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A New Oxidative Sensing and Regulation Pathway Mediated by the MgrA Homologue SarZ in *Staphylococcus aureus*

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Summary

Oxidative stress serves as an important host/environmental signal that triggers a wide range of responses from the human pathogen *Staphylococcus aureus*. Among these, a thiol-based oxidation sensing pathway through a global regulator MgrA controls the virulence and antibiotic resistance of the bacterium. Herein, we report a new thiol-based oxidation sensing and regulation system that is mediated through a parallel global regulator SarZ. SarZ is a functional homologue of MgrA and is shown to affect the expression of ~87 genes in *S. aureus*. It uses a key Cys residue, Cys13, to sense oxidative stress and to coordinate the expression of genes involved in metabolic switching, antibiotic resistance, peroxide stress defense, virulence, and cell wall properties. The discovery of this SarZ-mediated regulation, mostly independent from the MgrA-based regulation, fills a missing gap of oxidation sensing and response in *S. aureus*.

Keywords

sarZ; MgrA homologue; Transcription profiling; Oxidative sensing; Virulence; Antibiotic resistance

Introduction

Staphylococcus aureus is a Gram-positive human pathogen responsible for most wound and hospital-acquired infections (Lowy, 1998; Boyce, 1997). The coordinated expression of the organism's virulence factors is regulated by global regulators such as Agr, SarA, and MgrA (Novick, 2003; Bronner *et al*., 2004; Cheung *et al*., 2004). Elucidating the detailed mechanisms by which these proteins regulate gene expression and their regulatory pathways is crucial to gain a comprehensive picture of *S. aureus* pathogenesis and may contribute to new strategies for the therapeutic intervention of infections.

The SarA or MarR family proteins are a common class of regulatory proteins in *S. aureus*. The global regulators SarA and MgrA and their homologues belong to this family of

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proteins (Bronner *et al*., 2004; Cheung *et al*., 2004; Hong *et al*., 2005; Chen *et al*., 2006; Cheung *et al*., 2008). Both SarA and MgrA affect expression of hundreds of genes in *S. aureus* and play major roles that control various properties of the bacterium (Dunmun *et al*., 2001; Luong *et al*., 2006). While the exact molecular mechanism leading to SarA signaling is still unclear, we recently have demonstrated that thiol-based oxidation is responsible for MgrA signaling (Chen *et al*., 2006). A Cys residue located at the dimerization domain of the MgrA dimer is redox sensitive. Its oxidation leads to dissociation of MgrA from its promoter DNA. A similar redox-sensing mechanism has been shown for OhrR type proteins in other gram positive bacteria (Fuangthong and Helmann, 2002; Lee *et al*., 2007; Newberry *et al*., 2007). However, MgrA is the first example of utilizing this mechanism to regulate antibiotic resistance and expression of virulence factors in a pathogen. MgrA contributes to the regulation of over 300 genes (Luong *et al*., 2006) including genes encoding virulence factors (for example: capsular polysaccharide, nuclease, α-toxin, coagulase, protease, and protein A), genes involved in autolysis (for example: *lytM* and *lytN*), other global regulatory genes (for example: *agr, lytRS, arlRS, sarS*, and *sarV*), and genes encoding efflux pumps such as *norA, norB*, and *tet38* that confer resistance to antibiotics (Ingavale *et al*., 2003; Luong *et al*., 2003; Truong-Bolduc *et al*., 2003; Ingavale *et al*., 2005; Kaatz *et al*., 2005; Truong-Bolduc *et al*., 2005).

A search for MgrA homologues in the whole genome of *S. aureus* identified MgrH1 (MgrA Homologue 1) which shares a high sequence identity with MgrA and OhrR proteins (Fig. 1). MgrH1 is also known as SarZ due to its homology to SarA (Cheung *et al*., 2004). Most noticeably, the lone Cys residue that is key to oxidative sensing in MgrA and OhrR is conserved in MgrH1. Meanwhile, residues that form a hydrogen bonding network around this Cys and a hydrophobic pocket near the Cys are conserved among MgrA, OhrR, and MgrH1. These observations prompted us to propose that MgrH1, a member of MarR family proteins in *S. aureus*, could serve as a functional homologue of the global regulator MgrA and employ a similar oxidative sensing mechanism to regulate gene activation. In fact, a recently published study by Sekimizu and colleagues (Kaito *et al*., 2006) showed that this protein plays multiple roles as a regulator of both virulence factors and hemolysis genes in *S. aureus*. In the present study, we will use the name SarZ instead of MgrH1. In this paper, we show that SarZ affects the expression of 87 genes. Most importantly, we demonstrate that SarZ is a thiol-based oxidation sensing protein. SarZ senses oxidative stress and exerts a global regulatory role on metabolism, antibiotic resistance, oxidation resistance, autolysis, and virulence in *S. aureus*.

Results and Discussion

Sequence similarity between SarZ and MgrA

A search for close homologues of MgrA in the GenBank database identified the SarZ protein. The sequence similarity between MgrA and SarZ is 71% with 34% identical residues (Fig. 1). The MgrA redox sensitive residue, Cys12, is conserved as the sole Cys residue, Cys13, in SarZ. Cys12 of MgrA is recognized by a network of hydrogen bonds involving Tyr26, Tyr38, and Ser113 from the other monomer (Chen *et al*., 2006). This network is also conserved in SarZ and provided by residues of Tyr27, Tyr38, and Ser113 (Fig. 1). In addition, residues that form a hydrophobic pocket in MgrA appear to be conserved in the sequence of SarZ. Collectively, these common features suggest that SarZ is a functional homologue of MgrA and uses its Cys13 residue to sense oxidative stress for gene regulation.

A recent report showed that *sarZ* regulates hemolysin production in *S. aureus* and affects expression of certain virulence factors (Kaito *et al*., 2006). However, the regulatory

mechanism of SarZ and its effect on global gene activation in *S. aureus* have not been elucidated.

Identification of sarZ-regulated genes by microarray

First, transcription profiling of mRNAs was performed using Affymetrix GeneChips for the wild-type *S. aureus* Newman and the isogenic *sarZ* mutant (Bae *et al*., 2004) to identify genes controlled by *sarZ*. Transcription profiling of several other staphylococcal global regulators (*agr, arlRS, mgrA, sarA, rot*, and *sigB*) has already been used to evaluate their respective regulons (Dunman *et al*., 2001; Saïd-Salim *et al*., 2003; Bischoff *et al*., 2004; Liang *et al*., 2005; Luong *et al*., 2006). In addition, transcription profiling following perturbations such as heat, cold, stringent stress, SOS stress, mild acidic pH, H_2O_2, HOCl, NO, peracetic acid, and salicylate have been obtained and have revealed valuable information on stress responses of *S. aureus* (Weinrick, *et al*., 2004; Anderson *et al*., 2006; Chang *et al*., 2006a; Chang *et al*., 2006b; Richardson *et al*., 2006; Chang *et al*., 2007; Riordan *et al*., 2007).

In triplicate, *S. aureus* strain Newman and isogenic *sarZ* mutant cells were grown to both log (2 h, OD_{600}=0.6) and early stationary (5 h, OD_{600}=2.0) phases to take into account the possibility of growth phase-dependent regulation, then total RNA of each strain was isolated from each growth phase. Each RNA sample was subsequently labeled, hybridized to an *S. aureus* Affymetrix GeneChip and processed, according to manufacturer recommendations. Following normalization, replicates were averaged and the transcript titers of each mRNA species were compared across strains at each time point, as previously described (Beenken *et al*., 2004; Bischoff *et al*., 2004; Weinrick *et al*., 2004; Luong *et al*., 2006). Genes with decreased expression in the *sarZ* mutant as compared to wild-type were grouped as genes downregulated in the *sarZ* mutant (Table 1), while genes with increased expression were grouped as genes upregulated in the *sarZ* mutant. Real-time RT-PCR was used to verify GeneChip results for five genes from Table 1. As shown in Table 2, there was reasonable correlation between both measurements; although, the fold changes were much more profound in real-time RT-PCR.

