aerobically is limited in the absence of carbon dioxide. In prokaryotes, the most favourable generation of energy is aerobic respiration where NADH, generated from glycolysis and the Krebs cycle, donates electrons to the electron transport chain. The electrons are terminally accepted by oxygen to generate water. Some facultative anaerobes undergo aerobic respiration in the presence of oxygen but shift to another terminal electron acceptor in the absence of oxygen. The energy generated is dependent on the terminal electron acceptor utilized. Lactic acid bacteria, however, have a very different metabolism in that they cannot produce heme and thus do not have a classic electron transport chain. Lactic acid bacteria produce and secrete the aforementioned acids into their extracellular environment to create a proton gradient across their cellular membranes, generating ATP using an ATP synthase. This fermentative metabolism, though energetically unfavourable in comparison to other facultative anaerobes, can be beneficial in polymicrobial associations (Stacy et al. 2016; Stacy et al. 2014). *S. gordonii* and *Aggregatibacter actinomycetemcomitans* are found in

the oral cavity and are associated with focal oral infections in other parts of the body (Gendron et al. 2000). Lactic acid, produced as a by-product by *S. gordonii*, is used as a nutrient by *Aggregatibacter actinomycetemcomitans* (Stacy et al. 2016; Stacy et al. 2014). The mechanisms involved in the interactions between the two bacteria have not been fully characterized.

1.3.2 Polymicrobial associations of SMG species in humans

Colonization of human tissues by SMG species are influenced by a number of factors, including bacterial attachment to host surface, aggregation or growth, physical factors (pH and oxygen availability), nutrient availability and the host immune system (Stacy et al. 2015). Streptococci have a number of surface proteins and secreted factors that mediate primary attachment to host surfaces (discussed later). Following primary attachment, bacterial growth can take place and this allows for polymicrobial associations. The spatial organization of bacteria in a polymicrobial environment is niche and bacteria dependent (Stacy et al. 2015).

SMG are often found in polymicrobial environments with anaerobes, in both commensal and pathogenic associations. The development of polymicrobial communities has been described in models of oral cavity colonization. Within four hours of professional teeth cleaning, up to 85% of the cultivable cells from teeth are streptococci (Nyvad and Kilian 1987). After 12 hours post cleaning, this develops into a polymicrobial community. Primary attachment to the host is facilitated by streptococci through surface proteins that bind to the tooth surface and surface polysaccharides (Nobbs et al. 2009). Secondary colonizers include obligate anaerobes such as *Fusobacterium* and *Prevotella*

species (Takahashi 2005; Aas et al. 2005; Zaura et al. 2009; Welch et al. 2016). As niches within the oral cavity are diverse (tongue surface/ tooth surface/ gums), there is differential colonization and communities that develop at each site (Zaura et al. 2009). Polymicrobial associations can also lead to disease downstream. Cases of dissemination of bacteria from the oral cavity leading to invasive system infection have been described (Li et al. 2000b; Hsiao et al. 2012).

The following sections will describe factors that affect a polymicrobial community involving streptococci. These specifically deal with the environment, *Streptococcus* strain dependent response to the environment and how this can affect other bacteria in the immediate environment.

1.3.2.1 Oxygen related metabolism and role in competition

Streptococci vary in their tolerance and use of oxygen. Some strains of *S. mutans* are extremely sensitive to oxygen, while others can exhibit an oxygen dependent growth enhancement (Higuchi 1984). The specific genes involved in oxygen related metabolism are many and each *Streptococcus* strain, even within a species, can carry a unique combination of oxygen related metabolic tools. This section will describe genes involved in oxygen related metabolism in the genus *Streptococcus* as these have not been characterized in SMG as yet.

1.3.2.1.1 Reactive oxygen species (ROS) and putative resistance mechanisms in SAG

ROS can be generated by the transfer of electrons to oxygen leading to the production of superoxide ion (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl radical ($^{\bullet}OH$) (Yesilkaya et al. 2013). Streptococci are thought to have ways to detoxify each of these,

and while some mechanisms are known (discussed below), other resistance mechanisms have been observed but are yet to be characterized.

Superoxide ions attack iron sulfur clusters in proteins (Kuo et al. 1987; Flint et al. 1993). Superoxide dismutase (*sodA*) facilitates the conversion of superoxide ions to hydrogen peroxide and oxygen (Fridovich 1978). There are four types of superoxide dismutases in prokaryotes depending on the cofactor (Mn, CuZn, Fe, Ni) (Zelko et al. 2002). Streptococci predominantly carry the Mn dependent *sodA*. SodA is required for virulence and aerobic growth of *S. pneumoniae* (Yesilkaya et al. 2000). Knockouts of *sodA* had a lower *in vitro* aerobic growth rate, exhibited attenuated virulence and had a higher susceptibility to paraquat, which catalyzes superoxide formation (Yesilkaya et al. 2000; Poyart et al. 2001). A by-product of SodA is hydrogen peroxide, which can also be toxic to the cell. Another way streptococci deal with peroxides and free radicals is with scavengers including the tripeptide glutathione (L-glutamyl-L-cysteinyl-glycine) (Potter et al. 2012). A knockout of the glutathione transporter/reductase genes (*gshT*/*gor*) increased *S. pneumoniae* sensitivity to superoxides (Potter et al. 2012).

Hydrogen peroxide (H_2O_2) targets sulfur atoms in cysteine and methionine protein residues and causes oxidative damage to DNA and cell membranes (Falcioni et al. 1998; Winterbourn and Metodiewa 1999; Griffiths et al. 2002). Intracellular H_2O_2 is toxic to all streptococci as even *S. pneumoniae* (considered a high producer of H_2O_2) is at risk of increased mutations and decreased survival in the presence of H_2O_2 (Regev-Yochay et al. 2007). H_2O_2 can be detoxified using the alkylhydroperoxidase (AhpCF) system in most streptococci. There are other genes also associated with peroxide resistance. Deletion of

the operon with cytochrome c-type biogenesis protein (*ccdA)*, thioredoxin like family protein (*tlpA)* and methionine sulfoxide reductase A/B (*msrAB)* increases *S. pneumoniae* susceptibility to H_2O_2 by ten times (Andisi et al. 2012). Most bacteria carry the catalase gene to neutralize H_2O_2 but this is lacking in streptococci.

The hydroxyl radical is formed from H_2O_2 in the presence of metal ions. Hydroxyl radicals can cause extensive DNA damage (Imlay and Linn 1988). The iron binding protein Dpr prevents hydroxyl formation and affects oxygen tolerance in streptococci (Yamamoto et al. 2000; Yamamoto et al. 2002).

1.3.2.1.2 Oxygen usage

The use of oxygen in lactic acid bacteria is very different from other facultative anaerobes. In streptococci, oxygen is used through substrate phosphorylation by several oxidases. NADH oxidase (Nox-2) is essential for aerobic growth in most streptococci (Higuchi et al. 1999; Yu et al. 2001; Chong and Nielsen 2003; Derr et al. 2012). Nox-2 catalyzes the oxidation of NADH while reducing oxygen to form water. Oxidation of NADH is important in regenerating NAD^+ for glycolysis. A number of oxidases use oxygen to produce H_2O_2 as a means for competitive inhibition of other bacteria.

1.3.2.1.3 H_2O_2 production in streptococci and competition

In polymicrobial communities, *Streptococcus* production of competitive inhibitors such as H_2O_2 inhibit obligate anaerobes and even some streptococci (Takeuchi et al. 2000; Kreth et al. 2008; Zheng et al. 2011a; Zhu and Kreth 2012). There is some variation in *Streptococcus* H_2O_2 resistance (Kreth et al. 2008; Zheng et al. 2011a). Pyruvate oxidase (*spxB*) in *S. pneumoniae* facilitates the conversion of pyruvate to acetyl

phosphate while using oxygen to produce H_2O_2 (Regev-Yochay et al. 2007). Deletion of *spxB* increases *S. pneumoniae* sensitivity to H_2O_2 implying that *spxB* mediates H_2O_2 resistance as well, through an unknown mechanism (Pericone et al. 2003). Lactate oxidases uses lactate to produce pyruvate while converting oxygen to H_2O_2 (Seki et al. 2004). Both pyruvate and lactate oxidases are found in select *S. anginosus* strains. However, most SMG strains do not produce H_2O_2.

1.3.2.2 *Streptococcus* interspecies competition

Streptococci are the dominant species in the oral cavity and as primary colonizers compete for localized space. Aside from H_2O_2, streptococci are thought to produce other inhibitors to compete with closely related species. For example, the *Streptococcus* invasion locus (*sil*) system is thought to control the production of short peptide inhibitors called bacteriocins. The *sil* system was first identified in GAS, where they found that a pheromone peptide (SilCR) controlled expression of bacteriocins (Eran et al. 2007; Belotserkovsky et al. 2009; Armstrong et al. 2016). Components of this system have been found in SMG (Olson et al. 2013). The putative bacteriocins subtilosin (locus tag: SIR_RS16420) and lactococcin (locus tag: SIR_RS11095) have also been identified in SMG, though these are independent of the *sil* system and are yet to be characterized. Subtilosin is thought to target Gram-positives while lactococcin mainly targets lactococci (Babasaki et al. 1985; Holo et al. 1991). Salvaricin is another putative bacteriocin that can inhibit other streptococci (Ross et al. 1993).

1.3.3 Pathogenic associations

Due to SMG species being overlooked as etiological agents, literature surrounding the underlying mechanisms involved in SMG pathogenicity is lacking. This area is actively being researched by the Surette laboratory. Putative SMG virulence associated genes based on the current pathogenicity mechanisms in other streptococci are described below.

1.3.3.1 Virulence factors

Virulence factors in SMG species were divided into groups based on their putative functions **(Figure 1.3)**. The first group includes SMG surface proteins, associated with attachment and colonization in the host. SMG have putative collagen, fibronectin, fibrinogen (fbp) and laminin (lmb) binding proteins that allow attachment to these specific host surface molecules (Olson et al. 2013). SMG also carry putative enzymes including sialidase, pullulanase and hyaluronidase that are thought to degrade sialic acid, pullulan polysaccharides and hyaluronic acid respectively, found on the host cell surface (Olson et al. 2013). Our lab has previously shown that clinically isolated SMG strains have hyaluronidase activity (Grinwis et al., 2010). A number of SMG putative surface proteins have LPXTG domains that allow them to be anchored to the cell wall by sortases (Löfling et al. 2011). A number of these are thought to be involved in attachement and colonization. SMG also have putative genes for pili, though these proteins are yet to be characterized (Olson et al. 2013).

The second group includes putative cytotoxic proteins that are secreted by SMG (**Figure 1.3**). *S. anginosus* and *S. constellatus* streptolysin S homologues were shown to

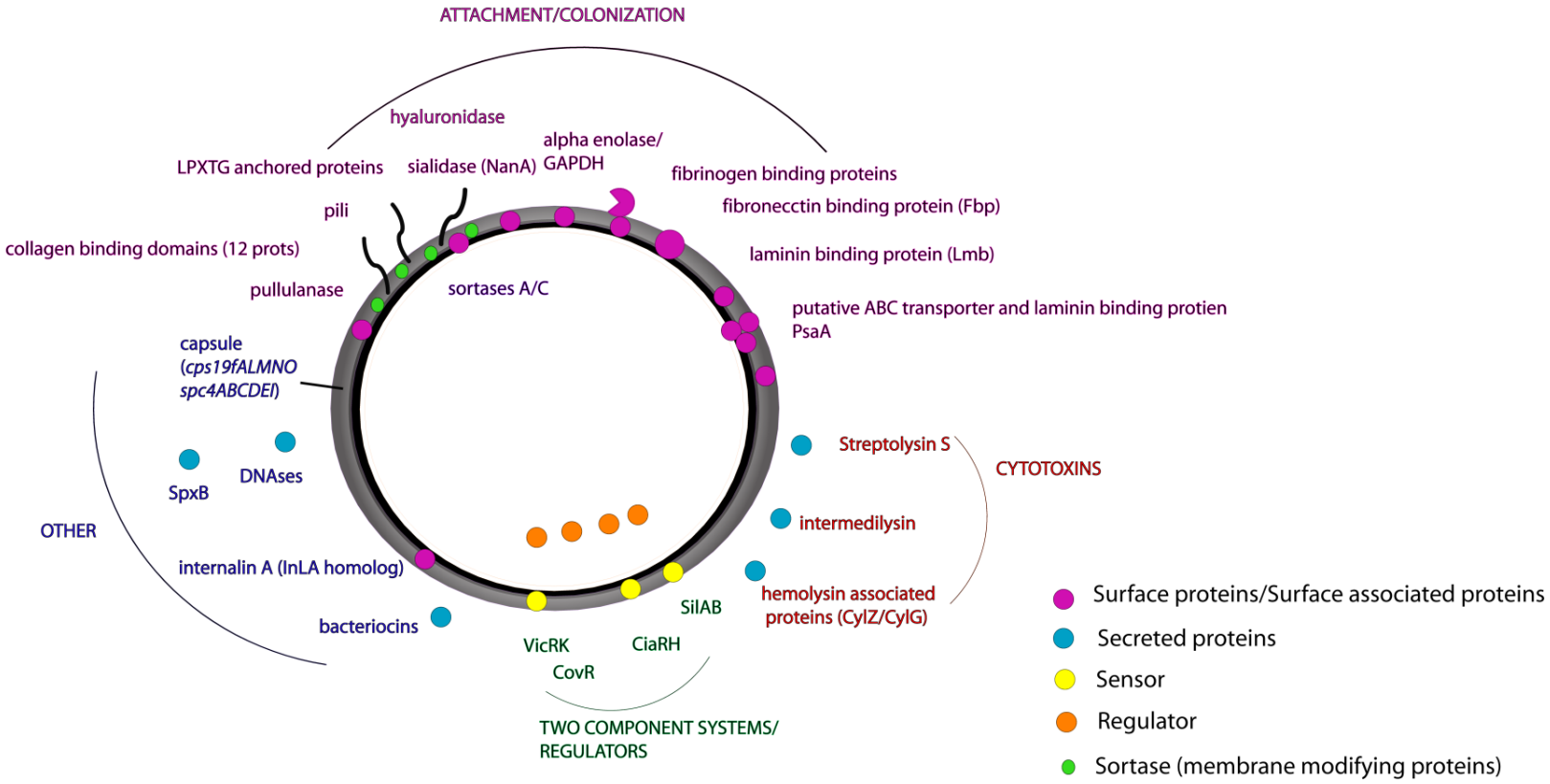

Figure 1.3: Putative virulence factors found in the *Streptococcus* Anginosus Group. Virulence factors were divided into four groups (1) Attachment/Colonization (purple text), (2) Cytotoxins (red text), (3) Two component systems/regulators (dark green text) and (4) Other (dark blue text).

have β-hemolytic and cytotoxic activity (Tabata et al. 2014). Streptolysin S has additional pathogenic functions in other streptococci including inhibiting phagocytic clearance (Datta et al. 2005). *S. intermedius* strains have a human specific cytotoxin called intermedilysin that binds to human antigen CD59 (cluster of differentiation - 59) and facilitates complement lysis (Nagamune et al. 1996; Grinwis et al. 2010; Olson et al. 2013). In addition to these, homologs for *cylZ* and *cylG* were also found in SMG and these have homology with fatty acid biosynthesis genes (Olson et al. 2013; Rosa-Fraile et al. 2014). The *cyl* operon is associated with β-hemolysis in GBS (Rosa-Fraile et al. 2014). The role of these homologues in cytotoxicity has not been determined in SMG.

The third group includes sensor and regulator systems that putatively affect the expression of virulence associated genes based on observations in other streptococci (**Figure 1.3**). The aforementioned *Streptococcus* invasion locus was first associated with pathogenicity when a transposon insertion in the pheromone peptide gene *silCR* attenuated virulence in GAS (Hidalgo-Grass et al. 2002). The SilA regulator and SilB sensor are part of this system. CovR is considered a global virulence regulator in GAS and GBS (Graham et al. 2002; Lamy et al. 2004; Gusa and Scott 2005; Alam et al. 2013). The VicRK system regulates bacteriocin production, biofilm development and cell death in some streptococci (Ng et al. 2005; Duque et al. 2011; Senadheera et al. 2012). The CiaRH system regulates competence and affects nasopharyngeal colonization in certain streptococci (Echenique et al. 2002; Sebert et al. 2002; Li et al. 2011).

The last group includes putative genes with a broad spectrum of associations with virulence (**Figure 1.3**). Certain SMG strains have capsules. The capsules in these strains

were found to inhibit phagocytosis by human PMNs (Kanamori et al. 2004). SMG species also carry genes for putative bacteriocins and these can indirectly affect pathogenesis through polymicrobial interactions. We have previously shown that SMG species can produce DNases (Grinwis et al. 2010). This can be beneficial in degrading neutrophil extracellular traps during infection (Derré-Bobillot et al. 2013). A gene with homology to internalin A (*inlA*) in *Listeria monocytogenes*, which promotes internalization, was also found in SMG (Lecuit et al. 1997; Olson et al. 2013). In addition to these, SMG have ROS resistance systems as described in previous sections. These can be beneficial in removing ROS species and reducing cellular damage during respiratory burst.

1.3.3.2 Models of polymicrobial infection

Several SMG infection models have demonstrated that SMG can act synergistically with other bacteria. In mice, infection of the respiratory tract with *S. constellatus* and *Prevotella intermedia* resulted in 60% mortality in comparison to 10% in each mono-infection (Shinzato and Saito 1994). A subcutaneous abscess model has also been used for this purpose. Co-infection with *S. constellatus* and *Fusobacterium nucleatum* resulted in a larger abscess and higher bacterial colony forming units than infection with either bacteria alone (Nagashima et al. 1999). These studies suggest that SMG species have a synergistic relationship with certain obligate anaerobes.

1.4 Aims and hypothesis

SMG species differ in their pathogenic associations and polymicrobial interactions. To further understand the basic mechanisms involved in SMG polymicrobial

interactions and infection, *S. intermedius* was chosen as a model organism. *Prevotella melaninogenica* was chosen as the obligate anaerobe to study SMG polymicrobial interactions because it is a clinical isolate, previously isolated from a CF patient with SMG and polymicrobial etiology. The goal of my thesis is to study *S. intermedius* polymicrobial interactions *in vitro* and *in vivo*.

Hypothesis: *Streptococcus intermedius* promotes the growth of obligate anaerobes, such as *Prevotella melaninogenica,* while inhibiting the growth of competing organisms such closely related streptococci and other SMG strains

Thesis Aims

1) **To characterize the oxygen dependent metabolism of *S. intermedius* for growth optimization**

   Chapter 2: Metabolic and Transcriptomic Profiling of *Streptococcus intermedius* during Aerobic and Anaerobic Growth. These findings were published in the following manuscript.

   Fei, F., Mendonca, M.L., McCarry, B.E., Bowdish, D.M.E., Surette, M.G.. 2016. Metabolic and transcriptomic profiling of *Streptococcus intermedius* during aerobic and anaerobic growth. Metabolomics 12:46. doi:10.1007/s11306-016-0966-0

2) **To establish *in vitro* conditions for polymicrobial culture of obligate anaerobe *Prevotella melaninogenica* with *S. intermedius***

   Chapter 3: *Streptococcus* dependent survival of obligate anaerobe *Prevotella melaninogenica* in the presence of oxygen

3) **To establish a model for *S. intermedius* polymicrobial infection that addresses pathogenicity mechanisms and host defences**

   Chapter 4: Characterization of a polymicrobial subcutaneous abscess model involving *Streptococcus intermedius* and *Prevotella melaninogenica.*

4) **To characterize a competitive mechanism used by *S. intermedius* against other SMG strains**

   Chapter 5: The *sil* locus in *Streptococcus* Anginosus Group: Interspecies killing and a hotspot of genetic diversity.

Details regarding author contributions can be found in the preface preceding each chapter.

CHAPTER 2

METABOLIC AND TRANSCRIPTOMIC PROFILING OF *STREPTOCOCCUS INTERMEDIUS* DURING AEROBIC AND ANAEROBIC GROWTH

Metabolic and Transcriptomic Profiling of *Streptococcus intermedius* during Aerobic and Anaerobic Growth

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Preface: The research in this chapter was conducted from 2012 to 2015. I am a co-first author on the paper. Experiments were designed by my supervisor Dr. Michael G. Surette, collaborators Dr. Dawn Bowdish and Dr. Fan Fei, and myself. I conducted growth experiments and transcriptome analysis. Dr. Fan Fei conducted the metabolomic analysis. Dr. Fan Fei and I wrote the manuscript and collaborated the mapping of *S. intermedius* metabolism.

Title: Metabolic and Transcriptomic Profiling of *Streptococcus intermedius* during Aerobic and Anaerobic Growth

Abbreviated Title: Aerobic and anaerobic growth in *S. intermedius*

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Abstract

Streptococcus intermedius, *S. constellatus*, and *S. anginosus* comprise the *Streptococcus* Anginosus/Milleri Group (SMG). They are facultative anaerobic bacteria that asymptomatically colonize the upper respiratory, gastrointestinal and urogenital tracts. They are also common pathogens in pyogenic invasive infections, as well as pulmonary and urinary tract infections. Most SMG infections are polymicrobial and associated with co-infecting obligate anaerobic bacteria. To better understand the effect of oxygen on the growth and physiology of these organisms, we compared the global metabolomic and transcriptomic profiles of *S. intermedius* strain B196 under aerobic and anaerobic conditions. The largest transcriptional changes were associated with induction of oxidative stress response genes under aerobic conditions. Modest changes in expression of genes associated with primary metabolism were observed under the two conditions. Intracellular and extracellular metabolites were measured using HILIC-LCMS. Differences in the abundance of specific metabolites were correlated with observed transcription changes in genes associated with their metabolism, implying that metabolism is primarily regulated at the transcriptional level. Rather than a large shift in primary metabolism under anaerobic conditions our results suggest a modest tuning of metabolism to support the accelerated growth rate of *S. intermedius* strain B196 in the absence of oxygen. For example, under anaerobic conditions, purine metabolism, pyrimidine *de novo* synthesis and pyrimidine salvage pathways were up-regulated at metabolic and transcriptional levels. This study provides a better understanding of differences between *S. intermedius* anaerobic and aerobic metabolism. The results reflect

the organism's predilection for anaerobic growth consistent with its pathogenic association with anaerobes in polymicrobial infections.

Keywords (3-6)

Streptococcus intermedius, transcriptomics, metabolomics, aerobic, anaerobic, *Streptococcus* Milleri/Anginosus Group

Introduction

The *Streptococcus* Milleri/Anginosus Group (SMG) is comprised of three distinct but closely related species of facultative anaerobic Gram-positive bacteria (*S. anginosus*, *S. constellatus*, and *S. intermedius*) (Gossling 1988). The SMG are often considered as commensal human microbiota and can be found asymptomatically colonizing the oral cavity, upper respiratory tract, urogenital tract and gastrointestinal tract in healthy individuals (Gossling 1988; Whiley et al. 1992). However, the SMG are also recognized pathogens in pyogenic infections including soft tissue abscesses, pleural empyema, brain and liver abscesses, and respiratory infections (Ruoff 1988; Whiley et al. 1992; Coman et al. 1995; Shinzato and Saito 1995; Laupland et al. 2006; Ripley et al. 2006; Parkins et al. 2008; Sibley et al. 2008; Siegman-Igra et al. 2012; Asam and Spellerberg 2014). Phenotypic heterogeneity in this group can make their identification challenging and recent studies suggest that the SMG are under appreciated pathogens with incidence rates for pyogenic infections comparable to Group A and Group B *Streptococcus* combined (Laupland et al. 2006; Siegman-Igra et al. 2012). The SMG have been primarily associated with adults with respect to both carriage and infection, however they may be underestimated in pediatric disease (Lee et al. 2010).

Most infections associated with the SMG are polymicrobial with a significant burden of obligate anaerobic bacteria present in the infection site. This has been observed in lower airway infections (Shinzato and Saito 1995; Parkins et al. 2008; Sibley et al. 2008; Filkins et al. 2012), pleural empyema (Van der Auwera 1985; Wong et al. 1995; Sibley et al. 2012) and abscesses (Gossling 1988; Shinzato and Saito 1994; Hirai et al.

2005; Sibley et al. 2012). Understanding how SMG adapts to aerobic and anaerobic environments may provide insight into the mechanisms used by *S. intermedius* for survival and persistence in the host during colonization and disease progression.

In this study we examined the *in vitro* growth of *S. intermedius* strain B196 in aerobic (5% CO_2) and anaerobic (90%N_2, 5% CO_2, 5% H_2) conditions using growth kinetics, transcriptomics (RNA-seq), and both intracellular and extracellular metabolomics. The effect of oxygen on the growth, physiology and metabolism of *S. intermedius* provides insight in understanding its pathogenic association with anaerobes in polymicrobial infections. Moreover, understanding the metabolic regulation of *S. intermedius* under various oxygenated environment through comprehensive metabolomic studies can provide insights modulating its commensal or pathogenic activities in human host.

Materials and Methods

Chemicals

HPLC grade methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), and water (H_2O) were purchased from Caledon laboratories (Georgetown, ON, Canada). Ammonium acetate and formic acid were purchased from Fisher Scientific Company (Ottawa, ON, Canada). 2.0mm steel chrome ball bearings were purchased from Bearing & Oil Seals Specialists Inc. (Hamilton, ON, Canada). The isotopically labelled standards for recovery determination (RS) and for peak intensity normalization (IS) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Lipid standards were purchased from Avanti® Polar Lipids, Inc. (Alabaster, AL, USA), and other chemical

standards for LC-MS were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Biolog Inc. (Hayward, CA, USA).

Bacterial Strain, Media and Growth Conditions

S. intermedius B196 is an invasive isolate from the hip abscess of a cystic fibrosis patient. A complete genome sequence is available for this strain (Olson et al. 2013). *S. intermedius* was grown on Todd Hewitt agar supplemented with 0.5% yeast extract (THY) at 37ºC in a 5% CO_2 incubator for 3 days. A single colony was inoculated into 5 mL THY broth for overnight static growth under the above conditions. For growth kinetics, overnight cultures were inoculated into 5 mL THY broth at an initial OD_{600nm} of 0.05 and cultured aerobically (5% CO_2) and anaerobically (90% N_2, 5% CO_2, 5% H_2) with optical density as well as colony forming units (CFU) recorded every hour (detailed procedures included in Supplementary Material ESM 1). For RNA-seq and metabolomics, the same overnight cultures were inoculated into THY broth at an initial OD_{600nm} of 0.1 and grown under either aerobic and anaerobic conditions with samples collected at mid-exponential phase (OD_{600nm}= 0.7) .

Strand-specific RNA-seq

Three biological replicates were prepared for *S. intermedius* under aerobic and anaerobic conditions. A 2 mL culture from each replicate at OD_{600nm}= 0.7 was centrifuged. The cell pellets were collected and stored in RNAprotect bacteria reagent (Qiagen, Venlo, Netherlands) for later use at -80ºC. Total cellular RNA was isolated and purified using TRIzol (Invitrogen, Carlsbad, CA, USA) and RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Ribosomal RNA (rRNA) was depleted using Ribo-Zero rRNA

removal Kit for bacteria (Epicentre, Madison, WI, USA). cDNA was prepared using the Superscript III first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Strand specific RNA sequencing libraries were prepared using the dUTP approach (Parkhomchuk et al. 2009). The NEB-Next library preparation modules for Illumina were used for library preparation with a separate index used per biological replicate. The libraries were submitted to the McMaster Genomics Facility (McMaster University, Hamilton, Canada) for quality control (QC) and sequencing using standard Illumina protocol (Illumina HiSeq1000, San Diego). QC included assessment of fragment size using BioAnalyzer and routine qPCR quantification to quantify the amount of cDNA. The libraries were converted to FastQ format using Illumina's Casava software (version 1.8.2) with no index mismatches during demultiplexing. Approximately 20 million reads were obtained per sample with 16-17 million reads per biological replicate mapping back to the genome of *S. intermedius* B196 with high stringency. A detailed procedure can be found in Supplementary Material ESM 1.

RNA-seq Data Analysis

The transcriptomic data were aligned, assembled, analyzed and graphed using the Bowtie2: Tophat2: Cufflinks: Cuffdiff: CummeRbund pipeline (Trapnell et al. 2012). A total of 1815 genes were analyzed. Differential gene expression analysis was done using Fragments per kb per million (FPKM) values generated using the pipeline. This value takes into consideration the number of reads mapping back to each gene and normalizes that to the total number of reads generated from the sequencing run. Statistical analysis was included in the Cuffdiff analysis. Genes were classified into pathways based on

BioCyc database (Caspi et al. 2014). The transcriptomic (RNA-seq) data is summarized in Table 2.S1 and the dataset is included in Supplementary Material ESM 2 (Fei et al. 2016).

Extraction Protocol for Intracellular and Extracellular Metabolites

The extraction procedures for intracellular and extracellular metabolomics were based upon previously published work (Fei et al. 2014). Cells from a 2 mL cell culture at 0.7 OD_{600nm} were pelleted by centrifugation at 4 ºC, washed once with 1 mL PBS for intracellular metabolomic analysis; from the same culture, 20 µL of the culture supernatant after centrifugation was collected for extracellular metabolomic analysis. Prior to extraction, 10 µL RS consisting of 770 µM L-methionine-d_3 and 378 µM L-tryptophan-d_5 were added to the cell pellet and THY supernatant. For intracellular metabolomics, the cell pellet was extracted with 100 µL cold methanol/ethanol/water (MeOH/EtOH/H_2O, 2:2:1) and two 2.0 mm chrome steel beads using the Powerlyzer 24 (MO BIO Laboratories Inc., Carlsbad) for 2 min. The cell extract supernatant was collected after centrifugation at 9500 x g for 3 min. The cell debris (consisting of precipitated protein and particulates) was extracted with 50 µL MeOH/EtOH/H_2O two more times, under the same condition. For extracellular metabolomics, 20 µL THY supernatant was extracted with 80 µL MeOH/EtOH (1:1). The solution mixtures were vortex mixed for 2 min and centrifuged at 9500x g for 3 min. The clear supernatants were collected and diluted with 100 µL 60 % v/v ACN/H_2O. IS with 252 µM L-phenylalanine-d_8, 151 µM glycine-phenylalanine, and 88 µM diphenylalanine were added to the total 150 µL cell extracts and 200 µL supernatant extracts. Five separate controls for THY

medium were also extracted as above. Both intra- and extracellular extracts were stored in -80 ºC during extraction processes and before LC-MS analyses. Five biological replicates were collected for aerobic and anaerobic growth conditions and LC-MS was performed in sextuplicate for each sample.

HILIC-TOF-MS Analysis for Intracellular and Extracellular Metabolites

The HILIC-TOF-MS method and parameters were based upon previously published work (Fei et al. 2014). The intracellular and extracellular extracts were analyzed in two separate batches using an Agilent Technologies 1200 RR Series II liquid chromatograph (LC) coupled to a Bruker MicrOTOF II Mass Spectrometer. A 2 µL injection was separated on a 50 mm × 2.1 mm Kinetex 2.6 µm HILIC column of pore size of 100 Å (Phenomenex, CA, USA). The column temperature was maintained at 40 ºC, and the auto sampler storage tray was set at 4ºC. The mobile phases were acetonitrile (A) and 10 mM ammonium acetate in HPLC grade water adjusted to pH 3 with formic acid (B). The flow rate was kept at 0.2 mL/min during a 24-min run with the following gradient: 95 % A for 0.5 min to 35 % A at 12.5 min with an extra 0.5 min hold, then to 95% A at 14 min. The column was equilibrated at 95% A for 10 min before the next injection.

The extracts were analyzed in both ESI+ and ESI- modes. The samples were acquired in random order. A quality control pooled sample was prepared by combining 5 µL extracts from all samples in either the intracellular or extracellular extract batches. The pooled samples were injected seven times at the beginning of each analysis and also

after every 5 samples. MeOH/EtOH/H_2O blank and a standard mixture containing IS and RS were also run after every 10 samples.

LCMS Data Analysis and Metabolite Identification

The data processing and analysis were modified from a previously published protocol (Fei et al. 2014). Post-acquisition internal calibration using intracellular sodium formate clusters in both ESI+ and ESI- were performed with Bruker's DataAnalysis 4.0 SP4. The LC-MS data files were converted to .mzXML format using Bruker CompassXport. The metabolic features were extracted and aligned using open source XCMS with centWave algorithm (Smith et al. 2006); adducts, isotopic ions, and in-source fragments were identified using CAMERA (Kuhl et al. 2010).

To get the final metabolite feature list, metabolite features with apparent retention factor (k_{app}') lower than 0.7 were removed. Isotopic ions, features resulting from IS, RS, and sodium formate clusters were also removed. The metabolite features were normalized with IS eluted closest to their retention time (i.e. features eluted before 7.40 min were normalized by phe-phe; features eluted between 7.40 and 8.30 min were normalized by L-phenylalanine-d_8; features eluted after 8.30 min were normalized by gly-phe). Features with greater than 20% variance in the pooled sample were removed to get the final metabolite feature list.

Metabolite features were identified based on accurate mass and retention time of authentic standards or compound analogs (for lipid identification only) with two identification points (Creek et al. 2014). There were 105 metabolites identified from 1885 intracellular metabolic features, 66 metabolites were identified with level 1

metabolomics standard initiative (MSI)(Sumner et al. 2007), 10 metabolites were putatively annotated with level 2 MSI, and 29 metabolites were assigned to compound classes with level 3 MSI. There were 26 phospholipids (PLs) identified to two phosphatidylcholines (PCs), one phosphatidylethanolamine (PEs), 20 phosphatidylglycerols (PGs), and three lyso-PGs in the intracellular metabolome based on the accurate mass and retention time of lipid analogs (Zheng et al. 2010; Fei et al. 2014). Similarly, there were 116 metabolites identified from 3382 extracellular metabolite features, with 82, 8 and 2 metabolites identified with level 1, 2, and 3 MSI, respectively. Both intra- and extracellular metabolomic data were summarized in Table 2.S1 and the datasets are included in Supplementary Material ESM 3 and 4, respectively (Fei et al. 2016).

Multivariate Statistical Analyses

Both transcriptomic and metabolomic data were subjected to principal component analysis (PCA) and orthogonal partial least-squares discriminative analysis (OPLS-DA) after pareto scaling using SIMCA-P+ 12.0.1 (Umetrics, Kinnelon, NJ). The metabolomic data, 79 and 92 identified metabolites from *S. intermedius* intracellular and extracellular metabolome respectively, were also analyzed using MetaboAnalyst 3.0 for pathway analysis (default setting) based on the *Staphylococcus aureus* metabolic pathway (Xia et al. 2015) (Supplementary Material ESM 5; Fei et al. 2016). Intracellular and extracellular metabolite features and genes were assessed by univariate analyses such as Student's t test (two-tailed, unpaired heteroscedastic) and one-way ANOVA using Microsoft Excel 2010 and MetaboAnalyst 2.0, respectively. Metabolic features and genes with p value less

than 0.05 (from Student`s t test or one-way ANOVA) and fold change greater than 1.5 between conditions were considered significantly differentiated. The pathway maps were constructed based on BioCyc data for *Streptococcus intermedius* strains B196 and JTH08.

Results and Discussion

S. intermedius metabolism is affected by aerobic or anaerobic growth conditions

The intracellular and extracellular metabolomes and the transcriptome were used to characterize and differentiate the responses of *S. intermedius* to anaerobic and aerobic growth conditions. Comprehensive multivariate OPLS-DA analyses were conducted on the 1885 intracellular and 3382 extracellular metabolic features of *S. intermedius*, which included polar metabolites and phospholipids. There were robust metabolomic differences between aerobic and anaerobic growth environments **(Fig. 2.1b-c)** with prediction statistic (Q^2) above 0.85 (Broadhurst and Kell 2006). The extracellular metabolomic profiles of *S. intermedius* grown in aerobic or anaerobic conditions were distinctly different from each other, and the THY medium control, implying distinct nutrient consumption and metabolite release under these growth conditions. Twenty-eight major metabolic pathways were found using MetaboAnalyst based on identified metabolites **(Fig. 2.2)**.

The presence of oxygen affected both the intracellular and extracellular metabolome of *S. intermedius*. Over 37.7% of the intracellular metabolite features (710/1885 metabolite features) were differentially produced when comparing aerobic and

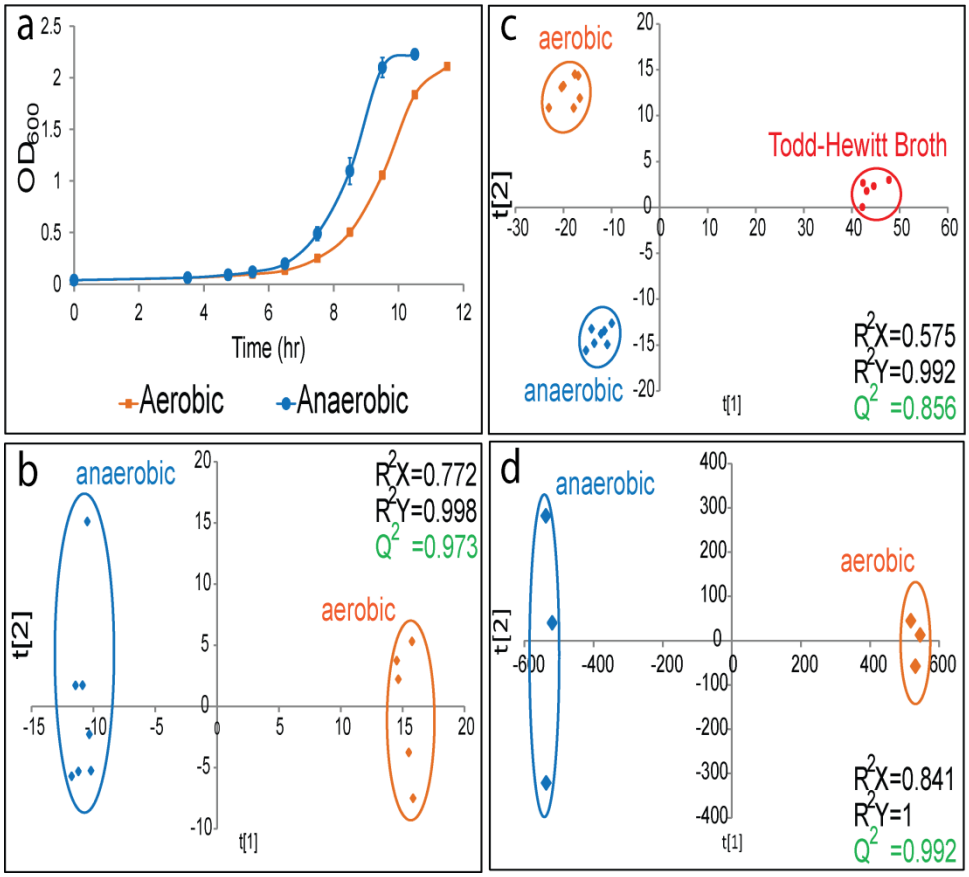

Fig. 2.1 (a) The growth curves of *S. intermedius* cultured in either aerobic (in orange) or anaerobic (in blue) environment. The doubling time in aerobic condition was 54 min and the doubling time in anaerobic condition was 41 min. The optical density of the cells was measured every hour in triplicate. OPLS-DA score plots summarizing **(b)** 1885 metabolite features found in the intracellular extracts between aerobically and anaerobically cultured *S. intermedius*, **(c)** 3382 metabolite features found in extracellular medium of aerobically and anaerobically cultured *S. intermedius* and Todd Hewitt growth media, and **(d)** gene expression differences including 1815 transcripts obtained from RNA-seq of *S. intermedius* grown in either aerobic or anaerobic conditions. The samples belonging to the same treatment were highlighted with circles.

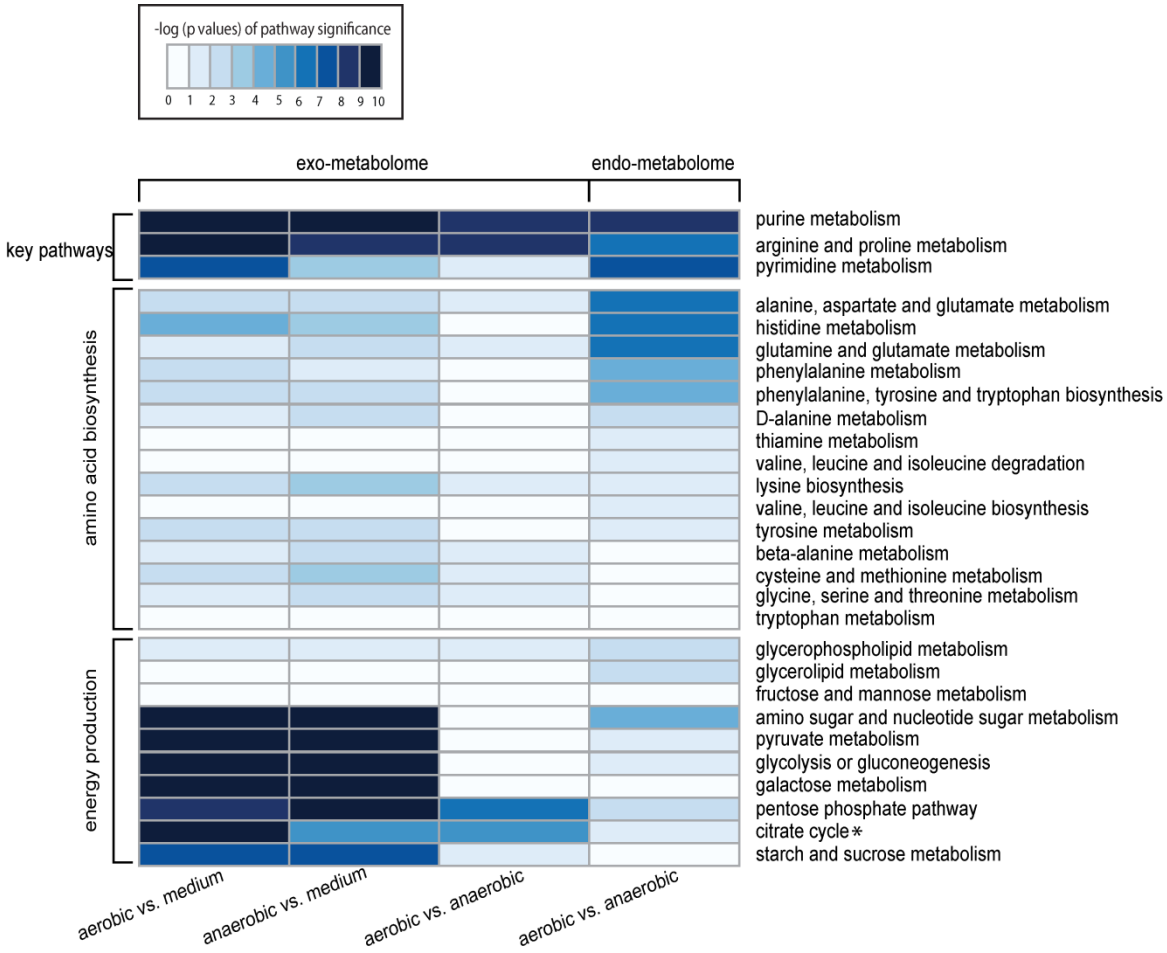

Fig. 2.2 A heatmap of 28 pathways and how they were affected by aerobic or anaerobic growth conditions**,** based on identified metabolites in the intracellular and extracellular metabolomes of *S. intermedius*. The p value of each pathway was computed by pathway analysis using MetaboAnalyst 2.0 using Gram-positive *Staphylococcus aureus* as the model organism. Rows were metabolite pathways; columns were comparisons between treatments including aerobic and anaerobic growth conditions and the Todd Hewitt media blank**.** Comparisons were based on either intracellular *S. intermedius* cell extracts of 79 identified metabolites (exclude phospholipids) or extracellular supernatant of 92 identified metabolites. The color key indicates the $-\log_{10}$ of p values for pathway significance (refer to the color scale). The energy production pathways were calculated mostly based on C5, C6 monosaccharide and disaccharide abundances. A list of identified metabolites used for the analyses was included in the supplementary material.**S. intermedius* does not have the tricarboxylic acid cycle (TCA); the TCA pathway shown here was based on *Staphylococcus aureus* metabolism.

anaerobic growth environments. Among these, 327 features were more abundant under anaerobic condition, and 383 features were less abundant (**Fig. 2.S1a**). Similarly, 38.6% (1307/3382 features) of the extracellular metabolite features were significantly different among aerobic, anaerobic supernatants and THY medium controls. Although the majority of changes occurred between THY and growth conditions, 3.4% (114/3382) of the features were differentially expressed between aerobic and anaerobic supernatants (28 increased and 86 decreased under anaerobic growth conditions (**Fig. 2.S1 b-d**).

The RNA-seq dataset of *S. intermedius* B196 captured the transcription of 1815 genes based on the current annotation (Olson et al. 2013). These genes were classified to 58 gene pathways. The transcription profiles under aerobic and anaerobic growth were distinct as shown in OPLS-DA score plot (**Fig. 2.1 d**). There were 625 genes that significantly affected with p values $\leq 10^{-4.5}$ (the Cuffdiff threshold cut-off) (Trapnell et al. 2012). Of these, 297 genes had greater than 2-fold changes in gene expression (**Fig. 2.S2**). **Figure 2.3** illustrates the percentage of genes in each pathway that were up-regulated or down-regulated with a change greater than 2-fold. The pathways can be divided into four groups based on the responses seen: (1) Aerobic Response only, where genes in pathway were up-regulated only in the presence of oxygen (2) Anaerobic Response only, where genes were up-regulated in the absence of oxygen, (3) Mixed Response, which includes a subset of genes up-regulated and a subset down-regulated under each condition, and (4) An unaffected group, where there was no oxygen dependent response. The data confirms that aerobic and anaerobic growth conditions can lead to global metabolic and transcriptional changes in *S. intermedius.*

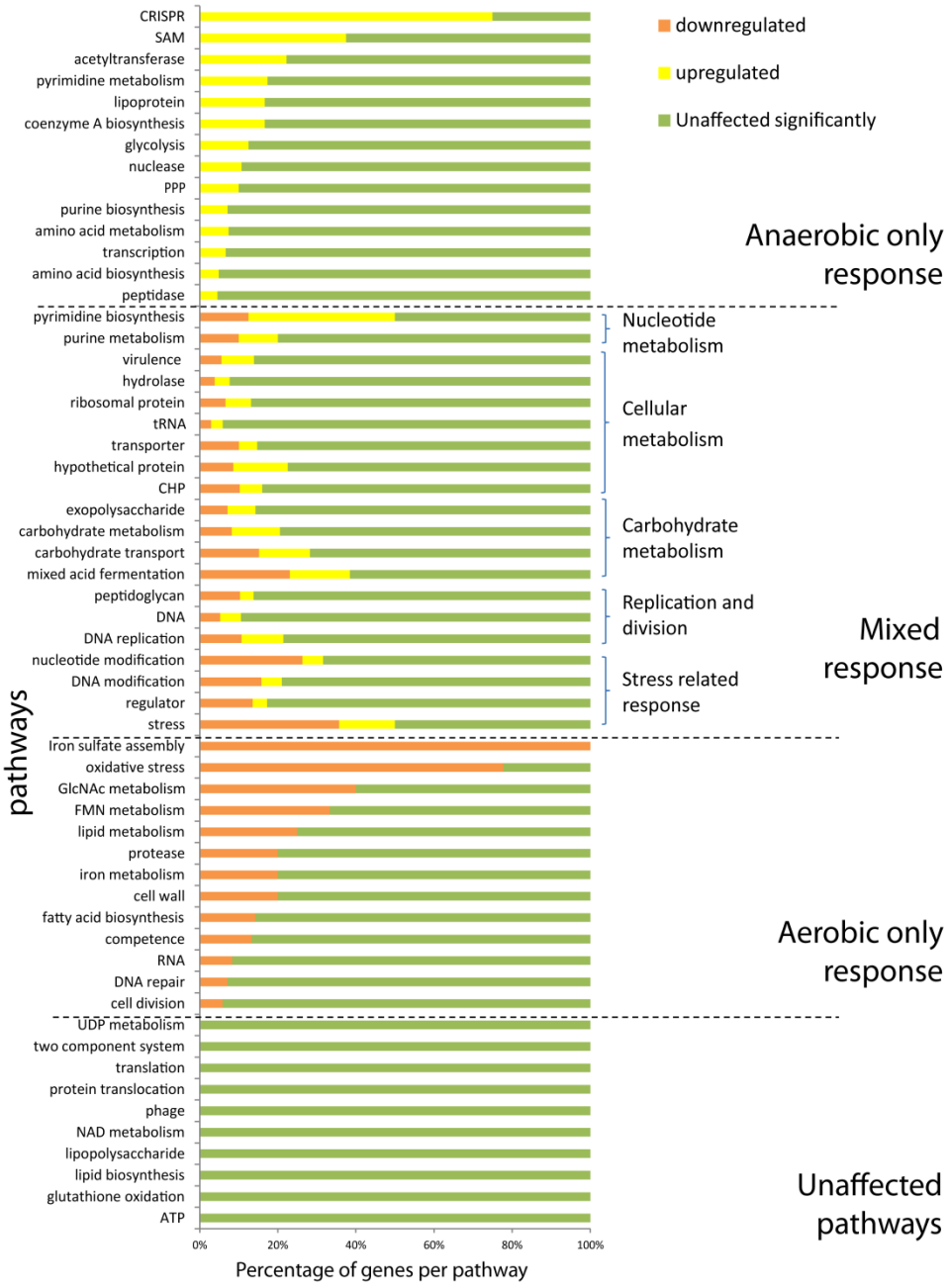

Fig. 2.3 The effect of the presence of oxygen on gene expression of *S. intermedius.* Anaerobic gene expression was compared to aerobic and visualized with down-regulated, upregulated and unaffected genes coloured in orange, yellow and green respectively. Genes were classified into pathways and assembled into 4 groups based on response, namely, anaerobic response, mixed response, aerobic response and unaffected pathways. CRISPR: clustered regularly interspaced short palindromic repeats; SAM: S-adenosyl methionine; PPP: Pentose phosphate pathway; CHP: conserved hypothetical protein; GlcNAc: N-acetylglucosamine; FMN: flavin mononucleotide; UDP: uridine diphosphate; NAD: nicotinamide adenine dinucleotide.

Adaptations affecting growth

S. intermedius exhibited differential growth kinetics in the presence and absence of oxygen. Under aerobic conditions, *S. intermedius* exhibited an extended lag phase and slower overall growth rate compared to anaerobic growth conditions (Fig. 2.1a, 2.S3). The doubling time during the logarithmic phase was 41 minutes under anaerobic conditions and 54 minutes under aerobic conditions based on CFUs (Fig. 2.S3). Consistent with the increased growth rate under anaerobic conditions, pathways associated with central carbon metabolism, the arginine deaminase pathway, pyrimidine and purine metabolism were found to be increased under anaerobic conditions using both transcriptomics and metabolomics.

Central carbon metabolism of S. intermedius was up-regulated during anaerobic growth

Many facultative anaerobes such as *E. coli*, can adapt to different oxygen environments by switching from aerobic to anaerobic respiration or fermentative metabolism under oxygen-deficient conditions (Trotter et al. 2011). In aerobic respiration, oxygen is used as the terminal electron acceptor in the electron transport chain, which generates the proton gradient across the cell membrane and allows ATP to be generated by the cell. Lactic acid bacteria, including SMG, lack heme, the main component of the cytochromes in the electron transport chain. To compensate, lactic acid bacteria generate NAD^+ and acidic byproducts (lactate, acetate and formate) via mixed acid fermentation (Crow and Pritchard 1977). This occurs under both aerobic and anaerobic conditions. The acidic byproducts are exported and generate a proton gradient across the cell membrane, allowing ATP synthesis. Here, the effect of oxygen on

glycolysis and mixed acid fermentation in *S. intermedius* were analyzed by transcriptomics and metabolomics (**Fig. 2.4**).

