An evolutionary path to altered cofactor specificity in a metalloenzyme

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Almost half of all enzymes utilize a metal cofactor. However, the features that dictate the metal utilized by metalloenzymes are poorly understood, limiting our ability to manipulate these enzymes for industrial and health-associated applications. The ubiquitous iron/manganese superoxide dismutase (SOD) family exemplifies this deficit, as the specific metal used by any family member cannot be predicted. Biochemical, structural and paramagnetic analysis of two evolutionarily related SODs with different metal specificity produced by the pathogenic bacterium Staphylococcus aureus identifies two positions that control metal specificity. These residues make no direct contacts with the metal-coordinating ligands but control the metal’s redox properties, demonstrating that subtle architectural changes can dramatically alter metal utilization. Introducing these mutations into S. aureus alters the ability of the bacterium to resist superoxide stress when metal starved by the host, revealing that small changes in metal-dependent activity can drive the evolution of metalloenzymes with new cofactor specificity.
Metalloproteins are critical to all aspects of life. They are ubiquitous, with as many as half of all enzymes requiring a metal cofactor for function. Metalloenzymes are often highly specific for their cognate metal ion, exhibiting reduced activity with non-native metal cofactors in vitro and in vivo. As a result, understanding the principles that govern the specificity of metal-protein interactions is relevant to nearly all aspects of biology, medicine and biotechnology. Yet the underlying biochemical principles that determine metal cofactor specificity, and the evolutionary processes that drive the optimization of enzyme active sites to utilize a specific metal cofactor remain unclear. This in turn limits our ability to predict the metal used by proteins from their sequence, to produce synthetic metalloenzymes to perform novel reactions for industrial applications, and to rationally design metalloenzyme inhibitors for medicine.

Our limited appreciation of how metalloprotein architecture dictates metal speciﬁcity is exempliﬁed by the superoxide dismutases (SODs). SODs detoxify superoxide, a reactive oxygen radical produced by aerobic metabolism and by the mammalian immune system in response to infection. The biological importance of SODs is illustrated by their presence in most organisms, including obligate anaerobes, and the independent evolution of three distinct dismutase protein families. These SOD families are deﬁned by the cofactor they utilize as nickel SODs, copper and zinc SODs, and manganese (Mn) or iron (Fe) SODs. Manganese-dependent SODs are the most common, and historically were thought to be strictly either manganese- or iron-speciﬁc. We recently established that a Mn/Fe SOD from the bacterium Staphylococcus aureus exhibits equal activity with either manganese or iron. These versatile enzymes are termed cambialistic SODs (camSODs). In addition to the camSOD (SodM), S. aureus also possesses a second, manganese-dependent SOD (Soda). Although cambialistic SODs had previously been described, their biological importance was questioned. However, the S. aureus camSOD contributes to infection by enabling the bacterium to maintain a defense against superoxide when manganese starved by the host. All members of the Mn/Fe SOD family are related in sequence, exhibit identical protein folds, and coordinate their metal ion using identical ligands, making it unclear why some enzymes absolutely require manganese for catalysis (MnSOD), while others require iron (FeSOD), and still others show metal cofactor ﬂexibility (camSOD).

The metal used by a protein is not permanently ﬁxed, and can change in response to environmental pressures. For example, iron was readily soluble in the anaerobic oceans during life’s early evolution and early organisms are thus thought to have been iron-philic. However, oxygenation by early photosynthetic organisms reduced the availability of iron. The resulting biological iron deﬁciency would have imposed selective pressure to adapt iron-dependent enzymes to use non-iron cofactors. While supported by bioinformatic analyses, no experimental evidence has been presented demonstrating the evolutionary process by which a change in metal speciﬁcity has evolved through iterative mutation.

Here, we exploit the close relationship between the staphylococcal SODs to understand how evolutionary changes in metal utilization occur. Genomic analysis shows the camSOD likely evolved from a manganese-speciﬁc predecessor that subsequently underwent neofunctionalization, a deﬁned evolutionary process in which mutations rapidly accumulated in the duplicated gene during a period of functional redundancy, resulting in gain of a new beneﬁcial function. Integrated structural, biochemical, and electron paramagnetic resonance (EPR) studies reveal that two such mutations have altered amino acid residues in close spatial proximity to the SOD active site, driving the change in camSOD metal speciﬁcity. When these residues are reciprocally swapped, the metal speciﬁcities of the MnSOD and camSOD are largely interconverted. Remarkably, these residues possess non-polar sidechains located in the metal’s secondary coordination sphere, and make no direct contacts to the metal-coordinating ligands. These subtle changes regulate the electronic structure and redox properties of the catalytic metal ion, dictating which metals the enzymes can use. Leveraging these ﬁndings reveals that small increases in iron-dependent catalysis by camSOD enhance the ability of S. aureus to overcome the immune response. Collectively, our data show how subtle changes to metalloenzyme architecture can dramatically alter the metal ion’s reactivity and drive the evolution of isozymes with new cofactor speciﬁcity.

### Table 1 Enzymatic activity of all SOD forms from this study.

| Enzyme | Mutation | Mn activity | Fe activity | Cambialism ratio |
|--------|----------|-------------|-------------|-----------------|
| MnSOD  | Wild type | 1836 ± 79   | 4 ± 1       | 0.002           |
|        | F19I     | 1734 ± 61   | 5 ± 1       | 0.003           |
|        | G159L    | 942 ± 69    | 39 ± 4      | 0.041           |
|        | L160F    | 1167 ± 92   | 19 ± 13     | 0.016           |
|        | G159L-L160F | 173 ± 21    | 84 ± 12     | 0.486           |
|        | F19I-G159L-L160F | 526 ± 49    | 57 ± 7      | 0.108           |
|        | L159G    | 808 ± 17    | 24 ± 3      | 0.030           |
|        | F160L    | 342 ± 5     | 111 ± 20    | 0.323           |
|        | L159G-L160F | 687 ± 33    | 111 ± 1     | 0.013           |
|        | L19F-L159G-L160F | 789 ± 54    | 39 ± 2      | 0.049           |

Enzymatic activities of all variants of S. aureus MnSOD and camSOD, in each of their metal-loaded forms, were assayed using a commercial SOD Activity Assay kit (Sigma). Each enzyme was assayed in triplicate using independent biological replicates, and error values quoted represent the standard deviation (SD) from the mean value given. All samples were assayed n = 3 except for three forms of the triple mutant variants that were assayed n = 4. Source data are provided as a source data file.

