Coordinated regulation of transcription by CcpA and the Staphylococcus aureus two-component system HptRS

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Abstract

The success of Staphylococcus aureus as a pathogen is due in part to its ability to adapt to changing environmental conditions using signal transduction pathways, such as metabolite-responsive regulators and two-component systems. S. aureus has a two-component system encoded by the gene pair sav0224 (hptS) and sav0223 (hptR) that regulate the hexose phosphate transport (uhpT) system in response to extracellular glucose-6-phosphate. Glycolytic intermediates such as glucose-6-phosphate are important carbon sources that also modulate the activity of the global metabolite-responsive transcriptional regulator CcpA. Because uhpT has a putative CcpA binding site in its promoter and it is regulated by HptR, it was hypothesized the regulons of CcpA and HptR might intersect. To determine if the regulatory domains of CcpA and HptRS overlap, ccpA was deleted in strains SA564 and ΔhptRS and growth, metabolic, proteomic, and transcriptional differences were assessed. As expected, CcpA represses hptS and hptR in a glucose dependent manner; however, upon CcpA derepression, the HptRS system functions as a transcriptional activator of metabolic genes within the CcpA regulon. Importantly, inactivation of ccpA and hptRS altered sensitivity to fosfomycin and ampicillin in the absence of exogenous glucose-6-phosphate, indicating that both CcpA and HptRS modulate antibiotic susceptibility.

Introduction

Staphylococcus aureus is a common commensal bacterium and a versatile pathogen that can infect nearly any anatomic site. As a commensal, S. aureus persistently colonizes the nares of 12–30% of the population, while 16–70% of people are intermittent carriers [1–3]. The prevalence of S. aureus and its increasing antibiotic resistance contributed to a 50% increase in diagnoses from 9.17 to 13.79 per 1000 hospitalizations from 1999 to 2005 [4]. This prevalence and
the success of \textit{S. aureus} as a pathogen are strong indicators of its ability to adapt and survive in diverse conditions.

\textit{S. aureus} survival in a host requires nutrients, avoidance of the immune system, and proliferation within a changing environment. To survive environmental challenges, \textit{S. aureus} must be able to respond to changing environmental cues [5–7]. Rapid responses to environmental challenges are achieved by alterations in enzymatic activity and through transcriptional and translational initiation changes [6]. Feedback and feedforward enzymatic changes are induced by changes in substrate, cofactor, and/or inhibitor concentrations, while transcriptional and translational changes are mediated through metabolite, metal ion, and/or cofactor-responsive regulators (e.g., CcpA, Fur, Rex), small RNAs (e.g., RsaE), alternative sigma factors (e.g., \(\sigma^B\)), and two- or three-component regulators (e.g., KdpD-KdpE) [8–17].

The metabolite-responsive carbon catabolite protein A (CcpA) belongs to the LacI protein family and regulates transcription in response to changes in metabolite concentrations such as glucose, fructose, and glycerol [8, 18]. These carbon sources can be converted into the activated glycolytic intermediates glucose-6-phosphate and fructose-1,6-bisphosphate through glycolysis or gluconeogenesis. Glucose-6-phosphate and fructose-1,6-bisphosphate promote phosphorylation and activation of the CcpA co-regulator, the histidine containing protein (HPr) [12, 13, 19]. CcpA activity is also regulated through phosphorylation by serine/threonine protein kinase 1 (Stk1) in response to changes in intracellular organic phosphate [20–22]. Other transcriptional regulators directly bind to metal ions such as Fur (Fe\(^{2+}\)) or dinucleotides such as Rex (NAD\(^+\)/NADH) [14, 15, 18]. Together these regulators alter transcription of genes involved in diverse cellular processes such as metabolism, virulence factor synthesis, and antibiotic resistance. There are also non-protein regulators that respond to environmental stimuli such as small regulatory RNAs (e.g., RsaE), which bind to RNA and alter translation initiation or stability of target mRNAs [16, 17]. Interestingly, transcription of several members of the Rsa family of sRNAs (rsaA, rsaD, rsaF, rsaE) are regulated by the alternative sigma factor B (\(\sigma^B\)), which confers promoter specificity for RNA polymerase to target genes, in response to physical and chemical stresses [16, 17, 23, 24]. Environmental stresses also activate other regulators such as two- and three-component systems.

