The SAV1322 gene from *Staphylococcus aureus*: genomic and proteomic approaches to identification and characterization of gene function

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**Abstract**

**Background:** Bacterial two-component regulatory systems (TCRS) are associated with the expression of virulence factors and antibiotic susceptibility. In *Staphylococcus aureus*, 16 TCRS types have been identified. The histidine kinase/response regulator SAV1321/SAV1322 in *S. aureus* shares considerable homology with the TCRS DesKR in *Bacillus subtilis*. However, a function for the SAV1322 locus has not yet been assigned.

**Results:** Deletion of the SAV1322 locus in *S. aureus* results in reduced growth when cultured under low (25 °C) and high (46 °C) temperature conditions. The *sav1322* deletion mutant is more tolerant to oxidative stress in vitro and is less pathogenic in a murine infection model when compared with wild-type parent strain Mu50. Furthermore, the *sav1322* mutant exhibits lower MICs for gentamicin, tetracyclines and glycopeptides, increased autolysis, and a thinner cell wall when compared with the wild-type strain. Microarray and proteomic analyses show that the expression of cell-wall-associated genes *glmS* and *murZ* are lower, and the expression of heat shock and stress-related genes (*hrcA, ctsR, dnaK, dnaJ, grpE, clpB*, and *clpC*) are higher in the *sav1322* mutant when compared with the wild-type strain. In addition, the *sav1322* mutant displays altered expression of proteins involved in carbohydrate/energy metabolism, cell wall metabolism, and stress or heat shock response, as well as other metabolic processes including lipid metabolism, amino acid biosynthesis, purine or pyrimidine metabolism, transcription, and protein biosynthesis.

**Conclusions:** The *S. aureus* SAV1322 locus plays a pronounced role in temperature adaptation, antibiotic resistance, and virulence by regulating a wide range of genes and proteins involved in metabolism and stress tolerance.

**Keywords:** Heat shock proteins, Microarray analysis, Microbial drug resistance, Physiological stress, Proteomics, *Staphylococcus aureus*

**Abbreviations:** CFU, Colony-forming unit; MRSA, Methicillin-resistant *S. aureus*; ORF, Open reading frame; PBS, Phosphate-buffered saline; TCRS, Two-component regulatory system; TSA, Tryptic soy agar; TSB, Tryptic soy broth; VISA, Vancomycin-intermediate *S. aureus*; VRSA, Vancomycin-resistant *S. aureus*
Background

*Staphylococcus aureus* is a major cause of nosocomial infections, resulting in increased morbidity and mortality worldwide. Commonly reported methicillin-resistant *S. aureus* (MRSA) exhibits resistance to multiple chemotherapeutic agents, including β-lactams, quinolones, and aminoglycosides, while community-acquired MRSA is associated with serious infectious disease, sepsis, and pneumonia [1, 2]. More recently, vancomycin-intermediate or vancomycin-resistant *S. aureus* (VISA or VRSA) strains have emerged in several countries. These factors have rendered successful treatment of these infections increasingly problematic.

*S. aureus* has survived and thrived over the years in part because of its adaptability and stress response capabilities. It is resistant to a variety of environmental factors, including oxidative, pH, osmotic, antibiotic and temperature stressors [3]. Two-component regulatory systems (TCRSs) act in response to a stimulus that allows cells to sense and respond to changes in many different environmental conditions. Most *S. aureus* strains are endowed with 16 sets of genes that encode TCRSs. An additional TCRS is present in the staphylococcal cassette chromosome mec in MRSA, and this is linked to the induction of methicillin resistance [4]. The well-studied TCRS Agr is a positive regulator of exoproteins, including proteases, hemolysins, and toxins [5, 6]. Other TCRSs, such as SaeSR and ArlSR, influence the expression of some virulence factors [7, 8]. Another system, SrrAB, is homologous to the *Bacillus subtilis* TCRS ResDE; it is involved in the modulation of anaerobic gene expression and sensitivity to oxygen tension [9]. The *S. aureus* TCRS VraSR is homologous to *B. subtilis* YvqEC, and it modulates cell wall biosynthesis and increased resistance to vancomycin [10]. Another *S. aureus* TCRS, NreBC, controls nitrate reductase and nitrite reductase operons [11]. More recently, it was reported that the *S. aureus* TCRS WalKR is involved in cell wall metabolism, cell survival, and vancomycin resistance [12, 13].

Although most TCRSs identified in *S. aureus* have been well studied, the functions of a few remain elusive or only partially explained. Of these, the uncharacterized SAV1321/SAV1322 (Mu50) TCRS demonstrates homology with *B. subtilis* DesKR. DesKR has been described as a regulatory system involved in the maintenance of membrane fluidity in response to temperature downshift. With decreasing temperature, the membrane-bound sensor kinase DesK phosphorylates its corresponding response regulator, DesR, which then binds to a specific recognition sequence in the promoter region of the des gene to activate its transcription [14]. Finally, activity of the membrane-located fatty acid desaturase Des maintains membrane fluidity in the cold [15].