A total of 87 genes were found to be affected by *sarZ* at either 2 h and/or 5 h sampling times. Among them, 32 genes were downregulated and 55 genes were upregulated in the *sarZ* mutant. The number of genes downregulated in the *sarZ* mutant is much less at 2 h than at 5 h; conversely, many more genes are upregulated in the *sarZ* mutant at 2 h than at 5 h. Only two genes (*sarZ* and SA0166) were upregulated by *sarZ* mutation at both log and stationary phases. Thus, the *sarZ* mutation appears to primarily lead to upregulation of genes in the log-phase cells and downregulation of many genes in the post-exponential cells.

Like MgrA, SarZ appears to be a pleiotropic regulator. Three genes (*pbuX, nuc, pyrR*) that are downregulated in the *sarZ* mutant have been reported to be downregulated in the *mgrA* mutant as well (Luong *et al*., 2006). Only 6 genes (*lacD, norB, scrA*, SACOL1476, SACOL1847, SACOL1849) out of 55 that are upregulated in the *sarZ* mutant in the log phase are also upregulated in the *mgrA* mutant (Luong *et al*., 2006). Other than these overlaps, the genes appear to be regulated by *sarZ* in a manner independent of *mgrA*.

Genes involved in many metabolic processes are affected by *sarZ*. For example, genes for the intermediary (*acs, pflAB, pckA*, SACOL0177) and amino acid metabolisms (*argGH, ilvD, lysC, hisC*), genes for fatty acid synthesis (*fabG*, SACOL1661, SACOL0212), and genes for sugar metabolism (*gntRK, lacD, malA, treC*) show significant changes in the *sarZ* mutant. In particular, *pflA* (pyruvate formate lyase activating enzyme, Buis and Broderick 2005) and *pflB* (pyruvate formate lyase, Lindmark *et al*., 1969; Lehtio and Goldman 2004) showed the most dramatic changes with 37-fold and 19-fold increases, respectively, in the

sarZ mutant compared to the wild-type at the 2 h sampling time. These genes encode two enzymes of the first committed steps of anaerobic respiration (Sawers and Bock 1988; Fuchs *et al*. 2007), indicating that *sarZ* may affect switching from oxygen-dependent to anaerobic metabolisms. The *acs* gene encodes acetyl-CoA synthetase that converts acetate to acetyl-CoA. All of *acs*, *pflB*, and *pckA* are involved in pyruvate metabolism. Acetic acid induces the expression of *lrgAB* (Rice *et al*., 2005). Pyruvate is a key metabolite for amino acid and fatty acid metabolism. Thus, *sarZ* might induce pleiotropic effects by affecting a key metabolite. The *sarZ* gene also affects 3 regulatory genes (*pyrR*, *gntR*, *gntR*-like gene) involved in regulating pyrimidine and gluconate metabolisms. Expression of 21 transporters for ammonium, purine, amino acids, and sugars were affected by *sarZ* disruption. In addition, two drug transporters (*norB*, *tet38*) showed an increased expression in the *sarZ* mutant. These transporters have been reported to be regulated by *mgrA* as well (Truong-Bolduc *et al*., 2005).

Many genes encoding cell surface proteins including *isdC*, *epiEF*, *lrgB*, *efb*, *fib*, *tcaA*, SACOL0507, SACOL1164, and SA0167 were affected by the *sarZ* mutation, as well as exonuclease *nuc* and 4 different exotoxins. A recent study showed that SarZ protein plays multiple roles as a regulator of both virulence factors and hemolysis genes in *S. aureus* (Kaito *et al*., 2006). Our microarray analysis did not find significant changes (at least 2-fold change) of *hla*, *hlb*, and RNAIII transcription levels in the *sarZ* mutant strain. Either the changes are small or, very likely, the difference is due to the use of strains (RN4220, NCTC8325-4) that carry the *rsbU* mutation which caused a reduction of *sigB* expression (Giachino *et al*., 2001; Horsburgh *et al*., 2002) in the previous study (Kaito *et al*., 2006).

Notably, 17 genes affected by *sarZ* mutation are also H_2O_2-responsive genes in *S. aureus* (Chang *et al*., 2006a). For instance, an *ohr* (organic hydroperoxide resistance) like gene (SACOL0872) shows a 2.7-fold increase in the *sarZ* mutant strain. The *ohr* gene was first isolated from *Xanthomonas camperstris* (Mongkolsuk *et al*., 1998) and studied extensively in *B. subtilis* (Volker *et al*., 1998; Fuangthong *et al*., 2001; Fuangthong and Helmann, 2002; Helmann *et al*., 2003, Hong *et al*., 2005; Lee *et al*., 2007). The organic hydroperoxide resistance gene *ohrA* in *B. subtilis* is located next to the cognate negative regulator *ohrR* that senses peroxide stress. The *S. aureus ohr*-like gene does not reside close to *sarZ* in the genome yet it appears to be regulated by SarZ. The *pflA* and *pflB* transcripts that were upregulated by *sarZ* disruption are also dramatically induced by H_2O_2 stress. These observations, together with the high sequence similarity (and conservation of the key Cys residue) between SarZ and MgrA/OhrR, strongly indicate that SarZ is a sensor and regulator for oxidative stress in *S. aureus*. To test this hypothesis we performed a series of biochemical and microbiological experiments.

Cys13 oxidation in SarZ

To evaluate the redox activity of Cys13 in SarZ, the protein was cloned (with an N-terminal His_6 tag), overexpressed in *E. coli*, and purified over Ni-NTA column. The purified His_6-tagged SarZ protein was reduced completely with DTT. The excess DTT was removed. Reduced SarZ was incubated with 4 equiv of cumene hydroperoxide (CHP) per SarZ monomer. The transient sulfenic acid intermediate (SarZ-Cys13-SOH) could be trapped by 4-chloro-7-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) that reacts with sulfenic acid to give a product that absorbs at 347 nm (Fig. 2A), whereas the reduced thiol reacts with NBD-Cl to form a thiol-NBD conjugate that absorbs at 420 nm in a control with reduced SarZ. We further quantified Cys13 oxidation by measuring the free thiol content per SarZ monomer using 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). Since there is only one Cys residue in the entire SarZ protein, we should observe 1 equiv of thiol per protein monomer in the absence of oxidation and no thiol after oxidation. As shown in Fig. 2B, both CHP and H_2O_2 (4 or 8

equiv per SarZ monomer) effectively oxidized Cys13 to give less than 0.2 equiv of free thiol per SarZ monomer after 10 min treatment.

To further support SarZ Cys13 oxidation *in vivo*, we employed a Cys alkylation based assay (Lee *et al.*, 2004) with the use of a 600-Da alkylator (PEG-alk, Scheme S1) that efficiently alkylates Cys13 in SarZ *in vitro* and *in vivo*. Cell extracts containing His_6-tagged SarZ expressed in the *sarZ* mutant were treated with PEG-alk. The protein extracts were separated by SDS-PAGE, transferred to membrane and the SarZ protein could be detected with an anti-His antibody. In the absence of peroxide stress, alkylation of SarZ led to a 600-Da molecular weight increase that was detected on SDS-PAGE gel. If *S. aureus* cells were first treated with 10 mM H_2O_2 for 20 min prior to the alkylation assay, the Cys13 residue of SarZ was modified by oxidation and could not be alkylated by the Cys alkyator as shown in Fig. S1. SarZ C13S mutant protein in H_2O_2 treated and untreated cells showed the same mobility as the unalkylated SarZ. Thus, these results show that Cys13 in SarZ is modified by exposure to H_2O_2 inside *S. aureus*.

Binding of SarZ to ohr promoter is affected by Cys13 oxidation

Next, we assessed whether oxidation of Cys13 affected SarZ's ability to bind DNA. Since *ohr* is downregulated by *sarZ*, we examined binding of SarZ to the *ohr* promoter fragment with an electrophoretic mobility shift assay. A DNA fragment containing the *ohr* promoter was radioactively labeled, and mixed with purified SarZ. Binding complexes were separated on gel. Low mobility bands were observed with increasing concentrations of SarZ in the gel shift experiment. Approximately 8-fold excess of SarZ was required to completely shift 1 pmol of radioactively labeled promoter DNA (Fig. S2). The *ohr* promoter sequence has AT-rich palindromic sequences (See Experimental procedures), which is also observed in other many promoters of expression-changed genes in Table 1 (Data not shown).