Two- and three-component signal transduction systems, such as the \textit{S. aureus} KdpD-KdpE two-component system, serve as a stimulus response coupling mechanism that allows bacteria to alter transcription of genes necessary for survival in response to changing environmental conditions [10, 11]. The two-component systems are generally comprised of a sensor histidine kinase and a response regulator [11]. Signal sensing is through the input domain in the sensor histidine kinase that activates phosphorylation of a histidine in the autokinase domain. The phosphoryl group is subsequently transferred to an aspartate in the receiver domain of the response regulator, increasing output domain activity, and altering the transcription of response-regulated genes [11]. In the example of the KdpD-KdpE two-component system, changes in environmental potassium can alter the concentration of intracellular potassium and phosphate, affecting the ionic strength of the cytoplasm, and/or the ATP concentration [25]. In response, the KdpD-KdpE two-component system alters transcription of potassium ion transport genes (i.e., \textit{kdpA}, \textit{kdpB}, and \textit{kdpC}) [26, 27]. Activation of KdpD results in autophosphorylation of histidine 673, which then transfers the phosphoryl group to aspartate 52 on the response regulator KdpE [27]. In addition to activating transcription of the \textit{kdpABC} operon, KdpE alters transcription of \textit{S. aureus} virulence genes, such as protein A, \(\alpha\)-toxin, and aureolysin [27–29]. Interestingly, \textit{kdpDE} transcription is under the control of another two-component system (AgrC-AgrA) that responds to cell density and metabolic signals [29]. In total, \textit{S. aureus} are hypothesized to have 16 two-component systems, many of which are named and at least partially described [9, 30]. Of the partially defined two-component systems
in *S. aureus*, one is encoded within the gene pair *sav*0224 (*hptS*) and *sav*0223 (*hptR*). The HptRS two-component system is in a predicted three-gene operon downstream of *sav*0225 (*hptA*), a putative metal binding protein. Recently, it was suggested that HptS and HptR regulate transcription of the hexose phosphate transport gene (*uhpT*) in response to changes in the availability of exogenous glucose-6-phosphate [31, 32]. The hexose phosphate transport system functions as an inorganic phosphate-coupled hexose phosphate sugar transporter for sugars such as glucose-6-phosphate, fructose-6-phosphate, and mannose-6-phosphate [33, 34]. This transport system is medically relevant because it is one of only two known transporters (i.e., UhpT and GlpT) that mediate the uptake of the antibiotic fosfomycin [35, 36]. In an *S. aureus* USA300 lineage strain, inactivation of either *hptS* or *hptR* increased fosfomycin resistance, reduced transcription of *uhpT*, and reduced growth when cultivated in a chemically defined medium containing glucose-6-phosphate as the primary carbon source [31]. Interestingly, in *S. aureus* strain NCTC8325, it was demonstrated that HptA binds to glucose-6-phosphate and may function to activate the HptRS two component system [32]. Although cultivation in a chemically defined medium containing glucose-6-phosphate indicated that HptRS regulated the UhpT transporter, it is likely that cultivation on a single carbohydrate source caused widespread changes in metabolism. Commonly, carbohydrate-dependent metabolic adaptation is regulated in part by CcpA [8, 13], thus linking separate regulatory systems that balance *S. aureus* responses to the availability of rapidly catabolizable carbon sources.

Not only is glucose-6-phosphate the proposed effector molecule of HptA, it is also an effector of CcpA [13]. This is noteworthy because the *uhpT* gene promoter contains both a HptR (-67 to -96 bp), and a putative CcpA binding site (cre site; -61 to -46 bp), suggesting *uhpT* gene transcription is coordinately regulated by HptR and CcpA [18, 32]. Support for CcpA-mediated transcriptional regulation of *uhpT* was observed when inactivation of CcpA abolished the glucose-dependent increase in *uhpT* mRNA [8]. Interestingly, transcription of the *hptR*, *hptS*, and *hptA* operon is induced in *S. aureus* strain COL under anaerobic cultivation, which is consistent with reduced CcpA activity during anaerobic growth due to a decrease in phosphorylation of the HPr co-regulator [8, 37, 38]. These observations indicate the CcpA and HptR regulons overlap. In total, it is likely that CcpA and HptR form an undefined, complex regulatory network that responds to environmental changes to regulate *uhpT* and other genes. To determine if HptRS is within or interacts with the CcpA regulon, Δ*ccpA*, Δ*hptRS*, and Δ*hptRS*/*ccpA* mutants were constructed in *S. aureus* strain SA564 and were assessed for growth, metabolic, proteomic, and transcriptional differences.