Genes associated with glycolysis including glucokinase (*glcK*, 2.83-fold) and fructose 1,6-bisphosphate aldolase (*fba*, 2.69-fold), were up-regulated under anaerobic conditions (**Fig. 2.4 a**). Additionally, expression of the glycogen biosynthesis operon (*glgABCD*) was also increased by more than 2-fold under anaerobic growth, implying that glucose utilization exceeds energy requirements and is therefore stored in the form of glycogen. There was also a decreased expression of genes involved in the synthesis of acetoin, namely acetolactate synthase (SIR_RS12085, 5.32-fold) and aldehyde dehydrogenase (*aldB*, 6.13-fold) as has been found in other lactic acid bacteria such as *Lactococcus lactis* (Bassit et al. 1993). Regulation of enzymatic activity by glycolytic intermediates and other metabolites could alter affect carbohydrate metabolism and contribute to the increased growth kinetics observed under anaerobic conditions. This will not be reflected in the transcriptional profile.

The presence of oxygen in the growth environment is known to affect genes associated with carbohydrate uptake in non-SMG streptococci (Ahn et al. 2007; Ahn et al. 2009). The utilization of C5 and C6 sugars (e.g. ribose, glucose, trehalose) from the extracellular medium was equivalent between growth conditions. However, eight genes involved in fructose, ascorbate, glucose, mannose and N-acetylgalactosamine transport were up-regulated under anaerobic conditions (**Fig. 2.S4**). Conversely, the expression of seven genes involved in the uptake of trehalose, lactose, starch and glycerol as well as six putative carbohydrate uptake genes were down-regulated during anaerobic growth. These

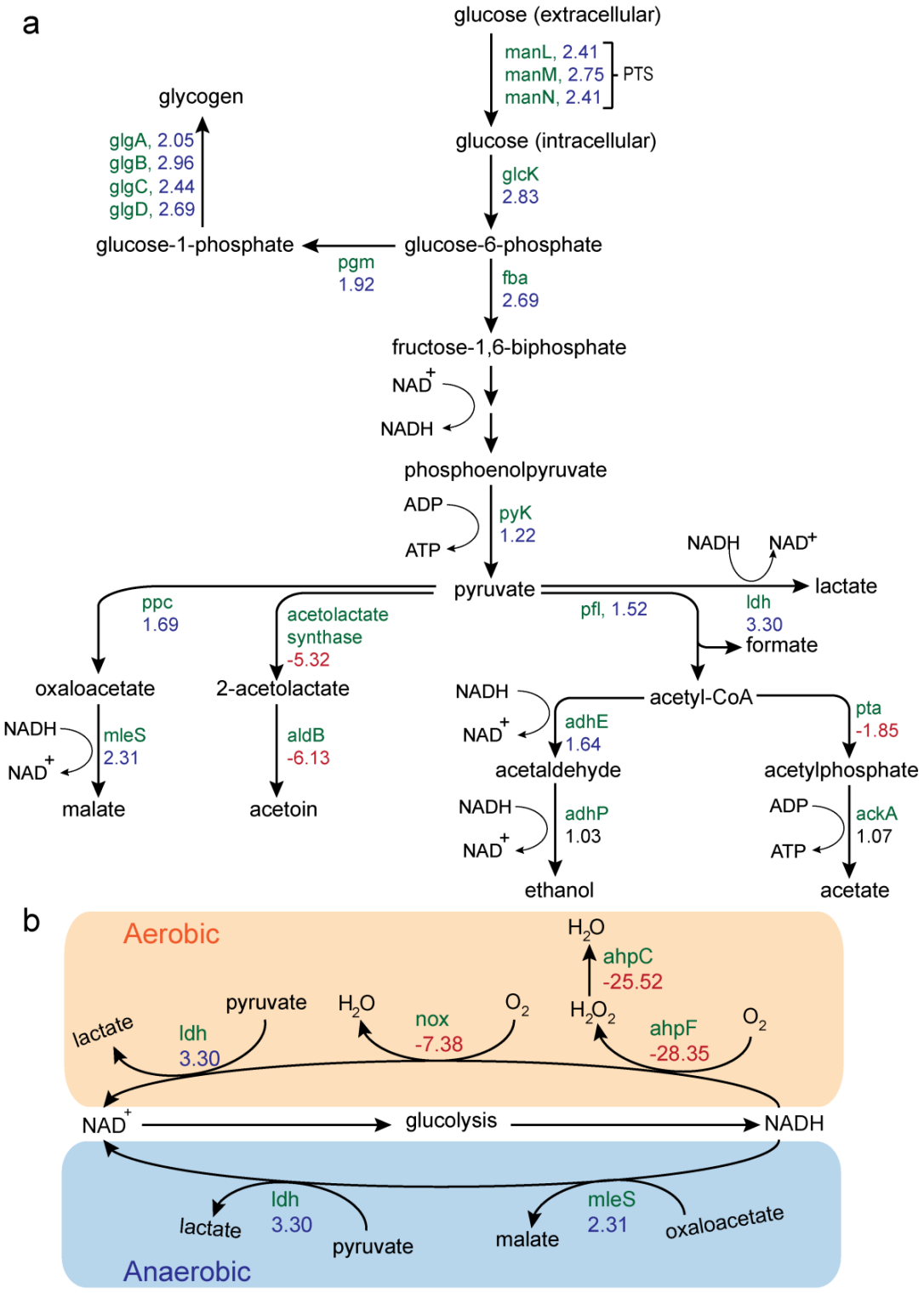

Fig. 2.4 Overview of **(a)** glycolysis and mixed acid fermentation pathways and **(b)** NAD^+/NADH cycling pathway of *S. intermedius*, which were affected by aerobic and anaerobic growth conditions. Genes that were up-regulated in anaerobic conditions were

indicated in blue; genes that were down-regulated in anaerobic condition were indicated in red; genes which expression was not statistically significant were indicated in black (Student's t test, $p>0.05$). PTS, phosphotransferase system; *manL*, putative phosphotransferase system, mannose-specific EIIAB; *manM*, PTS system, mannose-specific IIC component; *manN*, PTS system, mannose-specific IID component; *glcK*, glucokinase putative; *fba*, fructose biphosphatealdolase; *pyK*, pyruvate kinase; *pgm*, putative phosphoglucomutase/phosphomannomutase; *glgA*, glycogen synthase, ADP-glucose type; *glgB*, glycogen branching enzyme; *glgC*, glucose-1-phosphate adenylyltransferase; *glgD*, glucose-1-phosphate adenylyltransferase, *GlgD* subunit; *ldh*, L-lactate dehydrogenase; *pfl*, formate C-acetyltransferase; *pta*, phosphate acetyl/butaryltransferase; *ackA*, acetate kinase; *adhE*, bifunctional acetaldehyde-CoA/alcohol dehydrogenase; *adhP*, alcohol dehydrogenase; *aldB*, alpha-acetolactate decarboxylase; *ppc*, phosphoenolpyruvate carboxylase; *mleS*, malate dehydrogenase; *nox*, NADH oxidase; *ahpC*, alkyl hydroperoxide reductase subunit C; *ahpF*, alkyl hydroperoxide reductase subunit F.

data suggest that regulation of carbohydrate uptake is also a feature of *S. intermedius* metabolism when grown under different oxygen levels.

The NAD^+/NADH cycling pathway and carbohydrate metabolism are inextricably linked in *S. intermedius* (**Fig. 2.4 b**). Under anaerobic conditions, lactate dehydrogenase (*ldh*, 3.30-fold) and malate dehydrogenase (*mleS*, 2.31-fold) were up-regulated with glycolysis genes to allow regeneration of NAD^+ (**Fig. 2.4 b, 2.S4**). However, under aerobic conditions, *nox* and *ahpCF* are up-regulated while *ldh* and *mleS* are down-regulated, implying a change in the mechanism of NAD^+/NADH cycling. Along with a lower expression of glycolysis genes under aerobic conditions, these two features may contribute to slower growth.

Up-regulation of arginine deiminase pathway in an anaerobic growth environment could lead to increase in de novo synthesis of pyrimidine

In host-pathogen interactions, the ability of bacteria to compete for nutrients with host cells is essential for bacterial colonization and pathogenesis. Amino acids have been used as the primary carbon source by bacteria in rich media (Prub et al. 1994; Sezonov et al. 2007). However, we observed minimal net change in amino acid concentrations while comparing the growth conditions to the original THY medium aside from arginine (Fig. 2.S5). Thus oxygen had little impact on the metabolism of other amino acids.

Arginine is known to be required for optimal SMG growth (Rogers et al. 1987) and was consumed during *S. intermedius* growth in our experiments. The arginine deiminase (ADI) pathway is used for energy production and also feeds into *de novo* synthesis of pyrimidine via carbamoyl phosphate (Zúñiga et al. 2002; Gruening et al. 2006; Cusumano and Caparon 2015). There are four enzymes involved in the arginine deiminase (ADI) pathway: arginine deiminase (*arcA*), ornithine carbamoyltransferase (*arcB*), carbamate kinase (*arcC*) and arginine/ornithine antiporter (*arcD*) (Gupta et al. 2013) (Fig. 2.5). The production of ammonia and ATP from carbamoyl phosphate via ArcC provides energy and protection against acid stress (Marquis et al. 1987; Cotter and Hill 2003). The gene expression of *arcC* was similar under both growth conditions. Therefore, the energy generation or acid stress resistance provided by carbamoyl phosphate and ADI is comparable under both conditions. On the other hand, the expression of *arcA*, *arcB* and *arcD,* which lead to the synthesis of carbamoyl phosphate, were all increased significantly under anaerobic growth conditions. Additionally, the

intracellular concentrations of arginine and ornithine (the carbamoyl phosphate by-product) were also elevated in the anaerobic conditions. Overall, this implies that under anaerobic condition, there is a greater conversion of arginine to carbamoyl phosphate for *de novo* synthesis of pyrimidine via the ADI pathway. Though the overall consumption of arginine was slightly greater in aerobic conditions, the arginine was preferentially metabolized to citrulline and exported, which resulted a three-fold greater extracellular citrulline concentration. Thus, up-regulation of the ADI pathway under anaerobic conditions contributed to the increased production of carbamoyl phosphate and consequently, the up-regulation of pyrimidine *de novo* synthesis. Many pathogens use the consumption of arginine as a means to impair the host's ability to produce nitric oxide (Stadelmann et al. 2013; Cusumano et al. 2014) and this may be a strategy used by *S. intermedius* in infections.

Anaerobic growth conditions enhance pyrimidine and purine metabolism in S. intermedius

The intracellular concentrations of cytosine, cytidine, adenosine, adenine, guanosine, and uridine were all higher during anaerobic growth, while their extracellular abundances were much lower implying greater cellular uptake in comparison to the aerobic culture (**Fig. 2.S6, 2.S7**). In agreement with the metabolic data, the salvage and *de novo* nucleotide synthesis pathways were also both up-regulated anaerobically at the transcription level

The pyrimidine *de novo* synthesis pathway was elevated during anaerobic growth. This was indicated by the increase of intracellular levels of orotate, a pathway

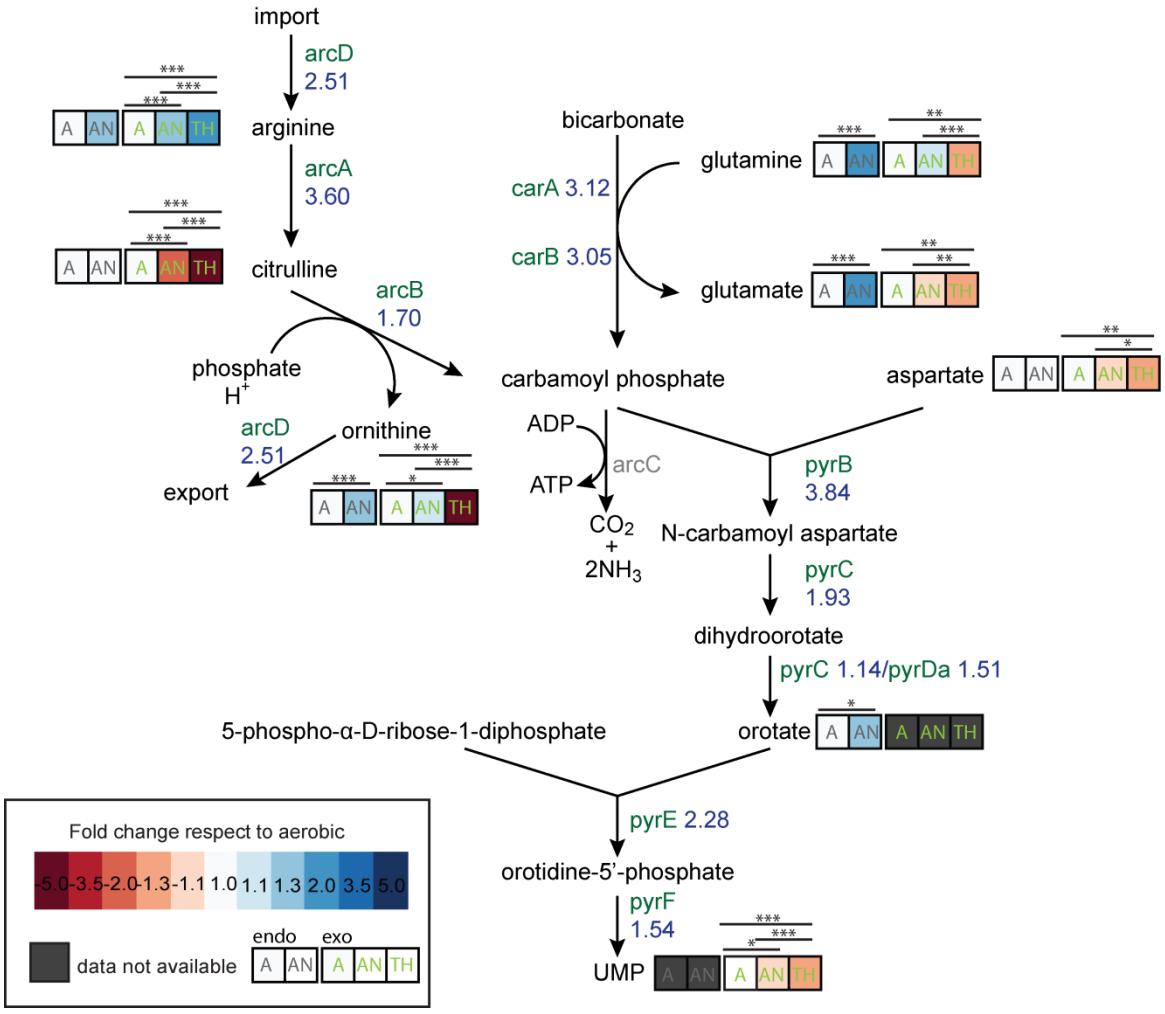

Fig. 2.5 The arginine deiminase and pyrimidine *de novo* synthesis pathways**.** Pathway was constructed based on the BioCyc database for *S. intermedius* B196 and JTH08. The metabolite names were written in black and the gene names were written in green. The fold changes of metabolite expression were indicated in color scaled boxes for *S. intermedius* grown in aerobic (A) and anaerobic (AN) conditions and the Todd Hewitt media blank (TH). The endo-metabolome was colored in grey and the exo-metabolome was light green, fold changes in gene expressions were indicated by numerical values. The undetected metabolites were indicated with a black filled box. The fold changes in metabolite or gene levels were calculated respective to aerobic growth conditions for either endo- or exo-extracts, where an increase was shown in blue and a decrease was shown in red. n=7 except for intracellular cell extract in aerobic conditions and Todd Hewitt media. *p<0.05, **p<0.005, ***p<0.0001.

intermediate, and the increased expression of pyrimidine synthesis genes (*pyrBCDEF*) (**Fig. 2.5, 2.S8**). Pathways for production of carbamoyl phosphate (ADI pathway), aspartate (aspartate aminotransferase), and bicarbonate (*carAB*), precursors for pyrimidine *de novo* synthesis, were all up-regulated anaerobically. Moreover, the purine metabolism pathways were also elevated anaerobically. Intracellular adenine level had shown great disparity according to the aerobic and anaerobic growth condition. Compared to aerobic condition, more than three-fold increase had been observed for intracellular adenine when *S. intermedius* was anaerobically cultured. Though the adenine level in the spent media was reduced in both growth conditions, a greater reduction was noted in the anaerobic growth. It may suggest greater influx of adenine under anaerobic growth. The genes involved in the inter-conversion between nucleosides and nucleotides were also up-regulated anaerobically to adjust to the high demand of intracellular metabolites (**Fig. 2.S6, 2.S7**). The enhanced expression of nucleoside and nucleotide metabolism genes may contribute to the increased growth rate of *S. intermedius* under anaerobic conditions.

Adaptation to oxidative stress

Protection against oxidative stress from both internally produced and exogenous reactive oxygen species is important for the streptococci (Higuchi et al. 2000; Jakubovics et al. 2002). Oxidative stress can cause damage to iron-sulfur cluster containing proteins as well as DNA (Imlay 2013). Genes involved in oxidative stress response were the most differentially expressed genes in our study. Under aerobic conditions, NADH oxidase (*nox*, 7.38-fold) was up-regulated in comparison to anaerobic conditions, as was the alkyl-hydroperoxidase system (*ahpCF*, 25.52- and 28.35-fold), the peroxide resistance

protein (*dps*; 5.74-fold) and superoxide dismutase (*sodA*; 4.96-fold). Dps removes free iron from the cell, preventing the generation of peroxides and SodA degrades superoxides while generating hydrogen peroxide which can then be reduced to water by *ahpCF* via NAD^+/NADH cycling pathway. These adaptations to oxidative stress have been observed in *S. mutans* (Higuchi 1984; Higuchi et al. 2000; Ahn et al. 2007).

Redox balance is important for cell homeostasis, and it is in part maintained through NAD^+/NADH cycling pathways, where NADH is oxidized to NAD^+ for glycolysis (**Fig. 2.4b**). We also observed that under aerobic conditions, genes involved in iron-sulfur cluster and iron metabolism were up-regulated in *S. intermedius* (**Fig. 2.S9**) as well as genes in several DNA-repair pathways including competence (uptake of extracellular DNA), RNA metabolism, and DNA modification and DNA repair enzymes.

Other

Oxygen had minor effects on expression of virulence genes

Despite the fact that the SMG is associated with anaerobic infections (e.g. abscesses), only a minority of genes associated with virulence are differentially regulated under oxygen varying growth conditions. Under anaerobic conditions, genes in oxidative stress pathway such as sialidase (*nanA*, 2.00-fold), pullulanase (*pulA2*, 2.17-fold), and a putative membrane toxin regulator (2.57-fold) were up-regulated (**Fig. 2.3**). *nanA* and *pulA2* are associated with binding to host surfaces in streptococci (Hytönen et al. 2006; Brittan et al. 2012). On the other hand, some potential virulence genes such as proteases (**Fig. 2.3**), the bacteriocin accessory protein (*bta*, 4.00-fold) and a metallobetalactamase family protein (SIR_RS10820, 2.11-fold) were down-regulated anaerobically. The

expression of CRISPR system (cas1, 2.53-fold; cas2, 2.01-fold; csn2, 2.70-fold) and nucleases (*rnc*, 2.01 fold; SIR_RS13205, 2.33 fold; *hsdR*, 2.93 fold) were increased anaerobically. These are involved in resistance to uptake of foreign genetic elements and phage infection (Marraffini and Sontheimer 2010; Midon et al. 2011; Sapranauskas et al. 2011; Derré-Bobillot et al. 2013). An association of the CRISPR system with oxidative stress tolerance has been previously seen in streptococci (Serbanescu et al. 2015). Overall, the expression of virulence associated genes was not strongly regulated by presence or absence of oxygen and these genes may be regulated by additional host specific signals.

Cellular membrane composition re-modelled to adapt to different environmental conditions

The major phospholipid (PL) class detected in *S. intermedius* was phosphatidyl glycerol (PG) and lyso-PG. There were 13 PGs and 2 lyso-PGs that varied significantly between aerobic and anaerobic growth conditions (**Fig. 2.6**). The cellular membrane of *S. intermedius* was largely composed of saturated short-chain PLs during aerobic growth. Consistent with the observed lipid profiles, the transcriptomic analysis found enoyl-CoA hydratase protein *fabM* (also referred to as *phaB*, 2.85-fold) and beta-ketoacyl-acyl carrier protein synthase III (*fabH*, 2.50-fold), associated with the synthesis of unsaturated and branched chain lipids respectively, were down-regulated under anaerobic conditions (Choi et al. 2000; Marrakchi et al. 2002; Fozo and Quivey 2004). It is unknown whether these metabolic and transcriptomic changes could affect the cellular membrane rigidity

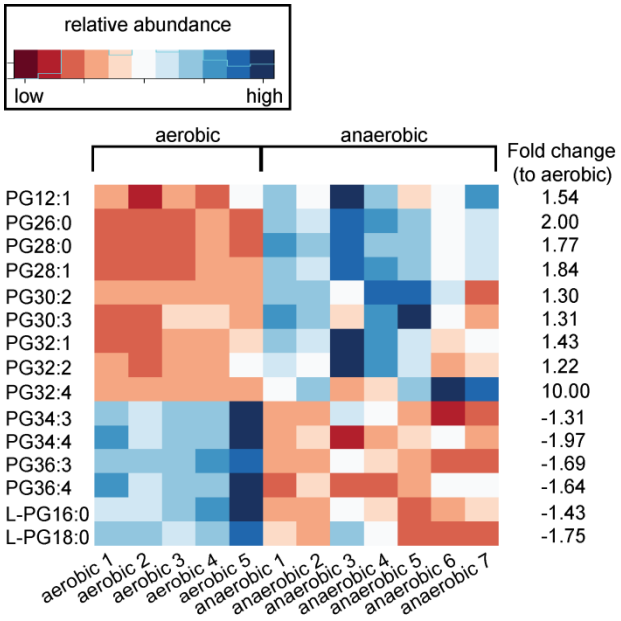

Fig. 2.6 The heatmap of 15 statistically significantly affected phospholipids ($p<0.05$) found in the intracellular metabolome of *S. intermedius* cultured in either aerobic or anaerobic conditions. The phospholipids were listed according to their alkyl chain length and saturation. The relative abundances of phospholipids were illustrated using a color scale, with blue indicating high abundances and red indicating low abundances.

and permeability in *S. intermedius*. Increased levels of unsaturated fatty acids has been reported in *E. faecalis* during aerobic growth (Portela et al. 2014).

Concluding Remarks

This study examined the global physiologic, metabolic and transcriptomic adaptations of *S. intermedius* grown in aerobic and anaerobic environments. Our study demonstrates that while *S. intermedius* is able to adapt to either condition, the anaerobic growth condition is favored with a 24% faster growth rate which also correlated with the up-regulation of the central carbon metabolism, the arginine deaminase pathway and the nucleotide *de novo* synthesis/salvage pathways. The largest transcriptional responses we observed were related to oxidative stress response under aerobic conditions. Overall, as a facultative anaerobe, *S. intermedius* is able to grow under varying oxygen tensions and may facilitate its colonization of distinct mucosal surfaces within the human host (upper respiratory, gastrointestinal and urogenital tracts). Moreover, its accelerated growth and adaption to anaerobic conditions may reflect its propensity for polymicrobial pyogenic infections with anaerobic bacteria. This adaptability allows *S. intermedius* to coexist in complex polymicrobial environments, both as a commensal and a pathogen.

Acknowledgement

This work is dedicated to the late Prof. Brian McCarry (1946-2013). FF was supported by an Ontario Graduate Scholarship. MGS and DMEB are supported by the CIHR and hold Canada Research Chairs. Work in the Bowdish laboratory is supported by the McMaster Immunology Research Centre (MIRC) and work in the Bowdish and Surette labs are supported by the M.G. DeGroote Institute for Infectious Disease Research

(IIDR). The authors would like to thank the Center for Microbial Chemical Biology (CMCB) at McMaster for access to the LC-MS.

Conflict of Interest Disclosure

All authors declared no conflict of interest.

Compliance with Ethical Standards

This study was funded by a grant from the Canadian Institutes of Health Research to DMEB and MGS (Grant # 108032). FF was supported by an Ontario Graduate Scholarship. MGS and DMEB are supported by Canada Research Chairs. This article does not contain any studies with human participants or animals performed by any of the author

Supplementary Material

ESM I

Growth Curve Measurement for *S. intermedius*

Optical density measurements

Samples were taken from the culture and used to measure the absorbance at 600 nm using cuvettes and blanking against the media. The Nanodrop 2000 (ThermoFisher Scientific, Waltham, USA) was used to measure the absorbance.

Colony forming units (CFU) measurements

Colony forming units were measured by taking a sample of the culture and serially diluting it before plating for colonies. A 1:10 serial dilution was used in volumes of 100

uL. A 5 uL volume of each dilution was plated and colonies counted to quantify the amount of bacteria.

RNA-seq Transcriptomics

RNA Purification

Three biological replicates were analyzed per condition tested (aerobic vs. anaerobic). The *S. intermedius* cell pellet was collected from samples with OD_{600} 0.7. Broth cultures were centrifuged and pellets were resuspended in 700 µL RNAprotect bacteria reagent (Qiagen, Venlo, Netherlands) with 100 µg/mL rifampicin and incubated for 10 minutes at room temperature before freezing at -80ºC. Frozen samples were defrosted at room temperature and centrifuged for 20 minutes at 4ºC. The cell pellet were resuspended in 700 µL RNase free water with lysozyme (10 µL of 100 mg/mL) and mutanolysin (5µL of 10 U/µL). The suspension was incubated at 37ºC for 45 minutes. Cells were then centrifuged and treated with 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA). The aqueous phase was collected and an equal volume of 70%v/v ethanol was added, and the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) to isolate DNA-free total cellular RNA. Ribosomal RNA (rRNA) was depleted using Ribo-Zero rRNA removal Kit for Bacteria (Epicentre, Madison, WI, USA) according to the manufacture's protocol. Briefly, resuspended magnetic beads were washed and prepared. A 15 µL volume of purified RNA was treated with 4 µL RiboZero reaction buffer, 10 µL of RiboZero RNA removal solution and 11 µL of RNase free water and incubated at 68ºC for ten minutes. The treated RNA was added to the magnetic beads and vortexed. It was then incubated at 50ºC for 7 minutes before placing on the magnetic stand to separate the beads from the

rRNA free supernatant. The RNA from the supernatant was purified using the Agencourt RNAClean XP Beads (Beckman Coulter, Brea, USA) as per directions. Agencourt RNAClean beads (180 μL) were added to the supernatant (85 μL). RNA was eluted from the beads with 32 μL of RNase free water. An Experion RNA StdSens chip was used to confirm depletion of rRNA. This was followed with a DNase digestion using TURBO DNase (Life Technologies, Carlsbad, USA). The reaction consisted of 30 μL of RNA, 1.5 μL of DNase and 3.5 μL of 10x Buffer. The reaction was incubated for 30 minutes at 37ºC. The RNA was purified again using the Agencourt RNAClean XP beads. To the DNase reaction (35 μL), 70 μL of magnetic beads were added and the final elution of RNA was done with 12 μL of RNase free water.

cDNA Synthesis from RNA

The RNA was then converted to cDNA using the Superscript III first strand cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). Random hexamers were used as primers for cDNA synthesis. Single strand cDNA was purified using the Agencourt RNAClean XP beads (2x) and eluted with 22 μL RNAse free water. Strand specific RNA sequencing was carried out (Parkhomchuk et al. 2009). Complementary second strand of cDNA incorporated uridine instead of thymidine. To synthesize this, the purified single stranded cDNA (22 μL) and 7.5 mM of each dNTP (dATP, dCTP, dGTP and dUTP) was treated with RnaseH and Klenow Fragment DNA polymerase (Invitrogen, Carlsbad, CA, USA) in a final volume of 40 μL at 16ºC for 2 hours. The double stranded cDNA was purified using the Agencourt AMPure XP beads (Beckman Coulter, Brea, USA) with 2x volume of beads added to the DNA.

cDNA Library Preparation

The cDNA was fragmented into ~ 300 bp lengths using the covaris S220 ultrasonicator with 175 W peak power, 10% duty factor, 200 cycles/burst for 430 seconds (Covaris, Woburn, Massachusetts, USA). Fragmented cDNA ends were repaired using the NEBNext End Repair Module (New England Biolabs, Ipswich, Massachusetts, USA) as per directions. The AMPure XP beads were used to purify cDNA by adding 1x volume of beads and eluting with 32 μL of RNAse free water. To facilitate ligation of adaptors, dA-tailing of the cDNA fragments was conducted using the NEBNext dA-tailing Module (New England Biolabs, Ipswich, Massachusetts, USA). AMPure DNA XP bead purification was again conducted with 1x beads and cDNA eluted with 25 μL RNAse free water. The NEBNext Adaptor Ligation Module (New England Biolabs, Ipswich, Massachusetts, USA) was next used as per directions and followed with adding USER enzyme to the reaction. The USER enzyme (New England Biolabs, Ipswich, Massachusetts, USA) generates gaps where uracil is found in the cDNA. This was done for the adaptor as well as the second strand of cDNA**,** which consisted of uracils**,** in order for the second strand of cDNA to be degraded. Agencourt AMPure bead purification was then conducted with 1x beads and cDNA eluted with 20 μL RNAse free water. The final preparation step consisted of a Phusion High fidelity PCR (Life Technologies, Carlsbad, USA) with the primer index (specific for each biological replicate and condition) and the universal primer for 8 cycles using the directions for the NEBNext Kit.

DNA Sequencing

The libraries were submitted to the McMaster Genomics Facility (McMaster University) for quality control and sequencing. QC included assessment of fragment size on the BioAnalyzer and qPCR quantification. The libraries were then pooled in equimolar amounts, denatured, and diluted to 12 pM; sequencing was performed using 40% of one lane on the HiSeq 1000 with 101 bp paired end reads according to standard Illumina protocols. Following sequencing, the libraries were converted to FastQ format using Illumina's Casava software (version 1.8.2, San Diego, California, USA). No index mismatches were allowed during demultiplexing. Approximately 20 million reads were obtained per condition for each biological replicate, of which between 16 to 17 million reads mapped back to the genome.

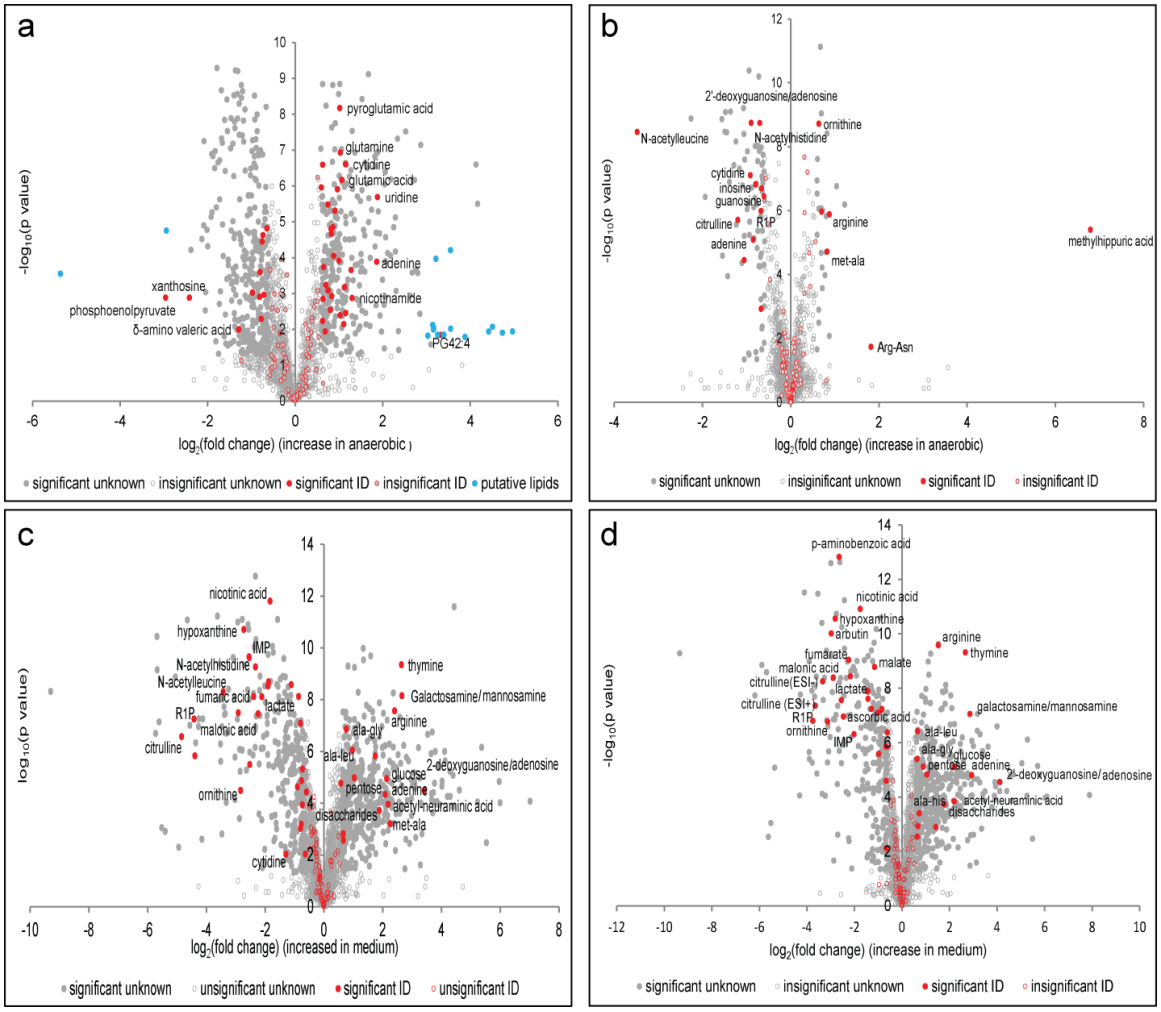

Fig. 2.S1 Global metabolomic differences between aerobically and anaerobically cultured *S. intermedius* shown by volcano plots, comparing **(a)** intracellular metabolome profiles of aerobic and anaerobic conditions, **(b)** extracellular metabolome profiles of aerobic and anaerobic conditions, **(c)** extracellular metabolome profiles of aerobic condition and Todd Hewitt media blank, and **(d)** extracellular metabolome profiles of anaerobic condition and Todd Hewitt media blank. Significant metabolite features with $p<0.05$, and greater than 1.5 fold changes were indicated with solid circles, others were labelled with open circles. Identified metabolite features were colored in red, putative lipids were indicated in blue, and the unknowns were colored in grey. Some known significant metabolite features were labelled with their chemical names. Some metabolites appear multiple times in one volcano plot due to detection in both ESI- and ESI+ modes or as adduct ions

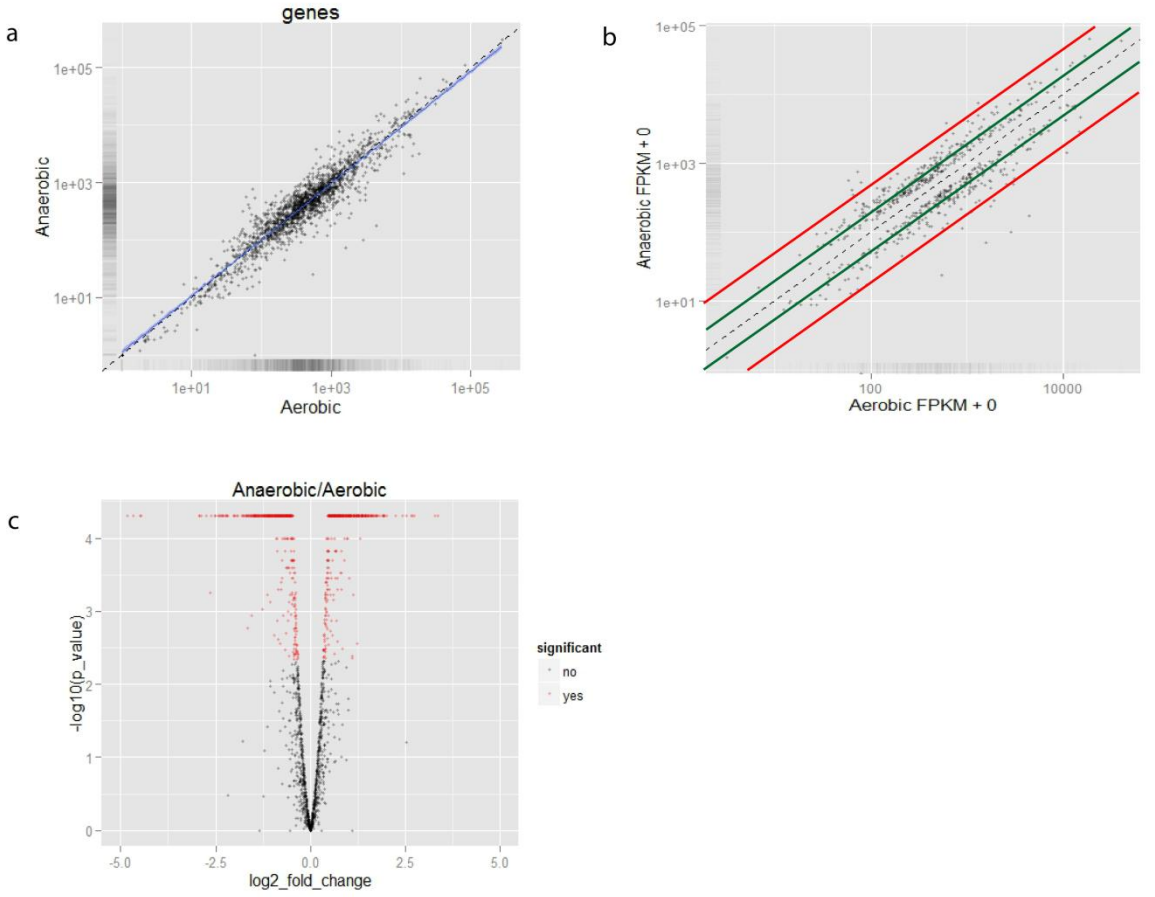

Fig. 2.S2 Overview of transcriptomic results for *S. intermedius* growth under aerobic and anaerobic conditions using CummeRbund. The scatter plot in **(a)** depicts the expression of all genes (1815 genes) under aerobic and anaerobic conditions. The theoretical correlation for equivalent expression under the two conditions (Blue) overlaps with the actual correlation. Scatter plot analysis of significant genes (625 genes) with p-value of $10^{-4.5}$ is shown in **(b)**. The lines correlating to 2 fold and 4 fold changes in gene expression are shown. There are few genes upregulated above 4 fold, with the majority of genes being upregulated under aerobic conditions. The volcano plot in **(c)** highlights statistically significant genes in red. Figures were generated using CummeRbund (Trapnell et al. 2012)

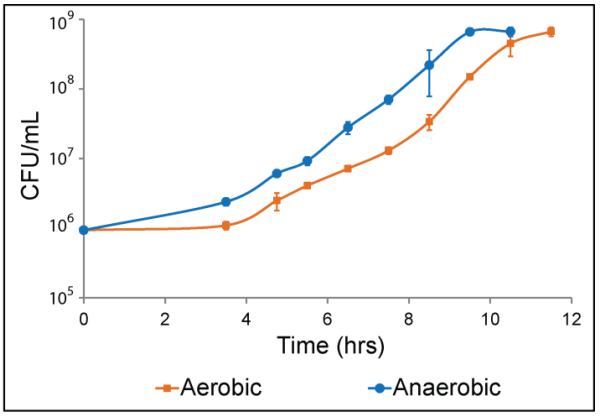

Fig. 2.S3 The growth curves (CFU/mL over time) of *S. intermedius* cultured in either aerobic (black) or anaerobic (blue) environment

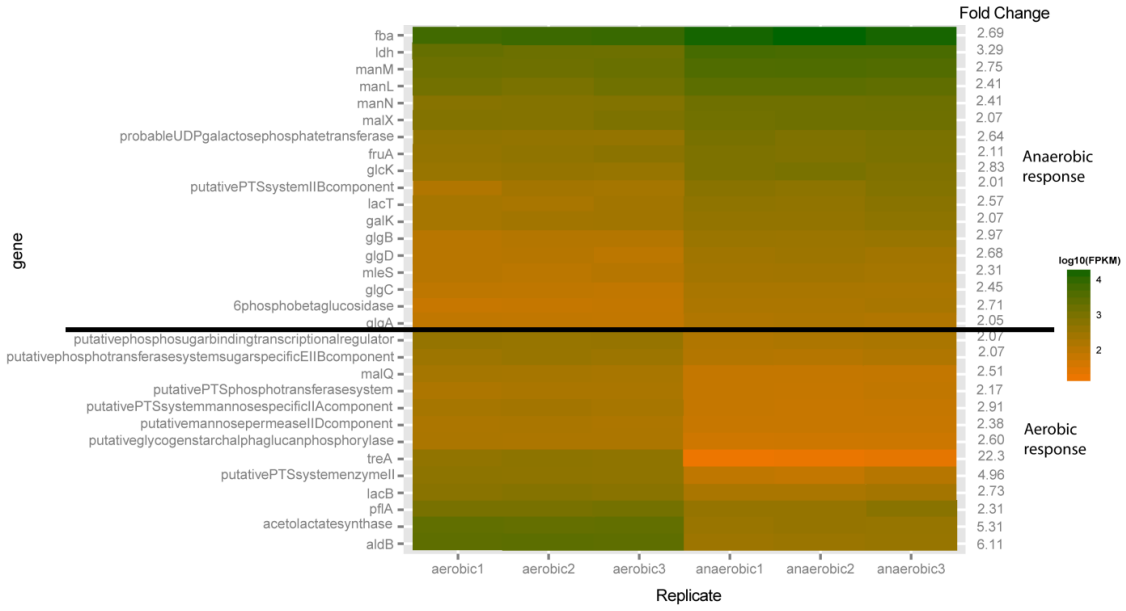

Fig. 2.S4 Variation in expression of genes involved in carbohydrate metabolism in the presence/absence of oxygen. Genes with statistically significant fold changes above 2 were classified either as an "Anaerobic response" or an "Aerobic response"**,** based on the condition wherein they are upregulated. Data was FPKM values generated from Cufflinks (Trapnell et al. 2012)

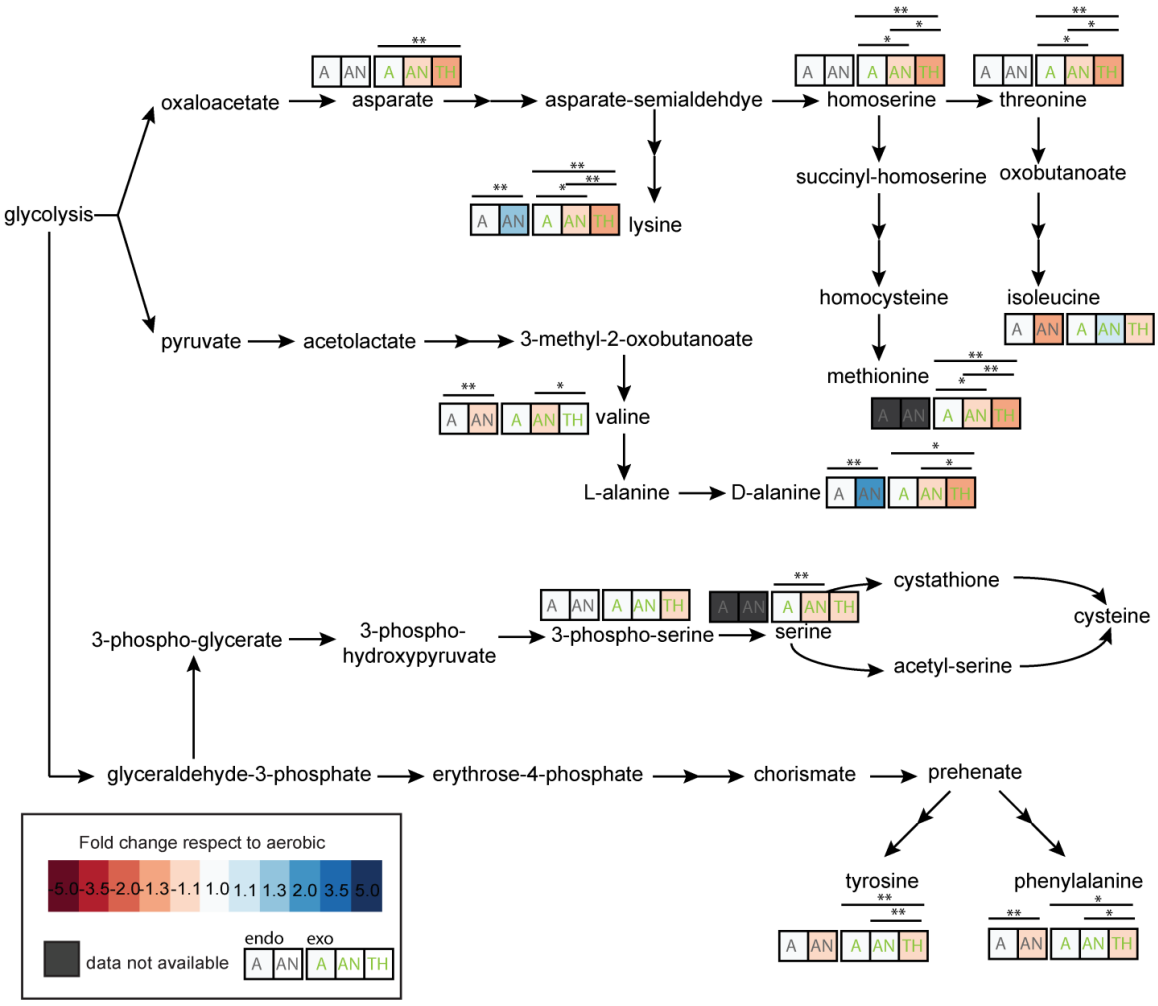

Fig. 2.S5 Amino acid metabolism of strain *S. intermedius* B196**,** constructed based on the BioCyc database for *S. intermedius* B196 and JTH08. The metabolite names were written in black and the gene names were written in green. The fold changes in metabolite expression were indicated in color scaled boxes for *S. intermedius* grown in aerobic (A) and anaerobic (AN) conditions and the Todd Hewitt medium control (THY). The intracellular metabolome was colored in grey and the extracellular metabolome was in light green; fold changes in gene expressions were indicated by numerical values. The undetected metabolites were indicated with a black filled box. The fold changes in metabolite or gene levels were calculated respective to aerobic growth conditions for either intra- or extra-cellular extracts, an increase was shown in blue and a decrease was shown in red. n=7 except for intracellular cell extract in aerobic conditions and Todd Hewitt media. *p<0.05, **p<0.005, ***p<0.0001

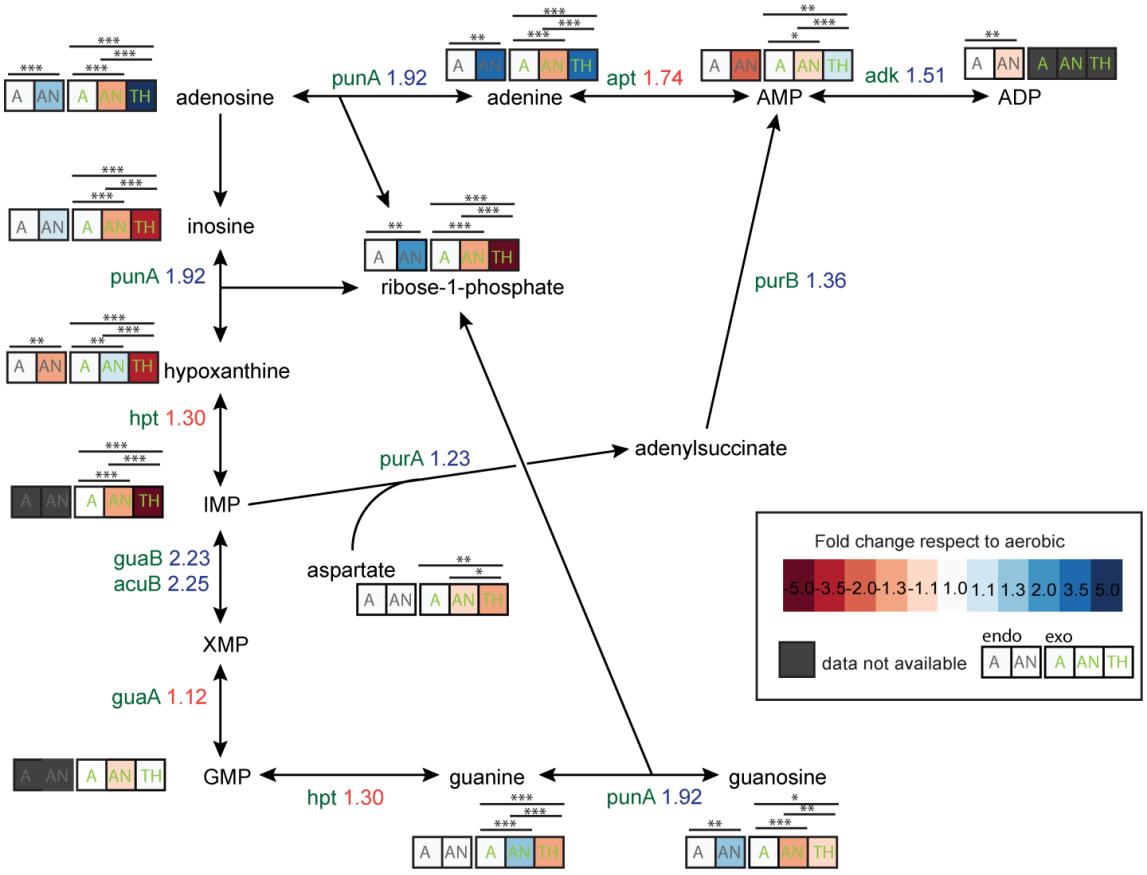

Fig. 2.S6 The purine metabolism pathway**,** constructed based on the BioCyc database for *S. intermedius* B196 and JTH08, as discussed in **Fig. 2.S5**

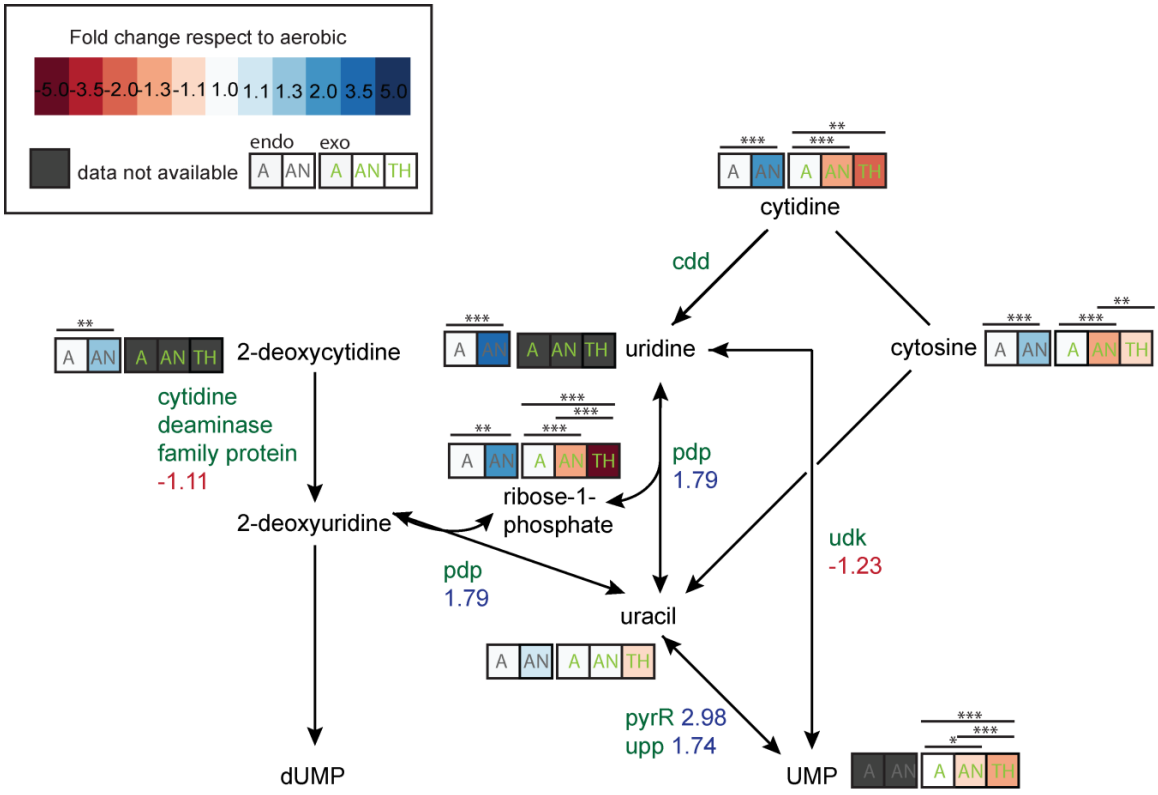

Fig. 2.S7 The pyrimidine salvage pathway**,** constructed based on the BioCyc database for *S. intermedius* B196 and JTH08, as discussed in **Fig. 2.S5**

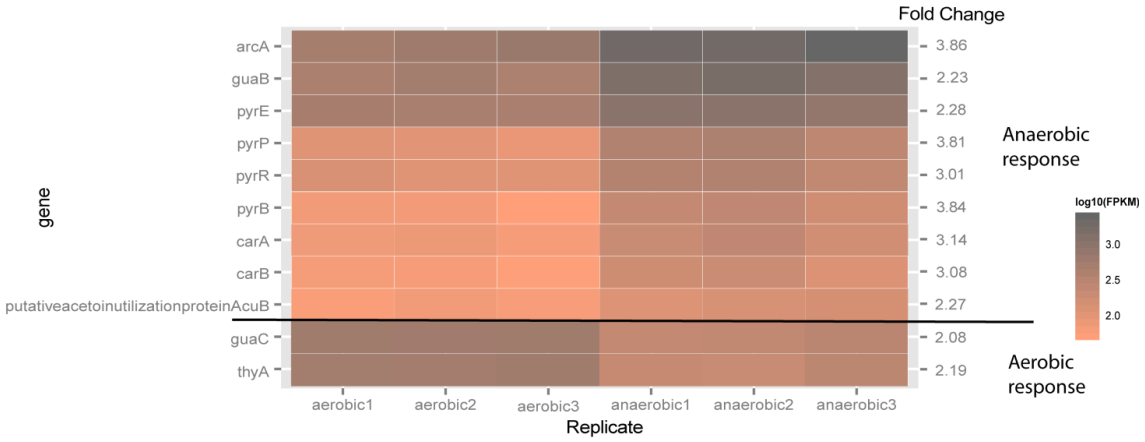

Fig. 2.S8 Genes involved in pyrimidine metabolism were affected by the presence/absence of oxygen. The response can be divided into "Anaerobic" or "Aerobic" based on the condition wherein they are up-regulated. Data was obtained using Cufflinks for RNAseq analysis (Trapnell et al. 2012).

