The relationship of the lipoprotein SsaB, manganese and superoxide dismutase in *Streptococcus sanguinis* virulence for endocarditis

Katie E. Crump,[§] Brian Bainbridge,[†§] Sarah Brusko, Lauren S. Turner,[‡] Xiuchun Ge, Victoria Stone, Ping Xu and Todd Kitten*
Philips Institute for Oral Health Research, Virginia Commonwealth University, Richmond, VA 23298, USA.

Summary

***Streptococcus sanguinis* colonizes teeth and is an important cause of infective endocarditis. Our prior work showed that the lipoprotein SsaB is critical for *S. sanguinis* virulence for endocarditis and belongs to the LraI family of conserved metal transporters. In this study, we demonstrated that an *ssaB* mutant accumulates less manganese and iron than its parent. A mutant lacking the manganese-dependent superoxide dismutase, SodA, was significantly less virulent than wild-type in a rabbit model of endocarditis, but significantly more virulent than the *ssaB* mutant. Neither the *ssaB* nor the *sodA* mutation affected sensitivity to phagocytic killing or efficiency of heart valve colonization. Animal virulence results for all strains could be reproduced by growing bacteria in serum under physiological levels of O_2. SodA activity was reduced, but not eliminated in the *ssaB* mutant in serum and in rabbits. Growth of the *ssaB* mutant in serum was restored upon addition of Mn^{2+} or removal of O_2. Antioxidant supplementation experiments suggested that superoxide and hydroxyl radicals were together responsible for the *ssaB* mutant's growth defect. We conclude that manganese accumulation mediated by the SsaB transport system imparts virulence by enabling cell growth in oxygen through SodA-dependent and independent mechanisms.**

Accepted 20 April, 2014. *For correspondence. E-mail tkitten@vcu.edu; Tel. (+1) 804 628 7010; Fax (+1) 804 828 0150. Present addresses: †Department of Oral Biology, University of Florida, Gainesville, FL 32610, USA. ‡Commonwealth of Virginia Division of Consolidated Laboratory Services, Richmond, VA 23219, USA. §These authors contributed equally to this work.
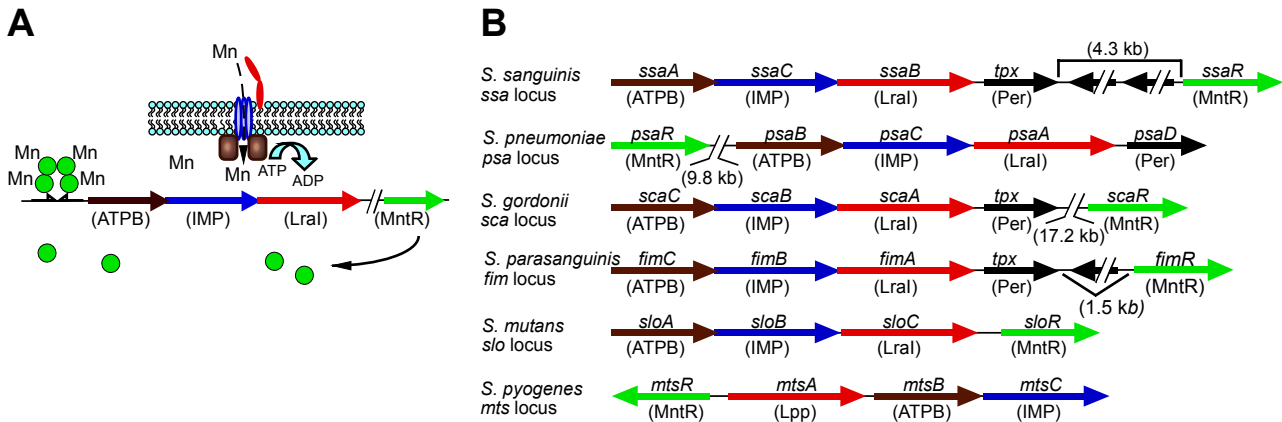
Introduction

More than 700 bacterial species have been identified in the mouth, with streptococci being the most abundant (Aas *et al.*, 2005). While most oral streptococci are harmless or even beneficial in the mouth (Belda-Ferre *et al.*, 2012), they can cause a wide array of extra-oral diseases, most notably, infective endocarditis. This disease is a serious infection of the heart valves or endocardium, with complications that include congestive heart failure, aneurysm and stroke (Bashore *et al.*, 2006). Even with antibiotic availability and improved treatments, recent studies report endocarditis mortality rates ranging from ~ 12–45% (Thuny *et al.*, 2012; Bor *et al.*, 2013).

Bacterial endocarditis is thought to occur when blood-borne bacteria colonize pre-existing cardiac 'vegetations' composed predominantly of platelets and fibrin and formed in response to injury, as can occur with certain congenital cardiac conditions. Oral bacteria, especially oral streptococci (Bor *et al.*, 2013), are a frequent cause of endocarditis. Among these species, *Streptococcus sanguinis* is particularly important (Di Filippo *et al.*, 2006). Due to their habitat, oral bacteria are a frequent source of transient bacteraemia resulting from dental treatment; therefore, antibiotic prophylaxis to reduce bacteraemia is a long-held practice for invasive dental procedures performed on high-risk patients (Wilson *et al.*, 2007). However, it now appreciated that poor oral health and routine activities such as chewing or brushing can also lead to bacteraemia, and that these bacteraemias account for more cases of endocarditis than dental procedures (Wilson *et al.*, 2007; Wray *et al.*, 2008). The constant prophylaxis that would be required to protect against these cases is not practical with standard antibiotics. Therefore, identifying mechanisms involved in *S. sanguinis* virulence is important not only for understanding *S. sanguinis* pathogenesis but also for designing better drugs for the prevention and treatment of endocarditis.

The many differences between the oral cavity, blood and cardiac vegetation suggest that *S. sanguinis* may possess virulence factors that are important for endocarditis pathogenesis, but not for oral colonization. However, very few genes required for endocarditis virulence have as yet been

Fig. 1. LraI operon organization and predicted gene functions.
A. Model of a generic LraI operon, including repression mediated by Mn^{2+}-bound MntR.
B. Comparison of LraI operons from representative streptococcal species. Genes depicted in the same colour are orthologous. ATPB, ATP-binding protein; IMP, integral membrane permease; LraI, substrate-binding lipoprotein; Per, thiol peroxidase; MntR, MntR-family regulator. Sequences taken from accession numbers NC_009009, AE005672, NC_009785, NC_017905, AF232688 and NC_004070, in the order depicted.

identified in *S. sanguinis* by us or others (Paik *et al.*, 2005; Ge *et al.*, 2008; Turner *et al.*, 2009b; Callahan *et al.*, 2011; Fan *et al.*, 2012). In a study examining 52 predicted lipoproteins, we found that the SsaB lipoprotein was singularly important for virulence; mutation of the *ssaB* (SSA_0260) gene reduced *S. sanguinis* competeness > 1000-fold in a rabbit endocarditis model (Das *et al.*, 2009).

Sequence comparisons suggest that SsaB serves as the substrate-binding protein for an ATP-binding cassette (ABC) transport system. Such systems are composed of an ATP-binding protein (ATPB), which provides energy for transport; an integral membrane permease (IMP) through which the substrate passes; and a substrate binding protein, which in Gram-positive bacteria is a lipoprotein (Fig. 1A) (Davidson *et al.*, 2008). SsaB belongs to a family of orthologous metal-transport proteins termed 'LraI' for Lipoprotein Receptor Antigen I (Jenkinson, 1994; Claverys, 2001) and is closely related to the LraI proteins from *Streptococcus pneumoniae*, *Streptococcus gordonii*, *Streptococcus parasanguinis* and other streptococci (Fig. 1B). All the systems illustrated have been shown to transport manganese (Dintilhac *et al.*, 1997; Kolenbrander *et al.*, 1998; McAllister *et al.*, 2004); however, transport of both manganese and iron has been reported in some cases (Spatafora *et al.*, 2001; Oetjen *et al.*, 2002; Janulczyk *et al.*, 2003; Paik *et al.*, 2003).

Previous studies have shown the importance of LraI proteins for streptococcal virulence (Berry and Paton, 1996; Marra *et al.*, 2002; Janulczyk *et al.*, 2003; Smith *et al.*, 2003; Johnston *et al.*, 2004; McAllister *et al.*, 2004), including for infective endocarditis (Burnette-Curley *et al.*, 1995; Kitten *et al.*, 2000; Paik *et al.*, 2003; Das *et al.*, 2009), and also for resistance to oxidative stress

(Kolenbrander *et al.*, 1998; Jakubovics *et al.*, 2002; Marra *et al.*, 2002; Tseng *et al.*, 2002; Janulczyk *et al.*, 2003; Paik *et al.*, 2003; Johnston *et al.*, 2004; McAllister *et al.*, 2004). It has been hypothesized that the increased sensitivity to oxidative stress is responsible for the reduced virulence of streptococcal LraI mutants; however, this hypothesis has yet to be formally tested. Moreover, it is not clear which reactive oxygen species (ROS) might be involved. Therefore, in this study, we examined the role of manganese, the LraI transporter SsaB, and the manganese-dependent superoxide dismutase (SodA) in *S. sanguinis* virulence. We report novel findings related to manganese and iron accumulation, the contribution of SodA to virulence, the identification of growth conditions that replicate the results of animal studies, and the contribution of superoxide to the O$_2$ sensitivity of an *ssaB* mutant.

