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The auxiliary protein complex SaePQ activates the phosphatase activity of sensor kinase SaeS in the SaeRS two-component system of *Staphylococcus aureus*

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Summary

In bacterial two-component regulatory systems (TCSs), dephosphorylation of phosphorylated response regulators is essential for resetting the activated systems to the pre-activation state. However, in the SaeRS TCS, a major virulence TCS of *Staphylococcus aureus*, the mechanism for dephosphorylation of the response regulator SaeR has not been identified. Here we report that two auxiliary proteins from the *sae* operon, SaeP and SaeQ, form a protein complex with the sensor kinase SaeS and activate the sensor kinase's phosphatase activity. Efficient activation of the phosphatase activity required the presence of both SaeP and SaeQ. When SaeP and SaeQ were ectopically expressed, the expression of coagulase, a *sae* target with low affinity for phosphorylated SaeR, was greatly reduced, while the expression of alpha-hemolysin, a *sae* target with high affinity for phosphorylated SaeR, was not, demonstrating a differential effect of SaePQ on *sae* target gene expression. When expression of SaePQ was abolished, most *sae* target genes were induced at an elevated level. Since the expression of SaeP and SaeQ is induced by the SaeRS TCS, these results suggest that the SaeRS TCS returns to the pre-activation state by a negative feedback mechanism.

Keywords

Bacteria; Membrane proteins; Lipoproteins; Negative-feedback; Phosphatase

Introduction

Two-component systems (TCSs) are a major sensory-regulatory mechanism by which bacteria respond to various environmental cues such as nutrient concentrations, ionic strength, and membrane disturbances (Stock *et al.*, 2000, Beier & Gross, 2006, Hoch, 2000). A prototypical TCS is composed of a histidine kinase (HK) sensor protein and a response regulator (RR). Activation of the HK leads to autophosphorylation of a conserved histidine residue. The phosphoryl group is subsequently transferred to the conserved aspartic acid

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residue in the receiver domain of the cognate RR, altering the function of its output domain (Hoch, 2000). Often the RR is a DNA binding protein and its activation brings about transcriptional changes (Hoch, 2000, West & Stock, 2001).

To synchronize TCS activity with changing environmental cues, a bacterium must be able to dephosphorylate the phosphorylated RR (P-RR) and return the TCS to the pre-activation state. Studies showed that the level of P-RR is determined by the stability of P-RR (i.e., autodephosphorylation) and/or by dephosphorylation of P-RR by discrete phosphatases or by a bi-functional HK (Bourret *et al.*, 2010, Zhao *et al.*, 2002, Gao & Stock, 2009). The phosphatase activity of a bi-functional HK can be regulated by other auxiliary proteins. In the NtrBC (NRII/NRI) TCS of *E. coli*, which controls expression of the nitrogen regulon, the phosphatase activity of the sensor kinase NtrB (NRII) is activated by the binding of PII protein, a small homotrimeric signal transduction protein (Keener & Kustu, 1988, Pioszak *et al.*, 2000, Ninfa & Jiang, 2005). By binding to the kinase domain of NtrB, PII activates the phosphatase activity of NtrB while it inhibits the autokinase activity of the HK (Ninfa & Jiang, 2005, Pioszak *et al.*, 2000). In *Mycobacterium tuberculosis*, the lipoproteins LprF and LprJ were suggested to modulate the phosphatase activity of KdpD, the HK of KdpDE TCS (Steyn *et al.*, 2003). In *Firmicutes* bacteria, the membrane protein LiaF negatively regulates the LiaRS TCS (Jordan *et al.*, 2006, Jordan *et al.*, 2007, Suntharalingam *et al.*, 2009). Recently, in *Listeria monocytogenes*, LiaF was suggested to stimulate the phosphatase activity of LiaS (Fritsch *et al.*, 2011). In *Bacillus subtilis*, YycFG is an essential TCS that connects cell division to cell wall homeostasis (Fabret & Hoch, 1998, Fukushima *et al.*, 2011, Fukushima *et al.*, 2008). The sensor kinase YycG is negatively regulated by two membrane auxiliary proteins, YycH and YycI, by forming a ternary protein complex (Szurmant *et al.*, 2008, Szurmant *et al.*, 2007, Szurmant *et al.*, 2005), where the negative regulation is carried out by the transmembrane helices of the proteins (Szurmant *et al.*, 2008). Unlike the regulation by PII, however, the molecular details of the negative regulation by those lipoproteins and membrane proteins still remain to be determined.

Staphylococcus aureus, an important human pathogen, has 16 TCSs (Cheung & Zhang, 2002). Among them, the SaeRS TCS is induced by human neutrophil peptides (HNPs) and plays a key role in virulence by activating the production of major virulence factors such as alpha-hemolysin (Hla), coagulase (Coa), and fibronectin binding proteins (Giraud *et al.*, 1997, Goerke *et al.*, 2005, Liang *et al.*, 2006, Xiong *et al.*, 2006, Voyich *et al.*, 2009, Rogasch *et al.*, 2006). The *sae* operon is composed of four ORFs (*saeP*, *saeQ*, *saeR*, and *saeS*) and two promoters (P1 and P3) (Fig. 1A). The P3 promoter transcribes *saeR* and *saeS*, encoding the RR and HK, respectively, with a fairly constitutive activity. On the other hand, P1, whose activity is 2 – 30 times higher than P3, can transcribe all four ORFs, and, because it has two SaeR binding sites, its transcription is induced by the SaeRS TCS (i.e., autoinduction) (Geiger *et al.*, 2008, Li & Cheung, 2008, Sun *et al.*, 2010b, Nygaard *et al.*, 2009). The HK SaeS has two transmembrane domains separated by 9 a.a. extracellular residues (Adhikari & Novick, 2008). Since the 9 a.a. acid segment is too small to bind to a ligand, SaeS is classified as an intramembrane sensing HK (Mascher, 2006). The SaeS in the strain Newman carries a L18P mutation in the first transmembrane domain and shows constitutively active kinase activity, resulting in high level expression of its target genes including SaeP and SaeQ even in the uninduced condition (Adhikari & Novick, 2008, Mainiero *et al.*, 2010, Schafer *et al.*, 2009). On the other hand, the wild type SaeS in other strains requires external stimuli (e.g., HNPs) for its activation (Geiger *et al.*, 2008). SaeP and SaeQ are predicted to be a 146 a.a. lipoprotein and a 157 a.a. membrane protein, respectively. However, except for the fact that they are not necessary for target gene expression (Adhikari & Novick, 2008), the role of these two proteins is completely unknown.

Previously, we observed that phosphorylated SaeR (P-SaeR) has a long half-life (over 4 h), even in the presence of the cytoplasmic domain of SaeS (SaeS^C), (Sun et al., 2010b), suggesting that P-SaeR is very stable and that SaeS^C does not possess phosphatase activity. Therefore, in this study, we examined the possibility that the two proteins, SaeP and SaeQ, provide phosphatase activity to the SaeRS TCS and found that the proteins induce phosphatase activity of SaeS by forming a protein complex.

Results

MBP-SaeS does not show detectable phosphatase activity

Although, in our previous study, SaeS^C did not show phosphatase activity, it is possible that the lack of phosphatase activity of SaeS^C is due to the absence of N-terminal transmembrane domains in SaeS^C. To test this possibility, we expressed and purified two full length SaeS proteins, SaeS^L (the wild type) and SaeS^P (the L18P mutant), in the form of maltose binding protein (MBP) fusions. As a control HK, we used His-tagged AirS, an iron-sulfur cluster containing HK that responds to oxidative stresses in low oxygen conditions (Sun *et al.*, 2012). Despite the fact that SaeS is a membrane protein, the MBP-SaeS fusion proteins were soluble in *E. coli*. As shown in Fig. 1B, while the half-life of P-AirR alone was 47 min, it was 19 min in the presence of AirS, suggesting that AirS has P-AirR phosphatase activity. In the presence of either MBP-SaeS^L or MBP-SaeS^P, however, the level of P-SaeR continued to increase until 40 min and remained steady until 60 min. These results indicate that, at least in an MBP fusion form, full length SaeS does not have detectable phosphatase activity.

SaeP, SaeQ, and SaeS form a protein complex

SaeP contains a lipo box (LXXC) and is predicted to be a lipoprotein (Hutchings *et al.*, 2009, Juncker *et al.*, 2003), while SaeQ is predicted to be a membrane protein with three transmembrane domains (Fig. 2A). To confirm the nature and localization of the proteins, we fractionated the strain Newman cells and carried out Western blot analysis for SaeP and SaeQ. We used the strain Newman because, due to its constitutively active SaeS^P, the strain constantly produces SaeP and SaeQ (Adhikari & Novick, 2008). As expected, both SaeP and SaeQ were detected in the cell membrane fraction (Newman in Fig. 2B). More importantly, when lipidation of lipoprotein was blocked by disruption of *Igt* (prolipoprotein diacylglycerol transferase) (Hutchings *et al.*, 2009), SaeP was secreted into the culture supernatant, confirming its lipoprotein nature (*Igt* in Fig. 2B).