### Results

The two S. aureus SODs exhibit extensive similarity. Initially, we comprehensively characterized which metals the MnSOD and camSOD could use. The MnSOD was active only with manganese, while the camSOD was active with manganese or iron (Supplementary Fig. 1 and Table 1). As with other characterized cambialistic SODs, the activity of S. aureus camSOD is signiﬁcantly lower than that of the MnSOD, suggesting that cambialism represents an evolutionary compromise, where catalytic activity is sacriﬁced at the expense of enabling cofactor ﬂexibility. Alignment of the S. aureus SOD amino acid sequences demonstrated that the two proteins share 75% identity (Supplementary Fig. 2). Next, we examined the S. aureus SODs for structural differences using circular dichroism (CD) spectroscopy. The iron- and manganese-loaded forms of both proteins showed very similar CD spectra (Fig. 1a), consistent with both SODs containing comparable secondary structure composition in solution (Supplementary Table 1). We then determined three dimensional crystal structures of the iron-loaded forms of each SOD by X-ray diffraction and compared them with structures of the manganese-loaded counterparts (Supplementary Table 2). All four forms adopted near-identical architectures (Fig. 1b and Supplementary Fig. 3) whose polypeptide backbones could be overlaid with only minor deviations (Supplementary Table 3).
Crucially, the metal-binding ligands adopted near-identical spatial positions in all four structures (Fig. 1c), with insignificant changes to metal-ligand bond lengths or angles within the crystallographic resolution (Supplementary Tables 4 and 5). This is consistent with our previous EPR study that showed the positions of the protons on the metal-coordinated solvent and histidine ligands are identical24.

Identification of residues proximal to the active site. The structural similarity of the two SODs suggested that subtle differences must explain their different metal specificity. By overlaying the crystal structures of MnSOD and camSOD, all 50 amino acids that differed between the two proteins were spatially localized (Fig. 2a). Few of these were at the dimerization interface (Supplementary Fig. 4), consistent with this region being highly conserved in SODs (Fig. 2b), and most were surface localized (Fig. 2c). However, we identified three residues (19, 159, and 160) internal to the protein that varied between MnSOD and camSOD that were spatially close (<10 Å) to the metal (Fig. 2d), where non-polar sidechains (Leu or Ile for Phe, or Gly for Leu) were swapped. Notably, these sidechains in the secondary coordination sphere made no direct contacts with the metal-coordinating sidechains in the primary coordination sphere.

Metal-specificity is determined by sequence positions 159/160. To study their role in metal specificity, we prepared variant forms

![Fig. 1 Extensive structural similarity of S. aureus MnSOD and camSOD.](image)

**Fig. 1** Extensive structural similarity of *S. aureus* MnSOD and camSOD. **a** Overlaid CD spectra (n = 1) of the (upper panel) manganese-loaded (MnSOD in blue; camSOD in gray) and (lower panel) iron-loaded (MnSOD in gold; camSOD in teal) isoforms, demonstrating similar secondary structure content of the two isozymes in solution. **b** Superimposed crystal structure cartoons of (upper panel) manganese-loaded forms24 and (lower panel) iron-loaded forms of each enzyme, with the proteins colored as per panel (a), and with manganese ions, iron ions, and waters shown as purple, orange, and red spheres, respectively. Analysis of all four structures show that the polypeptide backbones of all isoforms of the staphylococcal SODs are essentially identical to within the ~2 Å resolution of the structural data (Supplementary Tables 3–5). **c** No significant differences within the structural resolution were detectable in the metal coordination environment that could explain the disparate metal specificity of their catalysis.

![Fig. 2 Mutation of two residues located close to the active site inverts specificity.](image)

**Fig. 2** Mutation of two residues located close to the active site inverts specificity. **a** Superimposed ribbon representation structures of the polypeptide backbones showing conserved (C) regions of the manganese-loaded forms of MnSOD (blue) and camSOD (gray), with all variable (V) residues highlighted (yellow and orange, respectively). **b** Analogous ribbon representation of the structure of *S. aureus* MnSOD, illustrating the localization of variable (cyan) and conserved (purple) regions of SOD sequence based on ConSurf analysis59 of 500 SOD sequences (calculated conservation scores represented by the color spectrum key). Approximate dimerization interface is indicated (gray). **c** Superimposed space-filling models of MnSOD and camSOD, colored as in panel (a), illustrating that >85% of sidechains that vary between the *S. aureus* SODs are surface-exposed (2.5 Å² surface exposure cutoff used). **d** Magnified view of the superimposed cartoon representations of MnSOD and camSOD structures, colored as in panel (a), showing the spatial location of the three residues targeted for mutagenesis in proximity to the manganese ion (purple).
of MnSOD and camSOD in which the amino acids at the three candidate positions were reciprocally swapped. The recognition that SOD metal specificity in vitro and in vivo is better described as a spectrum than as binary possibilities highlights the need for a quantitative measure of cofactor plasticity to be used alongside absolute activity. Herein, we define the term cambialism ratio (CR) as such a measure, being the ratio of iron-dependent activity to manganese-dependent activity detectable using a spectrophotometric assay; this value is close to zero for MnSODs (CR = 0.002 for *S. aureus* MnSOD), close to unity for camSODs (CR = 0.996 for *S. aureus* camSOD), and has large values for FeSODs (CR > 800 for *E. coli* FeSOD).

Reciprocal mutations introduced at position 19 had negligible effects on the metal specificity of either enzyme (Table 1). Conversely, exchanging the residues at position 159 significantly impacted metal specificity. In the MnSOD, conversion of Gly159 to Leu (MnSOD Gly159Leu) increased its Fe-dependent activity ~10-fold relative to wild-type MnSOD while decreasing its manganese-dependent activity ~2-fold (Table 1). While remaining a highly manganese-specific enzyme, this variant exhibited slightly increased cambialism (CR = 0.048). The reciprocal mutation in camSOD, converting Leu159 to Gly (camSOD Leu159Gly), diminished its iron-dependent activity >10-fold relative to wild-type camSOD while increasing its manganese-dependent activity 3-fold, resulting in an enzyme variant with a MnSOD-like activity profile (CR = 0.030) (Table 1). These results indicate that the residue at position 159 has a major effect on the ability of the *S. aureus* SODs to use iron and manganese, but the contribution of this residue alone was insufficient to fully explain the disparate metal specificities of the two *S. aureus* enzymes.