Results

SsaB mediates manganese and iron accumulation and is under the control of a manganese-dependent regulator

To investigate the metal specificity of the SsaABC system, we performed inductively coupled plasma-optical emission spectroscopy (ICP-OES). Figure 2A demonstrates that wild-type *S. sanguinis* strain SK36 accumulated 0.12 μg manganese, 0.47 μg iron and 10.86 μg magnesium per mg of cellular protein. The *ssaB* mutant showed significant reductions in both manganese and iron content, to 0.014 μg mg^{-1} and 0.12 μg mg^{-1} respectively. There was no significant difference in the amount of magnesium (9.37 μg mg^{-1}), indicating that the mutation did not have a
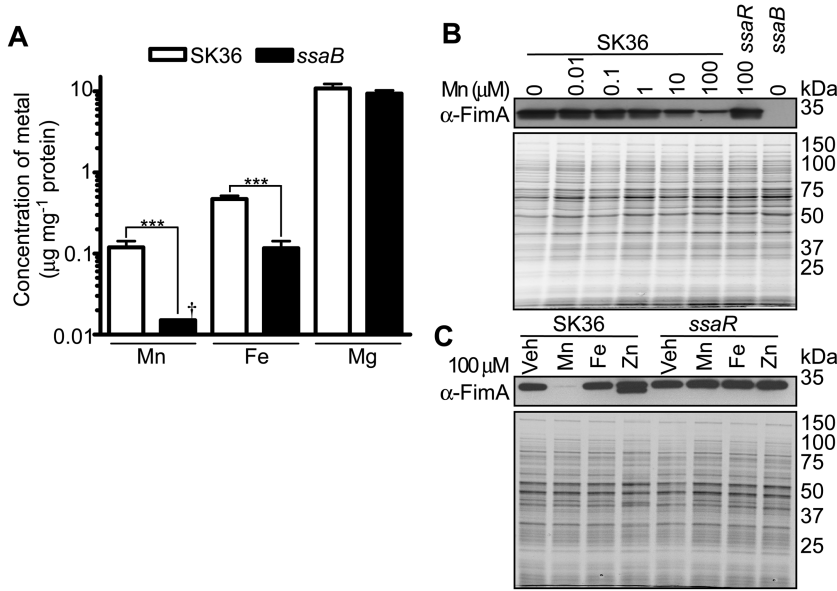
Fig. 2. SsaB mediates manganese and iron accumulation and is under the control of an Mn^{2+}-dependent regulator.
A. SK36 and *ssaB* mutant cells analysed by ICP-OES. Means and standard deviations from *n* = 3 independent experiments are shown. The symbol '†' represents value below the limit of detection (0.016 µg mg^{-1} cellular protein). Significance was determined by an unpaired *t*-test for iron and magnesium or by one-sample *t*-test in comparison to the limit of detection for manganese. ****P* < 0.001.
B. Western blot analysis of SsaB expression in SK36, *ssaR* and *ssaB* mutant strains cultured in BHI broth supplemented with the indicated concentrations of Mn^{2+}. SYPRO Ruby staining of a duplicate SDS-PAGE gel is shown as a loading control.
C. Western blot and stained gel as in (B), except that cells were cultured in Chelex-treated BHI broth alone or supplemented with 100 µM Mn^{2+}, Fe^{2+}, or Zn^{2+}. Blots in (B) and (C) are representative of *n* = 3 independent experiments.

global effect on divalent metal content. In addition, no significant differences were observed between the two strains in the concentrations of 14 additional metals for which there were standards (including zinc, nickel and copper), nor in signal intensities for 60 other elements analysed for which there were no standards (data not shown).

Because we detected higher concentrations of iron compared with manganese in both strains, unlike results obtained previously with *S. pneumoniae* (Jacobsen *et al.*, 2011), we wanted to determine whether this finding was due entirely to the lower concentration of manganese relative to iron in BHI broth (∼ 0.2 µM versus ∼ 15 µM respectively) (Jacobsen *et al.*, 2011). SK36 and the *ssaB* mutant were grown in APT broth, which is rich in both metals (890 and 270 µM manganese and iron, respectively, as determined by ICP-OES). These strains were compared with APT-grown *Escherichia coli*, which is known to preferentially accumulate iron over manganese (Anjem *et al.*, 2009), and *Lactobacillus plantarum*, which is known to accumulate high levels of manganese and little or no iron (Archibald and Fridovich, 1981). Figure S1 demonstrates that when both metals were abundant, *E. coli* incorporated more iron than manganese as expected, and both *S. sanguinis* strains incorporated more manganese than iron. *L. plantarum* also accumulated more manganese than iron, but the difference between the two metals was much greater than for *S. sanguinis*. The *ssaB* mutant incorporated less manganese and iron than SK36, but the difference was less than in BHI and, for iron, was not significant. The combined results suggest that manganese is acquired via SsaB-dependent and independent mechanisms when manganese is relatively abundant. Taken together, these data demonstrate that *ssaB* muta-

tion reduces manganese and iron accumulation, and that the relative cellular abundance of these two metals in *S. sanguinis* varies dramatically depending on their relative abundance in the growth medium.

Previous studies have determined that LraI operons are negatively controlled by a MntR-like metal-dependent regulator (Que and Helmann, 2000; Jakubovics and Jenkinson, 2001). Sequence similarity and proximity to the *ssaACB* operon suggests that *ssaR* (SSA_0256) encodes an *S. sanguinis* MntR-family regulator (Fig. 1). As with transport, negative regulation has been reported to be mediated by Mn^{2+}, Fe^{2+}, or both (Jakubovics *et al.*, 2000; Paik *et al.*, 2003; Hanks *et al.*, 2006; Johnston *et al.*, 2006; Rolerson *et al.*, 2006; Kloosterman *et al.*, 2008; Chen *et al.*, 2013). To examine the regulation of SsaB in *S. sanguinis*, we performed western blot analysis of SsaB expression in BHI-grown cells using antiserum raised against the SsaB orthologue from *S. parasanguinis*, FimA (Das *et al.*, 2009). SsaB protein levels decreased with increasing Mn^{2+} concentrations up to 100 µM (Fig. 2B). The Mn^{2+}-dependent repression of SsaB was lost in an *ssaR* mutant, confirming the identity of SsaR as the Mn^{2+}-dependent regulator of SsaB. To determine whether the regulation was specific for Mn^{2+}, SsaB levels were also assessed in the presence of Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, or Fe^{3+} at concentrations of 100 µM each. None of these metals had a detectable effect on SsaB expression (data not shown).

We next considered that effects of some metals might have been obscured in this experiment by divalent cations already present in BHI. Therefore, SsaB levels were also assessed in cells grown anaerobically in BHI treated with Chelex 100 to reduce divalent cation levels, then supplemented with Mg^{2+}, Ca^{2+}, and 100 µM of either Mn^{2+}, Fe^{2+}, or

Zn^{2+}. Mn^{2+} appeared to reduce SsaB expression more completely in this medium than in standard BHI (Fig. 2C). This could be due to partial de-repression of *ssaB* in standard BHI by Zn^{2+}, whose concentration was 26 µM as measured by ICP-OES, and which was shown to de-repress *psaBCA* expression in *S. pneumoniae* (Kloosterman *et al.*, 2008; Jacobsen *et al.*, 2011). Neither Fe^{2+} nor Zn^{2+} had any apparent effect on intensity of the main SsaB band in SK36 or the *ssaR* mutant. Interestingly, a second band was apparent below the main band in the Zn^{2+}-treated SK36 sample. The identity of this band is under investigation. Finally, given that previous studies have determined that components of LraI operons are necessary to combat ROS, we wanted to determine whether SsaB expression was regulated by O_2. Fig. S2 demonstrates that similar SsaB expression levels were observed at 0%, 6% and ambient O_2 levels. Thus, our results suggest that SsaB expression is controlled by SsaR in a Mn^{2+}-dependent fashion, but not by O_2.

Because we observed that *ssaB* mutation affects both manganese and iron accumulation, we wanted to determine whether SsaB-dependent uptake of both metals was required for growth. SK36 cultured for 24 h in 6% O_2 in BHI pre-treated with Chelex as above reached an OD_{660} of 0.33 ± 0.08 ($n = 3$). In contrast, when we assessed *ssaB* mutant growth at 24 h in the same medium, the OD_{660} reading was significantly less: 0.004 ± 0.003 ($P = 0.003$, paired *t*-test, $n = 3$). Supplementation of the medium with 2 µM Mn^{2+} improved the growth of both strains (OD_{660} of 0.83 ± 0.08 for SK36 and 0.79 ± 0.12 for *ssaB*) and eliminated the significant difference between the two. This suggests that the added Mn^{2+} restored growth by uptake through a lower-affinity transporter. Fe^{2+} added to 2 or 10 µM did not reproducibly restore the growth of either strain, nor did Co^{2+}, Cu^{2+}, Ni^{2+}, or Zn^{2+} (not shown). While SsaB is required for accumulation of wild-type levels of manganese and iron, these results and Fig. 2 suggest that Mn^{2+} alone governs SsaB expression and restores the aerobic growth of an *ssaB* mutant.

