A Superoxide Dismutase Capable of Functioning with Iron or Manganese Promotes the Resistance of *Staphylococcus aureus* to Calprotectin and Nutritional Immunity

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Abstract

*Staphylococcus aureus* is a devastating mammalian pathogen for which the development of new therapeutic approaches is urgently needed due to the prevalence of antibiotic resistance. During infection pathogens must overcome the dual threats of host-imposed manganese starvation, termed nutritional immunity, and the oxidative burst of immune cells. These defenses function synergistically, as host-imposed manganese starvation reduces activity of the manganese-dependent enzyme superoxide dismutase (SOD). *S. aureus* expresses two SODs, denoted SodA and SodM. While all staphylococci possess SodA, SodM is unique to *S. aureus*, but the advantage that *S. aureus* gains by expressing two apparently manganese-dependent SODs is unknown. Surprisingly, loss of both SODs renders *S. aureus* more sensitive to host-imposed manganese starvation, suggesting a role for these proteins in overcoming nutritional immunity. In this study, we have elucidated the respective contributions of SodA and SodM to resisting oxidative stress and nutritional immunity.

In vitro analysis demonstrated that SodA is strictly manganese-dependent whereas SodM is in fact cambialistic, possessing equal enzymatic activity when loaded with manganese or iron. Cumulatively, these studies provide a mechanistic rationale for the acquisition of a second superoxide dismutase by *S. aureus* and demonstrate an important contribution of cambialistic SODs to bacterial pathogenesis. Furthermore, they also suggest a new mechanism for resisting manganese starvation, namely populating manganese-utilizing enzymes with iron.
Author Summary

During infection, pathogens must overcome the restriction of essential nutrients such as manganese by the host, while simultaneously coping with other host defenses such as the oxidative burst. Using the host protein that limits manganese availability during infection and mice lacking this effector, we determined that acquisition of a second superoxide dismutase that is capable of using either manganese or iron enhances the ability of *Staphylococcus aureus* to cause infection. When manganese-starved by the host, this cambialistic enzyme enables *S. aureus* to maintain superoxide dismutase activity and survive when exposed to oxidative stress. These results reveal the important contribution of cambialistic superoxide dismutases to bacterial pathogenesis and represent a new mechanism for resisting manganese starvation during infection.

Introduction

The spread of antibiotic resistance amongst bacteria has led both the Centers for Disease Control and Prevention and the World Health Organization to state that infections represent a serious threat to human health [1, 2]. This threat is exemplified by *Staphylococcus aureus*, a Gram-positive bacterium that asymptomatically colonizes one third of the population and is a leading cause of antibiotic-resistant infections [3–5]. A promising area of investigation is elucidating how pathogens overcome host defenses such as the active withholding of essential nutrients and the oxidative burst of immune cells.

During infection, pathogens must obtain all of their nutrients from the host, including the essential metal ions that are needed for the approximately one-third of all bacterial proteins that require a metal cofactor [6–8]. This requirement is exploited by the host, which restricts the availability of these essential nutrients, a defense termed nutritional immunity [9–13]. The canonical example of nutritional immunity is the iron (Fe)-withholding response [10, 11]. In addition to Fe, the host also restricts the availability of manganese (Mn) and zinc (Zn) [9, 12–14]. The prototypical example of Mn and Zn restriction is the staphylococcal abscess, which is rendered virtually free of these metals during infection [9, 14]. A critical component of the Mn- and Zn-withholding response is the host protein calprotectin (CP) [9, 12, 14]. This innate immune effector is highly expressed in neutrophils in which it comprises 40–60% of the cytoplasmic protein, and at sites of infection it can be found in excess of 1 mg/ml [15, 16]. Loss of CP results in host defects in metal sequestration and increased sensitivity to a number of bacterial and fungal pathogens, including *S. aureus* [9, 14, 17–19]. In culture, CP inhibits the growth of a similarly wide range of pathogens [16–20]. The antimicrobial activity of CP is dependent on binding of metal ions to its two transition metal-binding sites [14, 20, 21]. The first site or ‘Mn/Zn site’ is comprised of six histidines and is capable of binding either Mn or Zn with nanomolar and picomolar affinities (K_d), respectively [14, 20–22]. The second site or ‘Zn site’ is comprised of three histidines and an aspartic acid and binds Zn with picomolar or sub-picomolar affinity [14, 20, 22].

In order to cause disease, invading pathogens must not only overcome nutrient starvation but also simultaneously cope with other host defenses, such as the oxidative burst of neutrophils and other immune cells [23]. Bacteria defend themselves from the oxidative burst by numerous mechanisms, including enzymes such as superoxide dismutases (SODs) that detoxify the damaging reactive oxygen species with which they are bombarded [24–27]. The activating metal cofactor divides the SOD enzymes into several families, with the most common amongst bacteria belonging to a single protein superfamily, which utilizes either Mn or Fe as
cofactor. It has proven exceptionally difficult to predict which metal is utilized by a given Mn/Fe-dependent SOD [24, 28], in part due to the fact that Fe- and Mn-SODs coordinate their metal cofactor using the same protein ligands within an identical protein fold [28, 29]. This structural similarity also enables both Fe- and Mn-SODs to bind the other, non-cognate metal, but this usually results in an inactive enzyme; most members of this protein family are strictly dependent on their cognate metal for catalysis [28–31]. Notably, a subset of the Mn/Fe-dependent SOD family is active when loaded with either Fe or Mn [32–40]. While these ‘cambialistic’ SODs are present in a diverse group of microbes, analysis of their activity has largely been limited to in vitro studies, limiting our understanding of how cambialism benefits microbes and the contribution of these enzymes to colonization of the host.

*S. aureus* possesses two SODs, SodA and SodM, both of which are cytoplasmic and are reported to be Mn-dependent [41–43]. While all staphylococci possess SodA, SodM is unique to *S. aureus* [44]. Highlighting their importance to virulence, loss of either SodA or SodM in a skin model of infection or loss of both SODs in a systemic mouse model, reduces the ability of *S. aureus* to cause disease [14, 42]. However, a molecular explanation for the advantage that *S. aureus* gains by expressing two apparently Mn-dependent SODs is unknown. Host-imposed Mn starvation mediated by CP reduces total staphylococcal SOD activity, both in culture and during infection, which renders *S. aureus* more sensitive to oxidative stress and neutrophil-mediated killing [13, 14, 20]. Yet paradoxically, the simultaneous loss of both SodA and SodM renders *S. aureus* more sensitive to CP [14], indicating that SodA and/or SodM somehow enhance the ability of *S. aureus* to resist metal starvation.

Given the importance of the two staphylococcal SODs to infection, we have elucidated their respective contributions to resisting oxidative stress and nutritional immunity. This analysis revealed that SodA is important for resisting oxidative stress and infection when Mn is abundant, whereas SodM is important under Mn-deplete conditions. Our data demonstrate that SodM is in fact cambialistic, possessing equal enzymatic activity when loaded with Mn or Fe. We propose that the ability of SodM to utilize Fe enables *S. aureus* to retain SOD activity when starved of Mn by the host, thereby enhancing the ability of the bacterium to overcome nutritional immunity, resist oxidative stress, and ultimately cause infection.

**Results**

*Mn availability differentially regulates the expression of the *S. aureus* SODs*

Several prior studies have examined the impact of Mn availability on the expression of SodA and SodM; however, different conclusions were reached [41, 42, 45]. In light of this ambiguity, we initially assessed the impact that CP and oxidative stress have on *sodA* and *sodM* expression. When normalized to optical density in order to account for differences in growth, high levels of *sodA* transcription were observed regardless of whether CP was present (Fig 1A), whereas the presence of CP enhanced *sodM* expression independent of the presence of oxidative stress (Fig 1B). These results indicate that *sodM*, but not *sodA*, is induced in response to Mn or Zn limitation. To clarify which metal gave rise to this effect, we used mutant CP variants that lack either the Mn/Zn site (ΔMn/Zn site mutant, which does not bind Mn) or the Zn site (ΔZn site mutant, which binds both Mn and Zn) [20]. As expected, neither mutant induced the expression of *sodA* (Fig 1C). The increased expression of *sodM* observed with wild type (WT) CP is lost in the presence of the ΔMn/Zn site mutant, but not the ΔZn site mutant, indicating that *sodM* is induced in response to Mn limitation (Fig 1D).

