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An oxidation-sensing mechanism is used by the global regulator MgrA in *Staphylococcus aureus*

Peng R Chen[1], Taeok Bae[2,4], Wade A Williams[2], Erica M Duguid[1], Phoebe A Rice[3], Olaf Schneewind[2] & Chuan He[1]

Staphylococcus aureus **is a human pathogen responsible for most wound and hospital-acquired infections[1,2]. The protein MgrA is both an important virulence determinant during infection and a regulator of antibiotic resistance in *S. aureus*[3–7]. The crystal structure of the MgrA homodimer, solved at 2.86 Å, indicates the presence of a unique cysteine residue located at the interface of the protein dimer. We discovered that this cysteine residue can be oxidized by various reactive oxygen species, such as hydrogen peroxide and organic hydroperoxide. Cysteine oxidation leads to dissociation of MgrA from DNA and initiation of signaling pathways that turn on antibiotic resistance in *S. aureus*. The oxidation-sensing mechanism is typically used by bacteria to counter challenges of reactive oxygen and nitrogen species[8–12]. Our study reveals that in *S. aureus*, MgrA adopts a similar mechanism but uses it to globally regulate different defensive pathways.**

S. aureus, a Gram-positive bacterium, is the leading cause of a variety of human infections ranging from minor skin infection to life-threatening endocarditis, pneumonia and septicemia[1,2]. MgrA, a homolog of the MarR family of multiple-antibiotic-resistance proteins[13,14], is a key global regulator in *S. aureus*. A recent study indicates that MgrA controls expression of ~350 genes[15] including those that encode a wide variety of virulence factors (for example, capsular polysaccharide, nuclease, α-toxin, coagulase, protease and protein A), genes involved in autolysis (for example, *lytM* and *lytN*) and other global regulatory genes (for example, *agr*, *lytRS*, *arlRS*, *sarS* and *sarV*)[3,5,16]. MgrA also negatively regulates the expression of efflux pumps such as NorA, NorB and Tet38 (refs. 6,7), which confer resistance to quinolone-type antibiotics such as ciprofloxacin (**1**) and norfloxacin (**2**). A link between MgrA and glycopeptide resistance has been suggested on the basis of transcription profile studies[15,17]. How does MgrA, a protein with 147 amino acids, have such diverse regulatory roles? What is the activation mechanism of this protein? The present study addresses these questions.

We first confirmed that MgrA is indeed responsible for resistance to fluoroquinolones and vancomycin (**3**) in *S. aureus* using the *S. aureus* Newman strain and an isogenic mutant strain with a transposon insertion in *mgrA* (ref. 18). The minimum inhibition concentration (MIC) measured for the *mgrA* mutant strain is two-fold higher than that of the wild-type Newman strain for fluoroquinolones (ciprofloxacin and norfloxacin) and vancomycin (**Table 1**). This result agrees with previous findings that MgrA serves as a repressor for an efflux pump involved in the export of fluoroquinolones[6,7], and it confirms the hypothesis that MgrA also regulates vancomycin resistance in *S. aureus*[15].

Next, we investigated whether MgrA contributes to the pathogenesis of *S. aureus* infections using a murine abscess model. We intravenously injected bacteria (10^6 colony-forming units (CFU)) into mice (ten mice per strain) and killed the mice 4 d after infection. We then removed the kidneys and liver and measured CFU of *S. aureus* in each organ by colony formation on agar plates[18]. The *mgrA* mutant strain showed a 10,000-fold reduction of CFU in kidneys and a 100-fold reduction in livers compared to the isogenic parent, *S. aureus* strain Newman (**Fig. 1**). This indicates that MgrA is a key virulence determinant in *S. aureus*.

Hoping to reveal the regulatory mechanism of MgrA, we solved the crystal structure of a truncated version of the protein (with deletion of the second through fifth residues and the last four residues of the protein) at 2.86 Å by multiwavelength anomalous dispersion (**Supplementary Figs. 1** and **2** and **Supplementary Table 1** online). The overall structure of MgrA resembles those of *Escherichia coli* MarR and its homologs[19–21]. It consists of a dimer related by a crystallographic two-fold rotation (**Fig. 2a**). The dimer is rich in α helices and is triangular in shape with two winged-helix DNA binding

Table 1 Susceptibilities of strains to fluoroquinolones and vancomycin

	MIC (μg ml^{-1})		
Strain	CIP (**1**)	NOR (**2**)	VCM (**3**)
Wild type (Newman)	0.35	0.7	1.2
mgrA mutant (ΦNΞ 3040)	0.7	1.4	2.4

CIP, ciprofloxacin; NOR, norfloxacin; VCM, vancomycin.