Interconversion of the residue at position 160 alone also influenced metal specificity (Table 1). The MnSOD variant (Leu160Phe) was similar to the MnSOD Gly159Leu variant, exhibiting diminished manganese-dependent activity and increased iron-dependent activity (CR = 0.016) but to a lesser extent than in Gly159Leu, whereas the camSOD variant (Phe160Leu) showed slightly modified activity with both metals but remained largely cambialistic (CR = 0.032). Crucially, combining this mutation with that at position 159 further inverted the enzymes’ metal specificity. The MnSOD double mutant (Gly159Leu-Leu160Phe) showed a substantial difference in metal utilization from that of the wild type or the single-mutant variants. The iron-dependent activity of the MnSOD double mutant was increased >20-fold relative to wild-type MnSOD, resulting in a highly cambialistic enzyme that displayed activity with manganese and with iron that differed only ~2-fold (CR = 0.486). It’s notable that, like in wild-type camSOD, this highly cambialistic variant exhibited diminished activity with manganese, illustrating that gaining cofactor flexibility requires a compromise with catalytic efficiency. It further demonstrates the importance of comparing both the absolute activity and the cambialism ratio when assessing metal specificity. Conversely, the camSOD double mutant (Leu159Gly-Phe160Leu) had greater activity with manganese and reduced activity with iron relative to the wild type or the single mutant (camSOD Leu159Gly). This shift in camSOD metal preference resulted in a highly manganese-specific enzyme (CR = 0.016) (Table 1). Cumulatively, these results demonstrate that just two mutations in the secondary coordination sphere largely interconvert the metal specificity of camSOD and MnSOD.

Next, triple variant mutants, in which residues 19, 159 and 160 were all swapped, were evaluated. Unexpectedly, the MnSOD triple mutant (Phe19Ile-Gly159Leu-Leu160Phe) exhibited greater activity with manganese and less activity with iron (CR = 0.108) than the double mutant (Table 1). The camSOD triple mutant (Ile19Phe-Leu159Gly-Phe160Leu) had increased activity with both metals relative to the double mutant (camSOD Leu159Gly-Phe160Leu). These studies demonstrate that positions 159 and 160 are important in regulating the reactivity of the metal cofactor. Strikingly, mutations at these positions produced profound effects on metal specificity, yet did so with essentially no changes to the protein backbone structure, at least within the resolution of the crystal structures (Supplementary Fig. 5). The physical structures of the metals’ primary ligand spheres were the same, as were the substrate access channels and hydrogen-bonding networks that are important for proton-coupled electron transfer.

**Metal utilization correlates with the metal’s electronic properties.** Despite the similarity of the crystal structures, the mutations did have direct effects on the electronic structure and electrochemical properties of manganese centers. High-field electron paramagnetic resonance (HFEPR) spectra of the Mn (II)-loaded *S. aureus* SOD wild-type forms24 and the six variants were distinct (Fig. 3a). These spectra are determined by the D and E values of the zero-field interactions (ZFI) of the Mn(II) centers (Supplementary Fig. 6, Supplementary Eqs. 1–6, and Supplementary Note 1). Since the ZFI arises from the spin-orbit and magnetic interactions of the five unpaired Mn(II) electrons, this unambiguously demonstrated that the electronic structures of the metal centers were different. The sum, |D|+E, which measures the size of the Mn(II) ZFI, has been shown to correlate semiquantitatively with manganese-dependent activity, with cambialistic SODs exhibiting intermediate values between the low values of Mn(II)-loaded FeSODs and the high values of MnSODs. As reported previously, the wild-type *S. aureus* proteins were consistent with this trend24, but the current measurements shed greater light. The values of |D|+E and the manganese-dependent enzymatic activities of seven of the enzyme forms (Supplementary Table 6) exhibited a significant linear correlation (Supplementary Fig. 7). The furthest departure from this correlation was camSOD Leu159Gly-Phe160Leu. This exception notwithstanding, the significance of this correlation can be interpreted from the results of studies on Mn(II/III)- and Fe(II/III)-containing, 4′-substituted 2,2′:6′:2′″-terpyridine complexes. These compounds are structurally homologous and exhibit metal-specific or cambialistic SOD-like activity depending on the electron-donating/withdrawing strength of the 4′ substituent, quantified by its Hammett σ values27. The reduction potentials of both the manganese and iron complexes exhibit a linear dependence on σ, as do the ZFI of the Mn(II) complexes26. Hence, the relationship between metal-specific activity, redox tuning through indirect secondary coordination sphere electronic effects, and Mn(II) ZFI in the enzymes is entirely consistent with the effects of subtle charge polarization in the simpler, better understood chemical systems.

Further evidence that the secondary coordination sphere residues influence the redox properties of the manganese cofactor comes from observations of their auto-oxidation properties. Mn (III) weakly absorbs in the 400–600 nm region25,28,29. The spectra of the two wild-type SODs in their resting state (equilibrated with ambient oxygen) had similar shapes, but the MnSOD peak intensity was greater (Fig. 3b). This demonstrated the MnSOD was more susceptible to auto-oxidation under these conditions than the camSOD, whereas the converse was true for the double mutants (Fig. 3b). These trends in auto-oxidation correlated with manganese-dependent enzyme activity (Table 1). One half reaction catalyzed by SODs is the oxidation of superoxide to molecular oxygen, and auto-oxidation is formally the reverse of this reaction (Supplementary Eq. 7). If auto-oxidation proceeds by the reverse mechanism (Supplementary Eqs. 8–12), then any changes in metal reduction potential will affect this reaction in
both directions (Supplementary Note 2). There are other factors that could differentially affect the two reactions, namely substrate accessibility and proton availability. However, within the limits of the resolution of the crystal structures, we found no evidence of changes in structure that were likely to affect superoxide/dioxygen motion to/from the metal ion or proton availability, with the positions of the residues around the active site remaining unchanged (Supplementary Fig. 5). While our structural analysis suggested these alternative possibilities were unlikely, we also measured the susceptibility of the oxidized form of the manganese-loaded SODs to reduction by absorption spectroscopy. Each enzyme was oxidized with permanganate, and then titrated with increasing concentrations of dithionite while monitoring the disappearance of the oxidized Mn(III) species using UV/visible detection (Supplementary Fig. 8). The camSOD was fully reduced at lower concentrations of dithionite than the MnSOD, consistent with the proposed difference in reduction potentials between the wild-type isozymes (Supplementary Eqs. 13–17). Importantly, this trend was also reversed in the double mutant variants, with MnSOD G159L-L160F becoming reduced at lower concentrations of dithionite than camSOD L159G-F160L. The SOD metal site is only accessible via a narrow solvent channel, making small redox-active molecules inefficient electrochemical mediators for SODs, thus precluding direct reduction potential measurements.