We also evaluated the impact of oxidative stress on the expression of the SODs using the superoxide-generating compound paraquat (PQ). Similar to previous studies [42], PQ induced
the expression of sodA in metal-replete media (Fig 1A). However, PQ did not alter the induction of expression of sodM observed with CP (Fig 1B), nor did it change the sodA and sodM expression pattern observed with the ΔMn/Zn or ΔZn site mutants (Fig 1E & 1F). Cumulatively, these observations suggest that the expression of SodM increases when S. aureus is Mn-limited regardless of level of oxidative stress experienced by S. aureus.

The activity of the staphylococcal SODs does not correlate with gene expression

The propensity of Mn/Fe-SODs to acquire the wrong metal can result in discordance between expression levels and enzymatic activity [46]. In order to determine if SodA and SodM activity

Fig 1. Expression of sodA and sodM varies by Mn availability. S. aureus carrying (A, C, & E) pSodA and (B, D, & F) pSodM YFP reporter constructs were grown in the presence of either wild type CP (A-F), the ΔMn/Zn site mutant (C-F), or the ΔZn site mutant (C-F), and in the (A, B, E, & F) presence or (A-D) absence of 0.1 mM PQ. Expression data are normalized to growth. Error bars indicate SEM (n = 3 or more). (A & B) # = p < 0.05 via two-way ANOVA with Tukey’s post-test for the indicated comparison. * = p < 0.05 via two-way ANOVA with Tukey’s post-test relative to bacteria grown in the presence of paraquat without CP. ^ = p < 0.05 via two-way ANOVA with Tukey’s post-test relative to bacteria grown in the absence of both paraquat and CP (C-F). * = p < 0.05 via two-way ANOVA with Dunnett’s post-test when compared to wild type CP.

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correlated with expression, total and individual SOD activity were assessed in the presence and absence of CP and PQ. Consistent with prior results, CP significantly reduced total staphylococcal SOD activity [14] (S1A Fig). In the absence of CP, the predominant activity comes from SodA (Fig 2A & 2B). The presence of the SodA/SodM heterodimer [41, 43] indicates that SodM is present in Mn-replete media, although the SodM homodimer’s activity is barely detectable in the gel. In the presence of CP, the relative contribution of SodA to total SOD activity decreased while that of SodM increased (Fig 2A & 2B). Notably, not only did the fractional contribution of SodM change, the absolute level of SodM activity also increased (Figs 2A & S1C). Consistent with prior studies, the addition of PQ increased total staphylococcal SOD activity [14] (S1A Fig). However, the addition of PQ did not change the impact that Mn availability had on the relative contributions of SodA and SodM to total staphylococcal SOD activity (Figs 2B, S1B & S1C). Together, these results indicate that in Mn-replete environments SodA is the primary source of SOD activity but SodM becomes the predominant SOD when S. aureus experiences Mn starvation.

SodA and SodM differentially contribute to resisting oxidative stress based on Mn availability

To test the respective contribution of each SOD to resisting Mn starvation, the ability of ΔsodA and ΔsodM single mutants, as well as a ΔsodAΔsodM double mutant, to grow in the presence of CP was assessed. Similar to previous results [14, 42], ΔsodAΔsodM was profoundly more sensitive to CP and PQ than wild type (Fig 3A & 3B), while ectopic expression of either SodA or SodM reversed this sensitivity (S2 Fig). In the absence of PQ, ΔsodA grew as well as wild type S. aureus in both the presence and absence of CP (Fig 3A), whereas the ΔsodM mutant, although it did not reach significance, displayed consistent reduced growth relative to wild type at high levels of CP (Fig 3A). Given the role of SodA and SodM in detoxifying superoxide, we also evaluated the impact of Mn availability on the ability of ΔsodA and ΔsodM to resist oxidative stress. Consistent with the activity analysis and its reported role as the primary SOD expressed by S. aureus [41, 42], loss of SodA resulted in increased sensitivity to PQ in the absence of CP (Fig 3A). However, at high concentrations of CP ΔsodA was no more sensitive to PQ than wild type S. aureus (Fig 3B). When compared to ΔsodA, the impact that CP had on the sensitivity of ΔsodM to oxidative stress was reversed; the ΔsodM mutant was no more sensitive to PQ than wild type bacteria in the absence and presence of low concentrations of CP, but the mutant was significantly more sensitive at high concentrations (Fig 3B). Notably, in the presence of intermediate CP concentrations in which both SodA and SodM are active, neither ΔsodA nor ΔsodM is more sensitive than WT S. aureus to oxidative stress. Utilization of the CP metal-binding site mutants revealed that both in the presence and absence of PQ, the increased sensitivity of ΔsodM is due to Mn sequestration (Figs 3C, 3D, S2C & S2D). Cumulatively, these results indicate that in Mn-replete environments SodA is primarily responsible for protecting S. aureus from oxidative stress, whereas SodM is critical for protecting S. aureus from oxidative stress in Mn-deplete environments. They also suggest that SodM promotes resistance to nutritional immunity by facilitating the retention of SOD activity and resistance to oxidative stress.

SodM is cambialistic

Paradoxically, our results indicate that the reportedly Mn-dependent enzyme SodM promotes resistance to host-imposed Mn starvation. In light of these observations, we analyzed the metal specificities of recombinant SodA and SodM. To facilitate these studies, the SODs were expressed in and purified from E. coli grown in iron-replete media. Following expression in E. coli and consistent with negligible Mn accumulation by E. coli in the absence of oxidative stress...
[46], inductively coupled plasma mass spectrometry (ICP-MS) analysis revealed that both of the purified recombinant staphylococcal SODs were loaded with Fe when recovered from the heterologous host (S3 Fig). Substantial activity was observed with purified Fe-SodM (210 +/- 21 U/mg protein), but negligible activity was detected from Fe-SodA (4 +/- 1 U/mg). Each of the recombinant proteins were denatured in the presence of metal chelators and then refolded in vitro, with successful elimination of Fe and loading with Mn confirmed by ICP-MS (S3 Fig). Enzymatic analysis revealed that the Mn-SodA form has substantial activity (1594 +/- 81 U/mg) in contrast to the Fe form. Surprisingly, Mn-SodM was also active (215 +/- 21 U/mg) and to a degree similar to that of the Fe-SodM, although both forms display activity substantially lower than that of Mn-SodA. The comparable activity of the Mn- and Fe-loaded forms of SodM indicate that it is not Mn-dependent, as previously suggested, but cambialistic [43].
Cambialism enables SodM to retain activity when *S. aureus* is Mn-starved

The cambialistic properties of SodM raise the possibility that in Mn-deficient conditions, including those induced by the presence of CP, Fe-loaded SodM predominates in the cell. We took advantage of the fact that Fe-dependent SODs can be selectively inactivated by hydrogen peroxide to evaluate if both the Mn- and Fe-loaded forms of SodM are present in *S. aureus* [31]. For these experiments, SodM was expressed from a plasmid in ΔsodA ΔsodM and SOD activity was assessed following growth in Fe- and Mn-replete media. Control experiments using purified protein confirmed that the Fe-loaded form of SodM, but not the Mn-loaded forms of SodA or SodM, is sensitive to peroxide poisoning (Fig 2A). Consistent with prior studies [43], following growth in Mn-replete media SodM activity was not affected by peroxide indicating that the protein is loaded with Mn. However, when grown in Fe-replete media SodM activity was sensitive to hydrogen peroxide, indicating that it was Fe-loaded (Fig 4). These results indicate that in *S. aureus* SodM can be active with either Mn or Fe. CP has been observed to bind Fe$^{2+}$, although it is unclear if this binding contributes to antimicrobial activity [13, 47]. As such, it raises the possibility that when exposed to CP *S. aureus* may be incapable of populating SodM with Fe. To evaluate the metallation state of SodM, lysates from cells cultured in the presence of CP were treated with H$_2$O$_2$ and assayed for SOD activity. In the
absence of CP there was no reduction in activity associated with the heterodimer following peroxide treatment indicating that SodM is loaded with Mn. However, in the presence of CP, peroxide treatment eliminated almost all SodM activity, indicating that it is loaded with Fe (Fig 2A). Oxidative stress did not change the metal that was associated with SodM in both the presence and absence of CP (S1B Fig). Cumulatively, these observations suggest that the cambialistic nature of SodM enables \textit{S. aureus} to resist host-imposed Mn starvation by facilitating the retention of SOD activity.