In this study, we generated an *S. aureus* mutant lacking the SAV1322 locus to examine its role in bacterial physiology and virulence. Comparative microarray and proteomic analyses were performed to determine putative members of the SAV1322 regulon.

Results

Construction and characterization of the *S. aureus sav1322 knockout mutant*

A *sav1322* knockout mutant strain was created from the wild-type (WT) *S. aureus* Mu50 using homologous recombination. Re-introducing the SAV1322 locus into the *sav1322* mutant generated a complementation strain. The WT SAV1322 locus is shown in Fig. 1a. To confirm allelic replacement, chromosomal DNA was isolated from the WT, *sav1322* mutant, and complementation strains. PCR analysis, sequencing, and RT-PCR confirmed the mutation (Fig. 1b).

To determine whether deletion of the *S. aureus* SAV1322 locus had any impact on bacterial growth and morphology, growth curves and colony forming units (CFU) were compared at 37 °C. We observed no difference in growth between the WT and the *sav1322* mutant strains at this temperature. Similarly, no obvious differences in colony morphology were observed (data not shown).

Temperature stress

To examine the role of the SAV1322 locus in response to heat and cold stress, we compared the growth rates of the WT, *sav1322* mutant, and complementation strains at 25 and 46 °C (Fig. 2). The *sav1322* mutant grew less at 46 °C when compared with the WT strain. The WT strain exhibited a normal growth curve from inoculation until 3 h, at which point a decline in growth was noted. The complementation strain exhibited a growth curve that was similar to the WT; however, a growth decline was noted after 1 h (Fig. 2a). At 25 °C, growth of the WT and mutant strains started to increase at 3 h, yet the growth rate for the WT strain was faster than that of the *sav1322* mutant strain. Growth of the complementation strain increased continuously, beginning immediately after inoculation at 25 °C (Fig. 2b).

Oxidative stress

To examine the role of the SAV1322 locus during oxidative stress, a disk diffusion assay with two different
concentrations of hydrogen peroxide (H$_2$O$_2$; 15 and 30 %) was performed. When compared with the WT strain, the sav1322 mutant strain displayed greater resistance to both hydrogen peroxide concentrations tested (Fig. 3). Sensitivity of the complementation strain to oxidative stress was similar to that of the WT strain for both hydrogen peroxide concentrations tested (Fig. 3).

Microarray transcriptional analysis
All of the microarray data have been deposited in the NCBI’s Gene Expression Omnibus (GEO) under accession number GSE85824. A comparison of the Mu50 and the sav1322 mutant transcription profiles revealed differential expression of 17 genes (Table 1). Notably, expression of two genes associated with cell wall peptidoglycan synthesis...
(murZ, encodes UDP-N-acetylglucosamine 1-carboxylvinyl transferase and glmS, encodes glucosamine-fructose-6-phosphate aminotransferase), was lower in the sav1322 mutant strain. Conversely, transcription of the chaperone genes dnaJ, dnaK, and grpE, as well as the negative heat shock regulator genes, hrcA and ctsR, was higher in the sav1322 mutant strain. Furthermore, transcript levels of two genes encoding Clp ATPases, clpB and clpC, were higher in the sav1322 mutant strain.

Two-dimensional gel electrophoresis of the WT and sav1322 mutant strains
Two-dimensional gel electrophoresis was used to examine differences in protein profiles between WT and sav1322 cell lysates (Additional file 2). We observed that proteins associated with carbohydrate metabolism, including those involved in the tricarboxylic acid (TCA) cycle exhibit lower expression in the sav1322 mutant when compared with the WT strain (Table 2). Moreover,

Table 1: Representative genes differentially expressed in the WT and sav1322 mutant strains

| Gene locus | Gene | Function | Fold change in sav1322 mutant |
|------------|------|----------|--------------------------------|
| SAV1147    | sdhC | Succinate dehydrogenase cytochrome b-558 | −1.65                          |
| SAV2123    |      | Hypothetical protein                      | −1.64                          |
| SAV2124    | murZ | UDP-N-acetylglucosamine 1-carboxylvinyl transferase | −2.02                          |
| SAV2125    | glmS | Glucosamine-fructose-6-phosphate aminotransferase | −1.64                          |
| SAV0052    | emaA | Adenine N-6-methyltransferase              | 1.55                           |
| SAV0522    | ctsR | Transcriptional regulator CtsR             | Stress response                 |
|            |      | Stress response                           | 1.76                           |
|            |      | Transcription regulation                  |                                |
| SAV0523    |      | Hypothetical protein                      | 2.01                           |
| SAV0524    |      | ATP:guanido-phosphotransferase            | 1.92                           |
| SAV0525    | clpC | ATP-dependent Clp protease ATP-binding subunit ClpC | Stress response | 1.72 |
| SAV0975    | clpB | ClpB chaperone homologue                  | Stress response                | 2.13 |
| SAV1350    | citB | Aconitate hydratase                       | Tricarboxylic acid cycle       | 1.55 |
| SAV1579    | dnaJ | Chaperone protein DnaJ                    | Stress response                | 1.56 |
| SAV1580    | dnaK | Chaperone protein DnaK                    | Stress response                | 2.30 |
| SAV1581    | grpE | Heat shock protein GrpE                   | Stress response                | 3.96 |
| SAV1582    | hrcA | Heat-inducible transcriptional repressor  | Stress response                | 3.16 |
| SAV1799    |      | Hypothetical protein                      | 2.23                           |
| SAV2497    |      | Putative membrane protein                 | 1.82                           |
Table 2: Representative proteins differentially expressed in the WT and sav1322 mutant strains