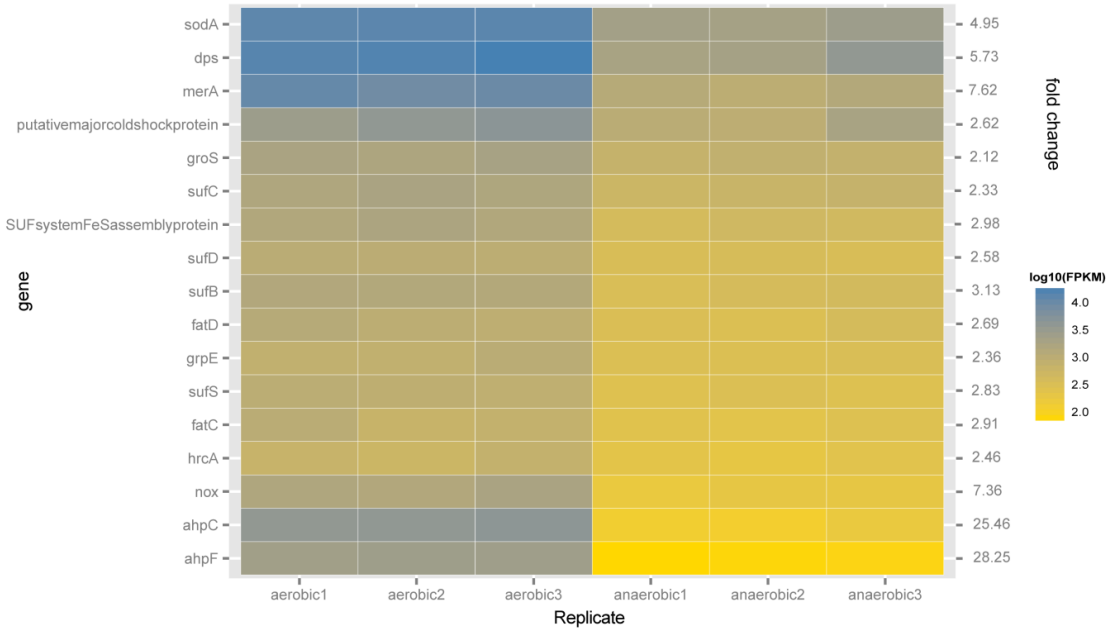

Fig. 2.S9 Induction of genes involved in oxidative stress under aerobic conditions. The heatmap includes genes that were induced aerobically above 2 fold**.** The up-regulated genes are involved in pathways including oxidative stress, iron metabolism and iron sulfur cluster assembly. The data is based on FPKM values generated from Cufflinks (Trapnell et al. 2012)

Table 2.S1 Summary of metabolomic and transcriptomic data

	Intracellular metabolome		Extracellular metabolome			Transcriptome	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Todd-Hewitt Broth	Aerobic	Anaerobic
Biological replicates	5	7	7	7	5	3	3
Percentage variance	22%	22%	14%	13%	14%	10.5%	13.7%
Number of metabolite/gene features	1885		3382			1815	
Percentage of recovery [a]	81-105%		104-105%			----	
Identified features	124		116			----	
Identified metabolites	105		93			----	

[a] determined based on tryptophan-d_5 in ESI positive mode

CHAPTER 3

STREPTOCOCCUS DEPENDENT SURVIVAL OF OBLIGATE ANAEROBE *PREVOTELLA MELANINOGENICA* IN THE PRESENCE OF OXYGEN

Streptococcus dependent survival of obligate anaerobe *Prevotella melaninogenica* in the presence of oxygen

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Preface: The research in this chapter was conducted from 2013 to 2016. The experiments were designed by Dr. Michael G. Surette and I. All experiments were conducted and analyzed by myself. I wrote the manuscript with help from Dr. Surette. The members of the Surette laboratory helped with the design and editing process.

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Abbreviated Title: Streptococci promote *Prevotella* survival

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ABSTRACT

Streptococci are common members of the human microbiome, many of which have both commensal and pathogenic lifestyles. Many streptococci have pathogenic associations with polymicrobial infections, which usually involve obligate anaerobes. We explored the ability of streptococci to promote the survival of obligate anaerobes in the presence of oxygen, focussing on the commensal *Streptococcus* Anginosus/Milleri group. Our analyses of eight *Streptococcus* species demonstrate that they can rapidly deplete oxygen to create an anaerobic environment *in vitro*. Some streptococci produce hydrogen peroxide as a by-product of oxygen depletion, inhibiting growth and/or killing obligate anaerobes. We also showed that strains that produce hydrogen peroxide, such as *S. pneumoniae*, inhibited growth of the anaerobe *Prevotella melaninogenica* under hypoxic conditions, while there was no inhibition anaerobically. However, in the presence of catalase, a hydrogen peroxide detoxifying enzyme, *P. melaninogenica* was not inhibited by *S. pneumoniae*. A knockout in the endogenous hydrogen peroxide detoxifying alkylhydroperoxidase (AhpCF) system in a non-hydrogen peroxide producer, *S. intermedius* B196, resulted in hydrogen peroxide detection in the mutant. The knockout decreased the viability of *Prevotella* in coculture under hypoxic conditions but complementation rescued growth. Our data provides an additional explanation for how streptococci can promote anaerobe colonization and infection in the presence of oxygen. It also emphasizes the role of AhpCF in scavenging endogenous hydrogen peroxide in the presence of oxygen, promoting anaerobe survival.

IMPORTANCE

Streptococci are often isolated along with obligate anaerobes from polymicrobial commensal and infection sites. Many factors contribute to anaerobe survival including oxygen exposure. Our study found that in environments where oxygen is present, most streptococci can create an anaerobic environment; however, they differ in their compatibility with obligate anaerobes. Alternatively, other streptococci such as *S. pneumoniae* deplete oxygen by producing H_2O_2 thus making an environment toxic to obligate anaerobes. The interactions taking place in polymicrobial infections are undoubtedly complex and dynamic. Our simplified system addresses just the effect of oxygen on such a community depending on its members. It increases our understanding of *Streptococcus* polymicrobial interactions and the requirements for building a polymicrobial community involving obligate anaerobes.

INTRODUCTION

The *Streptococcus* genus consists of a diverse group of species, including human commensals and pathogens. As commensals, they are common members of the human microbiome; colonizing the digestive tract, upper respiratory tract, urogenital tract and skin (Nobbs et al. 2009). Pathogenic streptococci are implicated in a range of diseases that vary by body location, symptoms and characteristics. They are associated with pyogenic infections such as abscesses and empyema, complex respiratory infections such as pneumonia, and other complex diseases (otitis media, heart disease, cancer, sepsis and necrotizing fasciitis) (Coman et al. 1995; Shiga et al. 2001; Van der Auwera 1985; Sasaki et al. 1998; Li et al. 2000; Narikiyo et al. 2004; Laupland et al. 2006; Peters et al. 2012; Walker et al. 2014; Elander et al. 2016).

Streptococci are facultative anaerobes that can grow in the presence of oxygen, but many grow better under anaerobic or hypoxic environments (Higuchi 1984; Fei et al. 2016). The effect of oxygen on a polymicrobial environment has been most studied in the oral cavity during colonization of the tooth enamel. Streptococci are suggested to be the initial colonizers in the oral cavity, allowing other bacteria to colonize and build a polymicrobial community (Kreth et al. 2009; Dewhirst et al. 2010; Ma et al. 2014). As the community matures, an oxygen gradient is created allowing the obligate anaerobes to colonize. One oxygen depletion strategy used by streptococci is the production of hydrogen peroxide (H_2O_2), which affects biofilm development through competition and increases extracellular DNA in the matrix (Kreth et al. 2009; Itzek et al. 2011). There are several ways streptococci can produce H_2O_2. The best studied mechanisms are pyruvate

oxidase (*spxB/pox*) and lactate oxidase (*lox/lctO*) (Pericone et al. 2003; Tong et al. 2007; Zheng et al. 2011b). An L-amino acid oxidase has also been identified, which is involved in the production of H_2O_2 from seven L-amino acids (Tong et al. 2008). These reactions require oxygen, thus little or no H_2O_2 is produced under anaerobic conditions (Jakubovics et al. 2002).

Aside from H_2O_2 producing oxidases, two NADH oxidases can be found in streptococci (Nox-1 and Nox-2) that are potentially involved in the depletion of oxygen. Nox-1 is encoded by *ahpF* and is part of the alkylhydroperoxidase system AhpCF (Poole et al. 2000b; Niimura et al. 2000). This system has low NADH oxidizing activity in *Streptococcus* strains tested but is critical in detoxification of H_2O_2. Nox-2 (*nox*) is critical for aerobic growth and has high NADH-oxidizing activity in streptococci (Higuchi et al. 1999). The role of the aforementioned oxidases in the depletion of oxygen has not been previously studied. Notably, streptococci differ from other facultative anaerobes as they do not undergo aerobic respiration using oxygen as the terminal electron acceptor. Thus, oxygen is not reduced to water at the end of the electron transport chain.

Many streptococci, including the *Streptococcus* Anginosus/Milleri Group (SMG), are typically isolated from polymicrobial infections along with anaerobes. The SMG includes three distinct but closely related species, *S. anginosus*, *S. intermedius* and *S. constellatus*. Polymicrobial SMG infections involving anaerobes include lower airway infections (Shinzato and Saito 1995; Parkins et al. 2008; Sibley et al. 2008; Filkins et al. 2012), pleural empyema (Hocken and Dussek 1985; Van der Auwera 1985; Wong et al.

1995) and abscesses (Gossling 1988; Shinzato and Saito 1994; Hirai et al. 2005). The isolation of obligate anaerobes from these infection sites suggests that there are established anaerobic microenvironments. It is currently unknown whether these local environments are generated by bacterial or host metabolism or a combination of both (Farber and Seltzer 1988; Sundqvist 1992; Campbell et al. 2014).

In this study, we examined the ability of commensal streptococci with a focus on the SMG group to deplete oxygen and support growth of an obligate anaerobe, *Prevotella melaninogenica*. While *Streptococcus* species vary in metabolism, synergistic/ competitive interactions and oxygen tolerance, the level of oxygen exposure and H_2O_2 production (or lack thereof) were found to be critical factors in their ability to support the viability of *Prevotella*.

METHODS

Bacterial strains

The bacterial strains used are listed in Table 3.1. Obligate anaerobe, *Prevotella melaninogenica* C1009, is a clinical strain that was isolated from the sputum of a cystic fibrosis patient. Nine *Streptococcus* strains (eight species) were used in this study. *S. intermedius* B196 is an invasive isolate from the hip abscess of a cystic fibrosis patient that was undergoing a pulmonary exacerbation. *S. pneumoniae* P1547 is a virulent, invasive strain. *S. anginosus* subsp. *anginosus* ATCC 33397 is a human throat isolate. *S. constellatus* subsp. *pharyngis* c1050 is an invasive isolate. *S. mutans* UA159 is a cariogenic dental pathogen and is available through the American Type Culture Collection (ATCC 700610). The *S. mitis* P2F12, *S. salivarius* P2B5, *S. mutans* P2A8, and

Table 3.1: Bacterial strains and plasmids used in analysis

Strain	**Isolation source**	**Reference**
P. melaninogenica C1009	Cystic fibrosis patient	N/A
S. intermedius B196	Hip abscess	(Parkins et al. 2008; Olson et al. 2013)
S. pneumoniae P1547	Blood	(Kim and Weiser 1998)
S. anginosus ATCC 33397	Throat	(Whiley and Beighton 1991)
S. constellatus subsp. *pharyngis* c1050	Invasive	(Olson et al. 2013)
S. mutans UA159	Dental carries	(Ajdić et al. 2002)
S. mitis P2F12	Asthma	N/A
S. oralis P2A8	Asthma	N/A
S. agalactiae P7G7	Asthma	N/A
S. salivarius P2B5	Asthma	N/A
Plasmids	**Antibiotic resistance marker**	**References**
pDL278	spectinomycin^R	(Dunny et al. 1991)
pUC19	Ampicilin^R	(Yanisch-Perron et al. 1985)
pBCSHM032	Tetracycline^R	(Henriques et al. 2013)

S. agalactiae P7G7 strains have been isolated in our lab from lower respiratory tract specimens and species determination was done by sequencing of the 16S rRNA genes.

Growth of Bacteria

P. melaninogenica C1009 colonies were grown on Kanamycin Vancomycin Laked Blood (KVLB) agar for 3 days in an anaerobe chamber (90% N_2, 5% H_2, 5% CO_2) at 37°C. Single colonies were used to inoculate Tryptic Soy broth with 0.5% Yeast Extract (TSY) and grown anaerobically at 37°C overnight. All streptococci except for *S. pneumoniae* were cultured in the anaerobe chamber by growing colonies on Todd Hewitt with 0.5% Yeast Extract (THY) agar for 2 days at 37°C and then using colonies to inoculate THY broth for overnight growth at 37°C. *S. pneumoniae* P1547 was grown in similar conditions on tryptic soy agar plates supplemented with 5% sheep blood and 10 µg/mL neomycin. *S. pneumoniae* broth cultures were grown similarly to other streptococci.

Oxygen Depletion

Broth cultures in a 96 well plate were used to determine if streptococci could deplete oxygen in a low oxygen diffusion environment. Oxygen levels were measured using 96 well Oxoplates (PreSens Precision Sensing GmbH), which have a fluorescent oxygen detector at the bottom of each well. Overnight cultures of streptococci with 3 biological replicates were diluted 12.5 fold in a final volume of 150 µL and overlaid with 100 µL of mineral oil. The plates were incubated (without shaking) in a Synergy plate reader overnight at 37°C with readings taken for growth (OD_{600nm}). We eliminated the shaking option as this would increase the system's oxygen exposure. Fluorescence was

measured for the indicator (excitation: 540 nm/ emission: 650 nm) and reference (excitation: 540 nm/ emission: 590 nm) dyes. The oxygen partial pressure (%) was calculated as per manufacturer's instructions.

Coculture experiments

Overnight cultures of *P. melaninogenica* and *Streptococcus* strains were used directly in coculture experiments and incubated in both the anaerobe chamber as well as the hypoxic chamber (0.2% O_2, 5% CO_2). Three or more biological replicates were used for all strains. In a 96 well sterile plate, 5 x 10^6 CFU/mL of streptococci and 1 x 10^6 CFU/mL of *P. melaninogenica* were incubated in 150 uL Brain Heart Infusion (BHI) broth. To determine the colony forming units for *P. melaninogenica*, 10 µL of coculture was serially diluted, plated on KVLB agar and incubated at 37°C in the anaerobe chamber. *S. pneumoniae* P1547 CFUs in cocultures were determined by plating on Tryptic Soy agar with 5% sheep blood and 10 µg/ml neomycin. Other streptococci were plated on McKay (Sibley et al. 2010) and THY agars to determine their colony forming units. For a more detailed analysis, *S. pneumoniae* P1547, *S. intermedius* B196 and *P. melaninogenica* were monitored hourly in a coculture for 6 hours and then at 24 hours.

Determination of hydrogen peroxide levels

Prussian Blue (PB) agar was used to detect H_2O_2 production on agar (Saito et al. 2007). A 2 µL volume of overnight broth culture of *Streptococcus* strains was spotted on PB plates and incubated under hypoxic, aerobic (5% CO_2, ambient O_2) and anaerobic conditions for 2 days. H_2O_2 was also detected in broth cultures using the Peroxidetect Kit (Sigma-Aldrich) as per instructions for aqueous solutions using the Synergy plate reader.

Knockout construction and complementation

Previous observations in our laboratory noted H_2O_2 production in an *S. intermedius* B196 transposon mutant with a mutation in the alkylhydroperoxidase system *ahpCF* (Margot Grinwis, personal communications). A deletion mutant of this system was constructed using gene replacement. The spectinomycin resistance marker from pDL278 along with its promoter were cloned into the *Bam*HI and *SalI* sites of pUC19 with primers SpecF and SpecR (Table 3.1, 2; Dunny et al. 1991). The upstream and downstream regions of *ahpCF* in *S. intermedius* were cloned on either side of the spectinomycin resistance marker in pUC19 using primers AhpCupF, AhpCupR, AhpFdownF and AhpFdownR (Table 3.2). The cassette (*ahpCF* upstream: *specR* : *ahpCF* downstream) was amplified using PCR with primers AhpCupF and AhpFdownR and purified. *S. intermedius* is naturally competent and can be transformed using the competence stimulating peptide, ComC (DSRIRMGFDFSKLFGK; Lacroix 2014). An overnight anaerobic THY broth culture was diluted 1000 fold in 500 µL THY and incubated for 2 hours in 5% CO_2 incubator before adding synthetic ComC peptide (10 ng) and 500 ng of purified cassette DNA. The reaction was incubated in normal growth conditions for an hour before plating the transformation reaction on THY + spectinomycin (75 µg/ml). Plates were incubated under anaerobic growth conditions. Colonies were screened using colony-PCR and sequencing of PCR products to verify the deletion. The plasmid pBCSMH032 was used as the complementation vector and was

Table 3.2: List of primers used

Primer Name	Primer sequence (5'-3')	Restriction enzyme
specF	AAAAAGGATCCGACGAAGAGGATGAAGAGG	*Bam*HI
specR	AAAAAGTCGACCCCAAATATTAAATAATAAAAC	*Sal*I
AhpCupF	AAAAAGCATGCGGTTGGACTGTTTATCTGTCTTT	*Sph*I
AhpCupR	AAAAAGTCGACGATAAATTTCCCGTTTCCAGC	*Sal*I
AhpFdownF	AAAAAGGATCCGCCTATAAACAAATCATTATTTCAATG	*Bam*HI
AhpFdownR	AAAAAGGTACCCCAAGTCCCTGTCTTTTTCTCTCAATC AGTCCAATTCC	*Kpn*I
ahpCFcompF	AAAAAGCATGCATGGTACTTATTTGTTAGCATC	*Sph*I
ahpCFcompR	TTTAGCGGCCGCATTTTCTTTATTATTGACGAATC	*Not*I

generously gifted by Dr. Sergio R. Filipe (Henriques et al. 2013). The plasmid replicates in *S. intermedius* B196 and has a tetracycline resistance marker. The plasmid was purified after an initial lysozyme (1 mg) and mutanolysin (50 U) culture pellet treatment for 30 minutes before using the Wizard Plus SV miniprep DNA purification system. The GFP gene in pBCSMH032 was replaced with the promoter and genes for *ahpCF* at the *Sph*I and *Not*I sites with primers AhpCFcompF and AhpCFcompR. The ligation was transformed into *S. intermedius ahpCF* knockout (*S. intermedius* Δ*ahpCF*) as described earlier and colonies selected on THY + spectinomycin (75 µg/ml) + tetracycline (1 µg/ml). Putative complemented strains were screened using PCR and sequencing. The complemented strain will be referred to as *S. intermedius* Δ*ahpCF* + *ahpCF*. Overnight cultures of *S. intermedius*, *S. intermedius* Δ*ahpCF* and *S. intermedius* Δ*ahpCF* + *ahpCF* were tested for H_2O_2 production on PB agar and in broth cultures. They were also used in coculture experiments with *P. melaninogenica*.

RESULTS

Streptococci deplete oxygen in a semi-permeable environment

Streptococci differ in their tolerance and consumption of oxygen (Thomas and Pera 1983; Higuchi 1984; Higuchi et al. 2000; Yamamoto et al. 2000; Henningham et al. 2015). To investigate oxygen depletion in streptococci, we chose eight species of streptococci namely, *S. agalactiae* P7G7, *S. anginosus* subsp. *anginosus* ATCC33397, *S. constellatus* subsp. *pharyngis* C1050, *S. intermedius* B196, *S. mitis* P2F12, *S. mutans* UA158, *S. mutans* P2A8, *S. pneumoniae* P1547 and *S. salivarius* P2B5 (**Table 3.1**). *S. pneumoniae* P1547 was included as a control as this species is known to produce large

amounts of H_2O_2 (Regev-Yochay et al. 2007). All of the tested strains are clinical isolates; the majority are from patients with chronic respiratory diseases. Cultures were grown in 96 well Oxoplates (PreSens Precision Sensing GmbH) to determine the amount of oxygen in the culture with an overlay of 100 µL of mineral oil. This volume of mineral oil was required to create a sufficient barrier to lower the diffusion of environmental oxygen into the media.

We demonstrated that all species of streptococci depleted oxygen and maintained an anaerobic environment with some notable differences (**Figure 3.1**). *S. anginosus* subsp. *anginosus* ATCC33397 and *S. agalactiae* P7G7 maintained an anaerobic environment for over 20 hours while *S. pneumoniae* P1547 and *S. salivarius* P2B5 maintained low O_2 for >10 hours. Other streptococci required longer to generate an anaerobic environment (eg. *S. constellatus* subsp. *pharyngis* C1050) or maintained the anaerobic environment for less time (*S. mitis* P2F12). After the anaerobic period, a gradual increase in oxygen levels was observed in most strains, which coincided with the onset of stationary phase as shown in the respective growth curves (**Figure 3.1**). An exception to this trend was *S. mutans*. Two *S. mutans* strains maintained 50% partial oxygen pressure well into stationary phase. These observed differences between *Streptococcus* species are likely multifactorial; possibly due to differences in growth rate, viability in stationary phase and differences in the presence and regulation of oxidases (Yu et al. 2001; Zheng et al. 2011a; Baker et al. 2014; Henningham et al. 2015).

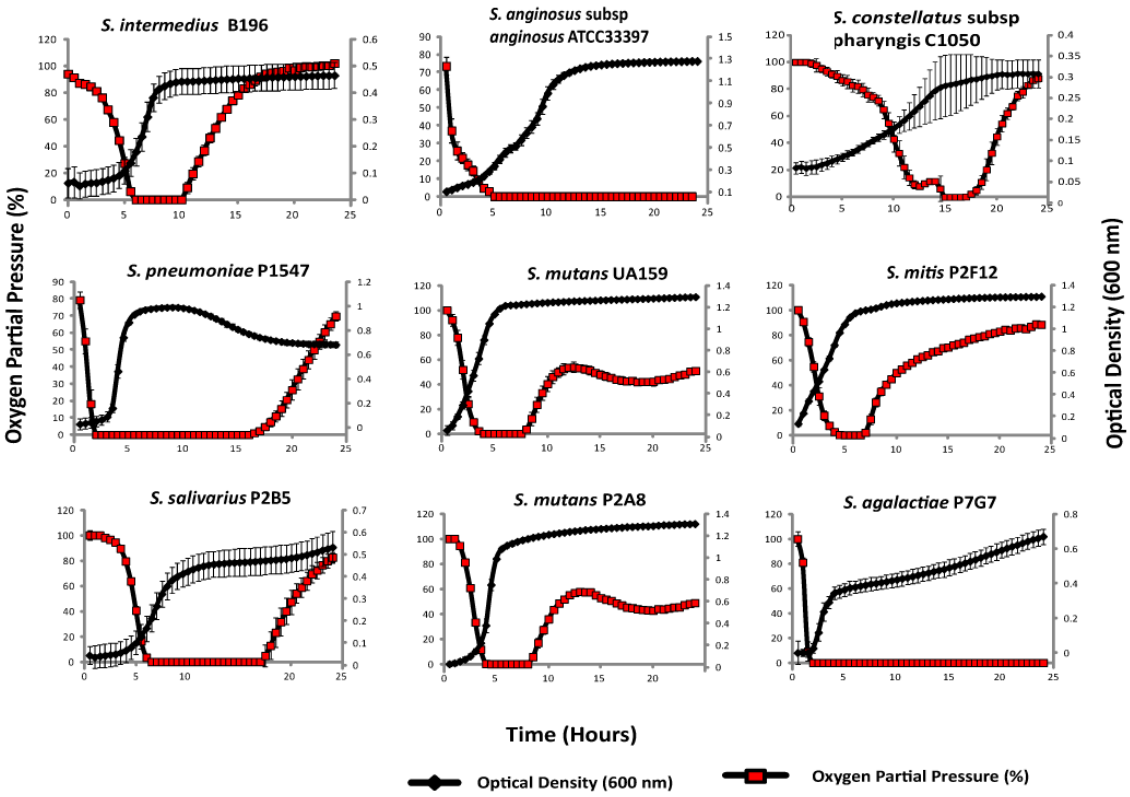

Figure 3.1: Streptococci deplete oxygen and create an anaerobic environment. Streptococci were monitored for their ability to deplete oxygen in an aerobic environment using an oxygen sensor built into Oxoplates (PreSens Precision Sensing GmbH). The partial pressure of oxygen (red) was determined using manufacturer instructions and monitored during growth measured by optical density (black). Error bars represent standard deviation between three biological replicates used for each strain.

Ability of streptococci to support *Prevotella melaninogenica* survival in low oxygen conditions

Given the ability of streptococci to deplete oxygen from low oxygen environments, we next sought to determine if they would allow survival of an obligate anaerobe, *Prevotella melaninogenica*, under these conditions. We expected that H_2O_2 production (from *S. pneumoniae* for example) would inhibit growth of *Prevotella*, so we compared growth of *Prevotella* in co-culture with streptococci in both anaerobic and hypoxic conditions (**Figure 3.2**). We also tested the streptococci for H_2O_2 production on Prussian Blue indicator plates (**Figure 3.2**).

Based on our observations of H_2O_2 production and growth with *Prevotella*, the strains were divided into 3 groups. The first group included strains which produced H_2O_2, potentially as the only inhibitor against *Prevotella*, and inhibited *Prevotella* under hypoxic but not anaerobic conditions. *S. pneumoniae* P1547, *S. salivarius* P2B5 and *S. anginosus* subsp. *anginosus* ATCC33397 were grouped as H_2O_2 producers. The higher *Prevotella* viability in coculture with *S. anginosus* subsp. *anginosus* ATCC33397 and *S. salivarius* P2B5 in comparison to *S. pneumoniae* P1547 may be due to decreased H_2O_2 production. Coculture with *S. anginosus* subsp. *anginosus* ATCC33397, in particular, had only modest impact on *Prevotella* growth under hypoxic conditions correlating with the lowest level of H_2O_2 production. The second group included non-H_2O_2 producers. These strains maintained viability of *Prevotella* under both hypoxic and anaerobic conditions. The non-H_2O_2 producers included *S. intermedius* B196, *S. constellatus* subsp. *pharyngis* c1050, *S. agalactiae* P7G7 and *S. mitis* P2F12 (**Figure 3.2**). Two *S. mutans*

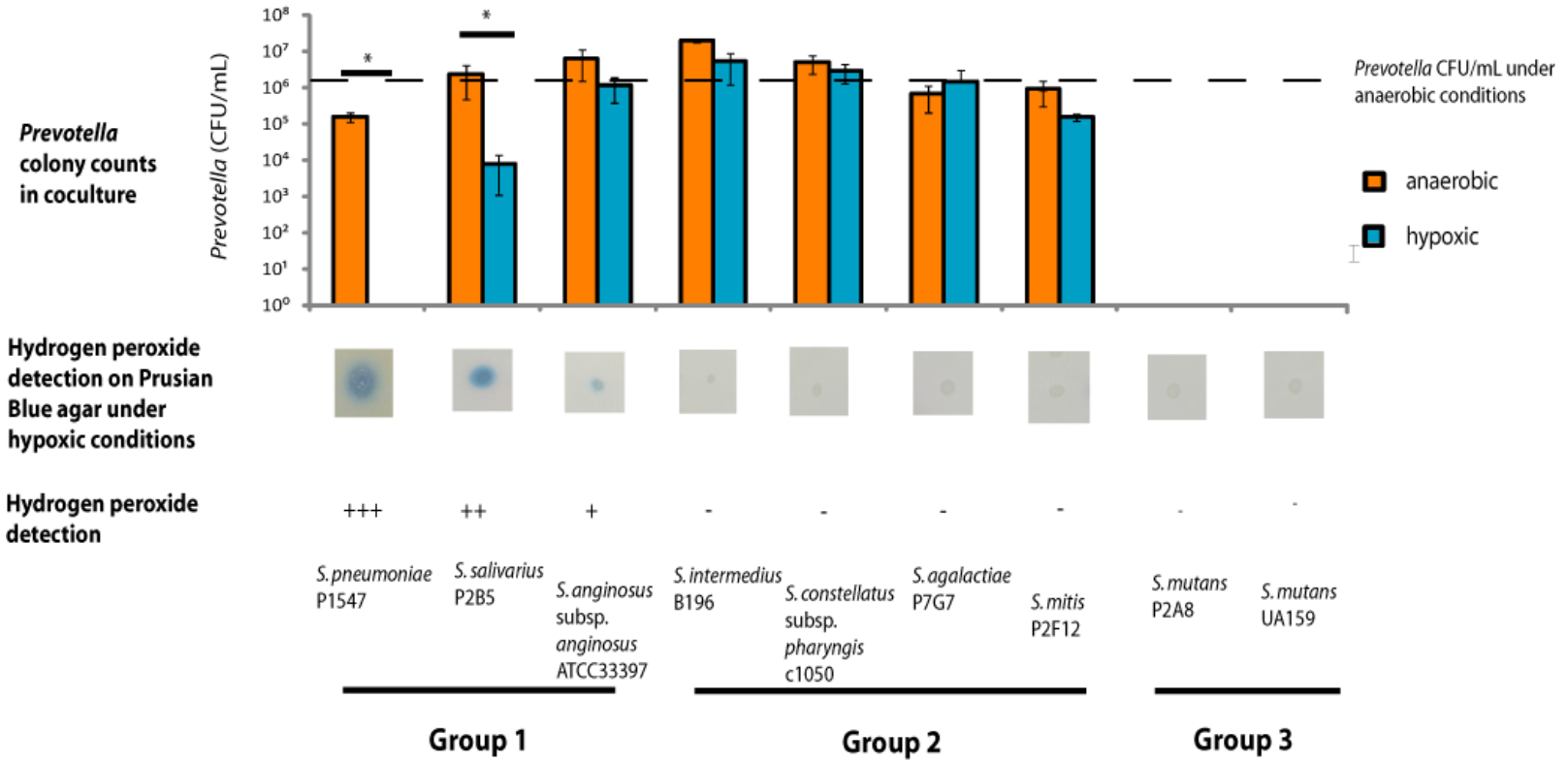

Figure 3.2: *Prevotella melaninogenica* viability is affected by the production of hydrogen peroxide and other inhibitors in *Streptococcus* cocultures under hypoxic conditions. *Prevotella* viability is shown in colony forming units/ml (CFU/ml) in coculture with streptococci (labelled at the bottom) under anaerobic and hypoxic conditions. *Streptococcus* strains were analyzed for hydrogen peroxide production using Prussian Blue agar under hypoxic conditions, with the blue zone indicating hydrogen peroxide production. Cocultures were incubated for 16 hours. *Prevotella* viability was significantly different under anaerobic and hypoxic conditions in *S. salivarius* ($p<0.05$) and *S. pneumoniae* ($p<0.005$) cocultures using the Student's t-test. Error bars represent standard deviation for three biological replicates used for each coculture.

strains inhibited *Prevotella* independent of oxygen presence (Group 3; *S. mutans* P2A8 and *S. mutans* UA159). The observed inhibition with these strains implicated a mechanism of inhibition other than H_2O_2.

To test whether H_2O_2 was the cause of *Prevotella* inhibition by the Group 1 H_2O_2 producers, we examined *Prevotella* survival in the presence of *S. pneumoniae* P1547 with or without catalase and used *S. intermedius* B196 as a control (**Figure 3.3**). When *Prevotella* is incubated alone under hypoxic conditions, a gradual decrease in its viability is observed over time, confirming its strict anaerobic classification (**Figure 3.3**). In a coculture with *S. pneumoniae* P1547, a rapid loss in *Prevotella* viability is observed (**Figure 3.3B**). This is prevented by adding catalase, which depletes H_2O_2 (**Figure 3.3A and B**). This confirms that H_2O_2 production by streptococci is responsible for the decrease in *Prevotella* in coculture. *Prevotella* viability is maintained in a coculture with the non-H_2O_2 producer *S. intermedius* B196, demonstrating the growth inhibitory effect of H_2O_2 on *Prevotella*.

The alkylhydroperoxidase system AhpCF detoxification of H_2O_2 promotes anaerobe viability

The alkylhydroperoxidase system (AhpCF) is used by streptococci to detoxify H_2O_2 (Higuchi et al. 1999; Poole et al. 2000a; Niimura et al. 2000). AhpF is also referred to as Nox-1, an NADH oxidase that was shown to have low NADH oxidase activity in streptococci (Higuchi et al. 1999). Our laboratory has previously observed that H_2O_2 was detected in an *S. intermedius* AhpCF transposon mutant, based on the Prussian Blue indicator agar test (Margot Grinwis, personal communication). This mutation

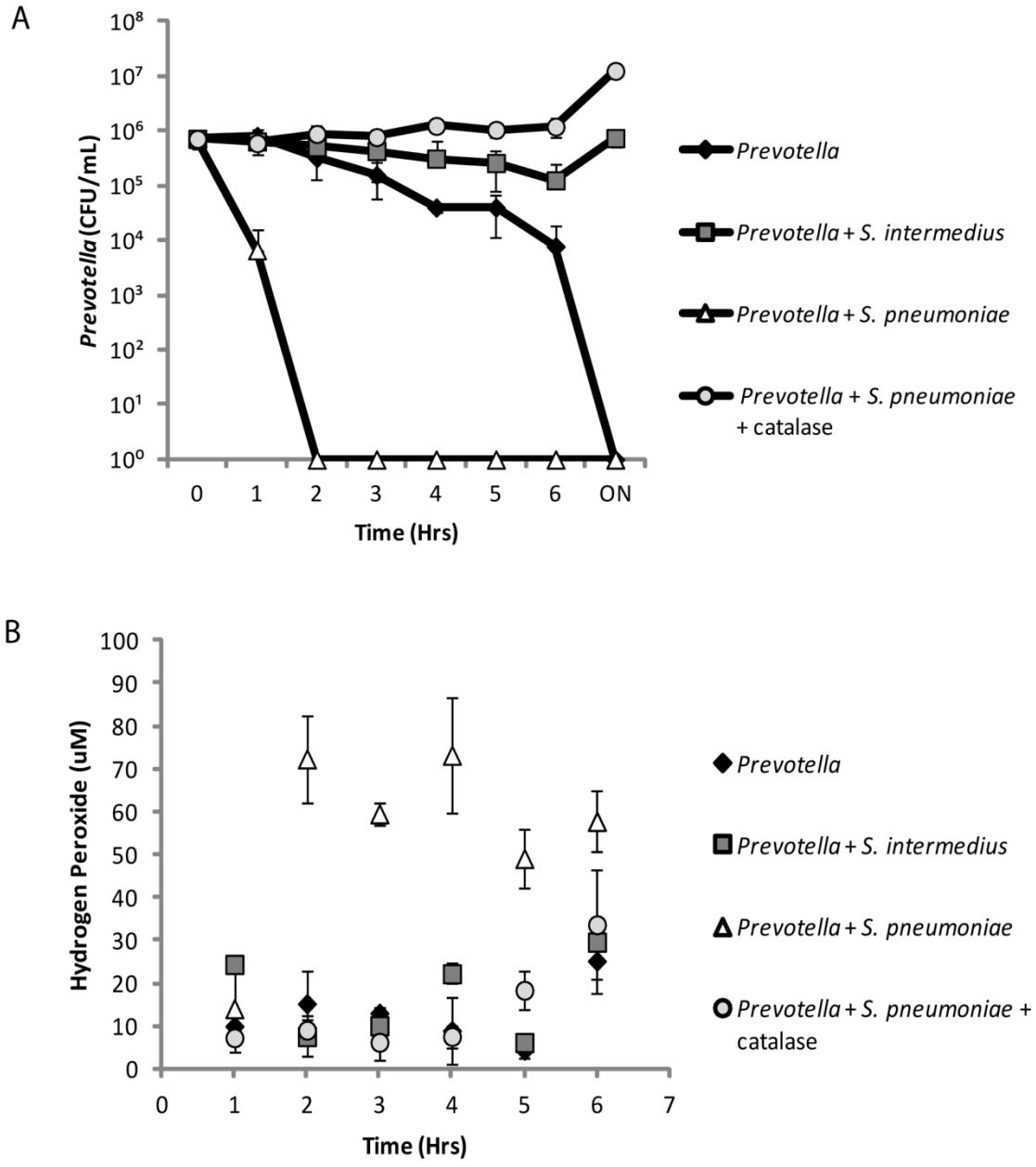

Figure 3.3: Catalase dependent detoxification of hydrogen peroxide produced by *Streptococcus pneumoniae* P1547 increases survival of *Prevotella melaninogenica* under hypoxic conditions. In A, the colony forming units (CFU/mL) of *P. melaninogenica* is shown in coculture with *S. intermedius*, *S. pneumoniae* and *S. pneumoniae* with catalase. The levels of hydrogen peroxide in the cocultures are shown in B. Hydrogen peroxide concentrations were determined using the Peroxidetect kit (Sigma-Aldrich). Error bars represent the standard deviation for the three biological replicates used for each sample group.

converted *S. intermedius* from a non-H_2O_2 producer to an H_2O_2-producer, though the source of H_2O_2 is unknown. An AhpC mutant in a catalase deficient *Escherichia coli* also accumulated H_2O_2 (Seaver and Imlay 2001). The authors argued that the H_2O_2 detected was a byproduct of aerobic metabolism that is usually scavenged by the alkylhydroperoxidase system.

To investigate the role of AhpCF in *S. intermedius* to promote *Prevotella* viability under hypoxic conditions, we created a knockout of AhpCF in *S. intermedius* B196 and complemented the knockout, herein referred to as *S. intermedius* Δ*ahpCF* and *S. intermedius* Δ*ahpCF* + *ahpCF* respectively. We hypothesized that detectable H_2O_2 in *S. intermedius* Δ*ahpCF* would decrease *Prevotella* viability, as was seen for *S. pneumoniae* coculture. Also, as catalase rescued *Prevotella* viability in an *S. pneumoniae* coculture, AhpCF complementation will rescue *Prevotella* viability in an *S. intermedius* Δ*ahpCF*+*ahpCF* coculture.

AhpCF is putatively involved in both H_2O_2 detoxification and oxygen depletion (Higuchi et al. 1999; Poole et al. 2000b; Niimura et al. 2000). Like *S. pneumoniae*, H_2O_2 in *S. intermedius* Δ*ahpCF* culture reduced its own viability if left for prolonged growth in the presence of oxygen. We characterized our strains for both H_2O_2 detoxification and oxygen depletion. First, we looked at the production of H_2O_2 by *S. intermedius* B196, *S. intermedius* Δ*ahpCF* and *S. intermedius* Δ*ahpCF* + *ahpCF* (**Figure 3.4A and B**). The levels detected in *S. intermedius* Δ*ahpCF* broth culture were lower than H_2O_2 levels known to be produced by *S. pneumoniae* (42.7 µM vs. 1 mM<, (Pericone et al. 2003); **Figure 3.4B)** These levels, though lower, would be sufficient to inhibit *Prevotella* based

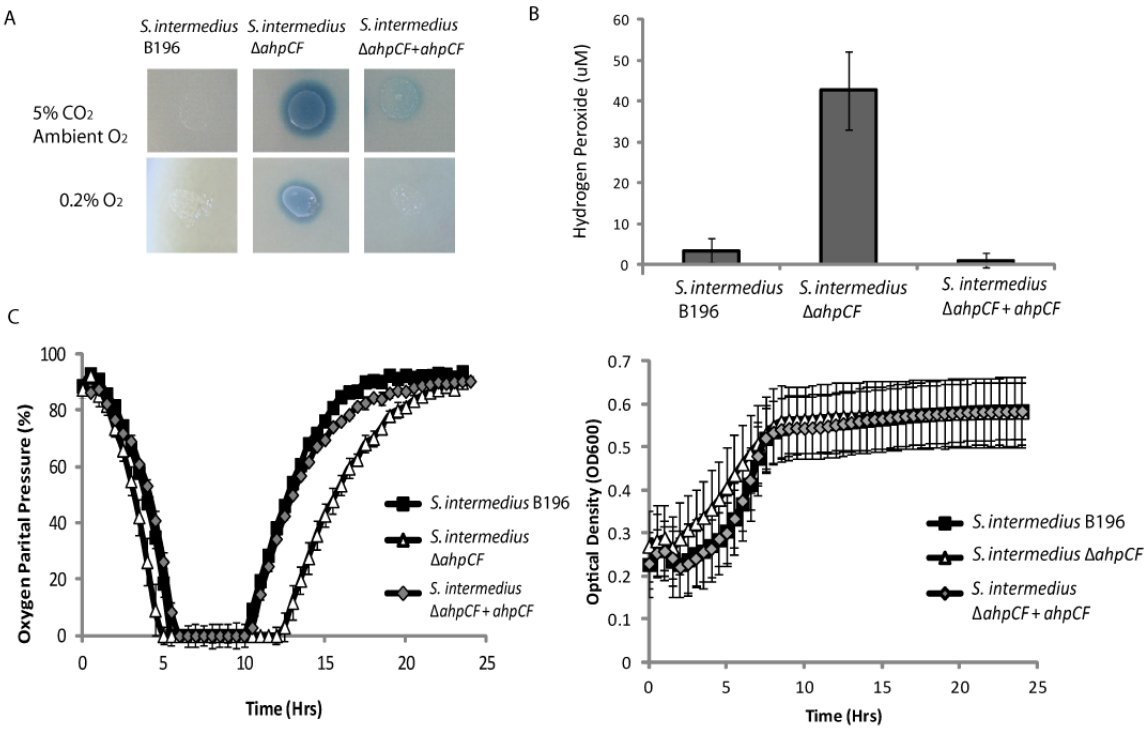

Figure 3.4: Phenotypic analysis of alkylhydroperoxidase knockout (Δ*ahpCF*) in *Streptococcus intermedius* B196. The colony morphology of *S. intermedius* strains on Prussian Blue agar is shown in A. The same strains were tested for production of hydrogen peroxide in broth in a 5% CO_2 incubator, shown in B. Hydrogen peroxide concentrations were determined using Peroxidetect (Sigma). In C, oxygen depletion of the strains was tested using Oxoplates (PreSens Precision Sensing GmbH). The respective growth curves during depletion are shown. Three biological replicates were used in B and C. Error bars represent standard deviation.

Table 3.3: Hydrogen peroxide concentration dependent inhibition of *Streptococcus intermedius*, *Streptococcus pneumoniae* and *Prevotella melaninogenica* under anaerobic conditions

	Hydrogen peroxide concentration (Colony forming units/mL ± standard deviation)					
Strain	**12 uM**	**25 uM**	**50 uM**	**100 uM**	**1 mM**	**10 mM**
S. intermedius	$5.33 \times 10^7 \pm 3.05 \times 10^7$	$1.00 \times 10^8 \pm 2.00 \times 10^7$	$6.67 \times 10^7 \pm 3.05 \times 10^7$	$6.67 \times 10^7 \pm 1.15 \times 10^7$	$2.86 \times 10^6 \pm 2.73 \times 10^6$	0
S. pneumoniae	$1.33 \times 10^7 \pm 9.02 \times 10^6$	$1.47 \times 10^7 \pm 6.42 \times 10^6$	$1.40 \times 10^7 \pm 2.00 \times 10^6$	$1.93 \times 10^7 \pm 6.43 \times 10^6$	$1.47 \times 10^7 \pm 1.10 \times 10^7$	0
P. melaninogenica	$6.00 \times 10^7 \pm 4.00 \times 10^7$	$3.07 \times 10^7 \pm 1.61 \times 10^7$	0	0	0	0

on its minimum inhibitory concentration (MIC) for H_2O_2 (Table 3.3). The MIC for H_2O_2 of *S. intermedius* is similar to that of *S. pneumoniae*. While H_2O_2 production is considered a virulence factor for *S. pneumoniae*, it comes at the cost of reduced viability after H_2O_2 accumulation (Regev-Yochay et al. 2007). We also looked at the activity of AhpCF in oxygen depletion. The *ahpCF* deletion did not significantly affect the rate of *S. intermedius* oxygen depletion; it did, however, increase the duration of the anaerobic period (**Figure 3.4C**). A minimal increase in growth rate was observed in the mutant, which resulted in increased oxygen depletion slightly earlier in the growth phase (**Figure 3.4C**).

Lastly, Δ*ahpCF* strains were cocultured with *Prevotella* under hypoxic conditions. The results, shown in **Figure 3.5**, were similar to the *S. pneumoniae* coculture in **Figure 3.3**. *Prevotella* viability decreased over time under hypoxic conditions, as was previously shown. In a coculture with *S. intermedius* Δ*ahpCF*, *Prevotella* viability was inhibited due to H_2O_2 production. The inhibition with this strain was lower in comparison to *S. pneumoniae* in **Figure 3.3** due to lower H_2O_2 levels. The difference in *Prevotella* viability alone compared to with *S. intermedius* Δ*ahpCF* is significant at 5 hours ($p<0.05$). Coculture with *S. intermedius* Δ*ahpCF* + *ahpCF* increased *Prevotella* viability to greater than that of wildtype *S. intermedius*. Thus, AhpCF mediated detoxification of H_2O_2 promotes viability of *Prevotella* in a hypoxic coculture. Our data implies detoxification of H_2O_2 is not only important for *S. intermedius* aerobic growth, but also for establishing polymicrobial communities.

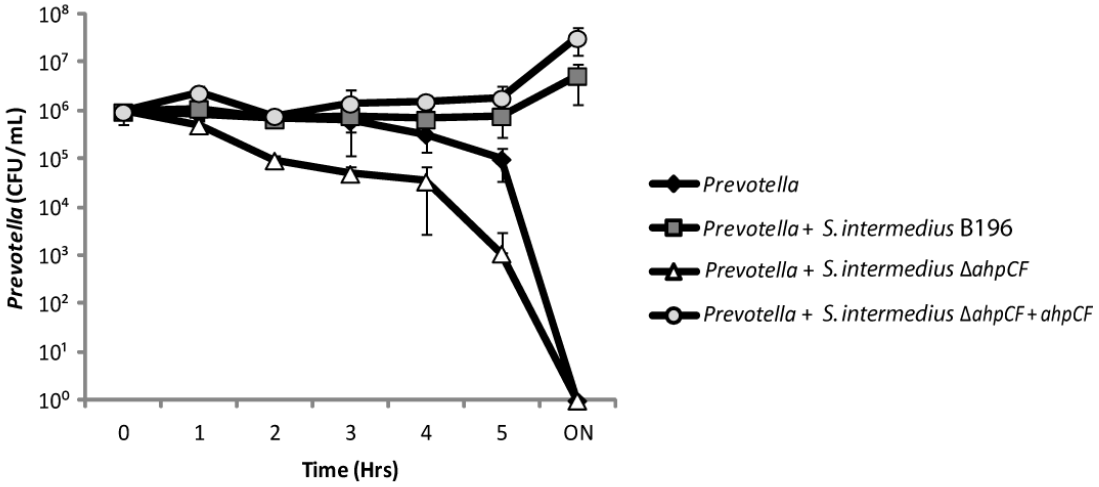

Figure 3.5: Detoxification of hydrogen peroxide by *Streptococcus intermedius* increases *Prevotella melaninogenica* survival under hypoxic conditions. The colony forming units (CFU/mL) for *P. melaninogenica* is shown in cocultures with *S. intermedius* (*S. intermedius* B196), an alkylhydroperoxidase mutant (*S. intermedius* Δ*ahpCF*) and complemented mutant (*S. intermedius* Δ*ahpCF* + *ahpCF*). At 5 hours, the *Prevotella* viability in coculture with the mutant is significant in comparison to *Prevotella* alone ($p<0.05$) using the Student's t-test. Three biological replicates were used for each sample group. The error bars represent standard deviation.

DISCUSSION

The polymicrobial interactions in *Streptococcus* colonization and infections are poorly understood. Obligate anaerobes are often isolated along with streptococci from complex microbial infections (Sibley et al. 2008; Parkins et al. 2008; Filkins et al. 2012; Sibley et al. 2012; Shweta and Prakash 2013; George et al. 2016). Here, we have demonstrated that streptococci promote anaerobe viability by depleting oxygen and creating an anaerobic environment. The genes involved in this activity are strain dependent. Oxygen depletion mechanisms in lactic acid bacteria are very different from other facultative anaerobes. *E. coli* undergo aerobic respiration in the presence of oxygen, reducing oxygen. As streptococci cannot make heme (a component of cytochromes in the electron transport chain), they do not undergo aerobic respiration. Instead, NADH and substrate oxidases are the only currently known oxygen consumption mechanisms. Recently, we showed that a minimal transcriptional and metabolic shift in metabolism is observed while comparing aerobic and anaerobic growth of *S. intermedius* B196 (Fei et al. 2016). The highest transcriptional fold change was observed in oxidative stress genes including the NADH oxidases, *ahpCF* and *nox*. While this SMG strain has no known H_2O_2 production mechanisms, these NADH oxidases appear to be critical in *S. intermedius* aerobic growth. It is evident from our data that oxygen depletion in polymicrobial environments is dependent on the strains present and their mechanisms of oxygen consumption.

The genus *Streptococcus* varies in competitive inhibitor production and interactions, even when dealing with a single obligate anaerobe. In our coculture

experiments, at least one additional inhibitory mechanism besides hydrogen peroxide was detected. Non-H_2O_2 mediated inhibition of *Prevotella* has been previously observed (Ma et al. 2014). Identification and characterization of these inhibitors is required to understand the mode of inhibition and potential clinical benefit in polymicrobial infections (Brook and Walker 1984).