**SodM enhances the ability of \textit{S. aureus} to resist Mn starvation during infection**

In order to evaluate if SodA or SodM differentially contribute to pathogenesis based on Mn abundance during infection, we took advantage of the difference in Mn availability in wild type and CP-deficient mice [9]. Initially, the respective contributions of SodA and SodM to systemic disease in wild type C57BL/6 mice, in which the staphylococcal abscess is devoid of Mn, was assessed. In wild type mice, infection with \( \Delta \text{sodA} \) resulted in a modest, but not significant, reduction in bacterial burden when compared to wild type \textit{S. aureus}. In contrast, in wild type mice infected with \( \Delta \text{sodM} \) there was a significant reduction in bacterial burden relative to wild type \textit{S. aureus} (Fig 5), indicating that in the absence of Mn, SodM is critical for staphylococcal infection. Next, we infected CP-deficient mice, which fail to sequester Mn from staphylococcal liver abscesses [9]. Consistent with prior results, higher bacterial burdens were recovered from CP-deficient mice (C57BL/6 S100A9-/-) than wild type C57BL/6 mice infected with wild type \textit{S. aureus} [9] (Fig 5). CP-deficient mice infected with \( \Delta \text{sodA} \) had significantly reduced bacterial burdens when compared to wild type \textit{S. aureus}. This result contrasts with CP-deficient mice infected with \( \Delta \text{sodM} \), which had bacterial burdens comparable to that of those infected with wild type \textit{S. aureus}. In total, these observations indicate that SodA, but not SodM, contributes to staphylococcal disease when Mn is abundant. They also support the hypothesis that SodM contributes to the ability of \textit{S. aureus} to resist host-imposed Mn starvation during infection.

**Discussion**

During infection the innate immune system combats invading microbes by restricting the availability of the essential nutrient Mn [9, 12, 14]. At the same time, \textit{S. aureus} and other

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\( \text{S. aureus} \)

\[ \begin{align*}
\text{SodM} & \quad +\text{Fe} \quad -\text{Mn} \\
\text{SodM} & \quad +\text{Mn} \quad \text{Mn} \quad \text{Fe}
\end{align*} \]

\[ \text{SodM} \quad -\text{H}_2\text{O}_2 \]

\[ \text{SodM} \quad +\text{H}_2\text{O}_2 \]

**Fig 4. SodM can be metallated with either Mn or Fe in \textit{S. aureus}**. The \textit{S. aureus} \( \Delta \text{sodA}\Delta \text{sodM} \) mutant expressing SodM from a plasmid was grown in NRPMI supplemented with either 1 \( \mu \text{M FeCl}_2 \) or 1 \( \mu \text{M MnCl}_2 \), and SOD activity was assessed in cell lysates (24.8 \( \mu \text{g of total protein} \)), with and without peroxide treatment. Peroxide treatment was used to inactivate Fe-containing SODs. Purified SodM (0.3 \( \mu \text{g} \)), loaded with either Mn or Fe, were included as controls. The experiment was repeated 3 times, and representative gels are shown. doi:10.1371/journal.ppat.1006125.g004
pathogens must also overcome other host defenses including the oxidative burst of immune cells [23]. Accomplishing this latter task is made more challenging, as host-imposed Mn starvation inactivates bacterial Mn-dependent SODs [14]. The current investigations revealed that the possession of a cambialistic SOD enables\textit{S. aureus} to counter these dual host threats both in culture and during infection. This strategy represents an entirely new mechanism for resisting host-imposed Mn starvation and establishes that cambialistic SODs contribute to bacterial pathogenesis.

The Fe/Mn superfamily of SODs is widely distributed in bacteria, archaea, and eukaryotes. Members of this family are generally thought to be reliant on either Mn or Fe for catalytic activity [28, 30, 48]. However, since the 1980s, predominantly \textit{in vitro} analyses have suggested that a subset of these enzymes, termed cambialistic SODs, are capable of using both Fe and Mn, [32–34, 38–40]. Cambialistic SODs have been reported in both Gram-positive and Gram-negative bacteria, including the human pathogens \textit{Porphyromonas gingivalis}, \textit{Streptococcus pneumoniae}, and \textit{Streptococcus mutans} and suggested to be present in other microbes including \textit{Bacteroides fragilis}, and \textit{Bacteroides thetaiotaomicron} [32–40, 49–52]. However, the lack of detailed \textit{in vivo} studies and the fact that many cambialistic SODs have greater activity when loaded with one or the other cofactor \textit{in vitro} has resulted in skepticism regarding the importance of cambialism [32, 36, 38–40]. As such, a false dichotomy exists that members of the Fe/ Mn SOD family must use either Mn or Fe but not both. This dichotomy has led to confusion over the biologically relevant metal utilized by several bacterial SODs, especially given the

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{SodM contributes to resisting Mn starvation during infection. Wild type C57BL/6 (C57) and C57BL/6 S100A9\textsuperscript{−/−} (CP\textsuperscript{−/−}) mice were infected with either wild type, ΔsodA or ΔsodM \textit{S. aureus}. Mice were sacrificed 96 h after infection, and bacterial loads in the livers were enumerated. p <0.05 as determined by Mann-Whitney test.}
doi:10.1371/journal.ppat.1006125.g005
\end{figure}
difficulty in predicting the cofactor utilized by this family of enzymes using bioinformatics [28]. The observation that SodM has equal activity with either Mn or Fe in vitro, can be activated with both metals in vivo, and promotes resistance to nutritional immunity during infection establishes a clear and important role for cambialistic SODs in facilitating resistance to host defenses. Given the ubiquity of CP and host-imposed metal starvation during infection, it seems likely that expression of a cambialistic SOD would provide a benefit to other pathogens as well. Cambialistic SODs are also found in a diverse collection of environmental microbes [32, 33, 35, 39], suggesting cambialism may represent a generalized strategy used by organisms to maintain a defense against superoxide in niches where Fe and Mn availability can fluctuate.

While metal-dependent mononuclear enzymes have historically been thought to utilize a specific cofactor, it has become apparent that there can be significant plasticity in the metal cofactor they can utilize, particularly in the case of Mn- and Fe-utilizing enzymes [53]. In response to peroxide stress E. coli and many other pathogens sequester intracellular Fe and increase the expression of Mn importers, which in turn leads to accumulation of this metal [53–55]. In E. coli, this action results in Fe-utilizing enzymes, such as ribulose-5-phosphate 3-epimerase, becoming populated with Mn [53, 56, 57]. This change in cofactors enables E. coli to both maintain enzymatic activity and prevent Fenton chemistry-induced damage, which can arise from the interaction of Fe$^{2+}$ with oxidants [53, 56, 57]. Notably, in many Fe-centric organisms, including E. coli, Salmonella typhimurium, and Yersinia pestis, Fe starvation increases the expression of Mn uptake systems and the accumulation of Mn [54, 58–60]. In addition to enabling bacteria to activate Mn-dependent isozymes [61], the increased Mn levels may also allow them to replace Fe with Mn in non-redox enzymes. These observations in conjunction with our findings suggest that populating metalloenzymes with an alternative yet catalytically active metal may be a general strategy used by bacteria to survive when a specific metal is limiting. While specific examples for metals other than Fe and Mn are currently lacking, conceptually this cofactor plasticity may enable microbes to maintain critical metabolic processes when limited for other essential metals.