Given the fact that both SaeP and SaeQ reside in the cell membrane as does SaeS, it is possible that these proteins directly interact with SaeS. Our recent finding that SaeQ stabilizes SaeS^P in strain Newman further supports the idea of the direct protein-protein interactions (Jeong *et al.*, 2011). We first examined this possibility with the bacterial two-hybrid system (Karimova *et al.*, 1998). We cloned each protein sequence as either bait or prey and tested whether the combination of the two plasmid constructs can increase LacZ expression, an indicator for the protein-protein interaction. As a control sensor kinase, we used BraS, a staphylococcal intramembrane sensor kinase responding to bacitracin stress (Hiron *et al.*, 2011, Kolar *et al.*, 2011). As shown in Fig. 3A, although lower than the positive control leucine zipper domain, any combination of SaeS, SaeP, and SaeQ showed higher LacZ activity than that of single protein controls or the combinations of BraS-SaeP and BraS-SaeQ, suggesting that SaeS, SaeP, and SaeQ form either a ternary complex or a binary complex in various combinations. The L18P mutation in SaeS^P did not affect the protein-protein interaction (compare S^P and S^L in Fig. 3A). Intriguingly, the plasmids carrying *saeP*, *saeQ*, and *saeS* sequences appeared to be unstable, and mutant plasmids without insert DNA sequences arose quickly, explaining, at least in part, the relatively low LacZ activity of the test strains. When the protein interactions were further investigated by

immunoprecipitation with either anti-SaeP or anti-SaeQ antibody, all three proteins were collected together (Fig. 3B), confirming the direct protein-protein interactions and the formation of a protein complex. It should be noted that, in the co-immunoprecipitation experiment, the membrane proteins were solubilized by a non-ionic detergent, and the co-precipitation was not due to intact cell membranes. A model for the protein complex is shown in Fig. 3C.

SaePQ suppresses the SaeRS-mediated signaling

Since SaeP and SaeQ form a protein complex with SaeS, we hypothesized that the two proteins affect the SaeRS-mediated signaling via SaeS. To investigate this hypothesis, we ectopically expressed SaeP and/or SaeQ from a plasmid using an anhydrotetracycline-inducible promoter, and measured their effects on the overall SaeRS-mediated signaling by monitoring the promoter activity of three well known *sae* targets: *hla* (alpha-hemolysin), *coa* (coagulase), and P1 of the *sae* operon (Mainiero et al., 2010, Jeong et al., 2011). At the inducer concentration we utilized (100 ng/ml), SaeP and SaeQ were expressed at a level similar to that seen with HNP-1 induction (Fig. S1). As a *sae* target gene with high affinity to P-SaeR (i.e., class II target), *hla* does not require induction of the SaeRS TCS for its transcription (Geiger et al., 2008), while *coa* and the P1 promoter of *sae* operon both do (class I targets) (Mainiero et al., 2010, Jeong et al., 2011). For this analysis, we used the strain of USA300-P23 producing wild type SaeS because the L18P mutation in Newman SaeS might skew the effect of SaePQ. When both SaeP and SaeQ were expressed in the absence of the inducer HNP-1, the activity of all three promoters was reduced; however, the reduction was more prominent in *coa* promoter and P1 promoter, and virtually no activity was detected (Fig. 4A). When SaeQ alone was expressed, only the activity of *coa* promoter was decreased. On the other hand, expression of SaeP alone had no effect. When we repeated the experiment in the presence of HNP-1, we obtained similar results except that *coa* promoter activity was not completely abolished (Fig. 4A). These results indicate that 1) SaeP and SaeQ suppress the SaeRS-mediated signaling, 2) the efficient suppression requires both SaeP and SaeQ, and 3) the low affinity targets, *coa* and P1 promoters, are more susceptible to the suppression.

To confirm the negative regulatory effect of SaeP and SaeQ on the SaeRS-mediated signaling at the protein level, we carried out Western blot analysis for Hla and Coa. As reported previously, without HNP-1, Coa was not detected (Coa in Fig. 4B) (Jeong et al., 2011, Mainiero et al., 2010). On the other hand, the expression of Hla was not significantly altered by SaePQ (Hla in Fig. 4B). When induced by HNP-1, Coa was detected; but its production was noticeably reduced by the expression of SaeP, SaeQ or SaePQ (Fig. 4B). Again, no significant difference was observed in Hla expression (Fig. 4B). Notably, the expression of SaeP and/or SaeQ from the plasmids did not significantly affect the level of the signaling molecules SaeR and SaeS (Fig. 4B), showing that the negative regulatory effect of SaePQ was not caused by alteration in the amounts of SaeR and SaeS.

The complex SaePQ specifically promotes dephosphorylation of P-SaeR

The protein complex formation in Fig. 3 strongly suggests that SaePQ suppresses the SaeRS-mediated signaling via SaeS. To determine the enzyme activity affected by SaePQ, we measured the autokinase, phosphoryl transfer, and phosphatase activity of SaeS in the presence or absence of SaePQ. To provide SaeP and/or SaeQ, we ectopically expressed SaeP and/or SaeQ in a *sae*-deletion mutant of strain Newman (NMA Δ sae), and purified the cell membranes. When cell membranes containing SaeP and/or SaeQ were added, neither the autophosphorylation of SaeS^c nor stability of P-SaeS^c was affected (Fig. S2). When the membranes were added to the phosphoryl transfer reaction, where the phosphoryl group in P-SaeS^c is transferred to SaeR, again no significant difference was observed until 10 min

into the reaction when the level of P-SaeR peaked (Fig. 5A), implying that SaePQ does not inhibit the phosphoryl transfer reaction either. However, the level of P-SaeR was significantly decreased at 40 min in the presence of SaeQ or both at 20 min and 40 min in the presence of SaePQ (Q and PQ in Fig. 5A and Fig. S3), strongly suggesting that SaePQ suppresses the SaeRS-mediated signaling via dephosphorylation of P-SaeR.

Assuming that the dephosphorylation of P-SaeR was carried out by SaeS^c, the promotion of P-SaeR dephosphorylation by SaePQ-containing membranes suggests that the transmembrane domains of SaeS are not required either for P-SaeR dephosphorylation or for interaction with SaePQ, and that SaeS-SaePQ interaction is probably mediated by the cytoplasmic domains of SaeS and SaeQ (Fig. 3C). Testing this hypothesis with the bacterial two hybrid system was unsuccessful because of the instability of the plasmid containing SaeS^c. Therefore, as an alternative approach, we examined the SaeS^c-SaeQ interaction by co-immunoprecipitation, where the intact membranes containing SaeP and/or SaeQ were mixed with SaeS^c and treated with anti-SaeQ antibody. As shown in Fig. 3D, when either SaeQ or SaePQ-containing membrane was present, SaeS^c was co-immunoprecipitated by anti-SaeQ antibody, suggesting that SaeQ and SaeS interact with each other via their cytoplasmic domains.

To investigate whether SaePQ can promote P-SaeR dephosphorylation in *in vivo* conditions, we added [³²P]-orthophosphate to the cells and compared the level of P-SaeR by immunoprecipitation. As shown in Fig. 5B, when SaePQ was present, the level of P-SaeR was reduced approximately by 40%, confirming the *in vivo* promotion of P-SaeR dephosphorylation by SaePQ.

SaePQ activates phosphatase activity of SaeS

Although unlikely, the previous experimental results do not exclude the possibility that dephosphorylation of SaeR is carried out by SaePQ, not by SaeS. To exclude that possibility, we mutated the following three amino acids in SaeS: H132, D133 and T136. H132 is the phosphorylated residue and is essential for kinase activity, whereas D133 and T136 were predicted to be critical for phosphatase activity (Huynh *et al.*, 2010). When these mutant SaeS^c proteins were subjected to the autophosphorylation reaction, the H132Q and D133A mutants showed no autokinase activity, while T136A mutant showed approximately 75% wild type activity (H/Q, D/A, and T/A in Fig. 6A). To measure the effect of the mutations on the phosphatase activity, first, we phosphorylated SaeR with wild type His-SaeS^c and eliminated His-SaeS^c with Ni-column chromatography. Then we mixed the purified P-SaeR with the mutant SaeS^c proteins in the presence of the cell membranes containing SaePQ. As a control, we also used membranes without SaePQ (PQ – in Fig. 6B). As can be seen, the wild type SaeS^c protein significantly dephosphorylated P-SaeR only in the presence of the membranes containing SaePQ (WT in Fig. 6B). On the other hand, the H132Q and D133A mutants of SaeS^c did not dephosphorylate P-SaeR even in the presence of SaePQ (H/Q and D/A in Fig. 6B), demonstrating that the phosphatase activity is provided by SaeS, not by the membranes containing SaePQ. Finally, the T136A mutant of SaeS^c showed approximately 25% wild type phosphatase activity (T/A in Fig. 6B), suggesting that, in SaeS, the autokinase and phosphatase activities are closely connected.