Oxygen is highly reactive and can gain electrons to form reactive oxygen species such as H_2O_2 (Yesilkaya et al. 2013). Catalase is one way that bacteria detoxify H_2O_2. Streptococci lack catalase and use systems like AhpCF to detoxify H_2O_2. Previously, catalase has been used to demonstrate that detoxification of H_2O_2 produced by streptococci facilitates growth of a secondary bacteria (Regev-Yochay et al. 2006). Our approach used an endogenous H_2O_2 detoxification system in streptococci to prove that in addition to mitigating endogenous oxidative stress, it promotes growth of surrounding bacteria by removing a competitive inhibitor.

H_2O_2 was detected in our *S. intermedius* ∆*ahpCF* strain. An *ahpC* mutant in a catalase deficient *E. coli* also accumulated endogenous H_2O_2 (Seaver and Imlay 2001). This was also observed in an *ahpC* mutant in *S. pyogenes* (Brenot et al. 2005). In *S. intermedius*, it is unknown whether the H_2O_2 detected is due to similar accumulation. As streptococci have several ways of generating H_2O_2, it is possible that an unknown mechanism exists in *S. intermedius*. Interestingly, an *ahpC* mutant in *S. pyogenes* was attenuated in virulence in a subcutaneous model, implying detoxification of ROS is critical for *S. pyogenes* infection. An increased anaerobic period was also observed in our *S. intermedius* ∆*ahpCF* mutant. Deletion of *ahpCF* in *S. mutans* results in increased

NADH oxidizing activity (Higuchi et al. 1999). The increased anaerobic period we observed could be due to higher Nox-2 activity in the mutant, similar to what was shown in *S. mutans*.

Complete AhpCF mediated detoxification of endogenous H_2O_2 is strain dependent. In strains that have both AhpCF and H_2O_2 producing oxidases, the H_2O_2 levels can be too high for AhpCF to detoxify completely. In *S. pyogenes*, AhpCF is unable to completely detoxify H_2O_2 produced by endogenous lactate oxidase (Saito et al. 2001; Seki et al. 2004; Pulliainen et al. 2008). *S. pneumoniae* lacks AhpCF (Henningham et al. 2015). These factors account for high levels of H_2O_2 and contribute to competition.

The observed interaction between SMG and *Prevotella* appears to be a commensal relationship where *Prevotella* benefits from SMG and SMG is unaffected. It is as yet unknown if there are additional interactions taking place between the two bacteria. Mouse models involving these bacteria suggest a synergy exists during co-infection (Shinzato and Saito 1994) as increased mortality was observed in co-infected mice (Shinzato and Saito 1994). It is also possible that streptococci could favour growth of beneficial anaerobes. We concluded from our data that the relationship between streptococci and anaerobes is complex, with some strains cooperating with anaerobes and others that do not and this can be significantly affected by the microenvironment. Our work does not confine the interaction between streptococci and anaerobes to be solely rescuing anaerobes from oxygen toxicity. It provides an additional layer to the complex interactions taking place in microbial communities. Indeed, while it is unknown how colonizers such as *Streptococcus* and *Prevotella* cause infections in distal parts of the

body, their propensity for interaction could facilitate infection and increase anaerobe viability in infected sites.

CHAPTER 4

CHARACTERIZATION OF A POLYMICROBIAL SUBCUTANEOUS ABSCESS MODEL INVOLVING *STREPTOCOCCUS INTERMEDIUS* AND *PREVOTELLA MELANINOGENICA*

Characterization of a polymicrobial subcutaneous abscess model involving *Streptococcus intermedius* and *Prevotella melaninogenica*

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Preface: The research in this chapter was conducted from 2012 to 2016. The experimental design was done by Dr. Michael Surette, Dr. Dawn Bowdish, Dr. Preethi Jayanth and myself. I conducted work involving bacterial growth for infection and enumeration of bacteria in abscesses. Dr. Preethi Jayanth conducted work with mice including infection, monitoring and obtaining abscesses. Dr. Preethi Jayanth conducted flow cytometry to enumerate neutrophils. I conducted the histology microscopy. Statistical analysis and figures were compiled by Dr. Preethi Jayanth and myself. I wrote this chapter with help from Dr. Dawn Bowdish.

Title: Characterization of a polymicrobial subcutaneous abscess model involving *Streptococcus intermedius* and *Prevotella melaninogenica*

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ABSTRACT

The *Streptococcus* Anginosus/Milleri Group is part of the human microbiome but also associated with polymicrobial pyogenic infections such as invasive abscesses and pleural empyema, often involving obligate anaerobes. We used a subcutaneous abscess model to investigate co-infection of group member *S. intermedius* and the obligate anaerobe *Prevotella melaninogenica*. *S. intermedius* abscesses were dominated by neutrophils that resolved in 21 days. In contrast, *P. melaninogenica* induced abscesses with lower immune cell infiltration in comparison and resolved in 5 days. While the abscesses formed in the co-infection were similar in size and immune cell composition to that of *S. intermedius* mono-infection, *P. melaninogenica* persisted in the co-infection. In order to characterize the immunopathology of the abscess, we depleted neutrophils in mice prior to infection. This resulted in decreased abscess size and weight loss in mice, indicating that neutrophils are required in immunity against *S. intermedius*. Our results demonstrate that the presence of neutrophils is a defining immunopathological feature of *S. intermedius* abscesses and that this environment favors the survival of *P. melaninogenica* in a polymicrobial infection.

INTRODUCTION

Streptococcus Anginosus/Milleri Group (SMG) strains were originally isolated from humans in the early 1900s (Andrewes and Horder, 1906; Prevot, 1924; Prevot, 1925) and currently includes three distinct but closely related species *S. anginosus*, *S. constellatus* and *S. intermedius*. These strains are believed to be commensals as they are commonly found in mucosal sites in humans such as the oral and nasal cavities, the gastrointestinal tract and female urogenital tract. They have also been isolated from numerous pyogenic infections involving the central nervous system, respiratory system and gastrointestinal system (Whiley et al. 1992; Clarridge et al. 2001; Junckerstorff et al. 2014). SMG infections are often polymicrobial. Obligate anaerobes such as *Prevotella* and *Fusobacterium* species have been isolated with SMG in lower airway infections (Shinzato and Saito 1995; Sibley et al. 2008; Parkins et al. 2008; Filkins et al. 2012), pleural empyema (Hocken and Dussek 1985; Van der Auwera 1985; Wong et al. 1995) and abscesses (Gossling 1988; Hirai et al. 2005). These clinical associations suggest there is a cooperative relationship between SMG and obligate anaerobes in humans.

Polymicrobial infection models demonstrate that SMG interacts with certain bacteria in a synergistic manner. In mice, a pneumonia model with *S. constellatus* and anaerobe *Prevotella intermedia* increased mortality from 10% in mono-infection to 60% in co-infection (Shinzato and Saito 1994). A subcutaneous abscess model in mice involving *S. constellatus* and *Fusobacterium nucleatum* has also been established (Nagashima et al. 1999). The authors found that both abscess size and bacterial numbers increased in co-infections (Nagashima et al. 1999). One way that anaerobes could be

affecting SMG is through secreted factors and nutrients. Anaerobe culture supernatants, thought to include these factors, promoted the growth of *S. constellatus in vitro* (Shinzato and Saito 1994; Nagashima et al. 1999). The culture supernatant also increased mortality from 10% to 20% in the pneumonia model (Shinzato and Saito 1994) and increased abscess size in the abscess model in comparison to infection with *S. constellatus* alone (Nagashima et al. 1999). The increase in disease severity in co-infections suggests bacterial synergy is taking place. An additional cooperation mechanism was demonstrated in Chapter 3, where we showed that SMG can promote the viability of *P. melaninogenica* under hypoxic conditions.

A clinical characteristic of SMG infections is neutrophil infiltration (Wong et al. 1995; Noguchi et al. 2015). Streptococci can activate neutrophils but the degree of activation is dependent on the strain (Snäll et al. 2016). The specific immunogens involved have not been fully characterized (Snäll et al. 2016). Studies with SMG and human neutrophils have shown that while able to internalize SMG species, their killing of SMG was slower and incomplete in comparison to killing of *Staphylococcus aureus* (Wanahita et al. 2002). They concluded that this allows SMG to form abscesses. Also, SMG strains with capsules were more likely to form abscesses in mice in comparison to those lacking capsules (Kanamori et al. 2004). SMG capsule was shown to be involved in inhibition of phagocytosis and neutrophil mediated killing (Kanamori et al. 2004). From the polymicrobial perspective, human neutrophil mediated killing of *S. constellatus* is suppressed by anaerobe culture supernatants (Shinzato and Saito 1994; Nagashima et al. 1999).

Previous mouse models of SMG infection have focussed on the role of anaerobes and their culture filtrate in increasing the virulence of SMG species and affecting acute SMG infections. Indeed, by-products of anaerobe metabolism do contribute to bacterial virulence, but not as much as when live bacteria are present. In this chapter, we attempted to identify the role of SMG in a subcutaneous co-infection with *Prevotella melaninogenica* and characterize the host response to co-infection in comparison to mono-infection.

METHODS

Bacterial strains and culture conditions

S. intermedius C1365 is an invasive strain, isolated from human blood. *P. melaninogenica* C1009 is a clinical strain, isolated from a cystic fibrosis patient. *S. intermedius* colonies were grown on Brain Heart Infusion (BHI) agar for 3 days at 37°C in a 5% CO_2 incubator. Colonies were used to grow broth cultures in BHI broth overnight at 37°C at 5% CO_2. As these bacteria grow in clumps, the broth culture was homogenized in its growth media with 2.8 mm ceramic beads at 1000 rpm for 2 minutes to break up the clumps formed during growth. *P. melaninogenica* colonies were grown on Kanamycin Vancomycin Laked Blood (KVLB) agar and broth cultures in BHI overnight at 37°C in the Bactron IV anaerobe chamber (5% CO_2, 5% H_2, 90% N_2). Bacterial cells were washed in PBS and prepared to the desired inoculums (1 x10^8 colony forming units (CFU) for *S. intermedius* and 4 x10^7 CFU *P. melaninogenica*). All washing and handling steps were performed at room temperature. Bacteria were resuspended in a final volume of 100 µL phosphate buffered saline (PBS).

Mouse strains and subcutaneous infection

Six to eight week old male BALB/c mice were purchased from Jackson Laboratory (Bar Habor, ME). Mice were anaesthetized and the area to be inoculated was shaven under sterile conditions. A 100 µl volume of bacterial cell suspension was used for both the mono- and co-inoculated groups and was subcutaneously inoculated into the backs of the mice. Control mice were injected with 100 µl of PBS. The weights of the mice and volume of the abscesses were monitored and recorded daily. The volume of the abscess was calculated by the width, length and depth using calipers.

Preparation of abscess for plating and staining

Mice were humanely euthanized according to Canadian Council on Animal Care guidelines. Abscesses were excised, weighed and homogenized in 1 ml of PBS. Serial dilutions were made and plated. Enumeration of *S. intermedius* was performed by plating serial dilutions on McKay agar and incubating for 48 hrs at 37˚C in 5% CO_2. Enumeration of *P. melaninogenica* was done similarly with plating on KVLB agar and incubated for 2 days in the anaerobe chamber at 37°C. A 100 µl volume of the abscess homogenate was stained with fluorescent antibodies for flow cytometry.

Depletion of Neutrophils

Neutrophils were depleted in mice by using the Gr-1 antibody which recognizes Ly6C and Ly6G. The control used was Anti Rat IgG antibody. The antibodies were produced by growing one RB6-8C5 hybridoma in serum-free media and isolating antibodies using a Protein L agarose column. The antibodies were administered by i.p. injections into the mice on day 1 and day 2 at a concentration of 150 µg. On day 3, the

mice were infected with either *S. intermedius* or PBS as a vehicle. The mice were bled on day 5 and the blood samples were analysed by flow cytometry.

Flow cytometry

Blood samples from the mice were collected by retro-orbital bleeding. The isolated cells were pretreated with 2.4G2 antibody to block nonspecific binding to FC receptors. Samples were stained with the following fluorescent antibodies: Ly6G:phycoerythrin, Ly6C:FITC, CD45:PacificBlue, F4/80: APC, MHCII/CD11c:Per-Cy5.5 and CD11b: phycoerythrin-Cy7. The abscess homogenate samples were also stained with the fluorescent antibodies Ly6G: phycoerythrin, Ly6C:FITC, CD45:PacificBlue, F4/80:APC, CD3: phycoerythrin-Cy7 and CD11b:Per-Cy5.5. The cells isolated from neutrophil depleted mice were stained with Ly6G: phycoerythrin, Ly6C:FITC, CD11b:phycoerythrin-Cy7 and CD45:PacificBlue. The stained cells were fixed with 1% paraformaldehyde and assayed using a BD LSRII flow cytometer (BD). Appropriate isotype controls were used to gate the cells and the data was analyzed by FlowJo (Tree Star Inc.)

Histology

Histological samples were prepared as follows. The excised abscesses were fixed in 10% neutral buffered formalin for 24 hours and were paraffin-embedded. The tissue blocks were cut into 3 µm sections stained with hematoxylin and eosin (H&E) and Gram stain at the Core Histology Facility, McMaster Immunology Research Centre. The same facility also prepared immunohistochemistry staining with Purified anti-mouse Ly-6G

(1A8)(Cedarlane) and cleaved caspase-3 (Asp175)(5A1E) Rabbit mAb (Cell Signalling Technology Inc.). Histology slides were imaged using the Leica DMI4000B microscope.

Degradation of NETs by DNase treatment

Six to eight week old BALB/c mice were mono-infected or co-infected subcutaneously along with 2 mg DNase1 (Roche). Control mice were infected with the bacterial cultures and inactive DNase. Inactive DNase was prepared by heating at 75°C for 5 minutes and vortexing for 1 minute. Mice were euthanized after 2 days of infection.

RESULTS

Subcutaneous abscess formation by *S. intermedius* infection is driven by neutrophil infiltration

Previous mouse models and clinical observations have found that SMG infections are characterized by neutrophil recruitment to the infection site (Shinzato and Saito 1994; Wong et al. 1995; Nagashima et al. 1999; Noguchi et al. 2015). To establish a working model to study polymicrobial infections, a subcutaneous abscess model in mice was used. This model has been used to study polymicrobial infections involving SMG (Nagashima et al. 1999). We characterized the early time points of abscess formation in an *S. intermedius* mono-infection (3 hours, 6 hours and 1 day). Our approach involved using histology and stains such as hematoxylin and eosin (H&E) (for all cells), the Gram stain (for bacteria), Ly6G immunochemistry (for neutrophils, monocytes and granulocytes) and cleaved caspase-3 (for apoptosis).

Specific staining for neutrophils and granulocytes (Ly6G) at the three time points is consistent with clinical reports and other mouse models which find that neutrophils are

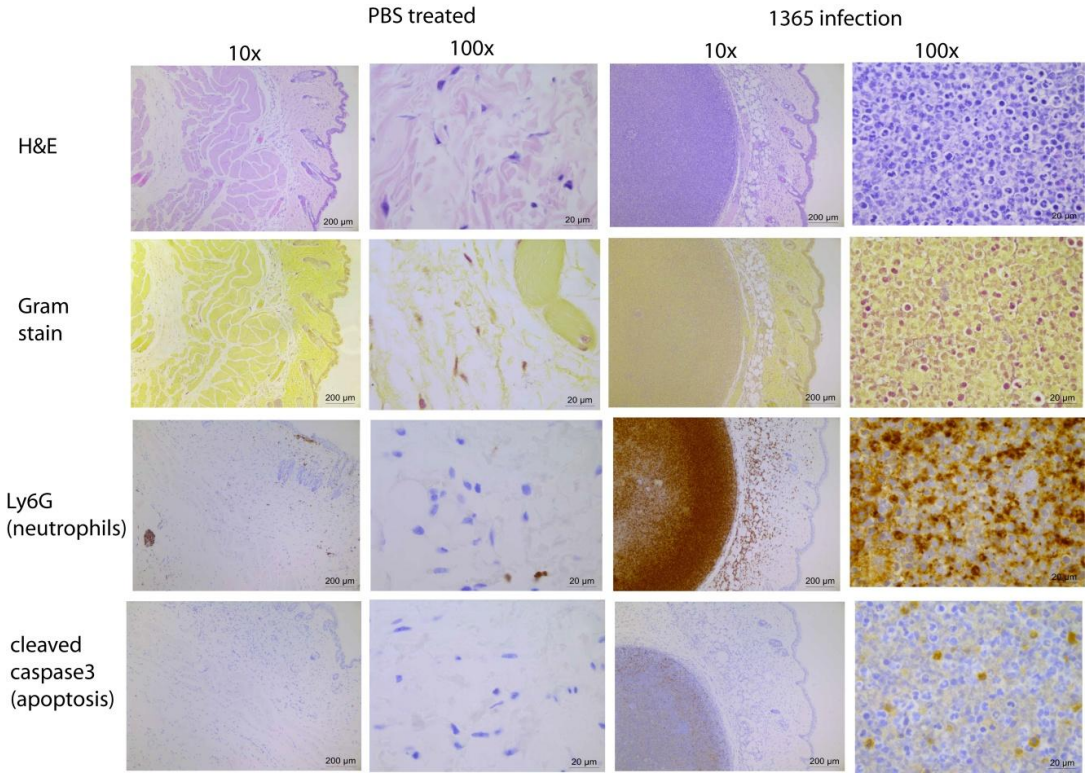

Figure 4.1: Development of abscess at 1 day post-infection with *S. intermedius* subcutaneously. Individual BALB/c mice were infected with 1 x10^8 CFU *S. intermedius* c1365 (n=3 per sample group). An abscess sample from an individual mouse was used for histological analysis with hematoxylin and eosin staining (H&E), Gram stain, and immunohistochemistry for Ly6G (neutrophils and granulocytes) and cleaved caspase3 (apoptosis). The scales are as indicated.

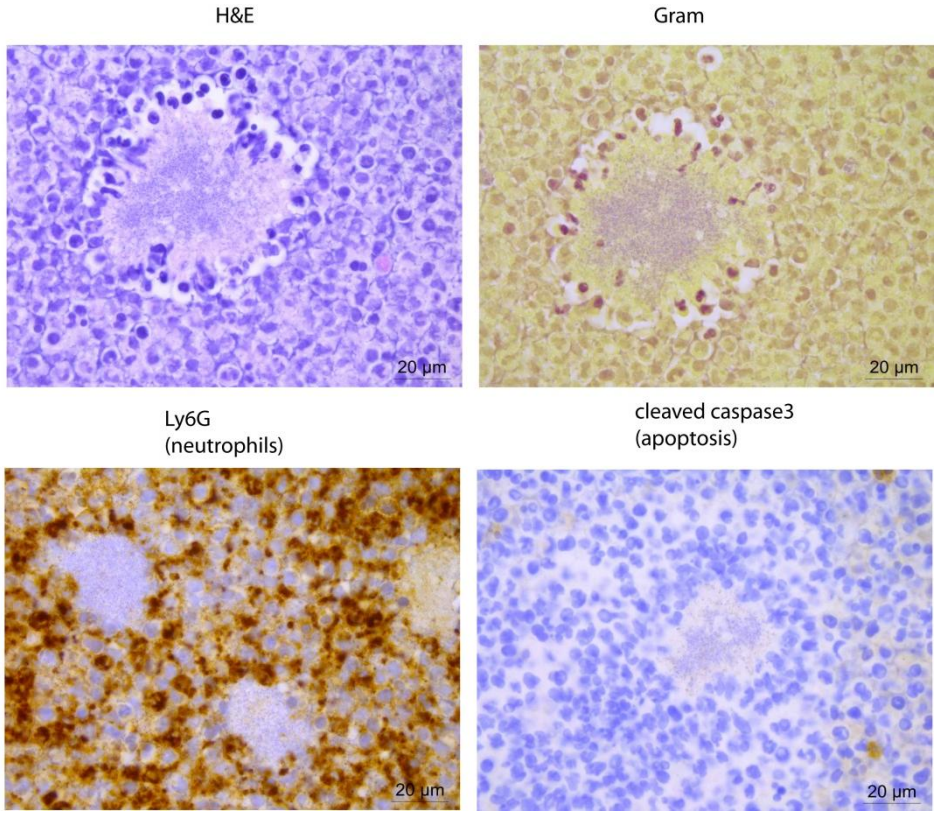

Figure 4.2: Neutrophils and granulocytes at the site of *S. intermedius* subcutaneous abscess infection. Individual BALB/c mice were infected with 1 x10^8 CFU *S. intermedius* c1365 (n=3 per sample group). An abscess sample from an individual mouse was used for histological analysis with hematoxylin and eosin staining (H&E), Gram stain, and immunohistochemistry for Ly6G (neutrophils and granulocytes) and cleaved caspase3 (apoptosis). The scales are as indicated

recruited to site of SMG infection (**Figure 4.1, 4.S1, 4.S2**). With the infiltration of neutrophils, a small abscess forms by 6 hours post infection and increases in size by 24 hours. Apoptosis occurred at 6 and 24 hours post-infection (cleaved caspase-3 in **Figure 4.S1, 4.S2, 4.1**). The core of the abscess did not stain optimally at 6 hours, which is consistent with necrotic tissue (**Figure 4.S2**). On further examination of the histological samples at day 1, we found sites in the centre of the abscess where the neutrophils appeared to surround *S. intermedius* (**Figure 4.2)**. The Gram stain clearly shows the Gram positive streptococci, surrounded by immune cells (**Figure 4.2**). Cellular debris (including DNA) was present (**Figure 4.1, 4.2**).

Abscess formation in co-infection is driven by *S. intermedius*

Next, we used our abscess model to determine whether a co-infection of *S. intermedius* with *P. melaninogenica* altered abscess size or leukocyte infiltration. We monitored the abscess size in mice daily as a measure of disease. Infection with *P. melaninogenica* alone resolved in 5 days and no *P. melaninogenica* were detected on day 6 (**Figure 4.3A, 4.B**). Thus, *P. melaninogenica* induced abscesses resolved successfully. *S. intermedius* abscesses did not resolve by Day 6. A decreasing trend was observed in both abscess volume and bacterial numbers from day 2 to day 6, though not significant. The abscess sizes and *S. intermedius* CFUs were similar in mono- and co-infected mice. In addition, *P. melaninogenica* numbers were higher in co-infected mice on day 2 and day 6 in comparison to *P. melaninogenica* infection alone **(Figure 4.3A)**.

Neutrophils and granulocytes were recruited in *S. intermedius* mono- and co-infection (**Figure 4.3C, 4.S3, 4.S4**). This was also seen in *P. melaninogenica* infected

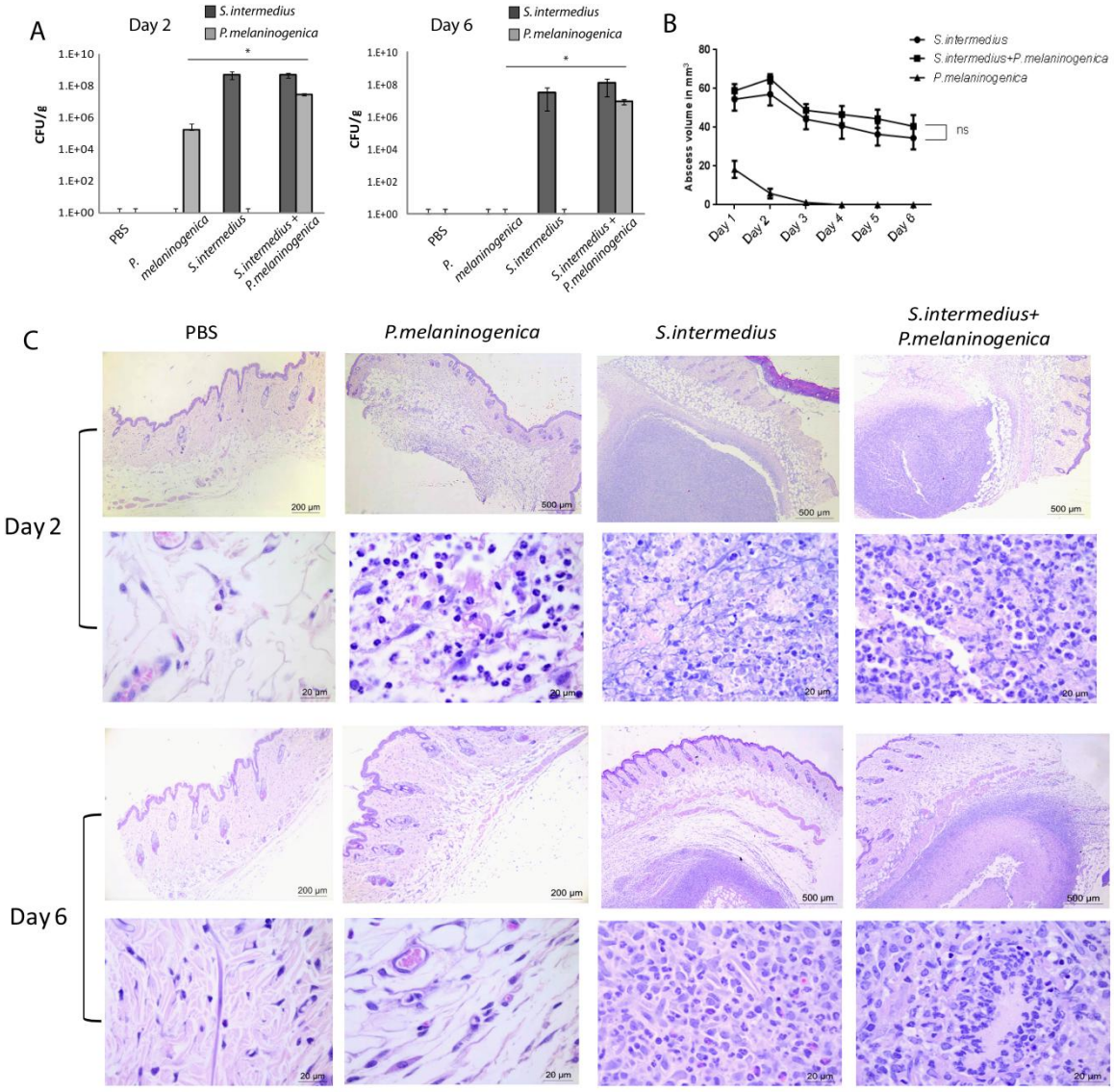

Figure 4.3: Persistence of *P. melaninogenica* in co-infection with *S. intermedius*. Individual BALB/c mice were infected with *S. intermedius* c1365 (1 x10^8 colony forming units (CFU)) and/or *P. melaninogenica* C1009 (4 x10^7 CFU) subcutaneously resulting in abscess formation (n=6 per sample group) (A) Abscesses were assessed for bacterial CFUs at Day 2 and Day 6 following infection (n=6). Statistical significance is displayed as $p<0.02$ by student T test. (B) Time course analysis of infection using abscess. Data shown is a representative of 3 experiments (C) Histological etiology in induced abscesses by *S. intermedius* alone and co-infection with *P. melaninogenica*. Hematoxylin and eosin (H&E) staining was used to visualize abscess formed (n=2). Histological analysis was conducted once.

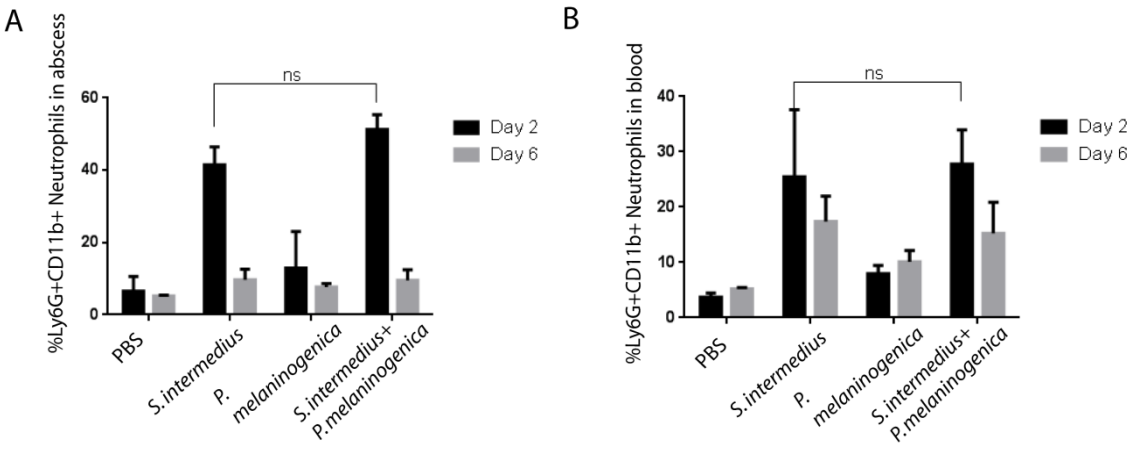

Figure 4.4: Increased neutrophil counts in response to subcutaneous infection. Individual mice were infected with *S. intermedius* c1365 (1 x10^8 CFU), *P. melaninogenica* c1009 (4 x10^7 CFU) or the combination (n=6 per sample group). (A) Abscesses from individual mice (n=3) were analyzed for the percentage of neutrophils (Ly6G+CD11b+/CD45+) on day 2 and day 6 of infection. (B) Blood samples from individual mice (n=3) were analyzed for the percentage of neutrophils (Ly6G+ CD11b+/CD45+) on day 2 and day 6. Statistical analysis was done using student T-test. Neutrophil counts were conducted once.

mice at day 2 but resolved by day 6 (**Figure 4.S3**). The high immune cell recruitment in *S. intermedius* abscesses did not result in clearance of the infection. Neutrophil counts in the abscess were higher on day 2 compared to day 6 in *S. intermedius* mono- and co-infected mice and these were higher in comparison to *P. melaninogenica* mono-infections (**Figure 4.4A**). Higher neutrophils counts were also seen in the blood on both day 2 and day 6 (**Figure 4.4B**), suggesting that circulating neutrophils may be continuously recruited to the infection site. It is unknown why the neutrophil counts decrease in the blood by day 6.

We used histology to determine the cause of cellular debris in the abscess. A low amount of apoptosis was seen (**Figure 4.S3, 4.S4**) in conjunction with necrosis based on tissue staining **(Figure 4.3C)**. There was also some evidence that neutrophil extracellular trap (NET) formation could be taking place (**Figure 4.3C, 4.S5**). To determine whether neutrophil NETs could be involved in this model, *S. intermedius* infection and co-infection were repeated with active and inactive DNase (**Figure 4.5**). Addition of exogenous DNase significantly decreased abscess size in the *Prevotella* mono-infection. Only modest effects of DNase were observed in the co-infection. These results are consistent with the production of DNase by *S. intermedius* which may account for the minimal effect of added DNase when *S, intermedius* was present. Addition of DNase had no effect on bacterial CFUs.

To analyze the role of neutrophils in containing the infection, we depleted neutrophils using GR-1 monoclonal antibodies prior to mono- and co-infections. Depletion of neutrophils decreased the abscess volume in both the *S. intermedius* mono-

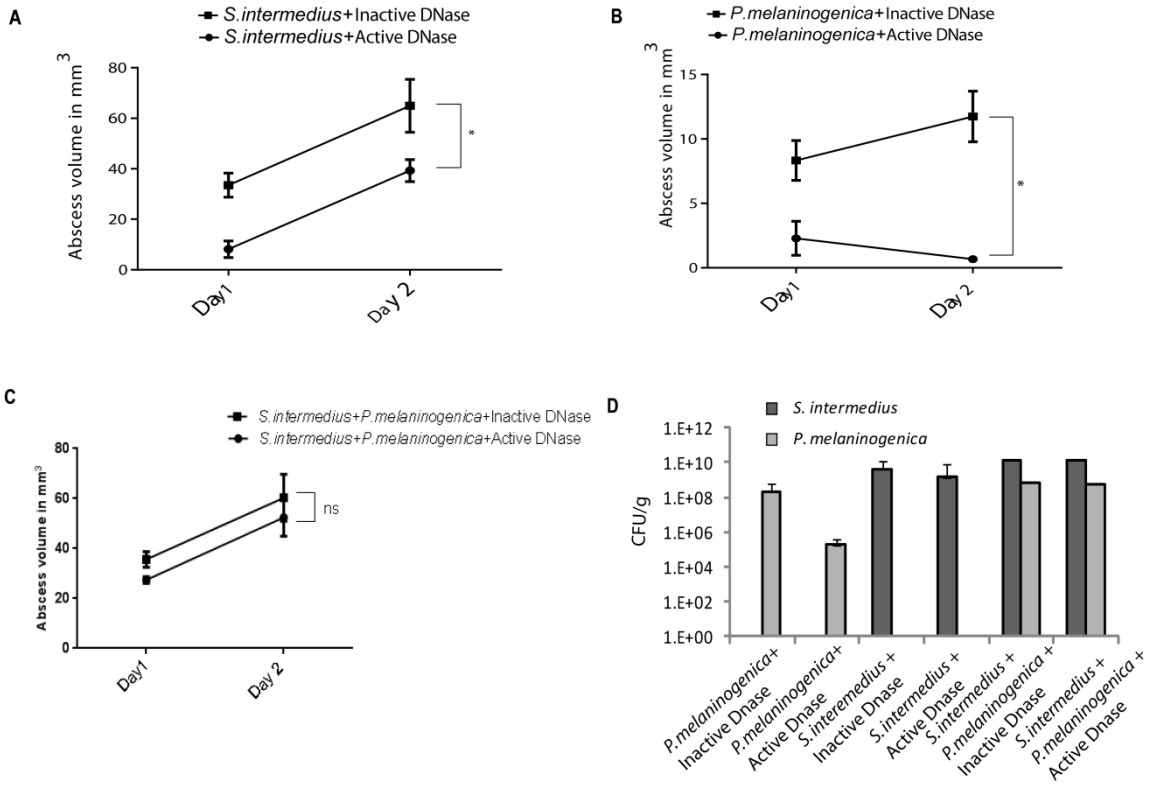

Figure 4.5: Affect of active DNase on abscess formation with *S. intermedius* and *P. melaninogenica*. Individual mice were subcutaneously infected with1 x10^8 CFU *S. intermedius* c1365 and/or 4 x10^7 CFU *P. melaninogenica* c1009 along with active or inactive DNase (n=4 per sample group). (A) Abscess volume monitoring of *S. intermedius* infected mice for 2 days. Statistical significance is displayed as $p<0.05$ by student T test. (B) Abscess volume monitoring for 2 days for *P. melaninogenica* infected mice. Statistical significance is displayed as $p<0.05$ by student T test. (C) Abscess monitoring in co-infected mice (D) Bacterial counts in abscesses on day 2. Experiment was conducted once.

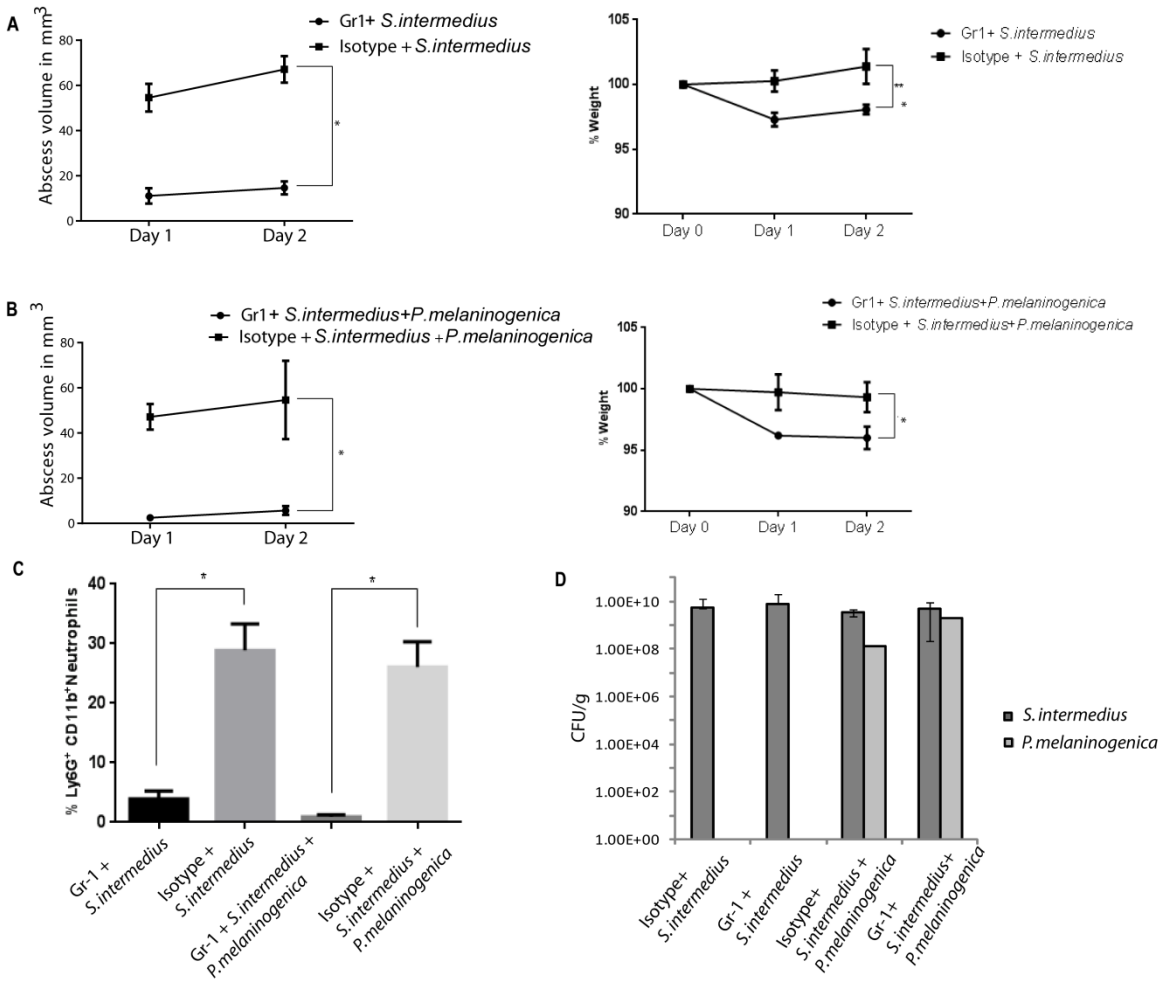

Figure 4.6: Neutrophil depletion in BALB/c mice affects abscess formation in mono- and co-infected mice. Gr-1 mediated neutrophil depleted and isotype treated mice were infected with 1 x10^8 CFU *S. intermedius* c1365 and/or 4 x10^7 CFU *P. melaninogenica* c1009 (n=4 per sample group). Mice were monitored for 2 days and sacrificed on day 2. (A) Abscess volume and weight change in mice infected with *S. intermedius* alone. Statistical significance is displayed as $p<0.05$ by student T test. (B) Abscess volume and weight change in co-infected mice. Statistical significance is displayed as $p<0.05$ by student T test. (C) Analysis of blood samples for neutrophils using flow cytometry and anti-Ly6G and anti-CD11b antibodies. Statistical significance is displayed as $p<0.005$ by student T test. Percentage of neutrophils was determined (Ly6G+CD11b+/CD45+) (D) Bacterial counts of abscesses on day 2. Experiment was done twice.

and co-infected mice (**Figure 4.6A, B**). Furthermore, depletion decreased the weight of infected mice by up to 5%, implying increased severity of infection in both groups (**Figure 4.6A, B**). Thus, a neutrophil mediated response to *S. intermedius* infection in mice appears to limit disease progression. Depletion did not affect the bacterial numbers significantly, though there is a higher count of *P. melaninogenica* in the GR-1 treated co-infected mice (**Figure 4.6D**).

Co-infection increases duration of abscess infection in mice

Anaerobes have been shown to increase disease severity in co-infection with SMG species (Shinzato and Saito 1994; Nagashima et al. 1999). Our co-infection studies with *S. intermedius* and *P. melaninogenica* did not increase mortality. To understand how co-infection and persistence of *P. melaninogenica* affects disease, we monitored the resolving time of abscesses in mice. *S. intermedius* mono-infected mice resolved infection in 21 days (**Figure 4.7A**). We sacrificed the co-infected mice at 30 days when they did not resolve the infection naturally. This difference in resolving time suggests that the persistence of *P. melaninogenica* prolonged the infection although *P. melaninogenica* was not detected in the abscesses of co-infected mice after 30 days while *S. intermedius* numbers remained significantly higher compared to the mono-infection (**Figure 4.7B**). The dynamics of infection over these 30 days are unknown, but it appears that *P. melaninogenica* numbers decrease, while *S. intermedius* having benefitted from *P. melaninogenica* persistence, sustains the infection. It has been previously shown that SMG numbers can decrease following anaerobe numbers decreasing (Nagashima et al. 1999). The co-infection abscess at 30 days still has intact neutrophils and granulocytes

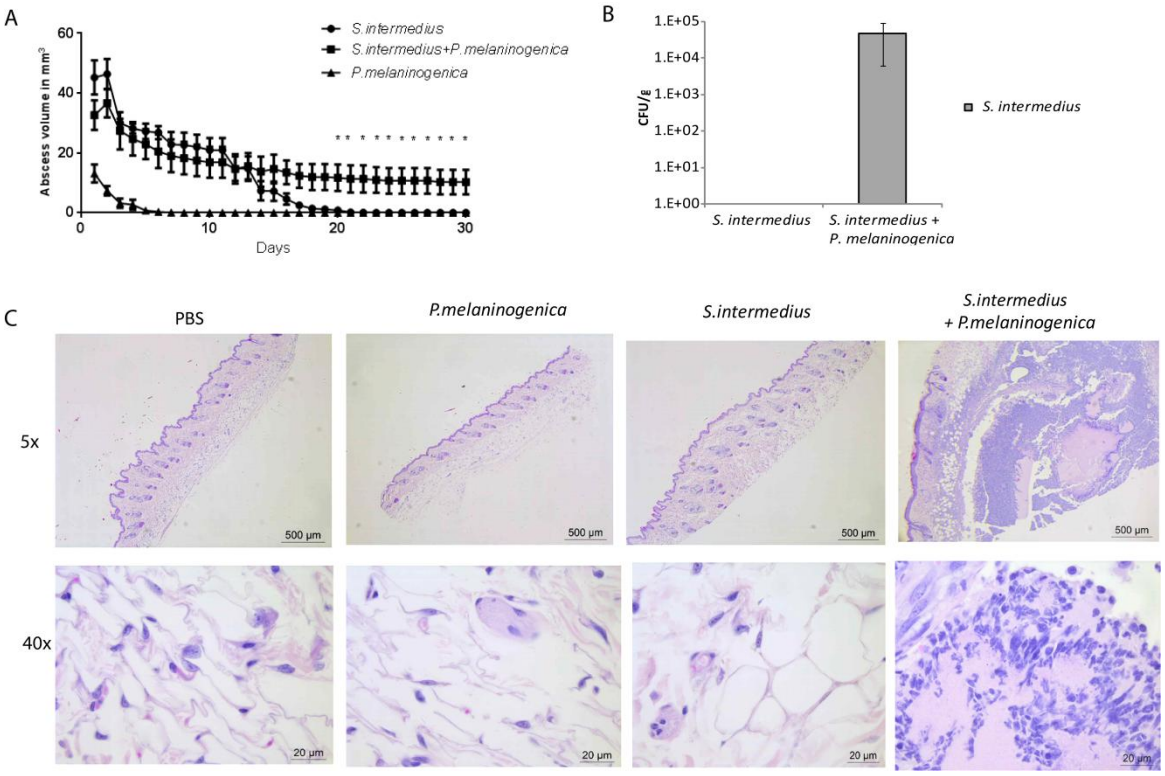

Figure 4.7: Prolonged co-infection disease duration in comparison to infection with *S. intermedius* alone. Individual BALB/c mice were infected with 1 x10^8 CFU *S. intermedius* c1365 and/or 4 x10^7 CFU *P. melaninogenica* c1009 (n=6 per sample group). Abscesses were allowed to resolve naturally in the mice. On day 30, abscesses persisted in co-infected mice alone. All mice were sacrificed on day 30. (A) Abscess did not resolve in co-infected mice. Statistical significance is displayed as $p<0.05$ by student T test. (B) Bacterial enumeration of co-infected abscesses on day 30 only detected *S. intermedius* c1365 and not *P. melaninogenica* (n=3 per sample group). (C) Hematoxylin and eosin (H&E) staining of abscess from individual mouse in each sample group (n=2 per sample group). Experiment was conducted once.

with extensive cellular debris (**Figure 4.7C**).

DISCUSSION

Obligate anaerobes are often isolated with SMG species from clinical pyogenic infections including abscesses and pleural empyema. The human response to infection by SMG species can vary depending on the bacterial strain and the individual being infected. Indeed, our laboratory has previously shown that cytokine response on exposure to different strains of SMG vary (Kaiser et al. 2014). In addition, some human red blood cells are resistant to the cytotoxic activity of *S. intermedius* (Giraldi 2015). *S. intermedius* C1365 is an invasive blood isolate that was previously shown to induce a high cytokine response on infection of PBMCs from three donors (Kaiser et al. 2014). We used an established SMG model for polymicrobial infection to study *S. intermedius* C1365 abscess formation (Nagashima et al. 1999).

Our histology and flow cytometry data is consistent with the neutrophil infiltration observed in SMG clinical and model infections. We observed that *S. intermedius* mono-infections took 20 days to resolve in spite of the influx of neutrophils to the infection site. However, neutrophils do improve the host response to *S. intermedius* as mice that underwent neutrophil depletion lost more weight than control mice. *S. intermedius* may be able to withstand and thwart neutrophil defense mechanisms. We are missing a control to see if neutrophil depletion made mice susceptible to their microbiome leading to a possible weight loss.

While specific immunity evasive mechanisms have not been characterized in *S. intermedius,* homologs for characterized mechanisms in streptococci have been found in

SMG (Olson et al. 2013). SMG have putative surface proteins involved in binding to fibronectin, fibrinogen, collagen and laminin (Olson et al. 2013). They also can putatively digest host polysaccharides including sialic acid, hyaluronic acid and pullulan (Grinwis et al. 2010; Olson et al. 2013). Another bacterial strategy is inhibition of phagocytosis by neutrophils. SMG capsule has been shown to inhibit phagocytosis by human PMNs (Kanamori et al. 2004). SMG species also have genes that provide resistance to reactive oxygen species produced during phagocytosis. These include genes that can degrade superoxides (superoxide dismutase, *sodA*) and hydrogen peroxide (alkyl hydroperoxidase, *ahpCF*) (Olson et al. 2013). A streptolysin-S like peptide has also been found in SMG with the potential to inhibit phagocytosis (Datta et al. 2005; Tabata et al. 2013). In addition to these, SMG carry a number of uncharacterized nucleases that have the potential to degrade neutrophil NETs (Olson et al. 2013). Clinically isolated SMG strains have been shown to degrade DNA (Grinwis et al. 2010).

A limitation of our study includes the lack of a *P. melaninogenica* mono-infection control in our neutrophil depletion analysis. Healthy mice were successfully able to clear a *P. melaninogenica* mono-infection. The immune response to this infection included neutrophils in addition to other unidentified cells. Due to this limitation, we cannot stipulate the role of neutrophils in clearance of a *P. melaninogenica* infection. However, the effect of DNAse on the *Prevotella* mono-infection suggests that neutrophils and neutrophil NETs may be one mechanism limiting *Prevotella* survival in the abscess. Our model was the first to show such a drastic difference in *Prevotella* bacterial numbers in the co-infection compared to *P. melaninogenica* mono-infection. This difference was

dependent on the presence of *S. intermedius*. The contribution of *P. melaninogenica* to the co-infection remains to be elucidated.

The general course of neutrophilic response upon bacterial infection is neutrophil recruitment and activation, followed by bacterial phagocytosis and degradation. Neutrophils usually undergo cell death through apoptosis, necrosis, autophagy or NET formation (Iba et al. 2013). Streptococci have been shown to induce necrosis (Zysk et al. 2000; Kobayashi et al. 2003; Ato et al. 2008), apoptosis (Kobayashi et al. 2003; Bordon et al. 2013) and NET formation (Sumby et al. 2005; Buchanan et al. 2006; Zhao et al. 2016) in human PMNs. The optimal outcome would be PMN apoptosis as this occurs even in healthy neutrophils, allowing them to be cleared by scavenging macrophages without excessive tissue damage. Apoptotic neutrophils that are not cleared undergo secondary necrosis and this can cause extensive tissue damage due to ROS and antimicrobial granules (Reviewed by Silva 2010). Apoptosis and secondary necrosis have been previously seen in PMN response to *S. pyogenes* (Kobayashi et al. 2003). The authors found that infection of human PMNs *in vitro* with *S. pyogenes* induced a distinct cellular response compared to infection with *Borellia hermsii*, *Listeria monocytogenes*, *Burkholderia cepacia*, and *Staphylococcus aureus* (Kobayashi et al. 2003). They showed that *S. pyogenes* induced both apoptosis and necrosis in human PMNs and uniquely altered neutrophil cell death in comparison to other pathogens. It could be that secondary necrosis is exacerbating *S. intermedius* infection by delaying scavenging of apoptotic neutrophils, thereby increasing recruitment of immune cells. Our data shows that the abscesses formed with *S. intermedius* initially appear to have viable neutrophils but

mature to have extensive cellular debris, with a small portion of neutrophils undergoing apoptosis. Based on the extensive debris, necrosis appears to be occurring as well. Thus, our data is very similar to what is seen in *S. pyogenes* infections. Amidst the debris in SMG abscesses, there is evidence of NETs in the centre of mature abscesses (>2 days post infection). Based on the size of the abscess, NET formation occurred in a small fraction of neutrophils. We thought that neutrophil NETs could be important in immunity, so we injected mice with DNAse along with inoculum. Our data was inconclusive as a different trend was observed in *S. intermedius* mono- and co-infections. However, our histological data clearly shows the formation of NETs and this merits further study. We concluded that while neutrophils are critical in limiting disease progression, they cannot clear *S. intermedius*.

In our analysis, *S. intermedius* appears to efficiently manipulate the neutrophil response in mice. There appears to be sub-populations of neutrophils undergoing different forms of cell death. In addition, the secondary necrosis could favour *S. intermedius* during infection while causing extensive host damage. It is unknown if this occurs in clinical abscesses as well. While both *S. pyogenes* and SMG cause pyogenic infections, both are unable to cause infection after intranasal inoculation (Kasper et al. 2014). However, an *S. pyogenes* intranasal infection of a humanized MHC-II mouse resulted in nasopharyngeal infection (Kasper et al. 2014). As SMG are often associated with infection of the respiratory tract, and as SMG and *S. pyogenes* infections share some similarities, this model might answer questions as to whether human MHC-II plays a role in respiratory infections caused by SMG.

Our understanding of the mechanisms involved in *S. intermedius* infection is lacking. There are numerous bacterial and host factors involved. A systemic approach targeting mechanisms on both fronts is warranted to decipher how each factor impacts the infection at large. In addition, a number of the SMG evasion mechanisms are specific for humans, making it difficult to recreate what is observed in humans without surgical injection of bacteria (Shinzato and Saito 1994; Olson et al. 2013). The development of better models of SMG infection in combination with a mechanistic approach will broaden our understanding.