Amongst the staphylococci, S. aureus is the most pathogenic species and the only one that expresses two SODs [41–44], with SodM presumably being gained through duplication and subsequent divergence. However, the advantage that S. aureus gains by expressing two Mn-dependent SODs, which are 75% identical at the level of their primary sequence, had not been apparent. Our current studies found that SodM is induced by CP-imposed Mn-starvation. The observation that SodM is cambialistic and enables S. aureus to maintain SOD activity when Mn starved by the host provides a rationale for its acquisition. It also suggests a model in which the Mn-dependent SodA is important during the initial colonization of a tissue, while SodM becomes important later during infection following the imposition of Mn starvation by the host immune response. Notably, S. aureus is not the only pathogen to express multiple superoxide dismutases that initially appear to be functionally redundant in culture, but upon subsequent analysis possesses properties that enhance fitness in the context of pathogenesis [62, 63]. For example, a second Cu/Zn SOD expressed by some Salmonella is protease-resistant and binds to peptidoglycan, which enables it to retain activity and promote survival within the phagolysosome [63]. Cumulatively, these observations and our results emphasize the importance of evaluating the contribution of apparently redundant SODs to resisting oxidative stress in the context of the other stressors that an organism encounters within its ecological niche.

The antimicrobial activity of CP is generally thought to be mediated by the sequestration of Mn$^{2+}$ and Zn$^{2+}$ [9, 20, 21]. However, CP was recently shown to bind Fe$^{2+}$, resulting in the suggestion that Fe restriction is a primary driver of its antimicrobial activity [47]. Notably, CP does not bind Fe$^{3+}$, the ionic state that exists in oxidizing environments such as sites of infection [9, 20, 47]. Additionally, several experimental lines of evidence suggest that Mn limitation
contributes to the antimicrobial activity of CP both in culture and during infection. In both *Acinetobacter baumannii* and *S. aureus*, CP reduces intracellular Mn but not Fe levels [17, 64]. The current observation that *S. aureus* replaces Mn in SodM with Fe even in the presence of concentrations of CP approaching 1 mg/ml further supports the idea that in culture CP is not imposing Fe limitation on *S. aureus*. Furthermore, in wild type mice loss of MntABC and MntH, the two Mn importers expressed by *S. aureus*, results in a substantial reduction in virulence; however, this defect is completely reversed in CP-deficient mice [13]. The observation that SodM is critical for infection in wild type but not CP-deficient mice further supports the idea that Mn but not Fe sequestration by CP contributes to controlling infection. Perhaps not surprisingly, given the myriad of high affinity staphylococcal Fe acquisition systems [65], it also suggests that during infection *S. aureus* more successfully competes with the host for Fe than Mn. Cumulatively, these findings strongly support, at least in the case of *S. aureus* and *A. baumannii*, that Mn and not Fe sequestration significantly contributes to the antimicrobial activity of CP.

Antibiotic resistance is a serious and growing threat to human health, with multiple agencies calling for the development of new approaches to treat bacterial infections [1, 2]. Understanding how pathogens overcome innate immune defenses has the potential to reveal new opportunities for therapeutic intervention. Our studies reveal a new mechanism by which bacteria can overcome a two-pronged attack by the host. They also clearly demonstrate a role for cambialism in resisting nutritional immunity and bacterial pathogenesis. Moreover, these results provide newfound importance for a neglected family of proteins that is widely distributed throughout the tree of life.

**Methods**

**Ethics Statement**

All animal work was approved by the Vanderbilt University Institutional Animal Care and Use Committee (protocol #M1600123) and was performed in accordance with United States Public Health Service Policy on Humane Care and Use of Laboratory Animals and the US Animal Welfare Act.

**Bacterial Strains**

*Staphylococcus aureus* strain Newman was used unless otherwise indicated. All strains and plasmids used in this study are listed in Tables 1 and 2. *S. aureus* was routinely grown in tryptic soy broth (TSB) and on tryptic soy agar plates (TSA), while *E. coli* was routinely cultivated in Luria Broth (LB) and on Luria agar plates. Both species were grown at 37˚C. As needed for plasmid maintenance or gene inductions, 10 µg/ml of chloramphenicol, 50 µg/ml of kanamycin, 100 µg/ml of ampicillin, or 10 ng/ml of anhydrotetracycline was included in the media used. All strains were stored at -80˚C in media containing 30% glycerol.

**Construction of staphylococcal mutants and plasmids**

The Δ*sodA* and Δ*sodM* mutants were created via Phi85 transduction of the *sodA::tet* and *sodM::erm* alleles from RN6390 [43]. The staphylococcal SodA and SodM expression constructs were created by amplifying *sodA* and *sodM* using the primers indicated in Table 3 and then cloning these fragments into the anhydrotetracycline-inducible plasmid pRMC2 [66]. To generate the YFP reporter plasmids the promoters for *sodA* and *sodM* were amplified with the primers listed in Table 3 and then cloned using standard techniques into pAH5 [67]. To generate the promoterless YFP construct pAH5 was digested with *Pst*I and *Kpn*I to remove the existing promoter, blunted and then self-ligated.
Calprotectin growth assays were performed largely as previously described [13, 20], with the exception that overnight cultures were performed in Chelex-treated RPMI + 1% casamino acids (NRPMI) supplemented with 1 mM MgCl$_2$, 100 μM CaCl$_2$, and 1 μM FeCl$_2$. These cultures were diluted 1:100 into 100 μl of culture medium in a 96-well round-bottom plate and incubated at 37˚C and with shaking at 180 rpm. The culture medium consisted of 38% TSB and 62% CP buffer (3 mM CaCl$_2$, 20mM Tris base, and 100 mM NaCl, 10 mM β-mercaptoethanol, pH 7.5) supplemented with 1 μM MnCl$_2$ and 1 μM ZnSO$_4$. Where indicated 0.1 mM PQ was added to the media. The same growth conditions were utilized for the expression studies. For both growth and expression assays, optical density (OD$_{600}$) and fluorescence was assessed after 8 hrs of growth.

SOD activity

Total and individual superoxide dismutase (SOD) activity were assayed using a water-soluble tetrazolium salt assay and a gel-based nitro blue tetrazolium assay, respectively, as previously described [14, 68]. For both assays, the bacteria were grown as for the CP assays and harvested in exponential phase (OD$_{600}$ of ~ 0.3–0.35). The cells were collected and then resuspended in 0.5 mM KPO$_4$ buffer at pH 7.8 with 0.1 mM EDTA [46]. The bacteria were then lysed via mechanical disruption and centrifuged to remove insoluble material. The protein concentration in the cell lysate was determined via BCA assay (Pierce). Total SOD activity was assessed using the SOD Assay Kit (Sigma-Aldrich), per the manufacturer’s instructions. To evaluate the individual

| Strain | Description | Reference |
|--------|-------------|-----------|
| Wild type | S. aureus Newman | This Study |
| ΔsodA | S. aureus Newman sodA::tet | This Study |
| ΔsodM | S. aureus Newman sodM::erm | This Study |
| ΔsodAΔsodM | S. aureus Newman sodA::tet sodM::erm | [14] |
| WT pRMC2 | S. aureus Newman carrying pRMC2 | This study |
| ΔsodAΔsodM pRMC2 | ΔsodAΔsodM carrying pRMC2 | This study |
| ΔsodAΔsodM pRMC2-sodA | ΔsodAΔsodM carrying pRMC2-sodA | This study |
| ΔsodAΔsodM pRMC2-sodM | ΔsodAΔsodM carrying pRMC2-sodM | This study |
| WT pEmpty | S. aureus Newman carrying pAH5-empty | This study |
| WT pSodA | S. aureus Newman carrying pAH5-pSodA | This study |
| WT pSodM | S. aureus Newman carrying pAH5-pSodM | This study |

