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Investigation into the Role of Manganese in the Growth of the Opportunistic Pathogen Streptococcus sanguinis

Brittany Spivey
Virginia Commonwealth University

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Abstract

While *Streptococcus sanguinis* plays a beneficial role in the oral cavity as a competitor of *Streptococcus mutans* and *Streptococcus sobrinus*, the bacteria that produce dental caries, it can cause deadly infective endocarditis if given the opportunity to colonize the vegetations that form over damaged endocardial tissue. Pre-existing heart conditions, surgery, and intravenous drug use predispose individuals to endocarditis. *S. sanguinis* growth and consequential virulence is significantly impeded by restriction to manganese. This is due to the resulting overwhelming oxidative stress and formation of reactive oxygen species which damage DNA and other cellular components. Manganese is essential for *S. sanguinis* proteins involved in DNA synthesis and is predicted to play other important roles. This study investigates the importance of the previously identified manganese transporter SsaACB, of the Lral family of conserved metal transporters, in combination with other proteins, such as ComCDE, SSA_0872, LguL, SSA_1625, and NrdD, for strain survival *in vitro*. These proteins of interest were selected because notable accumulation or reduction of associated metabolites in a metabolomic study suggested causation for the loss of virulence of the *ssaACB* knockout strain *in vivo*. A serum growth study of strains with single and double knockout mutations incubated at physiological conditions was conducted and the results were further supported by observation of fermenter culture growth after chelation of free manganese ions to exacerbate the effects of manganese starvation. This study identified combinations of proteins which are not essential for manganese-dependent *S. sanguinis* growth.

Keywords: opportunistic pathogen, pathogen, *Streptococcus sanguinis*, infective endocarditis, endocarditis, oral microbiology, oral health

Introduction

Endocarditis, which affected an estimated 13 of every 100,000 Americans in 2009, is the infection and inflammation of the endocardium, the lining of the inner chambers and valves of the heart (Bor et al. 2009). The risk of infection soars when the endocardium is damaged, often by heart conditions, surgery, and intravenous drug use (Habib 2006). Microbes can infect the normally sterile vegetations, composed of platelets and fibrin, which form over the damaged heart tissue as well as prosthetic parts. These opportunistic microbes, which may be bacteria or fungi, originate from the environment or other parts of the body, such as the mouth, and reach the endocardium through the bloodstream. This colonization begins with microbe deposition on and binding to the platelet-fibrin thrombi that compose much of the vegetation. The colonized vegetation grows until significantly exceeding normal size (Paik et al. 2005). The infected vegetations physically interfere with cardiac function and, when dislodged, cause embolisms and cerebrovascular accidents. Although *S. sanguinis* secretes low levels of hydrogen peroxide, resulting chemical damage to heart tissue has not been observed. Cases of endocarditis are rising, largely due to increasing prevalence of heart conditions and subsequent operations as well as rising antibiotic resistance. Though endocarditis is associated with severe illness, complications, and death, early diagnosis and appropriate antibiotic administration dramatically increase chances of survival (Millar and Moore 2004).

Streptococci, a genus of bacteria named for the twisting, bead-like formation of their cell clusters, are involved in 24.7% of documented endocarditis cases (Bor et al. 2009). *S. sanguinis*, is a microaerobic gram-positive bacterium which has the capability to enter the bloodstream from the oral cavity when disturbed, for instance by a routine dental procedure (Richey et al.

2008). It belongs to the viridans group of streptococci, named for the distinct green cultures they form on blood agar. *S. sanguinis* is one of the most common species of streptococci to cause endocarditis (Philippo et al. 2006). It is estimated that the human mouth hosts a range of over 700 species of microbes, comprised of bacteria, archaea, and yeasts (Aas et al. 2005). These diverse microbes form complex multi-species communities and many play beneficial roles in oral health (Zhu et al. 2018). *S. sanguinis* itself is normally a competitor of *S. mutans* and *S. sobrinus*, the bacteria which produce lactic acid and adhesive extracellular polysaccharides from sucrose and cause dental caries (Kreth et al. 2005).