Supplementary Material

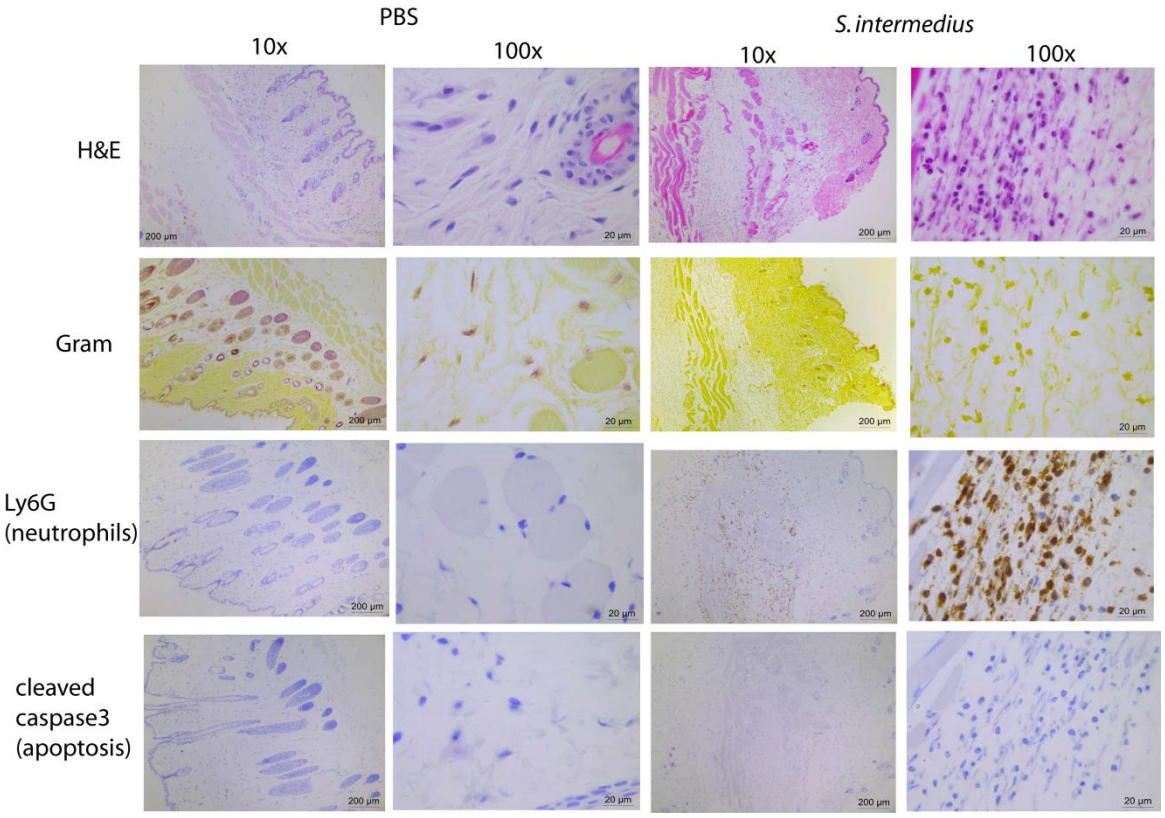

Figure 4.S1: Infiltration of neutrophils at 3 hours post infection with *S. intermedius*. Individual BALB/c mice were injected with 1 x10^8 CFU *S. intermedius* c1365 (n=3 per sample group). An abscess sample from the injected site in an individual mouse was used for histological analysis with hematoxylin and eosin staining (H&E), Gram stain, and immunohistochemistry for Ly6G (neutrophils) and cleaved caspase3 (apoptosis). The scales are as indicated.

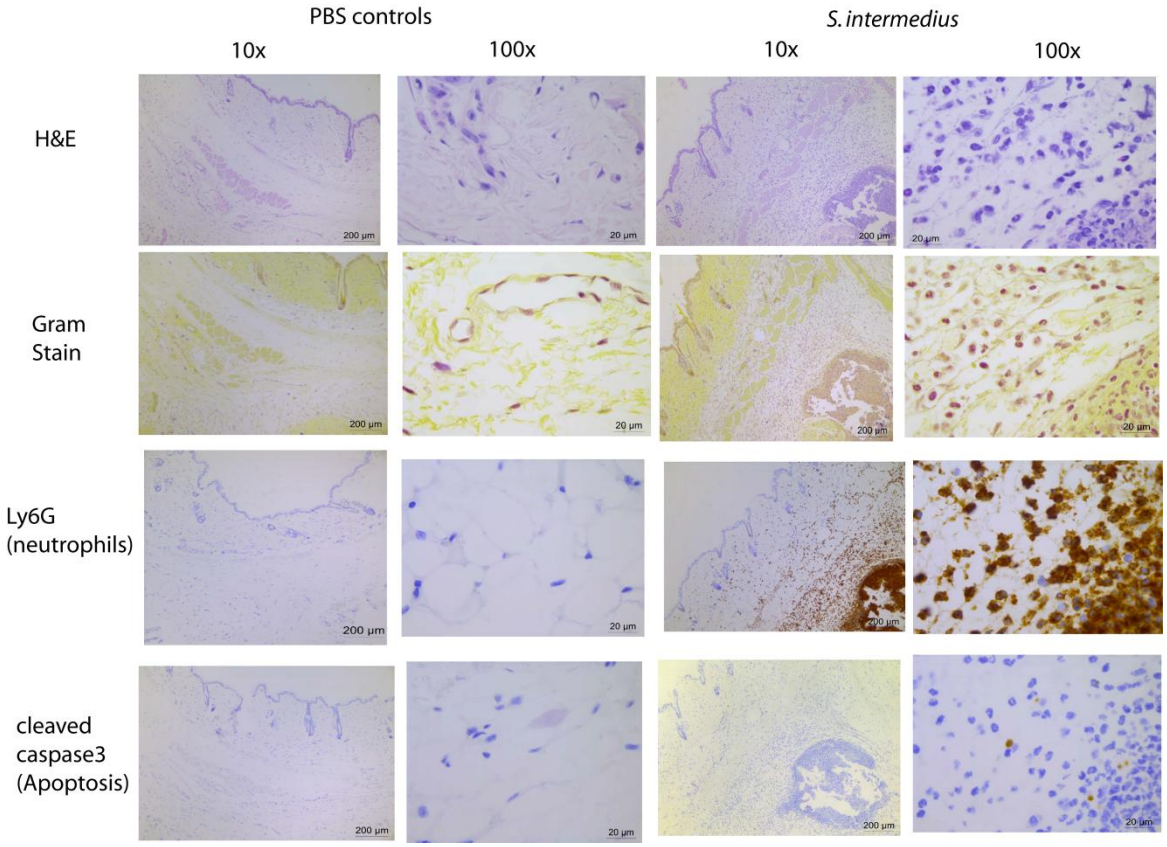

Figure 4.S2: Development of abscess at 6 hours post-infection with *S. intermedius* subcutaneously. Individual BALB/c mice were infected with 1 x10^8 CFU *S. intermedius* c1365 (n=3 per sample group). An abscess sample from an individual mouse was used for histological analysis with hematoxylin and eosin staining (H&E), Gram stain, and immunohistochemistry for Ly6G (neutrophils) and cleaved caspase3 (apoptosis). The scales are as indicated.

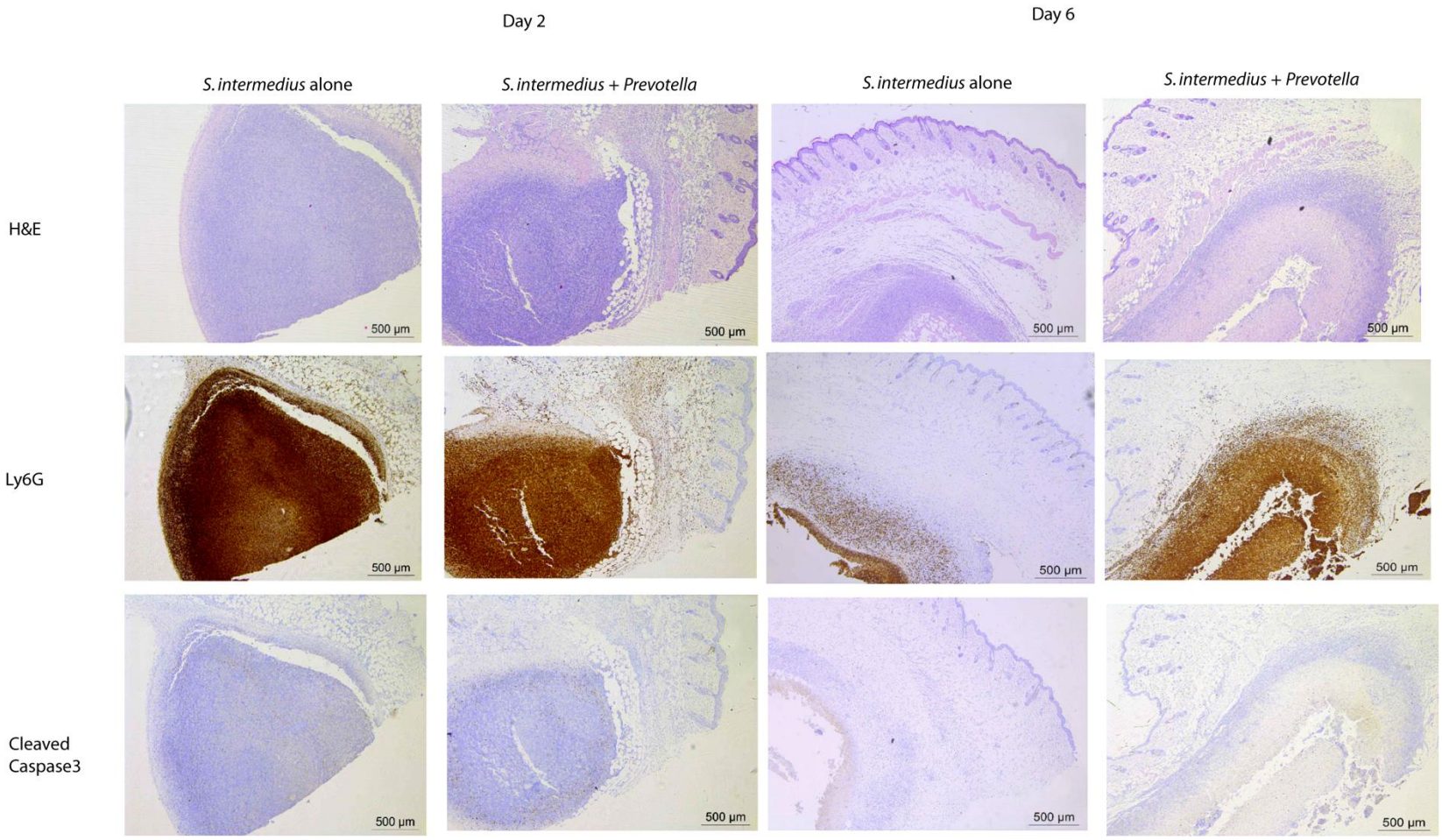

Figure 4.S3: Hisotological analysis of abscess induced by *S. intermedius* c1365 alone and in combination with *P. melaninogenica* on day 2 and day 6. Individual BALB/c mice were infected with *S. intermedius* c1365 (1 x10^8 colony forming units (CFU)) and/or *P. melaninogenica* C1009 (4 x10^7 CFU) subcutaneously resulting in abscess formation (n=6 per sample group). Hematoxylin and eosin (H&E), Ly6G immunochemistry and cleaved caspase3 immunochemistry staining was used to visualize abscess formed (n=2). Histological analysis was conducted once.

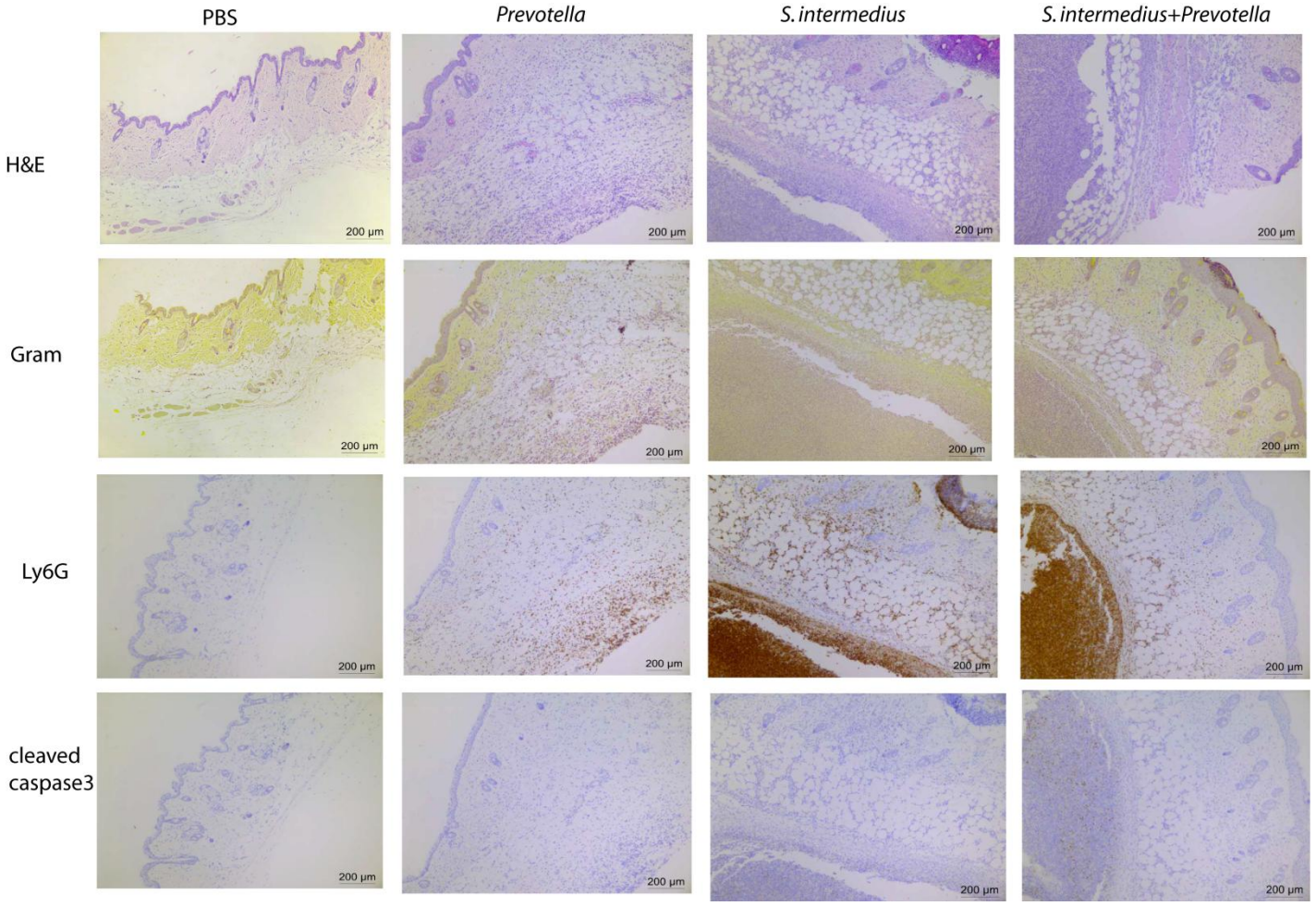

Figure 4.S4 Hisotological analysis of abscess induced by *S. intermedius* c1365 alone and in combination with *P. melaninogenica* on day 2 post infection. Individual BALB/c mice were infected with *S. intermedius* c1365 (1 x10^8 colony forming units (CFU)) and/or *P. melaninogenica* C1009 (4 x10^7 CFU) subcutaneously resulting in abscess formation (n=6 per sample group). Hematoxylin and eosin (H&E), Gram, Ly6G immunochemistry and cleaved caspase3 immunochemistry staining was used to visualize abscess formed (n=2). Histological analysis was conducted once.

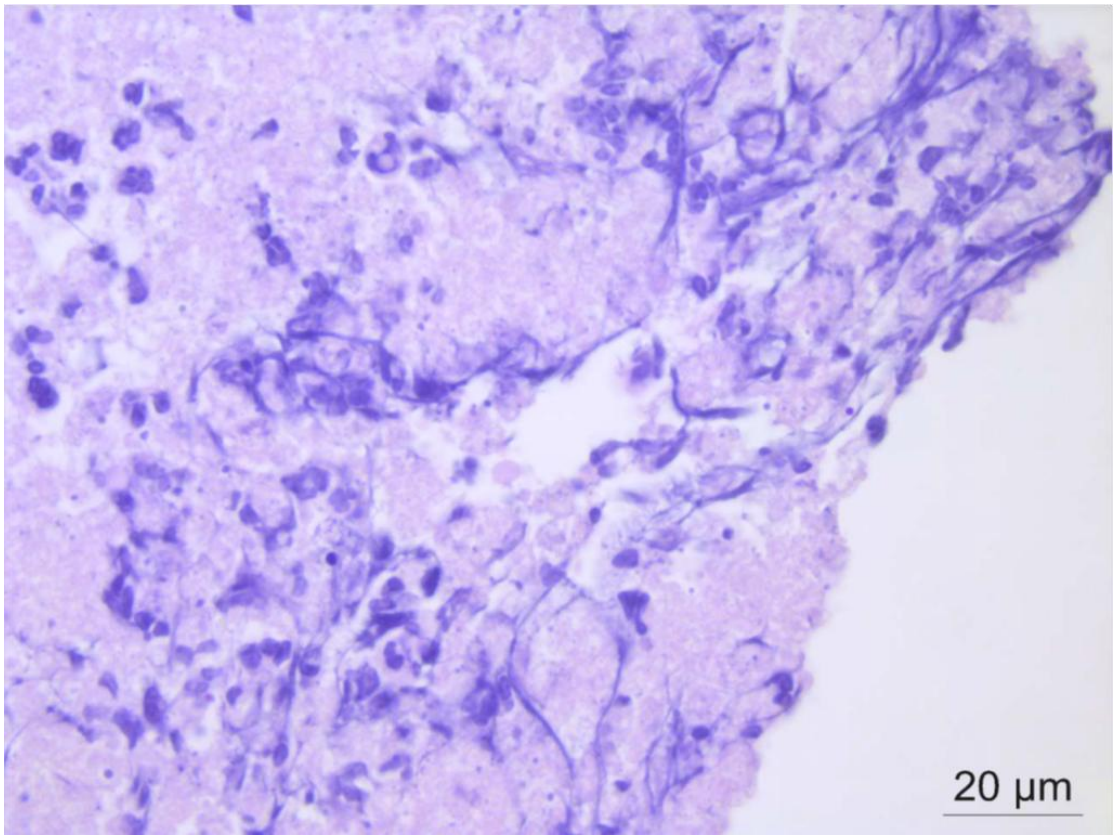

Figure 4.S5: Histological staining with hematoxylin and eosin of abscess induced with *S. intermedius* and *P. melaninogenica* 6 days post infection.

CHAPTER 5

THE *SIL* LOCUS IN *STREPTOCOCCUS* ANGINOSUS GROUP: INTERSPECIES COMPETITION AND A HOTSPOT OF GENETIC DIVERSITY

The *sil* locus in *Streptococcus* Anginosus Group: Interspecies competition and a hotspot of genetic diversity

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Preface: This research was conducted from 2013 to 2016. The experiments were designed by Dr. Michael Surette, Jake C. Szamosi, Anne-Marie Lacroix and myself. Bioinformatic analysis of *Streptococcus* Anginosus genomes was conducted by Jake C. Szamosi. I conducted the wet lab work including experiments with the mutant and inhibition assays. Jake C. Szamosi and I worked together to analyze the data and make the figures. Dr. Michael Surette, Jake C. Szamosi and I worked on writing the manuscript. Dr. Dawn Bowdish was a helpful resource while working on this research.

Title: The *sil* locus in *Streptococcus* Anginosus Group: Interspecies competition and a hotspot of genetic diversity

Abbreviated Title: Genetic diversity of the sil locus in *Streptococcus* Anginosus Group

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Abstract

The *Streptococcus* Invasion Locus (*sil*) was first described in *Streptococcus pyogenes* and *S. pneumoniae*, where it has been implicated in virulence. The two-component peptide signaling system consists of the SilA response regulator and SilB histidine kinase along with the SilCR signalling peptide and SilD/E export/processing proteins. The presence of an associated bacteriocin region suggests this system may play a role in competitive interactions with other microbes. Comparative analysis of 43 *Streptococcus* Anginosus/Milleri Group (SMG) genomes reveals this to be a hot spot for genomic variability. A cluster of bacteriocin/immunity genes is found adjacent to the *sil* system in most SMG isolates (typically 6-10 per strain). In addition, there were two distinct SilCR peptides identified in this group, denoted here as $SilCR_{SMG\text{-}A}$ and $SilCR_{SMG\text{-}B}$, with corresponding alleles in *silB*. Our analysis of the 43 *sil* loci showed that $SilCR_{SMG\text{-}A}$ is only found in *S. intermedius* while all three species can carry $SilCR_{SMG\text{-}B}$. In *S. intermedius* B196, a putative SilA operator is located upstream of bacteriocin gene clusters, implicating the *sil* system in regulation of microbe-microbe interactions at mucosal surfaces where the group resides. We demonstrate that *S. intermedius* B196 responds to its cognate $SilCR_{SMG\text{-}A,}$ and, less effectively, to $SilCR_{SMG\text{-}B}$ released by other Anginosus group members, to produce putative bacteriocins and inhibit the growth of a sensitive strain of *S. constellatus*.

Keywords

Streptococcus Milleri Group, *Streptococcus* Anginosus Group, *sil* system, quorum sensing, bacteriocins, cell-cell signaling

Introduction

The *Streptococcus* Anginosus/Milleri Group (SMG) is a group of three distinct yet closely related species: *S. anginosus*, *S. constellatus* and *S. intermedius*. The species *S. anginosus* has two subspecies, *S. anginosus* subsp. *anginosus* and *S. anginosus* subsp. *whileyi* (Jensen et al. 2013). The species *S. constellatus* has three subspecies, *S. constellatus* subsp. *constellatus*, *S. constellatus pharyngis* and *S. constellatus* subsp. *viborgensis* (Jensen et al. 2013). In humans, the SMG lead a dual lifestyle as both commensals and pathogens. These strains can be found asymptomatically colonizing the oral cavity as well as the gastrointestinal and urogenital tracts (Poole and Wilson 1979; Whiley et al. 1992; Jacobs et al. 1995). However, they are also important pyogenic pathogens involved in empyema and soft tissue abscesses (Ruoff 1988b; Whiley et al. 1992; Coman et al. 1995; Laupland et al. 2006; Ripley et al. 2006; Siegman-Igra et al. 2012; Asam and Spellerberg 2014) as well as infections of the lower airways (Shinzato and Saito 1995; Sibley et al. 2008; Parkins et al. 2008). The SMG can be difficult to culture and identify (Ruoff 1988b; Sibley et al. 2010) and are underappreciated pathogens. They have been associated with more invasive pyogenic infections than Group A and Group B *Streptococcus* combined (Laupland et al. 2006; Siegman-Igra et al. 2012).

Host colonization involves competition with resident microorganisms. One mechanism by which streptococci inhibit closely related bacteria is through short peptides called bacteriocins (Dawid et al. 2007). Variable bacteriocin and putative bacteriocin genes are found adjacent to the *sil* locus in GAS, and these are predicted to be under the

regulatory control of SilA (Belotserkovsky et al. 2009). The Streptococcus Invasion locus (*sil*) system was first identified in Group A Streptococci (GAS), where a transposon insertion in the *sil* system attenuated virulence in a murine model (Hidalgo-Grass et al. 2002). The core signaling system has been characterized and contains the cell-cell signaling peptide SilCR, a peptide processing/export system (SilD/E), and two component sensing system (SilA/B). Upon sensing pheromone peptide SilCR, the histidine kinase SilB phosphorylates the response regulator SilA, upregulating genes involved in SilCR production. These include the SilCR peptide itself and ABC transporters, SilD and SilE. SilC is encoded on the antisense strand of *silCR* and its expression represses expression of SilA-inducible genes in GAS (Hidalgo-Grass et al. 2002; Eran et al. 2007). SilA/B transcriptional regulation is uncharacterized, but induction of the *sil* system is dependent on its presence and expression (Eran et al. 2007). The system also includes a putative CAAX protease, thought to be involved in immunity against or maturation of bacteriocins.

The *sil* system has been implicated in virulence in GAS (Hidalgo-Grass et al. 2002; Salim et al. 2008). Disruption of *silC* leads to attenuation in a murine model (Hidalgo-Grass et al. 2002). As SilC represses SilCR induction, the lack of the pheromone peptide appears to favour pathogenesis. However, interspecies communication occurring between GAS and Group G streptococci (GGS) via SilCR peptide positively regulates SilCR and bacteriocins (Belotserkovsky et al. 2009). Thus, regulation of this system in streptococci is complex and can impact pathogenesis in multiple ways.

The *sil* system has not been characterized in SMG. Here we describe the *sil* system in 43 SMG genomes, outlining species-specific variation and identification of novel putative bacteriocins. We note this locus as a hotspot for genetic variability with most strains featuring a large cluster of putative bacteriocin/immunity genes. In addition, we have explored SilCR-mediated competition between SMG strains using *S. intermedius* B196 as a representative strain.

Materials and Methods

Strains

Forty-five assembled SMG genomes were used for our analysis (Table 5.S1 in Supplementary Files). Seven of these were previously published complete, closed genomes (C1050, C1051, C818, C232, C270, C238, B196; (Olson et al. 2013)), and 26 were unpublished draft genomes (see below). The strains of these 26 new genomes have been described in Kaiser et al. 2014. The *sil* regions will be deposited in GenBank (accession numbers to be available soon). Twelve draft genomes were also downloaded from NCBI (Benson et al. 2015) (ADME01000005.1, AP013072.1, ATFK01000001.1, AJKN01000015.1, AICP01000048.1, AFXO01000004.1, NC 018073.1, AFUP01000001.1, AICQ01000033.1, AFXN01000007.1, AREF01000001.1, AECT01000012.1),

Assembly

The in-house draft genomes were all sequenced using NexteraXT libraries and Illumina MiSeq using either 150 or 250 bp paired-end reads. The sequences were assembled using our in-house genome assembly pipeline (Surette Lab Assembly Pipeline,

or SLAP). SLAP preprocesses the reads by trimming adapters with Cutadapt (Martin 2011) and performing quality trimming and error correction with sga preprocess and sga correct (Simpson and Durbin 2012). It then estimates an optimal kmer size using an in-house adaptation of the program Kmergenie (Chikhi and Medvedev 2014), and calls four separate assemblers (MaSuRCA (Zimin et al. 2013), IDBA (Peng et al. 2011), SPAdes (Bankevich et al. 2012), and Velvet (Zerbino and Birney 2008)) to produce four candidate assemblies. The assemblies are then scaffolded using SSPACE (Boetzer et al. 2011) and quality metrics are provided to the user via FastQC (Andrews, 2010) for read quality and QUAST (Gurevich et al. 2013) for assembly quality. For our purposes, the best assembly was chosen for each strain based on N50 value and total assembly length. All in-house code is available from the authors upon request.

Identifying the putative *sil* region in SMG

The putative *sil* system was identified in the published genome *S. intermedius* B196 (Accession number: NC_022246.1) based on its structural similarity to the GAS and GGS *sil* locus. We used megablast (Altschul et al. 1997) to find each gene from this putative *sil* locus in the 44 additional SMG genomes. We found the genes together on a single contig in 43 genomes (aside from *S. intermedius* B196) and split across multiple contigs in two genomes (M1 and C424, which we excluded from our analysis). A list of the 43 strains used for our analysis is provided in Table 5.S1. The 43 putative *sil* regions were annotated from the BLAST hits using in-house Python and Biopython scripts (van Rossum and Drake, 2001; Cock et al. 2009) with manual adjustments, and the annotations were visualized and compared using Geneious (Kearse et al. 2012).

Phylogenetic trees

A phylogenetic tree of the *sil* locus was generated using MrBayes (Huelsenbeck and Ronquist 2001; Altekar et al. 2004). Each gene (*silA* through *silD*) was aligned individually using MUSCLE (Edgar 2004) and the alignments were concatenated (with gaps in place of the nucleotides for missing genes), providing a single alignment of all genes. The genes were each assigned their own partition in the MrBayes model, with a 4-by-4 General Time Reversible evolutionary model (Tavare 1986) and invariant/gamma rates distribution (Yang 1994) for each partition. Since many sequences were missing individual genes, the presence or absence of each gene was encoded as binary "standard" data in a separate partition (Lewis 2001). The trees were run until the average standard deviation of the split was less than 0.01 and the consensus tree was visualized using the Interactive Tree of Life (iTOL) (Letunic and Bork 2016)

Annotating the bacteriocin region

The putative bacteriocin region adjacent to the *sil* region was annotated using the online version of antiSMASH (Blin et al. 2013; Blin et al. 2014) accessed November of 2013. Only the results of the antiSMASH-internal GLIMMER (Delcher et al. 1999) annotation were used. In order to determine how variable the accessory regions are, the sequences from the GLIMMER hits were translated and preliminary clustering was undertaken using OrthoMCL (Fischer et al. 2011). This produced 16 putative orthologous groups, here referred to as ORF1 through ORF16. To identify any pseudogenes or related ORFs not discovered by GLIMMER, we manually divided each cluster into smaller groups based on the MUSCLE alignment of the cluster (if multiple groups were apparent)

and generated a consensus sequence for each group. We searched naively for each of these consensus sequences in all of the bacteriocin regions (tolerant to 20% mismatch at the amino acid level).

We generated a tree for each of the 16 ORF clusters produced by OrthoMCL using FastTree (Price et al. 2009). The location of each putative accessory gene in each strain was marked and patterns of synteny were manually examined.

We searched for each ORF in the non-redundant protein sequences (nr) database with Blastx (Altschul et al. 1997) to identify putative functions. In addition, each ORF was searched against the BACTIBASE database (Hammami et al. 2007; Hammami et al. 2010) of bacteriocins to identify hits with existing bacteriocins.

Bacterial culturing conditions

We cultured *S. intermedius* B196 and *S. constellatus* M505 on Todd Hewitt agar with 0.5% yeast extract (THY) and incubated at 37°C in a 5% CO_2 incubator for 3 days. We inoculated THY broth with colonies and incubated either at 5% CO_2 or anaerobically (5% CO_2, 5% H_2 90% N_2) at 37°C overnight. We used broth cultures to conduct further experiments. THY supplemented with 75 μg/ml spectinomycin (THY-spec) was used to grow our knockout strain (see below). We used *Escherichia coli* Top10 chemically competent cells (Life Technologies) during cloning. *E. coli* carrying desired knockout constructs grew on Luria-Bertani agar with 100 μg/mL spectinomycin (LB-spec).

Identification of SilA binding sites in *S. intermedius* B196

Binding of response regulator SilA to direct repeats in GAS and GGS affects expression of *sil* locus components as well as putative bacteriocins (Hidalgo-Grass et al.

2002). The SilA binding site in GAS (ACCATTCATG-11bp-ACCTTTTAAG) (Belotserkovsky et al. 2009) was used as a query to identify putative sites in *S. intermedius* B196 using the motif search in Geneious (Kearse et al. 2012). The ClustalW 2.1 alignment of these sites (Goujon et al. 2010) was used to generate an *S. intermedius* B196 consensus sequence, visualized using WebLogo (Crooks et al. 2004).

SilCR knockout construction

We constructed a deletion mutant of *silCR* in *S. intermedius* B196. First, we cloned the spectinomycin resistance marker from pDL278 (Dunny et al. 1991) along with its promoter into pUC19 with primers (specF - aaaaaggatccgacgaagaggatgaagagg (*Bam*HI) and specR -aaaaagtcgacccaaatattaaataataaaac (*Sal*I)) with restriction sites underlined. We then cloned the upstream and downstream regions of *silCR* in *S. intermedius* on either side of a spectinomycin resistance marker in pUC19 using primers (SilCRupF –aaaaagagctccaaaaagcaatactgaagt (*Sac*I), SilCRupR – aaaaggatcccatgtattattataacccgac (*Bam*HI) and SilCRdownF – aaaagtcgacctaaaaatacaaataacctcattg (*Sal*I), SilCRdownR – aaaagcatgccttctctctatttttacatcag (*Sph*I)) with restriction sites underlined. We amplified the cassette (*silCR* upstream: *specR* : *silCR* downstream) using PCR with primers SilCRupF and SilCRdownR and purified it. Our laboratory has found that *S. intermedius* B196 is naturally competent and can be transformed using the competence stimulating peptide, ComC (DSRIRMGFDFSKLFGK; Lacroix 2014). An overnight THY broth culture was diluted 1000 fold in 500 μL THY and incubated for 2 hours at 37°C in 5% CO_2 before adding peptide (10 ng) and purified cassette DNA (500 ng). The reaction was incubated in

normal growth conditions for an hour before plating the transformation reaction on THY + spectinomycin (75 μg/ml). We incubated the plates for 2 days anaerobically at 37°C and screened colonies using PCR and sequencing to verify the deletion.

Bacteriocin activity assays

To assess bacteriocin activity, we adapted top-agar overlay experiments (Kormin et al. 2001; Maricic and Dawid 2014). *S. intermedius* B196 (the bacteriocin producer) and *silCR* mutant strain were grown in an overnight anaerobic broth culture as previously described. A volume of 4 μL of the overnight culture was spotted on THY agar and incubated in the anaerobe chamber at 37°C for 2 days. In some cases, synthetic SilCR peptide was spotted along with culture. The amount of peptide added was dependent on the experiment. *S. constellatus* M505, which lacks the bacteriocin cluster and *caax* gene, was used as a bacteriocin-sensitive strain. This was grown anaerobically in THY for 24 hours. Top agar (THY) was prepared using 1.5% agar as this higher percentage improved the visualization of the zone of clearing. A 100 μL of overnight broth culture of *S. constellatus* M505 was added to 5 mL molten Top agar and inverted 3 times before pouring onto the agar plates with *S. intermedius* spots. Plates were incubated for 1-2 days at 5% CO_2 at 37°C.

Results

Identification of the *sil* system in *S. intermedius* B196

We have previously sequenced and annotated the genome for the clinically isolated strain *S. intermedius* B196 (Olson et al. 2013). We identified a region with structural similarity to the *sil* region previously identified in GAS and GGS. This region

contains five of the six genes included in the GAS/GGS *sil* locus (*silA, silB, silCR, silD,* and *silE*), with the same relative positioning and orientation (**Figure 5.1A**). The locus is bounded by a putative D-Ala-D-Ala carboxypeptidase (SIR_RS15545) at one end and *sodA* at the other. Adjacent to the β-lactamase is a conserved hypothetical protein (SIR_RS15550) that has a thioredoxin-like domain. This is followed by 13 small ORFs containing putative bacteriocins, followed by a gene with homology to CAAX proteases. Downstream of the CAAX protease are the *sil* genes including *silA*, *silB*, *silCR*, *silD* and *silE*. We did not find evidence for a counterpart to *silC* in *S. intermedius* B196 based on nucleotide similarity to GAS *silC*. While there is an open reading frame on the antisense strand of SilCR, there is no evidence that it is transcribed or plays any role in *sil* regulation. More research is required to conclude if this is *silC*.

Comparison between the *S. intermedius* B196 and GAS *sil* systems revealed sequence divergence despite the overall similar organization of the *sil* loci. Although the putative *sil* region in *S. intermedius* B196 shares its structure with that found in *S. pyogenes* AF493605.1 (Hidalgo-Grass et al. 2002), these genes have low rates of sequence identity. *S. intermedius* B196 SilA is only 49.4% identical (85.7% similar) to GAS SilA in amino acid sequence (60.0% nucleotide identity). B196 SilB is 34.6% identical and 73.9% similar to GAS SilB (53.7% nucleotide identity). B196 SilCR is 28.0% identical and 68.0% similar to GAS SilCR (47.8% nucleotide identity). B196 SilD is 56.2% identical and 90.3% similar to GAS SilD (63.6% nucleotide identity). Finally, B196 SilE is 74.5% identical and 88.6% similar to GAS SilD (68.5% nucleotide identity).

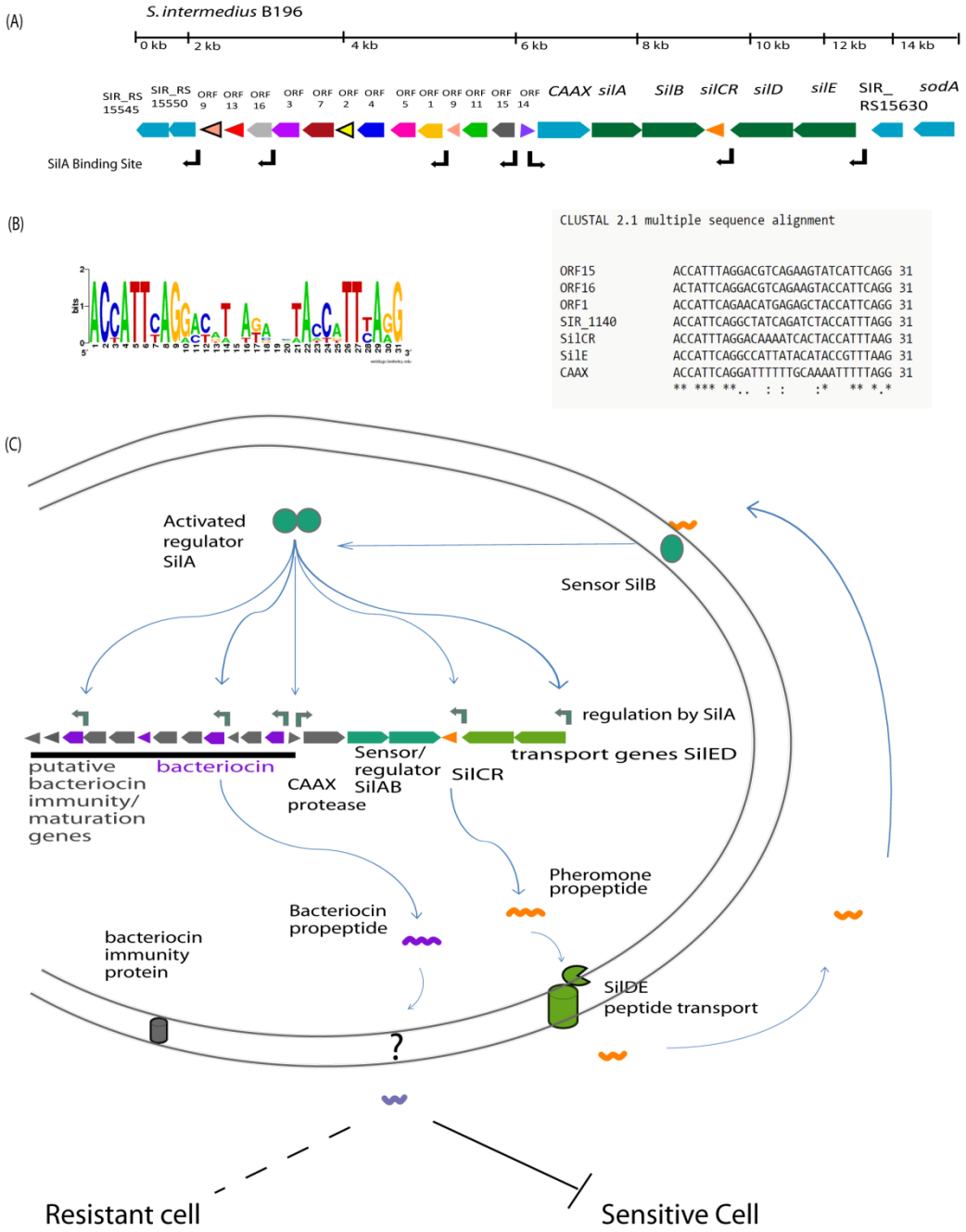

Figure 5.1: The predicted *sil* system in *S. intermedius* B196. (A) The organization of the *sil* locus showing the signal peptide gene (SilCR) and signaling transduction genes (*silAB* and *silED*) in green. The associated locus genes including putative CAAX protease is shown in blue and bacteriocin associated gene clusters are indicated in color. The positions of predicted SilA binding sites are indicated below. (B) The predicted SilA binding site and consensus sequence. (C) Schematic illustration of the *sil* regulatory network in the *Streptococcus* Anginosus Group based on the *sil* system in Group A *Streptococcus* (Eran et al. 2007; Belotserkovsky et al. 2009).

In other streptococci, the *sil* system and putative bacteriocins are transcriptionally regulated by response regulator SilA and the SilCR pheromone peptide (Hidalgo-Grass et al. 2002). The SilA binding site in GAS (ACCATTCATG-11bp-ACCTTTTAAG) was used to find putative SilA binding sites in the *S. intermedius* B196 *sil* locus, as highlighted in **Figure 5.1A**. Direct repeats were found upstream of *silCR*, *silE*, *caax*, *SIR_RS15550* and in the accessory region upstream of *ORF1*, *ORF15* and *ORF16*. The predicted operator was conserved in all of these genes except CAAX protease, which had inconsistencies in the second repeat (**Figure 5.1B**).

A schematic for the hypothetical regulation of the *sil* system in SMG is shown in **Figure 5.1C**. SilCR can be produced by SMG species and exported. Sensing of extracellular SilCR peptide induces SilB to autophosphorylate and phosphorylate response regulator SilA. SilA, in turn, induces expression of the seven genes indicated in **Figure 5.1B**. Induction of these genes is predicted to amplify the response via SilCR dependent autoregulation and induce production of bacteriocins which can inhibit closely related bacteria.

Bacteriocin activity in *S. intermedius* B196 is regulated by SilCR

We investigated the role of the *sil* system in inter-species competition using *S. intermedius* B196 as a model. A putative SilA binding site was found upstream of putative bacteriocins ORF1, ORF15 and ORF 16 (**Figure 5.1**). To investigate whether SilCR regulates bacteriocin expression in *S. intermedius* B196, we constructed a *silCR* deletion mutant. We assayed bacteriocin activity by spotting *S. intermedius* B196 and its mutant on THY agar and after growth, applying a top agar overlay of a sensitive strain, *S.*

constellatus M505. Strain M505 was chosen because it lacks the *sil* accessory region (see below) and we therefore predicted it would lack immunity genes and be sensitive to SilCR-dependent bacteriocins produced by B196. When M505 was overlaid in top agar over B196, a clear zone of growth inhibition was observed (**Figure 5.2A**). This activity is lost in the B196 Δ*silCR* mutant, (*S. intermedius* B196 Δ*silCR*; **Figure 5.2A**). Exogenous addition of the synthetic SilCR peptide from B196 ($SilCR_{SMG\text{-}A}$) restored the wildtype phenotype, demonstrating that SilCR induces production of an inhibitor of strain M505.

Comparative genomic analysis of the *sil* locus separates SMG strains into two *silCR* peptide groups

We investigated the distribution of the *sil* system in SMG. Forty five SMG strains were used in our analysis: a combination of in-house sequenced genomes and those available online. Only genomes containing the *sil* genes on a single contig were analyzed (43 of the 45 genomes; as listed in **Table 5.S1**).

Initial investigation of the sequences divided SMG strains into two groups. In 24 of the 43 genomes, both *silB* and *silCR* appeared truncated; *silB* at the 5' end and *silCR* at the 3' end. Further examination, however, found that the 5' end of *silB* was well conserved in 22 of the 24 strains. In addition, results from a BLAST search of this region showed high identity with histidine kinases, which is consistent with the putative function of SilB. Indeed, the gene had been annotated as such in at least one of the published genomes in our data set (protein accession YP 008494978 from genome C232), implying that we had found a new variation of *silB*. These 22 strains also had a well-conserved

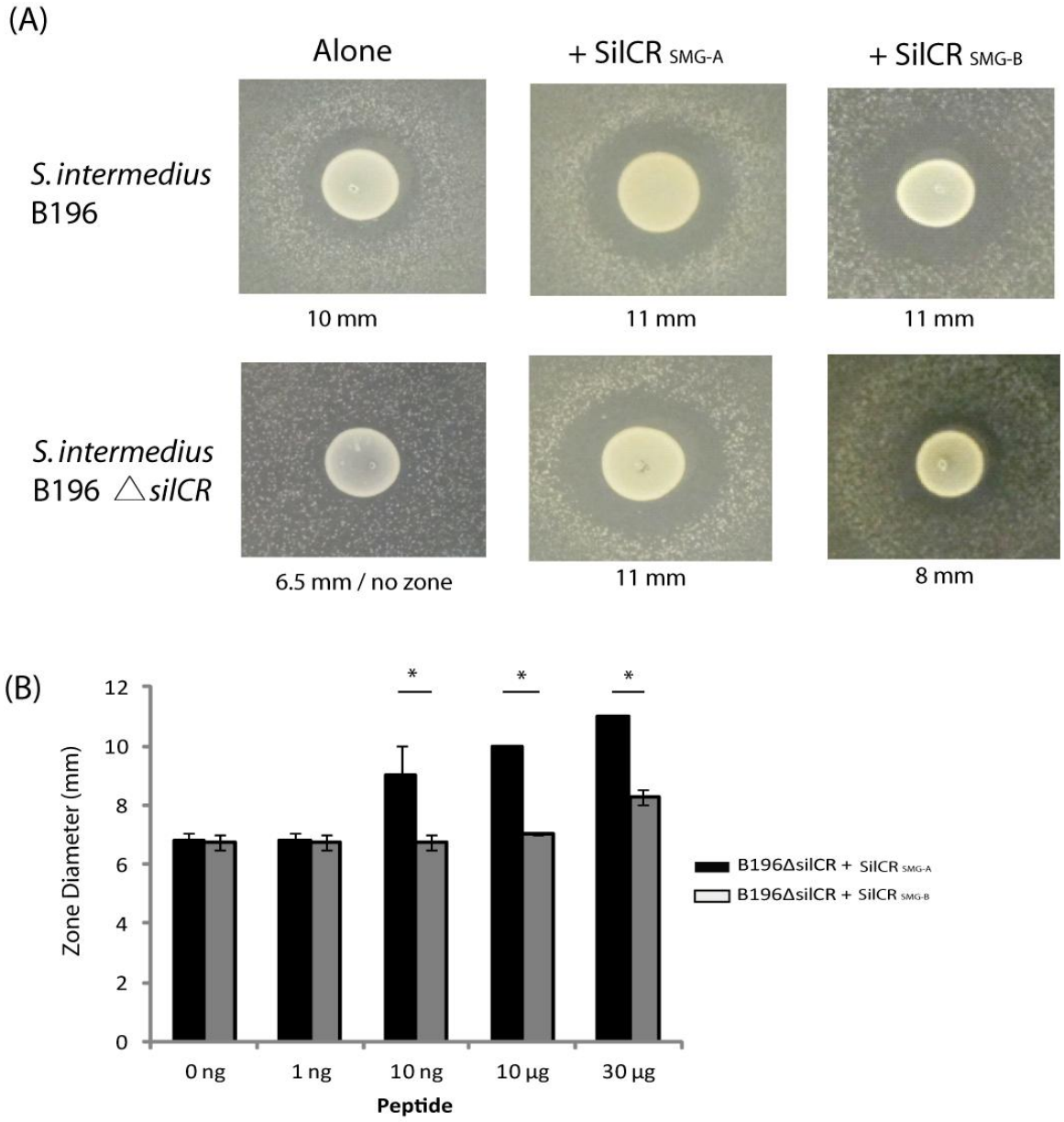

Figure 5.2: Bacteriocin-mediated competition in *Streptococcus intermedius* is controlled by the SilCR peptide. The gene for the pheromone peptide SilCR was deleted in *S. intermedius* B196 (*S. intermedius* B196 Δ*silCR*). (A) Competition in the wildtype and mutant was ascertained using an overlay of sensitive strain *S. constellatus* M505. The two identified SMG SilCR peptides were exogenously added to wildtype and mutant and the diameter of the inhibition zone measured as shown. (B) A concentration dependent induction of inhibition by $SilCR_{SMG-A}$ and $SilCR_{SMG-B}$ in *S. intermedius* B196. Note the B196 colony diameter is ~6.5mm. $SilCR_{SMG-A}$ induced zones are significantly larger than those with $SilCR_{SMG-B}$ ($p<0.005$)

region following their truncated *silCR*. A stop codon located 150 bp from the start of *silCR* was used to define the "new" putative *silCR*. Thus, we have identified two distinct SilCR peptides in SMG, $SilCR_{SMG-A}$ and $SilCR_{SMG-B}$ (previously truncated version), with predicted mature amino acid sequences GWLEDLLKHFSGYNSLTKGDSNNTLG and GWLEDLFSPYLKKYKLGKLGQPDLG, respectively. Notably, each SilCR peptide allele associated exclusively with a corresponding variant of histidine kinase SilB. This suggests that the response by a strain should be dependent on the kinase variant. The conserved 5' end of SilCR propeptide is hypothetically cleaved at the double glycine residues to produce the mature SilCR peptide.

To investigate whether *S. intermedius* B196 could respond to the alternative peptide, $SilCR_{SMG-B}$ was added to the knockout (**Figure 5.2A**). Despite high peptide concentrations, $SilCR_{SMG-B}$ induced a smaller inhibitory zone in M505 than did $SilCR_{SMG-A}$ (8mm vs. 11 mm). To test the sensitivity of strain B196 to the two peptides, several concentrations of each peptide were used. We found that higher concentrations of $SilCR_{SMG-B}$ were required to produce inhibitory zones comparable to those produced by $SilCR_{SMG-A}$ (**Figure 5.2B**). Thus, strain B196 can sense and respond to both pheromone SilCR peptides produced by SMG. However, its inhibitor production is SilCR peptide- and concentration- dependent. It requires a higher concentration of the non-cognate signaling peptide ($SilCR_{SMG-B}$) to elicit a response.

To investigate the phylogeny of the *sil* locus in the 43 SMG genomes, the gene presence, sequence, and orientation were used to construct a tree (**Figure 5.3**). The tree of *sil* genes corresponded well with SMG species. Six different organizations of the *sil* locus

were noted in SMG, as depicted in the locus arrangement legend in **Figure 5.3**. The type of SilCR peptide the strain produced ($SilCR_{SMG\text{-}A}$ vs. $SilCR_{SMG\text{-}B}$) is also indicated in the figure. Strains encoding $SilCR_{SMG\text{-}A}$ have low diversity overall and are monophyletically nested within *S. intermedius*, while all three species can encode $SilCR_{SMG\text{-}B}$. In 33 of the 43 *sil* loci, we identified the canonical arrangement of the *sil* locus (locus arrangement A in **Figure 5.3**). This arrangement is found in strains carrying $SilCR_{SMG\text{-}A}$ and $SilCR_{SMG\text{-}B}$ and is the arrangement seen in *S. intermedius* B196 (**Figure 5.1A**). The additional *sil* locus arrangement groups are characterized by the loss of genes and when SilCR is present, only included $SilCR_{SMG\text{-}B}$. Locus group B is defined by an intact *sil* region but is altered next to *silE* with *sodA* absent and includes a single *S. anginosus* strain. The other arrangements of the *sil* region (C-D) are represented by deletions leading to loss of core *sil* genes. Group C consists of a single *S. constellatus* strain and is defined by missing *silE* and *sodA*. Group D is represented by one *S. constellatus* strain and one *S. intermedius* strain and is characterized by missing a number of *sil* locus components including the putative bacteriocin region, CAAX protease and *silA* response regulator. Group E is composed of only *S. anginosus* strains, and is characterized by the lack of all *sil* genes and accessory proteins except *silD*, *silE*, and *sodA*, with an inversion in *silD* and *silE* relative to *sodA*. Group F includes a single *S. anginosus* strain and is identical to Group E but missing *silE*. Overall, the *S. anginosus* strains had the most variation in composition of the *sil* locus with all locus groups represented except Groups C and D.