To test whether SaePQ activates the phosphatase activity of native SaeS in the membrane, we carried out P-SaeR dephosphorylation with membranes containing SaeS and/or SaePQ. Without induction by HNP-1, wild type cells do not produce SaePQ at early exponential growth phase (Jeong et al., 2011); therefore, membranes from wild type USA300-P23 or its P1 promoter mutant (P1m) at the early exponential growth phase contain only SaeS (Jeong et al., 2011). On the other hand, membranes from the *sae*-deletion mutant of strain Newman carrying pAT-PQ contain only SaePQ. The membranes from wild type cells induced by

HNP-1 or transformed with pAT-PQ contain both SaeS and SaePQ. When P-SaeR was mixed with membranes containing SaePQ alone, the half-life of the protein was 160 min (Δ +PQ in Fig. 6C). The half-life was reduced to 50 min when mixed with the membranes containing SaeS alone (WT and P1m in Fig. 6C), suggesting that the native form of SaeS in the membrane might have a low level of P-SaeR phosphatase activity. However, when P-SaeR was mixed with the membranes containing both SaeS and SaePQ, the half-life of P-SaeR was greatly reduced to approximately 10 min (11 min for +HNP and 9.5 min for +PQ in Fig. 6C), demonstrating that SaePQ can activate the phosphatase activity of the native SaeS in the membrane.

SaeP and SaeQ enable differential target expression by the SaeRS TCS

When ectopically expressed, SaePQ deactivated the expression of *sae* target genes in a selective manner (Fig. 4). To confirm the selective deactivation in natural conditions, we induced the strain USA300-P23 with HNP-1 and measured the promoter activity of *hla*, *coa* and *sae* P1 by promoter-*lacZ* reporter assays. Upon HNP-1 induction, the *hla* promoter activity was gradually increased, while the *coa* promoter activity was increased until 1.5 h, but decreased at 2 h, which coincides with the induction of P1 promoter activation, an indicator for SaePQ expression (WT in Fig. 7). To verify that SaePQ is responsible for the decreased activity of the *coa* promoter, we repeated the experiment with the P1 promoter mutant USA300-P1m that does not produce SaePQ (Jeong et al., 2011). In this strain, when the P1 promoter was induced at 1.5 h post induction, the activity of the *coa* promoter was not decreased (P1m in Fig. 7), indicating that SaePQ is probably responsible for the decreased activity of the *coa* promoter.

Most *sae* target genes are affected by SaePQ

To examine the global effect of SaePQ on *sae* target gene expression, we treated the wild type strain of USA300-P23 and its P1 promoter mutant with HNP-1 and analyzed the transcription of *sae* target genes by microarray assays (Fig. 8). SAUSA300_1587, which has a perfect SaeR binding site (GTTAAN₆GTAA, where N represents any nucleotide) (Nygaard et al., 2009, Sun et al., 2010b), was repressed by HNP-1 (Fig. 8 and S4A); however, the transcription of most other *sae* target genes was induced (Fig. 8). More importantly, except for alpha-hemolysin and staphylokinase, the induction level was significantly lower in wild type than in the P1 promoter mutant (WT vs. P1m in Fig. 8 and S4B), demonstrating that the SaePQ-induced phosphatase activity of SaeS imposes global effects on the expression of *sae* target genes.

Discussion

In *S. aureus*, the SaeRS TCS has been extensively studied due to its critical role in virulence factor production and bacterial pathogenesis. Although it is well established that activation of SaeS leads to phosphorylation of SaeR, and that SaeR phosphorylation is essential for its DNA binding and transcriptional activation (Nygaard et al., 2009, Sun et al., 2010b), the molecular basis underlying dephosphorylation of P-SaeR has not been known. In this study, we showed that the direct binding of the SaePQ complex to SaeS activates phosphatase activity of SaeS, which leads to dephosphorylation of P-SaeR and deactivation of *sae* target gene expression.

SaeQ is predicted to be a membrane protein with three transmembrane domains and two cytoplasmic domains (Fig. 2A and 3C). Since SaeQ in the membrane is co-immunoprecipitated with the soluble SaeS^c (Fig. 3D), it is likely that the SaeQ-SaeS interaction occurs via their cytoplasmic domains. Conformational changes by the SaeQ binding might expose the key amino acids for the phosphatase activity without altering

autokinase activity (Fig. 6 and S2). Protein sequence analysis with SMART (Simple Modular Architecture Research Tool, <http://smart.embl.de/>) showed that SaeQ has the DoxX domain (8 a.a. to 109 a.a.), whose function is unknown (Letunic *et al.*, 2012). It remains to be determined whether the domain has a role in the interaction with SaeS or SaeP. Unlike SaeQ, most parts of SaeP, except for the membrane anchoring region, are expected to reside outside of cytoplasmic membrane (Fig. 3C). Therefore, SaeP is expected to interact with SaeQ and SaeS via their extracytoplasmic domains, and this interaction appears to be required for full activation of the phosphatase activity of SaeS (Fig. 4 and 5). The SMART analysis showed that SaeP contains the DM13 domain (55 a.a. – 146 a.a.), whose function might involve a redox reaction with a conserved cysteine residue (Iyer *et al.*, 2007). At this time, it is unknown whether the domain is involved in interactions with SaeQ and SaeS. Since both SaeP and SaeQ have relatively long half-lives, 90 min for SaeP and 41 min for SaeQ (Fig. S5), once the proteins are produced, they are expected to induce the phosphatase activity of SaeS persistently even in the absence of inducer.

When induced by HNP-1, the wild type strain carrying pAT-PQ showed very little P1 activity (P1-lacZ in Fig. 4A), while the strain without the plasmid showed much higher P1 promoter activity (P1 in Fig. 7). These results can be explained by the distinct concentrations of SaePQ in those strains. pAT-PQ produces SaePQ at a level similar to HNP-1 induction (Fig. S1); therefore, upon induction by HNP-1, the cells with pAT-PQ will produce twice the SaePQ than cells without the plasmid. The increased level of SaePQ is expected to induce higher phosphatase activity, resulting in almost abolished P1 promoter activity (P1-lacZ in Fig. 4A). Intriguingly, compared with the *coa* promoter, the P1 promoter is induced and deactivated more slowly (Fig. 7), making it an ideal promoter for the negative feedback system. The slow induction of P1 would initially allow expression of other *sae* regulons; then, once SaeP and SaeQ are expressed, the slow deactivation of P1 activity along with the long half-lives of SaeP and SaeQ (Fig. S5) would ensure a long-lasting inhibitory effect on the expression of the low affinity *sae* targets.

The activity of TCS is often controlled via its sensor kinase and, in some cases, the molecular mechanism of the control is known. For instance, PII, an inhibitor of the nitrogen regulator NtrBC (NRII/NRI) TCS, reduces the activity of NtrBC by inhibiting the autokinase activity and activating the phosphatase activity of sensor kinase NtrB (Jiang & Ninfa, 1999, Pioszak *et al.*, 2000, Pioszak & Ninfa, 2003). On the other hand, in FixLJ TCS of *Sinorhizobium meliloti*, a TCS involved in induction of nitrogen fixation and respiratory genes, FixT inhibits the activity of the TCS either by blocking autokinase activity or by destabilizing the phosphorylated form of the sensor kinase (Garnerone *et al.*, 1999). It was suggested that FixT acts by mimicking a response regulator (Crosson *et al.*, 2005). Unlike PII or FixT, however, the SaePQ complex does not seem to affect the autokinase activity of SaeS or the stability of P-SaeS (Fig. S2) and only activates the phosphatase activity of the HK (Fig.6). In addition, while both PII and FixT are located in cytoplasm, the SaePQ complex is in membrane, demonstrating the diverse regulation strategies among bacterial TCSs. In the NtrBC TCS, as a signal transducing protein itself, PII senses α -ketoglutarate, ATP, ADP, and glutamine, and its activity is also controlled by covalent modification (Jiang & Ninfa, 2009a, Jiang & Ninfa, 2009b, Ninfa & Jiang, 2005); therefore, by involving PII in the signaling process of NtrBC TCS, *E. coli* can integrate multiple cellular cues to make decisions in nitrogen assimilation (Jiang & Ninfa, 2009a, Keener & Kustu, 1988). At this stage, however, there is no evidence that SaeP or SaeQ is capable of sensing other environmental cues.

Recent studies suggest that PAS (Per-Arnt-Sim) domain and affinity to ADP play an important role for phosphatase activity of a HK (Yamada *et al.*, 2009, Yeo *et al.*, 2012, Gutu *et al.*, 2010). PAS domain is a widespread protein module found in signaling proteins and

involved in ligand binding and protein-protein interactions (Henry & Crosson, 2011, Huang *et al.*, 1993, Moglich *et al.*, 2009). In their study on the WalRK of *Streptococcus pneumoniae*, an essential TCS controlling cell wall homeostasis and response to antibiotic stress, Gutu, A.D. et al showed that deletion of the PAS domain in WalK greatly reduces the phosphatase activity of the sensor kinase (Gutu *et al.*, 2010). Likewise, in their structural study of ThkA/TrrA TCS system in *Thermotoga maritima*, Yamada, S. et al also showed that deletion of the PAS domain abolished P-TrrA phosphatase activity of the ThkA, the sensor kinase of the system (Yamada *et al.*, 2009). On the other hand, in their study on the bi-functional HK PhoQ of *Salmonella enterica*, Yeo, W-S. et al demonstrated that the main determinant for phosphatase activity of PhoQ is the HK's affinity to ADP that was generated by the hydrolysis of ATP in the nucleotide binding pocket of the HK (Yeo *et al.*, 2012). Since SaeS does not contain a PAS domain, the phosphatase activity of SaeS should be activated in a different manner from that of WalK in *S. Pneumoniae*. However, the ADP affinity model of PhoQ provides a testable hypothesis on the role of SaePQ in the activation of phosphatase activity of SaeS: SaePQ might induce the phosphatase activity of SaeS by increasing the affinity of SaeS to ADP.