Oxidation of SarZ with CHP (1 and 10 μM) or H_2O_2 led to weakened binding of the protein to DNA (lanes 4 and 7, Fig. 3 and Fig. S2); a recent study did indicate that the sulfenic acid form of OhrR retains DNA-binding activity and it is the further modified OhrR that loses DNA affinity (Lee *et al*., 2007). The sulfenic acid or other Cys13 oxidized form of SarZ generated in this case may be stable and resist further oxidation. Addition of excess reducing agent (DTT) restored the binding of SarZ to promoter DNA to its original level (lanes 8, Fig. 3). Control experiments showed that the C13S mutant SarZ, with Cys13 mutated to Ser, binds the promoter DNA with a similar affinity as the wild-type SarZ (Fig. 3); however, this binding was not affected by treatment with oxidants (Lane 13 and 15, Fig. 3) which confirms that Cys13 serves as the redox sensing residue. In addition, SarZ treated with a low concentration of CHP (1 μM) was sensitive to Coenzyme A (CoA) on DNA binding (Lane 5, Fig. 3). CoA is the major low molecular weight thiol in *S. aureus* (delCardayré *et al*., 1998). The SarZ-SOH intermediate may form a mixed disulfide with CoA which induces its dissociation from DNA as reported for OhrR (Lee *et al*., 2007). Overall, the electrophoretic mobility shift assay results confirmed that oxidation of Cys13 regulates binding of SarZ to DNA. The exact form of Cys13 oxidation that leads to weakened binding of SarZ to DNA is still unclear and will be studied in the future.

Gene expression induced by H_2O_2 in Newman and sarZ mutant strains

To support the biochemical observation that SarZ is an oxidation sensor, we evaluated expression levels of genes regulated by SarZ under normal and H_2O_2-challenged growth conditions. Expression of *ohr* was monitored in wild-type, *sarZ* and *mgrA* mutant strains treated with and without 10 mM H_2O_2 using real-time RT-PCR (Table 3). As expected, *ohr* was induced by addition of H_2O_2 to cultures of strain Newman. This gene is constitutively activated in *sarZ* mutant but not in *mgrA* mutant. It is known that *ohr* genes are negatively

regulated by OhrR in *B. subtilis* and related bacteria, and confer resistance to peroxides (Fuangthong *et al*., 2001;Sukchawalit *et al*., 2001;Chuchue *et al*., 2006;Oh *et al*., 2007). Our result indicated that this gene is also activated in *S. aureus* upon peroxide challenge. It appears to be regulated by SarZ instead of MgrA.

Peroxide and antibiotic resistance assay

Plate assays were employed to test the potential resistance of the *sarZ* mutant strain towards various peroxides and antibiotics. As shown in Fig. 4, *sarZ* mutant exhibited higher resistance towards CHP as compared to the wild-type. This phenotype could be complemented by expressing SarZ (from a plasmid) in the *sarZ* mutant strain. We also observed a slight resistance of *sarZ* mutant towards H_2O_2, and again the phenotype could be complemented by expressing SarZ and SarZ C13S mutant in the *sarZ* mutant strain. In addition, we found that the *sarZ* mutant strain is more resistant to some antibiotics such as vancomycin and chloramphenicol (Fig. 4). Although no specific resistance gene was found to be regulated by *sarZ*, this protein does affect expression of many cell-wall related proteins. We speculate that this resistance phenotype is due to changes of bacterial cell wall properties, which may render staphylococci more resistant to cell wall lysis induced by antibiotic or reduced permeability of the cell wall. These phenotypes could be complemented by expressing SarZ in the *sarZ* mutant strain. When SarZ C13S was expressed in the *sarZ* mutant strain, a slightly decreased resistance to vancomycin was observed as compared to the complementary experiment with the wild-type SarZ. This suggests involvement of Cys13 in the regulation mechanism.

Regulation of autolysis by sarZ

To test whether the *sarZ* mutation changes the bacterial cell wall properties towards autolysis, we performed autolysis assay induced by Triton X-100, a common reagent used to promote autolysis. Four stains were assayed, including wild-type Newman, *sarZ* mutant, *sarZ* mutant complemented with a vector carrying the *sarZ* gene, and *sarZ* mutant carrying the empty vector as a control. As shown in Fig. 5, upon Triton X-100 treatment the *sarZ* mutant strain showed reduced autolysis rate compared to wild-type Newman. Expression of SarZ in the mutant restored its susceptibility towards Triton X-100 to levels similar to wild-type Newman. The empty vector control gave the same phenotype as the mutant. Thus, *sarZ* mutation does affect the bacterial cell wall properties, which may account for the observed resistance towards certain antibiotics.

The autolysis rates of all four strains against Triton X-100 were retarded after oxidative challenge with 400 μM H_2O_2 as shown in Fig. 5B. Wild-type Newman and *sarZ* complementary strains showed a significant decrease of autolysis activity to a similar level as *sarZ* mutant strain. Inactivation of SarZ leads to upregulation of autolysis regulators such as *lrgB* (Table 1), which is a holin-like protein negatively controling autolytic genes in *S. aureus* (Groicher *et al*., 2000). The real-time PCR analysis of *lrgB* also showed that the *lrgB* gene was induced by addition of 10 mM H_2O_2 to cultures of strain Newman (7.55 ± 2.06 fold) and constitutively expressed in *sarZ* mutants like *ohr* gene (4.81 ± 0.49 fold). This effect may yield a decreased autolysis rate. The autolysis activities of *sarZ* mutant and the complementary control also decreased, but to a lesser extent than wild-type Newman, suggesting that other autolysis regulators might be affected by SarZ-independent oxidation regulation pathways.

Contribution of sarZ to virulence

Previously, we reported that MgrA is a major virulence determinant in *S. aureus* and *mgrA* mutation leads to a dramatic drop of virulence (4-6 logs) (Chen *et al*., 2006). It had also been suggested that mutation of *sarZ* decreases the virulence of *S. aureus* but the attenuation

(~10 fold) is much less significant than the *mgrA* mutant strain (Kaito *et al*., 2006). Since MgrA and SarZ regulations in *S. aureus* are largely independent of each other, despite sharing a similar oxidation sensing mechanism, we wondered whether deletions of *sarZ* and *mgrA* may be additive for virulence. A *sarZ/mgrA* double mutant strain was constructed and its effect on *S. aureus* virulence was compared with *sarZ* or *mgrA* single gene mutant in the murine abscess model (Fig. 6). Bacteria (Newman, *sarZ*, *mgrA*, *mgrA/sarZ*; 10^6 CFU, colony-forming units) were injected intravenously into mice (ten mice per strain). Five days post infection, animals were killed, and kidneys and livers were removed to measure bacterial loads in organs. Colony forming units were counted on agar plates after plating organ pulps. As expected, the *mgrA* mutant strain exhibited a dramatic reduction of virulence as bacterial loads were reduced by 3-5 and 4 logs in livers and kidneys, respectively. The *sarZ* mutant displayed a statistically insignificant decrease in virulence as compared to wild-type (a repeat of this experiment did show a statistically significant decrease in virulence in agreement with the previous report; Fig. S3) (Kaito *et al*., 2006). However, a double *sarZ/mgrA* mutant exhibited an epistatic phenotype over the *mgrA* mutation. Bacterial loads in kidneys and livers were further decreased as compared to infection with the *mgrA* mutants (Fig. 6). This result indicates that *mgrA* and *sarZ* regulate virulence pathways independently and perhaps can be targeted synergistically since a similar mechanism is used to induce the activity of both regulations.