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| Table 2. Plasmids used in this study. |
|-------------------------------|-------------------------------|---------|
| Plasmid | Description | Reference |
| pRMC2 | Anhydrotetracycline-inducible plasmid | [66] |
| pRMC2-sodA | sodA cloned into pRMC2 | This study |
| pRMC2-sodM | sodM cloned into pRMC2 | This study |
| pAH5 | YFP reporter plasmid | [67] |
| pAH5-pSodA | pAH5 with the sodA promoter driving YFP expression | This study |
| pAH5-pSodM | pAH5 with the sodM promoter driving YFP expression | This study |
| pEmpty | pAH5 without a promoter driving YFP expression | This study |
| pET29a-sodA | SodA expression vector | This study |
| pET29a-sodM | SodM expression vector | This study |

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activity of each SOD, the cell lysates were normalized to total protein concentration and resolved on 10% native polyacrylamide gel. The gels were incubated in buffer containing 0.05 M potassium phosphate pH 7.8, 1 mM EDTA, 0.25 mM nitro blue tetrazolium chloride, and 0.05 mM riboflavin and then exposed to light, as previously described [68]. To evaluate if SOD activity was due to iron-loading, prior to assessing activity the gels were incubated with 20 mM H$_2$O$_2$ or water for 20 minutes. Gels were imaged using a BioRad imager Universal Hood II and the fractional distribution of SOD activity was determined using the BioRad Quantity One software.

Cloning of the sodA and sodM genes for heterologous expression

The sodA and sodM genes were amplified by PCR from S. aureus genomic DNA using Pfu polymerase (NEB) and the primer pairs sodA_for and sodA_rev and sodM_for and sodM_rev, respectively, which incorporated 5' NdeI and 3' BamHI restriction sites. PCR products were A-tailed with Taq polymerase (NEB) and cloned into the pGEM-T vector (Promega) to yield pGEM-T-sodA and pGEM-T-sodM, respectively. An internal NdeI site in the sodM sequence was silently mutated by site-directed mutagenesis using the primer pair sodMqc1_for and sodMqc1_rev to yield pGEM-T-sodMqc. The genes were sub-cloned through NdeI/BamHI (NEB) digestion of the pGEM-T constructs, purification of the gene inserts by agarose gel electrophoresis, and subsequent ligation into NdeI/BamHI-digested pET29a vector (Novagen) to yield pET29a-sodA and pET29a-sodM constructs. Both pET29a constructs were sequenced (GATC Biotech, Germany). A sequence error detected in pET29a-sodM (deletion of base A570) was subsequently corrected through site-directed mutagenesis using primers sodMqc2_for and sodMqc2_rev, and the final construct confirmed through sequencing.

Expression, purification and quantification of recombinant SodA and SodM

The pET29a-sodA and pET29a-sodM constructs were transformed into Escherichia coli BL21 (DE3) cells and selected on LB agar plates containing 50 μg/ml kanamycin. For each cell type,
cells were inoculated into M9 medium containing 10 μM FeSO₄ and 50 μg/ml kanamycin and cultured overnight at 37˚C with 180 rpm orbital shaking. This overnight culture was used to inoculate 1 L M9 medium containing 10 μM FeSO₄ and 50 μg/ml kanamycin, cultured at 37˚C with 180 rpm orbital shaking. At OD₆₀₀ ~0.5, protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) plus a further 20 μM FeSO₄ and incubation for 4 h under the same conditions. Cells were harvested by centrifugation (20 min, 4,000 g, 4˚C), washed in 20 mM Tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA), followed by a further wash in 20 mM Tris, pH 7.5, 150 mM NaCl and stored at -20˚C.

Cells were resuspended in 20 mM Tris, pH 7.5 and lysed by sonication (6 x 10 s, with 1 min intervals, on ice) and the lysate clarified by centrifugation (20 min, 19,000 g, 4˚C). The soluble lysate was loaded onto a 5 ml HiTrap Q HP anion exchange chromatography (AEC) column (GE Healthcare), the column was washed with 5 column volumes (CV) of buffer (20 mM Tris, pH 7.5), followed by elution with a 9 CV linear NaCl gradient (0–1 M NaCl) in the same buffer, collecting 2 ml fractions, using an Äkta fast performance liquid chromatography (FPLC) system (GE Healthcare). Fractions were analyzed for protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SodA eluted from AEC at ~230 mM NaCl, whereas SodM eluted at ~248 mM NaCl. Aliquots (1 ml) of the peak AEC fractions containing the recombinant protein were further purified using the Äkta FPLC by size exclusion chromatography (SEC) on a Superdex 200 16/60 column (GE Healthcare), resolved in 20 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.5 at 1 ml/min, collecting 2 ml fractions.

Concentrations of purified recombinant SodA and SodM were determined from A₂₈₀nm measurements, using the empirically determined extinction coefficients (ε₂₈₀nm) of 62,681 M⁻¹ cm⁻¹ for SodA and 64,949 M⁻¹ cm⁻¹ for SodM, each derived from quantitative amino acid analysis (Alta Bioscience, UK). The metal content of the purified proteins was assessed by ICP-MS, as described below.

Unfolding and refolding of recombinant SOD proteins

To reconstitute recombinant SodA and SodM, which contained primarily Fe when purified from E. coli, with exclusively Mn they were unfolded and refolded in excess Mn, as previously described [69], with modifications. Unfolding was performed in 2.5 M guanidine hydrochloride, in the presence of 5 mM EDTA and 20 mM 8-hydroxyquinoline to remove bound metal ions, followed by refolding through several rounds of dialysis against 20 mM Tris, 100 mM NaCl, 10 mM MnCl₂, pH 7.5, to yield protein containing exclusively manganese. To analyze the bound metal, aliquots of each purified protein (~2 mg in 0.5 ml) were resolved on a Superdex 200 Increase 10/30 column (GE Healthcare) in 20 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.5, resolved at 0.75 ml/min and collecting 0.5 ml fractions using the Äkta FPLC. Eluant fractions were analyzed for protein by A₂₈₀nm and by SDS-PAGE, for elemental composition by ICP-MS, and for enzyme activity by in-gel activity assay.

Elemental analysis by inductively coupled plasma mass spectrometry

For elemental analysis via ICP-MS, protein-containing samples were diluted 50-fold into a solution of 2.5% HNO₃ (Suprapur, Merck) containing 20 μg/l Co and Pt as internal standards. Matrix-matched elemental standards (containing analyte metal concentrations of 0–500 μg/L) were prepared by serial dilution from individual metal standard stocks (VWR) with identical solution compositions, including the internal standard. All standards and samples were analyzed by ICP-MS using a Thermo x-series instrument operating in collision cell mode (using 3.0 ml/min flow of 8% H₂ in He as the collision gas). Isotopes $^{55}$Mn, $^{56}$Fe, $^{59}$Co, $^{66}$Zn, and $^{195}$Pt...
were monitored using the peak-jump method (100 sweeps, 20–30 ms dwell time on 3–5 channels per isotope, separated by 0.02 atomic mass units) in triplicate, and metal concentrations determined from the standard curve.

Animal infections

Nine-week-old female black C57BL/6 and CP-/- (C57BL/6 S100A9-/-) mice were infected using a retro-orbital infection model [9, 13, 14]. Livers were harvested and homogenized 96 hours post-infection. Serial dilutions were then plated and counted for colony forming units.