Two sequence motifs were identified in the DHp (dimerization and histidine phosphotransfer) domain of HK to be critical for phosphatase activity: DxxxQ motif in HisKA_3 subfamily and E/DxxT/N motifs in HisKA subfamily (Huynh *et al.*, 2010). Both motifs reside right next to the phospho-accepting His residue. According to the current hypothesis by Huynh *et al*, in the DxxxQ motif, the Asp residue interacts with a Lys residue in the receiver domain of the RR while the Gln residue coordinates the nucleophilic water molecule for hydrolysis of the phosphoryl group (Huynh *et al.*, 2010, Huynh & Stewart, 2011). On the other hand, in the E/DxxT/N motif, Glu/Asp residues are not expected to play a critical role for phosphatase activity, whereas Thr/Asn residues are essential for phosphatase activity by coordinating the water molecule for an in-line nucleophilic attack of the phosphoryl group (Huynh & Stewart, 2011). SaeS has the DHp domain belonging to HisKA subfamily and contains the DxxT motif. In our study, however, the mutational change of Asp to Ala (D/A) abolished the phosphatase activity of SaeS, while the mutation of Thr to Ala (T/A) did not (D/A and T/A in Fig. 6B). This suggests that, in SaeS, the Asp residue is essential for phosphatase activity, and that the Thr residue might not be involved in coordination of the nucleophilic water molecule, although we cannot formally exclude the possibility that the T/A mutation allowed an alternative amino acid to participate in the dephosphorylation reaction. Nevertheless these results suggest that, in bacteria, bi-functional HKs dephosphorylate their cognate P-RR in more diverse ways than we currently appreciate.

Although the SaeRS TCS is also found in other staphylococci (such as *S. epidermidis*, *S. capitis*, and *S. carnosus*) only *S. aureus* has multiple virulence genes under the control of this TCS (Ravcheev *et al.*, 2011). Since the sensor kinase SaeS is activated by human neutrophil peptides, the *sae* regulon seems to have evolved to defend the bacterium from attacks by neutrophils. Indeed, the *sae* regulon includes CHIPS (SAUSA300_1920), which inhibits neutrophil chemotaxis toward C5a and formylated peptides (Rooijackers *et al.*, 2006, de Haas *et al.*, 2004), and the two-component toxins LukAB (SAUSA_1975-1974) and HlgACB (SAUSA_2365-2367), which are important in killing neutrophils (Malachowa *et al.*, 2011, Dumont *et al.*, 2011) (Fig. 8). In addition, the staphylokinase binds to HNPs and protects *S. aureus* from being killed by antimicrobial peptides (Jin *et al.*, 2004). Since the *sae* regulon also includes virulence factors involved in other aspects of defense (e.g., fibrinogen and fibronectin-binding proteins, serine proteases, and IgG-binding protein Sbi, etc), it seems that the role of SaeRS in staphylococcal pathogenesis has been further expanded and plays a key role in host-pathogen interactions. Therefore, further studies on the role of the TCS in host-pathogen interaction as well as the molecular mechanism by which SaePQ

activates phosphatase activity of SaeS will greatly enhance our understanding of the bacterial pathogenesis and facilitate the development of therapeutic measures.

Experimental Procedures

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. aureus* were grown in Luria-Bertani broth and tryptic soy broth (TSB), respectively. However, for transduction of plasmids, heart infusion broth (HIB) supplemented with 5 mM CaCl₂ was used. When necessary, antibiotics were added to the growth media at the following concentrations: ampicillin, 100 µg/ml; erythromycin, 10 µg/ml (for *S. aureus*) and 100 µg/ml (for *E. coli*); and chloramphenicol, 5 µg/ml, kanamycin, 50 µg/ml.

DNA manipulation

Unless stated otherwise, all restriction enzymes and DNA modification enzymes were purchased from New England Biolabs. Plasmids and genomic DNA were extracted with Zippy™ plasmid miniprep kit (Zymo Research) according to the manufacturer's instructions. Plasmid DNA was introduced into *E. coli* by the method of Hanahan and Meselson (Hanahan, 1983) and into *S. aureus* RN4220 by electroporation (Kraemer & Iandolo, 1990) with a gene pulser (Bio-Rad). Subsequent transduction of the plasmids into target strains of *S. aureus* was carried out with ϕ 85.

Construction of plasmids

To generate the expression vector for Maltose binding protein (MBP) fusions for SaeS^L, and SaeS^P, the genes *saeS^L* and *saeS^P* were PCR-amplified with the primer pairs P1637/P1058 (Table S1) from USA300-P23 and Newman, respectively. The amplified fragments were digested with *KpnI* and *BamHI*, and then inserted at the same sites of pMCSG19 (Donnelly *et al.*, 2006). For production of His-AirS and His-AirR, *airS* and *airR* were PCR-amplified with the primer pairs airS-F-LIC/airS-R-LIC and airR-F-LIC/airR-R-LIC (Table S1); and the resulting PCR products were treated with T4 DNA polymerase in the presence of dCTP for 30 min at room temperature. The target vector pMCSG7 (Donnelly *et al.*, 2006) was digested with *SspI*, gel-purified, and then treated with T4 DNA polymerase in the presence of dGTP at 16°C for 15 min. The PCR products and vector were purified, mixed, and incubated at room temperature for 5 min; then the mixture was transformed first into *E. coli* DH5 α and subsequently into BL21 star (DE3).

To generate *lacZ* fusion constructs for the coagulase promoter (Pcoa) and the alpha-hemolysin promoter (Phla), the promoter DNA sequences were amplified with the primer pairs P1161/P1162 and P931/639 (Table S1), respectively. The amplified fragments were digested with *KpnI* and *EcoRI*, and ligated with pCL-*lacZ* (Sun *et al.*, 2010b) digested with the same enzymes, resulting in pCL-Pcoa-*lacZ* and pCL-Phla-*lacZ*. Since pCL-*lacZ* integrates into *geh* (glycerol ester hydrolase) of staphylococcal genome, no antibiotics are required to maintain the plasmid constructs.

To construct expression vectors for SaeP, SaeQ, and SaePQ, insert DNA sequences were PCR-amplified with the primer pairs P1146/P1147, P1148/P1149, and P1146/P1149, respectively. The amplified fragments were digested with *KpnI*, and then inserted between *KpnI* and *EcoRV* sites of pYJ335 under the control of anhydrotetracycline (ATc) inducible promoter (Ji *et al.*, 1999). DNA fragment containing both the insert DNA and the ATc-inducible promoter module was cut out from the plasmid by *SaII* and *KpnI* digestion and inserted into pAT18 (Trieu-Cuot *et al.*, 1991) digested with the same enzymes, resulting in pAT-P, pAT-Q and pAT-PQ.

To produce mutant SaeS^c proteins, two DNA fragments were PCR-amplified from the Newman genome with the forward primer pair (P1353, P1522, or P1524)/P1527 for the first fragment and the complement primer pair (P1354, P1523, or P1525)/P1526 for the second fragments (Table S1). The first and second fragments were mixed and further subjected to PCR amplification with primer P1526 and P1527. The final PCR products were treated with T4 DNA polymerase and inserted into pMCSG19 as described above, resulting in pSaeS^c-H132Q, pSaeS^c-D133A, and pSaeS^c-T136A. The plasmids were transformed first into *E. coli* DH5 α and subsequently into BL21 star (DE3) carrying pRK1037 (Donnelly et al., 2006).

To generate the *lacZ* promoter fusion for SAUSA300_1587, we used a ligation independent cloning method (Donnelly et al., 2006, Aslanidis & de Jong, 1990). First, vector DNA was PCR-amplified from pCL-lacZ using the primers P1728 and P1729 (Table S1), while the promoter region of SAUSA300_1587 was amplified using the primers P1754 and P1755 (Table S1). Phusion (NEB), a PCR enzyme with high fidelity, was used for the amplifications. The PCR products were treated with T4 DNA polymerase in the presence of dCTP (vector) or dGTP (insert DNA) and mixed together. The DNA mixture was used to transform *E. coli* DH5 α . The resulting plasmid, pCL-P1587-lacZ, was electroporated into *S. aureus* strain RN4220 and then into USA300-P23 or USA300-P23 containing a transposon insertion in *saeR* (USA-01594, Table S1). USA-01594 was generated by transducing the *saeR* transposon in Φ NE-01594 into USA300-P23 with ϕ 85.