[1]Department of Chemistry, The University of Chicago, 5735 South Ellis Avenue, Chicago, Illinois 60637, USA. [2]Department of Microbiology, The University of Chicago, 920 East 58th Street, Chicago, Illinois 60637, USA. [3]Department of Biochemistry and Molecular Biology, The University of Chicago, 929 East 57th Street, Chicago, Illinois 60637, USA. [4]Present address: Indiana University School of Medicine Northwest, 3400 Broadway, Gary, Indiana 46408, USA. Correspondence should be addressed to C.H. (chuanhe@uchicago.edu).

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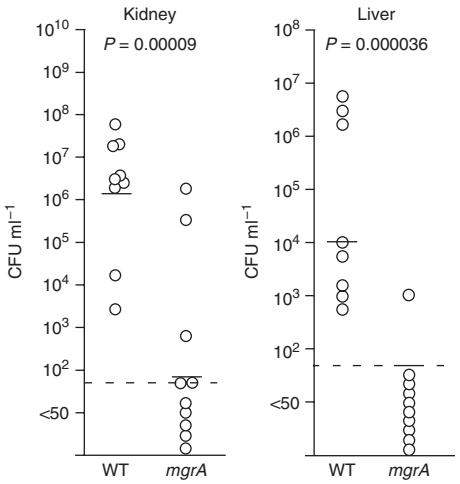


Figure 1 Effect of the *mgrA* mutation on the virulence of *S. aureus* as tested using the murine abscess model of infection. *S. aureus* strain Newman (wild type, WT) and *mgrA* mutant strain were used to infect ten mice each via retro-orbital injection. After 4 d, mice were killed and organs (kidneys and liver) were removed. Homogenized tissues were incubated on agar medium for *S. aureus* colony formation and enumeration. Each circle stands for one animal experiment. The horizontal bars indicate the mean (the CFU number was converted to log[CFU] and the arithmetic mean was obtained), and the dashed line represents the limit of detection, which is 50 CFU ml^{-1} in this case, as 20 μl of homogenates (1 ml total) were used for colony enumeration.

domains (β1-α3-α4-β2-W1-β3, **Fig. 2a**). This DNA binding domain is connected to the dimerization domain through helices α1, α5 and α6. The dimerization domain consists of helices α1, α6 and α7 with hydrophobic residues buried at the interface. The two DNA-binding helices (α4) in the MgrA dimer are oriented parallel to each other and separated by 34 Å. This spacing allows each α4 helix to be nicely buried in the major groove one turn apart in a continuous DNA duplex, as shown in a model of the MgrA–DNA complex (**Supplementary Fig. 3** online).

A careful inspection of the MgrA structure revealed that the only cysteine residue found in the MgrA sequence, Cys12, is located in the N-terminal helix α1 in the dimerization domain. This residue is presented by each monomer to be recognized (through hydrogen bonding) by Ser113 and Tyr38 of the other monomer (**Fig. 2b**). Cys12 is also surrounded by a pocket that sterically fits its side chain. This organization of the dimer interface is strikingly similar to that observed in the crystal structure of OhrR (ref. 22), a peroxide-sensing transcription factor that controls an organic hydroperoxide resistance gene (*ohr*) in *Bacillus subtilis*[23,24]. The lone cysteine residue in OhrR, Cys15, also lies in the interface of the OhrR dimer and is recognized by residues from the other monomer. Oxidation of Cys15 in OhrR by hydrogen peroxide or organic hydroperoxide leads to dissociation of OhrR from the promoter DNA and activation of the *ohr* gene to counter peroxide stress[23].

A sequence alignment shows conservation of the cysteine residue, the amino acids that form the cysteine recognition pocket, and some of the DNA-binding residues between MgrA and OhrR (**Supplementary Fig. 4** online). The structure and sequence similarities between these two proteins seem to suggest that MgrA is an oxidation sensor. The hydrogen bond network around Cys12 may be disrupted by oxidation, which could trigger dissociation of MgrA from DNA, as proposed for OhrR.