Contributions of sodA and ssaB to endocarditis virulence

O_2 sensitivity of LraI mutants has been associated with reduced activity of superoxide dismutase (SOD) (Jakubovics *et al.*, 2002; Tseng *et al.*, 2002; Johnston *et al.*, 2004), an enzyme that converts superoxide to hydrogen peroxide (H_2O_2) and O_2. Streptococci possess a single SOD, SodA, which is classified as Mn^{2+}-dependent (Bishop *et al.*, 2009). The reduced SodA activity in LraI mutants could therefore be due to inadequate levels of the Mn^{2+} cofactor, although reduced SodA expression due to manganese limitation has also been reported (Jakubovics *et al.*, 2002; Eijkelkamp *et al.*, 2014). To begin to assess

the relationship between SodA and SsaB, we examined a *sodA* mutant of SK36 created previously (Xu *et al.*, 2011). Compared with SK36 and the complemented mutant (*sodA sodA⁺*), the *sodA* mutant survived significantly less well in 50 mM paraquat, a compound that generates intracellular superoxide in the presence of O_2 (Fig. S3A). Additionally, the enzymatic activity of SodA was decreased or absent in the mutant, whether cells were grown in the presence or absence of O_2 (Fig. S3B and C). The inactivation of the *sodA* gene thus rendered the bacteria more susceptible to superoxide and eliminated the activity of the enzyme as expected, and both phenotypes were complemented by the *sodA* gene, confirming the integrity of the *sodA* mutant.

As with LraI proteins, SodA has been demonstrated to contribute to virulence in multiple streptococcal species (Yesilkaya *et al.*, 2000; Poyart *et al.*, 2001; Tang *et al.*, 2012). However, to our knowledge, isogenic *sodA* and LraI mutants have never been tested together, making it impossible to accurately determine the relative contribution of each protein to virulence. To address this issue for the endocarditis virulence of *S. sanguinis*, we co-inoculated JFP36, a derivative of SK36 that contains an erythromycin resistance gene at the same ectopic chromosomal site used for *sodA* complementation and which has virulence indistinguishable from that of SK36 (Turner *et al.*, 2009a), along with the *sodA* mutant, an *ssaB* mutant, or the *sodA*-complemented *sodA* mutant (*sodA sodA⁺*) into rabbits catheterized to induce the formation of sterile vegetations. After 20 h, both the *sodA* and *ssaB* mutants showed significantly decreased competitiveness compared with JFP36 (Fig. 3). Importantly, the *sodA* mutant was significantly more competitive than the *ssaB* mutant. Complementation of the *sodA* mutation restored its competitiveness to that of JFP36. In streptococcal endocarditis, pathology results primarily from growth of bacteria within the vegetation (Durack, 1975); thus, growth is tantamount to virulence. Taken together, the data demonstrate that both mutants are reduced in virulence, but that the *sodA* mutant is more virulent than the *ssaB* mutant in the endocarditis model. This suggests that manganese transported by the SsaABC system has an important role in virulence apart from serving as a cofactor or inducer for SodA.

Previous studies have shown that streptococcal endocarditis in the rabbit model initiates with attachment of bacteria to a sterile vegetation, followed by envelopment with a protective layer of platelets and fibrin and, finally, growth of bacteria and the vegetation (Durack and Beeson, 1972; Durack, 1975). Because both mutants showed decreased virulence in the rabbit model, we wanted to determine the stage at which each was affected. Rabbits were triply inoculated with JFP36, the *ssaB* mutant and the *sodA* mutant. Figure 4A shows that at the early time point
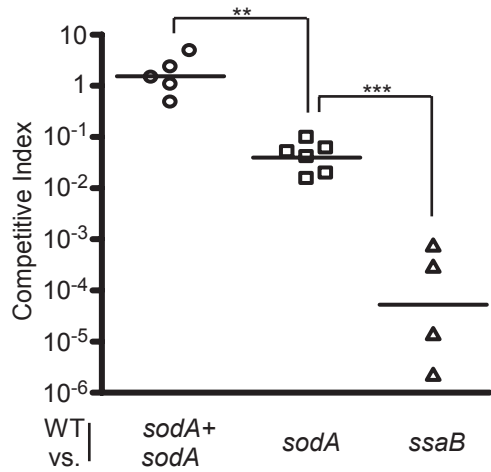
Fig. 3. Examination of *S. sanguinis ssaB* and *sodA* mutant virulence in a rabbit model of IE. Rabbits were co-inoculated with JFP36 (virulent competitor) in combination with the *sodA* or *ssaB* mutant, or the *sodA* complemented *sodA* mutant (*sodA sodA*⁺). Bacteria were harvested from aortic valve vegetations 20 h later and plated for bacterial counts. Symbols indicate competitive index values from 4–6 rabbits from $n = 2$ independent experiments. Geometric means are indicated by horizontal lines. Statistical significance was assessed by a one-way ANOVA with a Tukey–Kramer post-hoc test. **$P < 0.01$, ***$P < 0.001$.

killing (Poyart *et al.*, 2001; Tang *et al.*, 2012). We therefore examined sensitivity of the strains to killing by polymorpho-nuclear leucocytes (PMNs). When incubated with human PMNs, all three strains were killed to a similar extent (Fig. 4B). To confirm that we were using appropriate conditions, we performed the same assay with strains of *S. gordonii* shown previously to be resistant or sensitive to killing by PMNs (Young Lee *et al.*, 2006). Figure 4C shows that we reproduced the results of the previous study, demonstrating that the DL1 strain of *S. gordonii* is significantly more resistant to PMN killing than *S. gordonii* strain SK12. The experiment therefore confirmed that all three *S. sanguinis* strains were sensitive to PMN killing. Taken together, our data demonstrate that the reduction in virulence of the *sodA* and *ssaB* mutants in the rabbit model of endocarditis was not due to alterations in early bacterial colonization or survival.

To further investigate the relationship between *ssaB*, *sodA* and *S. sanguinis* virulence for endocarditis, a *sodA ssaB* double mutant was created and co-inoculated with the *ssaB* mutant and JFP36 into catheterized rabbits. At 2.5 h, there was a significant decrease in the number of *sodA ssaB* mutant bacteria recovered compared with the wild-type and *ssaB* mutant strains (Fig. 5). By 20 h post inoculation, the number of wild-type bacteria recovered had increased from a mean of 380 colony-forming units (cfu) to ∼ 10⁷ cfu per animal. In contrast, the *sodA ssaB* and *ssaB* mutants showed no significant growth in the same six animals. In addition, the *sodA ssaB* double mutant was not recovered from three of the six animals (gray symbols in Fig. 5), whereas the other two strains were recovered from all six. The data thus suggest that
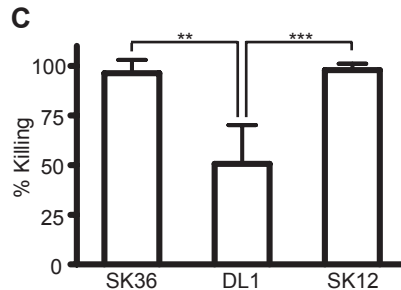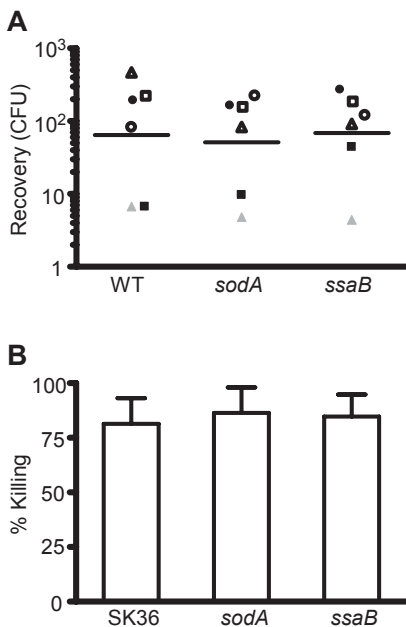
of 2.5 h, the numbers of recovered bacteria were similar for all strains, suggesting that decreased virulence was not due to alterations in early bacterial attachment. It has been shown in the rabbit model that immediately after attachment, many streptococci are engulfed by phagocytes (Durack, 1975). Moreover, SOD activity has frequently been associated with bacterial resistance to phagocytic

A

B

C

Fig. 4. Reduction in virulence is not due to alterations in early bacterial or immunological events.
A. Rabbits were inoculated with JFP36 and the *sodA* and *ssaB* mutants as in Fig. 3, except that all three strains were co-inoculated into each animal and bacteria were harvested 2.5 h after inoculation. Individual values and geometric means of recovered bacteria from 6 rabbits from 2 independent experiments are shown. Like symbols indicate strains recovered from the same rabbit. Gray symbols represent the limit of detection for rabbits from which no bacteria were recovered.
B and C. SK36 compared with its *sodA* or *ssaB* mutants (B) or with the DL1 and SK12 strains of *S. gordonii* (C) in a PMN survival assay. Means and standard deviations of the percent killing from $n = 3$ independent experiments are shown. Statistical significance was assessed by a repeated-measures ANOVA with a Tukey–Kramer post-hoc test. **$P < 0.01$, ***$P < 0.001$.
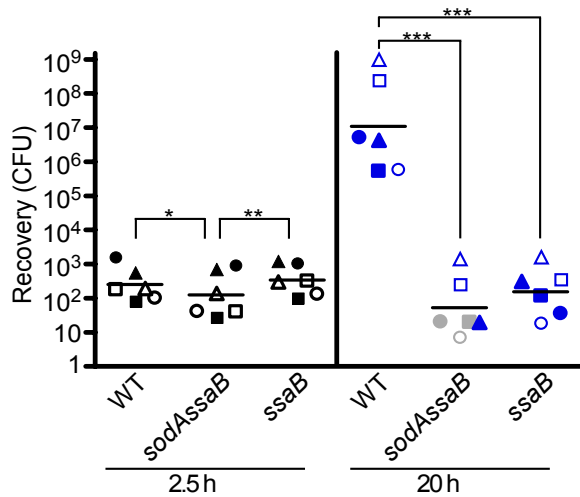
Fig. 5. Examination of *sodA* and *ssaB* single and double mutants in a rabbit model of *IE*. Rabbits were triply inoculated with JFP36, the *sodA ssaB* double mutant, and the *ssaB* mutant and sacrificed at 2.5 or 20 h post infection. Individual values and geometric means of recovered bacteria from 6 rabbits from 2 independent experiments are shown. Like symbols indicate strains recovered from the same rabbit. Gray symbols represent the limit of detection for rabbits from which no bacteria were recovered. Statistical significance was assessed by a repeated-measures ANOVA with a Tukey–Kramer post-hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the *sodA ssaB* double mutant is reduced in virulence compared with the wild-type and *ssaB* mutant strains. This indicates that despite its reduced virulence compared with a *sodA* mutant, an *ssaB* mutant retains biologically relevant levels of SodA activity *in vivo*.