| Spot ID | Accessiona | Protein name                                      | Protein Score | Gene | Locusb | Expression change |
|---------|------------|---------------------------------------------------|---------------|------|--------|-------------------|
| A023    | gi|15924402  | Dihydrolipoamide succinyltransferase              | 714           | odhB  | SAV1412 | Down              |
| A025    | gi|15924086  | Dihydrolipoamide dehydrogenase                    | 1153          | pxdD  | SAV1096 | Down              |
| A277    | gi|15924841  | Fumarate hydratase                                | 601           | fumC  | SAV1851 | Down              |
| A043    | gi|15924684  | Isocitrate dehydrogenase                          | 845           | citC  | SAV1694 | Down              |
| A286    | gi|15924412  | Glucose-specific enzyme II, PTS system A component | 297           | cir   | SAV1422 | Down              |
| A287    | gi|15924701  | Acetate kinase                                     | 750           | ackA  | SAV1711 | Up                |

**Carbohydrate metabolism and transport**

| A029    | gi|15925597  | Malatequinone oxidoreductase                       | 1353          | mqa2  | SAV2607 | Down              |
| A055    | gi|15923231  | L-lactate dehydrogenase                            | 298           | lctE  | SAV2041 | Down              |
| A056    | gi|15923246  | Xylitol dehydrogenase                              | 78            | SAV0256 |        | Down              |
| A063    | gi|15924332  | Transketolase                                      | 684           | tkt   | SAV1342 | Down              |
| A071    | gi|15925406  | Phosphoglyceromutase                               | 364           | gpmA  | SAV2416 | Down              |
| A183    | gi|15924687  | Pyruvate kinase                                    | 433           | pykA  | SAV1697 | Down              |
| A008    | gi|15923216  | Formate acetyltransferase                          | 1155          | pfB   | SAV0226 | Up                |
| A058    | gi|15925514  | D-lactate dehydrogenase                            | 972           | Ddh   | SAV2524 | Up                |
| A078    | gi|15925115  | Fructose-bisphosphate aldolase                     | 62            | fbaA  | SAV2125 | Up                |
| A281    | gi|15923595  | Alcohol dehydrogenase                              | 1305          | Adh   | SAV0605 | Up                |

**Energy metabolism**

| A027    | gi|15924921  | Glycerol kinase                                    | 1037          | glpK  | SAV1301 | Down              |
| A054    | gi|15923640  | Dihydroxyacetone kinase subunit DhaK              | 420           | SAV0650 |        | Down              |
| A067    | gi|15924001  | Enoyl-(acyl carrier protein) reductase             | 526           | fabl  | SAV1011 | Down              |
| A072    | gi|15923029  | Glycerophosphoryl diester phosphodiesterase-like protein | 616       | SAV0039 |        | Down              |
| A089    | gi|15925088  | (3R)-hydroxymyristoyl-ACP dehydrogenase           | 125           | fabZ  | SAV2098 | Up                |

**Energy metabolism**

| A039    | gi|15923948  | NAD-specific glutamate dehydrogenase              | 970           | gudB  | SAV0958 | Down              |
| A046    | gi|15923947  | Ornithine–oxo-acid transaminase                    | 1027          | rocD  | SAV0957 | Down              |
| A045    | gi|15925103  | Serine hydroxymethyltransferase                    | 365           | glyA  | SAV2113 | Down              |
| A049    | gi|15924192  | Carbamoyl phosphate synthase small subunit         | 923           | pyrAA | SAV1202 | Up                |
| A051    | gi|15924159  | Ornithine carbamoyltransferase                     | 556           | argF  | SAV1169 | Up                |
| A060    | gi|15924160  | Carbamate kinase                                   | 990           | arcC1 | SAV1170 | Up                |
| A280    | gi|15924727  | Bifunctional 3-deoxy-7-phophoheptulonate synthase/chorismate mutase | 434       | SAV1737 |        | Up                |