Recombinant protein expression and purification

For expression of MBP-SaeS proteins (i.e., MBP-SaeS^L and MBP-SaeS^P), the BL21 star (DE3) strain carrying the corresponding plasmid was grown in LB to exponential growth phase (OD₆₀₀ = 0.6); then 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added. The culture was further incubated at room temperature overnight. The MBP-SaeS fusion proteins were expressed as a soluble protein and purified with MBPTrap HP column (GE Healthcare) by following the column manufacturer's recommendations. Expression of AirS and AirR was carried out as described above except that the cells were induced at 16°C overnight. His-AirS and His-AirR were purified from the cells with HisTrap column (GE Healthcare). The purified protein was added with 20% glycerol and stored at -80 °C. The expression and purification of His-SaeR were carried out as described previously (Sun et al., 2010b).

AirR dephosphorylation reactions

His-tagged AirS (3 μ M) was autophosphorylated with 0.5 μ l [γ -³²P] ATP in 20 μ l of phosphorylation buffer (10 mM Tris HCl, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 10% glycerol) at room temperature for 10 min; then free [γ -³²P] ATP was eliminated with a Micro Bio-SpinTM Chromatography Column (Bio-rad). The His-tag of His-AirR was removed by TEV (tobacco etch virus) protease (Sigma) according to the manufacturer's recommendations. Phosphorylated His-AirS was mixed with the purified AirR (9 μ M) in 20 μ l of phosphorylation buffer and, if necessary, phosphorylated His-AirS was eliminated with a Ni-NTA affinity column (GenScript). At various time points (0, 1, 5, 10, 20, 40, and 60 min), 20 μ l of the sample was mixed with an equal volume of 2 \times SDS-PAGE loading buffer and analyzed by 13 % SDS-PAGE and autoradiography.

Fractionation of cell components

Test strains were grown in TSB to exponential growth phase (OD₆₀₀ = 1.0) and cells were collected by centrifugation. The supernatant (1 ml) was collected and designated culture supernatant. The cell pellet was washed with TSM (50 mM Tris HCl, 0.5 M sucrose, 10 mM MgCl₂, pH 7.5), suspended in 500 μ l TSM containing lysostaphin (40 μ g/ml), and

incubated at 37°C for 20 min. After centrifugation (4,600 g, 5 min), supernatant was obtained and designated cell wall fraction. The remaining protoplast in the pellet was suspended in 1 ml of membrane buffer (100 mM Tris HCl, 100 mM NaCl, 10 mM MgCl₂, pH 7.5). After sonication on ice for 10 seconds, the sample was subjected to ultracentrifugation (120,000 g) using SW50.1 rotor (Beckman) at 4°C for 30 min; then the supernatant was obtained and designated cytoplasm fraction, while the remaining pellet was designated membrane fraction. Except for the membrane fraction, proteins in all other fractions were precipitated by TCA (10% final concentration) and washed with acetone. All samples were finally suspended in 50 µl sample buffer (4% SDS, 0.5 M Tris HCl, pH 8.0) and then subjected to Western blot analysis.

Bacterial two hybrid protein interaction assay

Bacterial two hybrid tests were carried out by following the manufacturer's recommendations (EUROMEDEX). The *saeP*, *saeQ* and *saeS* gene sequences were amplified by PCR with primer pairs, P1276/P1213, P1214/P1215 and P1277/P1217 (Table S1). The amplified *saeP* and *saeQ* products were digested either with the *EcoRI* and *KpnI* (*saeP*) or with *EcoRI* and *BamHI* (*saeQ*) and then inserted into pKT25 to generate pKT25-*saeP* and pKT25-*saeQ*. In addition, the amplified *saeP* and *saeS* were digested with *EcoRI* and *KpnI*; then the digested fragments were ligated with pUT18C digested with the same enzymes to generate pUT18c-*saeP* and pUT18c-*saeS*. The test plasmids were co-transformed into *E. coli* DHM1. The interaction of the proteins was assessed by the β-galactosidase activity of the test strain.

β-galactosidase assays

The β-galactosidase assays were carried out as described by Sun et al (Sun et al., 2010b) with minor modifications. For bacterial two-hybrid assays, the test strains were grown in LB containing 100 µg/ml of ampicillin and 30 µg/ml of kanamycin at 30°C for 16 h. The cells were collected by centrifugation and suspended in AB buffer (100 mM potassium phosphate, 100 mM NaCl, pH 7.0) and treated with lysozyme (0.1 µg/ml) at 37°C for 15 min. For *S. aureus* stains, cells were grown in TSB and lysed with lysostaphin (0.1 µg/ml) treatment. After the addition of 900 µl of ABT buffer (AB buffer containing 0.1% Triton X-100) to the lysed cells, 50 µl of the cell lysate was mixed with 10 µl of MUG (4-methylumbelliferyl β-D-galactopyranoside, 4 mg/ml, Sigma) and incubated at room temperature for 1 h. Then 20 µl of the reaction was mixed with 180 µl ABT buffer in a black 96-well plate and the emission of fluorescence was measured by a plate reader (355 nm excitation, 445 nm emission). The LacZ activity was normalized by cell density at 600 nm, and the activity was determined by AU (arbitrary unit), where 1 AU corresponds to the generation of 1.2×10^8 mole of MU/h⁻¹ ml⁻¹ OD -1 600. This assay was repeated at least twice, generating similar results.

Co-immunoprecipitation

The strain Newman was grown in 3 ml TSB at 37°C with shaking (250 rpm) overnight. The next day cells were diluted 100 times with fresh TSB (3 ml); then further incubated at 37°C with shaking for 4 h. Cells were collected by centrifugation and treated with lysostaphin (0.1 µg/ml) in 10 ml TSM (50 mM Tris HCl, pH 7.5, 0.5 M sucrose, 10 mM MgCl₂) at 37°C for 15 min. Protoplasts were collected by centrifugation, suspend in 10 ml lysis buffer provided in the Co-IP kit (Pierce), and broken by bead beating (Precellys 24, Berton). Since the lysis buffer contains 1% NP-40, a detergent, membrane proteins will be released into the solution. An aliquot of 250 µg protein was used for co-immunoprecipitation test, which was carried out with the Co-IP Kit (Pierce) following the manufacturer's recommendations. Briefly, 30 µl of SaeP or SaeQ antibody was immobilized to resins and mixed with the cell lysate. The

bound materials were eluted, resolved by SDS-PAGE, and then subjected to Western blot analysis.

Co-immunoprecipitation for SaeQ in membrane and SaeS^c

His-SaeS^c (3 μ M) was mixed with the membranes (25 μ g total protein) purified from NMD Δ sae containing either pAT18 (vector control), pAT-P, pAT-Q, or pAT-PQ. Then 30 μ l of SaeQ antibody was immobilized to resins and mixed with the cell lysate. The bound materials were eluted, resolved by SDS-PAGE, and then subjected to Western blot analysis.

Phosphoryl transfer reaction

Purified SaeS^c (3 μ M) in 20 μ l of phosphorylation buffer was mixed with 0.5 μ l [γ -³²P]ATP (5 μ Ci) to initiate autophosphorylation. The reaction proceeded at room temperature for 10 min. After elimination of free [γ -³²P]ATP with a Micro Bio-SpinTM Chromatography Column (Bio-Rad), the phosphorylated HK proteins was mixed with SaeR (9 μ M) and, if necessary, cell membranes (25 μ g total protein) also in 20 μ l of phosphorylation buffer. At various time points (0, 5, 10, 20, 40, and 60 min), 15 μ l of the sample was mixed with an equal volume of 2 \times SDS loading buffer and analyzed by 15% SDS-PAGE and autoradiography.

In vivo immunoprecipitation

Test strains were grown in TSB to exponential growth phase (OD₆₀₀ = 0.6). Cells were collected by centrifugation, suspended in 500 μ l TSM containing lysostaphin (40 μ g/ml), and further incubated at 37°C for 20 min. Protoplasts were collected by centrifugation and suspended in 1 ml of phosphate-depleted CDM medium, in which HEPES substitutes for phosphate (Hussain *et al.*, 1991). [γ -³²P] orthophosphoric acid (100 μ Ci, Perkin Elmer) was added to the protoplasts; then the protoplasts were further incubated at 37°C for 1 h, collected by centrifugation, and finally broken by bead beating. SaeR protein was immunoprecipitated with SaeR antibody and analyzed by SDS-PAGE and autoradiography.

Western blot hybridization

Western blot analysis of proteins was carried out as described previously (Sun *et al.*, 2010b). The alpha-hemolysin antibody was purchased from Sigma. The SaeR and SaeS antibodies were generated in our laboratory. All other antibodies were generated by Genscript.

SaeR dephosphorylation reactions

The purified SaeR (9 μ M) was mixed with His-SaeS^c (3 μ M) and 0.5 μ l [γ -³²P] ATP in 20 μ l of phosphorylation buffer and incubated at room temperature for 10 min. After elimination of the HK with a Ni-NTA affinity column, cell membranes (25 μ g) were added to the phosphorylated SaeR. At various time points (0, 1, 5, 10, 20, 40, and 60 min), 20 μ l of the sample was mixed with SDS loading buffer and analyzed by 13 % SDS-PAGE and autoradiography.