Indeed, we found that hydrogen peroxide (H_2O_2), an organic hydroperoxide (cumene hydroperoxide, CHP, **4**) and potassium superoxide (KO_2) all efficiently oxidize the thiol group of Cys12 to form cysteine sulfenic acid *in vitro* (**Fig. 3a**). The oxidized species was trapped by 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, **5**), which gave a product that absorbs at 347 nm, whereas the reduced protein reacted with NBD-Cl to form a thiol-NBD conjugate having an absorbance maximum at 420 nm. Because Cys12 is the only cysteine residue in the entire protein, we can quantitatively evaluate its oxidation by measuring the free thiol content per MgrA monomer using the DTNB (5,5-dithiobis(2-nitrobenzoic acid); **6**) assay. Cumene hydroperoxide, hydrogen peroxide and superoxide are all capable of oxidizing the free thiol of Cys12 (**Fig. 3**). Cumene hydroperoxide seems to be the most efficient oxidant in this group; the presence of hydrophobic residues near Cys12 in the MgrA structure may contribute to better recognition of this organic hydroperoxide.

Next we evaluated whether oxidation of Cys12 affects binding of MgrA to DNA. We performed electrophoretic mobility shift experiments using the promoter sequence of *sarV*, which has been shown to interact with MgrA (**Fig. 3b**)[25]. Addition of excess amounts of MgrA to the DNA probe led to formation of the MgrA–DNA complex, and oxidation of MgrA with different oxidants dissociated the protein from DNA. Treating the oxidized MgrA with a reducing agent (DTT) regenerated the MgrA–DNA complex. We used an MgrA C12S mutant as a control, and this mutant protein remained bound to DNA under the same oxidation conditions that disrupt the wild-type MgrA-DNA interaction. This result confirms the hypothesis that oxidation of Cys12 regulates binding of MgrA to DNA.

How can the current finding be linked to the antibiotic resistance of the *S. aureus mgrA* mutant strain? Antibiotics such as fluoroquinolones

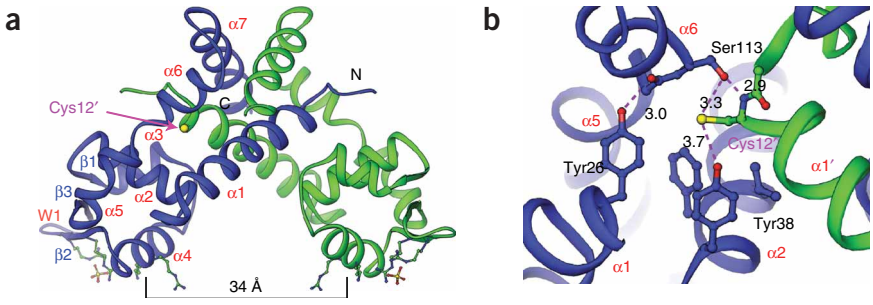


Figure 2 Crystal structure of MgrA. (**a**) Ribbon representation of the MgrA dimer with one subunit colored blue and the dyadic mate colored green. The N and C termini and secondary structural elements of one monomer are labeled (α, α-helices; β, β-sheets; W1, the wing region). Numbering is according to MgrA primary sequence (**Supplementary Fig. 4**). Potential DNA-interacting basic residues on the DNA binding domain are shown together with one ordered sulfate anion per monomer. The distance between two α4 helices is ~34 Å, which correlates to the spacing between two consecutive sections of major groove on the same surface of a B-form duplex DNA. The location of Cys12 in one monomer (Cys12′) is highlighted in the figure. (**b**) Closeup of the reduced Cys12 site in MgrA. Nitrogen, oxygen and sulfur atoms are colored blue, red and yellow, respectively. The Cys12′ residue from an α-helix (α1′) of one monomer (green) is recognized by residues from the other monomer (blue). Residues Cys12′, Tyr26, Tyr38 and Ser113 are shown, and potential hydrogen bonds involved are drawn as dashed lines with distances (in Å) labeled. Additional hydrophobic side chains on α2 are also shown.