Contributions of sodA and ssaB to serum growth

Because our results suggested that neither decreased attachment nor decreased resistance to phagocytic killing were responsible for the reduced virulence of *sodA* or *ssaB* mutants, we sought to examine growth under conditions simulating the *in vivo* environment. We first assessed growth of the *ssaB* mutant in pooled normal rabbit serum which, like human serum (Versieck, 1985), possesses nanomolar levels of manganese (Liu *et al.*, 2005). Cultures were incubated with 6% O_2 – our standard microaerobic condition. As shown in Fig. S4A, the *ssaB* mutant grew to a significantly lower density at 24 h than SK36. Heating of serum to 56°C for 30 minutes to inactivate complement had no effect on *ssaB* mutant growth (data not shown). Complementation of the mutant with the *ssaB* gene inserted into the same site behind the same promoter as the *sodA* complementation construct improved growth significantly, although not to the level of SK36. The incomplete complementation could be due to a polar effect of the *ssaB* mutation on expression of the downstream *tpx* (thiol peroxidase) gene (Fig. 1); however,

a separate *ssaB* mutant created using an allelic exchange strategy shown to generate non-polar mutations (Xu *et al.*, 2011) displayed the same serum growth defect as the *ssaB* mutant used here (data not shown). In addition, previous studies with other streptococci have indicated that the *tpx* gene is transcribed from its own promoter, in addition to being co-transcribed with the preceding genes (Fenno *et al.*, 1995; Novak *et al.*, 1998) and, in *S. gordonii*, is oppositely regulated from the upstream LraI genes in response to manganese (Jakubovics *et al.*, 2000; 2002). It is also possible that the incomplete complementation may be due to SsaB being expressed in the complemented strain at one-third the level of SK36 as determined by quantitative western blot (data not shown). This mirrors a previous finding in which a different chromosomal construct that expressed SsaB at 20–25% of wild-type levels produced an *in vivo* CI value of 0.19, while a plasmid-borne construct that fully restored expression restored the CI to 0.53, versus 1.7×10^{-5} for the *ssaB* mutant (Das *et al.*, 2009). Yet another chromosomal construct restored the *in vivo* CI to 0.49 compared with 5.7×10^{-5} for the *ssaB* mutant (Turner *et al.*, 2009a). Thus, loss of SsaB expression is overwhelmingly, if not completely, responsible for the virulence defect in the *ssaB* mutant. Finally, addition of $MnSO_4$ to 2 μM resulted in complete restoration of *ssaB* mutant growth (Fig. S4B), confirming that the *ssaB* mutant did not suffer from an undetected defect in an unrelated gene.

We next performed a similar serum growth experiment in which the *sodA* and *sodA ssaB* double mutants were also assessed. Fig. 6B shows that in 6% O_2, the *sodA ssaB* mutant showed no growth at 24 h, and could not be recovered at all in some experiments, in agreement with our findings in the rabbit model (Fig. 5). However, the *sodA* mutant grew indistinguishably from the wild type at 24 h, which contrasted with our *in vivo* findings. When we assessed bacterial growth in 12% O_2, representative of the O_2 found in arterial blood (Atkuri *et al.*, 2007), which flows past the aortic valve, we observed results similar to those *in vivo*, with a significant decrease in growth of the *sodA*, *ssaB* and *sodA ssaB* mutants at 24 h (Fig. 6C). Moreover, the hierarchical growth of the different strains at 12% O_2 reflected our *in vivo* results, with the *sodA ssaB* mutant exhibiting the greatest growth defect, followed by *ssaB* then *sodA*. Figure 6D demonstrates that increasing the O_2 concentration to super-physiological levels (21% O_2) by growth in room air resulted in poor growth of all strains and statistically indistinguishable growth of the three mutant strains, again contrasting with our *in vivo* findings. Finally, Fig. 6A shows that in 1% O_2, all strains grew indistinguishably from wild-type, indicating that neither *ssaB* nor *sodA* is required for serum growth under low-O_2 conditions. These results suggest that *in vivo* conditions are modelled well by growth in normal rabbit serum at 12% O_2, and that reduced
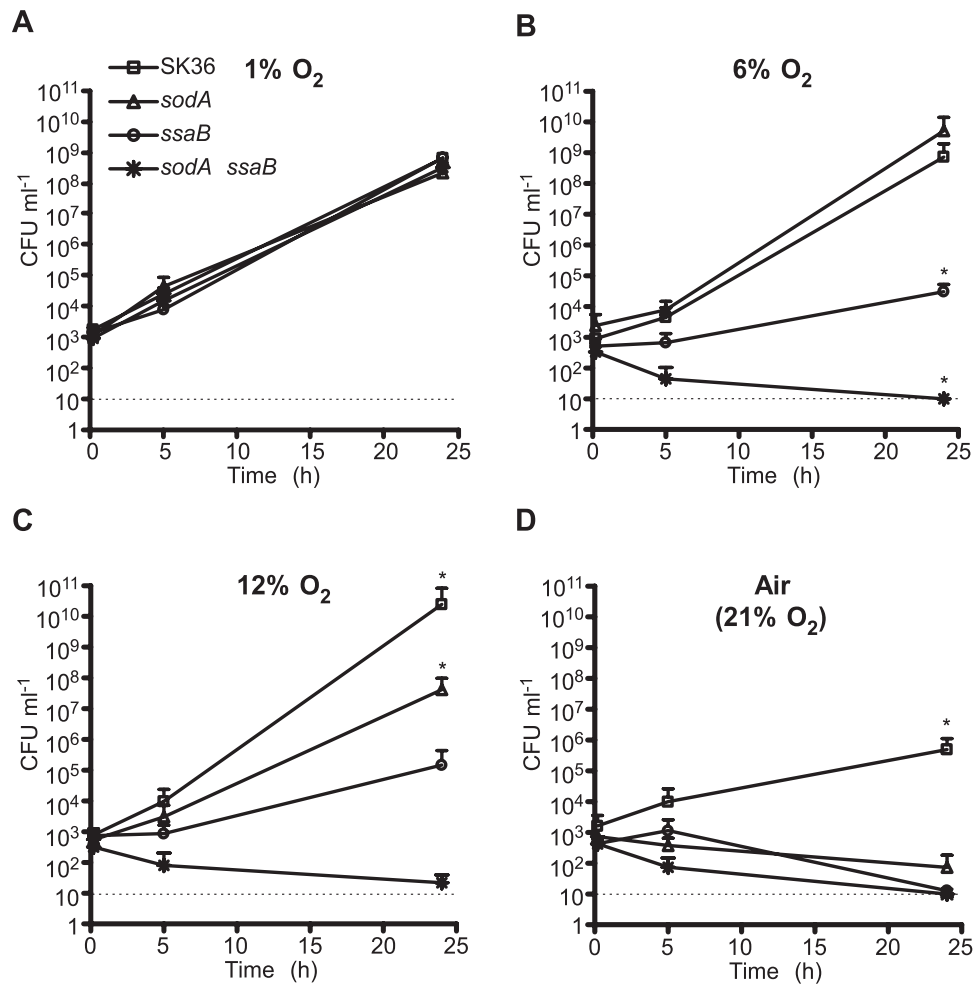
Fig. 6. Growth of *S. sanguinis* strains in rabbit serum. The strains indicated were cultured for 0, 5 or 24 h at 37°C in the indicated atmospheres, then serially diluted and plated for bacterial counts. The mean plus the standard deviation is shown for each time point from a minimum of $n = 3$ independent experiments. The dotted line represents the limit of detection. Statistical significance at 24 h was assessed by a repeated-measures ANOVA with a Tukey–Kramer post-hoc test. *$P < 0.05$ compared with all other strains. In (B), SK36 and the *sodA* mutant were significantly different ($P < 0.05$) from all strains except each other. In (C), the *ssaB* and *sodA ssaB* mutants were significantly different ($P < 0.05$) from all strains except each other. All other differences in all panels were non-significant.

growth under these conditions is sufficient to explain the observed virulence reductions of all three mutants.

Examination of SOD activity in serum-grown cells

Previous studies have shown that LraI mutants exhibit decreased SodA activity, but these studies were not performed under physiological conditions (Martin *et al.*, 1984; Niven *et al.*, 1999; Jakubovics *et al.*, 2002; Tseng *et al.*, 2002; Janulczyk *et al.*, 2003). Moreover, the reduced virulence of the *sodA ssaB* double mutant relative to the *ssaB* mutant (Fig. 5) indicates that biologically relevant levels of SodA activity are retained in an *ssaB* mutant *in vivo*. We therefore examined SodA activity using our physiological growth conditions. Fig. 7A shows significantly greater SodA activity in SK36 grown in rabbit serum and 12% O₂

compared with the *ssaB* mutant ($P < 0.05$). The *sodA* mutant demonstrated significantly decreased SOD activity compared with both SK36 and the *ssaB* mutant. Activity was restored to SK36 levels by complementation.