### Materials and methods

**Bacterial strains and cultivation conditions**

Bacterial strains, bacteriophage, and plasmids used in this study are listed in Table 1. *Escherichia coli* strain DH5α was cultivated in lysogeny broth (LB) or on LB containing 1.5% agar. *S. aureus* strains were grown in tryptic soy broth (TSB) containing 0.25% dextrose (BD Biosciences), TSB without dextrose (TSB-dex; BD Biosciences), or on TSB containing 1.5% agar (TSA). Bacterial pre-cultures were inoculated 1:100 from overnight cultures into TSB or TSB-dex, incubated at 37 °C, aerated at 225 rpm using a flask-to-medium ratio of 10:1, and grown for 2 h. These exponential growth phase pre-cultures were centrifuged for 5 min at 5,000 rpm (4,272.5 X g) at 22 °C and suspended in 1–2 mL of medium. Primary cultures were inoculated into 100 mL pre-warmed TSB or TSB-dex, at an absorbance at 600 nm (*A*<sub>600</sub>) of 0.01, and incubated at 37 °C, aerated at 225 rpm, with a flask-to-medium ratio of 10:1 (aerobic) or 10:8 (microaerobic/anaerobic). The *A*<sub>600</sub> and pH were recorded hourly. The data were analyzed...
using a 2-way ANalysis Of VAriance (ANOVA) using SigmaPlot version 11.1, from Systat Software, Inc. (San Jose California USA).

For antibiotic disk diffusion assays, bacterial strains were inoculated from a single colony into 2 mL TSB in a 14 mL culture tube and incubated overnight at 37 °C and aerated at 225 rpm. A 2 h pre-culture was prepared as described. After pre-incubation, the $A_{600}$ were recorded and bacteria were diluted to 0.5 McFarland units (i.e., $A_{600} = 0.08$; 1–5 x 10$^8$ CFU/mL). This suspension was diluted 1:10 and 100 μL was spread onto TSA plates and antibiotic disks (Becton, Dickinson and Company) were placed on each plate. Plates were incubated at 37 °C overnight. Zone of inhibition was recorded by measurement of the space between the edge of the disk and the first colony. The data were analyzed using a 2-way ANOVA.

For antimicrobial susceptibility tests, the broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI) was utilized, with the exception that no glucose-6-phosphate was added to the medium [43]. Briefly, bacterial strains were inoculated from a single colony on TSA into cation adjusted Mueller Hinton broth (CAMHB) with 50 mg/L Ca$^{2+}$ and 12.5 mg/L Mg$^{2+}$, and incubated at 37 °C under aeration at 225 rpm [43]. 2 h pre-cultures were prepared as described. The pre-cultures were diluted to produce a final inoculum of 1–5 x 10$^5$ CFU/mL in 96-well flat bottom cell culture plates. The plates were incubated at 37 °C for 20 h and the absorbance at 595 nm ($A_{595}$) was recorded in a plate reader. The minimum inhibitory concentration (MIC), as defined by CLSI, is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in microdilution wells as detected by the unaided eye [43]. To eliminate human error, an $A_{595} \leq 0.05$ was considered to reflect no bacterial growth; hence, the MIC was defined as the lowest concentration of antimicrobial agent that inhibits growth at $A_{595} \leq 0.05$. The data were analyzed using a 2-way ANOVA.

**Construction of hptRS and ccpA mutants in S. aureus**

Primers (Table 2) used for PCR amplification of S. aureus strain SA564 genomic DNA were designed using the SA564 genome sequence (NCBI Reference Sequence: NZ_CP010890.1) as a template. Inactivation of hptRS in strain SA564 was accomplished by deleting a 2,380 bp region, which included all of sav0223 (hptR) and the majority of sav0224 (hptS), using the gene splicing by overlap extension technique [44]. Primers MUT0223CF2 and MUT0223Y were used to amplify a 1,416 bp region upstream of sav0223 (fragment 1) and primers MUT0224AR

| Strain, Plasmid, Phage | Description | Source |
|------------------------|-------------|--------|
| Strains                |             |        |
| RN4220                 | S. aureus Restriction-deficient mutant of strain 8325–4 | [39] |
| SA564                  | S. aureus Clinical isolate wildtype (blaZ‘) | [7] |
| SA564 ccpA::ermB       | S. aureus CcpA knockout | [12] |
| Newman ccpA::tetM      | S. aureus CcpA knockout | [40] |
| SA564 hptRS::ermB      | S. aureus Two-component system double knockout | This Study |
| SA564 hptRS::ermB ccpA::tetM | S. aureus Triple knockout | This Study |
| DH5(α)                | E. coli cloning host | Invitrogen |
| Phages                 |             |        |
| φ11                    | Transducing Phage | [39] |
| 80α                    | Transducing Phage | [41] |
| Plasmid                |             |        |
| pBT2                  | Temperature-sensitive shuttle vector | [42] |