Bacteria are capable of living in both planktonic and biofilm forms (Donlan 2002). Planktonic bacteria function independently of their neighbors and succumb relatively easily to environmental stress. Due to technological limitations, microbiological research has historically focused only on bacteria in their planktonic form that can be grown in single species cultures. This is beginning to change as the limitations of traditional culturing methods are now recognized (Xuesong et al. 2010). Biofilms form under stressful conditions and in environments not conducive to planktonic bacteria, such as the dynamic, saliva-covered oral cavity. Oral bacteria are notably adhesive as their survival depends on remaining within their characteristic site in the oral cavity in addition to maintaining their position within the biofilm complex. Biofilm bacteria form extensive microchannels for nutrient flow and gas exchange by inducing a type of programmed cell death among certain bacteria and a nutrient and gas gradient develops (Bayes 2014). This gradient explains the layered organization among bacterial species within the biofilm complex. Primary colonizers such as *S. sanguinis* reside deeper within the biofilm matrix and evolved to grow optimally at microaerobic oxygen concentrations. Optimal anaerobic and

microaerobic species culture growth depends on incubation under respective characteristic oxygen concentrations.

Manganese plays a uniquely critical role in *S. sanguinis* survival and virulence, making it a promising target for antibiotic design. This is because manganese is essential for the function of a protein involved in DNA synthesis and repair. Such metal ions also serve as enzymatic cofactors. Additional functions of manganese in the metabolism of *S. sanguinis* are not yet fully understood (Makhlynets et al. 2013). In the laboratory strain of *S. sanguinis*, SK36, a knockout mutation of the SsaACB manganese transporter in the cell membrane inhibited cell growth under aerobic conditions in manganese-limited media, including serum, and also severely reduces endocarditis virulence (Crump et al. 2014, Das et al. 2009). *In vitro*, the effect of this mutation was exacerbated with ethylenediaminetetraacetic acid (EDTA), a chelating agent which binds with free extracellular metal ions, further depriving cells of manganese. In a metabolomics study, EDTA-treated *ssaACB* knockout mutants were recently found to exhibit several oxidative-stress responses. Oxidative stress is defined by formation of reactive oxygen species (Ezraty et al. 2013). These reactive species may cause a wide range of damage, especially to DNA.

We hypothesized that by combining additional mutations of proteins involved in or greatly affected by oxidative stress with the *ssaACB* mutation, we could confirm or disprove the contribution of additional selected proteins to *S. sanguinis* growth. The ComCDE proteins are involved in competence which oxidative stress can trigger. Species capable of this horizontal gene transfer pose additional challenges for targeting antibiotic design. The SSA_0872 proteins are putative zinc-dependent hydrolases and glyoxalases predicted to be involved in the glutathione pathway. The LguL protein is involved in oxidative stress management by leading to

production of the metabolite glutathione which reduces harmful reactive oxygen species. The SSA_1625 protein is a putative lactoglutathione lyase also anticipated to play a part in the glutathione pathway. The NrdD protein subunit serves as an anaerobic iron-dependent component of a protein involved in production of dNTP, from which DNA is synthesized. Interestingly, downstream metabolite products accumulated in manganese-deprived mutants despite the fact that the NrdEF component, which forms an aerobic ribonucleotide reductase, requires manganese. Further investigation will be needed to determine the reason for this. We further hypothesized that the set of mutant strains used in this study would either grow normally or would exhibit significant growth inhibition in a serum growth study at 12% O_2. The 12% O_2 concentration resembles the physiological oxygen found in the *in vivo* model.