Previous studies have suggested that SodA proteins from some streptococci (Martin *et al.*, 1986; De Vendittis *et al.*, 2012; Eijkelkamp *et al.*, 2014) exhibit *in vitro* activity with either Mn^{2+} or Fe^{2+} cofactors. By treating lysates to eliminate bound metal, then reconstituting with Mn^{2+} or Fe^{2+}, we determined that this was also true for *S. sanguinis* SodA (data not shown). We therefore wanted to determine whether the residual SOD activity in the *ssaB* mutant was due to Mn^{2+} or Fe^{2+} serving as the SodA cofactor. In previous studies, SODs have exhibited greater sensitivity to inactivation by H_2O_2 when cofactored with Fe^{2+} than with Mn^{2+} (Yamakura *et al.*, 1998; Tabares *et al.*, 2003; De
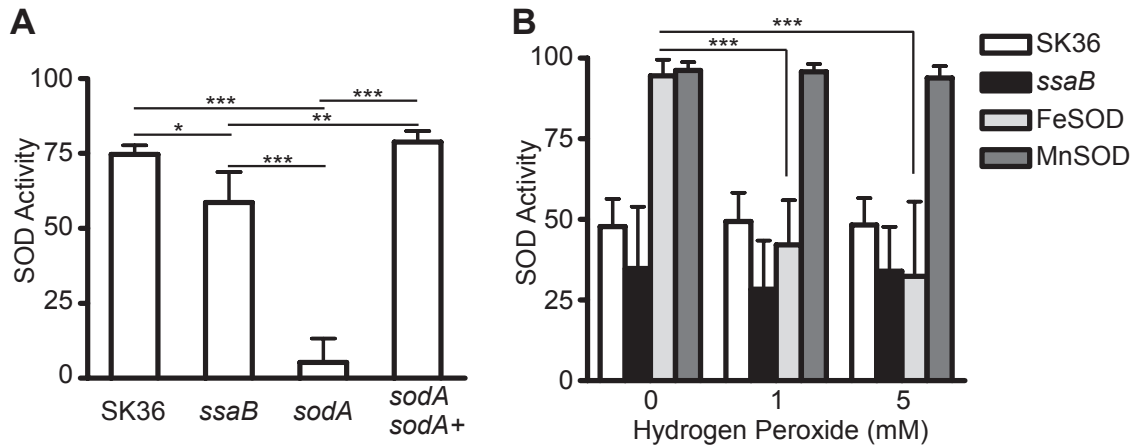
A

B

Fig. 7. SOD activity in *ssaB* and *sodA* mutants.
A. The strains indicated were cultured in rabbit serum with 12% O_2, and lysates tested for SOD activity. Activity is expressed as percent inhibition of WST-1 formazan formation.
B. *S. sanguinis* cell lysates cultured as in (A) and purified *E. coli* FeSOD and MnSOD proteins were incubated in the indicated concentration of H_2O_2 prior to determination of SOD activity. Means and standard deviations from $n = 3$ independent experiments are shown. Like samples were statistically compared at different H_2O_2 concentrations. For both panels, statistical significance was assessed by a repeated-measures ANOVA with a Tukey–Kramer post-hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Vendittis *et al.*, 2010). SK36 and *ssaB* mutant lysates from serum-grown cells were therefore incubated with various concentrations of H_2O_2 prior to the SOD activity assay. H_2O_2 treatment of control *E. coli* FeSOD sharply reduced SOD activity while treatment of the MnSOD had no effect (Fig. 7B), as shown previously (Beyer and Fridovich, 1987). SOD activity was not affected by H_2O_2 treatment in SK36 or the *ssaB* mutant. These results suggest that the residual SodA activity in the *ssaB* mutant grown under physiological conditions is due to Mn^{2+}, not Fe^{2+}, in the active site.

Examination of the effects of antioxidants on serum growth

Because we observed that the *ssaB* mutant had an increased level of SOD activity relative to the *sodA* mutant, while at the same time exhibiting poorer aerobic growth and virulence, we sought to examine the contribution of other forms of ROS to *S. sanguinis* growth under *in vivo*-like conditions. SK36 and the *ssaB* and *sodA* mutants were incubated in the presence of varying concentrations of tiron or deferoxamine mesylate (DM) in normal rabbit serum and 12% O_2 for 24 h, then plated for enumeration. Tiron (1,2-dihydroxybenzene-3,5-disulphonic acid) has been used as a cell-permeable scavenger of superoxide (Bors *et al.*, 1979; Krishna *et al.*, 1992). Tiron also enhances the rate of autooxidation of Fe^{2+} to Fe^{3+} and sequesters Fe^{3+} (Krishna *et al.*, 1992). DM is a cell-permeable chelator that binds Fe^{2+} and Fe^{3+} and may also promote oxidation of Fe^{2+} to Fe^{3+} (Keberle, 1964; Goodwin and Whitten, 1965). DM has been used to lessen formation of HO• via the Fenton

reaction: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO\bullet + OH^-$ (Liu *et al.*, 2011). Addition of tiron to 0.001 mM had little effect on *ssaB* mutant growth (Fig. 8A). Growth in concentrations ranging from 0.05 to 1 mM for the *ssaB* mutant were not significantly different from SK36 vehicle but were significantly different relative to *ssaB* vehicle ($P < 0.01$) suggesting tiron restored *ssaB* mutant growth. However, higher concentrations (10–25 mM) were required to significantly improve growth of the *sodA* mutant ($P < 0.01$). When we assessed the effect of iron chelation alone by adding DM, we found that all concentrations tested significantly improved growth of the *ssaB* mutant ($P < 0.01$ versus vehicle control) while having no significant effect on the *sodA* mutant (Fig. 8B). Taken together, these data suggest that increased oxidative stress from superoxide and HO• contributes to the *ssaB* mutant growth defect and attenuated virulence *in vivo*. In contrast, the *sodA* mutant appears to exhibit increased superoxide levels, but not increased sensitivity to HO•.

Discussion

In this study, we examined the relationship of manganese, SsaB, and the manganese-dependent SodA in *S. sanguinis* growth and virulence. We report five observations that we believe are novel for any *Streptococcus*. First, we demonstrate that wild-type and *ssaB* mutant *S. sanguinis* strains exhibited widely varying cellular manganese and iron levels and ratios depending on the growth medium. Second, a side-by-side comparison in an animal model revealed that both the *ssaB* and *sodA* mutants exhibited reduced virulence, with the *ssaB* mutant significantly less
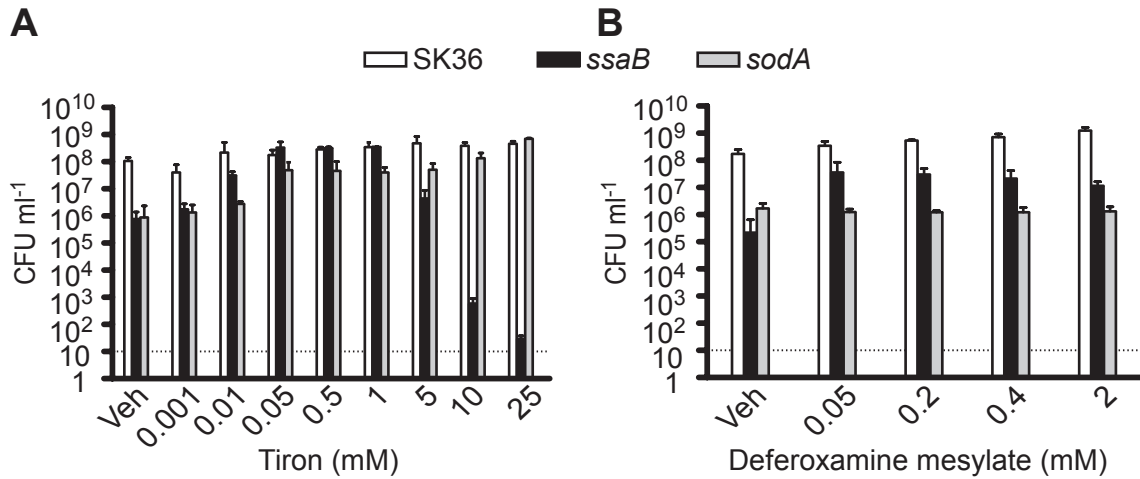
Fig. 8. Antioxidant treatment of serum-grown cells. The strains indicated were cultured in 12% O_2 in rabbit serum containing the vehicle solvent (Veh) or the indicated concentrations of (A) tiron or (B) DM for 24 h. Means and standard deviations from a minimum of $n = 3$ independent experiments are shown. The dotted line represents the limit of detection. Statistical significance (in text) was assessed by a one-way ANOVA with a Dunnett post-hoc test for comparison against the vehicle control.

virulent than the *sodA* mutant. Third, the *sodA ssaB* double mutant was reduced in virulence compared with the *ssaB* mutant, suggesting the presence of biologically relevant levels of SodA activity in a *ssaB* mutant *in vivo*. Fourth, we recapitulated *in vivo* virulence results using an *in vitro* serum growth system with physiologically relevant O_2 levels. Fifth, we demonstrated that addition of an iron chelator partially restored serum growth of the *ssaB* mutant and had no effect on the *sodA* mutant, while a compound with superoxide scavenging and iron-chelating properties completely restored serum growth to both mutants.