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and MUT0224X were used to amplify a 1,849 bp region that included 147 bp at the beginning of \textit{sav}0224 and the region downstream of \textit{sav}0225 (fragment 2). Primers MUT0224AF and MUT0223CR, were used to sew fragments 1 and 2 together to create the spliced gene fragment with KpnI and BamH1 restriction sites for digestion and ligation into plasmid pBT-2. The size

Table 2. Primers used in this study.

| Primers for knockout construction | S. aureus Mu50 Open Reading Frame | Sequence |
|----------------------------------|----------------------------------|----------|
| MUT0224X                         | SAV0226                          | GAAGTTGCTTGTCTGAGTCTCAGAATCCTTGC |
| MUT0224AR                        | SAV0224                          | GATGAAATCGTACGATGCTCATAATC |
| MUT0223CF2                       | SAV0222                          | ATCGTACGATTCATGCTGAGACGAGGAGATCGTATG |
| MUT0223Y                         | SAV0222                          | ATTCGAACACTTTAAGCCGAGGAGATCGTATG |
| MUT0224AF                        | SAV0222                          | AGTCAGGGATCTGTACGAGGAGGAGATCGTATG |
| MUT0223CR                        | SAV0222                          | AGTCAGGGATCTGTACGAGGAGGAGATCGTATG |
| SAV0224-ermB-f                   | SAV0222                          | AGTCAGGGATCTGTACGAGGAGGAGATCGTATG |
| SAV0224-ermB-r                   | SAV0222                          | AGTCAGGGATCTGTACGAGGAGGAGATCGTATG |
| PCR and DNA Sequencing confirmation Primers |                                  |          |
| SAvermB375R                      | SAV0222                          | TTTGGTTTAGGATGAAAGCATTCAGGAGGAGATCGTATG |
| SAv0222-6seq500                  | SAV0222                          | CATGACACTTGACGATGCTCAGGAGGAGATCGTATG |
| SAv0222-6seq1000                 | SAV0222                          | GTGCAACACGACGATGCTCAGGAGGAGATCGTATG |
| MUT0223CF2                       | SAV0222                          | GGAATTATGATCTGAGACGAGGAGATCGTATG |
| SAMUT02235R400                   | SAV0225                          | CTGTAAACACACACACACACAAAGGAGGAGATCGTATG |
| SA0224ermB2222F                  | SAV0222                          | GCCATACCAAGAGTTCAGGAGGAGATCGTATG |
| MUT0224AF                        | SAV0222                          | AGTCAGGGATCTGTACGAGGAGGAGATCGTATG |
| acuc-R                           | SAV0222                          | CGGCTATGGAACACTGTGGAATAG |
| SAV1652-F                        | SAV0222                          | GGTGAGGGATGACGACCAGGAGGAGATCGTATG |
| SAV0222-R                        | SAV0222                          | CAAGCCGCCTGACGATGCTCAGGAGGAGATCGTATG |
| SAV0222-F                        | SAV0222                          | CAAGCCGCCTGACGATGCTCAGGAGGAGATCGTATG |
| pFB-F                            | SAV0222                          | ACGACTTCAACGACGATGCTCAGGAGGAGATCGTATG |
| pFB-R                            | SAV0222                          | TTTGGTTTAGGATGAAAGCATTCAGGAGGAGATCGTATG |
| ADH-F                            | SAV0222                          | ACGACTTCAACGACGATGCTCAGGAGGAGATCGTATG |
| ADH-R                            | SAV0222                          | TTTGGTTTAGGATGAAAGCATTCAGGAGGAGATCGTATG |
| rocA-F                           | SAV0222                          | TTTGGTTTAGGATGAAAGCATTCAGGAGGAGATCGTATG |
| rocA-R                           | SAV0222                          | TTTGGTTTAGGATGAAAGCATTCAGGAGGAGATCGTATG |
| DHA-1439F                        | SAV1439                          | GCTATTGACGACGAGGAGGAGATCGTATG |
| DHA-1439R                        | SAV1439                          | GCTATTGACGACGAGGAGGAGATCGTATG |
| ccpA4F                           | SAV1736                          | CAGGAATCATTGCTGCAATGCTGCAATGCTGCAATG |
| ccpA4R                           | SAV1736                          | CAGGAATCATTGCTGCAATGCTGCAATGCTGCAATG |
| uhpTA2-F                         | SAV0222                          | CCTCGAATGACGAGGAGGAGATCGTATG |
| uhpTA2-R                         | SAV0222                          | CCTCGAATGACGAGGAGGAGATCGTATG |
| SAV0225RTF                       | SAV0225                          | CCTCGAATGACGAGGAGGAGATCGTATG |
| SAV0225RTR                       | SAV0225                          | CCTCGAATGACGAGGAGGAGATCGTATG |
| SAV0224RTF                       | SAV0224                          | GATGTTACGACGCATGCTGCAATGCTGCAATG |
| SAV0224RTR                       | SAV0224                          | GATGTTACGACGCATGCTGCAATGCTGCAATG |
| SAV0223RTF                       | SAV0223                          | GATGTTACGACGCATGCTGCAATGCTGCAATG |
| SAV0223RTR                       | SAV0223                          | GATGTTACGACGCATGCTGCAATGCTGCAATG |