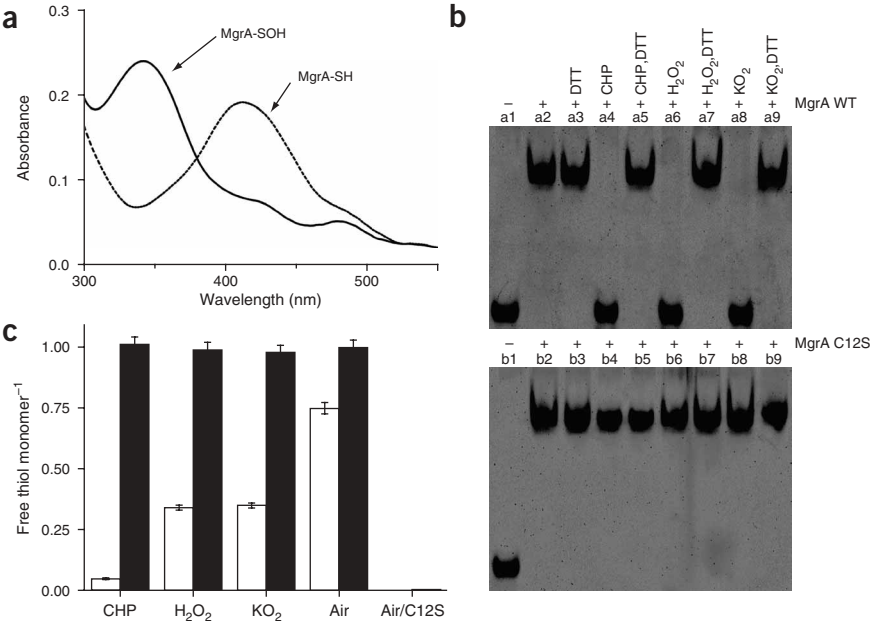


Figure 3 Oxidation of Cys12 dissociates MgrA from DNA *in vitro*. (**a**) Cysteine sulfenic acid formed *in vitro* from Cys12 oxidation was trapped by the NBD-Cl assay. Reaction of the oxidized MgrA with NBD-Cl (solid line): Cys-S(O)-NBD absorbs at 347 nm; reaction of the reduced MgrA with NBD-Cl (dashed line): Cys-S-NBD absorbs at 420 nm. (**b**) Electrophoretic mobility shift assay showing the effect of oxidation on the DNA binding of MgrA. Purified MgrA (or MgrA C12S) protein was incubated with a 40-base-pair oligonucleotide (0.4 pmol per 20 μl reaction) containing the MgrA binding sequence on the *sarV* promoter region. Lane a1 (or b1) contained 0 μM MgrA (or MgrA C12S) and lanes a2–a9 (or b2–b9) contained 2 μM MgrA (or MgrA C12S). Either CHP (7.5 mM) or H_2O_2 (15 mM) was added to the binding assay, which was incubated for 30 min at room temperature. When indicated, 50 mM DTT was then added into the solution and incubation was continued at room temperature for 30 min before samples were used for the shift assay. (**c**) Quantification of free thiol in MgrA upon oxidation (open bars) and reduction (filled bars) by the DTNB assay. The reduced form of purified MgrA contains one free thiol per monomer protein. This form of protein was treated with 4 equiv. of CHP, H_2O_2 or KO_2 for 10 min or with air for over 3 h to generate the oxidized MgrA. An MgrA C12S mutant protein was also assayed as the control. Error bars are s.d.