What are the implications of these findings? The first concerns a role for SsaB as an adhesin. 'SsaB' derives its name from '*S. sanguinis* adhesin B,' so named because purified SsaB was found to inhibit the adhesion of *S. sanguinis* cells to the model tooth surface saliva-coated hydroxyapatite (Ganeshkumar *et al.*, 1988). Our data showing no reduction in the number of *ssaB* mutant bacteria recovered from vegetations early after inoculation strongly argues against SsaB acting as an adhesin in endocarditis. It would be interesting to test the effect of *ssaB* mutation on oral adhesion, but this has not been done.

Previous studies have hypothesized that the reduced virulence of streptococcal *sodA* mutants is due to sensitivity to phagocytic killing (Poyart *et al.*, 2001; Tang *et al.*, 2012). Although this may yet prove true in other species (Young Lee *et al.*, 2006) or even other *S. sanguinis* strains, our results showed that in the *S. sanguinis* SK36 background, *ssaB* and *sodA* mutants are no more sensitive to PMN killing than their parent. This finding, together with the serum growth studies, suggests that reduced growth and virulence of the *sodA* or *ssaB* mutants is not due to

increased sensitivity to exogenous ROS from immune cells.

Our finding that mutation of *ssaB* reduced cellular levels of both manganese and iron is in agreement with previous studies of LraI mutants of *S. parasanguinis*, *S. mutans* and *Streptococcus pyogenes* (Oetjen *et al.*, 2002; Janulczyk *et al.*, 2003; Paik *et al.*, 2003). While the simplest explanation of these results is that the SsaABC system transports both metals, we cannot rule out the possibility that the effect of *ssaB* mutation on iron uptake is indirect, due for example, to an effect of cellular manganese levels on the expression of dedicated iron transporters, of which there are 2 or 3. Our previous study with *S. mutans*, which measured uptake of ^{55}Fe in the presence and absence of Mn^{2+} as a competitor, implicated the *S. mutans* LraI system (SloABC) in direct transport of both metals (Paik *et al.*, 2003). Similar studies will be required to determine whether this is also true for the *S. sanguinis* SsaABC system.

Adventitious production of ROS including superoxide and H_2O_2 during aerobic growth can be hazardous to any bacterium, especially when iron is present (Imlay, 2013). H_2O_2 reacts with Fe^{2+}, whether free in the cytoplasm or at solvent-accessible sites in proteins, to produce the highly reactive and deleterious ROS species HO• via the Fenton reaction. Superoxide can damage sensitive enzymes, many of which contain Fe^{2+}, resulting in release of additional free iron. Bacteria such as *E. coli* incorporate high levels of iron but avoid HO• damage by expressing catalase and alkyl hydroperoxide reductase to limit H_2O_2 levels to submicromolar levels (Imlay, 2013). In contrast, streptococci, including *S. sanguinis* (Carlsson *et al.*, 1987; Chen *et al.*, 2011), and the closely related lactobacilli (Sedewitz

et al., 1984) produce H_2O_2 as a product of the reaction between O_2 and pyruvate catalysed by pyruvate oxidase. Yet, streptococci do not possess catalase. As a result, H_2O_2 levels in aerobic cultures can approach 1 mM (Uehara *et al.*, 2001). In agreement with our findings, previous work has determined that *L. plantarum* takes up high levels of manganese, but neither requires nor accumulates iron (Archibald and Fridovich, 1981). Thus, lactobacilli avoid HO• damage not by limiting H_2O_2, but by avoiding cellular iron. Our study demonstrated that while *S. sanguinis* favours manganese over iron, it can also grow aerobically with high iron : manganese ratios. Moreover, *S. sanguinis* possesses multiple proteins containing mononuclear iron or iron–sulphur clusters (Xu *et al.*, 2007). Thus, *S. sanguinis* must employ a third strategy for avoiding HO• damage that neither limits H_2O_2 nor dispenses with cellular iron.

What is this strategy? For one, *S. sanguinis* must limit access of cellular Fe^{2+} to H_2O_2. Like other species that produce H_2O_2 as a primary metabolic product, *S. sanguinis* appears to have evolutionarily adapted to its lifestyle by eliminating genes encoding many Fe^{2+}-containing proteins, including TCA cycle enzymes and cytochromes (Pericone *et al.*, 2003; Xu *et al.*, 2007). In addition, streptococci appear to possess ROS-adapted versions of some enzymes and systems, including the Suf sulphur cluster assembly apparatus. In *E. coli*, the Suf system is induced by H_2O_2 stress and iron limitation and is less sensitive to ROS than the housekeeping Isc system (Imlay, 2013). It has also been shown in other bacteria including other streptococci that the Dpr protein, Dps, sequesters free Fe^{2+} in an H_2O_2-dependent reaction to suppress HO• formation and support aerobic growth (Yamamoto *et al.*, 2000; 2002; Tsou *et al.*, 2008). This is likely to be true in *S. sanguinis* as well. Other H_2O_2-mediated damage may be repaired by glutathione and thiol peroxidases encoded by *S. sanguinis*.

How then does manganese protect against oxidative stress in *S. sanguinis*? A model is presented in Fig. 9. Our results indicate that Mn^{2+} is required for SodA activity under biologically relevant conditions. This may be due to H_2O_2 sensitivity of the Fe^{2+}-cofactored form of the enzyme. Manganese complexes formed with small compounds have been shown to disproportionate superoxide and in some cases, to dissociate H_2O_2 (Barnese *et al.*, 2012). It is not clear how important this is in *S. sanguinis*, given that *sodA* mutant lysates possessed far more superoxide-abolishing activity than *ssaB* mutant lysates in an assay that should have detected the activity of manganese complexes as well as SodA (Barnese *et al.*, 2012). Likewise, it seems unlikely that manganese compounds protect cells by dissociating H_2O_2 since H_2O_2 levels remain high during growth (Carlsson *et al.*, 1987; Chen *et al.*, 2011).

It has also been suggested that Mn^{2+} may non-specifically displace Fe^{2+} from critical biomolecules that
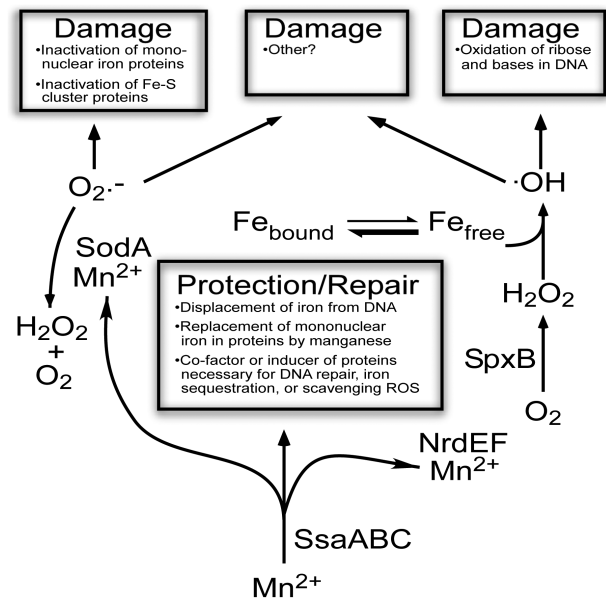

Fig. 9. The role of the SsaABC system, SodA and manganese in oxygen tolerance of *S. sanguinis*. Superoxide generated adventitiously from O_2 inactivates proteins with accessible mononuclear iron or iron–sulphur clusters unless converted to H_2O_2 and O_2 by Mn^{2+}-cofactored SodA. Enzymes containing mononuclear iron may be protected against superoxide and H_2O_2 damage by cofactor exchange with Mn^{2+}. High levels of H_2O_2 are produced from O_2 and pyruvate by pyruvate oxidase (SpxB). Free Fe^{2+} imported into the cell or released from damaged enzymes may be largely sequestered by ROS-induced iron-binding proteins, such as Dps. H_2O_2 and accessible Fe^{2+} together form HO• by the Fenton reaction and damage DNA and other cellular targets. Manganese transported through the SsaABC system may protect DNA by displacing bound iron and protect or repair other targets through induction of ROS-defence enzymes. In the presence of O_2 and an accessory protein, Mn^{2+} that is not bound to SodA or other cellular ligands forms a cofactor on NrdF, allowing for the production of deoxyribonucleotides needed for aerobic growth and DNA repair.

are sensitive to damage by Fenton-generated HO•, particularly DNA (Imlay, 2013). This would be consistent with our data showing the DM improves serum growth of the *ssaB* mutant. The fact that DM had no effect on growth of the *sodA* mutant suggests that manganese protects cells from Fenton-generated HO• even when superoxide levels are high. This result was somewhat surprising, because reaction of superoxide with proteins containing solvent-accessible mononuclear iron or iron–sulphur clusters can release free iron, fuelling Fenton-mediated damage (Imlay, 2013). One possibility is that this occurs, but damage is confined to the enzyme from which the iron is released.

Mn^{2+} may also help protect cells under aerobic conditions by replacing the Fe^{2+} cofactor in certain mononuclear enzymes. Evidence has been presented suggesting that such an exchange of cofactors explains why *E. coli* transports and benefits from increased levels of manganese when it experiences H_2O_2 stress (Sobota and Imlay,

2011). *S. sanguinis* possesses ribulose-5-phosphate 3-epimerase and isocitrate dehydrogenase, both of which are mononuclear iron enzymes that were implicated in the *E. coli* study.