Conclusion

Broad attention has been brought to the study of thiol-based redox sensing and regulatory processes (Hausladen *et al*., 1996; Choi *et al*., 2001; Pomposiello and Demple, 2001; Kim *et al*., 2002; Sevier and Kaiser, 2002; Finkel 2003; Wood *et al*., 2003; Chander and Demple, 2004; Green and Paget, 2004; Poole *et al*., 2004; Liu *et al*., 2005; Morris *et al*., 2005; Jones, 2006; Lee and Helmann, 2006; Sevier and Kaiser, 2006; D'Autréaux and Toledano, 2007; Ilbert *et al*., 2007; Lee *et al*., 2007). We have recently shown that oxidation of a single Cys in MgrA has a dramatic effect on the antibiotic resistance regulation and perhaps also virulence regulation in *S. aureus* (Chen *et al*., 2006). Here we show that a parallel oxidation sensing and regulation pathway mediated through an MgrA homologue, SarZ, exists in *S. aureus*. We show that SarZ uses a Cys-based oxidation sensing and regulatory mechanism resembling MgrA. The two proteins share similar oxidative sensing properties to peroxides. Transcription profiling of the *sarZ* regulon indicated that ~87 genes, encoding proteins that perform a variety of functions in *S. aureus*, are controlled by this global regulator.

Noticeably, SarZ controls genes involved in metabolic switching and peroxide response, which are mostly absent in the *mgrA* regulon. Previous study has indicated that such changes (for instance the turning on of *pflA*, *pflB* and *ohr*) should take place when *S. aureus* is challenged with H_2O_2 (Chang *et al*., 2006a); the bacterium is expected to change its cell wall thickness or morphology to protect itself and turn on anaerobic respiration upon challenges with oxidative stress. The observed dramatic upregulation of *pflA* and *pflB* and the change of cell wall properties (autolysis and resistance towards cell-wall targeting antibiotics) in the *sarZ* mutant strain are consistent with its role in oxidation sensing and response. These functions are actually regulated by SarZ instead of MgrA (Fig. 7). Therefore, the discovery of the SarZ-mediated regulation fills a missing gap for oxidation sensing and response in *S. aureus*. This study also suggested that thiol-based redox sensing and regulation may be more prevalent and play much broader roles in human pathogens as compared to many non-pathogenic bacteria (Collet and Bardwell, 2002; Imlay, 2003; Paget and Buttner, 2003; Poole *et al*., 2004; Poole, 2005; Imlay, 2008). Host immune systems respond to bacterial infections by generating reactive oxygen and nitrogen species. These oxidative stresses are sensed in parallel signaling pathways through SarZ and MgrA in *S. aureus* (Fig. 7). The high similarity of SarZ and MgrA and the synergistic effect of *sarZ* and

mgrA on virulence certainly indicate that both pathways can be targeted for developing new therapeutic agents to treat *S. aureus* infection.

Experimental procedures

Bacterial strains, plasmids and culture conditions

The human clinical isolate, *S. aureus* strain Newman, and its derivatives were used. Bacteria were grown with rotary shaking (250 rpm) in TSB (Tryptic soy broth, Difco) supplemented with 5 μg ml^{-1} nalidixic acid for Newman and 10 μg ml^{-1} erythromycin for mutant strains. *E. coli* strains DH5α and BL21 star (DE3) (Invitorogen) were used for DNA manipulation and protein expression, respectively. Vector pET28a (Novagen) was used for cloning in *E. coli*. *E. coli-S. aureus* shuttle vectors pYJ335 (Ji *et al*., 1999) and pHY300PLK (Takara) were used for complementary studies.

Construction of the mgrA-sarZ double mutant

In-frame deletions in the *sarZ* gene were generated by allelic replacement without antibiotic marker selection using pKOR1 (Bae and Schneewind 2006). DNA fragments containing 1 k b upstream and downstream sequences of *sarZ* were PCR-amplified from chromosomal DNA of Newman using the following primers: attB2-sarZ-up-F 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTATGAAGCAGGATCAGCAAATGGTA-3' and sarZ-up-HindIII 5'-ATAAGCTTAAGGTGATGGGCAGAAGTATGCATCGCTAT-3' for the upstream fragment, and attB1-sarZ-CF 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCACTAATGCAATAACAACTTGCAT-3' and sarZ-CR-HindIII 5'-ATAAGCTTCCAATCACTCCTTGTTAAAATAAACAATAT-3' for the downstream fragment. PCR products were digested with *Hin*dIII, mixed together and ligated by T4 DNA ligase (NEB). The ligation product was used for recombination with pKOR1 and recombination products were introduced to DH5α. The resulting plasmid pKORΔsarZ was transferred by electroporation first to *S. aureus* RN4220, and subsequently to Newman *mgrA*, generating strain Newman *mgrA*/pKORΔsarZ.

To integrate the plasmid, Newman *mgrA*/pKORΔsarZ was inoculated into 25 ml TSB with 10 μg ml^{-1} chloramphenicol (TSBCm10) and grown at 43 °C overnight. Culture aliquots were streaked on TSA (Tryptic soy agar, Difco) with 10 μg ml^{-1} chloramphenicol (TSACm10) and incubated at 43 °C overnight. From the resulting plate, one colony was picked, inoculated into 5 ml TSB and incubated at 30 °C overnight to facilitate plasmid excision. Cultures were then diluted 10^4-fold with sterile water and 0.1 ml aliquots were spread on TSA containing 0.1 μg ml^{-1} anhydrotetracycline (ATc, Clontech) (TSAATc) and incubated at 30 °C for 2 days.

To identify the desired deletion mutants on TSAATc, 8 large colonies were inoculated into TSB and incubated at 37 °C overnight. Chromosomal DNA was purified from the cultures and the *sarZ* gene was PCR-amplified using the primers attB2-sarZ-up-F and attB1-sarZ-CF. Four out of 8 picks carried a deletion. The allelic replacement mutants were isolated at frequencies of 50%.

RNA isolation

In triplicate, cultures of *S. aureus* strains grown overnight in BHI (Brain Heart Infusion, Difco)with appropriate antibiotics were diluted 1:100 into fresh BHI without antibiotics and grown at 37 °C with constant aeration for 2 hr and 5 hr respectively before being harvested. The method of harvesting samples for RNA isolation is to re-suspend bacterial culture with

an equal volume of ice-cold ethanol-acetone (1:1) solution and store the mixtures at -80°C for 20 min or until ready to prepare RNA. The 2 hr and 5 hr time spots were chosen as it is well known that a variety of genes' expression level in *S. aureus* are growth-phase dependent and dramatic changes had been observed between early-exponential and post-exponential growth phase conditions. The 2 hr samples represented the early-exponential growth phase with an OD_{600}=0.6 and a doubling time about 20 min. The 5 hr samples represented the post-exponential growth phase with an OD_{600}=2.0 and a doubling time about 4 hrs. OD data were obtained by using an Agilent 8453 UV-Vis Spectrophotometer with cuvettes of 1cm path length. For samples with OD_{600}>1.0, appropriate dilutions were applied before OD being read.

The harvested mixture samples for RNA isolation mentioned above were centrifuged at 10,000 × *g* at 4°C for 10 min and cell pellets were air dried and resuspended on ice in 0.5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). The cell suspensions were transferred to lysing matrix B tubes (MP Biomedicals) and processed in an FP120 FastPrep cell disruptor (MP Biomedicals) for 20 s at setting 5.0. After chilling on ice for 5 min, the tubes were repeated to process for 20 s at setting 4.5. The tubes were centrifuged at 17,000 × *g* in a tabletop centrifuge for 15 min at 4°C, and the upper phases were transferred to a 1.5-ml microtube. The RNA samples were further purified and treated with DNase I using the RNeasy mini kit (QIAGEN), according to the manufacturer's instructions. The RNA was spectrophotometrically quantified.

Microarray profiling

RNA was converted to cDNA, and microarray analysis was performed according to the manufacturer's instructions for antisense prokaryotic arrays (Affymetrix). To ensure reproducibility, three RNA samples from each strain and sampling time were prepared at each growth phase. Each RNA sample was independently hybridized to a GeneChip. One mutant 2 hr RNA sample was not included within microarray analysis, due to machinery malfunction; two replicates were used to analyze that particular strain/sampling time all others corresponded to three independent RNA sample measurements. For comparisons of each strain and sampling time, GeneChip signal intensity values were normalized to the total microarray signal, averaged, and compared using GeneSpring GX 7.3.1 software (Agilent Technologies), as previously described (Beenken *et al*., 2004; Bischoff *et al*., 2004; Weinrick *et al*., 2004; Luong *et al*., 2006). All microarray results have been deposited in the Gene Expression Omnibus (GEO) provisional number GSE13138. Genes with at least a two-fold difference (*t* test; $P = 0.05$) in RNA titer between the wild-type strain and the *sarZ* insertion mutant were considered differentially expressed in a *sarZ*-dependent manner.