Generation of probes for microarray experiments

DNA probes for microarray experiments were generated by adding 2 μ g of total RNA in a mixture containing 6 μ g of random hexamers (Invitrogen), 0.01 M dithiothreitol, an aminoallyl-deoxynucleoside triphosphate mixture containing 25 mM each dATP, dCTP, and dGTP, 15 mM dTTP, and 10 mM amino-allyl-dUTP (aa-dUTP) (Sigma), reaction buffer, and 400 units of SuperScript III reverse transcriptase (Invitrogen) at 42°C overnight. The RNA template then was hydrolyzed by adding NaOH and EDTA to a final concentration of 0.2 and 0.1 M, respectively, and incubating at 70°C for 15 min. Unincorporated aa-dUTP was removed with a QIAquick column (Qiagen). The probe was eluted with a phosphate

elution buffer (4 mM KPO₄, pH 8.5, in ultrapure water), dried, and suspended in 0.1 M sodium carbonate buffer (pH 9.0). To couple the aminoallyl cDNA with fluorescent labels, Cy3 or Cy5 (Amersham) was added for 1h. Uncoupled label was removed using the QIAquick PCR purification kit (Qiagen).

Microarray hybridization, scanning, image analysis, normalization, and analysis

Aminosilane-coated slides printed with a set of *S. aureus* open reading frame sequences (www.jcvi.org) were prehybridized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (Invitrogen), 0.1% sodium dodecyl sulfate, and 1% bovine serum albumin at 42°C for 60 min. The slides were then washed at room temperature with distilled water, dipped in isopropanol, and allowed to dry. Equal volumes of the appropriate Cy3- and Cy5-labeled probes were combined, dried and then suspended in a solution of 40% formamide, 5× SSC, and 0.1% sodium dodecyl sulfate. Suspended probes were heated to 95°C prior to hybridization. The probe mixture was then added to the microarray slide and allowed to hybridize overnight at 42°C. Hybridized slides were washed sequentially in solutions of 1×SSC-0.2% SDS, 0.1× SSC-0.2% SDS, and 0.1× SSC at room temperature, then dried in air, and scanned with an Axon GenePix 4000 scanner (<http://intranet.jtc.jcvf.org/sops/M008.pdf>). Individual TIFF images from each channel were analyzed with TIGR Spotfinder (available at <http://www.jcvi.org/software/tm4>) or Agilent Feature Extractor. Microarray data were normalized by LOWESS in TIGR MIDAS software (available at <http://www.jcvi.org/software/tm4>) and data visualized in TMEV. The results were deposited to the GEO repository (Accession number GSE35409).

Quantitative RT-PCR

qRT-PCR plates were prepared using 1:10 dilutions of a combined stock of forward and reverse primers at a concentration of 1.25 μM (Invitrogen). 10 μl of diluted 0.125 μM primers was added in quadruplicates to 384-well plates and were subsequently dried down. cDNA samples were generated using the overnight method described above to generate probes for microarray experiments without the use of aa-dUTP. 10 ng of each sample was then combined with 2.5 μl of 2× SYBR Green Master Mix (Roche) to a total reaction volume of 5 μl per well. qRT-PCR was performed in quadruplicate in a 5 μl reaction volume for all primer pairs (Table S1) and sample combinations. Median Cp values were calculated for the four qRT-PCR replicas. Quantile normalization was performed on median Cp values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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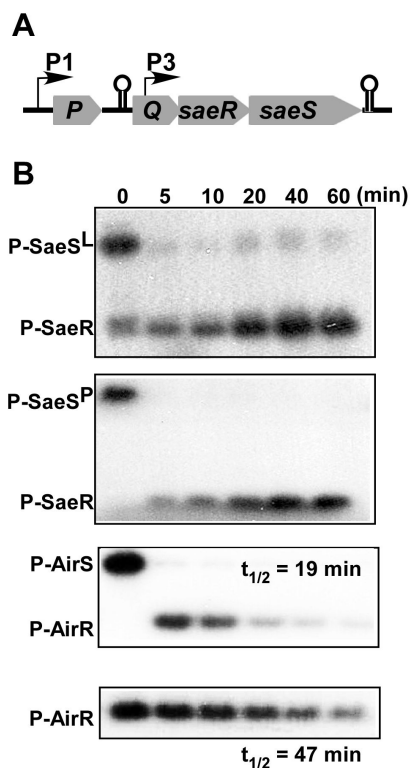


Figure 1. MBP-SaeS does not show detectable phosphatase activity

(A) A physical map for the *sae* operon. Stem-loop structures are indicated with lollipop shapes. Arrows represent P1 and P3 promoters. (B) Phosphoryl transfer reactions for the SaeRS TCS and AirSR TCS. Sensor kinases (MBP-SaeS and His-AirS) were autophosphorylated with [γ - 32 P]-ATP first; then response regulators (SaeR or AirR) were added and incubated at room temperature. The level of phosphorylated proteins was monitored by SDS-PAGE and autoradiography at the time points indicated. To measure the stability of P-AirR, AirS and [γ - 32 P]-ATP were eliminated; then P-AirR alone was incubated (the bottom panel). P-SaeS^L, phosphorylated MBP fusion of wild type SaeS; P-SaeS^P, phosphorylated MBP fusion of L18P mutant SaeS; His-AirS, His-tagged AirS; $t_{1/2}$, half-life. AirSR is also known as YhcSR (Sun *et al.*, 2005, Yan *et al.*, 2011, Sun *et al.*, 2012).

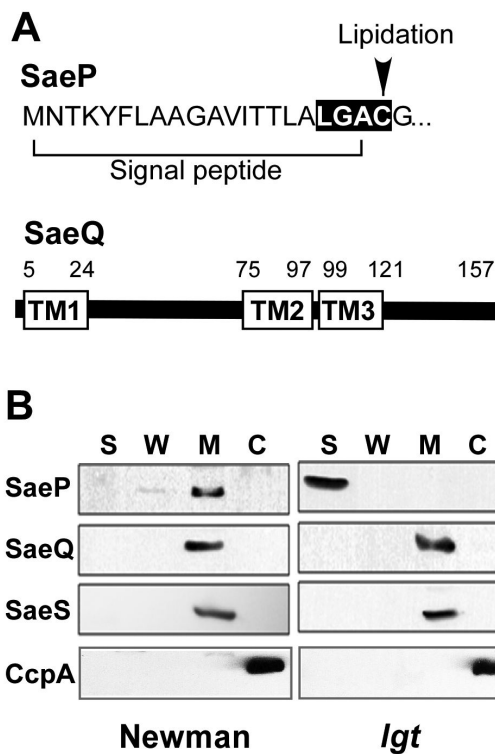


Figure 2. SaeP and SaeQ are in the cell membrane

(A) Lipobox in SaeP (top) and predicted transmembrane domains (TM) in SaeQ. The lipobox (LXXC) of SaeP was indicated with white letters in black background. The signal peptide and the predicted lipidation site (i.e., cysteine) are indicated. For SaeQ, transmembrane domains are indicated by white boxes with their locations in amino acid (a.a.). SaeQ is drawn to scale. SaeP sequence was analyzed with SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) and SaeQ with TMHMM v 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). (B) Localization of SaeP and SaeQ. Cell components were fractionated as described in Experimental Procedures. The subcellular location of SaeP and SaeQ was determined by Western blot analysis. SaeS, a known membrane protein, and CcpA (catabolite control protein A), a known cytoplasmic protein, were used as control proteins. S, culture supernatant; W, cell wall; M, cell membrane; C, cytoplasm; Newman, wild type Newman; *Igt*, Newman with a transposon insertion in *Igt* (prolipoprotein diacylglyceryl transferase).