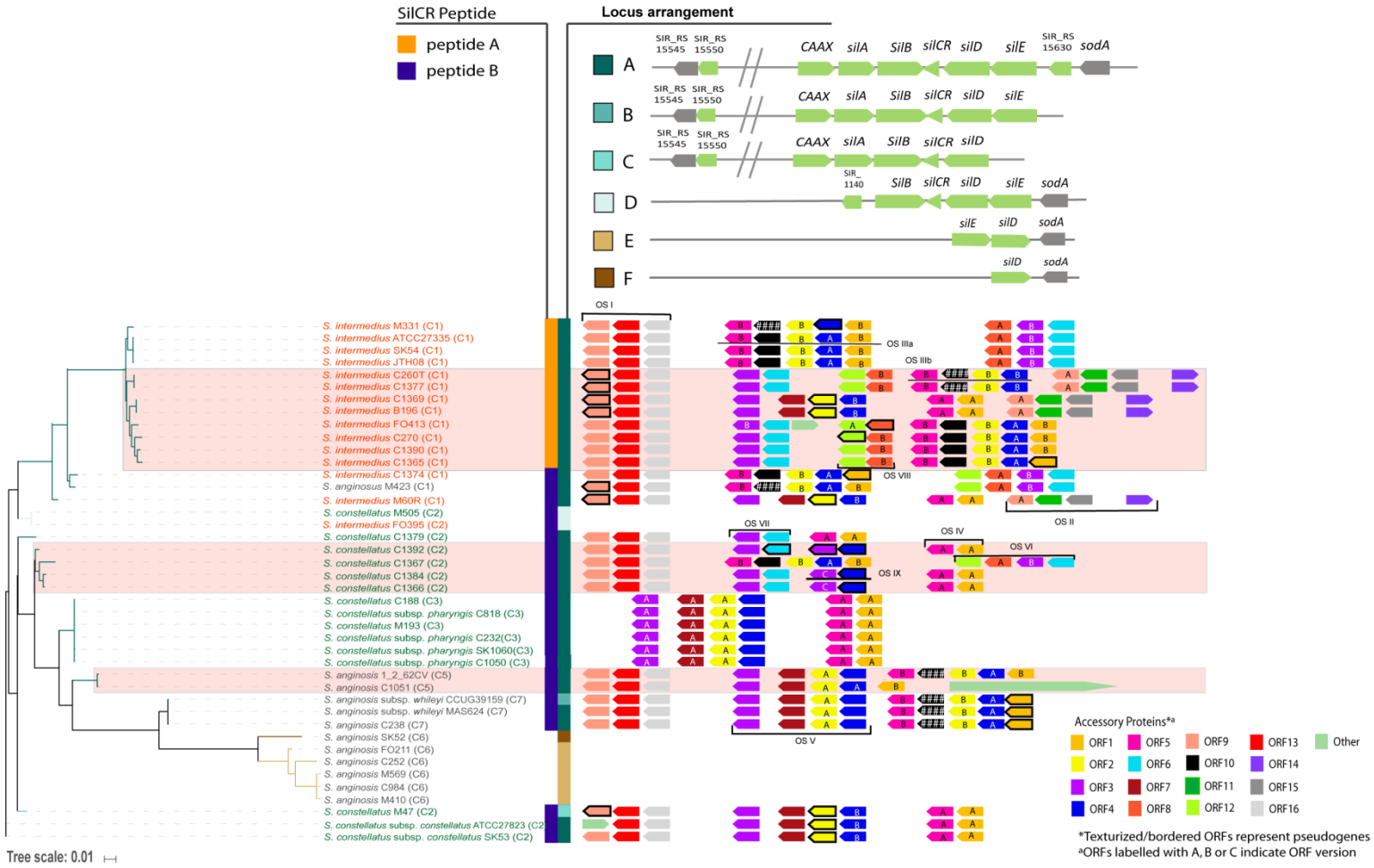

Figure 5.3: *sil* locus heterogeneity in the *Streptococcus* Anginosus Group (SMG). A tree was constructed based on *sil* genes in 43 strains. Six locus arrangements were found (shown in locus arrangement legend with the locus annotation). Hashes (//) in the annotation indicate a putative bacteriocin accessory region. Two SilCR peptides were identified, shown in the peptide legend. SMG species text is colour coded (*S. intermedius* in orange, *S. anginosus* in grey and *S. constellatus* in green). The accessory region ORFs are shown with the tree. Some ORFs have several versions, indicated by the letter inside the ORF.

Heterogeneity in the *sil* accessory bacteriocin region implies strain specific competition

The *Streptococcus sil* system controls expression of competitive bacteriocins in GAS (Eran et al. 2007; Belotserkovsky et al. 2009). In *S. intermedius* B196, we observe that bacteriocin activity is dependent on this signaling system. We discovered a highly variable accessory region in all strains carrying *sil* locus groups A-C (35 of the 43 genomes). We investigated the hypothetical open reading frames (ORFs) in this region for putative bacteriocin and immunity genes. Sixteen distinct orthologous groups were identified using OrthoMCL. A detailed similarity comparison within each orthologous group is included in the **Supplementary Files**. The amino acid identity heatmap for each ORF is shown in the supplementary figures. In general, while some ORFs are highly conserved within SMG, others are not. For example, ORF13 is conserved with 97% or higher amino acid identity across 28 strains while ORF2 is clearly divided into two groups with one group having 44% amino acid identity to the other. Using this classification scheme, it was apparent that some strains had two copies of predicted ORFs (e.g. ORF 4 in *S. anginosus* 1_2_62CV; **Figure 5.3**). It is important to note that the two copies are not identical and, given their patterns of synteny, it is not likely that they are functionally equivalent.

We assigned putative functions to the 16 ORFs identified based on similarity to hits in the non-redundant protein sequence database using BlastX (NCBI). These are summarized in **Table 5.1**. In total, six putative bacteriocins were identified (ORF1, ORF2, ORF6, ORF8, ORF15 and ORF16). Of the six bacteriocins, two were highly

Table 5.1: Bioinformatic analysis of identified putative bacteriocin accessory region ORFs using a sequence search against the non-redundant protein sequence database using BlastX (NCBI)

ORF	BlastX best hit	Cover	Identity	Score	Evalue	Accession
1	Bacteriocin	99%	99%	145	3e-43	WP_006267535.1
2	Bovicin 255 variant	71%	62%	85.9	2e-20	AAR02622.1
3	Role in replication	97%	67%	119	4e-32	WP_030127173.1
4	Bacteriocin secretion protein	57%	37%	46.2	7e-04	EWM55695.1
5	Hypothetical protein	98%	88%	145	5e-43	WP_020998893.1
6	Bacteriocins class II with double glycine leader peptide	88%	54%	67.8	1e-13	KEQ38561.1
7	Bacteriocin immunity protein	98%	99%	194	2e-61	WP_006267076.1
8	Bacteriocin class II with double glycine leader peptide family protein	88%	94%	137	4e-40	WP_009568291.1
9	No hits					
10	No hits					
11	Hypothetical protein	98%	100%	62.4	5e-11	WP_021002964.1
12	No hits					
13	Hypothetical protein	84%	53%	42.7	3e-4	EWM55551.1
14	tRNA (cytidine/uridine-2’-O-)- methyltransferase TrmJ	50%	46%	37.4	0.39	WP_059746670.1
15	ThmA bacteriocin	98%	63%	82	7e-19	KIQ75182.1
16	Bacteriocin BlpU	72%	70%	76.6	5e-17	WP_021143664.1

similar to existing bacteriocins (ORF2 is similar to bovicin 255 and ORF15 is similar to ThmA bacteriocin). The non-bacteriocin ORFs may have chaperone or immunity functions. ORFs 4 and 7 had high similarity with a bacteriocin secretion protein and a bacteriocin immunity gene, respectively. ORF3 has a putative role in replication. ORF14 shares some nucleotide identity with a tRNA (cytidine/uridine-2'-O-)- methyltransferase. A similar methyltransferase tRNA modification can suppress immunostimulation by *Escherichia coli* via Toll–like receptor 7 in addition to affecting tRNA stability and function (Gehrig et al. 2012). It is unknown whether ORF14 serves a similar function in SMG. Six of the putative ORFs did not have predicted functions (ORFs 5, 9, 10, 11, 12, and 13). Characterization of these genes is required to gain insight into their role. However, some speculation based on the patterns of co-occurrence with other genes is possible.

There were some clear patterns of synteny in the accessory regions of the *sil* loci (**Figure 5.3**). These became more apparent after taking the subclusters of each ORF into consideration. We describe the syntenic groupings of ORFs as "ORF sets" (OS) because the operon structures have not been established for these strains. In combination with our putative functional assignments, we attempted to characterize these ORF sets (**Figure 5.S1**) in order to group bacteriocins with co-occurring genes that may be functionally associated with them. All ORF sets except OS IX contain at least one putative bacteriocin (**Figure 5.S1**). Three ORFs occurred only once each across all strains and are labelled "other" (**Figure 5.3**).

A detailed description of each ORF set can be found in **Figure 5.S1**. Associations between ORFs were made based on observed synteny. Putative bacteriocin ORF1A is exclusively found adjacent to ORF5A, while ORF1B is separated from ORF5B by bacteriocin ORF2 and its associated genes. The two versions of putative bacteriocin ORF2 were found in two distinct clusters, OS III and OS V. ORF2 is always associated with a version of the putative secretion protein, ORF4. It is also always adjacent to either the putative immunity gene ORF7 or the unknown ORF10, suggesting an immunity function for the latter. Putative bacteriocin ORF6 is always found adjacent to ORF3, implying ORF3 may confer immunity against ORF6. A version of ORF3 is also found associated with ORF2 in OS V, albeit somewhat farther away. It may be that ORF3, rather than ORF7, confers immunity to ORF2A. Putative bacteriocin ORF8 was found adjacent to ORF12 in most strains (although ORF12 has been pseudogenized in some of these strains). ORF12 was not present in strains lacking ORF8 and may therefore be only required in the presence of ORF8. Uncharacterized ORF9 is associated with two putative bacteriocins (ORF15 and 16) in two separate ORF sets (OS I and OS II). In each case, the genes are also associated with a hypothetical protein of unknown function (ORFs 11 and 13, respectively). None of the strains carried all 6 putative bacteriocins, implying that each strain may be susceptible to at least one bacteriocin. A number of *S. intermedius* strains had five predicted bacteriocins (**Figure 5.3**) implying that this species has high competitive potential. *S. constellatus* C1367 and *S. anginosus* C423 also had five predicted bacteriocins.

The arrangements of ORF sets within the accessory regions correspond imperfectly with the *sil* tree, indicating a large amount of recombination or horizontal gene transfer occurring within this region. OS I is found in a majority of strains. The six strains lacking OS I form a monophyletic group with low genetic distance in the *sil* region, indicating a recent loss event. Areas of apparent recombination are highlighted with pink boxes in **Figure 5.3**. OS I occurs at one end of the bacteriocin accessory region in the strains where it is present. OS II is found on five genomes, in two positions relative to other ORF sets. OS IV typically appears at the downstream end of accessory regions where it is found (except in strains C1369 and B196), while OS III may be at the downstream end or may be upstream of OS VI. OS V occurs either upstream of OS II or OS III. In either case it is directly downstream of OS I.

DISCUSSION

In GAS and GGS, the streptococcus *sil* system senses and responds to pheromone peptide SilCR with induction of endogenous SilCR production and expression of bacteriocins. In some streptococci, expression of *silCR* is inhibited by expression of *silC*, which is encoded on the antisense strand at the 3' end of *silCR*. In those organisms it has been shown that *silC* expression can repress SilCR-activated promoters (Hidalgo-Grass et al. 2002; Eran et al. 2007). No conserved ORF across all *silCR*+ strains of SMG was found and more research is required to identify if there is a *silC* variant in SMG.

The prevalence of the *sil* locus within GAS is low, with only 4 out of the 19 fully sequenced genomes of GAS carrying it. Remnants of the locus have been left behind in strains that do not (Kizy and Neely 2009; Michael-Gayego et al. 2013; Jimenez and

Federle 2014). The prevalence is higher in GGS, with all sequenced strains carrying a functional SilCR gene (Belotserkovsky et al. 2009; Michael-Gayego et al. 2013). Our analysis of SMG showed that the majority of the strains sequenced carry the locus. Locus arrangement Group A and B in **Figure 5.3** shows that all components of the locus are present in most strains. The other groups included few strains and, like GAS, showed loss of some *sil* genes. Further study is needed to determine whether the lack of a fully functional *sil* system affects a strain's competitive fitness or ability to colonize or invade.

In SMG, considerably higher genetic heterogeneity was seen in the bacteriocin accessory region than in the *sil* gene region. *S. anginosus* displayed the most variation in the presence of *sil* genes, implying that the locus may not be under stabilizing selection and may not be required for competition in this species. Conversely, most *S. intermedius* and *S. constellatus* strains were included in locus group A, suggesting that the *sil* locus may be competitively necessary in these species, at least in the clinical context from which these strains were isolated.

The bacteriocins identified in the SMG *sil* locus have not been characterized or directly associated with intra- or interspecies competition; however, the identification of six putative bacteriocins within the *sil* locus in SMG is a new finding. Bacteriocins can mediate competition with closely related bacteria and can provide a competitive advantage during *in vivo* colonization experiments (Dawid et al. 2007). ORF2 is a homolog of bovicin 255, which has been shown to inhibit growth of select streptococci (Whitford et al. 2001). ORF15 is homologous to thermophilin 13 bacteriocin (ThmA), which is produced by *S. thermophilus* and can inhibit a broad range of Gram-positive

bacteria including spore formers (Marciset et al. 1997). In addition to these genes, four more putative bacteriocins were identified and remain to be characterized. The species selectivity of these putative bacteriocins in SMG is currently being investigated.

Interspecies SilCR induction of SilA-responsive genes has been described previously between GAS and GGS (Belotserkovsky et al. 2009). Inter- and intra- species competition in streptococci can occur in a polymicrobial environment such as the oral cavity. The hypothetical mature $SilCR_{SMG\text{-}A}$ and $SilCR_{SMG\text{-}B}$ peptides are 26 and 25 amino acids respectively, while the mature GAS peptide is 17. Both SMG immature peptides have the same 6 amino acids at their 5' end but vary at their 3' end. The SMG SilCR peptides are not very similar to the GAS and GGS SilCR peptides in either sequence or length and it remains to be determined if these species' *sil* systems are cross-reactive.

Our data demonstrate that *S. intermedius* B196 can sense and respond to $SilCR_{SMG\text{-}B}$, which can be carried by all three SMG species; however, a much higher concentration of the foreign peptide $SilCR_{SMG\text{-}B}$ was required to rescue the phenotype in B196Δ*silCR* than the B196-native version ($SilCR_{SMG\text{-}A}$). This implies a divergent co-evolution of the *silCR* and *silB* genes in the SMG-A clade, with selection for self-detection.

SilCR expression has been shown to attenuate virulence in a mouse model of necrotizing fasciitis (Hidalgo-Grass et al. 2002; Hidalgo-Grass et al. 2004). However, SilA has also been shown to promote expression of virulence-associated genes including streptolysin S, iron transporter SiaA and serine protease ScpC (Salim et al. 2008). It is unknown whether the *sil* system in SMG affects its pathogenicity. This system is unusual

in that it can contribute to both infection in the host and bacterial competition depending on the environment. Given the host-specificity of SMG, and the commensal and pathogenic roles it can play, further analysis of this system could deepen our understanding of SMG competition and its associated clinical diseases.

5. Authors Contributions

All authors listed have made substantial, direct and intellectual contribution to the work and approved it for publication

6. Conflict of interest statement

The research was conducted in the absence of any financial or commercial relationships that could be construed as conflicts of interest.

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Supplementary Files

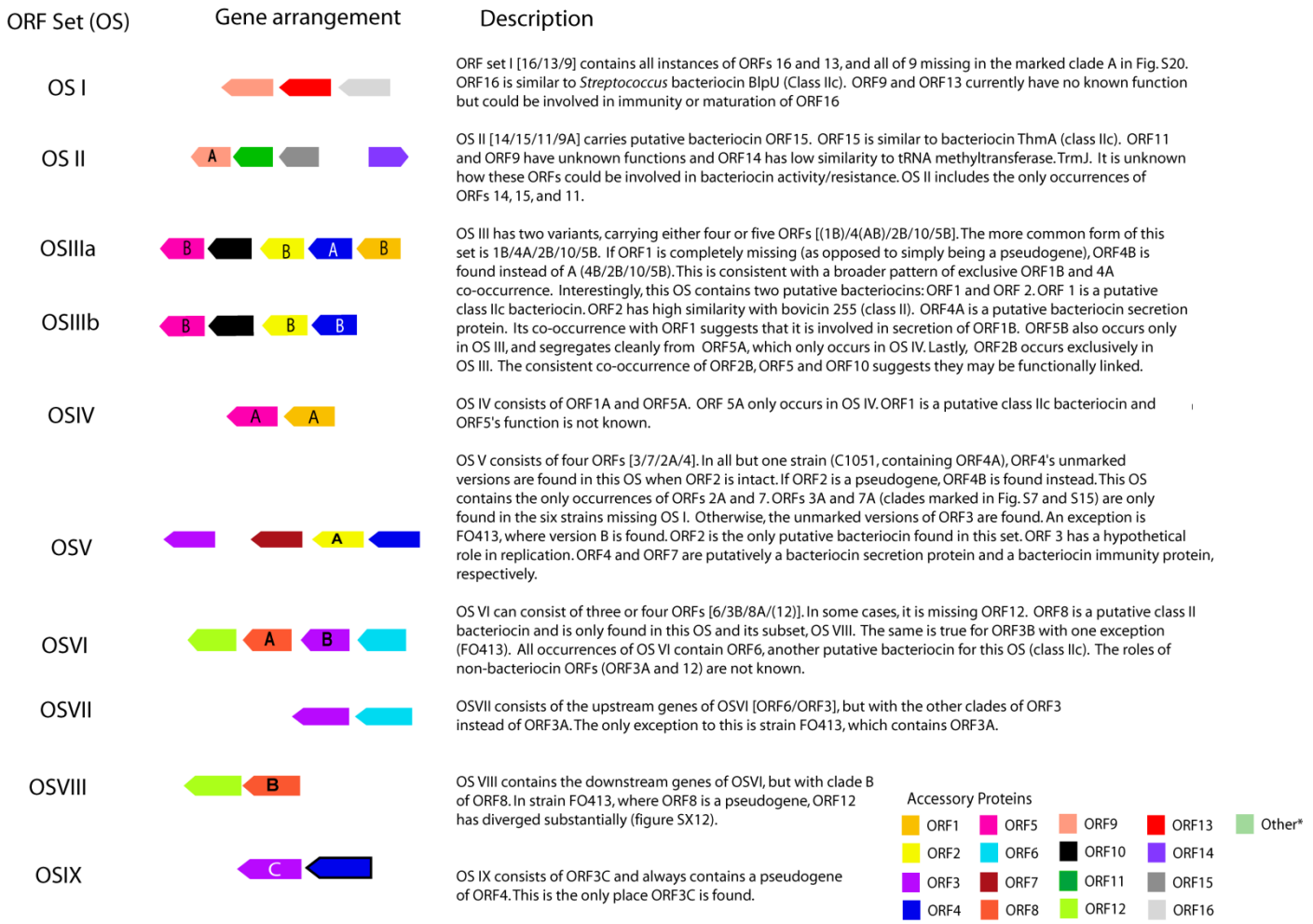

Figure 5.S1: Organization of ORF sets (OS) within the bacteriocin accessory region based on synteny and co-occurrence. antiSMASH Glimmer was used to identify putative ORFs within the bacteriocin accessory region (Delcher et al. 1999; Blin et al. 2013). Sixteen ORFs classes were identified using orthoMCL, which were further divided into subclusters based on genetic distance (A-C labelling inside ORF).

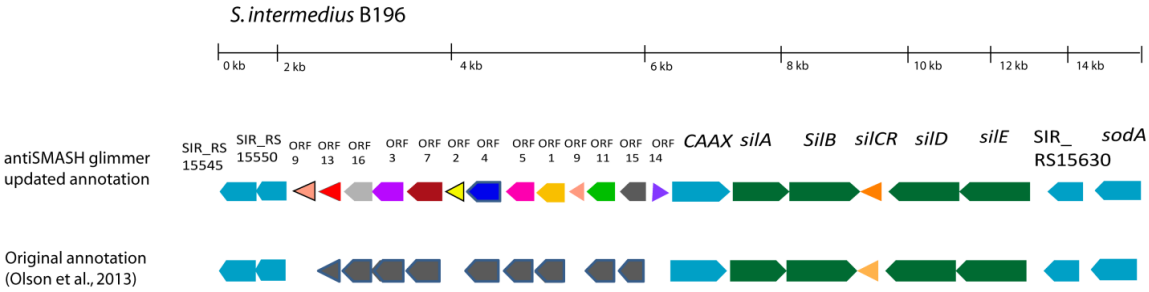

Figure 5.S2: Comparison of annotations for bacteriocin accessory region in *Streptococcus intermedius* B196. antiSMASH glimmer was used to identify all putative ORFs within the accessory region after cross-referencing with other *Streptococcus* Anginosus Group genomes (Delcher et al. 1999; Blin et al. 2013; Blin et al. 2014). This is compared to the original annotation as published in Olson et al., 2013.

Table 5.S1: Strains used in the analysis of *sil* loci in *Streptococcus* Anginosus Group

Strain Name	Isolated from	*sil* Classification	Genome Accession number	Sil Region Accession number
***S. intermedius* C270**	Airway	Group A	CP003858	KY315481
***S. intermedius* C1365**	Blood	Group A		KY315479
***S. intermedius* C1390**	Brain	Group A		KY315480
***S. intermedius* FO413 (AFX001000004)**	Dental Plaque	Group A	AFX001000004	KY315440
***S. intermedius* B196**	Hip abscess	Group A	CP003857	KY315459
***S. intermedius* C1369**	Blood	Group A		KY315460
***S. intermedius* C260**	Invasive	Group A		KY315471
***S. intermedius* C1377**	Blood	Group A		KY315470
***S. intermedius* M331**	Airway	Group A		KY315477
***S. intermedius* JTH08 (NC018073)**	N/A	Group A		KY315476
***S. intermedius* ATCC27335**	Invasive	Group A		KY315473
***S. intermedius* SK54 (AJKN01000015)**	invasive	Group A	AJKN01000015	KY315472
***S. intermedius* C1374**	Blood	Group A		KY315475
***S. intermedius* M60R**	Exacerbation	Group A		KY315461
***S. intermedius* FO395 (AFXN01000007)**	Dental plaque	Group D	AFXN01000007	KY315447
***S. anginosus* M423**	Airway	Group A		KY315478
***S. anginosus* M569**	Airway	Group E		KY315446
***S. anginosus* C984**	Airway	Group E		KY315444
***S. anginosus* M410**	Airway	Group E		KY315445
***S. anginosus* C252**	Invasive	Group E		KY315443
***S. anginosus* FO211 (AECT01000012)**	Nasopharynx	Group E	AECT01000012	KY315442
***S. anginosus* SK52 (AREF010000001)**	Throat	Group F	AREF010000001	KY315441
***S. anginosus* C238**	Exacerbation	Group A	CP003861	KY315458
***S. anginosus* subsp. *whileyi* MAS624 (AP013072)**	Sore throat	Group A	AP013072	KY315457
***S. anginosus* subsp. *whileyi* CCUG39159 (AICP01000048)**	Sore throat	Group B	AICP01000048	KY315456
***S. anginosus* C1051**	Blood	Group B	CP003860	KY315465
***S. anginosus* 1_2_62CV (ADME01000005)**	Crohn's disease	Group A	ADME01000005	KY315455
***S. constellatus* M505**	Airway	Group D		KY315448
***S. constellatus* subsp. *constellatus* SK53 (AICQ01000033)**	Purulent pleurisy	Group A	AICQ01000033	KY315463
***S. constellatus* subsp. *constellatus* ATCC27823**	Invasive	Group A		KY315464
***S. constellatus* M47**	Airway	Group C		KY315462
***S. constellatus* C1379**	Empyema	Group A		KY315469
***S. constellatus* C1392**	Brain	Group A		KY315468
***S. constellatus* C1367**	Blood	Group A		KY315474
***S. constellatus* C1384**	Empyema	Group A		KY315467
***S. constellatus* C1366**	Blood	Group A		KY315466
***S. constellatus* subsp. *pharyngis* SK1060 (AFUP01000001)**	throat	Group A	AFUP01000001	KY315449
***S. constellatus* M193**	Exacerbation[1]	Group A		KY315454
***S. constellatus* subsp. *pharyngis* C818**		Group A	CP003840	KY315453
***S. constellatus* subsp. *pharyngis* C1050**	Invasive	Group A	CP003859	KY315450
***S. constellatus* C188**		Group A		KY315451
***S. constellatus* subsp. *pharyngis* C232**	airway	Group A	CP003800	KY315452

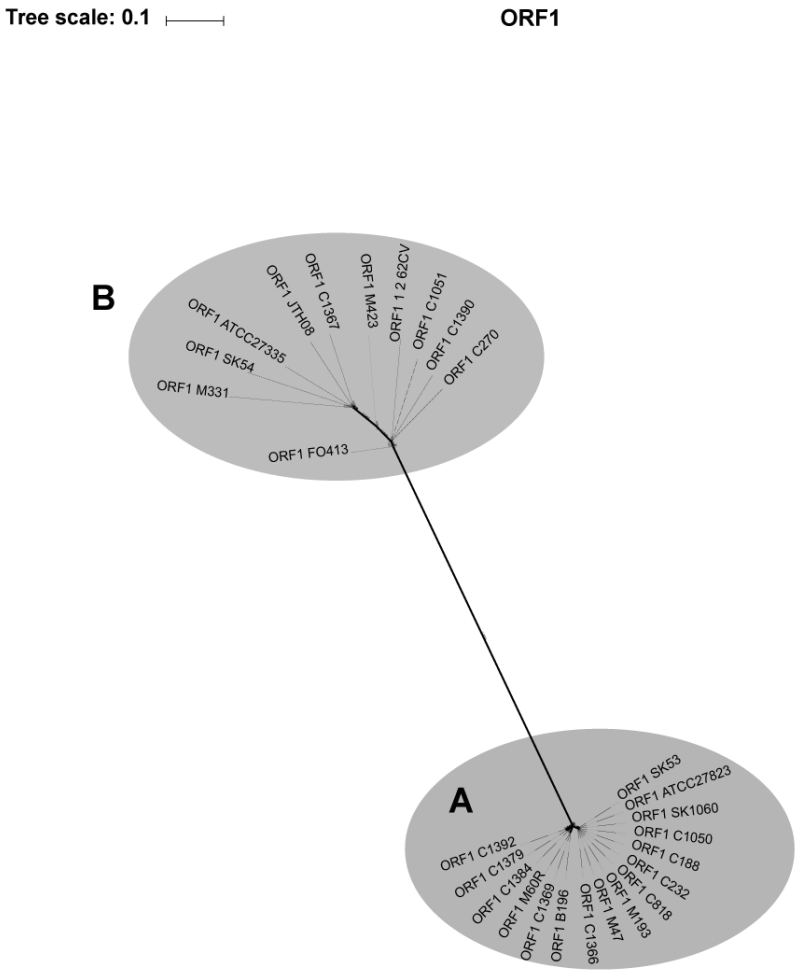

Figure 5.S3: Phylogenetic tree of ORF 1 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

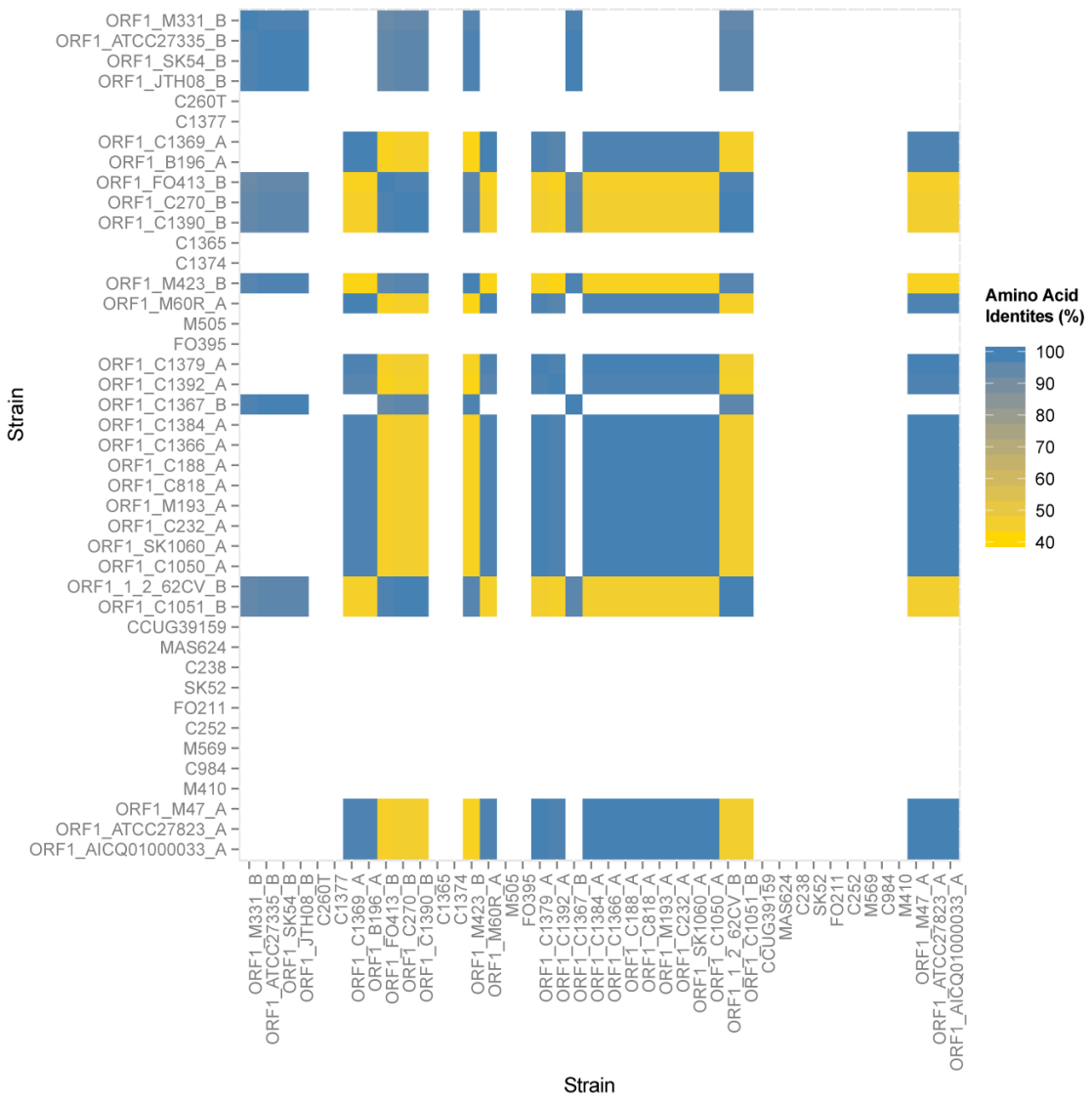

Figure 5.S4: Amino acid identity of ORF 1 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

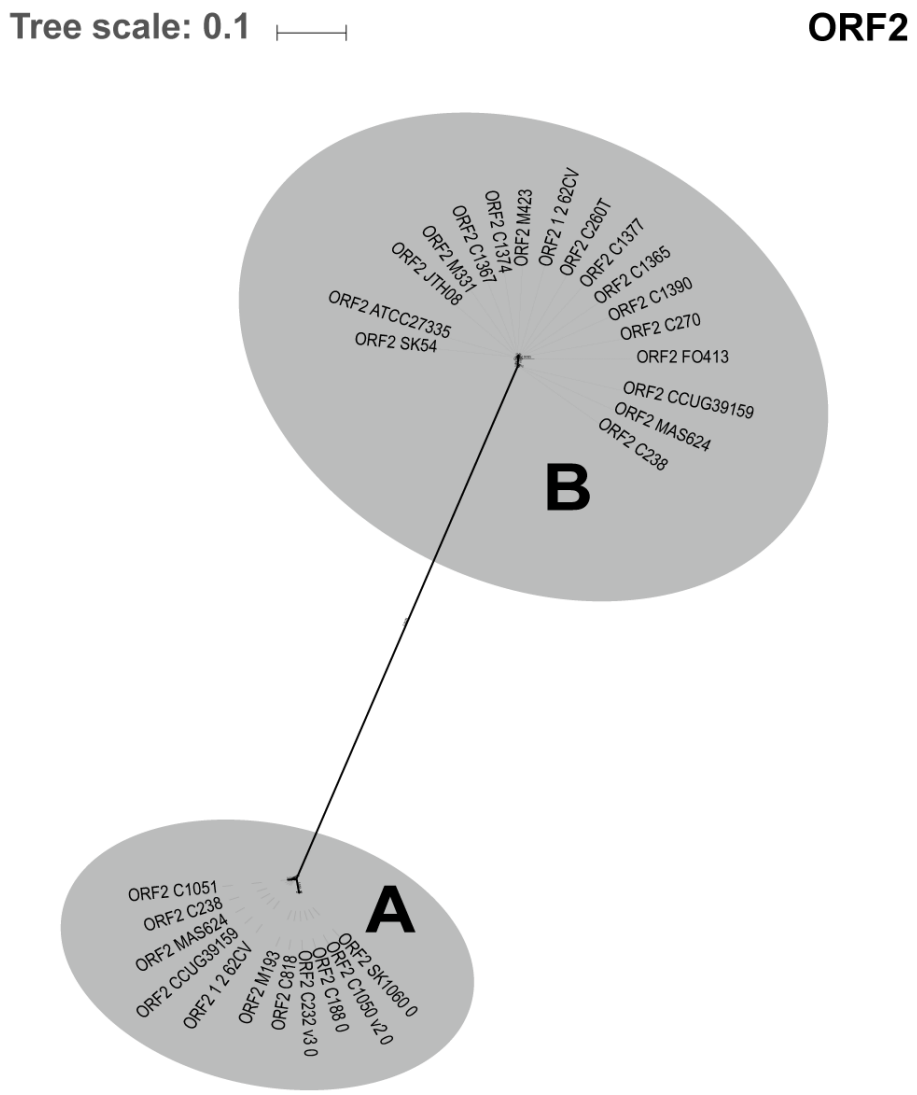

Figure 5.S5: Phylogenetic tree ORF 2 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

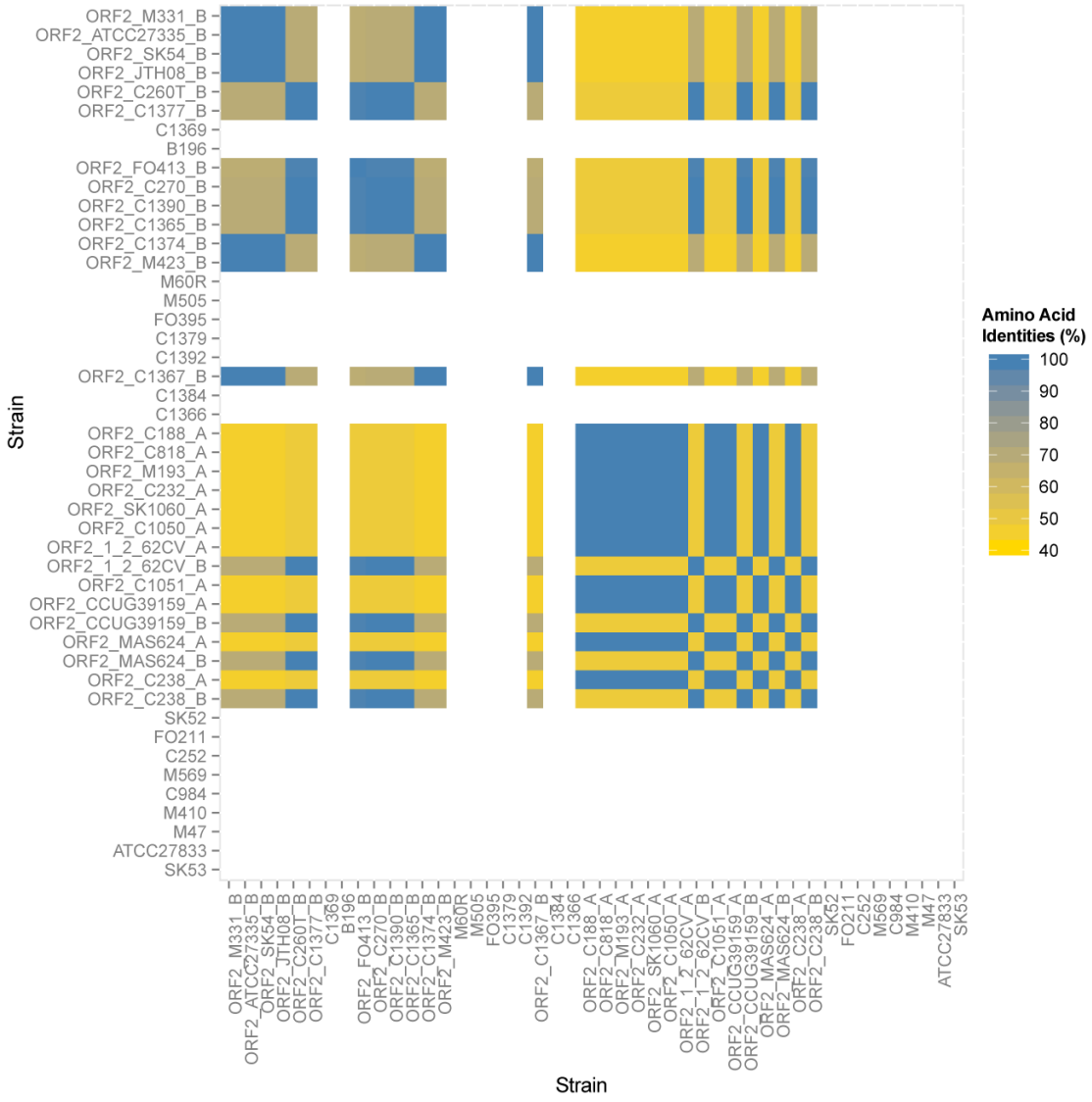

Figure 5.S6: Amino acid identity of ORF 2 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

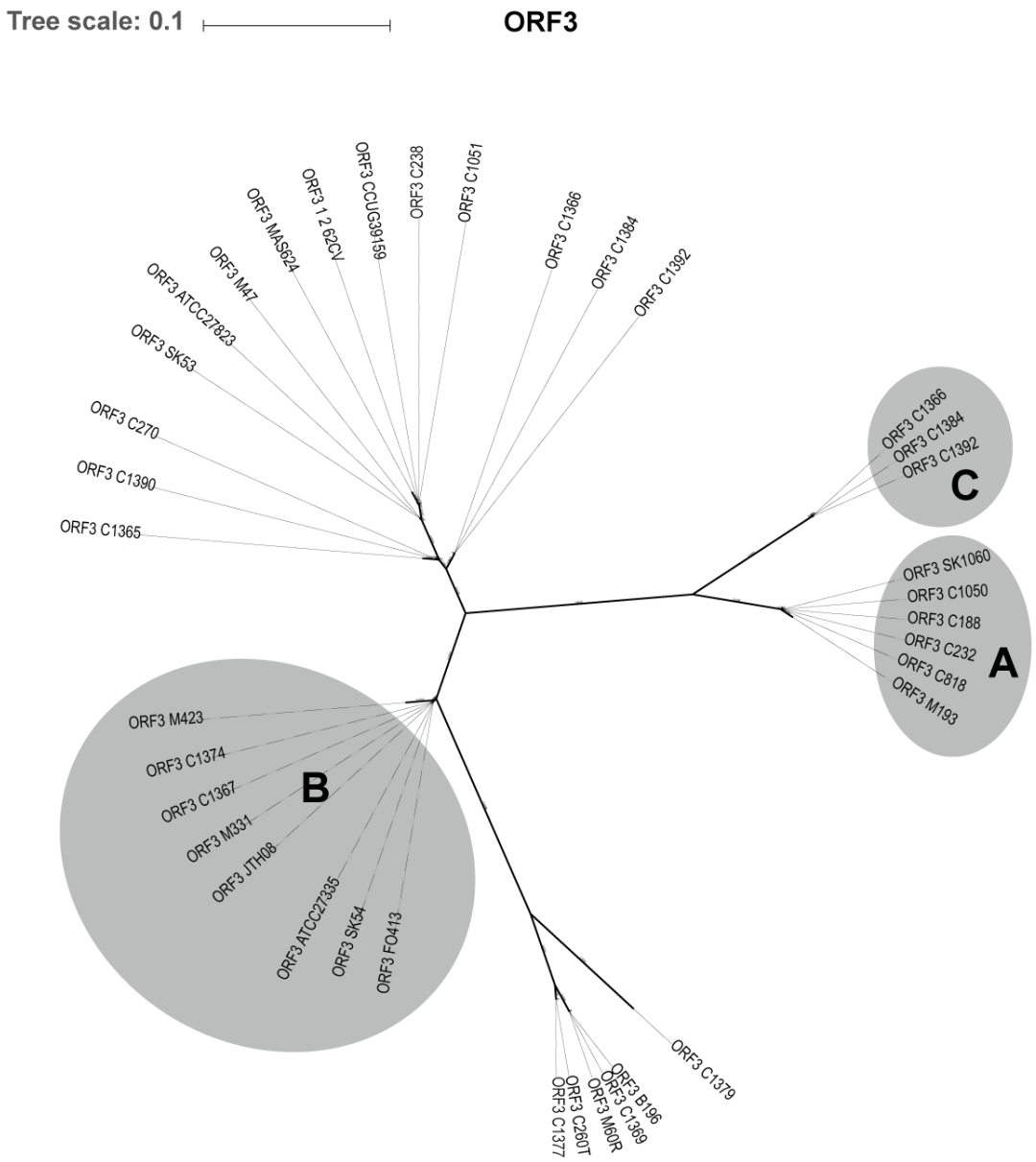

Figure 5.S7: Phylogenetic tree of ORF 3 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

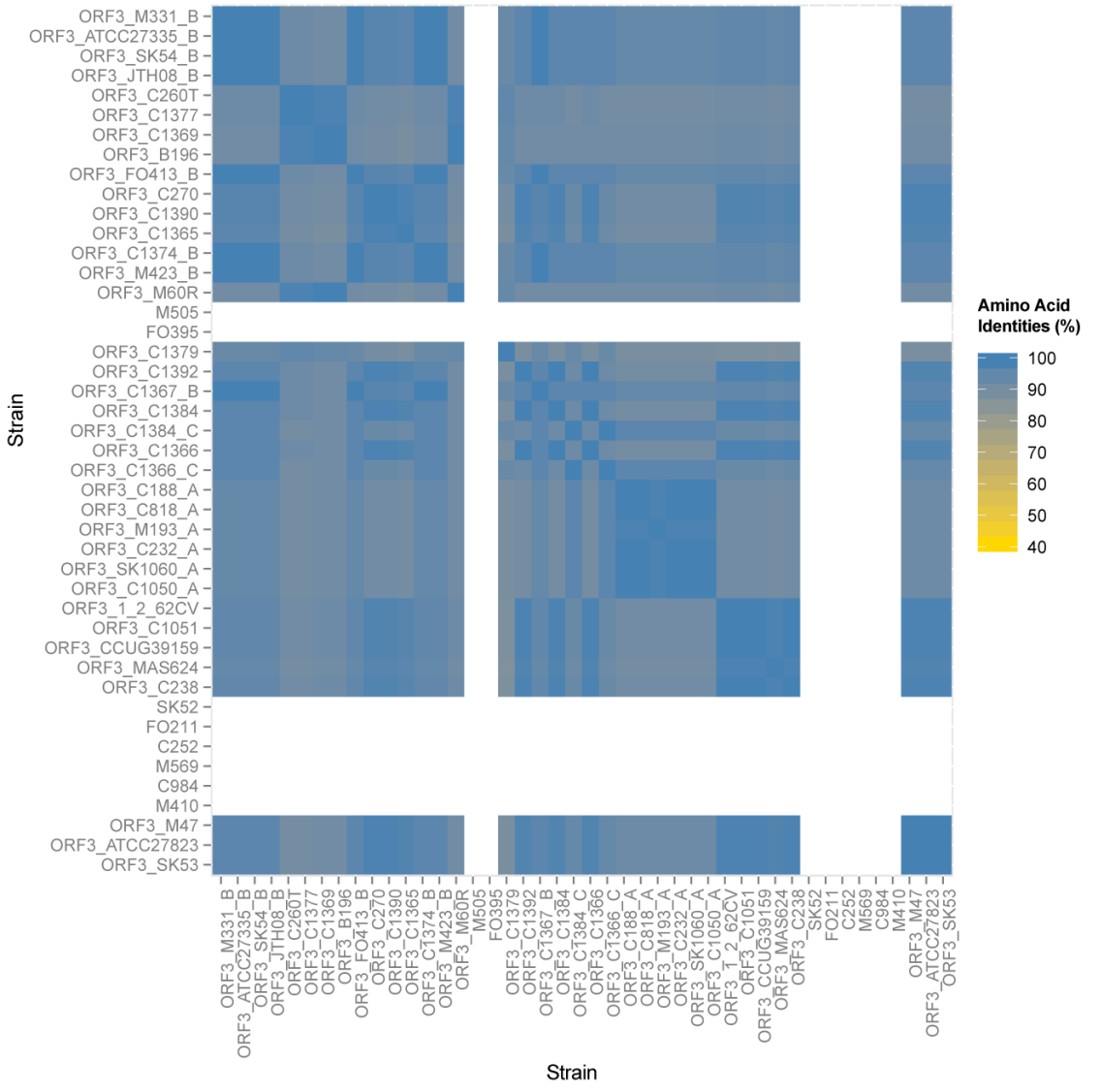

Fig 5.S8: Amino acid identity of ORF 3 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

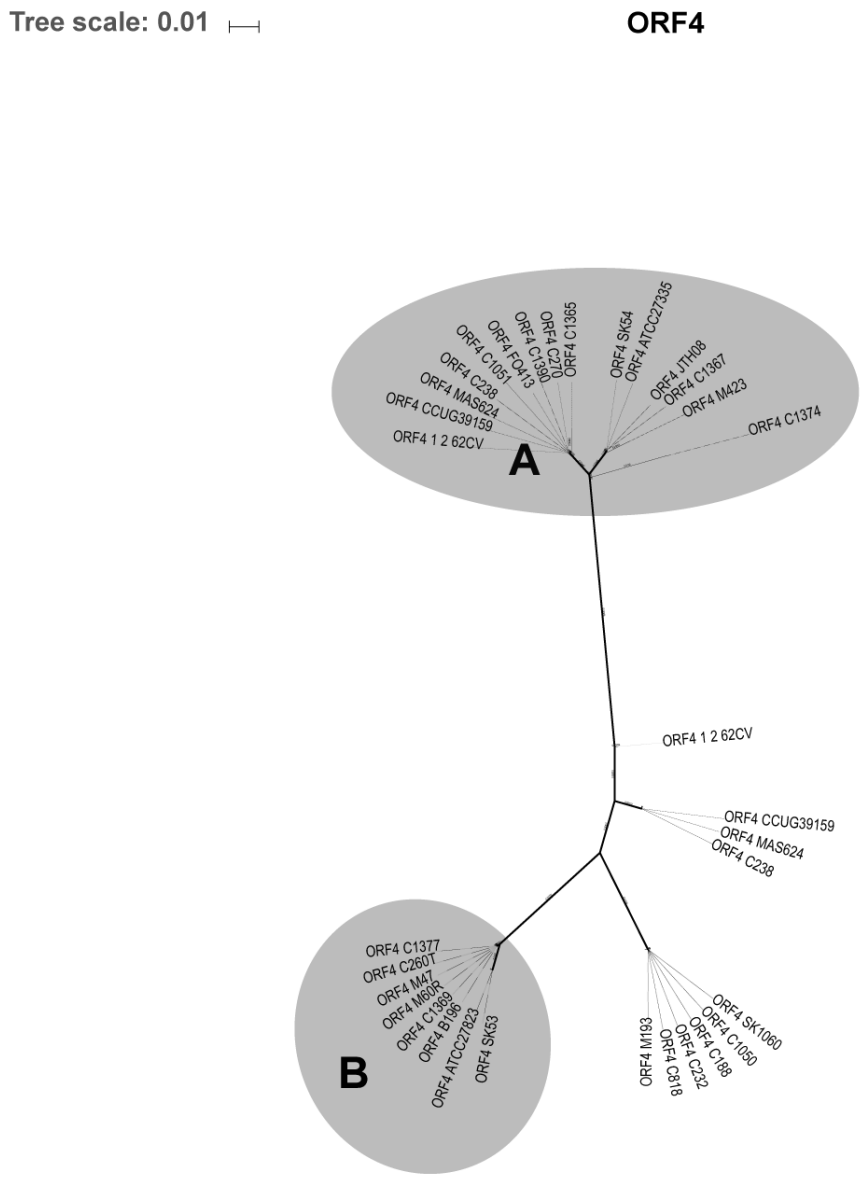

Figure 5.S9: Phylogenetic tree of ORF 4 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

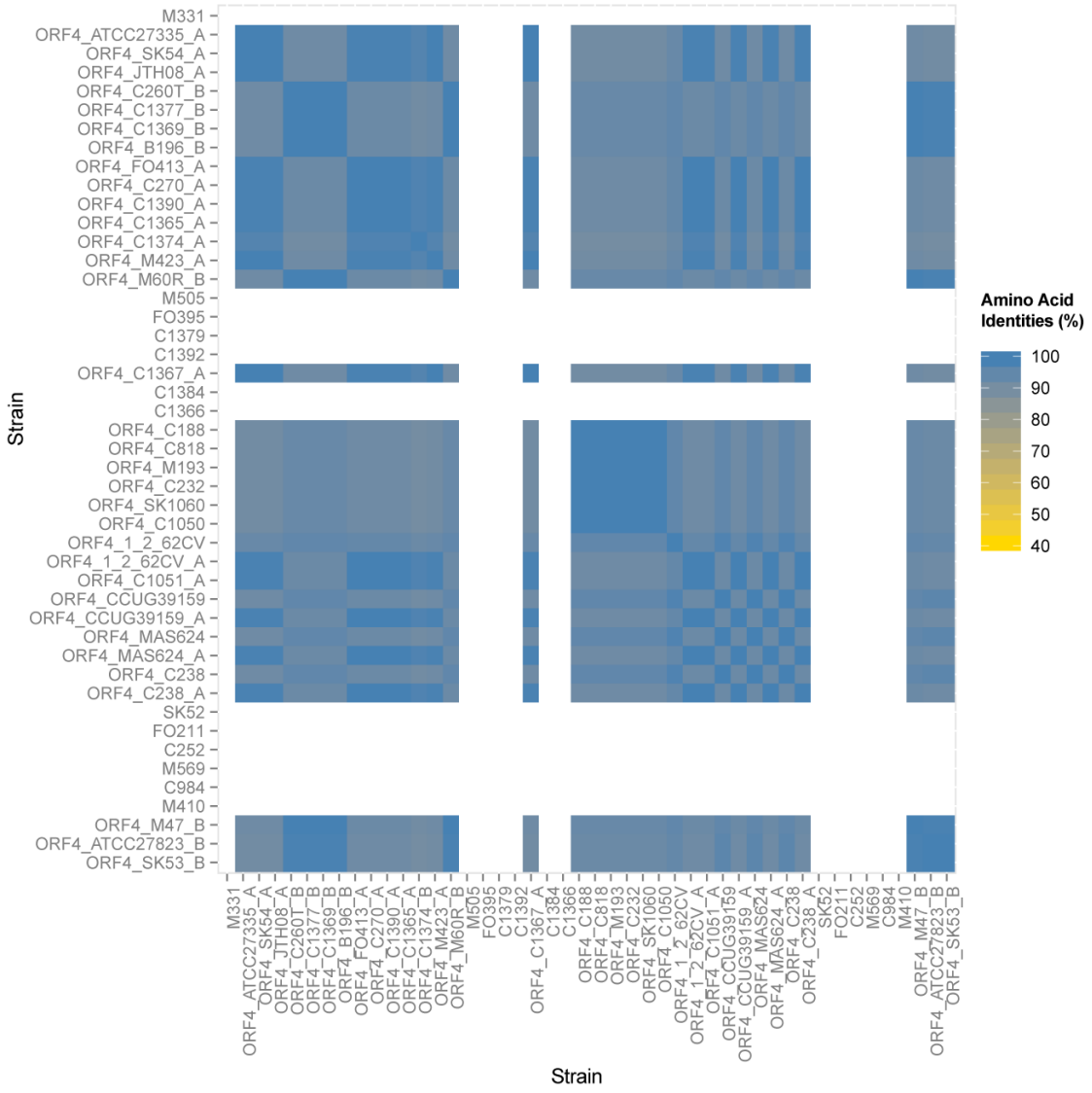

Figure 5.S10: Amino acid identity of ORF 4 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