Real-time RT-PCR

To confirm the microarray data, we selected genes from different functional categories to assay their relative expression levels by real-time RT (reverse transcription)-PCR. Briefly, one-step quantitative RT-PCR was performed by incubating DNase I-treated RNA with SuperScript III platinum SYBR Green One-Step qRT-PCR master mix (Invitrogen) using the ABI Prism 7300 detection system (Applied Biosystems). The cDNA was subjected to real-time PCR using the primer pairs listed in Table 2. Cycling conditions were 50 °C for 5 min and 95 °C for 5 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 35 sec and a dissociation step at 40 °C for 1 min, 95 °C for 15 sec, 60 °C for 30 sec, and 95 °C for 15 sec.

Chemical characterization and quantification of thiol modification

The reduced form of purified His_6-tagged SarZ (SarZ-SH) served as starting material and was generated by incubating with a large excess of DTT (100 mM) for 1 h, which was

followed by running the sample through desalting columns (HiTrap desalting column, Amersham Biosciences) three times at 4 °C. Assays were performed in a buffer with 100 mM KH_2PO_4/K_2HPO_4, 200 mM NaCl and 1 mM EDTA at pH 7.0. The SarZ-SOH modification was confirmed by the NBD-Cl assay (Ellis and Poole, 1997; Chen *et al*., 2006): aliquots of 100 μl reduced SarZ (50 μM in 10 mM Tris at pH 7.4 with 100 mM NaCl) were treated with 4 equiv of CHP (200 μM) at room temperature for 10 min followed by washing with thiol-assay buffer three times to generate the oxidized SarZ. All the washing steps in the DTNB assay and the NBD-Cl assay followed the same procedure by adding 500 μl thiol-assay buffer to 100 μl sample solution and concentrating down to the original volume with Microcon YM-10 (Amicon) ultrafiltration device (small molecules in the sample will be diluted by six fold after each time of washing). Both the oxidized and reduced SarZ were incubated with excess amounts of NBD-Cl (1 mM) for 1 h at room temperature in dark before being washed with thiol-assay buffer three times. The spectra were then taken in an Agilent 8453 UV-vis spectrophotometer against a blank of the buffer solution. Free thiol quantification was conducted by DTNB assay (Riddles *et al*., 1983; Chen *et al*., 2006): the reduced SarZ (50 μM) was treated with varying equivalents of CHP or H_2O_2 at room temperature for 10 min followed by washing with the thiol-assay buffer three times to generate the oxidized sample. The reduced SarZ can be regenerated by treating aliquots of the oxidized SarZ samples with 20 mM DTT for 1 h followed by removing extra DTT using Micro Bio-Spin 6 chromatography columns. Finally, 100 μl of all the oxidized and reduced SarZ samples were incubated with 1 mM DTNB at room temperature for 15 min before the UV-vis spectra were taken. The absorption at 412 nm from each sample was recorded and the free thiol concentrations were calculated by using the previously published methods (Riddles *et al*., 1983).

Electrophoretic mobility shift assays

The electrophoretic mobility shift experiments were performed by using 8-32 pmol of SarZ in 20 μl of binding buffer (20 mM Hepes-KOH at pH 7.6, 50 mM KCl, 5 mM Magnesium Acetate, 1 mM EDTA, 0.5 mg ml^{-1} BSA, 10 μg ml^{-1} Salmon sperm DNA, 0.005% Triton X-100 and 20% glycerol). The probe DNA was labeled using a T4 polynucleotide kinase (Invitrogen) and [γ-^{32}P] ATP (Perkin Elmer). DNA probe (1 pmol) was added to the mixture and incubated at room temperature for 30 min. CHP was added to the binding reaction and incubated for another 30 min at room temperature. When indicated, either 250 μM CoA or 1 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 8% native polyacrylamide gel was pre-run for 30 min before the binding samples were loaded, and the gel was then continuously run at 150 V and room temperature. The gels were dried and subjected to autoradiography using the storage phosphor screen (Image Screen-K, Kodak) and the Molecular Imager PharosFX Plus System (Bio-Rad). Oligonucleotides sequences used in the assays are: ohr1 5'-TTTTCGAATGGGTAAAGCATAAATGTATTTTAAATTAGGAGGTTATAAGT-3', ohr2 5'-ACTTATAACCTCCTAATTTAAAATACATTTATGCTTTACCCATTCGAAAA-3'.

Peroxide and antibiotic sensitivity assays

S. aureus wild-type Newman and the *sarZ* mutant strains were grown at 37 °C overnight in TSB with appropriate antibiotics to ensure plasmid maintenance. Overnight, cultures were diluted 100-fold into the same medium and grown at 37 °C for 3 h to reach an OD_{600} ~0.6. After further incubation at 37 °C for ~2 h, the numbers of bacterial cells from all strains were normalized to approximately 5×10^8 CFU ml^{-1} with fresh TSB, followed by six 10-fold serial dilutions. Then 5 μl of each strain of bacterial samples were spotted onto the TSA

plates containing CHP, H_2O_2, chloramphenicol (Cm), or vancomycin (Vm). All plates were incubated at 37 °C for 24 h before being read.

Autolysis assay

Triton X-100 induced autolysis assays were performed as previously described (Manna *et al*., 2004). In brief, overnight cultures were diluted 1:200 to fresh TSB containing 1 M NaCl and grown at 37 °C with shaking till OD_{600} reached 1.0. Cells were pelleted by centrifugation, washed twice with ice-cold water and then resuspended in the same volume of 50 mM Tris-HCl (pH7.2) supplemented with and without 0.05% (v/v) Triton X-100. The cells were then incubated at 30 °C with shaking and the lysis activity was monitored by measuring OD_{600} versus time. To assay Triton X-100 induced autolysis under the oxidants challenged conditions, the freshly diluted bacteria TSB culture containing 1M NaCl were allowed to grow at 37 °C with shaking for 3 hrs (OD_{600}=0.7). Then 400 μM H_2O_2 (final concentration)were added to the bacteria culture and the cells were incubated at 37 °C with shaking for another 30 min (OD_{600}=1.0) before being harvested. The pelleted cells were manipulated and assayed in the same way as mentioned above. All assays were performed in triplicate and the standard deviation of three independent experiments was <10%.

Murine abscess model

Newman and mutant strains were grown at 37 °C overnight in TSB or TSB containing 10 μg ml^{-1} of erythromycin (TSBerm10). The cultures were diluted 100-fold with fresh TSB or TSBerm10 and incubated at 37 °C for about 2 h until 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 plates to measure the infection dose (4×10^6 to 6×10^6 CFU). 0.1 ml of bacterial suspension was administered via retro-orbital injection into BALB/c nu/nu (nude) mice (6–8 weeks). Mice were killed by CO_2 asphyxiation 5 days after the injection, and kidneys and liver were removed. The organs were homogenized in 1 ml of PBS, and 10 μl of dilutions of the homogenates were plated on TSA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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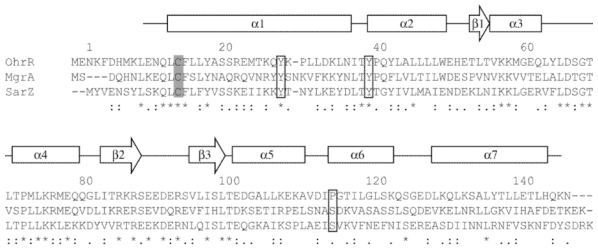

Fig. 1.