**Cell division, cell wall, cell envelope biogenesis**

| A017    | gi|15925144  | Glucosamine–fructose-6-phosphate aminotransferase | 167           | glmS  | SAV2154 | Down              |
| A032    | gi|15925072  | UDP-N-acetylMuramoyl-tripeptide–D-alanyl-D-alanine ligase | 792       | murF  | SAV2082 | Down              |
| A033    | gi|15925114  | UDP-N-acetylglucosamine-1-carboxylvinyl transferase | 1062      | murZ  | SAV2124 | Down              |
| A048    | gi|15923145  | Capsular polysaccharide synthesis enzyme Cap5G    | 518           | capG  | SAV0155 | Down              |
| A274    | gi|15924665  | Trigger factor                                     | 1278          | tig   | SAV1675 | Down              |

**Purine and pyrimidine metabolism**

| A079    | gi|15924058  | Phosphoribosylformylglycinamidine synthase         | 162           | purQ  | SAV1068 | Down              |
| A035    | gi|15924191  | Dihydroorotase                                     | 866           | pyrC  | SAV1201 | Up                |
| A059    | gi|15924190  | Aspartate carbamoyltransferase                     | 328           | pyrB  | SAV1200 | Up                |
| A080    | gi|15925102  | Uric acid phosphoribosyltransferase                | 206           | Upp   | SAV2112 | Up                |
several proteins involved in glycolysis/gluconeogenesis were differentially expressed; expression of fructose-biphosphate aldolase (FbaA) and alcohol dehydrogenase (Adh) proteins was greater in the sav1322 mutant, while expression of transketolase, phosphoglyceromutase (GpmA), and pyruvate kinase (PykA) was lower when compared with the WT strain. We also observed a lower expression levels for L-lactate dehydrogenase (LctE) and higher expression of D-

Table 2 Representative proteins differentially expressed in the WT and sav1322 mutant strains (Continued)

| Accession number | Description | Strain | Expression |
|------------------|-------------|--------|------------|
| A021 gi|15923537 | Elongation factor G | 411 | fusA | SAV0547 | Down |
| A031 gi|15923518 | Glutamyl-tRNA synthetase | 264 | gltX | SAV0528 | Down |
| A053 gi|15924247 | Elongation factor Ts | 317 | Tsf | SAV1257 | Down |
| A061 gi|15924246 | 3OS ribosomal protein S2 | 465 | rpsB | SAV1256 | Down |
| A065 gi|15923491 | 5OS ribosomal protein L2S | 424 | rplY | SAV0501 | Down |
| A069 gi|15923538 | Elongation factor Tu | 123 | Tuf | SAV0548 | Down |
| A070 gi|15925240 | 3OS ribosomal protein L3 | 516 | rplC | SAV2250 | Down |
| A076 gi|15924709 | 3OS ribosomal protein S4 | 532 | rpsD | SAV1719 | Down |
| A096 gi|15924253 | Prolyl-tRNA synthetase | 176 | proS | SAV1263 | Down |
| A108 gi|15925214 | DNA-directed RNA polymerase subunit alpha | 905 | rpoA | SAV2224 | Down |
| A276 gi|15924256 | Transcription elongation factor NusA | 931 | nusA | SAV1266 | Down |
| A030 gi|15924889 | Aspartyl/glutamyl-tRNA amidotransferase subunit B | 336 | gatB | SAV1899 | Up |
| A081 gi|15922999 | Seryl-tRNA synthetase | 379 | serS | SAV0009 | Up |
| A087 gi|15923529 | 5OS ribosomal protein L10 | 292 | rplJ | SAV0539 | Up |

**Stress response and heat shock**

| Accession number | Description | Strain | Expression |
|------------------|-------------|--------|------------|
| A066 gi|15923693 | Oxidoreductase | 242 | - | SAV0703 | Down |
| A084 gi|15924414 | Methionine sulfoxide reductase A | 605 | mtaA2 | SAV1424 | Down |
| A090 gi|15924413 | Methionine sulfoxide reductase B | 342 | mtaB | SAV1423 | Down |
| A138 gi|15925129 | General stress protein 20U | 519 | dps | SAV2139 | Down |
| A102 gi|15925538 | ATP-dependent Clp proteinase chain | 2194 | clpL | SAV2548 | Up |

**Others**

| Accession number | Description | Strain | Expression |
|------------------|-------------|--------|------------|
| A042 gi|15924396 | Tellurite resistance protein | 716 | - | SAV1406 | Down |
| A064 gi|15923958 | S-oxo-1,2,5-tricarboxilic-3-penten acid decarboxylase | 123 | - | SAV0968 | Down |
| A088 gi|15924853 | Bacterioferritin comigratory protein | 959 | - | SAV1863 | Down |
| A016 gi|15923990 | Thimot oligopeptidase-like protein | 733 | - | SAV1000 | Up |
| A020 gi|15925280 | Urease subunit alpha | 862 | ureC | SAV2290 | Up |
| A044 gi|15923092 | Aminoacylase | 628 | - | SAV0102 | Up |
| A091 gi|15925281 | Urease accessory protein UreE | 209 | ureE | SAV2291 | Up |
| A114 gi|15925318 | Dehydrogenase | 389 | - | SAV2328 | Up |
| A285 gi|15924697 | Metal-dependent hydrolase | 609 | - | SAV1707 | Up |