Materials and Methods

Bacterial strains and growth conditions

S. sanguinis wild type strain SK36 was used in this study. The *S. sanguinis ssaACB* knockout mutants were derived from the SK36 background and are listed in Table 1. T_0 cultures were cultivated in Brain Heart Infusion (BHI) (Difco, Moorslede, Belgium) broth at 6% O_2 for 18 hours at 37°C. BHI was chosen because it was previously found to support equivalent growth of all the strains used in this study. The 6% O_2 atmospheric condition was chosen to optimize culture growth. T_{24} cultures were cultivated in filter-sterilized pooled rabbit serum (Gibco, Gaithersburg, MD, USA) at 12% O_2 for 24 hours at 37°C. This serum was chosen to mimic rabbit blood, because the *in vivo* virulence model used in the lab is a rabbit model. The 12% O_2 atmospheric condition was chosen to approximate arterial O_2 concentrations found at the site of

the aortic valve, which is used in the rabbit model. The microaerobic atmospheric compositions used during culture incubation were generated with a programmable Anoxomat™ Mark II jar-filling system (Advanced Instruments, Norwood, MA, USA); for the anaerobic conditions used during plate incubation, a palladium catalyst was included in jars (Coy Laboratory Products Inc, Grass Lake, MI, USA). Prior to plating, samples were sonicated for 90 seconds to disrupt chains for colony forming units (CFU) count optimization. All cultures were plated using an Eddy Jet 2 spiral plater (Neutec Group Inc, Farmingdale, NY, USA) on BHI agar at 0% O_2 and incubated for 24 hours at 37°C. Cultures were inoculated from single-use aliquots of cryopreserved cells by 1000-fold dilution.

TABLE 1. Strains used in the study

Strain	Phenotype/Genotype	Source
SK36	Human oral plaque isolate	M. Kilian, Aarhus Univ., Denmark; (Xu et al., 2007)
JFP169	Δ*ssaACB* (Kan^R)	(Crump et al., 2014)
JFP173	Δ*ssaACB* (Tet^R)	This study
JFP49	Δ*comCDE*	(Rodriguez et al., 2011)
JFP239	Δ*ssaACB* Δ*comCDE*	This study
JFP240	ΔSSA_0872	(Xu 2011)
JFP241	Δ*ssaACB* ΔSSA_0872	This study
JFP242	Δ*lguL*	(Xu 2011)
JFP243	Δ*ssaACB* Δ*lguL*	This study
JFP244	ΔSSA_1625	(Xu 2011)
JFP245	Δ*ssaACB* ΔSSA_1625	This study
JFP246	Δ*nrdD*	(Rhodes et al. 2013)
JFP247	Δ*ssaACB* Δ*nrdD*	This study

Serum growth study

T_0 bacterial cultures were grown in five-mL of BHI at 6% O_2 for 18 hours at 37°C. T_0 cultures were plated and used to inoculate T_{24} five-mL 100% pooled rabbit serum with 10^6-fold dilution, pre-incubated at 12% O_2 and incubated further at 12% O_2 for 24 hours at 37°C. The exposure of T_{24} cultures to additional manganese was limited with the use of both serum and metal-free tubes. In addition, the T_0 cultures were serially diluted in phosphate-buffered saline (PBS) to reduce manganese contamination from BHI. At 24 hours post-inoculation, 1-mL

aliquots of bacteria were harvested, sonicated, and diluted in PBS for plating on BHI agar. Enumeration of streptococci was determined following 24-hour plate incubation in 0% O_2.