Sequence alignment of SarZ with its homologues MgrA and OhrR. Alignment of SarZ and MgrA sequences from *S. aureus* N315 and OhrR sequence from *B. subtilis* 168 was performed with CLUSTAL W ver. 1.83 program (Thompson *et al*., 1994). Asterisks, colons, and periods below the alignment indicate fully, strongly, and weakly conserved residues, respectively. The key Cys13 residues are shaded. Tyr26, Tyr38, Ser113 are denoted by squares. The secondary structure elements of MgrA are labeled as α and β to α helices and β sheet, respectively. Protein residue numbering based on the SarZ sequence.

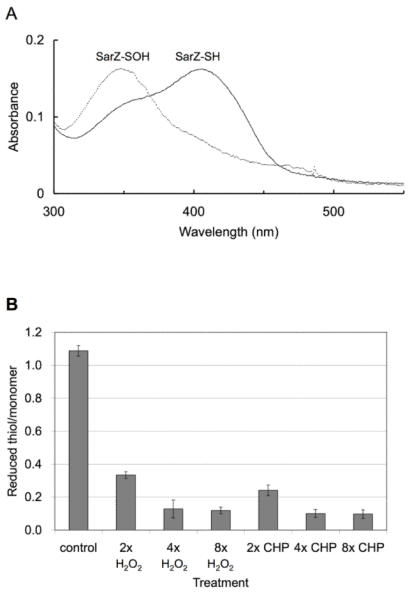

Fig. 2.

Monitoring oxidation of Cys13 *in vitro*. (A) Cysteine sulfenic acid formed *in vitro* from Cys13 oxidation by 4 equiv (per SarZ monomer) of CHP was trapped by the NBD-Cl assay. Reaction of the oxidized SarZ with NBD-Cl (solid line): Cys-S(O)-NBD absorbs at 347 nm; reaction of the reduced SarZ with NBD-Cl (dashed line): Cys-S-NBD absorbs at 420 nm. (B) Quantification of free thiol in SarZ upon oxidation and reduction by the DTNB assay. The reduced form of purified SarZ (control) contains one free thiol per monomer protein. This form of protein was treated with 2, 4 and 8 equiv (per SarZ monomer) of CHP or H_2O_2 for 10 min to generate the oxidized SarZ. Error bars are s.d.

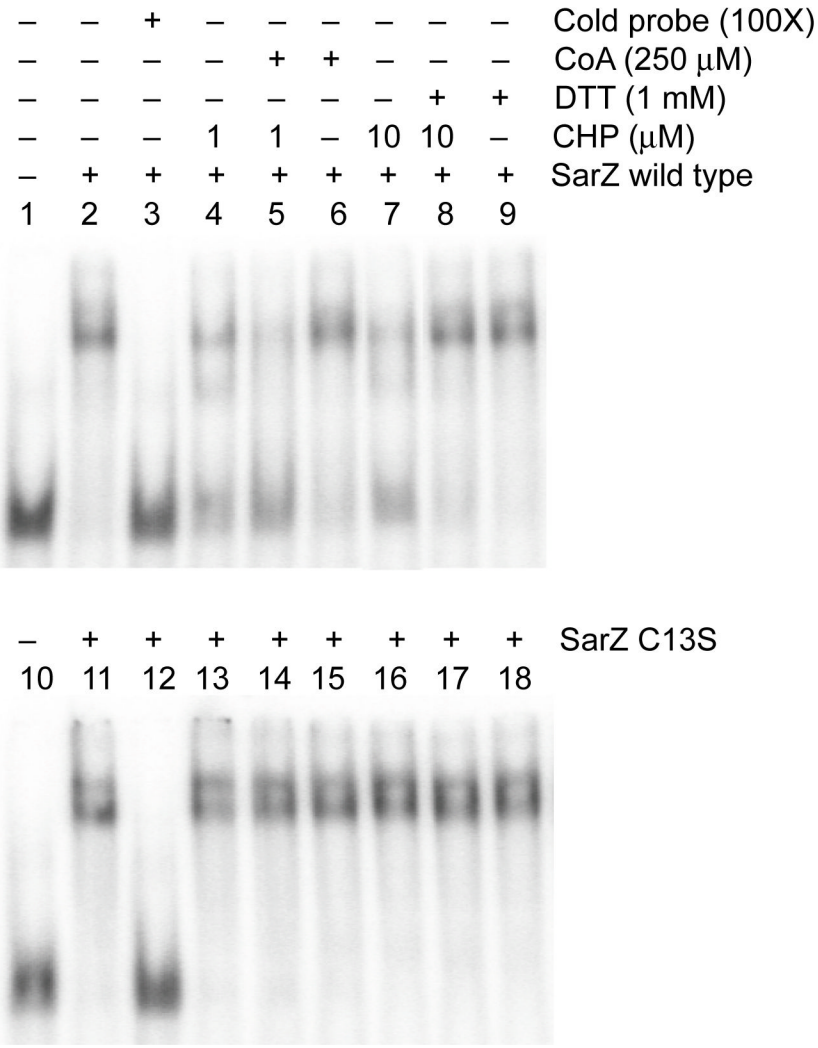

Fig. 3.

Electrophoretic mobility shift assay showing the effect of oxidation on the DNA binding of purified SarZ. Purified wild-type SarZ or SarZ C13S (32 pmol) was incubated with a radiolabeled nucleotide containing *ohr* promoter region (1 pmol, See Experimental procedures) in 20 μl reaction mixture. CHP was added to the reaction assay and incubated for 30 min at room temperature. When indicated, 1 mM DTT or 250 μM of CoA was added after 30 min incubation with oxidants for another 30 min at room temperature before samples were separated on the gel. All experiments were performed in the presence of 10 μg ml^{-1} Salmon sperm DNA.

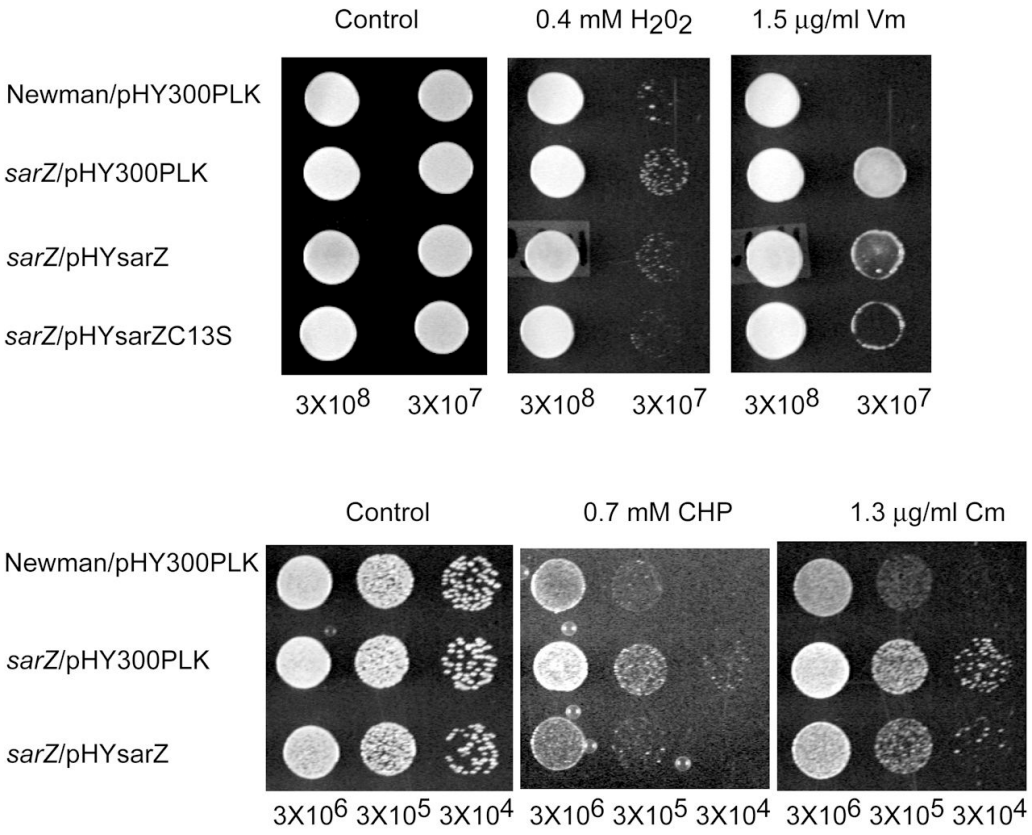

Fig. 4.