Supporting Information

S1 Fig. SodM is the predominant source of SOD activity in Mn-deplete environments regardless of oxidative stress. Wild type S. aureus and ΔsodAΔsodM were grown in the presence of CP, in the (A-C) presence and (A & B) absence of 0.1 mM PQ and (A) total SOD activity and (C) the individual contributions of SodA and SodM to SOD activity were determined (n = 3). (B) In-gel analysis of the individual activities of SodA and SodM following growth in the presence of CP and PQ. Hydrogen peroxide treatment was used to inactivate Fe-containing SODs. The experiment was repeated 3 times and representative gels are shown. * = p < 0.05 relative to no CP via two-way ANOVA with Tukey’s post-test. (TIF)

S2 Fig. Contribution of SodA and SodM to resisting oxidative stress and metal limitation. (A & B) The ΔsodAΔsodM mutant expressing either SodA or SodM from a plasmid was grown in various concentrations of CP in the (A) absence and (B) presence of 0.1 mM PQ. Growth (OD600) was measured after 8 h. * = p < 0.05 relative to WT containing empty vector via two-way ANOVA with Dunnett’s post-test. Error bars indicate SEM (n = 3 or more). (C-D) Wild type S. aureus, ΔsodA, ΔsodM, and ΔsodAΔsodM were grown in the presence of various concentrations of (C) the ΔMn/Zn site CP mutant or (D) the ΔZn site mutant in the absence of PQ. * = p < 0.05 relative to wild type at the same concentration of CP via two-way ANOVA with Dunnett’s post-test. Error bars indicate SEM (n = 3 or more). (TIF)

S3 Fig. Elemental analysis of purified and remetallated SodA and SodM. Aliquots (~2 mg in 0.5 ml) of purified recombinant (A) SodA and (B) SodM were resolved by analytical size exclusion chromatography and eluant fractions (0.5 ml) were analyzed for protein by A280nm (black), and for manganese (blue) and iron (red) content by ICP-MS. Both proteins contained exclusively iron when purified from the heterologous host. Each protein was then unfolded, stripped of iron, and refolded in the presence of manganese. The resulting proteins were analyzed identically, and the refolded (C) SodA and (D) SodM were found to contain exclusively manganese. (E) Each of the four resulting samples (Fe-SodA, Fe-SodM, Mn-SodA and Mn-SodM) were subjected to protein analysis by SDS-PAGE, and to both in-gel and spectrophotometric SOD activity analysis. (TIF)

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References
1. CDC. Antibiotic Resistance Threats in the United States. U.S. Centers for Disease Control and Prevention; 2013.
2. Organization WH. Antimicrobial resistance: global report on surveillance. 2014.
3. Lowy FD. *Staphylococcus aureus* infections. The New England journal of medicine. 1998; 339(8):520–32. Epub 1998/08/26. doi: 10.1056/NEJM199808203390806 PMID: 9709046
4. Kleven WS, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. Jama. 2007; 298(15):1763–71. Epub 2007/10/18. doi: 10.1001/jama.298.15.1763 PMID: 17940231
5. Said-Salim B, Matherna B, Kreiswirth BN. Community-acquired methicillin-resistant *Staphylococcus aureus*: an emerging pathogen. Infection control and hospital epidemiology: the official journal of the Society of Hospital Epidemiologists of America. 2003; 24(6):451–5. Epub 2003/06/28.
6. Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM. Metal ions in biological catalysis: from enzyme databases to general principles. Journal of biological inorganic chemistry: JBIC: a publication of the Society of Biological Inorganic Chemistry. 2008; 13(8):1205–18. Epub 2008/07/08.
7. Waldron KJ, Rutherford JC, Ford D, Robinson NJ. Metalloproteins and metal sensing. Nature. 2009; 460(7527):823–30. Epub 2009/08/14. doi: 10.1038/nature08300 PMID: 19675642
8. Waldron KJ, Robinson NJ. How do bacterial cells ensure that metalloproteins get the correct metal? Nature reviews Microbiology. 2009; 7(1):25–35. Epub 2008/12/17. doi: 10.1038/nrmmicro2057 PMID: 19079350
9. Corbin BD, Seeley EH, Raab A, Feldmann J, Miller MR, Torres VJ, et al. Metal chelation and inhibition of bacterial growth in tissue abscesses. Science. 2008; 319(5865):962–5. Epub 2008/02/16. doi: 10.1126/science.1152449 PMID: 18276893
10. Schäible UE, Kaufmann SH. Iron and microbial infection. Nature reviews Microbiology. 2004; 2(12):946–53. Epub 2004/11/20. doi: 10.1038/nrmicro1046 PMID: 15650940
11. Weinberg ED. Iron availability and infection. Biochimica et biophysica acta. 2009; 1790(7):600–5. Epub 2008/08/05. doi: 10.1016/j.bbagen.2008.07.002 PMID: 18675317
12. Kehl-Fie TE, Skaar EP. Nutritional immunity beyond iron: a role for manganese and zinc. Current opinion in chemical biology. 2010; 14(2):218–24. Epub 2009/12/18 PubMed Central PMCID: PMC2847644. doi: 10.1016/j.cbpa.2009.11.008 PMID: 20015678
13. Kehl-Fie TE, Zhang Y, Moore JL, Farrand AJ, Hood MI, Rathi S, et al. MntABC and MntH contribute to systemic *Staphylococcus aureus* infection by competing with calprotectin for nutrient manganese. Infection and immunity. 2013; 81(8):3395–405. Epub 2013/07/03. PubMed Central PMCID: PMC3754211. doi: 10.1128/IAI.00429-13 PMID: 23917815
14. Kehl-Fie TE, Chitayat S, Hood MI, Damo S, Restrepo N, Garcia C, et al. Nutrient metal sequestration by calprotectin inhibits bacterial superoxide defense, enhancing neutrophil killing of *Staphylococcus aureus*. Cell host & microbe. 2011; 10(2):156–64. Epub 2011/08/17. PubMed Central PMCID: PMC3157011.
15. Gebhardt C, Nemeth J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. Biochemical pharmacology. 2006; 72(11):1622–31. doi: 10.1016/j.bcp.2006.05.017 PMID: 16846921
16. Clohessy PA, Golden BE. Calprotectin-mediated zinc chelation as a biostatic mechanism in host defence. Scandinavian Journal of Immunology. 1995; 42(5):551–6.
17. Hood MI, Mortensen BL, Moore JL, Zhang Y, Kehl-Fie TE, Sugitani N, et al. Identification of an Acinetobacter baumannii zinc acquisition system that facilitates resistance to calprotectin-mediated zinc sequestration. PLoS pathogens. 2012; 8(12):e1003068. Epub 2012/12/14. PubMed Central PMCID: PMC3516566. doi: 10.1371/journal.ppat.1003068 PMID: 23236280

18. Bianchi M, Niemiec MJ, Siler U, Urban CF, Reichenbach J. Restoration of anti-Aspergillus defense by neutrophil extracellular traps in human chronic granulomatous disease after gene therapy is calprotectin-dependent. The Journal of allergy and clinical immunology. 2011; 127(5):1243–52 e7. doi: 10.1016/j.jaci.2011.01.021 PMID: 21376380

19. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goossmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS pathogens. 2009; 5(10):e1000639. PubMed Central PMCID: PMCPMC2763347. doi: 10.1371/journal.ppat.1000639 PMID: 19876394

20. Damo SM, Kehl-Fie TE, Sugitani N, Holt ME, Rathl S, Murphy WJ, et al. Molecular basis for manganese sequestration by calprotectin and roles in the innate immune response to invading bacterial pathogens. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(10):3841–6. Epub 2013/02/23 PubMed Central PMCID: PMC3593395. doi: 10.1073/pnas.1220341110 PMID: 23431180

21. Hayden JA, Brophy MB, Cunden LS, Nolan EM. High-affinity manganese coordination by human calprotectin is calcium-dependent and requires the histidine-rich site formed at the dimer interface. Journal of the American Chemical Society. 2013; 135(2):775–87. PubMed Central PMCID: PMCPMC3575579. doi: 10.1021/ja3096416 PMID: 23276281