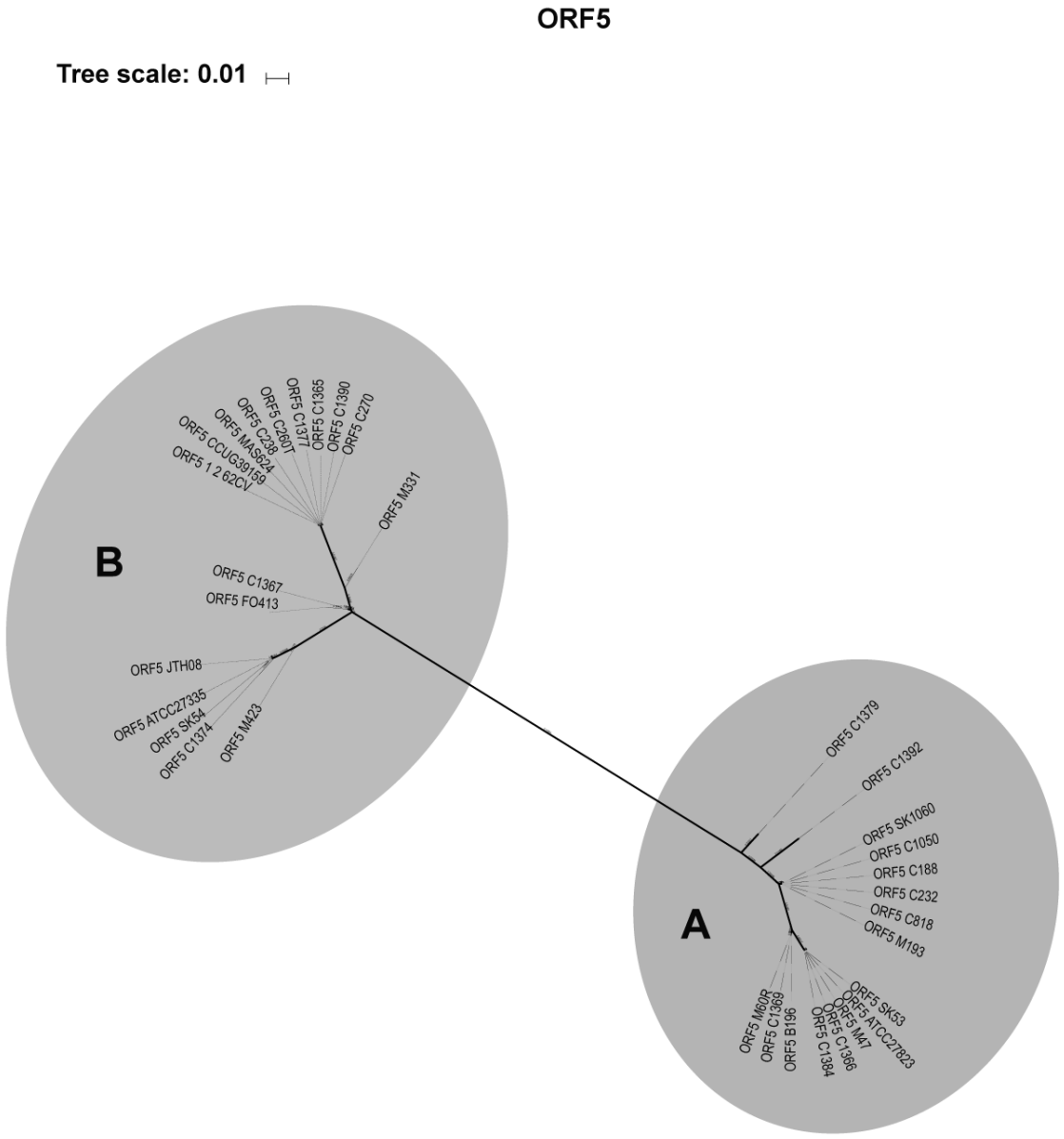

Figure 5.S11: Phylogenetic tree of ORF 5 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

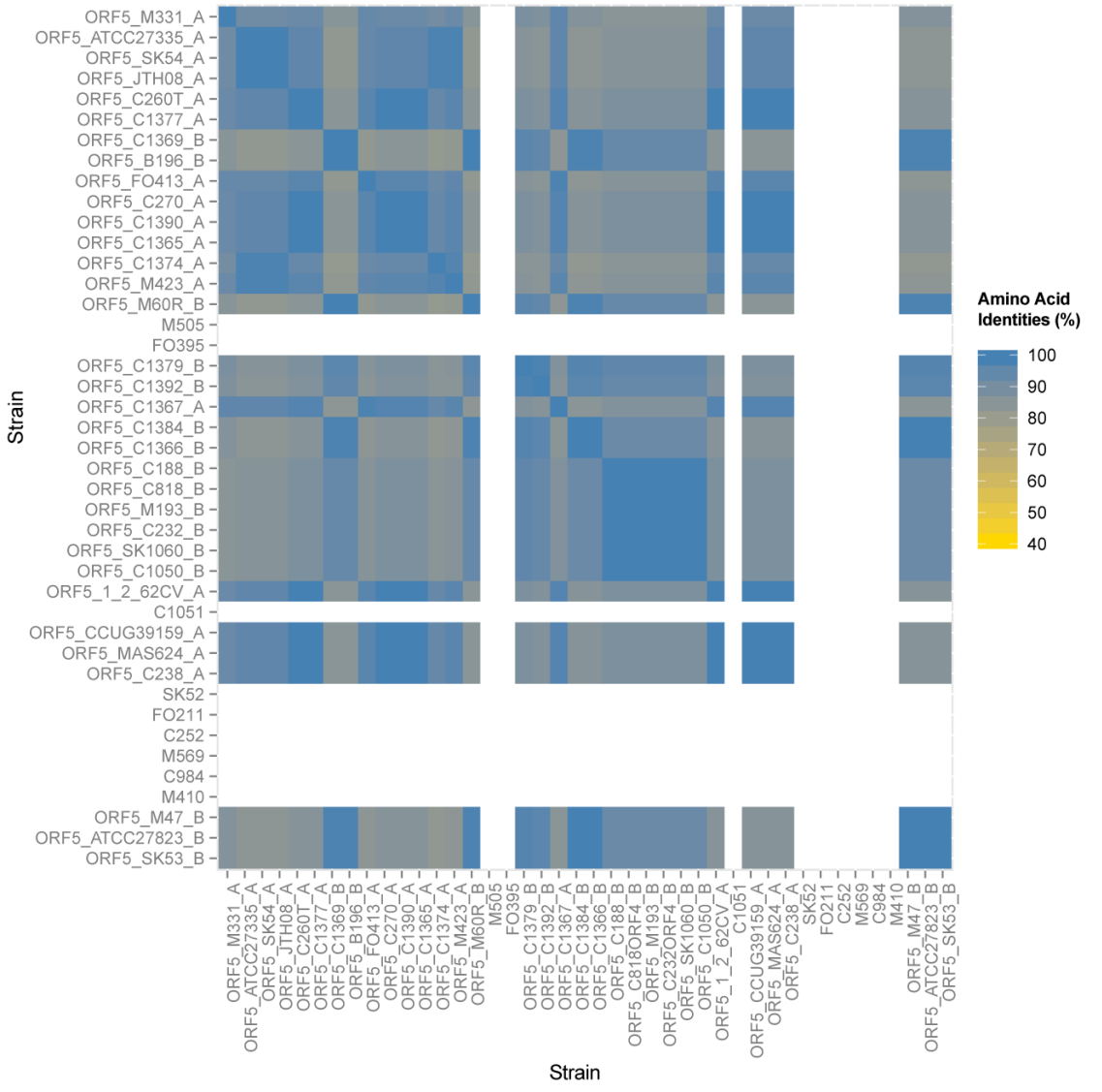

Figure 5.S12: Amino acid identity of ORF 5 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

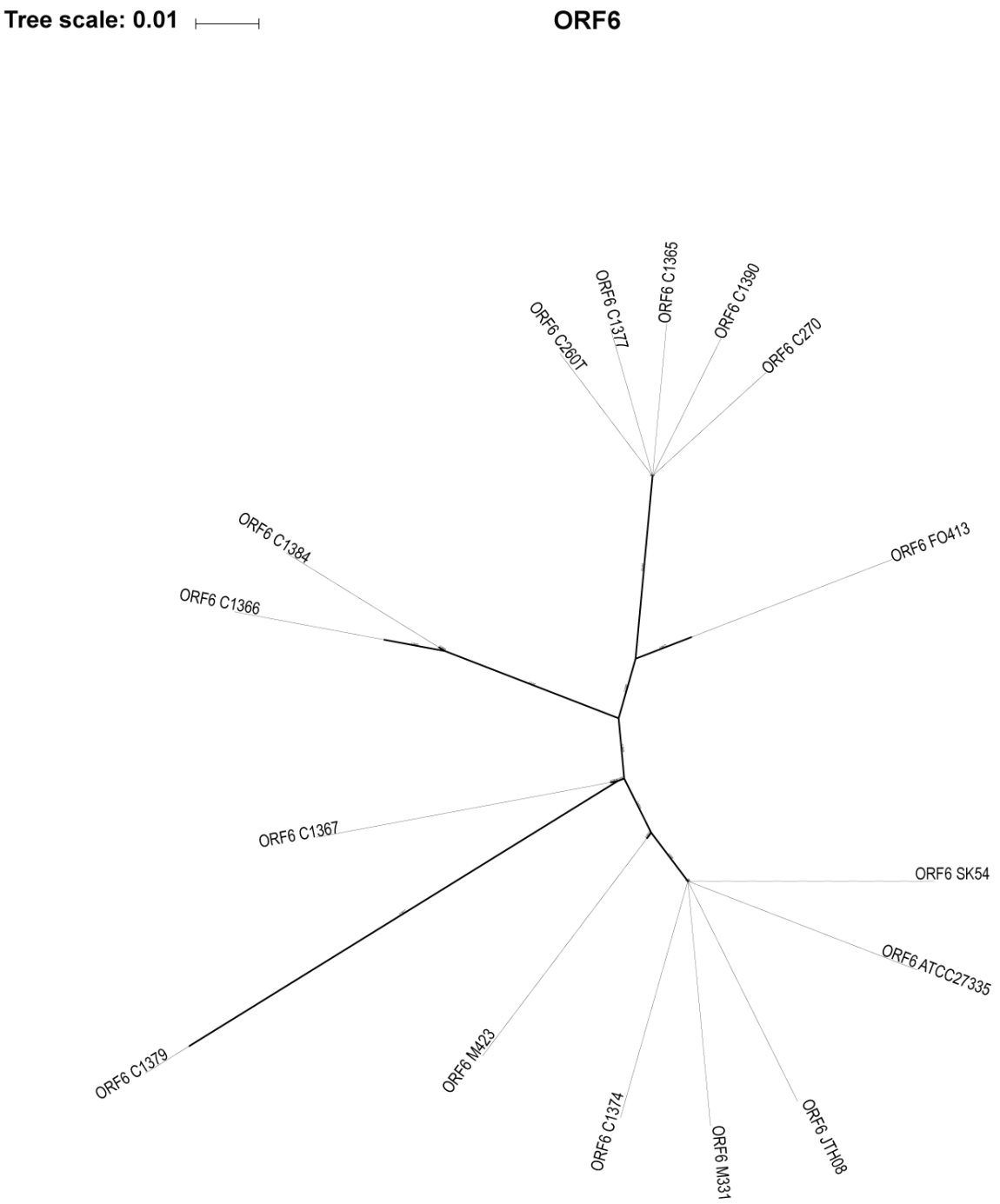

Figure 5.S13: Phylogenetic tree of ORF 6 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

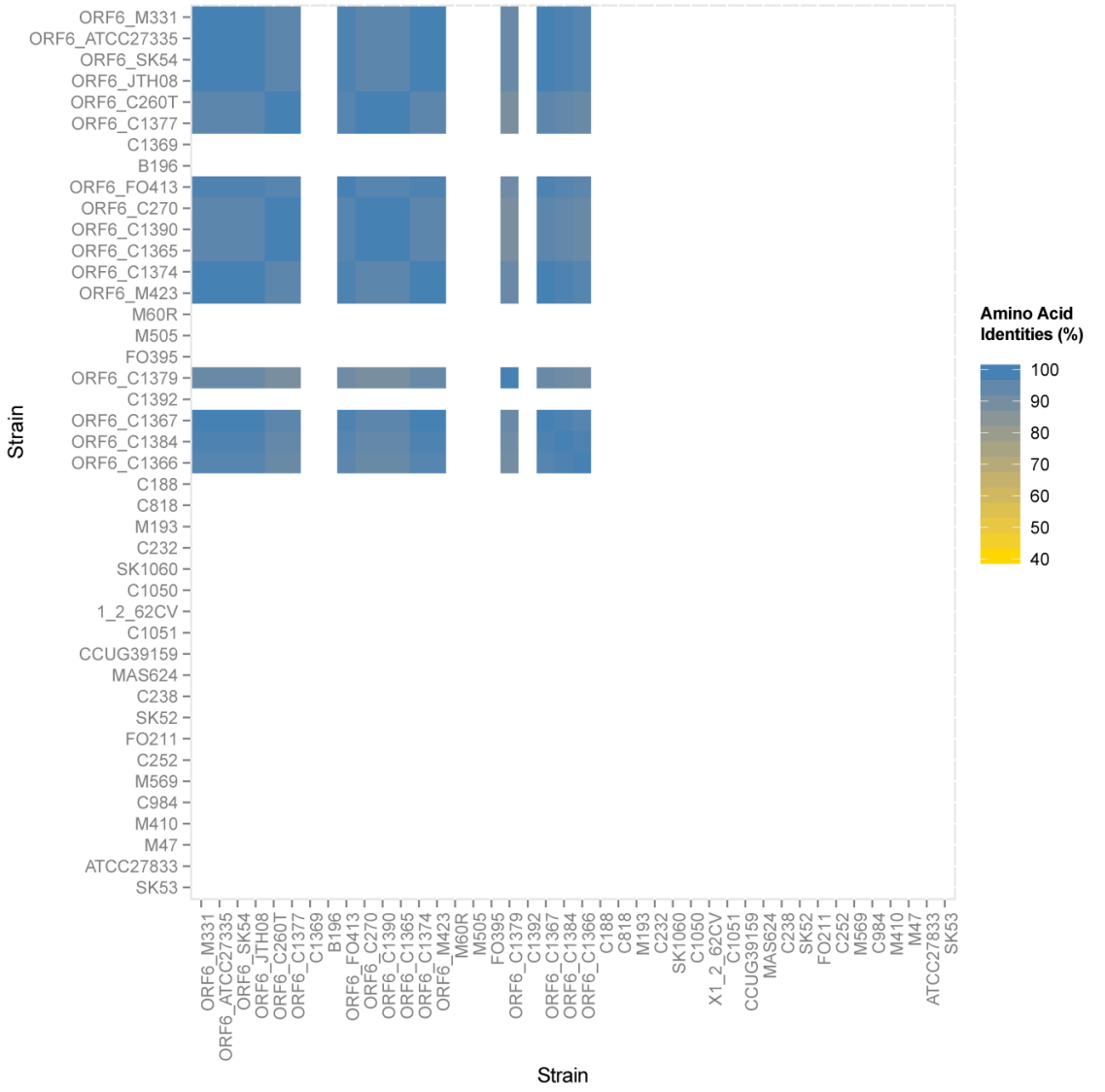

Figure 5.S14: Amino acid identity of ORF 6 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

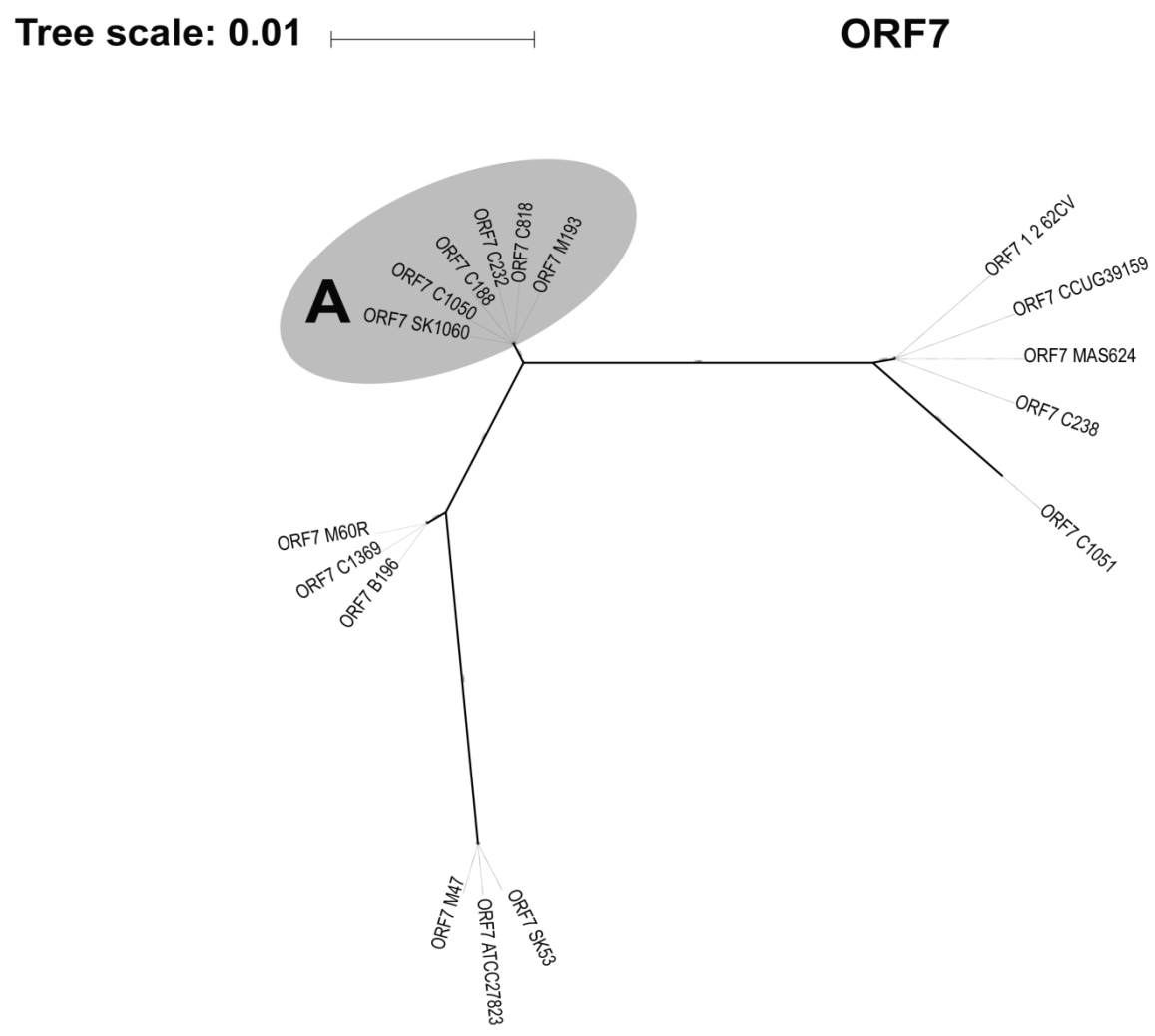

Figure 5.S15: Phylogenetic tree of ORF 7 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

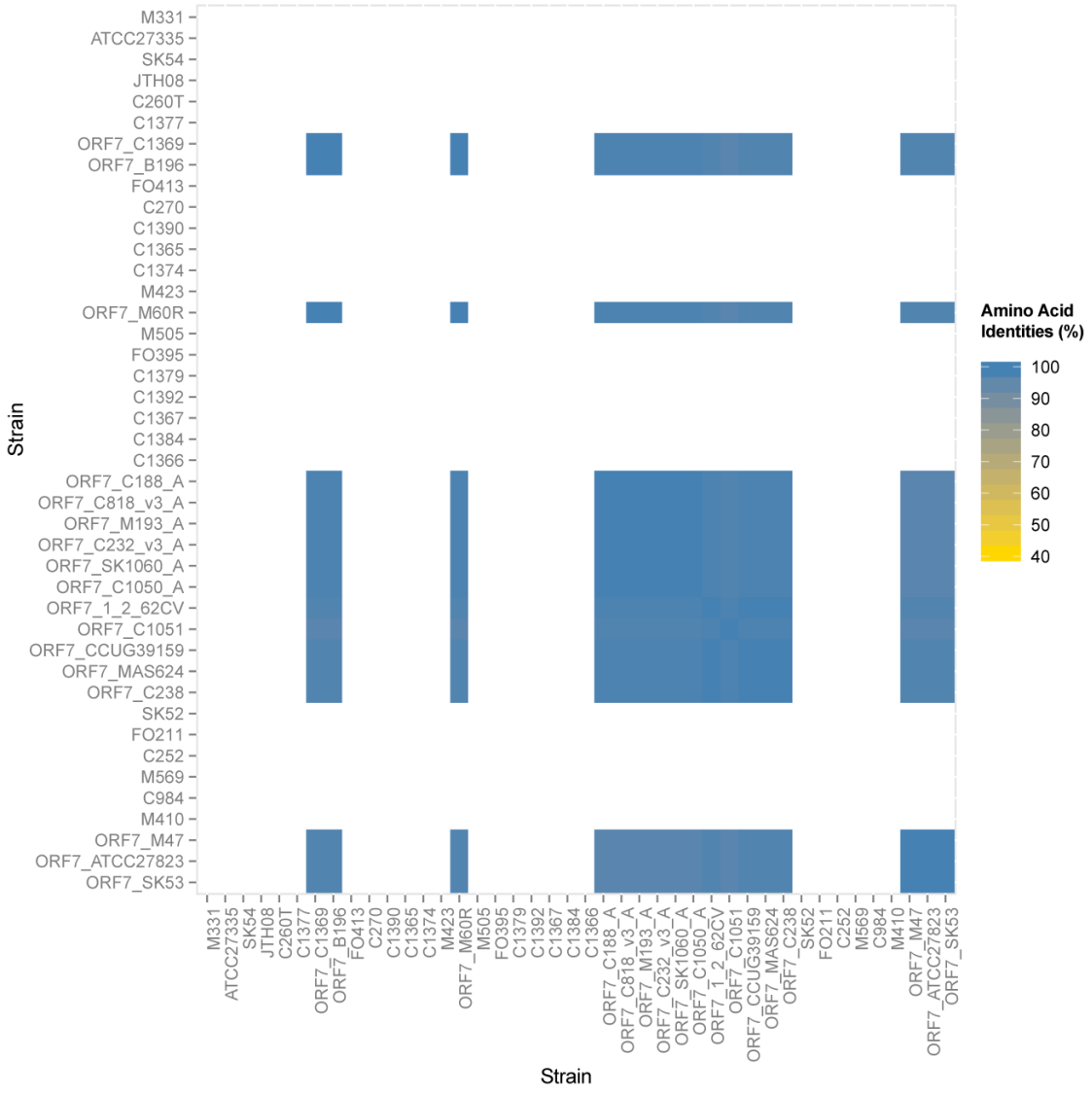

Figure 5.S16: Amino acid identity of ORF 7 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

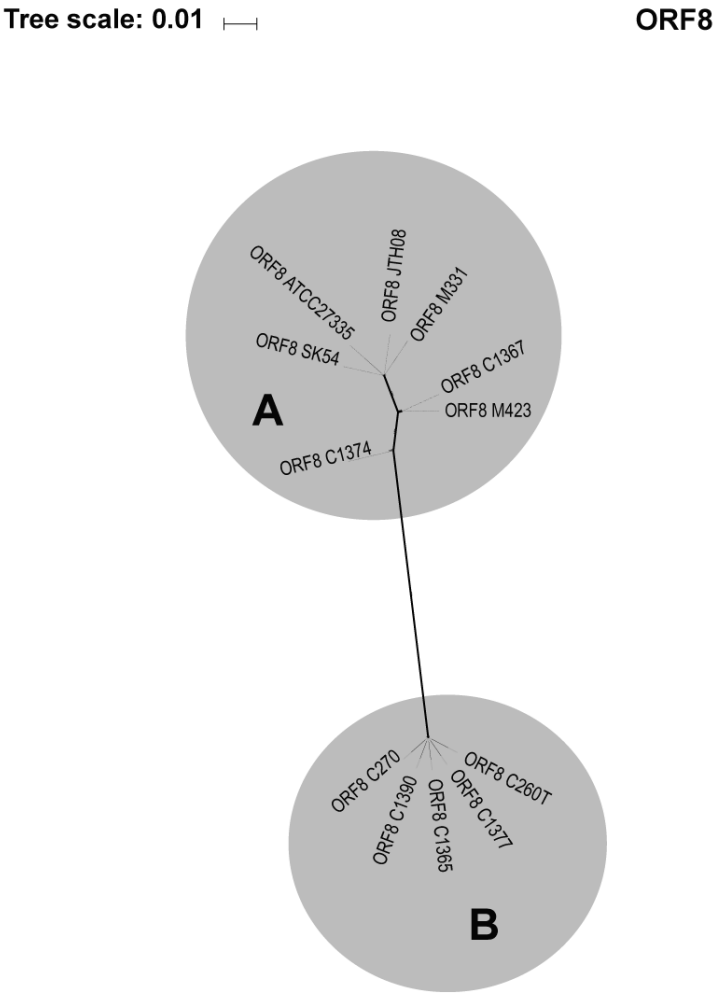

Figure 5.S17: Phylogenetic tree of ORF 8 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

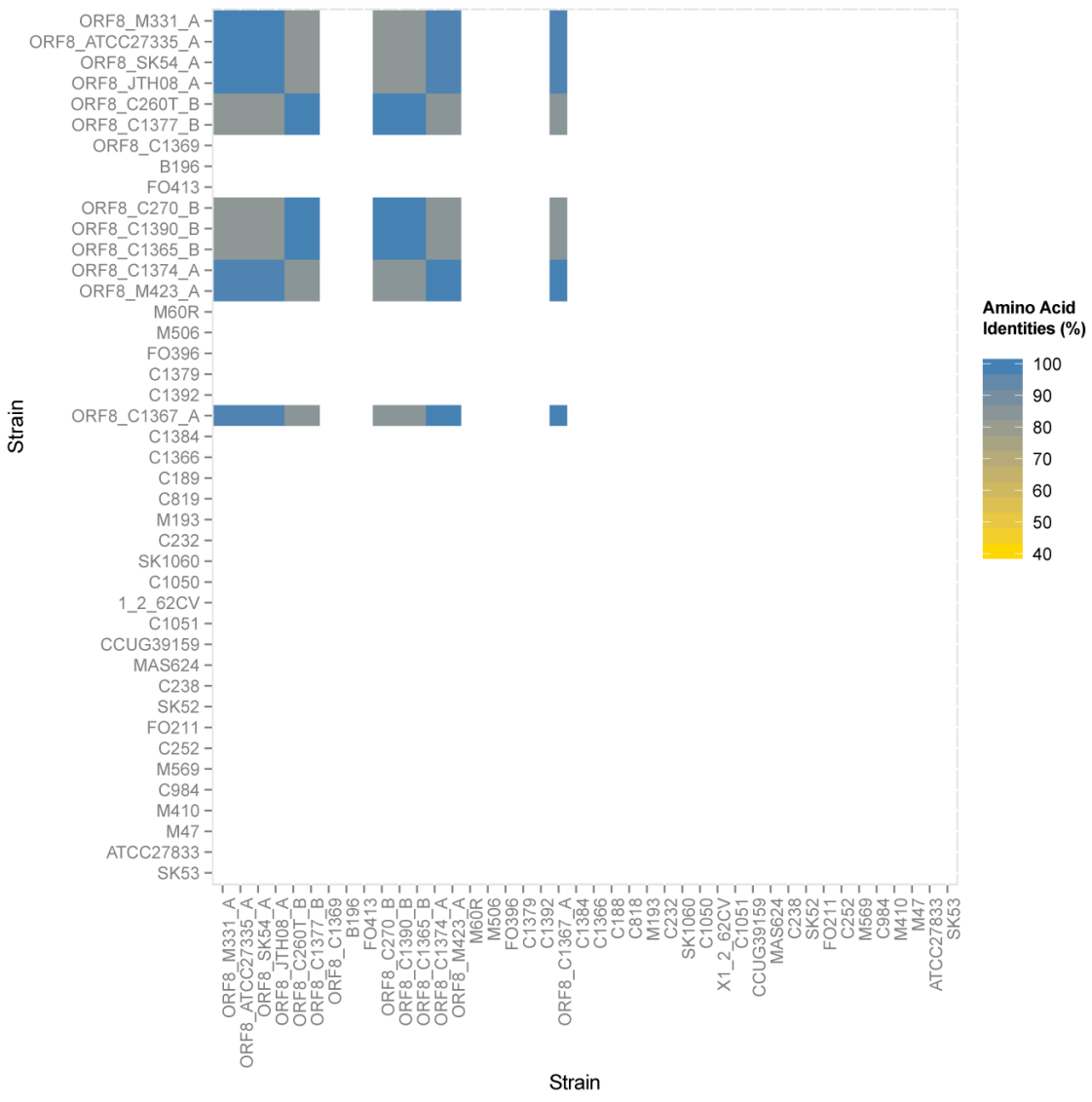

Figure 5.S18: Amino acid identity of ORF 8 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

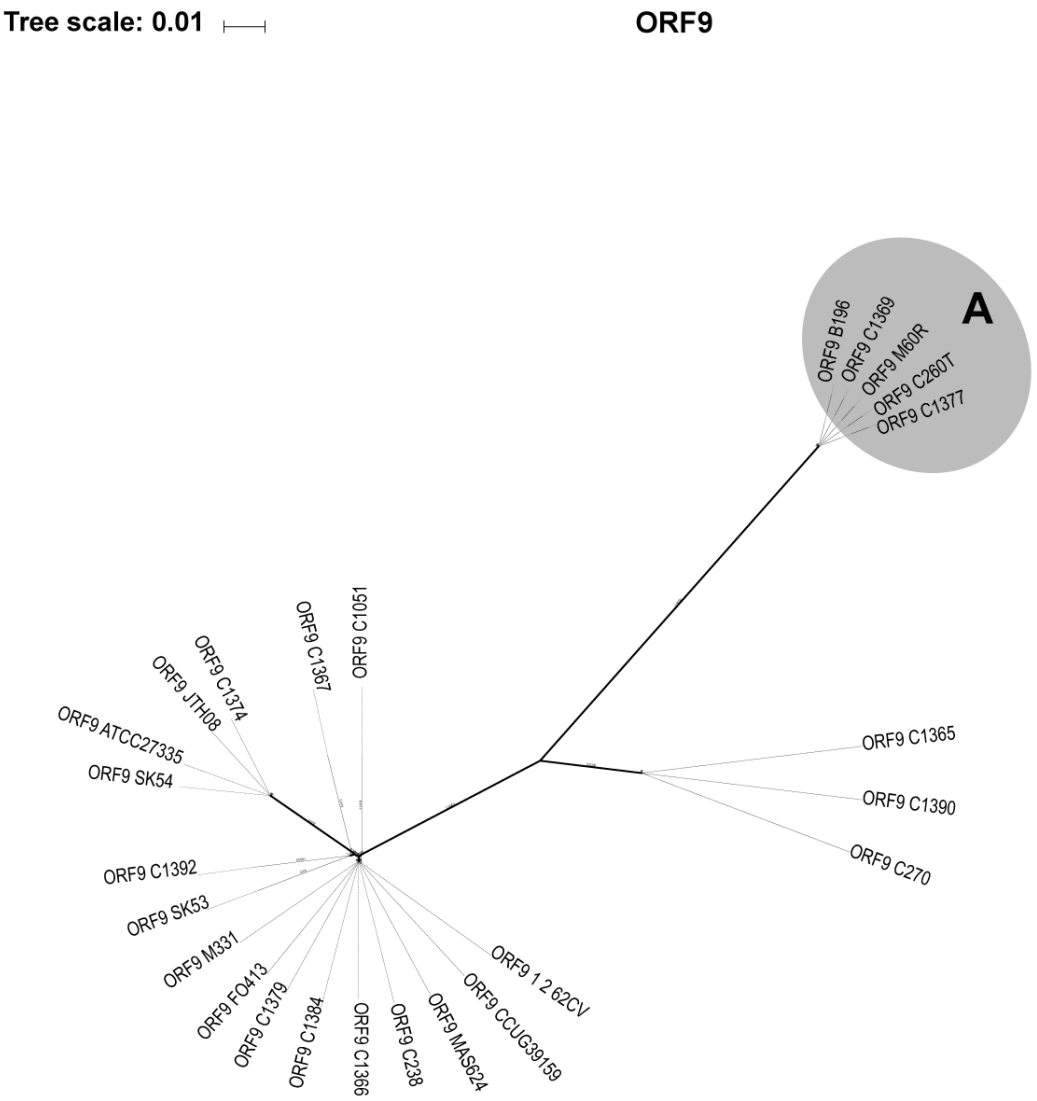

Figure 5.S19: Phylogenetic tree of ORF 9 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

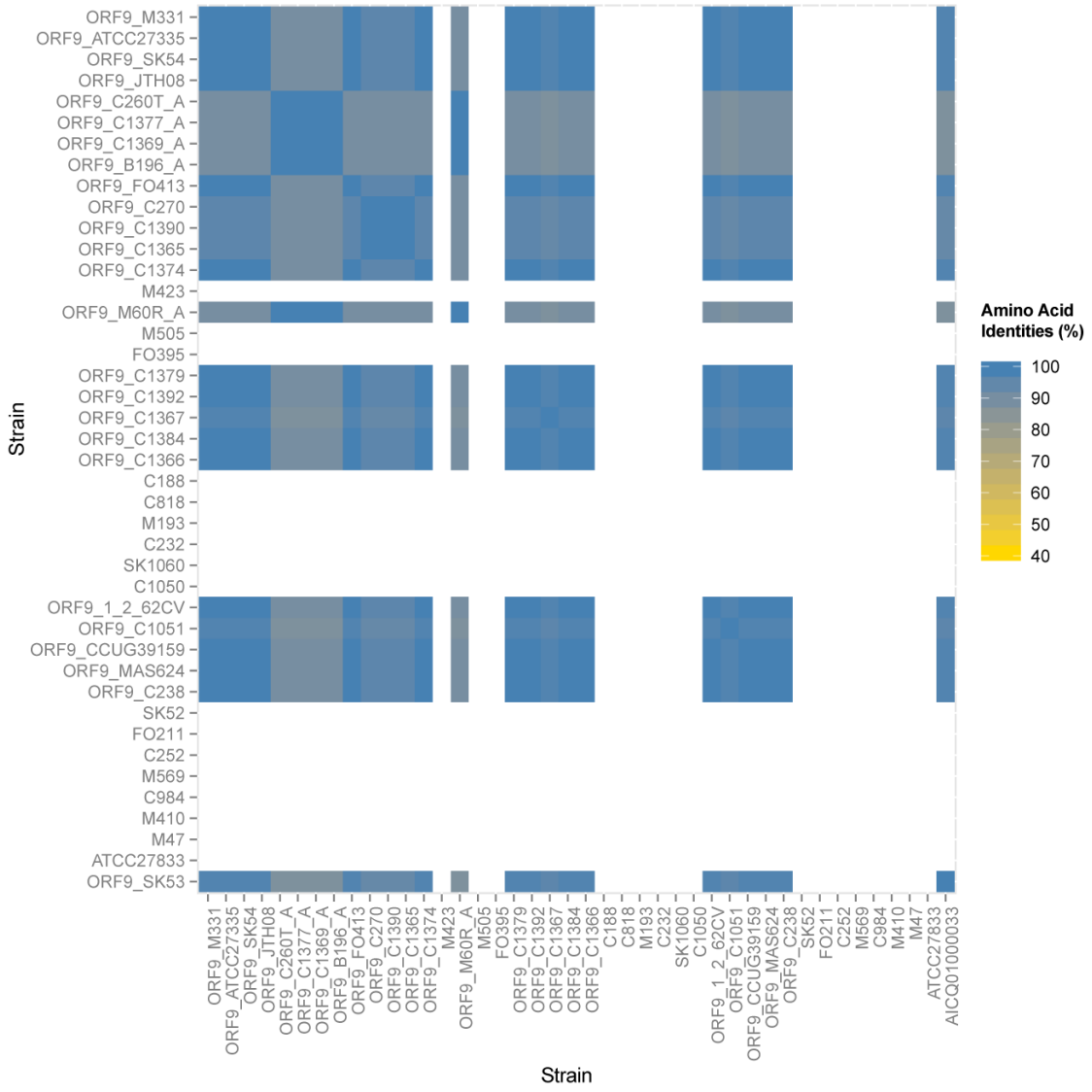

Figure 5.S20: Amino acid identity of ORF 9 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

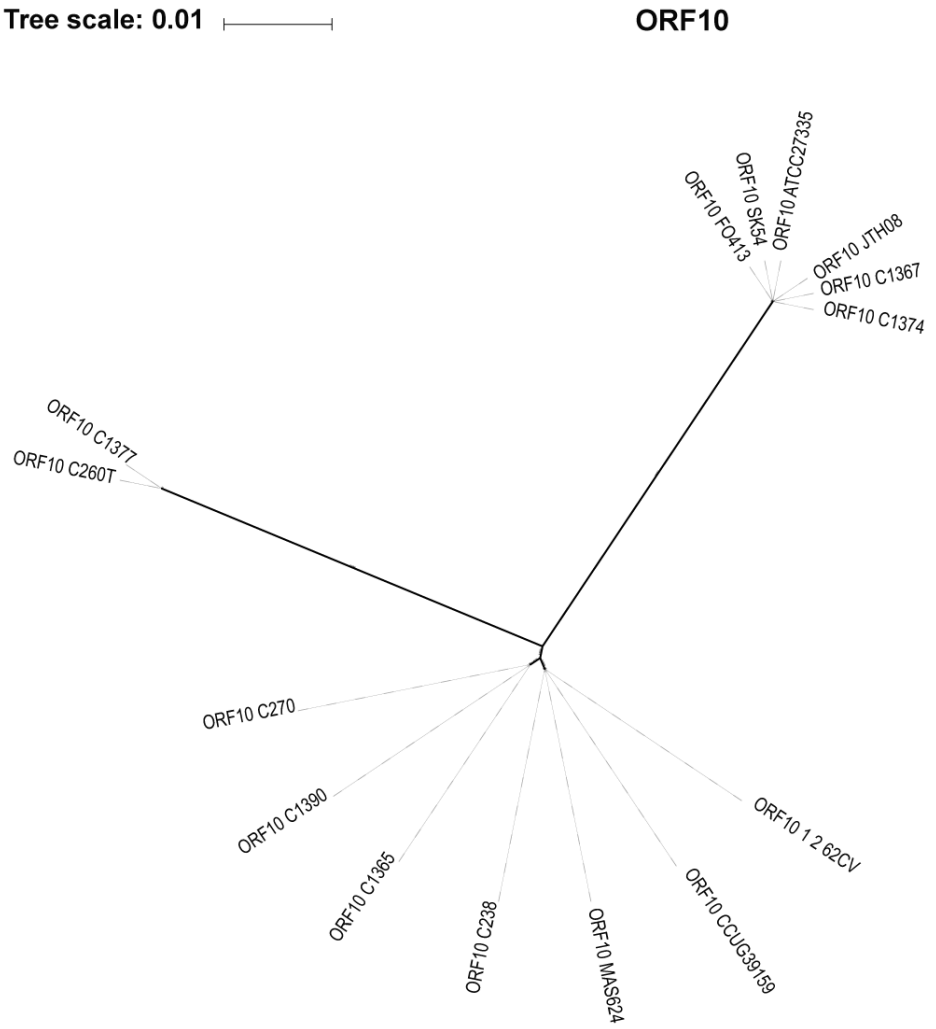

Figure 5.S21: Phylogenetic tree of ORF 10 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

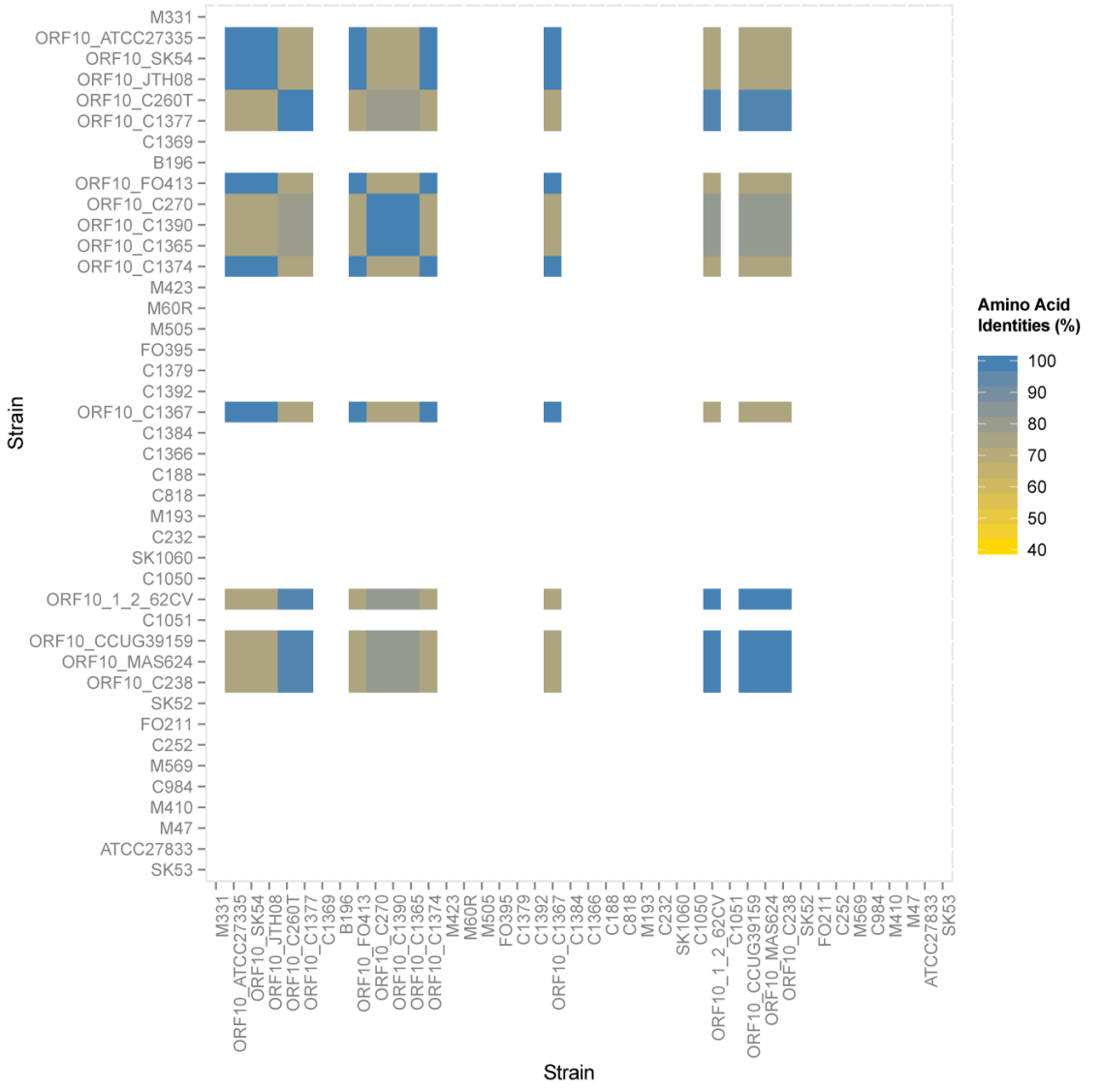

Figure 5.S22: Amino acid identity of ORF 10 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

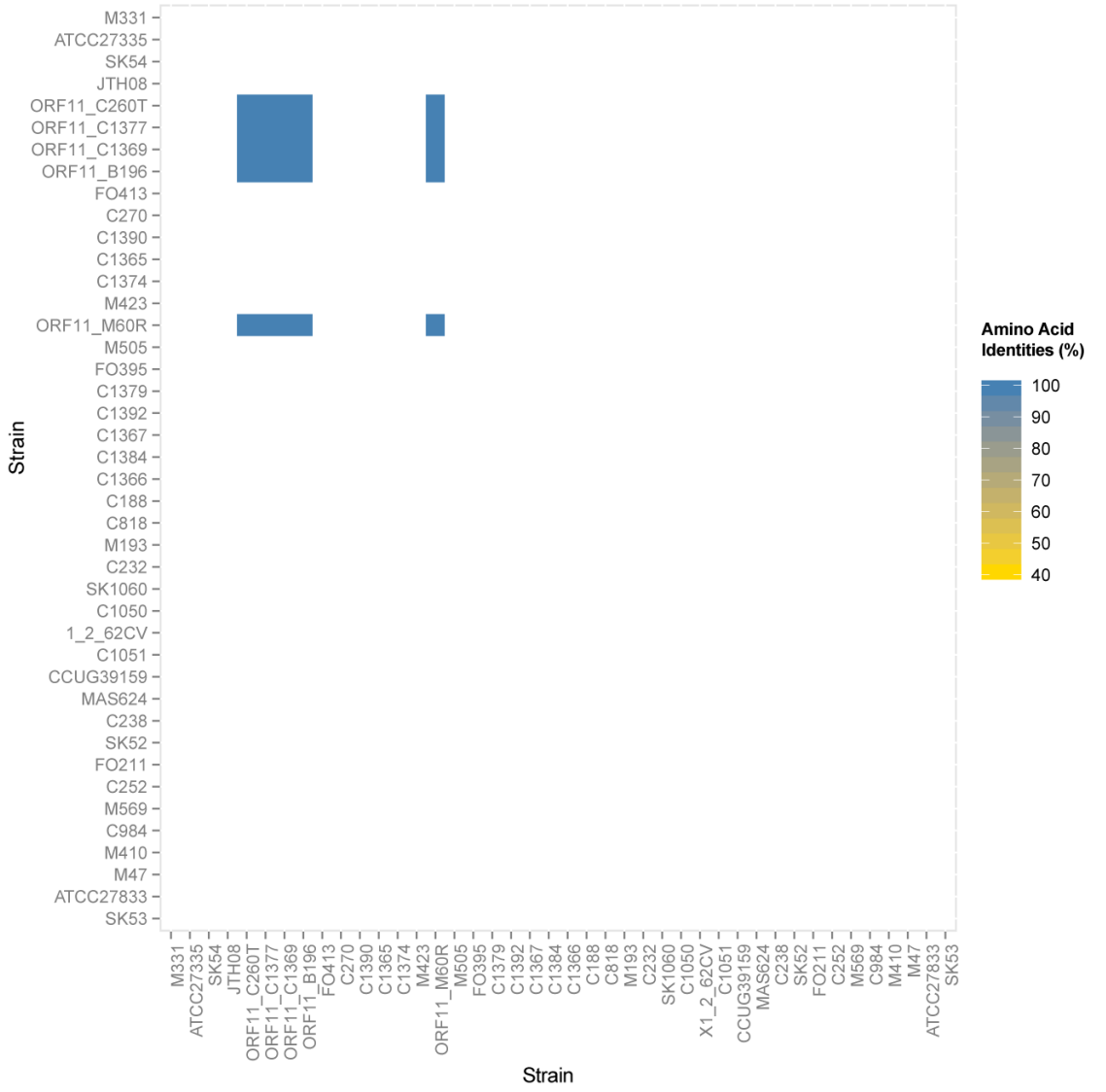

Figure 5.S23: Amino acid identity of ORF 11 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

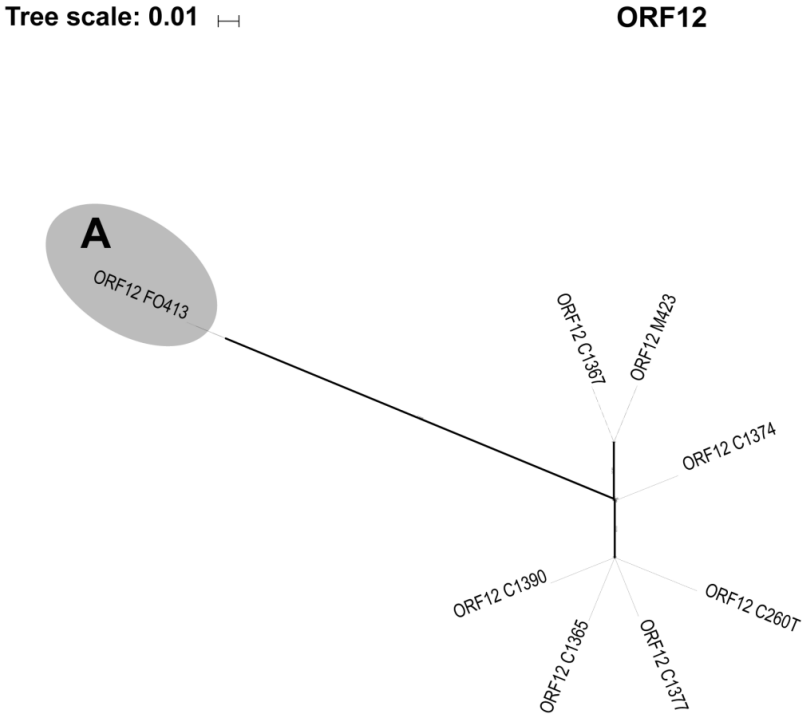

Figure 5.S24: Phylogenetic tree of ORF 12 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

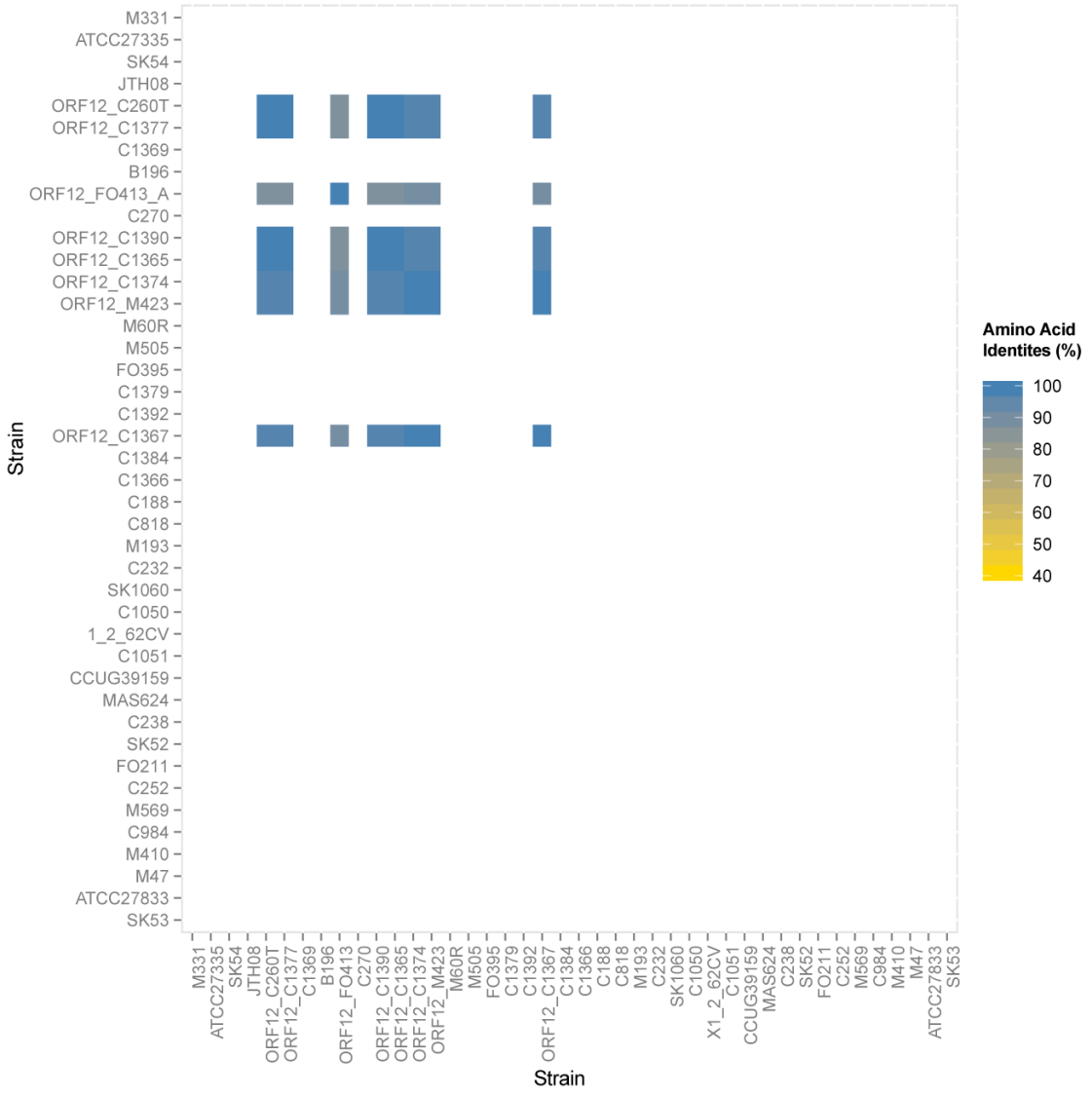

Figure 5.S25: Amino acid identity of ORF 12 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