The tiron data were also informative. Tiron should produce the same protective effect against HO• damage as DM by chelating free iron, with the added effect of scavenging superoxide. The fact that tiron fully restores serum growth to an *ssaB* mutant suggests that cells can grow normally with lower manganese levels as long as these two ROS are eliminated, which agrees with our data showing normal levels of growth of all the mutants in 1% O_2. Because the DM data indicate that the *sodA* mutant does not suffer from HO• damage, tiron must restore its growth through scavenging of superoxide. The increased concentration of tiron required to fully restore growth to the *sodA* mutant compared with the *ssaB* mutant is consistent with our other data suggesting that the *sodA* mutant possesses significantly less SOD activity than the *ssaB* mutant. Interestingly, high concentrations of tiron were toxic to the *ssaB* mutant. One explanation for this finding is that at these concentrations, tiron may exhaust *ssaB* mutant cells of already-depleted stores of Fe^{2+}, or more attractively, Mn^{2+}. Tiron forms complexes with Mn^{2+} that are less stable than those formed by Fe^{3+} (Courtney *et al.*, 1958), but are thought to be equivalent to those formed by Fe^{2+} (Krishna *et al.*, 1992).

Previous studies in other streptococci have suggested that Mn^{2+} upregulates genes encoding ROS-defence enzymes, including *sodA* and *tpx* (Jakubovics *et al.*, 2002; Eijkelkamp *et al.*, 2014). Because manganese binds most proteins with relatively low affinity, these proteins and non-proteinaceous cellular ligands must compete for available manganese (Cotruvo and Stubbe, 2012). SodA is an exception, as shown by its retention of bound manganese upon purification (Cotruvo and Stubbe, 2012). Given that SodA levels can reach 1.5–2% of total cellular protein (Jakubovics *et al.*, 2002), it would be expected that SodA overexpression could deplete the cell of free manganese, which would be harmful if manganese was needed for other critical functions. This could explain why SodA is expressed only when adequate stores of manganese are present. This rationale would not apply to Tpx, which is also induced by manganese and oxidative stress (Jakubovics *et al.*, 2002; Spatafora *et al.*, 2002; Hajaj *et al.*, 2012), because it does not possess a metal cofactor (Flohe *et al.*, 2011). This suggests a broader explanation: cells may be programmed to discontinue aerobic metabolism if manganese supplies are low.

Support for this hypothesis may come from a recent collaborative study involving our group. We determined that *S. sanguinis* possesses a class Ib (NrdEF) ribonucleotide reductase that is active *in vitro* with either iron or manganese as a cofactor (Makhlynets *et al.*, 2014), but which exhibits an absolute requirement for the manganese cofactor for aerobic growth and endocarditis virulence (Rhodes *et al.*, 2014). This enzyme, which requires O_2, Mn^{2+} and the NrdI accessory protein for assembly of the Mn^{3+}_2-tyrosyl radical cofactor, provides the deoxyribonucleotides essential for growth and DNA repair in the presence of O_2 because the cell's only other ribonucleotide reductase – the iron-dependent class III NrdDG enzyme – is inactivated by O_2 (Rhodes *et al.*, 2014). It is noteworthy that *E. coli* requires the class Ia, aerobic, iron-cofactored NrdAB ribonucleotide reductase under most conditions, but requires manganese-cofactored NrdEF, which it also encodes, under conditions of iron limitation and H_2O_2 stress (Martin and Imlay, 2011). Our DM study indicates that manganese-starved *ssaB* mutant cells experience HO•-mediated stress, which likely includes DNA damage (Imlay, 2013). Taken together, we hypothesize that in an *ssaB* mutant, manganese levels would be insufficient to support the ribonucleotide reductase activity needed for DNA repair. Therefore, the cell would benefit from halting other cellular functions until activity could be restored. This explanation could apply to all streptococci, since all species examined to date possess the same ribonucleotide reductases as *S. sanguinis* (Lundin *et al.*, 2009).

In conclusion, our results suggest that manganese accumulation by the SsaABC system is a unique virulence factor for streptococcal endocarditis and required for O_2 tolerance and growth. We provide experimental evidence that manganese exerts protection from oxidative stress by providing a cofactor for SodA. We have hypothesized various SodA-independent mechanisms for manganese activity including replacement of mononuclear iron, acting as a signal for gene induction, and as a cofactor for proteins critical for repair. Identification of the exact mechanisms by which manganese enables aerobic growth in pathogenic streptococci will be an important step in drug development. Antibiotics are currently the only form of prophylaxis available for people at high risk for endocarditis (Wilson *et al.*, 2007). Long-term antibiotic administration for daily prophylaxis is not feasible due to the promotion of antibiotic resistance. Highly conserved LraI transport systems represent a promising target for a new class of drugs that might inhibit streptococci in low-manganese environments such as blood, without introducing selective pressure in higher manganese environments such as the mouth (Paik *et al.*, 2003).

Experimental procedures

Bacterial strains and growth conditions

The *S. sanguinis* strains used in this study are provided in Table 1. All mutant strains were derived from the SK36 background. Bacteria were cultivated in brain heart infusion (BHI) broth and/or filter-sterilized pooled rabbit serum (Gibco) and

Table 1. Strains used in the study.

Strain	Phenotype/genotype	Source
SK36	Human oral plaque isolate	M. Kilian, Aarhus University, Denmark; Xu *et al.* (2007)
JFP36	Em^R; SSA_0169::pSerm	Turner *et al.* (2009a)
Ssx_0721	Kan^R; Δ*sodA*::*aphA-3*	Xu *et al.* (2011)
Ssx_0256	Kan^R; Δ*ssaR*::*aphA-3*	Xu *et al.* (2011)
JFP131	Kan^R Spc^R; Δ*sodA*::*aphA-3* SSA_0169::*sodA aad9*	This study
JFP133	Kan^R Cm^R; Δ*sodA*::*aphA-3 ssaB*::*mariner2*	This study
Lp14-12	Cm^R; *ssaB*::*mariner2*	Das *et al.* (2009)
JFP135	Cm^R Spc^R; *ssaB*::*mariner2* SSA_0169::*aad9*	This study
JFP155	Cm^R Spc^R; *ssaB*::*mariner2* SSA_0169::*ssaB aad9*	This study

plated on BHI agar (Difco). For some experiments, BHI was treated prior to inoculation with Chelex-100 resin (Bio-Rad) for 90 min, filter sterilized, and supplemented with CaCl$_2$ and MgSO$_4$ to 1 mM each. The following highly pure metals (100 µM) were then added to selected samples: MnSO$_4$, FeSO$_4$, or ZnSO$_4$ (Puratronic®, Alfa Aesar). Unless otherwise noted, overnight cultures were inoculated from single-use aliquots of cryopreserved cells by 1000-fold dilution. Cultures were then incubated for 20–24 h in either BHI or 80% BHI/20% rabbit serum under microaerobic conditions (6% O$_2$, 7% H$_2$, 7% CO$_2$ and 80% N$_2$) at 37°C. The latter medium was chosen to acclimate cells to serum and was found to support equivalent growth of all the strains used in this study. Atmospheric composition was adjusted using a programmable Anoxomat™ Mark II jar-filling system (AIG, Inc.); for anaerobic conditions, a palladium catalyst was included in jars or an anaerobic chamber (Coy Laboratory Products, Inc.) was used. For enumeration of streptococci, samples were sonicated for 90 seconds to disrupt chains prior to plating on BHI agar using an Eddy Jet 2 spiral plater (Neutec Group, Inc.). Transformation of *S. sanguinis* was accomplished in Todd-Hewitt broth (Difco) supplemented with 2.5% (v/v) heat-inactivated horse serum (Invitrogen) as described previously (Paik *et al.*, 2005). For selective *S. sanguinis* growth, chloramphenicol (Cm), erythromycin (Em), kanamycin (Kan) and spectinomycin (Spc) were used at concentrations of 5 µg ml^{-1}, 10 µg ml^{-1}, 500 µg ml^{-1} and 200 µg ml^{-1} respectively. *Escherichia coli* DH10B (ElectroMAX, Invitrogen) was used as a host for plasmid construction. All *E. coli* strains were cultured on Luria–Bertani (LB) agar plates containing Cm (5 µg ml^{-1}), Em (300 µg ml^{-1}), ampicillin (Ap; 100 µg ml^{-1}) or Kan (50 µg ml^{-1}) as needed.

Generation of recombinant strains

The *sodA* deletion mutant (Ssx_0721) was generated previously for a genome-wide deletion study (Xu *et al.*, 2011). It contains a Kan cassette disrupting the *sodA* gene (SSA_0721). To create the *sodA ssaB* double mutant, a 2360 bp DNA fragment containing the *ssaB* gene interrupted by a *mariner2* mini-transposon and flanking regions was amplified from the Lp14-12 strain using Lpp14-12 forward and reverse primers described previously (Das *et al.*, 2009). The resultant PCR product was used to transform the *sodA* mutant (Ssx_0721), essentially as described previously (Paik *et al.*,

2005), with selection on BHI agar containing Cm and Kan. The double mutant, JFP133, was PCR verified and contained both Cm and Kan cassettes at the expected locations.

The *aad9* gene encoding resistance to Spc was introduced into the SSA_0169 ectopic expression site (Turner *et al.*, 2009a) of Lp14-12 to create a Spc^R *ssaB* mutant, JFP135. This was accomplished by first modifying the plasmid pJFP56 (Turner *et al.*, 2009a) by deletion of *aphA-3* from the vector backbone by inverse PCR, DpnI digestion, and ligation. The resulting plasmid, pJFP96, was then introduced into Lp14-12 by transformation. A complemented *sodA S. sanguinis* strain, JFP131, was generated by fusing the intact *sodA* coding sequence to the *S. pneumoniae ami* promoter (Claverys *et al.*, 1995) and inserting it upstream of the *aad9* gene in pJFP96. This was accomplished by amplifying the entire *sodA* gene, the *ami* promoter, and recognition sites AscI and NotI using the primers: CTCTGCGGCCGCGAAAATTTGTT TGATTTTTAATGGATAATGTGATATAATGGTTGTCAATCTAT AATTCACATAAACAGAGGAGTAGAAAATGTCATTCGAATT ACCTGC and CGCAGGCGCGCCCTTATTATGCAGCGA GATTTTTCGCT. The PCR product was digested with AscI and NotI, ligated into pJFP96, and the reaction was transformed into *E. coli* DH10B. Purified plasmid DNA was then introduced into the *sodA* mutant by transformation. Selected transformants, which possessed the *sodA* and *aad9* genes in the SSA_0169 ectopic expression site, were confirmed by DNA sequencing and resistance to paraquat. An identical strategy was used to introduce the *ssaB* gene into the same SSA_0169 locus in the *ssaB* mutant under the control of the same expression signals to create strain JFP155. The construct was confirmed by DNA sequencing and western blot analysis.