Altogether, the spectroscopic data indicate that the two wild-type S. aureus SODs and the two double mutants differ in their manganese redox properties. The HFEPR spectra demonstrated that the electronic structure of their Mn(II) centers were predominantly manganese-dependent SODs 14,31 and the HFEPR and redox properties caused by the mutations. The double mutants gave rise to detectable differences in the enzymes’ HFEPR spectra and their ZFS (Supplementary Figs. 6–7 and Supplementary Table 6), demonstrating altered electronic structure and redox properties caused by the mutations. The camSOD exhibits greater auto-oxidation at rest than camSOD, illustrated by increased absorbance of Mn(III) at ~480 nm, a trend which is reversed in the double mutant variants that also exhibited reciprocal changes in metal speciation (Table 1). All samples (800 µM) were equilibrated with ambient aerobic conditions in 100 mM phosphate buffer, pH 7.5, 100 mM KCl, 1 mM EDTA before spectra were acquired. Spectra were collected on independent biological replicates (n = 2), mathematically converted to extinction coefficient (ε) using the Beer-Lambert law, and a representative spectrum is shown for each variant.

S. aureus camSOD likely arose after a duplication of MnSOD. A challenge in studying metalloenzyme neo-functionalization is a lack of knowledge regarding the properties of the predecessor from which it evolved. To evaluate the evolutionary relationship between the S. aureus SODs, we performed a maximum likelihood phylogenetic analysis using 2691 aligned Mn/Fe SOD sequences from bacteria, archaea and eukaryotes (Supplementary Data 1). The overall topology of the resulting phylogenetic tree was consistent with that derived from a previous analysis of a more limited sequence library19 (Fig. 4a). Combining amino acid correlation analysis (Supplementary Data 2) with the phylogenetics (Supplementary Fig. 9) showed that the analyzed SOD sequences could be grouped into two distinct subtypes based on the presence of highly conserved GGH or AAQ motifs, as well as a third, diverse group lacking either motif (Fig. 4a, b and Supplementary Fig. 10). We annotated known SOD metal specificities onto the tree, including only examples where activity with both manganese and iron cofactors were definitively tested, which demonstrated that the GGH subgroup contains predominantly manganese-dependent SODs 14,31 and the AAQ subgroup contains primarily iron-dependent SODs 23,25.
Nonetheless, confirmed cambialistic SODs were present in both subgroups and a FeSOD-containing enzyme has been shown to be an FeSOD and a GGH-containing enzyme has been shown to be an FeSOD (Fig. 4a, b), implying that the three metal specificities can and do interchange evolutionarily.

Inspection of the staphylococcal SOD protein similarity network and the tree (Supplementary Fig. 11) demonstrated that MnSOD is present in all staphylococcal genomes, whereas camSOD was identified only in the genomes of \textit{S. aureus}, and of \textit{S. argenteus} and \textit{S. schwitzi}, both recognized as early-branching lineages of the \textit{S. aureus} tree. As MnSOD is the closest homolog to camSOD, the most parsimonious explanations are that the sodM gene that encodes camSOD arose from a duplication of sodA, encoding MnSOD, or that sodM was acquired by horizontal gene transfer from a close relative. Regardless, either model predicts that the newly acquired sodM would have originally encoded a manganese-specific enzyme. Increased branch length indicates an increased rate of evolutionary change for the ancestral SodM, while SodA remained under purifying selection (Fig. 4c). The camSOD sequence is highly conserved across all \textit{S. aureus} (99% identity), and this homology extends to the second SOD found in \textit{S. argenteus} (97.5%) and \textit{S. schwitzi} (99%) (Supplementary Fig. 2). Notably, Leu159 and Phe160 in camSOD are strictly conserved between \textit{S. aureus}, \textit{S. argenteus}, and \textit{S. schweitzeri}, which are targeted for mutagenesis shown. Groups 1 and 2 of coevolving residues, identified using amino acid correlation analysis, were mapped onto the alignment, illustrating the GGH or AAQ motifs and associated correlated amino acids. The correlated residues, biological origin and metal specificity are indicated using the color scheme described in a, and sequence numbering is based on the \textit{S. aureus} SODs. c Sub-tree extracted from the SOD tree in panel a containing all identified staphylococcal SOD sequences. Support values correspond to maximum likelihood bootstrap values from 100 rapid bootstrap replicates, with values >90 shown in green. The scale bar indicates number of substitutions per site. Macrococcus and the oxidase-positive staphylococci form an out-group to a strongly supported grouping of oxidase-negative staphylococci, including \textit{S. aureus}, consistent with published Staphylococcaceae phylogenies. Within the oxidase-negative staphylococci, all MnSOD (yellow) grouped together while camSOD homologs (orange) formed an out-group to the MnSODs.