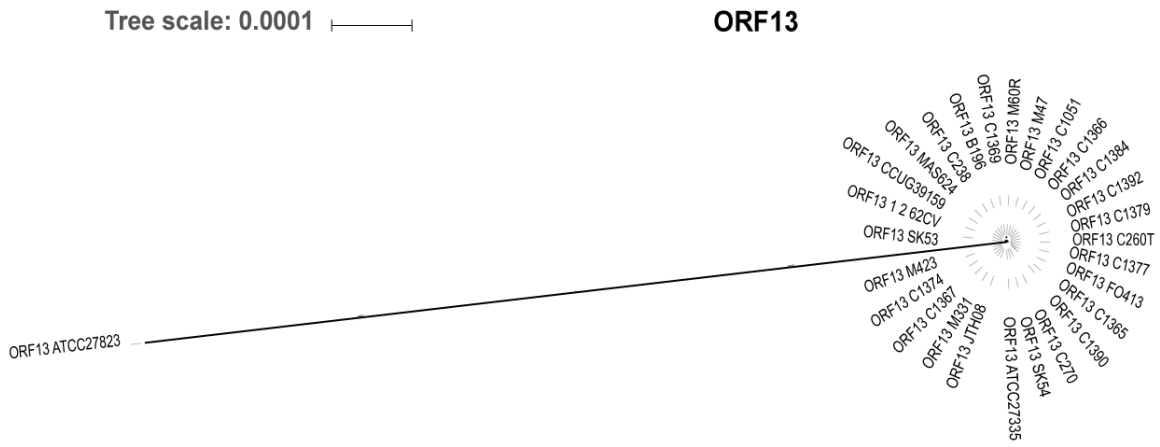

Figure 5.S26: Phylogenetic tree of ORF 13 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

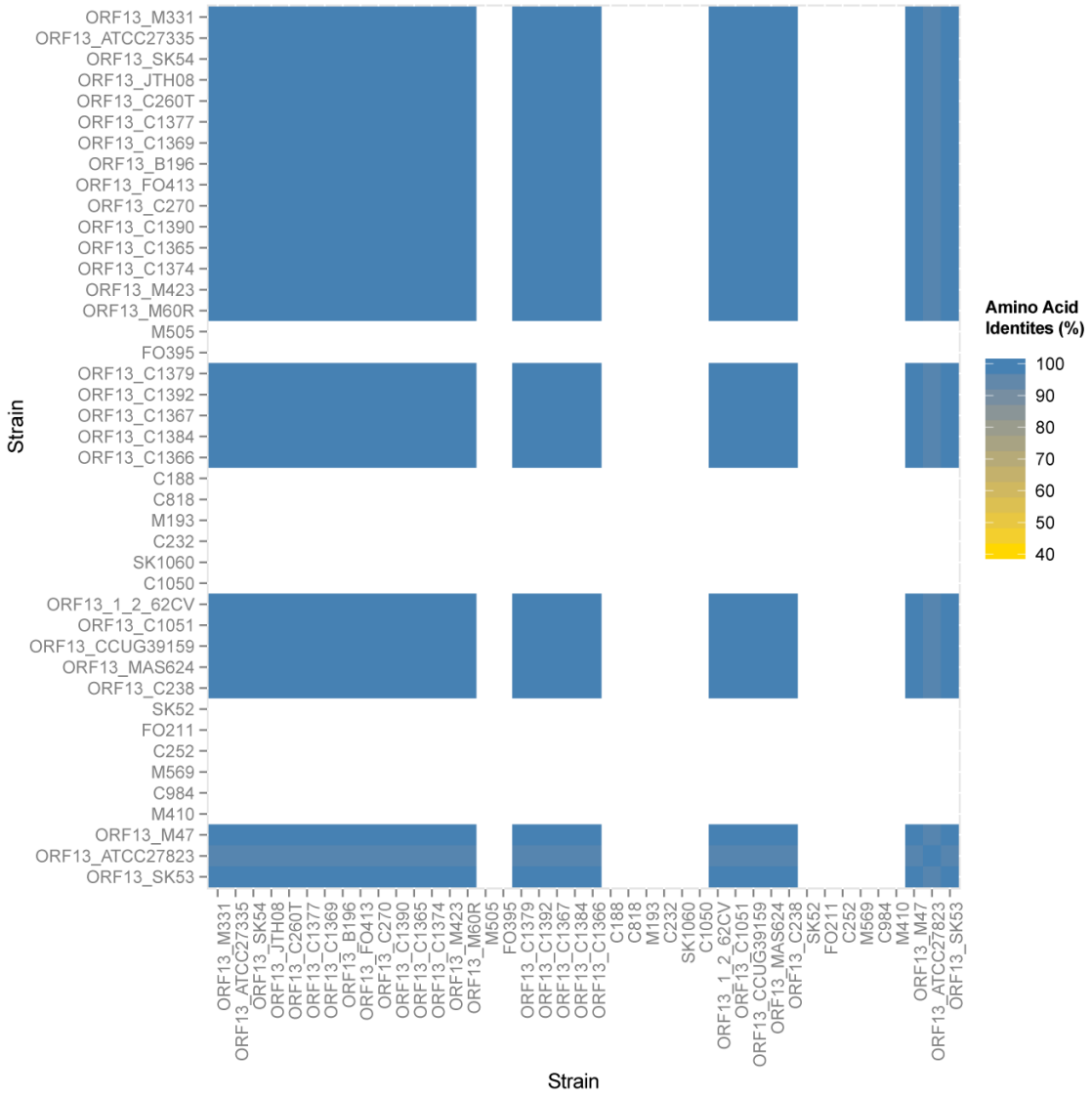

Figure 5.S27: Amino acid identity of ORF 13 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

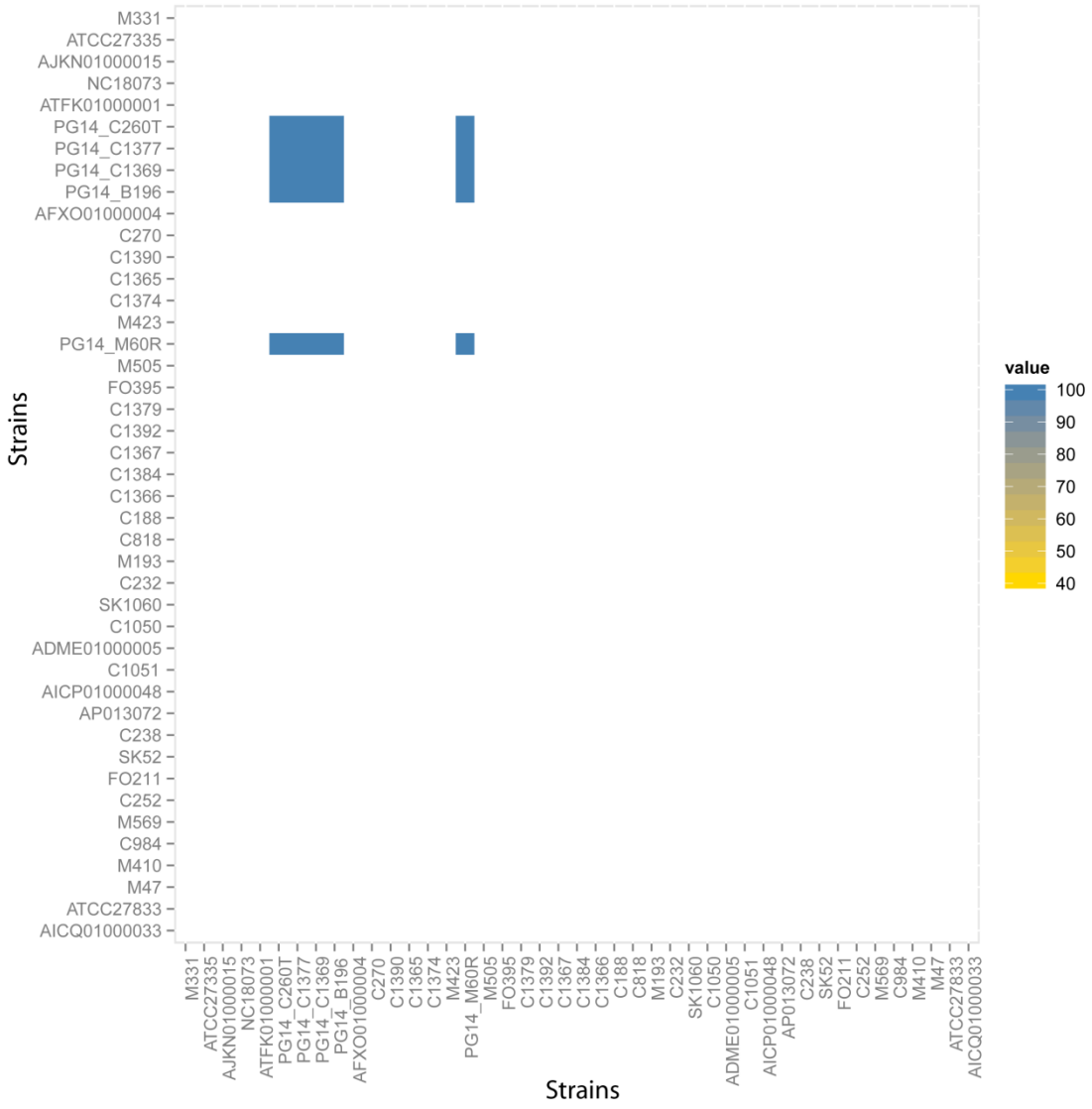

Figure 5.S28: Amino acid identity of ORF 14 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

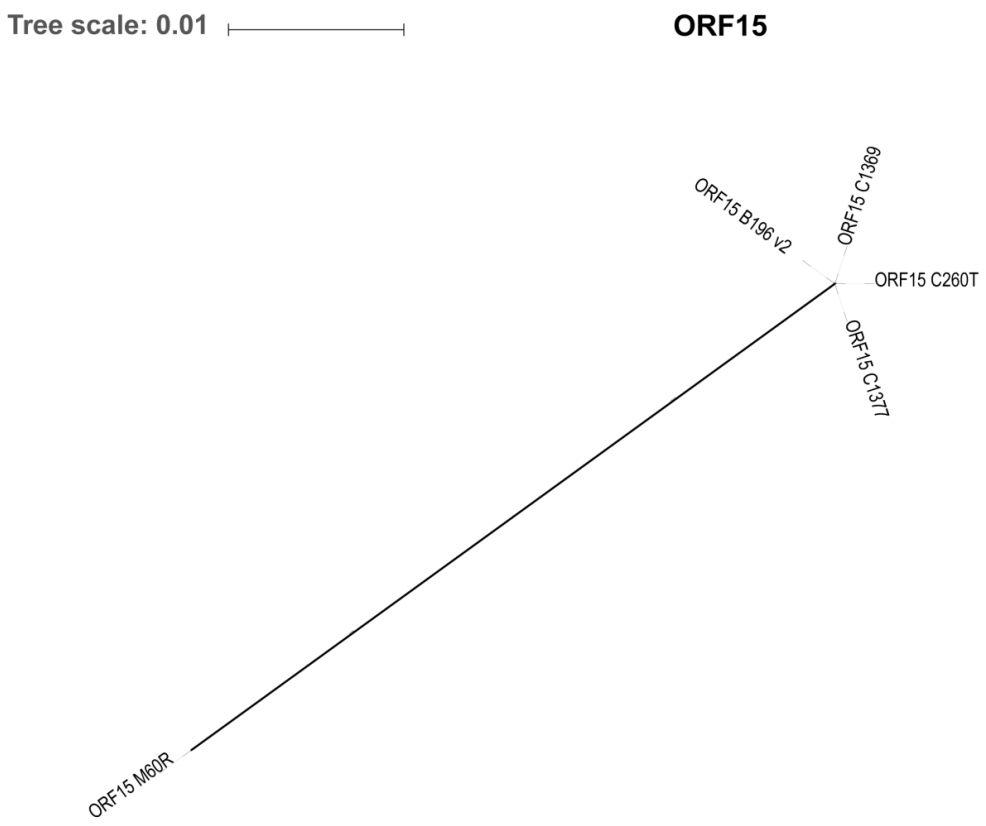

Figure 5.S29: Phylogenetic tree of ORF 15 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

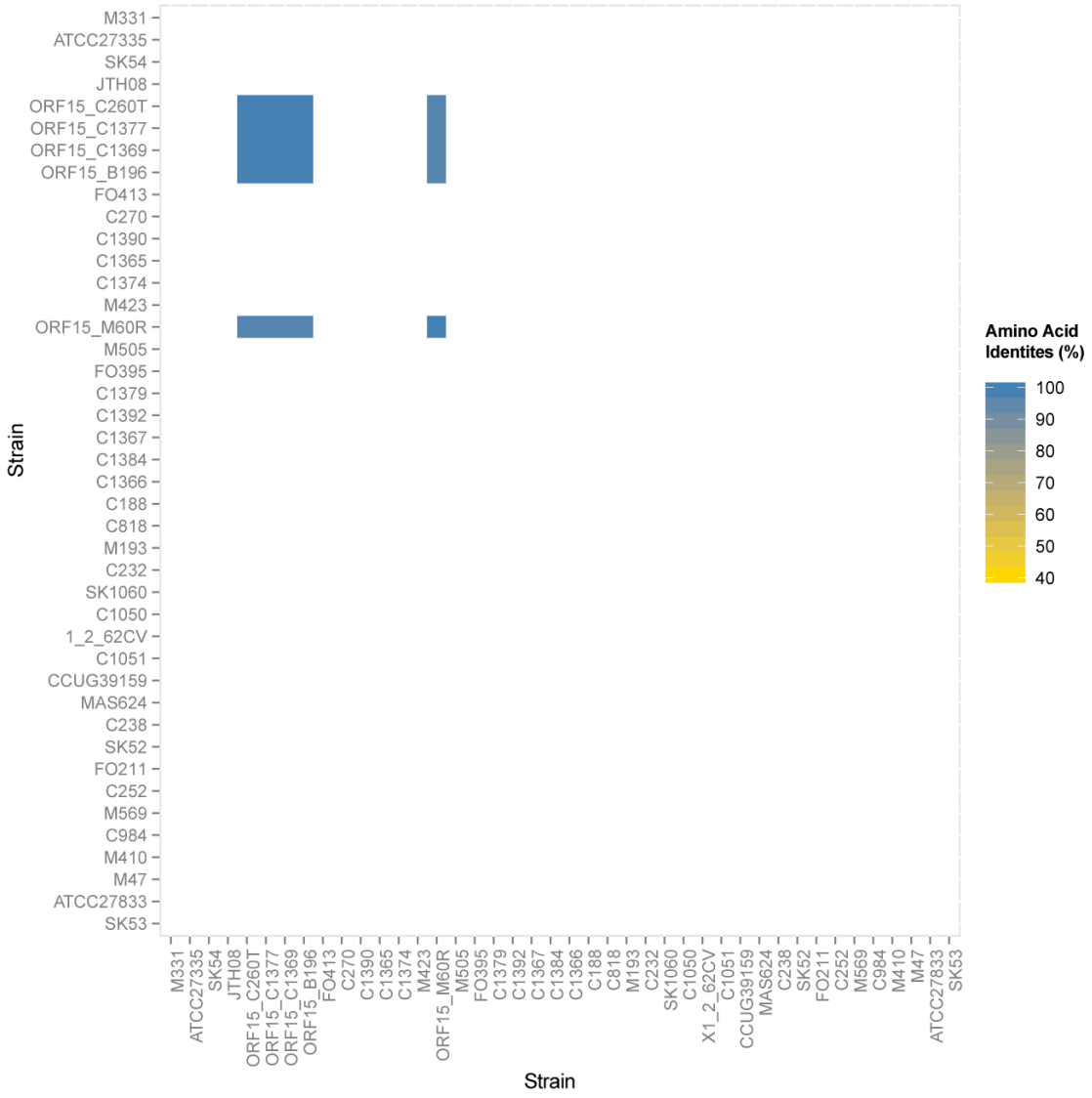

Figure 5.S30: Amino acid identity of ORF 15 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

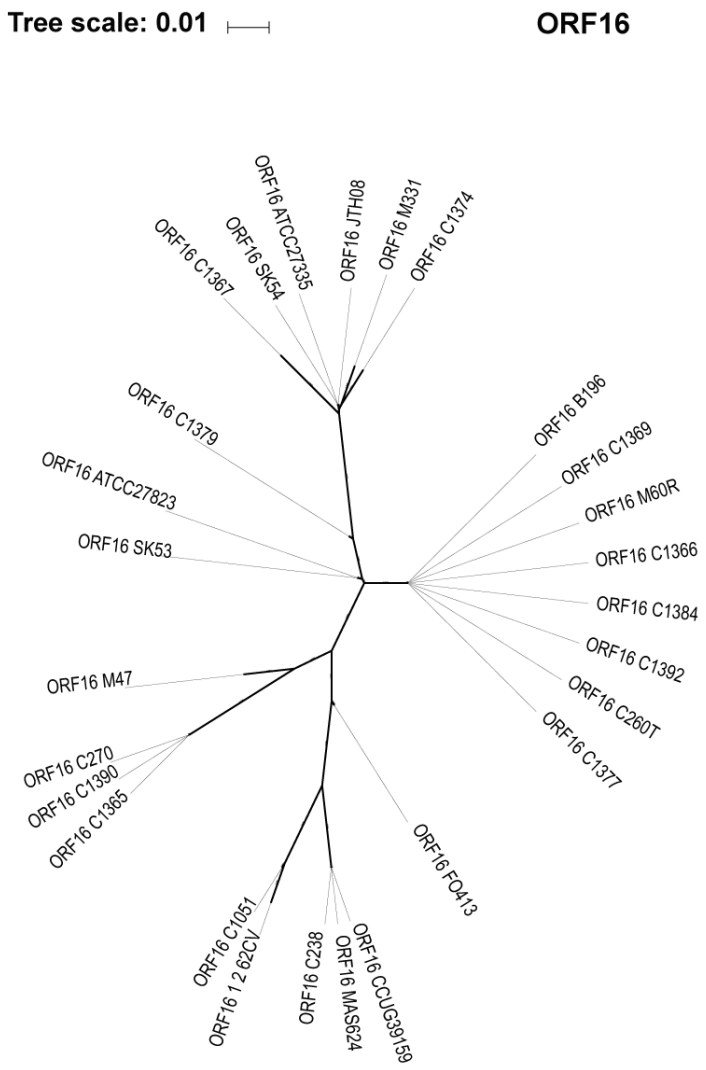

Figure 5.S31: Phylogenetic tree of ORF 16 in the *Streptococcus* Anginosus Group (SMG). The tree was generated using FastTree (Price et al. 2009). SMG strains are labelled with descriptions available in Table 5.1.

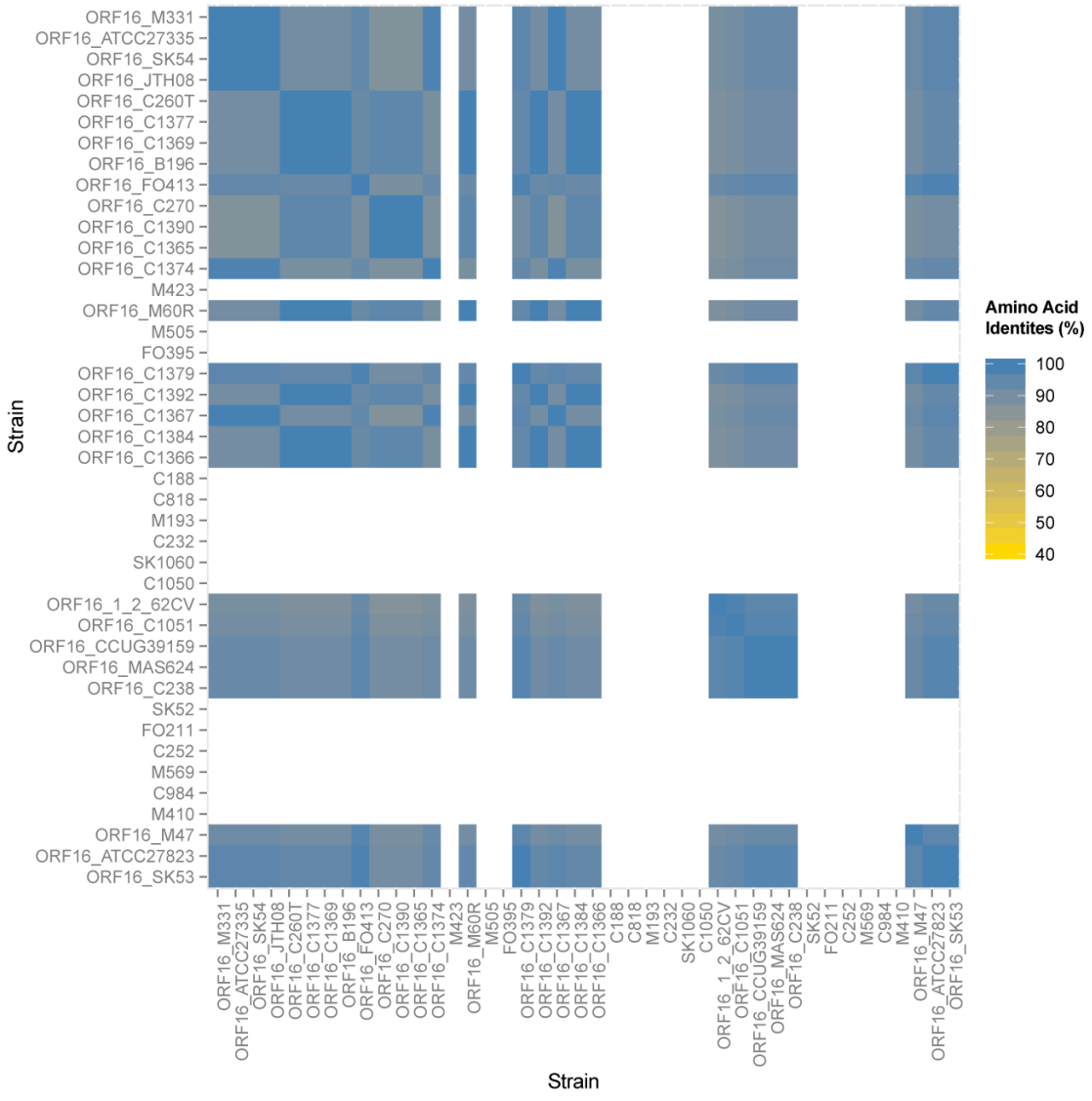

Figure 5.S32: Amino acid identity of ORF 16 in *Streptococcus* Anginosus Group (SMG) strains. The strains are described in Table 5.1. Strains are ordered as shown in Figure 5.3.

CHAPTER 6

DISCUSSION

6.1 Summary

SMG species lead dual lifestyles as both human commensals and pathogens. SMG associated infections are often polymicrobial, with obligate anaerobes being co-isolated. There is currently limited knowledge regarding the interaction between streptococci and obligate anaerobes. The diversity of anaerobes that are found with streptococci makes it difficult to form generalizations about the putative interactions and their associations with disease. **My goal was to investigate *S. intermedius* interactions with other microbes, to further our understanding of polymicrobial interactions.**

First, we characterized the basic metabolism of *S. intermedius* under aerobic and anaerobic growth conditions. An in depth transcriptome and metabolome analysis showed minimal upregulation of core pathways under anaerobic conditions, which we associated with the increased growth rate observed anaerobically. Under aerobic growth conditions, we observed an upregulation of oxidative stress genes. I demonstrated that *S. intermedius* favours anaerobic growth based on its growth rate. This chapter demonstrates that with minimal changes in gene expression, *S. intermedius* is able to adapt to changes in environmental oxygen.

The co-occurrence of obligate anaerobes with streptococci suggests that in such a community, there is an anaerobic microenvironment supporting the viability of obligate anaerobes (Mukae et al. 2016). It is unknown how such an environment is established. In Chapter 3, I demonstrated that streptococci can deplete oxygen, thereby creating an anaerobic environment. This is beneficial for *S. intermedius* as it grows better under anaerobic conditions (as shown in Chapter 2). There are several ways that streptococci

can deplete oxygen and for some strains, it involves the production of hydrogen peroxide (Yu et al. 2001; Zheng et al. 2011). This H_2O_2 production can inhibit come bacterial community members but be inconsequential to the producers as they have endogenous resistance mechanisms. We tested the ability of streptococci to support the viability of the obligate anaerobe *Prevotella melaninogenica* in the presence of oxygen. I demonstrated that under hypoxic conditions, certain streptococci can support *P. melaninogenica* viability, thereby allowing an anaerobe to grow in broth coculture exposed to oxygen. These streptococci (including *S. intermedius*) did not produce inhibitors such as H_2O_2. They had putative systems for oxygen depletion and removal of reactive oxygen species. To test whether *Streptococcus* detoxification of H_2O_2 is essential for *P. melaninogenica* survival in a hypoxic co-culture, a mutant in the alkylhydroperoxidase system *ahpCF* was created in *S. intermedius* B196. H_2O_2 was detected in the mutant culture although not to the same extent as *S. pneumoniae*, which has a dedicated enzyme for H_2O_2 production. In addition, the mutant inhibited *P. melaninogenica* viability in a hypoxic coculture. The mutant was able to deplete oxygen and create an anaerobic environment but unable to completely detoxify H_2O_2. This implicated the AhpCF system in endogenous H_2O_2 detoxification for *S. intermedius* but indirectly associated it with *P. melaninogenica* viability due to the communal effect of H_2O_2 detoxification. We also found that *S. mutans* has an H_2O_2 independent mechanism of inhibiting *P. melaninogenica*.

We demonstrated in chapter 3 that streptococci can promote *P. melaninogenica* survival *in vitro*. In chapter 4, we looked at how this interaction affects infection using a subcutaneous abscess model in BALB/c mice. Similar models in the past have

demonstrated that a synergy exists between the SMG species *S. constellatus* and *P. intermedia*, with higher mortality during co-infection in a pulmonary infection mouse model (Shinzato and Saito 1994). We observed that *S. intermedius* promoted *P. melaninogenica* survival in the co-infected abscesses in comparison to *P. melaninogenica* infection alone. This is in line with our *in vitro* observations of *S. intermedius* promoting *P. melaninogenica* viability. Co-infections also took longer to resolve in comparison to either infection alone. However, no mortality was observed. Using histology, we observed that the host response to *S. intermedius* infection is similar to that of Group A *Streptococcus* (GAS) infection, with neutrophil apoptosis, necrosis and NETosis occurring (Kobayashi et al. 2003; Buchanan et al. 2006). The range of neutrophil responses led us to conclude that SMG pathology is complex with several forms of host cell death taking place. The minimal bacterial synergism observed does not understate the validity of interaction taking place between *S. intermedius* and *P. melaninogenica*. *S. intermedius* promotes viability of *P. melaninogenica* under conditions where it would have otherwise naturally died off alone (*in vitro* and *in vivo*). We were unable to uncover the mechanisms involved in this bacterial interaction. The possible mechanisms involved will be discussed in the next sections.

In a polymicrobial community, one mechanism of *Streptococcus* competition with closely related bacteria is through the production of short bacteriolytic peptides called bacteriocins. In GAS, the *Streptococcus* invasion locus (*sil*) system controls bacteriocin production. A system with homology to the *sil* system has been identified in SMG (Olson et al. 2013). The system typically includes the pheromone peptide SilCR, the

sensor/regulator SilB/SilA, and ABC transporters SilD/SilE. In chapter 5, we identified components of the *sil* system in 43 SMG genomes. A bacteriocin cluster is associated with the *sil* locus in most strains and this is a hotspot for genetic variability in strains that carry them. We identified six putative bacteriocins in SMG out of sixteen putative open reading frames (ORFs) in the bacteriocin cluster. The remaining ORFs are thought to be involved in bacteriocin maturation or resistance mechanisms. In *S. intermedius*, we identified putative SilA binding sites upstream of select bacteriocins and *sil* genes, implicating the *sil* system and pheromone peptide SilCR in regulation of certain bacteriocins. We demonstrated that when the SilCR pheromone peptide gene is deleted in *S. intermedius*, a decrease in its bacteriocin-mediated inhibition of the sensitive strain *S. constellatus* M505 is observed. Adding the SilCR peptide exogenously to the mutant increased bacteriocin-mediated inhibition. Thus, we concluded that the pheromone peptide SilCR regulates certain bacteriocins in *S. intermedius*.

6.2 Interactions between *S. intermedius* and *P. melaninogenica*

We observed that *S. intermedius* promoted *Prevotella* viability *in vitro* and *in vivo*. This is not the first time that facultative anaerobes have been shown to increase obligate anaerobe survival in broth, in the presence of oxygen (Bradshaw et al. 1996). In the presence of three *Streptococcus* species, obligate anaerobes including *Prevotella* and *Fusobacterium* species had increased viability in mature broth chemostat cultures (Bradshaw et al. 1996). This was dependent on the presence of streptococci.

Oxygen is detrimental to *P. melaninogenica* viability (Takeuchi et al. 2000). *Streptococcus* oxygen depletion can create an anaerobic environment that we have shown

favours the viability of obligate anaerobes. It was previously shown that an anaerobic environment was created in a nine species polymicrobial community involving streptococci and various obligate anaerobes (Bradshaw et al. 1997). In addition to depleting oxygen, streptococci may also promote anaerobic viability by removing reactive oxygen species from the microenvironment. We demonstrated that a mutant in an H_2O_2 detoxification system inhibited *Prevotella* viability, even though the mutant could still deplete oxygen. The H_2O_2 produced did not inhibit *S. intermedius* under the conditions tested, but it did inhibit *P. melaninogenica*. This implies that *S. intermedius'* detoxification of H_2O_2 can have significant effects on a polymicrobial community.

The *Streptococcus* and *Prevotella* genera have unique metabolisms and there is some evidence of syntrophy. The anaerobic *P. intermedia* supernatant promoted *S. constellatus* growth *in vitro*, demonstrating that *Prevotella* can promote SMG viability through byproducts of its metabolism (Shinzato and Saito 1994). To address the specific mechanisms that could be involved in syntrophy, I looked deeper into the carbohydrate and amino acid metabolism of these two genera. I chose a broad scope as there is little species specific biochemical information currently available.

6.2.1 Amino acid metabolism

Streptococcus and *Prevotella* are both known to produce proteases, which supply peptides and amino acids as sources of nitrogen for the cells (Griswold et al. 1999; Byrne et al. 2010; Grinwis et al. 2010). **Figure 6.1** illustrates the use of amino acids by *P. intermedia* in the oral cavity (Takahashi 2005). The byproducts produced include volatile fatty acids in addition to other acids which could be used by other fermenters in the

community. One of the main byproducts of *Prevotella* amino acid metabolism is ammonia, which can neutralize the acidic environment created by both *Prevotella* and *Streptococcus* (see next section).

6.2.2 Carbohydrate and energy metabolism

Both *Streptococcus* and *Prevotella* are considered to be primary fermenters and can create acidic environments as a result of their carbohydrate metabolism (Abbe et al. 1991; Takahashi and Yamada 2000a). Streptococci are metabolically classified as lactic acid bacteria as they produce lactic acid, acetate, formate and alcohols as byproducts of their metabolism, with some storage of glycogen intracellularly (Yamada and Carlsson 1975; Abbe et al. 1991). *Prevotella* species can use environmental glucose to produce acetate, formate and succinate while also storing glycogen like products intracellularly (Takahashi and Yamada 2000a). One way *Prevotella* produces succinate is through a fumarate reductase cytochrome, part of a simple electron transport chain in *Prevotella* (Takahashi and Yamada 2000a). This allows them to reduce fumarate while regenerating NAD^+ during anaerobic respiration (Kroger et al. 1992). This anaerobic respiration involves other cytochromes and explains why *Prevotella* is dependent on heme (Robins et al. 1973; Chen and Wolin 1981). In our coculture analyses, we did not provide heme. It is possible that in the presence of heme, an increased viability would have been seen for *P. melaninogenica.*

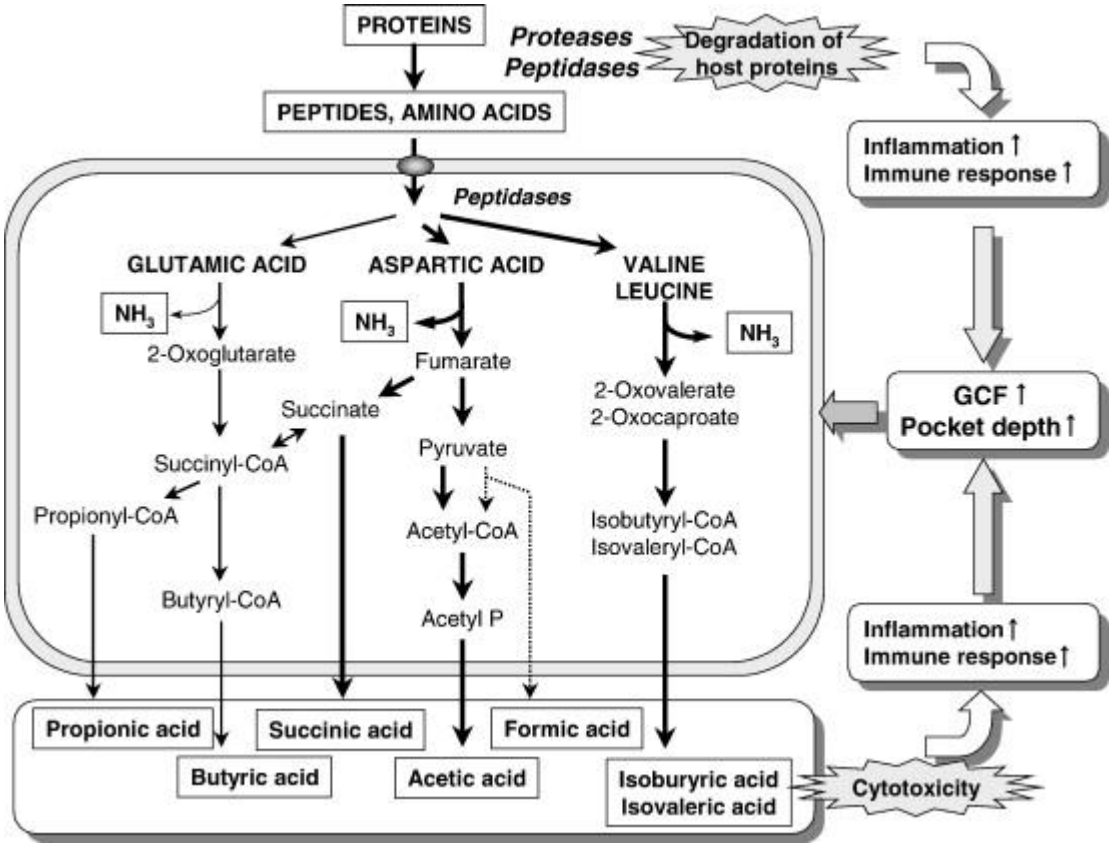

Figure 6.1: Amino acid metabolism of *Prevotella intermedia* in the oral cavity (taken from (Takahashi 2005). Environmental proteins can be degraded by extracellular proteases produced by *P. intermedia*. Amino acids and peptides are broken down into products that are part of carbohydrate metabolism, including pyruvate. Fumarate can be fed into the electron transport chain for ATP production. A number of acids, including volatile acids, are produced as byproducts that can affect the host immune system, increasing inflammation. This increases the gingival crevicular fluid (GCF) which is a rich source of proteins, creating a positive feedback loop.

Prevotella amino acid and carbohydrate metabolisms are highly linked. Like its close relative *Bacteroides*, *Prevotella* could potentially use environmental aspartate to produce fumarate and pyruvate (Takahashi and Yamada 2000b). The sequenced genome for *P. melaninogenica* ATCC 25845 (accession number: NC_014370.1) has an aspartate lyase AspA (locus tag: HMPREF0659_RS00370) that can putatively form fumarate from aspartate. *P. melaninogenica* ATCC 25845 also carries an aspartate oxidase (accession number: NC_014371.1; locus tag: HMPREF0659_RS09815) that allows it to produce oxaloacetate (another TCA cycle intermediate) from aspartate. These genes potentially allow *Prevotella* to convert metabolites from amino acid metabolism to carbohydrate metabolism and generate more ATP through the electron transport chain. These proteins are yet to be characterized in *Prevotella* but appear to be critical to metabolism.

A model for how the interaction takes place between *Streptococcus* and *Prevotella* has been described using colonization of the oral cavity (Takahashi 2005). The oxygen tolerant streptococci adhere to host surfaces and can ferment numerous carbohydrates to create an acidic environment. As streptococci deplete oxygen, obligate anaerobes such as *Prevotella* colonize. Proteases produced by both streptococci and *Prevotella* allow *Prevotella* to produce more metabolic byproducts to be used by other fermenters (**Figure 6.1**) (Takahashi 2005). In addition, *Prevotella* can respond to a limitation in glucose by producing additional proteases and cytotoxins (Saito et al. 2001). The ammonia produced by *Prevotella* neutralizes the pH of the environment. This benefits streptococci as they are inhibited when the acidity is below pH 4 (Takahashi and Yamada 1999).

6.3 Establishing polymicrobial environments and bacterial inhibition

The interactions occurring in polymicrobial environments are complex (Takahashi 2015). The metabolism of the bacteria involved changes with environmental nutrient sources, and each community member contributes to this. Bacteria have to survive on "tight incomes". This involves constant monitoring of the environment for resources to fulfill cellular requirements such as generating ATP, maintaining redox balance and acquiring carbon and nitrogen for making primary metabolites. Often, crossfeeding of metabolites takes place, making the system dynamic (Takahashi 2015). Some bacteria were found to coaggregate *in vitro*, suggesting direct interactions could take place *in vivo* during colonization (Kolenbrander et al. 1990; Levesque et al. 2003; Khemaleelakul et al. 2006). *Streptococcus* species have been shown to coaggregate with *Prevotella* (Levesque et al. 2003; Khemaleelakul et al. 2006). Using fluorescence microscopy, *P. melaninogenica* was shown to coaggregate with *S. gordonii* and other streptococci (**Figure 6.2**) (Khemaleelakul et al. 2006).

Bacteria often use inhibitors to shape their microenvironment. Broad spectrum inhibitors such as H_2O_2 are very effective against obligate anaerobes and this molecule is even used for intrageneric competition in streptococci (Regev-Yochay et al. 2006; Tong et al. 2007; Zheng et al. 2011a). A polymicrobial crossfeeding example involving hydrogen peroxide as an inhibitor is seen in the interaction between *S. gordonii* and the facultative anaerobe *Aggregatibacter actinomycetemcomitans* (Stacy et al. 2016; Stacy et al. 2014). *S. gordonii* produces lactate as a byproduct, which is useful to

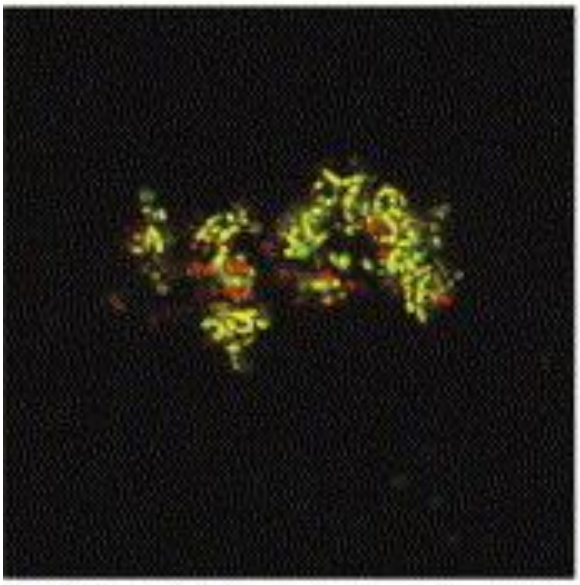

Figure 6.2: Coaggregation between *Prevotella melaninogenica* (red) and *Streptococcus gordonii* (green) (taken from (Khemaleelakul et al. 2006). Magnification is 2700x.*S. gordonii* were stained with SYTO-9, a green fluorescent nucleic acid dye. *P. melaninogenica* was stained with a red fluorescent nucleic acid dye, propidium iodide. Pure cultures were stained for 30 minutes and co-incubated for 2 hours before visualizing using confocal microscopy.

A. actinomycetemcomitans, but also produces H_2O_2, which inhibits it. The authors demonstrated that *A. actinomycetemcomitans* responds to H_2O_2 pressure by producing catalase to detoxify H_2O_2 but also physically "disperses" away from *S. gordonii*. This allows *A. actinomycetemcomitans* to be spatially oriented close enough to *S. gordonii* to benefit from its lactic acid production but far enough to not be inhibited by H_2O_2. This type of response to bacterial inhibitors might be common in a polymicrobial community.

Streptococci compete for the same niche since they require similar nutrients, can have similar associations with community members or the host and can have similar contributions to the development of a polymicrobial community. I have shown the direct competition of streptococci with each other (Chapter 5) and with an obligate anaerobe (Chapter 3). **Figure 6.3** illustrates what could occur in a polymicrobial community with both bacteriocin and H_2O_2 pressure being applied by streptococci. In this simplified model, the *Streptococcus* strain produces the inhibitor and affects the microenvironment. When no inhibitors are produced, a polymicrobial community can develop, including other streptococci and anaerobes. When only bacteriocins are produced, the bacteriocin producer may co-exist with obligate anaerobes (and other resistant bacteria), while inhibiting the bacteriocin-sensitive strain. It is possible for the inhibitor producer to propagate and spread. When only H_2O_2 is produced, streptococci and H_2O_2 resistant bacteria could form a mixed community, while inhibiting H_2O_2 sensitive strains. When both bacteriocins and H_2O_2 are produced, the producing bacteria will inhibit both H_2O_2- and bacteriocin-sensitive strains. This model does not take into consideration the

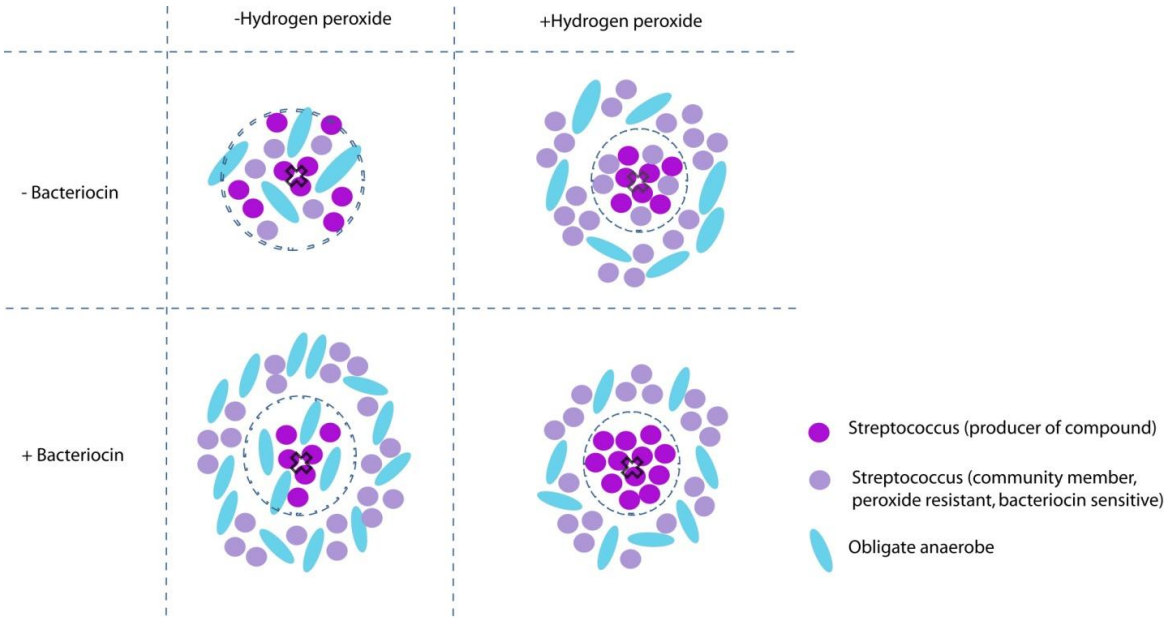

Figure 6.3: Model for inhibitor production by *Streptococcus* species and their effect on shaping a polymicrobial environment. The two inhibitors considered are bacteriocins (inhibits closely related streptococci in this case) and hydrogen peroxide (inhibits reactive oxygen sensitive strains, including obligate anaerobes). The inhibitor producer (shown in dark purple) produces inhibitors as indicated. Production of hydrogen peroxide inhibits obligate anaerobes but allows some streptococci to co-localize. Production of bacteriocins inhibits some Gram positive streptococci but allows obligate anaerobes to co-localize. Production of both can inhibit both streptococci and anaerobes. This model does not account for the level of oxygen exposure or the production of other inhibitors.

exposure to oxygen in this environment or the ability of the producer to spread. It also does not take into consideration other inhibitors that could be produced in the system. It is a simple approach to what could be occurring in a very complex system.

6.4 SMG Infection

The association of streptococci with both healthy and diseased individuals makes it difficult to clinically target the pathogen. Oral bacteria are often isolated from abscesses and other invasive infections (Li et al. 2000b; Hsiao et al. 2012; Arimatsu et al. 2014). This is thought to occur due to a break in the epithelial barrier in the oral cavity, allowing oral residents to systemically spread and cause infection in different body sites. It is possible that SMG species disseminate through the blood (Giuliano et al. 2012; Suzuki et al. 2016). However, it is unknown how dissemination takes place and culturing organisms from the blood can be difficult. More research is required to understand how these oral bacteria spread and cause polymicrobial infections.

In our mouse model, *P. melaninogenica* had increased viability in an *S. intermedius* co-infection in comparison to a mono-infection. We thought that the increased viability was due to the production of an anaerobic microenvironment in the abscess by *S. intermedius* that allowed *P. melaninogenica* to survive. However, it has been shown that neutrophils are capable of depleting oxygen during infection, depending on neutrophil density and respiratory burst (Campbell et al. 2014). Neutrophils use available oxygen to produce reactive oxygen species. In *P. melaninogenica* mono-infections, the abscesses were smaller in comparison to the co-infection and they visually appeared to have fewer neutrophils. It is possible that in the co-infection, both *S.*

intermedius and the neutrophils present deplete oxygen, allowing *P. melaninogenica* to survive. It could also be that *S. intermedius* depletion of oxygen limits neutrophil production of ROS and this allows *P. melaninogenica* to survive in the co-infection.

The abscesses in our mouse model are very different from the abscesses formed by *S. gordonii* and *A. actinomycetemcomitans* (Stacy et al. 2016). *S. gordonii* and *A. actinomycetemcomitans* are oral organisms associated with polymicrobial abscesses in humans. They are both facultative anaerobes. In a mono-infection with *A. actinomycetemcomitans*, abscesses had both oxic and anoxic microenvironments, with more anoxic *in vitro* transcriptome determinants aligning with the mono-infection. In a co-infection with *S. gordonii*, it was found that, again, both oxic and anoxic microenvironments were present. However, there was less alignment with the anoxic *in vitro* determinants than in the mono-infection. This combined with the known H_2O_2 production by *S. gordonii* led the authors to conclude that there is a greater availability of oxygen in the co-infected abscess and this shifts *A. actinomycetemcomitans* metabolism from fermentation to respiration. In our model, *S. intermedius* does not produce H_2O_2 and *P. melaninogenica* is sensitive to oxygen and reactive oxygen species. It is possible that our co-infected abscesses also have oxic and anoxic microenvironments and this allows *P. melaninogenica* to survive in the anoxic microenvironment.

6.5 Future directions

6.5.1 Interaction with anaerobes

To further analyze the interaction between SMG species and *P. melaninogenica* (or other anaerobes), several approaches can be taken. To visualize direct interaction,

microscopy can be used. Separate staining of each strain with nucleic acid fluorescent dyes SYTO-9 and propidium iodide and subsequent coculture for a short period can be done to visualize aggregation (Kobayashi et al. 2003). This would allow for a quick analysis for which bacteria SMG can directly interact with. The Surette laboratory has isolated multiple bacterial species along with SMG from clinical samples. This information can be used to see if direct interaction is taking place between these organisms.

To determine if metabolite based crosstalk is taking place between species, sterile supernatants from pure bacterial culture can be added to each strain and growth differences determined. Heat treatment of the supernatant can help identify if essential compounds are heat labile. A metabolomic analysis of the mono-culture supernatants and mixed co-culture supernantant can highlight which metabolites are being crossfed and if there are any unique metabolites produced in the co-culture. We currently know the exo-metabolites produced by *S. intermedius* in pure broth culture (Chapter 2). While there are probably differences in the exo-metabolites produced *in vivo*, this data is useful in predicting compounds that could be used by *Prevotella*. A transcriptomic analysis can be used to determine the changes in metabolism that occur between pure and mixed cultures. Our *in vitro* co-cultures did not provide heme for *P. melaninogenica*. This is required for growth of *Prevotella* species. With a supply of heme, it is possible that *P. melaninogenica* will have increased viability compared to what was observed. *In vivo*, *Prevotella* probably have mechanisms for obtaining heme from the host.

Our abscess model worked well for studying polymicrobial infection, but a humanized MHC-II mouse model with an intranasal infection route could be a more realistic model for determining how a commensal causes infection (Kasper et al. 2014). A co-infection can also be attempted with this model to see if this results in a polymicrobial infection. It would also be interesting to characterize the neutrophilic response to SMG species alone and in combination with anaerobes. *In vitro* stimulation of neutrophils with SMG species and detection of apoptosis or necrosis using flow cytometry can be useful for determining what is occurring during infection. Similar analyses using human neutrophils *in vitro* and microscopy determined that both apoptosis and necrosis were taking place after *S. pyogenes* stimulation (Kobayashi et al. 2003). Fluorescent SMG species (either through fluorescent dye staining or genetic modification) can allow for visualization of bacteria during phagocytosis using microscopy. Fluorescent staining of nucleic acids could possibly be used to visualize NETosis occurring in *in vitro* neutrophil stimulations.

6.5.2 The *sil* system and bacteriocin production

Chapter 5 introduces the SMG *sil* system and the bacteriocins without a mechanistic breakdown of how it functions. A systems approach to determine the function of each gene would be useful. This can be done by creating knockouts of each gene. We found that there are two SilCR peptides in SMG and that they can both affect bacteriocin production in *S. intermedius* B196. It is unknown whether this cross-reactivity occurs in all SMG strains. It would be interesting to determine if $SilB_{SMG\text{-}B}$ can be autophosphorylated by interaction with $SilCR_{SMG\text{-}A}$. In addition, outlining the

transcriptional regulation by SilA in *S. intermedius* B196 would be beneficial. We found 6 putative bacteriocins to be associated with the *sil* locus in SMG. Cloning and subsequent purification of these bacteriocins would be useful in *in vitro* analysis of activity and specificity in SMG and other Gram positive species. It could also be used to analyze the therapeutic potential of these bacteriocins as an oral bacteriocin delivery has been shown to be effective in clearing infection in a mouse model (Amer et al. 2014). The therapeutic potential of SMG-specific bacteriocins can be tested in an optimized SMG mouse model.

6.6. Conclusion

Polymicrobial interactions in the human microbiome are complex. Streptococci are associated with both healthy and diseased humans and their interactions can have adverse effects on polymicrobial communities. We demonstrated that *S. intermedius* can inhibit closely related SMG strains and promote interactions with certain obligate anaerobes. The mechanisms surrounding these interactions are not fully understood. They could involve direct bacterial binding, inhibitor production, syntrophy and immune system evasion. This thesis lays the groundwork for a deeper understanding into SMG polymicrobial interactions and how they can affect disease.

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