Plate sensitivity assay. Susceptibility of *S. aureus* strains toward antibiotics and oxidants were monitored using plate assay. Potential resistance of *sarZ* mutant strains to vancomycin (Vm, 1.5 μg ml^{-1}), chloramphenicol (Cm, 1.6 μg ml^{-1}), cumene hydroperoxide (CHP, 0.7 mM) or H_2O_2 (0.4 mM) were assayed as described in Methods, and compared with that of wild-type Newman. All plates were incubated at 37 °C for 24 h.

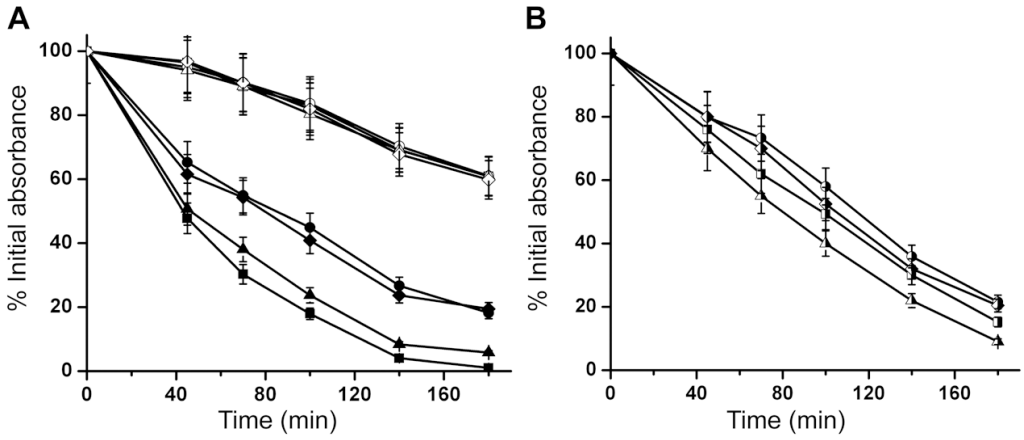

Fig. 5.

Autolysis Assay. Analysis of *sarZ* controlled autolytic activity in *S. aureus* strain Newman. (A) Effect of the *sarZ* mutation on Triton X-100 induced autolysis. □ and ■, wild-type *S. aureus* strain Newman; ● and ○, *sarZ* mutant strain; Δ and ▲, *sarZ* mutant strain supplemented with plasmid pYJ335 containing *sarZ* gene; ◊ and ◆, *sarZ* mutant strain supplemented with empty vector pYJ335. The lysis activity with (filled symbol) and without (open symbol) 0.05% Triton X-100 was monitored by measuring OD_{600} versus time. (B) Effect of oxidative stress on the SarZ-regulated autolysis in *S. aureus* strain Newman. Triton X-100 (0.05%) induced autolysis in *S. aureus* strains pre-treated with 400 μM H_2O_2 was monitored by recording the decline of OD_{600} in a period of three hours. The same bacteria strains from (A) are assayed and shown with the same symbol (half filler symbol). Results are mean ± s.d. from three independent experiments.

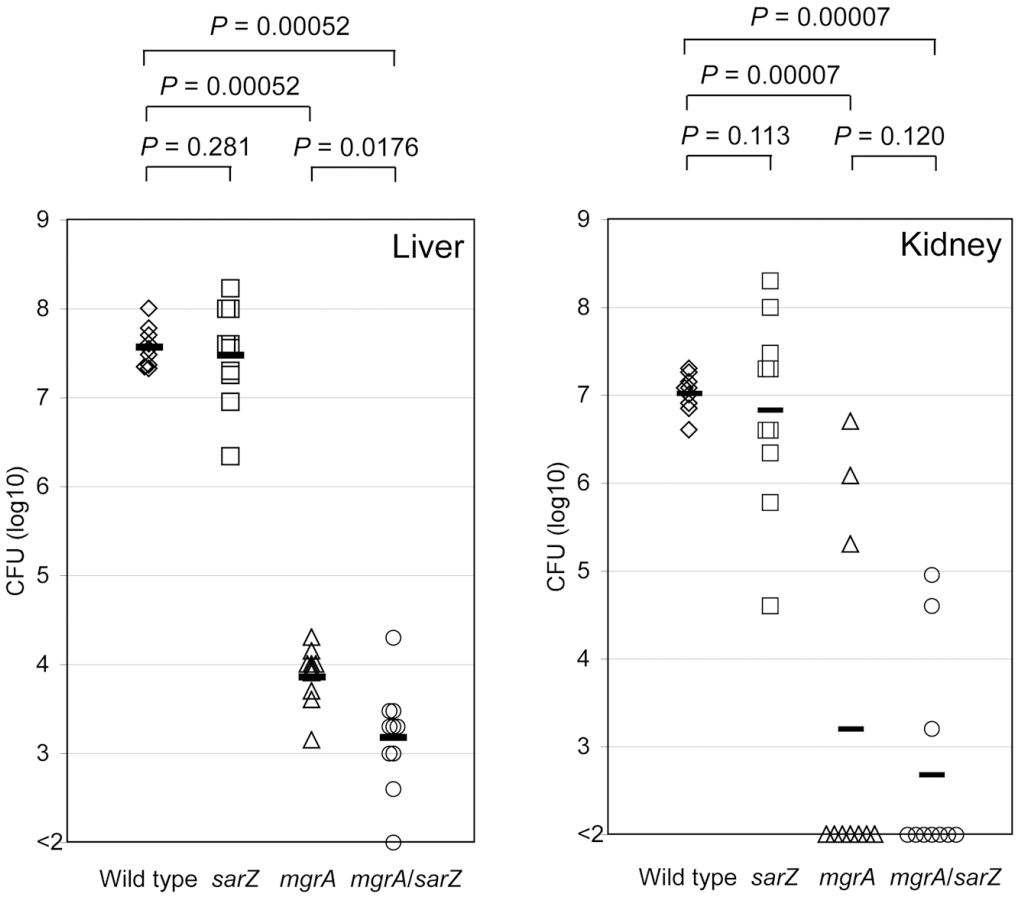

Fig. 6.

Effect of the *sarZ* mutation on the virulence of *S. aureus* as tested using the murine abscess model of infection. *S. aureus* strain Newman (wild-type), *sarZ*, *mgrA*, and *sarZ/mgrA* mutant strains were used to infect ten mice each via retro-orbital injection. After 5 days, mice were killed and organs (kidneys and livers) 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. The limit of detection was 100 CFU ml^{-1} in this case, as 10 μl of homogenates (1 ml total) were used for colony enumeration.

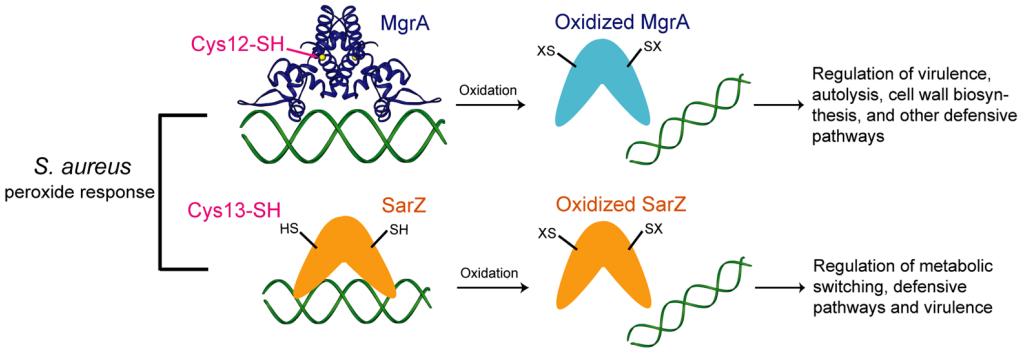

Fig. 7.

MgrA and SarZ play complementary roles in oxidation sensing and regulation in *S. aureus*. X stands for a covalent modification of the Cys resides.