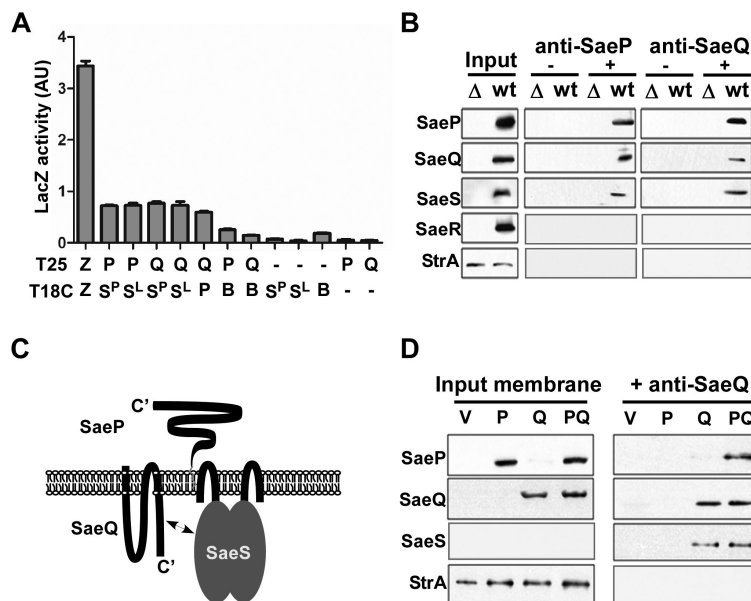


Figure 3. SaeP, SaeQ, and SaeS form a protein complex

(A) Bacterial two-hybrid assay for interactions between SaeP, SaeQ and SaeS. T25, T25 fusion; T18C, T18 fusion. Z, leucine zipper, a positive control; P, *saeP*; Q, *saeQ*; S^P, *saeS* encoding the L18P mutant SaeS^P; S^L, *saeS* encoding the wild type SaeS^L; B, *braS*, a negative control; -, no insert. (B) Co-immunoprecipitation with SaeP or SaeQ antibody. The Newman cell lysates were mixed either with anti-SaeP or anti-SaeQ antibody. Then the co-purified proteins were analyzed by Western blot analysis. -, no antibody; +, addition of antibody; wt, wild type Newman; Δ, *sae* deletion mutant Newman; SrtA; sortase A, a control membrane protein. (C) A model for the SaePQS ternary complex based on SaeQ sequence analysis with TMHMM v 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). The double-headed arrow indicates the interaction between SaeQ and the cytoplasmic domain of SaeS. (D) Co-immunoprecipitation of SaeS^c with SaeQ in membrane. Proteins were detected by Western blot analysis. V, vector control; P, SaeP; Q, SaeQ; PQ, SaePQ. Note that input membranes do not contain SaeS.

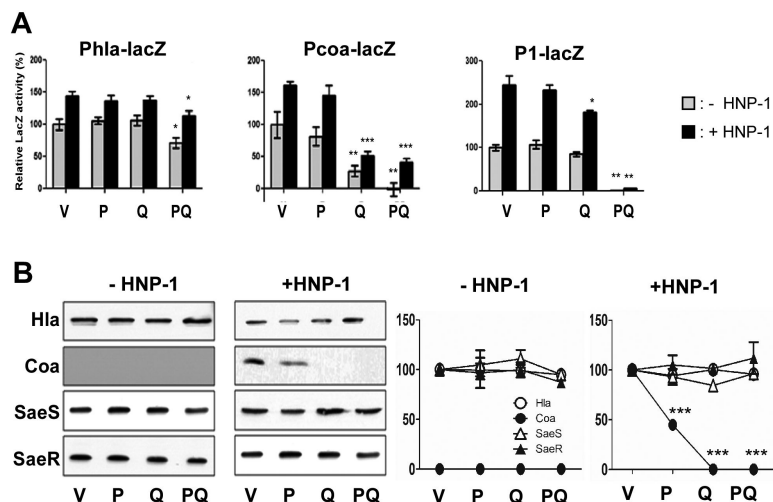


Figure 4. SaePQ suppresses the SaeRS-mediated signaling

(A) Effect of SaeP, SaeQ or SaePQ expression on the transcription activity of *hla*, *coa*, and P1 promoters. The promoter-*lacZ* fusion plasmids were generated with the single-copy reporter plasmid pCL-*lacZ* (Sun et al., 2010b), which is integrated in the chromosome of the strain USA300-P23. The test proteins were expressed from a tetracycline-inducible promoter in a multi-copy plasmid pAT18 (Trieu-Cuot et al., 1991). The promoter activity was measured by LacZ assays after 2 h growth in the presence (+) or absence (-) of human neutrophil peptide-1 (HNP-1, 5 μ g/ml). Each strain was compared with a control strain (V) and subjected to the *t*-test ($n = 3$). V, vector control; P, SaeP; Q, SaeQ; PQ, SaePQ. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (B) Effect of SaeP, SaeQ and SaePQ on the expression of alpha-hemolysin (Hla) and coagulase (Coa). Cells at the exponential growth phase were incubated in the absence or presence of HNP-1 for 2 h. Then Hla and Coa along with SaeS and SaeR were detected by Western blot analysis (left), quantified (right) and subjected to *t*-test ($n = 3$). ***, $p < 0.001$.

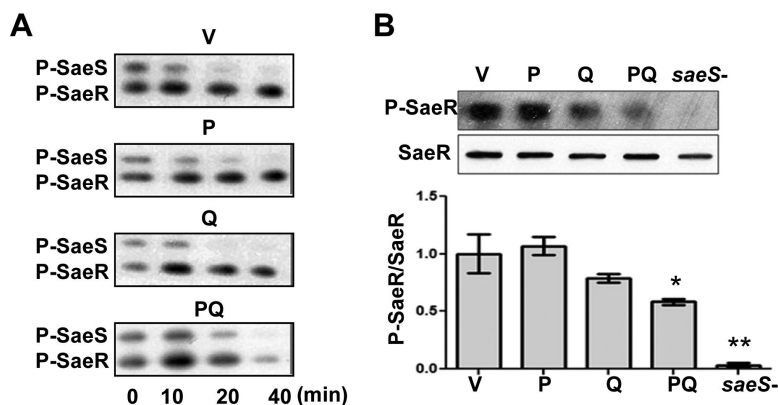


Figure 5. The complex SaePQ specifically promotes dephosphorylation of P-SaeR
(A) Effect of SaeP, SaeQ or SaePQ on the level of P-SaeR in the phosphoryltransfer reaction. The cytoplasmic domain of SaeS (SaeS^c) was phosphorylated with [γ -³²P] ATP; then SaeR and membrane fraction from test strains were added. V, vector control; P, SaeP; Q, SaeQ; PQ, SaePQ. The experiment was repeated three times, quantified and subjected to *t*-test (Fig. S3). **(B)** Effect of SaeP, SaeQ or SaePQ on phosphorylation level of SaeR in *in vivo* conditions. The protoplasts of USA300-P23 expressing SaeP, SaeQ, or SaePQ from plasmid were suspended in phosphate-depleted chemically defined medium, mixed with 100 μ Ci of [γ -³²P] orthophosphoric acid, and further incubated at 37°C for 1 h. After lysis of the protoplasts, SaeR proteins were immunoprecipitated. *saeS*⁻, a *saeS* transposon mutant strain of Newman. SaeR, loading control analyzed by Western blot analysis (top). The experiment was repeated three times, quantified and subjected to *t*-test (bottom). *, $p < 0.05$; **, $p < 0.01$.

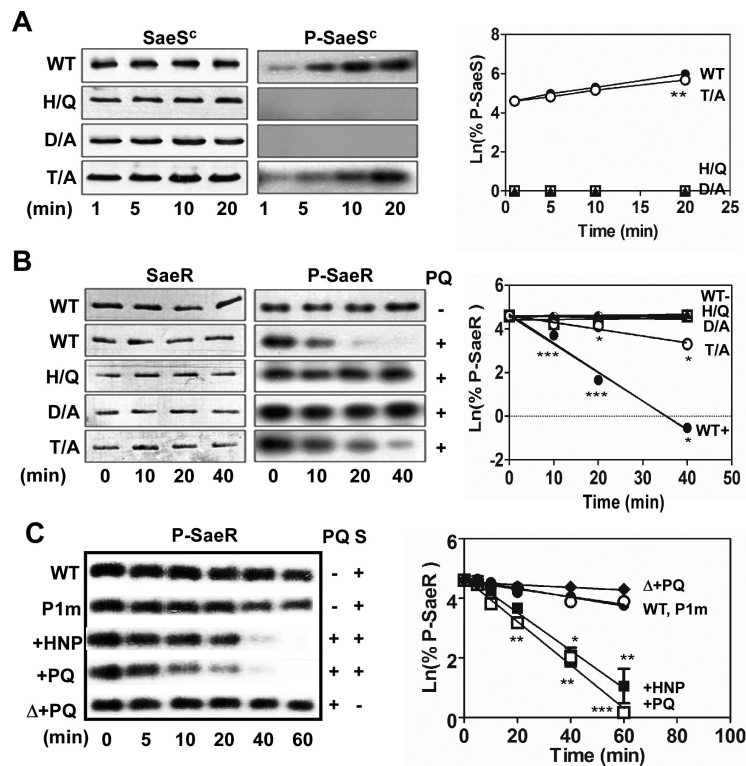


Figure 6. The complex SaePQ activates the phosphatase activity of SaeS

(A) Autokinase activity of mutant SaeS^c proteins. The proteins were incubated with [γ -³²P] ATP; then the phosphorylation was analyzed with SDS-PAGE and either Coomassie stain (left) or autoradiography (middle) at the time points indicated. The quantification results of the autoradiograph are also shown (right). WT, wild type SaeS^c; H/Q, H132Q mutant of SaeS^c; D/A, D133A mutant of SaeS^c; T/A, T136A mutant of SaeS^c. **, $p < 0.01$ by t -test ($n = 3$). (B) Phosphatase activity of the SaeS^c mutants in the presence of the membrane fractions containing SaePQ (PQ). To the purified P-SaeR, wild type or mutant SaeS^c proteins were added, and the reaction proceeded at room temperature and was analyzed with either Western blot analysis for SaeR (left) or autoradiography for P-SaeR (middle) at the time points indicated. WT, wild type SaeS^c; H/Q, H132Q mutant; D/A, D133A mutant; T/A, T136A mutant; -, the membranes purified from NM Δ sae without plasmid; +, the membranes purified from NM Δ sae carrying pAT-PQ. This experiment was repeated twice, quantified and subjected to t -test (right). *, $p < 0.05$; **, $p < 0.01$. (C) P-SaeR phosphatase activity of the membranes purified from USA300-P23 (WT), P1 mutant of USA300-P23 (P1m), USA300-P23 induced with HNP-1 (+HNP), USA300-P23 carrying pAT-PQ (+PQ), and *sae*-deletion mutant of the strain Newman carrying pAT-PQ (Δ +PQ). Membranes were mixed with purified P-SaeR; then their phosphatase activity was measured by SDS-PAGE and autoradiography. The experiment was repeated twice, quantified and subjected to t -test. PQ, SaePQ; S, SaeS; -, absence; +, presence. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