22. Brophy MB, Hayden JA, Nolan EM. Calcium ion gradients modulate the zinc affinity and antibacterial activity of human calprotectin. Journal of the American Chemical Society. 2012; 134(43):18089–100. Epub 2012/10/23. PubMed Central PMCID: PMCPMC3579771. doi: 10.1021/ja307974e PMID: 23082970

23. Rigby KM, DeLeo FR. Neutrophils in innate host defense against Staphylococcus aureus infections. Seminars in immunopathology. 2012; 34(2):237–59. PubMed Central PMCID: PMCPMC3271231. doi: 10.1007/s00281-011-0295-3 PMID: 22080185

24. Beyer W, Imlay J, Fridovich I. Superoxide dismutases. Progress in nucleic acid research and molecular biology. 1991; 40:221–53. Epub 1991/01/01. PMID: 1851570

25. Imlay JA. Pathways of oxidative damage. Annual review of microbiology. 2003; 57:395–418. Epub 2003/10/07. doi: 10.1146/annurev.micro.57.030502.090938 PMID: 14527285

26. Lynch M, Kuramitsu H. Expression and role of superoxide dismutases (SOD) in pathogenic bacteria. Microbes and infection / Institut Pasteur. 2000; 2(10):1245–55. Epub 2000/09/29.

27. Storz G, Imlay JA. Oxidative stress. Current opinion in microbiology. 1999; 2(2):188–94. Epub 1999/05/14. PMID: 10322176

28. Sheng Y, Abreu JA, Cabelli DE, Maroney MJ, Miller AF, Teixeira M, et al. Superoxide dismutases and superoxide reductases. Chemical reviews. 2014; 114(7):3854–918. PubMed Central PMCID: PMCPMC4317059. doi: 10.1021/cr4005296 PMID: 24684599

29. Miller AF. Superoxide dismutases: ancient enzymes and new insights. FEBS letters. 2012; 586(5):585–95. Epub 2011/11/15 doi: 10.1016/j.febslet.2011.10.048 PMID: 22079668

30. Clare DA, Blum J, Fridovich I. A hybrid superoxide dismutase containing both functional iron and manganese. The Journal of biological chemistry. 1984; 259(9):5932–6. PMID: 6371011

31. Bray RC, Cockle SA, Fielden EM, Roberts PB, Rotilio G, Calabrese L. Reduction and inactivation of superoxide dismutase by hydrogen peroxide. The Biochemical journal. 1974; 139(1):43–8. Epub 1974/04/01. PubMed Central PMCID: PMC1166249. PMID: 4377099

32. Yamano S, Sako Y, Nomura N, Maruyama T. A cambialistic SOD in a strictly aerobic hyperthermophilic archaeon, Aeropyrum pernix. Journal of biochemistry. 1999; 126(1):218–25. Epub 1999/07/07. PMID: 10393342

33. Lancaster VL, LoBrutto R, Selvaraj FM, Blankenship RE. A cambialistic superoxide dismutase in the thermophilic photosynthetic bacterium Chloroflexus aurantiacus. Journal of bacteriology. 2004; 186(11):3408–14. Epub 2004/05/20. PubMed Central PMCID: PMCPMC415758. doi: 10.1128/JB.186.11.3408-3414.2004 PMID: 15190226

34. Hiraoka BY, Yamakura F, Sugio S, Nakayama K. A change of the metal-specific activity of a cambialistic superoxide dismutase from Porphyromonas gingivalis by a double mutation of Gin-70 to Gly and Ala-142 to Gln. The Biochemical journal. 2000; 345 Pt 2:345–50. Epub 2000/01/06. PubMed Central PMCID: PMCPMC1220763. PMID: 10620511

35. Mandelli F, Franco Cairo JP, Citadini AP, Buchi F, Alvarez TM, Oliveira RJ, et al. The characterization of a thermostable and cambialistic superoxide dismutase from Thermus filiformis. Letters in applied microbiology. 2013; 57(1):40–6. Epub 2013/03/28. doi: 10.1111/lam.12071 PMID: 23630753
36. Eijkelkamp BA, Morey JR, Ween MP, Ong CL, McEwan AG, Paton JC, et al. Extracellular zinc competitively inhibits manganese uptake and compromises oxidative stress management in *Streptococcus pneumoniae*. PLoS one. 2014; 9(2):e89427. Epub 2014/02/22. PubMed Central PMCID: PMC3928430. doi: 10.1371/journal.pone.0089427 PMID: 24558498

37. De Vendittis A, Marco S, Di Maro A, Chambery A, Albino A, Masullo M, et al. Properties of a putative cambialistic superoxide dismutase from the aerotolerant bacterium *Streptococcus thermophilus* strain LMG 18311. Protein and peptidic letters. 2012; 19(3):333–44. Epub 2012/03/14. PMID: 22409500

38. Meier B, Sehn AP, Michel C, Saran M. Reactions of hydrogen peroxide with superoxide dismutase from *Propionibacterium shermanii*—an enzyme which is equally active with iron or manganese—are independent of the prosthetic metal. Archives of biochemistry and biophysics. 1994; 313(2):296–303. Epub 1994/09/01. doi: 10.1006/abbi.1994.1391 PMID: 8080276

39. Tabares LC, Bittel C, Carrillo N, Bortolotti A, Cortez N. The single superoxide dismutase of *Nakamura T, Torikai K, Uegaki K, Morita J, Machida K, Suzuki A, et al. Crystal structure of the cambialistic superoxide dismutase from *Aeropyrum pernix* K1—insights into the enzyme mechanism and stability. The FEBS journal. 2011; 278(4):598–609. Epub 2010/12/25. doi: 10.1111/j.1742-4658.2010.07977.x PMID: 21182595

40. Anjem A, Imlay JA. Mononuclear iron enzymes are primary targets of hydrogen peroxide stress. The Journal of biological chemistry. 2012; 287(19):15544–56. PubMed Central PMCID: PMC3346116. doi: 10.1074/jbc.M111.330365 PMID: 22411989

41. Meier B, Sehn AP, Michel C, Saran M. Reactions of hydrogen peroxide with superoxide dismutase from *Propionibacterium shermanii*—an enzyme which is equally active with iron or manganese—are independent of the prosthetic metal. Archives of biochemistry and biophysics. 1994; 313(2):296–303. Epub 1994/09/01. doi: 10.1006/abbi.1994.1391 PMID: 8080276

42. Karavolos MH, Horsburgh MJ, Ingham E, Foster SJ. Role and regulation of the superoxide dismutases of *Staphylococcus aureus*. Microbiology. 2003; 149(Pt 10):2749–58. Epub 2003/10/03. doi: 10.1099/mic.0.26353-0 PMID: 14523108

43. Valderas MW, Gatson JW, Wreyford N, Hart ME. The superoxide dismutase gene sodM of *Staphylococcus aureus* is a cambialistic, manganese-containing enzyme. Journal of bacteriology. 2003; 185(10):3223–7. Epub 2003/05/06. PubMed Central PMCID: PMC154076. doi: 10.1128/JB.185.10.3223-3227.2003 PMID: 12790184

44. Martin ME, Byers BR, Olson MO, Salin ML, Arceneaux JE, Tolbert C. A cambialistic superoxide dismutase from *Rhodobacter capsulatus* is a cambialistic, manganese-containing enzyme. Journal of bacteriology. 2003; 185(10):3223–7. Epub 2003/05/06. PubMed Central PMCID: PMC154076. doi: 10.1128/JB.185.10.3223-3227.2003 PMID: 12790184

45. Ballal A, Manna AC. Regulation of superoxide dismutase (sod) genes by SarA in *Staphylococcus aureus*. Microbiology. 2003; 149(10):3301–10. Epub 2003/03/17. PubMed Central PMCID: PMC2687179. doi: 10.1099/mic.0.26353-0 PMID: 12928603