**Unknown function**

| Accession number | Description | Strain | Expression |
|------------------|-------------|--------|------------|
| A040 gi|15924926 | Hypothetical protein | 700 | - | SAV1936 | Down |
| A062 gi|15923824 | Hypothetical protein SAV0834 | 537 | - | SAV0834 | Down |
| A074 gi|15924667 | Hypothetical protein SAV1677 | 436 | - | SAV1677 | Down |
| A075 gi|15925559 | Hypothetical protein SAV2569 | 203 | - | SAV2569 | Down |
| A083 gi|15924605 | Hypothetical protein SAV1615 | 91 | - | SAV1615 | Down |
| A086 gi|15924342 | Hypothetical protein SAV1352 | 256 | - | SAV1352 | Down |
| A085 gi|15925677 | Hypothetical protein SAV2687 | 746 | - | SAV2687 | Up |

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*aAccession numbers are from the NCBI protein database

*bDesignate ORF number/gene locus in the S. aureus Mu50 genome

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transketolase, phosphoglyceromutase (GpmA), and pyruvate kinase (PykA) was lower when compared with the WT strain. We also observed a lower expression levels for L-lactate dehydrogenase (LctE) and higher expression of D-
lactate dehydrogenase (Ddh) in the mutant; both of these proteins are associated with the interconversion of pyruvate and lactate.

Interestingly, several proteins associated with lipid metabolism and cell wall synthesis also displayed differential expression. In particular, expression of the glycerol kinase, dihydroxyacetone kinase subunit DhaK, enoyl-(acyl carrier protein) reductase and glycerophosphoryl diester phosphodiesterase-like protein was lower in the sav1322 mutant, whereas expression of (3R)-hydroxymyristoyl-ACP dehydrogenase was higher in the sav1322 mutant when compared with the WT strain. For cell wall synthesis, glucosamine-fructose-6-phosphate aminotransferase (GlmS), UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase (MurF), and UDP-N-acetylglucosamine-1-carboxyvinyl transferase (MurZ) exhibited lower expression levels in the sav1322 mutant when compared with the WT strain. GlmS is the key enzyme responsible for the synthesis of glucosamine-6-phosphate from fructose-6-phosphate [16], MurF catalyzes the addition of D-Ala-D-Ala to the nucleotide precursor UDP-N-acetylmuramic acid-L-Ala-γ-Glu-meso-diaminopimelate [17], and MurZ catalyzes the condensation of phosphoenolpyruvate with UDP-N-acetylglucosamine [18].

Lastly, markedly lower expression levels of proteins involved in the stress response were observed in the sav1322 mutant, including the general stress protein 20U (Dps), methionine sulfoxide reductase A (MsrA2), and methionine sulfoxide reductase B (MsrB). MsrA2 reduces the S isomer of methionine sulfoxide, and MsrB reduces the R form, providing protection against oxidative stress [19]. In contrast, expression of ClpL, a protein involved in thermostolerance, was up-regulated in the sav1322 mutant strain.

**In vivo assessment of the sav1322 mutant strain**

A mouse model of bacteremia was used to determine how deletion of the SAV1322 locus impacted staphylococcal pathogenicity. Systemic infection following tail vein inoculation of six-week-old female Balb/c mice with the sav1322 mutant produced a 2-log reduction in bacterial burden found in the lung, liver, and kidney when compared with mice inoculated with the WT strain (Fig. 4).

**Antibiotic susceptibility**

Epsilometer (E)-test strips were used to determine if the sav1322 mutant strain exhibited altered susceptibility or resistance to several antibiotics. Minimum inhibitory concentrations (MICs) of vancomycin and teicoplanin which target the bacterial cell wall were lower for the sav1322 mutant when compared to the WT strain (Table 3). We also performed a population analysis to obtain a more detailed evaluation of changes in susceptibility to vancomycin and teicoplanin. Consistent with a lower MIC for both of these antibiotics, we observed a decrease in vancomycin- and teicoplanin-resistant sub-populations compared with the WT strain (data not shown). However, those MICs were not recovered in the Com strain. Lower MICs for gentamicin, tigecycline, and tetracycline, which inhibit protein synthesis by binding the 30S subunit of ribosome, were also observed in the sav1322 mutant strain. The MICs of these antibiotics were fully recovered in Com strain. In addition, the
Heat-shock proteins (Hsps) are essential for stress tolerance and survival under protein-denaturing conditions. Four Hsp classes have been identified in Gram-positive bacteria. Class I Hsps comprise classical Hsps that are encoded by genes within the groESL and dnaK operons, and a cytoplasmic repressor HrcA controls expression of these proteins. Sig B controls Class II Hsps, while class II Hsps are Clp proteases that are typically controlled by the transcriptional repressor CtsR. Class IV Hsps are encoded by genes that are not controlled by HrcA, SigB, or CtsR [3, 20]. Interestingly, our data suggest that the sav1322 mutant strain is more sensitive to heat and cold than the WT strain. Microarray data further demonstrate that the transcription of class I Hsp and III Hsp genes. More specifically, hrcA, ctsR, dnaJ, dnaK, and grpE transcript levels are higher in the sav1322 mutant. However, expression of cold-shock genes (cspA and cspB) is not affected in the mutant. These results suggest that SAV1322 may play a role in temperature adaptation by regulating the expression of chaperone proteins. While CtsR represses the transcription of genes encoding class I and class III Hsps, however, we noted that expression of the class III chaperone genes clpC and clpB is higher in the sav1322 mutant strain.