Table 1

S. aureus genes affected by *sarZ* disruption[a]

ORF	Gene	Fold-change		H_2O_2 effect[b]	Description
		2 h	5 h		
Genes downregulated in the *sarZ* mutant					
SACOL1141	*isdC*	-2.1			Iron regulated Surface Determinant system
SACOL0860	*nuc*	-2.5		-	Thermonuclease precursor
SACOL0459	*pbuX*	-2.0			Xanthine permiase
SACOL1210	*pyrR*	-2.3			Pyrimidine operon regulatory protein
SACOL2384	*sarZ*	-3.4	-11.2		Staphylococcal accessory protein Z
SA0166		-2.1	-2.4		ABC transporter
SACOL1168	*efb*		-2.9	+	Fibrinogen-binding protein
SACOL1169	*fib*		-2.8		Fibrinogen-binding protein precursor related protein
SACOL0784	*hisC*		-2.0		Histidinol-phosphate aminotransferase
SACOL1076	*purS*		-2.5		Phosphoribosylformylglycinamide synthetase
SACOL2352	*tcaA*		-2.4		Teicoplanin resistance associated protein
SACOL0157			-3.0		α-helical coiled-coil SrpF–like protein
SA0167			-2.2		SrpL–like membrane lipoprotein
SACOL0159			-2.2		ABC transporter
SACOL0185			-2.5		Peptide ABC transporter
SACOL0186			-2.7		Dipeptide ABC transporter, DppC–like protein
SACOL0218			-2.0	+	Conserved hypothetical protein
SACOL0219			-2.5		Conserved hypothetical protein
SACOL0430			-2.6		Cystathionine β-lyase family protein
SACOL0431			-2.3		Cystathionine γ-synthase family protein
SACOL0505			-3.1		ABC transporter
SACOL0507			-2.3		N-acetylmuramoyl-L-Ala amidase precursor
SACOL0602			-2.3		Haloacid dehydrogenase-like protein
SACOL0883			-2.7		ABC transporter
SACOL0910			-3.0		Hypothetical protein
SACOL1164			-3.1		Fibrinogen-binding related protein

ORF	Gene	Fold-change		H_2O_2 effect[b]	Description
		2 h	5 h		
SACOL1996			-2.4		ABC transporter
SACOL1997			-2.7		GntR family protein
SACOL2295			-3.1		Staphyloxanthine biosynthesis protein
SACOL2709			-2.5		Conserved hypothetical protein
SACOL2710			-2.6		Conserved hypothetical protein
Genes upregulated in the *sarZ* mutant					
SACOL1783	*acs*	2.2		-	Acetyl-CoA synthetase
SACOL0964	*argG*	8.2			Arguininosuccinate synthetase
SACOL0963	*argH*	5.6			Arguininosuccinate lyase
SACOL1429	*asd*	4.4			Aspartate-semialdehyde dehyrogenase
SACOL0121	*deoD*	5.2		+	Purine nucreoside phospholylase
SACOL1872	*epiE*	3.0			Epidermin immunity protein E
SACOL1873	*epiF*	2.9			Epidermin immunity protein F
SACOL2482	*fabG*	4.1		+	3-oxoacyl-ACP reductase
SACOL0655	*fruA*	5.2			Fructose permiase
SACOL2525	*gntK*	6.5		+	Gluconokinase
SACOL2516	*gntR*	5.6		+	Gluconate operon repressor
SACOL2042	*ilvD*	3.2	-2.3		Dihydroxy-acid dehydrates
SACOL2183	*lacD*	2.0			Tagatose 1, 6-diphosphate aldrase
SACOL0248	*lrgB*	3.2			Holin–like protein
SACOL1428	*lysC*	4.4			Aspartokinase, α and β subunits
SACOL1551	*malA*	4.7			α-glucosidase
SACOL0192	*msmX*	2.1			Maltose ABC transporter
SACOL2397	*nirD*	3.0	-2.2		NAD (P) H Nitrite recuctase, small subunit
SACOL1475	*norB*	5.7			Drug transporter
SACOL2031	*nrgA*	4.7			Ammonium transporter family protein
SACOL0872	*ohr*	2.7		+	Organic hydroperoxide resistance protein
SACOL1838	*pckA*	2.6			Posphoenolpyruvate carboxykinase
SACOL0205	*pflA*	37.3		+	Pyruvate formate lyase activating enzyme
SACOL0204	*pflB*	18.8		+	Pyruvate formate lyase

ORF	Gene	Fold-change		H_2O_2 effect[b]	Description
		2 h	5 h		
SACOL2376	*scrA*	5.3			PTS system, Sucrose-specific IIBC component
SACOL0387	*set11*	9.1			Exotoxin 11
SACOL0122	*tet38*	5.1		+	Tetracycline resistance protein
SACOL0516	*treP*	6.7			PTS system, IIBC component
SACOL0517	*treC*	4.0			α, α phosphotreharase
SACOL0012		2.8			Homoserin O-acetyltransferase
SACOL0176		6.3		+	Conserved hypothetical protein
SACOL0177		4.6			Glucokinase-regulator-related protein
SACOL0183		3.4			Conserved hypothetical protein
SACOL0469		2.9			Exotoxin 1
SACOL0504		6.3	-3.0	+	ABC transporter
SACOL0708		2.8			DAK2 domain protein
SACOL0709		3.0			Conserved hypothetical protein
SACOL0882		2.9			ABC transporter
SACOL0884		2.1	-2.3		ABC transporter
SACOL1476		13.8			Amino acid permiase
SACOL1661		3.8		-	Acetyl-CoA carboxylase, *accC* homologue
SACOL1847		3.8			Conserved hypothetical protein
SACOL1849		2.3		-	Conserved hypothetical protein
SACOL1915		4.1			Glutamate ABC transporter
SACOL1916		3.8			Amino acid ABC transporter
SACOL2461		3.7		+	Conserved hypothetical protein
SACOL2579		3.6			Phytone dehydrogenase
SACOL2585		4.0	-2.0		*pfoS/R*–like regulatory protein
SACOL2619		3.1	-2.4		Amino acid permiase
SACOL2704		3.0			ATP phosphoribosyltransferase reguratory subunit
SAS0383		2.2			Exotoxin
SAV0894		2.2			ϕMu50B-like protein
SACOL0212			2.1		3-hydroxyacyl-CoA dehydrogenase

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[a] The entire microarray data were deposited to the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO, http://www.ncbi.nlm.nih.gov/geo/) with a provisional entry number GSE13138. Three uncharacterized ORFs were omitted in this table.

[b] Previously described ORFs affected by exposure to hydrogen peroxide (Chang *et al.*, 2006a).

+, Upregulation. -, Downregulation.

Table 2

Relative quantification of expression levels of selected genes controlled by SarZ determined by real-time PCR with primers and SYBR green probes

Gene	ORF	Real-time PCR	Primers
pflB	SACOL0204	146.0 ± 18.2	5'-AAAGCAGGCGTTATTACTGAAAGC-3' 5'-CGTCAATACCTACACCACCGATAG-3'
pflA	SACOL0205	56.0 ± 8.6	5'-TGACAAACATATTAGATTGACAGGAAAGC-3' 5'-ATCATCAGAATAACCAGGCACAAGG-3'
lrgB	SACOL0248	13.2 ± 2.7	5'-CTGTTATCCGTTATACCATTTTTC-3' 5'-CCACCTATTTTGTAAGTCTTATAC-3'
set11	SACOL0387	13.5 ± 1.0	5'-CTATAACGGTTCTAACGTTGTAC-3' 5'-CACCAACAGTAGATAGTCTAC-3'
ohr	SACOL0872	4.0 ± 0.1	5'-GAGCATTAGATATTGATATCGTTC-3' 5'-GTTAGTGTTACTTCTGGATGAG-3'

Table 3

Relative quantification of *ohr* expression by real-time PCR with primers and SYBR green probe

	Real-time PCR (fold)	
Strain	**−**	**+ H_2O_2**
Newman	1.00 ± 0.33	2.18 ± 0.82
Newman *mgrA*	0.68 ± 0.05	4.71 ± 2.44
Newman *sarZ*	3.41 ± 0.33	4.71 ± 0.18