46. Anjem A, Varghese S, Imlay JA. Manganese import is a key element of the OxyR response to hydrogen peroxide in *Escherichia coli*. Molecular microbiology. 2009; 72(4):844–58. Epub 2009/04/30. PubMed Central PMCID: PMC2776087. doi: 10.1111/j.1365-2958.2009.06699.x PMID: 19400769

47. Nakashige TG, Zhang B, Krebs C, Nolan EM. Human calprotectin is an iron-sequestering host-defense protein. Nature chemistry biology. 2015; 11(10):765–71. Epub 2015/08/25. PubMed Central PMCID: PMC4575267. doi: 10.1038/nchembio.1891 PMID: 26302479

48. Ose DE, Fridovich I. Manganese-containing superoxide dismutase from *Escherichia coli*; reversible resolution and metal replacements. Archives of biochemistry and biophysics. 1979; 194(2):360–4. PMID: 36037

49. Sugio S, Hiraoka BY, Yamakura F. Crystal structure of cambialistic superoxide dismutase from *Porphyromonas gingivalis*. European journal of biochemistry / FEBS. 2000; 267(12):3487–95. Epub 2000/06/10.

50. Gregory EM, Dapper CH. Isolation of iron-containing superoxide dismutase from *Bacteroides fragilis*: reconstitution as a Mn-containing enzyme. Archives of biochemistry and biophysics. 1983; 220(1):293–300. PMID: 6830240

51. Pennington CD, Gregory EM. Isolation and reconstitution of iron- and manganese-containing superoxide dismutases from *Bacteroides thetaiotaomicron*. Journal of bacteriology. 1986; 166(2):528–32. PubMed Central PMCID: PMC214636. PMID: 3700336

52. Nakamura T, Torikai K, Uegaki K, Morita J, Machida K, Suzuki A, et al. Crystal structure of the cambialistic superoxide dismutase from *Aeropyrum pernix* K1—insights into the enzyme mechanism and stability. The FEBS journal. 2011; 278(4):598–609. Epub 2010/12/25. doi: 10.1111/j.1742-4658.2010.07977.x PMID: 21182595

53. Anjem A, Imlay JA. Mononuclear iron enzymes are primary targets of hydrogen peroxide stress. The Journal of biological chemistry. 2012; 287(19):15544–56. PubMed Central PMCID: PMC3346116. doi: 10.1074/jbc.M111.330365 PMID: 22411989
54. Juttukonda LJ, Skaar EP. Manganese homeostasis and utilization in pathogenic bacteria. Molecular microbiology. 2015; 97(2):216–28. PubMed Central PMCID: PMCPMC4631260. doi: 10.1111/mmi.13034 PMID: 25898914

55. Kehres DG, Zaharik ML, Finlay BB, Maguire ME. The NRAMP proteins of Salmonella typhimurium and Escherichia coli are selective manganese transporters involved in the response to reactive oxygen. Molecular microbiology. 2000; 36(5):1085–100. PMID: 10846993

56. Sobota JM, Gu M, Imlay JA. Intracellular hydrogen peroxide and superoxide poison 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, the first committed enzyme in the aromatic biosynthetic pathway of Escherichia coli. Journal of bacteriology. 2014; 196(11):1980–91. PubMed Central PMCID: PMCPMC4010980. doi: 10.1128/JB.01573-14 PMID: 24659765

57. Sobota JM, Imlay JA. Iron enzyme ribulose-5-phosphate 3-epimerase in Escherichia coli is rapidly damaged by hydrogen peroxide but can be protected by manganese. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108(13):5402–7. PubMed Central PMCID: PMCPMC5010980. doi: 10.1073/pnas.1100410108 PMID: 21402925

58. Ikeda JS, Janakiraman A, Kehres DG, Maguire ME, Slauch JM. Transcriptional regulation of sitABCD of Salmonella enterica serovar Typhimurium by MntR and Fur. Journal of bacteriology. 2005; 187(3):912–22. PubMed Central PMCID: PMCPMC545731. doi: 10.1128/JB.187.3.912-922.2005 PMID: 15659669

59. Patzer SI, Hantke K. Dual repression by Fe(2+)–Fur and Mn(2+)–MntR of the mntH gene, encoding an NRAMP-like Mn(2+) transporter in Escherichia coli. Journal of bacteriology. 2001; 183(16):4806–13. PubMed Central PMCID: PMCPMC99535. doi: 10.1128/JB.183.16.4806-4813.2001 PMID: 11466294

60. Perry RD, Craig SK, Abney J, Bobrov AG, Kirillina O, Mier I Jr., et al. Manganese transporters Yfe and MntH are Fur-regulated and important for the virulence of Yersinia pestis. Microbiology. 2012; 158(Pt 3):804–15. PubMed Central PMCID: PMCPMC3352115. doi: 10.1099/mic.0.053710-0 PMID: 2222497

61. Martin JE, Imlay JA. The alternative aerobic ribonucleotide reductase of Escherichia coli, NrdEF, is a manganese-dependent enzyme that enables cell replication during periods of iron starvation. Molecular microbiology. 2011; 80(2):319–34 PubMed Central PMCID: PMCPMC3097424. doi: 10.1111/j.1365-2958.2011.07953.x PMID: 21383418

62. Gleason JE, Galaleldeen A, Peterson RL, Holloway SP, Waninger-Saroni J, et al. Candida albicans SOD5 represents the prototype of an unprecedented class of Cu-only superoxide dismutases required for pathogen defense. Proceedings of the National Academy of Sciences of the United States of America. 2014; 111(16):5866–71. Epub 2014/04/09 PubMed Central PMCID: PMC4000858. doi: 10.1073/pnas.1400137111 PMID: 24711423

63. Rushing MD, Slauch JM. Either periplasmic tethering or protease resistance is sufficient to allow a SodC to protect Salmonella enterica serovar Typhimurium from phagocytic superoxide. Molecular microbiology. 2011; 82(4):952–63. PubMed Central PMCID: PMCPMC3220996. doi: 10.1111/j.1365-2958.2011.07884.x PMID: 22023457

64. Radin JN, Kellihier JL, Párraga Solórzano PK, Kehl-Fie TE. The two-component system ArrS and alterations in metabolism enable Staphylococcus aureus to resist calprotectin-induced manganese starvation. PLoS pathogens. 2016; 12(11):e1006040. doi: 10.1371/journal.ppat.1006040 PMID: 27992777

65. Sheldon JR, Heinrichs DE. Recent developments in understanding the iron acquisition strategies of gram positive pathogens. FEMS microbiology reviews. 2015; 39(4):592–630. doi: 10.1093/femsre/fuv009 PMID: 25862688

66. Corrigan RM, Foster TJ. An improved tetracycline-inducible expression vector for Staphylococcus aureus. Plasmid. 2009; 61(2):126–9. Epub 2008/11/11. doi: 10.1016/j.plasmid.2008.10.001 PMID: 18996145

67. Malone CL, Boles BR, Lauderdale KJ, Thoendel M, Kavanaugh JS, Horsswill AR. Fluorescent reporters for Staphylococcus aureus. Journal of microbiological methods. 2009; 77(3):251–60. Epub 2009/03/07. PubMed Central PMCID: PMCPmc2693297. doi: 10.1016/j.mimet.2009.02.011 PMID: 19264102

68. McCord JM. Analysis of superoxide dismutase activity. Current protocols in toxicology / editorial board, Mahin D Maines. 2001; Chapter 7:Unit 3. Epub 2001/05/01.

69. Kirby T, Blum J, Kahane I, Fridovich I. Distinguishing between Mn-containing and Fe-containing superoxide dismutases in crude extracts of cells. Archives of biochemistry and biophysics. 1980; 201(2):551–5. PMID: 6994652