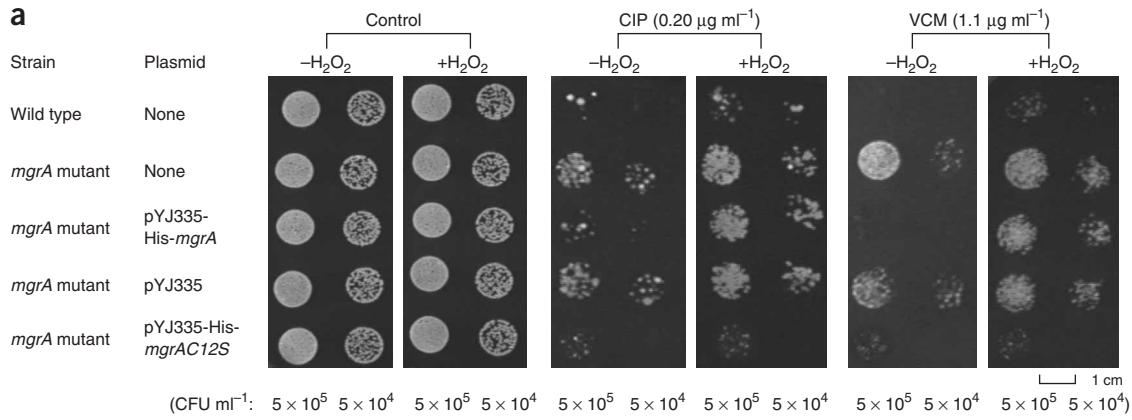
and vancomycin induce oxidative stress as a side effect[26–28]; MgrA may sense the oxidative stress and activate the resistance pathways in *S. aureus*. We tested the oxidation responses of five strains *in vivo*: the wild-type Newman, the *mgrA* mutant, the *mgrA* mutant complemented with *mgrA* cloned in plasmid pYJ335, the *mgrA* mutant complemented with *mgrAC12S* in pYJ335 and the control mutant strain with pYJ335. All strains grew without noticeable differences under normal conditions (**Fig. 4a**). The *mgrA* mutant strain and the mutant strain with plasmid pYJ335 showed resistance toward ciprofloxacin and vancomycin when treated with these antibiotics. Newman, the *mgrA* mutant strain complemented with pYJ335-His-*mgrA* and the *mgrA* mutant strain complemented with pYJ335-His-*mgrAC12S* were susceptible to these antibiotics under the same growth conditions; however, the Newman strain and the *mgrA* mutant strain carrying pYJ335-His-*mgrA* showed increased resistance comparable to that of the *mgrA* mutant strain under 100 μM H_2O_2. The resistance of the *mgrA* mutant strain carrying pYJ335-His-*mgrAC12S* was not noticeably different under oxidation conditions relative to normal conditions (the higher level of drug resistance shown by the *mgrAC12S* mutant strain relative to the Newman strain in the absence of oxidative stress may reflect weakened stability or DNA affinity of the MgrA C12S mutant protein *in vivo*). We also evaluated the susceptibility levels of all five strains toward ciprofloxacin and vancomycin in the absence and presence of paraquat (**7**), another reagent known to induce oxidative stress *in vivo* (**Fig. 4b** and **Supplementary Table 2** online). The Newman strain and the *mgrA* mutant strain complemented with pYJ335-His-*mgrA* showed greater antibiotic resistance in the presence of paraquat than in its absence, which indicates that oxidation of MgrA activates the resistance pathway.

We also constructed a *norA-lacZ* reporter fusion in the chromosomes of the wild-type and *mgrA* mutant strains (*norA* encodes a multidrug efflux transporter protein responsible for resistances to quinolones, and this gene is regulated by *mgrA* in *S. aureus*)[7], and we evaluated the β-galactosidase activity of these two strains in the presence and absence of oxidation challenges (H_2O_2, CHP and paraquat; **Fig. 4c**). The *mgrA* mutant strain showed high *norA* induction relative to the wild type under all conditions. Induction of *norA* was repressed in the wild-type Newman strain in the absence of oxidation challenge; however, we observed a consistent increase (two- to three-fold) in *norA* induction under the oxidation conditions. This *in vivo* result further confirms the proposed oxidation activation mechanism for MgrA.

Broad attention has been brought to the study of the mechanisms of antibiotic resistance in infectious diseases caused by bacterial pathogens such as *S. aureus*[29,30]. We show here that a global regulator, MgrA, uses an oxidation-sensing mechanism to regulate antibiotic resistance in *S. aureus*. This discovery reveals a clever strategy of *S. aureus* for potentially regulating a range of different defensive pathways using a single protein regulator. For instance, human immune systems respond to bacterial infections by generating reactive oxygen and nitrogen species to counter pathogens. This oxidative stress could be sensed through the MgrA-based signaling pathway to activate defensive responses from *S. aureus*. In fact, one of our recent studies indicates that autolysis of *S. aureus* is regulated through oxidation of MgrA *in vivo* as well (the wild-type Newman strain is more resistant to lysis under oxidation challenge; the *mgrA* mutant strain is less susceptible to lysis than the Newman strain). This signaling mechanism could be a general strategy used by other regulators in *S. aureus* and by other pathogens. Targeting this pathway may lead to alternative therapeutic treatments for infections.