**Fig. 4 S. aureus camSOD evolved from a manganese-specific predecessor.** a Amino acid correlation (defined in the text as the AAQ group, blue, and the GGH group, magenta) and unrooted maximum likelihood phylogenetic tree based on alignment of amino acid (a.a.) sequences of 2691 Mn/Fe SOD homologs from bacteria (green circles), eukaryotes (sky blue circles), and archaea (red circles). Known metal-specificities of characterized enzymes (MnSOD = yellow, FeSOD = brown; camSOD = orange) are annotated with triangles. b Fragment of the alignment of selected SOD sequences of known metal specificity, with the metal-binding residues (red) and three residues in \textit{S. aureus} camSOD (yellow) that were targeted for mutagenesis shown. Groups 1 and 2 of coevolving residues, identified using amino acid correlation analysis, were mapped onto the alignment, illustrating the GGH or AAQ motifs and associated correlated amino acids. The correlated residues, biological origin and metal specificity are indicated using the color scheme described in a, and sequence numbering is based on the \textit{S. aureus} SODs. c Sub-tree extracted from the SOD tree in panel a containing all identified staphylococcal SOD sequences. Support values correspond to maximum likelihood bootstrap values from 100 rapid bootstrap replicates, with values >90 shown in green. The scale bar indicates number of substitutions per site. Macrococcus and the oxidase-positive staphylococci form an out-group to a strongly supported grouping of oxidase-negative staphylococci, including \textit{S. aureus}, consistent with published Staphylococcaceae phylogenies. Within the oxidase-negative staphylococci, all MnSOD (yellow) grouped together while camSOD homologs (orange) formed an out-group to the MnSODs.

**Acquisition of camSOD coincided with virulence factor expansion.** To understand the wider context of camSOD emergence within the \textit{S. aureus} lineage, we performed whole genome comparisons and a protein similarity network analysis (Supplementary Data 3) of genomes sampled across the staphylococcal phylogenetic tree (Supplementary Data 4). The sodA gene encoding MnSOD was located within a genomic region rich in essential genes and that sodM was acquired by horizontal gene transfer from a close relative. Regardless, either model predicts that the newly acquired sodM would have originally encoded a manganese-specific enzyme. Increased branch length indicates an increased rate of evolutionary change for the ancestral SodM, while SodA remained under purifying selection (Fig. 4c). The camSOD sequence is highly conserved across all \textit{S. aureus} (99% identity), and this homology extends to the second SOD found in \textit{S. argenteus} (97.5%) and \textit{S. schwitzi} (99%) (Supplementary Fig. 2). Notably, Leu159 and Phe160 in camSOD are strictly conserved between \textit{S. aureus}, \textit{S. schwitzi}, and \textit{S. argenteus}. Thus, we conclude that the acquired sodM gene originally encoded a MnSOD, which subsequently evolved into the modern camSOD during the emergence of \textit{S. aureus}.
these factors having been primarily acquired via horizontal transfer of mobile elements. We screened the protein similarity networks to identify proteins with distributions similar to that of camSOD (Supplementary Data 3); proteins present in all S. aureus/S. argenteus but absent in other staphylococci, likely to have been present in their last common ancestor. We observed that the genomes that encoded camSOD possessed an expanded set of proteins involved in microbial competition, host interaction, immune evasion and virulence (Fig. 5c and Supplementary Data 3 and 5). Some of these were unique to S. aureus/S. argenteus, whereas others were present in other staphylococci but were enriched within this clade, and included proteins involved in capsule biosynthesis (Cap), immunomodulation (Fir, Erf, Emp), secreted effectors (Ess) and toxins (Luk). A component of the Isd heme-uptake system (IsdD) was also unique to this clade (Fig. 5c). Indeed, we observed that S. aureus genomes are enriched in components that function in iron uptake via heme or siderophores (Fig. 5c, Supplementary Fig. 12, and Supplementary Data 6). We conclude that the evolution of camSOD in the last common ancestor of S. aureus/S. argenteus coincided with the expansion of their repertoire of virulence genes, and that its genomic context is consistent with its established role in pathogenicity.

A single mutation alters resistance to host-derived stresses. The camSOD is a virulence factor that enables S. aureus to resist oxidative stress during infection when starved of manganese by the host. Our analysis suggested that camSOD likely evolved from a manganese-specific predecessor, implying that evolution subsequently adapted the manganese-specific precursor, resulting in the extant cambiastial enzyme. We thus aimed to leverage our ability to produce camSOD enzymes with altered metal specificity to test whether the camSOD’s iron-dependent catalysis directly contributes to S. aureus overcoming the host immune response, and if small changes in iron-dependent activity are sufficient to provide a functional advantage in the presence of natural stressors.

A single mutation was introduced into the S. aureus chromosome, converting the wild-type camSOD to its Leu159Gly variant, which shows reduced ability to utilize iron (Table 1). This strain was evaluated, alongside wild-type S. aureus and a ΔsodM mutant that lacks camSOD entirely, for its ability to resist conditions representative of dual host-imposed stresses, manganese starvation and oxidative stress. Manganese starvation was imposed using the manganese-binding immune effector calprotectin, which can accumulate at sites of infection at concentrations of 1 mg ml⁻¹, while superoxide was generated by addition of paraquat. Consistent with previous studies, the strain lacking camSOD was more sensitive to these stresses than wild type (Fig. 6a). The strain carrying the Leu159Gly variant camSOD enzyme was also more sensitive to these stresses (Fig. 6a), and its SOD activity showed altered susceptibility to inhibition by peroxide (Fig. 6b, c), consistent with altered metal loading. These results demonstrate that the
The diminished ability of the camSOD variant to use iron replicate. Black bars represent the mean, with error bars showing standard error analysis of independent biological replicates (labeled as SodA, camSOD as SodM). This is illustrated by the in-gel SOD activity assays using extracts of S. aureus (left) or recombinant enzymes (right) with H2O2 treatment of the extracts (left) or recombinant enzymes (right) with H2O2. The MnSOD camSOD was loaded with iron in wild-type cells. The MnSOD enzyme is compared to wild-type bacteria grown in the same concentration of calprotectin. Multiple independent clones of the Leu159Gly variant (lower panels) of calprotectin (CP) show reduced activity of wild-type SodA and SodM. The MnSOD camSOD shows greatly diminished growth in the presence of paraquat (PQ) relative to wild type (WT-blue circles) under manganese-depleted conditions imposed by the presence of the human protein complex calprotectin (CP)8. S. aureus cells expressing the Leu159Gly variant of camSOD from the native sodM locus (green triangles) also show reduced growth relative to the wild type. Note that the asterisk represents p < 0.05 via two-way ANOVA with Tukey’s post-test performed in Graphpad Prism when compared to wild-type bacteria grown in the same concentration of calprotectin. Multiple independent clones of the Leu159Gly variant (n = 8) were assayed at the same time as those of the wild type (n = 3) and the ΔsodM mutant strain (n = 3). Each data point represents an independent biological replicate. Black bars represent the mean, with error bars showing standard error of the mean (SEM). The diminished ability of the camSOD variant to use iron (Table 1) renders S. aureus less capable of overcoming the host immune response. b, c In-gel SOD activity assays using extracts (5.4 μg/ml) were prepared from wild type S. aureus and c the strain expressing the Leu159Gly camSOD variant, cultured under identical conditions to those used in a. Demonstrate that both forms of the SodM enzyme are detectable at similar levels (upper panels). Treatment of the extracts (left) or recombinant enzymes (right) with H2O2 (lower panels), a specific inhibitor of the iron-loaded form9, demonstrated that the camSOD was loaded with iron in wild-type cells. The MnSOD enzyme is labeled as SodA, camSOD as SodM. This is a representative gel from a triplicate analysis of independent biological replicates (n = 3). Note that molecular weight markers were not used in these native gels, with SOD bands identified in the cell extracts by comparison of their mobility with the purified recombinant proteins. Source data for all panels are provided as a source data file.