The clpC operon in S. aureus comprises four genes denoted ctsR, mcsA, mcsB, and clpC. One previous study has reported that a mutation in mcsB results in hypersensitivity to heavy metal, temperature, osmotic pressure, and oxidative stresses [21]. Clp proteases are implicated in stress resistance, and clpC and clpB transcription increases during acid shock in S. aureus [22]. In this study, the sav1322 mutant exhibited greater resistance to oxidative stress. This may be partially explained by the higher levels of clpC transcription observed in this strain.

Our proteomic data further revealed that the expression of 72 proteins is altered at least two-fold in the sav1322 mutant when compared with the WT strain. Many of these proteins are directly or indirectly involved in important cellular processes, including the TCA cycle. The TCA cycle have been implicated in antibiotic resistance, and, possibly, staphylococcal virulence [23, 24]. Inactivation of the TCA cycle delays the resolution of cutaneous ulcers in soft tissue infections of the mouse [25], and disruption of citZ (citrate synthase), citC (isocitrate dehydrogenase), and citB (aconitate hydratase) genes prevents bacterial capsule formation [26]. Surmann et al. analyzed survival and physiological adaptation of S. aureus after internalization by human lung epithelial cell lines A549. A number of TCA cycle enzymes including CitZ, CitC, SucC and SdhA increased in level upon internalization by this epithelial cell. Moreover, L-lactate dehydrogenase (LctE) increased in level after internalization by A549 and human embryonic kidney cells HEK 293, indicating

### Table 3 Antibiotic susceptibility profiles of the WT, sav1322 mutant, and complementation strains

| Antibiotics        | MIC (mg/L) | WT       | sav1322 mutant | Com       |
|--------------------|------------|----------|----------------|-----------|
| Daptomycin         | 3          | 0.19     | 0.125          |           |
| Erithromycin       | >256       | >256     | >256           |           |
| Gentamicin         | 0.5        | 0.064    | 0.25           |           |
| Imipenem           | >32        | 1        | 32             |           |
| Linezolid          | 2          | 2        | 2              |           |
| Oxacillin          | >256       | >256     | >256           |           |
| Penicillin         | >32        | >32      | 32             |           |
| Teicoplanin        | 8          | 0.5      | 0.5            |           |
| Tigecycline        | 0.5        | 0.064    | 0.25           |           |
| Tetracycline       | 16         | 1        | 8              |           |
| Vancomycin         | 8          | 0.75     | 0.75           |           |

MICs for the bacterial cell membrane-targeting lipopeptide antibiotic, daptomycin, were 3 mg/L and 0.19 mg/L for the WT and sav1322 mutant strains, respectively. Electron microscopy was used to determine if cell wall thickness was associated with the observed reduction in glycopeptide resistance in the mutant strain. The sav1322 mutant strain has a thinner cell wall (23.6 ± 3.1 nm) than the WT strain (36.1 ± 5.6 nm) (Fig. 5).

### Discussion

The SAV1321/SAV1322 TCRS system is a histidine kinase sensor/response regulator in the wild-type S. aureus Mu50. However, the functions of SAV1321/SAV1322 TCRS-associated genes are not yet known. With this study, we elucidate some of these functions by examining phenotypic, transcriptomic, and proteomic changes in a sav1322 mutant strain in response to different environmental stressors.

**Fig. 5** TEM images of representative S. aureus Mu50 (WT) and sav1322 mutant cells. Magnification: 100,000x. Data are expressed as mean ± SD values for cell wall thickness (nm). Asterisks denote statistical significance (p < 0.001) when compared with the WT strain.
the supplementary utilization of fermentative enzymes under microaerobic conditions [27]. Our proteomic results demonstrate that expression of proteins involved in TCA cycle, such as OdhB, CitC, PdhD, and FumC, is lower in the sav1322 mutant. Furthermore, several major fermentative and anaplerotic pathway components were also decreased in the mutant, in particular LctE, malatequinone oxidoreductase (Mqo2) and pyruvate kinase (PykA). These results suggest that the down-regulation of energy-providing pathways may be energetically less efficient in the mutant compared with the wild type strain, then the recovered CFU were lower in tissue of mice infected with the mutant (Fig. 4).