ICP-OES

Metal concentrations in media and bacterial cells were measured using ICP-OES. For experiments involving growth in BHI broth, cells were cultured overnight in 80% BHI/20% pooled rabbit serum in 6% O$_2$. Three ml of each culture was then added to 36 ml BHI broth that had been pre-incubated under the same conditions. Incubation was continued at 37°C for 5 h. For growth in APT, cells were cultured anaerobically overnight in APT broth (Difco), then diluted as above in prewarmed APT and incubated at 37°C for 5 h. *E. coli* cells were incubated with shaking (225 rpm), while the other strains

were incubated statically in tightly capped tubes containing anaerobically pre-incubated APT. Cells were harvested by centrifugation at 4°C for 10 min at 3743 *g* and washed twice in 10 ml cold PBS that had been pre-treated with Chelex and supplemented with 1 mM EDTA. Cell pellets were suspended in 1 ml concentrated HNO_3 (Fisher Scientific) and incubated at 95°C for 1 h; 900 μl of this was added to 9.1 ml dH_2O. Metal composition was determined using an MPX Vista inductively coupled plasma-optical emission spectrometer (Varian, Inc.). Concentrations were determined by comparison with a standard curve created with a 10 μg ml^{-1} multi-element standard (CMS-5; Inorganic Ventures) diluted in dH_2O and then twofold in 1% HNO_3 to generate dilutions ranging from 5.12 to 0.02 μg ml^{-1}. For protein determination, cell pellets from replicate cultures were suspended in 1 ml PBS and mechanically lysed using a FastPrep-24 instrument and Lysing Matrix B (MP Biomedicals) as described previously (Rhodes *et al.*, 2014). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce) as recommended by the manufacturer with BSA as a standard. To avoid contamination with metals, trace-metal grade HNO_3 was used, all dH_2O was treated with Chelex prior to use, and all glass and plastic vessels used for metal analysis were soaked overnight in 1 M HNO_3 prior to use.

Western blotting

Overnight bacterial cultures grown in media with or without supplemental metals were diluted 1:10 in pre-warmed media containing the same supplements, incubated for 3 h, harvested by centrifugation, washed twice with PBS, and resuspended in cold PBS. The bacteria were mechanically disrupted as above and protein concentrations were determined with a BCA assay kit (Pierce). Samples were separated on a 12.5% pre-cast SDS denaturing gel (Bio-Rad) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in PBS supplemented with Tween-20 (PBS-T, 0.1% Tween-20). The membrane was probed with 5% non-fat dry milk in PBS-T supplemented with rabbit antiserum raised against *S. parasanguinis* FimA (1:200) protein, which is cross-reactive with SsaB (Das *et al.*, 2009). Primary antibodies were detected using goat anti-rabbit IgG–HRP conjugate (Sigma-Aldrich) and visualized with a chemiluminescent substrate (Supersignal West Pico, Pierce) and film.

Paraquat survival assay

Overnight cultures of *S. sanguinis* were diluted 1:10 in BHI broth. Following an additional 3 h incubation at 37°C, bacteria were harvested by centrifugation. Bacteria were washed with cold PBS and diluted in PBS to ~ 10^7 bacteria ml^{-1} in the presence or absence of 50 mM paraquat. Following incubation at 37°C for 2 h in room air, samples were diluted and plated on BHI agar for enumeration.

SOD activity assays

Total SOD activity was determined by commercial xanthine oxidase assay according to the manufacturer's protocol (Sigma-Aldrich). Briefly, overnight cultures of bacteria in 80% BHI/20% rabbit serum were diluted 1:10 in pre-warmed 100% rabbit serum and grown in 12% O_2 for 3 h. Cells were harvested by centrifugation, washed twice with PBS, and lysed by mechanical disruption as above. Results were reported as activity (% inhibition of WST-1 formazan formation), as directed by the manufacturer. For some studies, an in-gel assay was also performed using native polyacrylamide gel electrophoresis (0.025 M Tris, 0.192 M glycine, pH 8.3 electrode buffer with Bio-Rad Criterion precast 12.5% acrylamide gels) followed by staining for SOD activity using nitro blue tetrazolium (NBT) as described (Beauchamp and Fridovich, 1971). For all assays, protein concentrations of lysates were determined by BCA assay, and equal amounts of protein were used. For some experiments, lysates and purified MnSOD (SodA) and FeSOD (SodB) from *E. coli* (Sigma-Aldrich) were treated with various concentrations of H_2O_2 for 60 min prior to assessing activity.

Virulence assays

Virulence assays were performed using a rabbit model of infective endocarditis (Rhodes *et al.*, 2014). Briefly, pathogen-free New Zealand white rabbits weighing 3–4 kg were purchased from RSI Biotechnology and allowed to acclimate to the vivarium 7 days prior to inoculation. The rabbits were anesthetized and a 19-gauge catheter (BD Bioscience) was inserted through the right internal carotid artery past the aortic valve to cause minor damage. The catheter was trimmed, sutured in place, and remained in the artery for the entire experiment. The incision was closed with surgical clips. Two days following catheterization, *S. sanguinis* strains were grown overnight in BHI in 6% O_2, diluted 20-fold into fresh BHI, incubated for 3 h, sonicated, washed and resuspended in PBS. Approximately ~ 10^7 cfu ml^{-1} each of JFP36 (a virulent competitor) and one or two additional strains were co-inoculated via peripheral ear vein. The inoculum was then diluted and plated on BHI agar with appropriate antibiotics for bacterial counts. At 2.5 or 20 h post inoculation, rabbits were euthanized by intravenous injection of Euthasol (Virbac AH). Following removal of the heart, catheter placement was verified and vegetations were removed. Vegetations were homogenized with PBS, sonicated, diluted, and plated on BHI agar with appropriate antibiotics as above. For competitive index (CI) assays, the CI value for each rabbit was calculated as the ratio of mutant/JFP36 in the vegetation homogenate divided by the mutant/JFP36 ratio in the inoculum. For experiments employing co-inoculation of three competing strains, the results were reported as recovered cfu per rabbit for each strain, with numbers normalized against ratios of the inoculated strains (generally near 1:1:1). All animal procedures were approved by Virginia Commonwealth University Institutional Animal Care and Use Committee and complied with applicable federal and institutional guidelines.

PMN killing assay

Overnight cultures of *S. sanguinis* and *S. gordonii* strains were diluted 1:10 in BHI broth and incubated for an additional 3 h at 37°C. Bacteria were harvested and diluted into serum-

free RPMI supplemented with glutamine (Gibco) at a concentration of 2×10^6 cfu ml^{-1}. Human PMNs were isolated from EDTA-anti-coagulated blood obtained from healthy individuals using Mono-Poly Resolving Medium (MP Biomedicals) as described by the manufacturer's instructions. The PMN cell layer was aspirated, washed two times with RPMI, and resuspended at a concentration of 2×10^6 cells ml^{-1}. A 0.5 ml aliquot of PMN and bacteria were mixed in a 1:1 ratio (v/v and cell number/cell number) in a 2 ml screw-cap polypropylene tube. The reactions were incubated at 37°C for 1 h in room air with gentle agitation. Following incubation, samples were sonicated for 90 s at 50% power using an Ultrasonic Homogenizer Model 150 VT sonicator (BioLogics, Inc.) to lyse PMNs and disrupt chains of streptococci. We determined previously that these sonication conditions maximize colony number when *S. sanguinis* cultures are plated (data not shown). Lysates were diluted in sterile PBS and plated for surviving bacteria on BHI agar. When bacteria were sonicated to disrupt clumps and chains before exposure to PMNs, similar results were obtained. Blood collection procedures were approved by the Virginia Commonwealth University Institutional Review Board.

Serum growth studies

Five-millilitre bacterial cultures were grown 20–24 h in 80% BHI/20% rabbit serum and 6% O_2, diluted 10^6-fold into 100% pooled rabbit serum pre-incubated in different levels of O_2, and incubated further at the same O_2 level. At 5 and 24 h 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 48 h plate incubation in 6% O_2. For antioxidant studies, overnight bacterial cultures were used to inoculate pooled rabbit serum as above in the presence of vehicle (water), tiron, or DM (Sigma) and incubated in 12% O_2. At 24 h post inoculation, 1 ml aliquots of bacteria were harvested, sonicated and diluted in PBS for bacterial counts on BHI agar as described above.

Statistics

All statistical tests were performed using InStat (GraphPad Software, Inc.). Significance was determined by *t*-test or ANOVA, as indicated in the text. A post-hoc test was employed when differences among groups were indicated as significant ($P < 0.05$) by ANOVA. *P*-values of < 0.05 for *t*-tests and post-hoc tests are indicated; *P*-values of ≥ 0.05 were considered non-significant.

Acknowledgements

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Supporting information

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