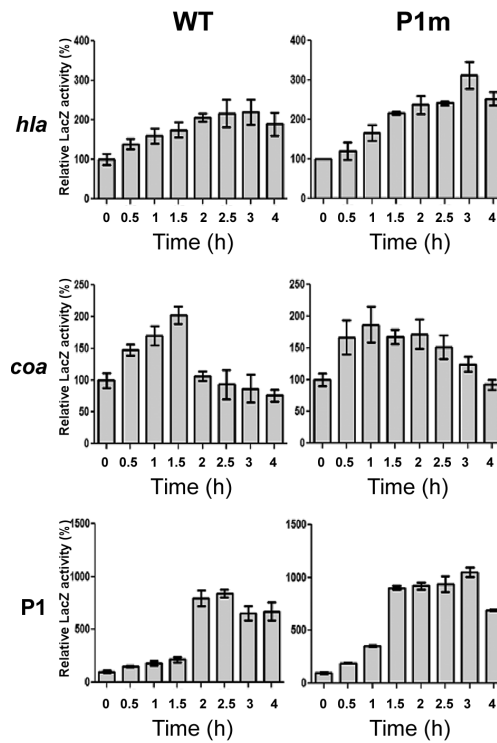


Figure 7. SaePQ renders differential target expression by the SaeRS system

The wild type (WT) and P1 mutant (P1m) strains of USA300-P23 were induced with HNP-1; then promoter activity of *coa*, *hla* and P1 was measured by *lacZ* reporter assays at the time points indicated. Error bars represent standard deviation (n = 3).

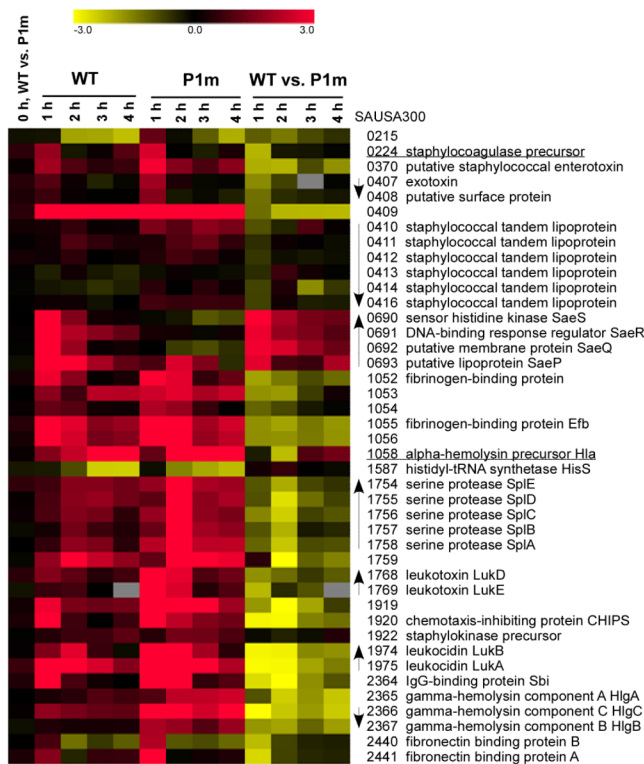


Figure 8. The expression of SaePQ downregulates most *sae* target genes
 The wild type (WT) and P1 mutant (P1m) strains of USA300-P23 were induced with HNP-1; then the *sae* target gene expression was measured by microarray analysis at the time points indicated. The first lane is for comparison of gene expression between wild type and the P1 mutant at 0 h. The gene numbers and function of the gene products are shown to the right of the picture. Operons are indicated with arrows pointing their transcription directions. The two *sae* targets, *coa* and *hla*, are underlined.

Table 1

Bacterial strains 746 and plasmids

Strain or plasmid	Relevant characteristic	Origin or reference
<i>E. coli</i>		
DH5 α	Plasmid free, Lac-	Startagene
DHM1	Reporter strain for BACTH assay	EUROMEDEX
<i>S. aureus</i>		
RN4220	Restriction deficient, prophage cured	(Kreiswirth <i>et al.</i> , 1983)
Newman	Clinical isolate, L18P substitution in SaeS	(Duthie & Lorenz, 1952)
NM Δ sae	Newman with deletion of the <i>sae</i> operon	(Sun <i>et al.</i> , 2010a)
USA300-P23	USA300-0114 without plasmid 2 and 3	(Jeong <i>et al.</i> , 2011)
USA300-P1m	USA300-P23 with P1 mutation	(Jeong <i>et al.</i> , 2011)
Φ NE-15201	Newman with transposon insertion in <i>lgt</i> , Em ^f	Phoenix library
Φ NE-09725	Newman with transposon insertion in <i>saeS</i> , Em ^f	Phoenix library
Φ NE-01594	Newman with transposon insertion in <i>saeR</i> , Em ^f	Phoenix library
USA-01594	USA300-P23 with transposon insertion in <i>saeR</i> , Em ^f	This study
<i>Plasmid</i>		
pMCSG19	A vector for high throughput protein productions	(Donnelly <i>et al.</i> , 2006)
pMCSG-sae ^{S^L} -FL	pMCSG19 carrying <i>saeS^L</i>	This study
pMCSG-sae ^{S^P} -FL	pMCSG19 carrying <i>saeS^P</i>	This study
pMCSG7	A vector for high throughput protein productions	(Donnelly <i>et al.</i> , 2006)
pMCSG-airS	pMCSG7 carrying <i>airS</i>	This study
pMCSG-airR	pMCSG7 carrying <i>airR</i>	This study
pCLitet	pCL55 carrying a tetracycline inducible promoter	(Grundling & Schneewind, 2007)
pCLitet-his-saeP	pCLitet carrying his-tagged <i>saeP</i>	This study
pKT25	low copy-number plasmid carrying T25 fragment of <i>cya</i>	EUROMEDEX
pUT18C	high copy-number plasmid carrying T18 fragment of <i>cya</i>	EUROMEDEX
pKT-saeP	pKT25 carrying <i>saeP</i>	This study
pKT-saeQ	pKT25 carrying <i>saeQ</i>	This study
pUT-saeP	pUT18C carrying <i>saeP</i>	This study
pUT-sae ^{S^P}	pUT18C carrying the <i>saeS</i> from Newman	This study
pUT-sae ^{S^L}	pUT18C carrying the <i>saeS</i> from USA300	This study
pYJ335	A shuttle vector for Gram ⁻ and Gram ⁺ , Erm ^f	(Ji <i>et al.</i> , 1999)
pAT18	A shuttle vector for Gram ⁻ and Gram ⁺ , Erm ^f	(Trieu-Cuot <i>et al.</i> , 1991)
pAT-P	pAT18 carrying <i>saeP</i> under tetracycline inducible promoter	This study
pAT-Q	pAT18 carrying <i>saeQ</i> under tetracycline inducible promoter	This study
pAT-PQ	pAT18 carrying <i>saePQ</i> under tetracycline inducible promoter	This study
pCL55	a single copy integration plasmid,	(Lee <i>et al.</i> , 1991)
pCL-lacZ	pCL55 carrying lacZ in its multi-cloning site	(Sun <i>et al.</i> , 2010b)
pCL-Phla-lacZ	pCL-lacZ carrying the <i>hla</i> promoter	This study
pCL-Pcoa-lacZ	pCL-lacZ carrying the <i>coa</i> promoter	This study
pCL-P1-lacZ	pCL-lacZ carrying the P1 promoter of <i>sae</i>	(Sun <i>et al.</i> , 2010b)

Strain or plasmid	Relevant characteristic	Origin or reference
pCL-P1587-lacZ	pCL-lacZ carrying the promoter region of SAUSA300_1587	This study
pSaeS ^c -H132Q	pMCSG19 producing SaeS ^c with H132Q mutation	This study
pSaeS ^c -D133A	pMCSG19 producing SaeS ^c with D133A mutation	This study
pSaeS ^c -T136A	pMCSG19 producing SaeS ^c with T136A mutation	This study

Ermr, erythromycin resistance.