Furthermore, LctE catalyzes the conversion from pyruvate to lactate. The L-lactate produced by this process allows S. aureus to maintain redox homeostasis during nitrosative stress caused by activated phagocytes and is essential for virulence [28]. In this study, the protein level of LctE was decreased in mutant compared with WT strain. This may resulted in decrease the production of L-lactate in the mutant and cannot survive from the attack of the host, including the radial nitric oxide.

Proteins involved in protein synthesis are also altered upon deletion of SAV1322 including 30S ribosomal protein S2, 30S ribosomal protein S4, and several aminoacyl-tRNA synthetase. In accordance with this proteomic result, the lower MICs of tetracycline, tigecycline, and gentamicin that bind 30S subunit of ribosome were observed in the sav1322 mutant, and fully restored in Com strain. These results suggest that SAV1322 may play a role in antibiotic resistance to tetracycline, tigecycline, and gentamicin, but further studies are needed to elucidate the underlying specific mechanism.

Proteins involved in cell wall biosynthesis are also altered upon deletion of SAV1322. Cell wall synthesis is crucial for bacterial division and growth, and it is an important target for antibiotics, including penicillin, vancomycin, and teicoplanin. As the number of reported MRSA strains increases, vancomycin has become the first-line antibiotic of choice for highly insoluble proteins that are difficult to analyze even in the presence of detergents.

Conclusions
This study is the first to provide a functional transcriptomic and proteomic analysis of the response regulator SAV1322 in S. aureus. SAV1322 plays a pronounced role in temperature adaptation, resistance to antibiotics, and virulence. In addition, it influences the expression of a large number of genes involved in the heat shock response, cell wall metabolism, energy metabolism, and response to other environmental stressors. Our findings provide valuable insight into antibiotics resistance and staphylococcal pathogenicity. Both may help shape future studies on antibiotic resistance trends and optimal antibacterial treatment strategies.

Methods
Construction of the sav1322 mutant and complementation strains
The S. aureus Mu50 was used to generate a SAV1322 knockout mutant strain using an allelic exchange method described previously [30]. Briefly, sequences flanking SAV1322 were amplified by PCR using primers that contained attB1 (5′-GGGGACAAGTTTGTACAAAAAAG CAGGCT-3′) and attB2 (5′-GGGGACCACTTTGTACACAA GAAAGCTGGGT-3′) sites on the respective up- and downstream target sequences. PCR fragments were cloned into the pKOR1 vector, and then introduced into S. aureus Mu50 by electroporation. To select for single-crossover mutants, electroporated clones were cultured overnight at 43 °C. Single-crossover mutants were selected and cultured in antibiotic-free broth medium to facilitate plasmid excision, then subjected to anhydrotetracycline induction to select a non-plasmid-carrying mutant. Successful deletion of SAV1322 was verified by PCR using the specific primers covering target sequences (UP FWD and DOWN REV)
and sequence analysis. For the complementation strain, a 1620-bp fragment of the SAV1322 gene was PCR-amplified using Mu50 genomic DNA as a template with the primer sets Com-F and Com-R (Fig. 1a), then cloned into the pYT3 vector and introduced into the sav1322 mutant strain as described previously [31]. To confirm of complementation, reverse transcription-PCR analysis was performed. Total RNA was isolated from the WT, mutant, and complementation strains and reverse-transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase for 60 min at 42 °C. Resulting cDNA fragments were used as templates for PCR amplification of the target gene.

Stress response analyses
Strains were cultured overnight in tryptic soy broth (TSB) at 37 °C and diluted to an OD_{600} reading of 0.3. Cells were then incubated at 25 and 46 °C with shaking, while OD_{600} readings were taken at 1 h intervals for 7 h. To investigate the oxidative stress response, WT and sav1322 mutant cultures grown overnight were plated on tryptic soy agar (TSA). A disk containing 10 μL H_{2}O_{2} (15 % or 30 %) was placed on the prepared agar plates, then incubated at 37 °C for 18 h.