Fig. 6 Iron-dependent camSOD activity enables S. aureus to resist host stresses. a The ΔsodM mutant strain (lacking the gene encoding camSOD-orange squares) of S. aureus shows greatly diminished growth in the presence of paraquat (PQ) relative to wild type (WT-blue circles) under manganese-depleted conditions imposed by the presence of the human protein complex calprotectin (CP)8. S. aureus cells expressing the Leu159Gly variant of camSOD from the native sodM locus (green triangles) also show reduced growth relative to the wild type. Note that the asterisk represents p < 0.05 via two-way ANOVA with Tukey’s post-test performed in Graphpad Prism when compared to wild-type bacteria grown in the same concentration of calprotectin. Multiple independent clones of the Leu159Gly variant (n = 8) were assayed at the same time as those of the wild type (n = 3) and the ΔsodM mutant strain (n = 3). Each data point represents an independent biological replicate. Black bars represent the mean, with error bars showing standard error of the mean (SEM). The diminished ability of the camSOD variant to use iron (Table 1) renders S. aureus less capable of overcoming the host immune response. b, c In-gel SOD activity assays using extracts (5.4 μg/ml) were prepared from wild type S. aureus and c the strain expressing the Leu159Gly camSOD variant, cultured under identical conditions to those used in a. Demonstrate that both forms of the SodM enzyme are detectable at similar levels (upper panels). Treatment of the extracts (left) or recombinant enzymes (right) with H2O2 (lower panels), a specific inhibitor of the iron-loaded form9, demonstrated that the camSOD was loaded with iron in wild-type cells. The MnSOD enzyme is labeled as SodA, camSOD as SodM. This is a representative gel from a triplicate analysis of independent biological replicates (n = 3). Note that molecular weight markers were not used in these native gels, with SOD bands identified in the cell extracts by comparison of their mobility with the purified recombinant proteins. Source data for all panels are provided as a source data file.
and cambialistic SODs, but Thr in iron-dependent SODs\textsuperscript{12,29,50}. Introduction of Thr in this position in manganese-specific or cambialistic SODs increased the cambialism ratio\textsuperscript{12,29}. This was previously explained by modification of a hydrogen-bonding network involving the mechanistically important metal-coordinated solvent\textsuperscript{29}. Our data confirm that a Gly residue in this position strongly favors manganese-specificity, but show that a small aliphatic sidechain at this position confers cambialism when combined with an aromatic sidechain on the neighboring residue at position 160. Importantly, our structural analyses of the staphylococcal SODs and their variants did not identify changes to the hydrogen-bonding network or substrate access channel, and \textsuperscript{1}H electron nuclear double resonance (ENDOR) spectroscopy demonstrated the positions of the solvent protons are unchanged between MnSOD and camSOD\textsuperscript{24}, and likely also in the mutants. These observations cast doubt on the hydrogen-bonding network explanation in our system, and highlight the potential of the staphylococcal SODs as a model system with which to uncover the mechanisms by which redox tuning is controlled.

Our data suggest that camSOD evolved from a MnSOD predecessor in the last common ancestor of \textit{S. aureus}/\textit{S. argenteus}/\textit{S. schweitzeri}. The acquisition and subsequent altered metal utilization of camSOD coincided with the acquisition of numerous other factors that contribute to infection\textsuperscript{8,39,41,42,51}. We exploited our ability to produce manganese-specific camSOD variants to determine whether its iron-dependent catalytic activity provides a direct benefit to \textit{S. aureus} during exposure to stresses encountered within the host. A \textit{S. aureus} strain expressing the Leu\textsuperscript{159}Gly variant camSOD showed reduced growth under manganese-restricted and oxidative-stress-exposed conditions relative to wild type. As this variant possesses reduced iron-dependent activity but increased catalysis with manganese, this result clearly demonstrates that \textit{S. aureus} growth under these conditions requires iron-dependent camSOD activity. We previously showed that camSOD is more important than MnSOD to the establishment of infection of wild-type mice\textsuperscript{8}. Collectively, these data support a model in which the manganese-specific camSOD antecedent, acquired by the last common ancestor of the extant \textit{S. aureus} clade\textsuperscript{41}, underwent neofunctionalization through selection for increased iron-dependent activity, a pressure likely imposed by the manganese starvation it experiences during infection.

This study presents experimental evidence of how a change in metal specificity has naturally evolved in a metalloenzyme. We provide evidence that the specificity switch was selected for by

**Fig. 7 Evolution of camSOD was driven by altered metal availability in the host.** Schematic model depicting the evolution of \textit{S. aureus} camSOD (orange) under selection pressure caused by exposure of the \textit{S. aureus} ancestor to host-imposed manganese-starvation mediated by calprotectin. Acquisition and evolution of camSOD enabled the ancestor of \textit{S. aureus} to maintain its antioxidant defense under these conditions using its iron-dependent catalysis, and coincided with its acquisition of numerous genes (green) involved in infection. Collectively, these acquired systems resulted in increased survival of \textit{S. aureus} during interaction with host immune mechanisms, creating pathogenic \textit{S. aureus}.
nutrient-starvation induced by the immune system, implying that metal availability can shape enzyme metal utilization6,8,25. Our data also demonstrate a critical role for secondary coordination sphere residues in mediating this specificity switch, with wider implications for the study of natural and synthetic metalloenzymes.