Live/dead assay

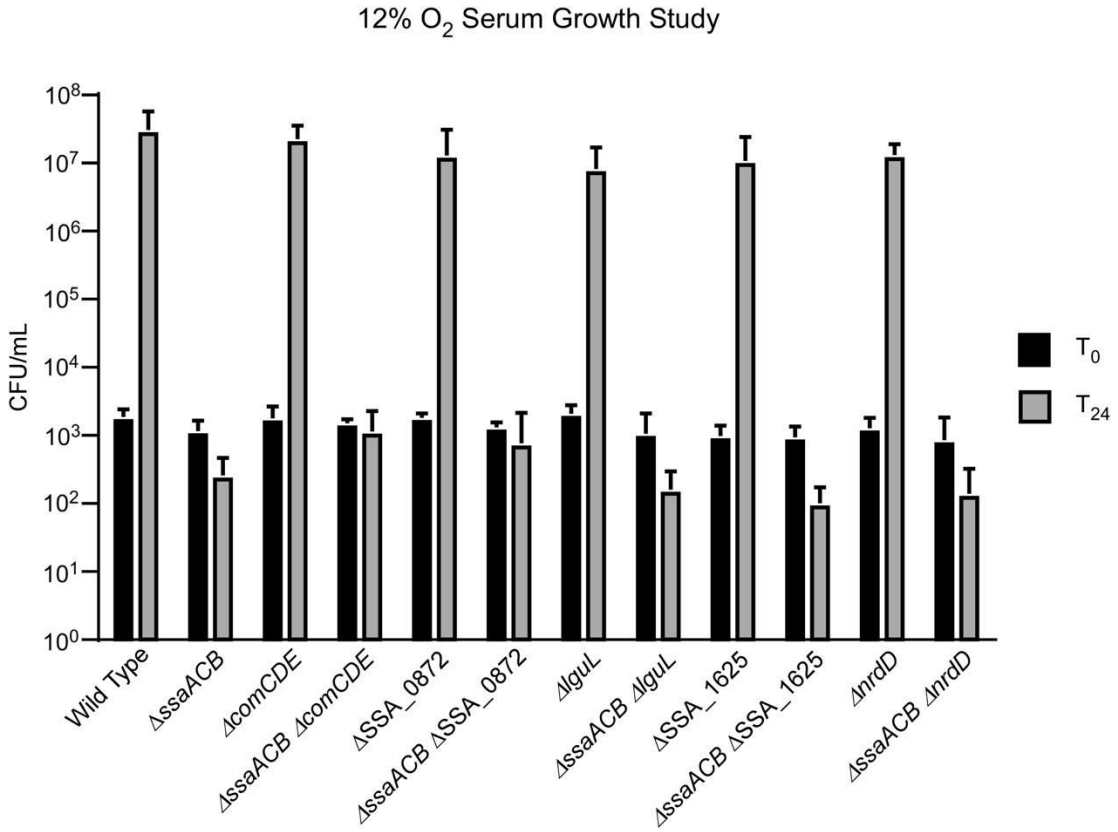
Four-hour cultures of planktonic *S. sanguinis* SK36 and JFP169 were separately grown at constant, optimal conditions in a fermenter with BHI. Samples were removed 20 minutes before and 10, 25, and 50 minutes after the addition of EDTA. These samples were placed into 96-well microtiter plates which were incubated with SYTO® 9 green-fluorescent nucleic acid stain and red-fluorescent nucleic acid stain propidium iodide (Thermo Fisher Scientific, Waltham, MA, USA). The fluorescence intensity of each marker was measured to determine the ratio of live to dead cells. This ratio represents culture viability indirectly as their binding depends on lack of cell membrane damage and membrane damage, respectively.

Statistics

All statistical tests were performed using InStat and Prism (GraphPad Software Inc, La Jolla, CA, USA). Significance was determined by t-test and with ANOVA, as indicated in the text and displayed in the graphs.