Microarray analysis
RNA extraction, cDNA labeling, hybridization, and microarray data analyses were carried out according to protocols described previously [32]. Cells were grown exponentially to an OD_{600} of approximately 0.5, then harvested. A customized S. aureus high-density synthetic oligonucleotide array was designed using 982 predicted open reading frames (ORF) with the GenBank accession number NC_002745 (NimbleGen Systems Inc., Madison, WI, US). The ORF listed in Additional file 3. Arrays were scanned with a NimbleGen MS 200 Microarray scanner set at 532 nm with a resolution of 2 μm to produce images (TIFF format) according to the manufacturer's instructions. Array data export processing and analysis was performed using NimbleScan v2.5 (Gene Expression RMA algorithm). Adjustments for batch effects were made with data-to-filter non-biological experimental variation (http://biosun1.harvard.edu/complab/batch/). A single raw intensity value was determined for each gene in every array by averaging spot replicates of all probes for each of the genes. Gene signal values were log2 transformed. Statistical significance of the expression data was determined using the local-pooled-error test and fold change in which the null hypothesis was that no difference exists between two groups. Adjusting P-values with a Benjamini-Hochberg algorithm controlled the false discovery rate. Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity.

Two-dimensional gel electrophoresis and protein identification
Cell extracts from overnight WT and sav1322 mutant cultures were separated by two-dimensional gel electrophoresis. Aliquots containing approximately 800-μg protein were diluted in 2D-PAGE rehydration buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 0.4 % DTT) to a final volume of 350 μL and centrifuged at 100,000 g for 30 min to remove insoluble material. For the first dimension, samples were run on pH 4–7 IPG strips (GE Healthcare Life Sciences, UK) on a Multiphor apparatus II instrument (Amersham Biosciences, UK) according to the manufacturer's instructions. Strips were incubated in equilibration buffer (6 M urea, 20 mM Tris-HCl, pH 8.8, 2 % SDS, 20 % glycerol, 2.5 % acrylamide, and 5 mM tributyl phosphate) for 20 min. The second dimension separation was performed using 8–16 % linear gradient SDS-polyacrylamide gels. Gels were stained with colloidal Coomassie solution (ProteomeTech, Seoul, Korea) according to the manufacturer's instructions. Differences in the expression profiles of spots were quantified using the ImageMasterTM 2D Platinum software (Amersham Biosciences, UK). In-gel digestion of the protein spots was performed as described previously [33]. Resulting tryptic peptides were analyzed using reversed phase capillary HPLC directly coupled to a Finnigan LCQ ion-trap mass spectrometer (LC-MS/MS) previously described [34]. Individual spectra from MS/MS were processed using TurboSEQUEST software (ThermoQuest, San Jose, CA). The generated peak list files were used to query the MSDB database or NCBI using the MASCOT program (http://www.matrixscience.com).

Antimicrobial susceptibility
MICs for different antimicrobial agents were determined with E-test strips (AB bioMérieux, Marcy l’Étoile, France). A sterile cotton swab was immersed in each bacterial culture grown to a 0.5 McFarland turbidity standard and streaked on Mueller-Hinton agar (Difco, Detroit, MI) plates. Plates were incubated at 37 °C, and MICs were measured after a 24-h incubation period according to the manufacturer's instructions.

Transmission electron microscopy (TEM)
WT and sav1322 mutant cells were prepared and visualized by TEM as described previously [35]. Morphometric evaluation of cell wall thickness was determined using photographs at 20,000× magnification. At least 30 cells from each strain with almost equatorial cut surfaces were measured and results are expressed as mean ± SD values. The statistical significance of cell wall thickness was evaluated by the Student's t-test (p < 0.001).
Mouse infection model
All animal experiments were conducted in accordance with guidelines and the approval of the Institutional Animal Care and Use Committee of Korea Centers for Disease Control and Prevention (KCDC-030-12-2A). WT and sav1322 mutant strains were grown and harvested at the mid-point of the exponential growth phase, washed in sterile PBS, and resuspended in PBS to a concentration of $1 \times 10^8$ cells per 0.1 ml. Female Balb/c mice (aged 5–6 weeks) were inoculated with this suspension or PBS via the tail vein. The experiment was performed with ten mice for each S. aureus strain and five mice for the PBS control. Mice were euthanized 24 h after infection, and lungs, livers, and kidneys were removed and homogenized in sterile PBS. Homogenates were diluted in PBS and plated on TSA, then incubated overnight in 37 °C. The bacterial burden in each organ was assessed by CFU counts. The student's t-test was used to determine statistical significance between samples.

Additional files

Additional file 1: Colony forming units (CFU) of the S. aureus Mu60 (WT), sav1322 mutant, and complementation (Com) strains at 37 °C, 46 °C, 25 °C. (DOCX 22 kb)

Additional file 2: 2-D PAGE of the S. aureus Mu50 (WT) and sav1322 mutant strains. (DOCX 568 kb)

Additional file 3: List of probes for microarray. (XLSX 58 kb)

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Availability of data and materials
All data generated or analysed during this study are included in this published article (and its supplementary information files). All of the microarray data have been deposited in the NCBI's Gene Expression Omnibus (GEO) under accession number GSE85824.

Authors’ contributions
JWK, HHK, and GSK performed laboratory work. JWK and JIY wrote the manuscript. YSL and JIY designed the study. IHK and HSK critically revised the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable

Ethics approval and consent to participate
Not applicable

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