Methods

Bacterial strains, culture conditions, and plasmids. Escherichia coli strains DE5a and BL21(DE3) were used for cloning and for recombinant protein expression, respectively, cultured at 37 °C in lysogeny broth (LB) or M9 medium supplemented with 200 µM FeSO₄ with 1.5% (w/v) agar for solid medium. S. aureus strains were cultured at 37 °C in tryptic soy broth (TSB), on tryptic soy agar plates (TSA), or in Tris minimal medium13. Where needed, tetracycline (10 µg ml⁻¹), erythromycin (10 µg ml⁻¹), kanamycin (50 µg ml⁻¹), ampicillin (100 µg ml⁻¹), or chloramphenicol (10 µg ml⁻¹) were added to the media. All strains were stored at −80 °C in media mixed with 25% (w/v) glycerol. The construction of the S. aureus SH1000 Δsoda (soda::Tn917-LITV), ΔsodM (sodM::Tn917-DI), ΔsodAΔsodM and Newman ΔsodA (soda::tet) and ΔsodM (sodM::erm) mutant strains have been previously described8,18. For pull-down analysis, the C-terminally StreptI-tagged variant of camSOD (including its Shine-Dalgarno sequence) was synthesized (IDT) and sub-cloned into the pSt10 shuttle vector44 using BamHI/PstI restriction cloning, and the resulting pSt10-Strep-sodM construct was introduced into S. aureus SH1000 using methods described previously24. Firstly, electrosusceptible S. aureus RN4220 cells were transformed with pSt10-Strep-sodM. Positive transformants, selected on TSA-agar plates supplemented with chloramphenicol, were used to generate p11 phage lysate. Finally, phage transduction was used to introduce pSt10-Strep-sodM into the S. aureus SH1000 background. The chromosomally integrated L159G mutation was introduced in S. aureus Newman via homologous recombination using the integration vectors55, pKOR1 and standard molecular approaches. This mutant was confirmed to be hemolytic by plating on blood agar prior to use.

Expression and purification of recombinant SODs. The cloning of the S. aureus soda and sodM, their expression in E. coli BL21(DE3) cells, the purification of the recombinant enzymes, the in vitro swapping of bound iron for manganese by unfolding/re-folding, and their quantitation, were all as previously described. Expression constructs of S. aureus soda and sodM were prepared by NdeI/BamHI restriction cloning into PET29a vector. Protein was expressed in E. coli BL21 cells, in M9 minimal medium supplemented with 200 µM ammonium iron sulfate or in standard LB broth, for 4 h post induction with 1 mM IPTG. Cell extracts were prepared by sonication of washed cell pellets in 20 mM Tris pH 7.5, 150 mM NaCl, 1x Complete EDTA-free protease inhibitor (Roche), followed by centrifugation at 19,000 × g. Cleared cell lysate was subjected to chromatographic separation using an AKTA purification system (GE Healthcare). Recombinant SOD proteins were purified using anion exchange chromatography (Hi Trap Q HP column, GE Healthcare) with a pH 7.5 buffer with 1 M NaCl gradient elution, and subsequently by size exclusion in 20 mM Tris pH 7.5, 150 mM NaCl buffer on a Superdex 200 (GE Healthcare) column. Metal cofactor exchange was achieved by unfolding SOD proteins in buffer containing 2.5 M guanidine hydrochloride in the presence of 5 mM EDTA and 20 mM 8-hydroxyquinoline to remove bound metal ions, followed by dialysis against 20 mM Tris pH 7.5, 100 mM NaCl, 200 mM MgCl₂, 1 mM EDTA, and 1–10 mM metal molar of interest. The constructs for recombinant expression variants of MnSOD and camSOD were mutagenized using the QuickChange site-directed mutagenesis approach (Stratagene), using the pET29a-soda and pET29a-sodM vectors55 and the primer pairs listed in Supplementary Table 7.

CD spectroscopy. Circular dichroism data were collected using a JASCO J-810 spectropolarimeter equipped with a PTC-4235 Peltier temperature controller. Circular dichroism data were collected using a JASCO J-810 spectropolarimeter equipped with a PTC-4235 Peltier temperature controller. Spectra were obtained by measuring the integrated amplitude of a standard two-pulse Hahn echo (τ/2 = 24 ns and interpulse time of 400 ns) as a function of the magnetic-field. The zero-field D and E values were determined using a modified version of the method described in our previous work24, because the shapes of the low-field edges of the 94 GHz spectra were different (Supplementary Note 1). To remove any arbitrariness in locating the magnetic-field of the zero-field edge, the spectra were measured under different conditions. The lowest-field inflection points were taken as the edge positions. This had the effect of taking into account the D values, which produced the different edge shapes, but also overestimated their values by 100 to 200 MHz in comparison to the previously reported values. The same approach was used for the high-field edges (E) as well.