METHODS

Bacteria strains, plasmids and culture conditions. *S. aureus* strains used in this work include Newman (wild-type), *mgrA* mutant of Newman (the isogenic transposon mutant, ΦNΞ 3040), *mgrA*-complemented strain (ΦNΞ 3040 carrying pYJ335-His-*mgrA*), *mgrAC12S* strain (ΦNΞ 3040 carrying pYJ335-His-*mgrAC12S*) and *mgrA*-complemented control strain (ΦNΞ 3040 carrying pYJ335). The *E. coli* strains used include DH5α for DNA manipulation and BL21(DE3) for protein overexpression. The plasmids used in this work are pYJ335 (*S. aureus–E. coli* shuttle vector containing a tetracycline-inducible promoter), pYJ335-His-*mgrA* (pYJ335 containing His-*mgrA* under control of the tetracycline-inducible promoter), pYJ335-His-*mgrAC12S* (pYJ335 containing His-*mgrAC12S* under control of the tetracycline-inducible promoter) and pET28a (*E. coli* cloning vector). *S. aureus* strains were grown in the



b

Strain	Plasmid	Highest concentration of visible growth (μg ml^{-1})			
		CIP		VCM	
		–PQ	+PQ	–PQ	+PQ
Wild type	None	0.25	0.45	1.0	1.4
mgrA mutant	None	0.65	0.65	2.2	2.2
mgrA mutant	pYJ335-His-*mgrA*	0.25	0.55	1.0	1.8
mgrA mutant	pYJ335	0.65	0.65	2.2	2.2
mgrA mutant	pYJ335-His-*mgrAC12S*	0.35	0.35	1.4	1.4

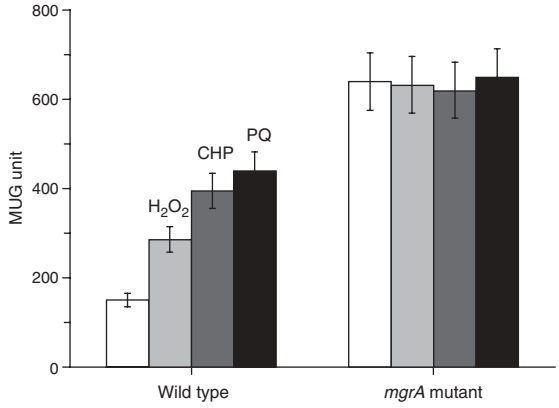


Figure 4 Monitoring *in vivo* effects of MgrA oxidation. (**a**,**b**) The change in susceptibility of *S. aureus* strains to ciprofloxacin (CIP) and vancomycin (VCM) under oxidative stress. The antibiotic resistance levels were tested in the absence (–H_2O_2) or presence (+H_2O_2) of 100 μM H_2O_2 by a plate sensitivity assay (**a**) and were also determined in the absence (–PQ) or presence (+PQ) of 25 μM paraquat by a 96-well plate sensitivity assay (**b**). Under normal growth conditions (control) the five strains did not show noticeable differences. The wild-type strain and the *mgrA* mutant strain complemented with pYJ335-His-*mgrA* showed higher susceptibility toward CIP and VCM. Under oxidation conditions both strains showed increased resistance, comparable to that of the *mgrA* mutant strain, toward these antibiotics. In control experiments, the pYJ335-His-*mgrAC12S*–containing mutant strain did not change its susceptibility toward CIP and VCM under normal versus oxidative conditions. (**c**) Induction of *norA*, a gene regulated by *mgrA*, by oxidative stress. β-Galactosidase activity of strains containing the *norA-lacZ* reporter fusion was determined in the wild-type (Newman) and *mgrA* mutant (ΦNΞ 3040) strains and expressed in MUG units (MUG, 4-methylumbelliferyl-β-D-galactopyranoside; 1 MUG unit = 1 pmol of MUG cleaved by β-galactosidase per min per OD_{600}). Empty bars are untreated cultures. Results are mean ± s.d. from three independent experiments performed in duplicate.

presence of 5 μg ml^{-1} nalidixic acid (for Newman), 10 μg ml^{-1} erythromycin (for *mgrA* mutant strain) or 10 μg ml^{-1} chloramphenicol (for strains containing pYJ335 vector).