Results

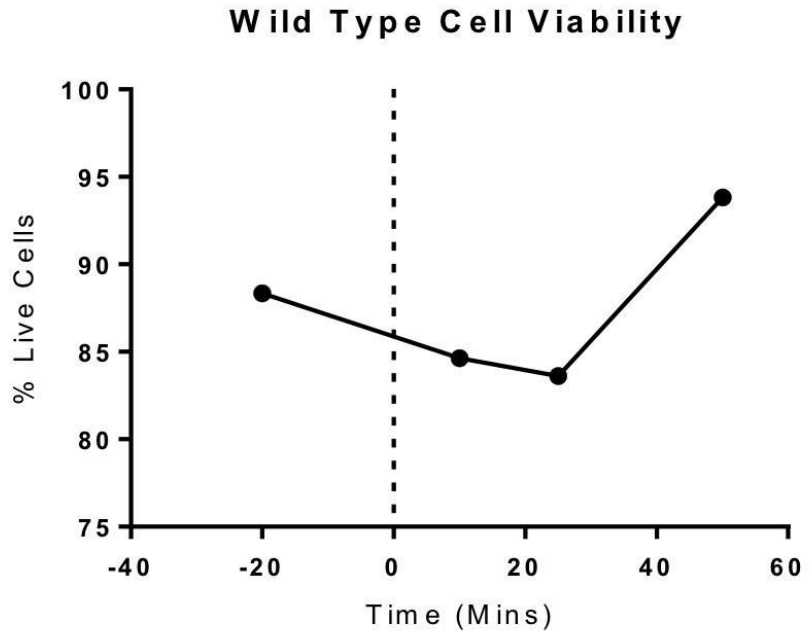
The effect of selected knockout mutations on *in vitro* growth. The effect of the *ssaACB* deletion in combination with *comCDE*, SSA_0872, *lguL*, SSA_1625, and *nrdD* on growth of *S. sanguinis* was investigated in this serum growth 12% O_2. The individual and coupled absence of these selected proteins did not significantly reduce growth. The double knockout mutants clearly grew more poorly in comparison with the wild type strain and single knockout mutants. However, no double knockout strain grew significantly differently than the *ssaACB* parent strain.

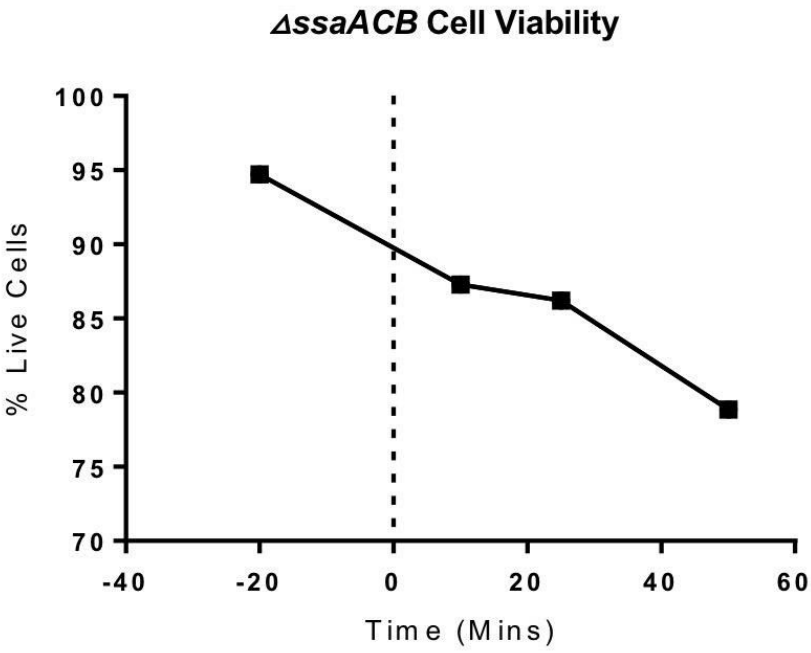


The data shown are representative of at least three experiments.

The effect of EDTA addition on fermenter-grown culture viability. The fermenter supported the growth of a larger volume of culture than that in the serum growth study, making it easier to

complete metabolomic and transcriptomic studies. EDTA chelation of manganese exacerbated the oxidative stress resulting from manganese starvation. The viability of the wild type culture population recovered from the EDTA treatment and even increased slightly from approximately 90% prior to EDTA treatment to 95% 50 minutes after addition of EDTA. The *ΔssaACB* mutant strain JFP169 (which bears the same *ΔssaACB* mutation as the JFP173 strain used in the serum growth study but has a different antibiotic resistance cassette) population saw a slight decrease after EDTA treatment consistent with the serum growth study results from approximately 95% prior to EDTA treatment to 80% 50 minutes after the addition of EDTA.