X-ray crystallography. Preparations of the iron-loaded forms of recombinant MnSOD and camSOD, and the manganese-loaded forms of both double mutant variants and of the triple mutant of camSOD (15C18A25D) were each subjected to crystallization screening and optimization as previously described24. Each protein preparation was confirmed to contain exclusively the target metal ion by ICP-MS prior to crystallization. Screening trays were set up using a Mosquito liquid handling robot (TTP Labtech) with commercially available screens: PACT, JCSG+ Structure (Molecular Dimensions) and Index (Hampton Research) in 96-well MRC crystallization plates (Molecular Dimensions) using the sitting drop of vapor-diffusion method, incubated at 20 °C. Optimization of initial conditions for salt, polyethylene glycol (PEG) precipitant concentration and pH was performed using the hanging drop of vapor-diffusion method, incubated at 20 °C, in 24-well Lambda, Molecular Dimensions, iron-loaded wild-type MnSOD in 200 mM MgCl₂, 100 mM Tris, pH 8.5 and 30% (w/v) PEG 4000, whereas iron-loaded camSOD was crystallized in 100 mM PCTP, pH 7.0 and 25% (w/v) PEG 1500. The manganese-loaded forms of the double mutant variant of MnSOD (Gly159Leu-Phe160Leu) was crystallized in 200 mM MgCl₂, 150 mM NaCl, pH 5.5 and 25% (w/v) PEG 3350, whereas the manganese-loaded form of the double mutant variant of camSOD (Leu159Gly-Phe160Leu) was crystallized in 30 mM MgCl₂, 30 mM CaCl₂, 39.1 mM NaCl, 60.9 mM Trizma, pH 8.5, 20% (w/v) PEG 550 MME and 10% (w/v) PEG 20,000. The manganese-loaded form of the triple mutant variant of camSOD (Ile199Phe-Leu159Gly-Phe160Leu) was crystallized in 100 mM potassium thiocyanate and 30% (w/v) PEG 2000 MME. All but the double mutant variant of camSOD were cryo-protected with 20% (w/v) PEG 400. X-ray diffraction data collection (Diamond Light Source, Didcot, UK) and processing, and model building and validation were all as previously described24, except that data processing also used xia2 and phasing also used Phaser58. The search model for iron-loaded wild-type MnSOD and manganese-loaded MnSOD Gly159Leu-Leu160Phe was PDB model 5N5E. The search model for iron-loaded wild-type camSOD and the manganese-loaded forms of camSOD Leu159Gly-Phe160Leu and SodM Ile199Phe-Leu159Gly-Phe160Leu was PDB model 5N57. The PDB codes for the structures of the iron-loaded forms of MnSOD and camSOD and the manganese-loaded forms of the camSOD triple mutant variants are 6EX3, 6EX4, and 6EX5, respectively. The data collection and refinement statistics are summarized in Supplementary Table 3. All crystallographic images were generated using Pymol Molecular Graphics System, Version 1.8 (Schrödinger, LLC). To illustrate structural regions where the sequence is conserved, ConSurf was used26 to analyze 500 HMMER homologs of the SOD family, sampled from the Uniref90 database. Conservation scores were calculated using the Bayesian method.

Pull-down assay. The StreptI-tagged camSOD was constitutively expressed in a S. aureus SH1000 ΔsodM strain18 and purified using affinity chromatography. Cell
extracts were prepared by freeze-grinding under liquid nitrogen in 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA. Thawed, ground material was centrifuged (4000 g for 20 min, 4˚C) to remove cell debris, and the resulting supernatant was filtered through a 0.45 μm membrane filter. The soluble protein extract was applied to a StreptTrap column (GE Healthcare), equilibrated and subsequently washed in 20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA. Elution (1 ml) used 2.5 mM chloride (Sigma), 50 mM potassium phosphate buffer pH 7.8, 1 mM EDTA, 0.25 mM nitroblue tetrazolium (w/v) native polyacrylamide gels. For staining, gels were incubated in 50 mM 6. The amino acid correlation network was visualized in Cytoscape71 (Supplementary Data 2) in the alignment of the 2,691 SOD sequences identified, 0.1 mM PQ was added to the media.

CIRCULAR GENOME COMPARISON. Circular genome comparison was generated with BRIG82, using S. aureus NCTC3825 genome83 as a reference, which was compared to 29 analyzed Staphylococcus genomes using BLASTN84 (Fig. 5). S. aureus NCTC3825 virulence factors (PATRIC85, and VIDO86 databases), and essential genes (DRAI87, and AuroVid88 databases) annotation (Supplementary Data 5) were downloaded from the respective databases on 29th March, 2019.

Protein network analyses of metal acquisition systems. Networks containing homologs of proteins known to be involved in manganese and iron import were isolated using iGraph89 and dnet90 packages in R. Protein networks not containing protein homologs from any of the camSOD-positive species, and none from the camSOD-negative species. To be defined in the group labeled as Enriched in S. aureus, each camSOD-positive species had to contain more protein homologs than any of the camSOD-negative species within the network. Heatmaps of the identified protein networks (Fig. 5 and Supplementary Fig. 12) were generated using gplots package (https://CRAN.R-project.org/package=gplots) within R.

Circular genome comparison. Circular genome comparison was generated with BRIG82, using S. aureus NCTC3825 genome83 as a reference, which was compared to 29 analyzed Staphylococcus genomes using BLASTN (Fig. 5). S. aureus NCTC3825 virulence factors (PATRIC85, and VIDO86 databases), and essential genes (DRAI87, and AuroVid88 databases) annotation (Supplementary Data 5) were downloaded from the respective databases on 29th March, 2019.

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Amino acid correlation analysis. Groups of coevolving amino acids were identified (Supplementary Data 2) in the alignment of the 2,691 SOD sequences sampled across the tree of life (Supplementary Data 1), using amino acid correlation analyses. This was implemented in Pfstat76 following the user guide. Pfstat correlations were trimmed with trimAl66 and used to generate phylogenies under LG + G model in FastTree86. Homologs of the proteins of interest were identified based on the network and tree topologies, together with manual inspection of protein sequence alignments, and used to generate the heatmap with gplots package.

Other bioinformatic methods. Multiple sequence alignments were visualized and annotated using Jalview87. Phylogenetic trees were visualized and annotated using FigTree (http://tree.bio.ed.ac.uk/software/figtree/), Archaeopteryx88, and BRIG82. Linear genome fragments were aligned and visualized using EasyFig89.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Structural data that support the findings of this study have been deposited in the Protein Data Bank with the accession codes PDB 6XX1, PDB 6XX2, PDB 6XX3, PDB 6XX4, and PDB 6XX5 (see Supplementary Table 2). Source data for Table 1, Fig. 6 and Supplementary Fig. 1–d are provided with the paper. There are no restrictions on any data within the manuscript. Biological materials will be provided on request.
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**Author contributions**

A.B.-S. designed and performed most in vitro experiments, with further contributions from E.S.M., E.T., and P.C. Y.M.G. performed *S. aureus* experiments. A.B. assisted A.B.-S. with X-ray crystallography, and K.M.S. performed all bioinformatic analyses with assistance from C.B. for network analysis. S.U. performed HFPEP experiments and analyzed these data, with input from L.C.T., who also prepared the auto-oxidation calculations. K.J.W. and T.E.K.-F. conceived and managed the study, and wrote the manuscript with S.U., with input from all authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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