Murine abscess model. Wild-type *S. aureus* Newman and *mgrA* mutant strains were grown at 37 °C overnight in TSB (Tryptic Soy Broth; for wild type) or TSB containing 10 μg ml^{-1} of erythromycin (TSB_{erm10}). The cultures were diluted 100-fold with fresh TSB or TSB_{erm10} and incubated at 37 °C for about 2.5 h until optical density at 600 nm (OD_{600}) reached 1.0. Bacteria were collected by centrifugation, washed and suspended in phosphate-buffered saline (PBS) to an OD_{600} of 0.4. Viable staphylococci were enumerated by colony formation on TSA (Tryptic Soy Agar) plates to measure the infection dose (4×10^6 to 6×10^6 CFU). 100 μl of bacterial suspension were administered via retro-orbital injection into BALB/c *nu/nu* (nude) mice age 6–8 weeks. Mice were killed by CO_2 asphyxiation 4 d after the injection, and kidneys and liver were removed. The organs were homogenized in 1 ml of PBS, and 20 μl of dilutions of the homogenates were plated on TSA.

Crystallization of the truncated MgrA (MgrAΔ). MgrAΔ and Selenomethionine (SeMet)-MgrAΔ proteins were crystallized at 4 °C by hanging-drop vapor diffusion. The protein solution (2 μl of 10 mg ml^{-1} protein in 200 mM NaCl, 2 mM DTT and 20 mM MES, pH 6.6) was mixed with 2 μl of reservoir buffer (1.6 M ammonium sulfate, 0.1 M MES, pH 6.0) and equilibrated with 0.8 ml of reservoir buffer. Crystals of MgrAΔ were rinsed in cryoprotectant solutions consisting of reservoir buffer with an added 10%, 20% and 25% glycerol. The cryoprotected crystals were flash frozen in liquid nitrogen, and diffraction data were collected at the BioCARS beamline 14-ID-B at the Advanced Photon Source, Argonne National Laboratory.

Electrophoretic mobility shift assays. The electrophoretic mobility shift experiments were performed by adding 0.4 pmol of a 40-base-pair *sarV* promoter region containing the MgrA-binding fragment to 20 μl of binding buffer (20 mM Tris-HCl at pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 50 μg ml^{-1} BSA, 10 μg ml^{-1} salmon sperm DNA, 0.2% Triton X-100 and 10% glycerol). The reduced form of MgrA was freshly prepared (**Supplementary Methods** online) and added to give a final concentration of 2 μM, and then the mixture was incubated at room temperature (~20 °C) for 30 min. Either 7.5 mM CHP or 15 mM H_2O_2 was added to the binding reaction and incubated for another 30 min at room temperature. We obtained samples treated with KO_2 using a different method (**Supplementary Methods**). When indicated, 50 mM DTT was added into the solution and incubation continued at room temperature for 30 min before the samples were used for the shift assay. The 6% nondenaturing polyacrylamide gel was pre-run for 30 min before the binding samples were loaded, and the gel was then continuously ran at 85 V

for 1 h. The band shifts were detected by staining the gel with SYBR Gold nucleic acid gel stain (Invitrogen) for 30 min, and the images were taken using the Gel Doc image system (Bio-Rad). Oligonucleotides sequences used in the assays are: 5′-GGTATAAATGTTGTCGAATAAACAACAAGTTGTCCAAAAG-3′ and 5′-CTTTTGGACAACTTGTTGTTTATTCGACAACATTTATACC-3′.

Other methods. Detailed information is available in **Supplementary Methods**.

Data deposition. The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2bv6).

Note: *Supplementary information is available on the Nature Chemical Biology website.*

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AUTHOR CONTRIBUTIONS

C.H. designed experimental strategies with the help of O.S. and P.A.R. P.R.C. performed all experiments with the help of T.B. (microbiology), W.A.W. (microbiology) and E.M.D. (crystallography).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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