Wild Type Cell Viability
100
95
90
85
80
75
% Live Cells
-40
-20
0
20
40
60
Time (Mins)


ΔssaACB Cell Viability
100
95
90
85
80
75
70
% Live Cells
-40
-20
0
20
40
60
Time (Mins)

Discussion

This study did not identify a debilitating knockout mutation in *S. sanguinis* and thus did not identify any new manganese-dependent proteins or pathways. Such a mutation would have demonstrated significantly poorer growth in comparison to the *ssaACB* knockout mutant in the serum growth study and exhibited a dramatic decrease in viability upon addition of EDTA in the fermenter culture. The genes selected for study in this experiment were chosen after a metabolomics study demonstrated notable associated metabolite accumulation or reduction during the oxidative stress resulting from manganese starvation. The SsaACB manganese transporter, used in the double knockout mutations, consists of lipoprotein permease and ATPase components. Its role is essential for physiologically relevant growth. The knockout mutants may have survived in BHI because *S. sanguinis* could have unidentified manganese transport systems.

The manganese transporter remains a sound target for antibiotic design because of the relatively small number of genes involved as well as its specificity to *S. sanguinis* (Crump et al. 2014). In contrast, a previous study concluded that *S. sanguinis* virulence does not depend on any single protein involved in adhesion, discouraging further antibiotic development targeting this particular group of proteins (Turner et al. 2009). Future studies may examine both the existence of additional manganese transporter proteins as well as test the viability of more complex combinations of knockout mutations. Once key manganese-dependent proteins are identified, additional studies of *S. sanguinis* in both its planktonic and biofilm forms would follow.

As we study endocarditis treatment, methods for prevention and diagnosis are being reevaluated (Richey et al. 2008). The current practice of prophylactic antibiotic administration

for patients with heart problems prior to initiation of dental procedures has been called into question due to the limited protection current antibiotics provide as well as the danger of unnecessary antibiotic exposure (Wilson et al. 2007). Furthermore, sources of bacteremia are not restricted to dental procedures and include unavoidable daily activities (Paik et al. 2005). Nevertheless, rates of endocarditis rose significantly in the United Kingdom after cessation of prophylactic antibiotic prescription (Thornhill et al. 2016). Endocarditis is particularly challenging to diagnose, and physicians rely on extensive blood tests that meet the Duke Criteria for Clinical Diagnosis of Infective Endocarditis and heart imaging, which may show the substantial infected vegetations characteristic of advanced infection. Careful diagnosis is important because administration of improper or unnecessary antibiotics gives rise to antibiotic resistant bacteria (Habib 2006). Widespread antibiotic abuse has resulted in global entry in what the World Health Organization has termed the "post-antibiotic era." While antibiotics have saved countless lives and enabled complex surgeries, inevitable antibiotic resistance is rapidly outpacing new antibiotic production. This is attributed not only to antibiotic over-prescription and improper administration but also to unnecessary antibiotic distribution among livestock and low economic incentive for investment in development of new antibiotics. This last reason can be explained by the fact that clinical trials for antibiotics are expensive due to the need for large patient samples to produce statistically significant results in comparison to control groups which receive existing drugs instead of placebos for ethical reasons, the low price of antibiotics, and the certainty of eventual antibiotic resistance (Ventola 2015). Therefore, comprehensive, multifaceted understanding and solutions are needed to combat such public health threats.

As the dangers of antibiotic abuse are being realized, a new appreciation for the human microbiome is arising. The Human Microbiome project has marked the start of a new age of advancement in microbiology and medicine. The significance of the astounding diversity of microbe populations throughout an individual human, the changes in microbial populations over the course of the lifetime of an individual, and the large differences in microbial populations between individuals is beginning to be appreciated (Turnbaugh et al. 2007). Infections and diseases like caries and periodontitis are now viewed as the result of oral microbiome dysbiosis (Zhu et al. 2018). Future approaches to infection may focus on resetting the normal balance of microbial species with probiotics (Xuesong et al. 2010).

Ultimately, an antibiotic for *S. sanguinis* endocarditis infections could be designed to prevent translation, transcription, or protein function of the essential manganese-dependent proteins.

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