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of the spliced gene fragment was confirmed by restriction digestion. The ermB cassette from plasmid pEC4 was amplified using primers SAV0224ermB-F and SAV0224ermB-R and inserted between the cloned sav0225 and sav0222 DNA by digestion with BsiW1 to create plasmid pBT2-Δsav0223/0224:ermB. This plasmid was electroporated into E. coli strain DH5α, harvested, and transformed into S. aureus strain RN4220. S. aureus strain RN4220Δsav0224/0223:ermB was constructed using the temperature shift protocol of Foster et al. [45]. The Δsav0223/0224:ermB mutation from strain RN4220-Δsav0224/0223::ermB was transduced into S. aureus strain SA564 using S. aureus phage φ11 to make strain SA564-Δsav0224/0223::ermB. The resulting transductants were selected for erythromycin resistance and confirmed by PCR. To reduce the likelihood of spurious mutations from selection and transduction, the Δsav0224/0223::ermB allele was backcrossed into wild-type strain SA564 using transducing phage 80α to create SA564-ΔhptRS::ermB (ΔhptRS). Deletion of genes sav0224/0223 were confirmed by DNA sequencing (Eurofins MWG Operon) using primers SAvermB375R, SAV0222-f-seq500, SAV0222-fseq1000, MUT0223CF, SAMUT0223R400, SAMUT0224ermB222F, and SAMUT0224AF. The ccpA::tetM allele was transduced from S. aureus strain Newman ccpA::tetM using phage 80α into strain SA564-ΔhptRS::ermB to make strain SA564-ΔhptRS::ermB::ccpA::tetM (ΔhptRS/ccpA). Successful transduction was confirmed by PCR of both ccpA and hptRS mutant regions using primers acuC-R, SAV1652-F and SAV0225-R, SAV0222-F.

Determination of acetate, glucose, and lactate levels in medium supernatant
Bacterial cultures (1 mL) were harvested hourly during cultivation and centrifuged for 5 min at 20,800 × g at 4 °C. The cell-free media were transferred to microfuge tubes and stored at -20 °C until needed. Glucose, acetate, and ammonia concentrations in the culture media were determined (n = 3) with kits purchased from R-biopharm.

Reverse transcription PCR
For quantification of mRNA by reverse transcription PCR (RT-qPCR), RNA isolation and RT-qPCR were performed as described [46]. Briefly, cDNA (20 ng/reaction) was used for real-time amplification using primers listed in Table 2. 16S rRNA was used as an internal reference and transcript levels relative to 16S RNA were determined by the comparative threshold (ΔΔC_T) method (Bio-Rad) using two technical replicates per plate. All experiments were performed in biological triplicate for each gene. Experimental setup and data analysis were carried out using a Bio-Rad CFX96 real-time PCR detection system and CFX Manager software version 3.1 following the minimum information for the publication of quantitative real-time PCR (MIQE) guidelines [47]. The data for each biological replicate (n = 3) was compiled using the gene study tool within the BioRad CFX manager software package. In order to assess significance, a p-value cutoff of 0.05 (p ≤ 0.05), and fold change greater than 1.5 (FC ≥ 1.5), were utilized to determine if there was a statistically significant difference between the transcript abundance of the gene when strains were compared (Tables A-D in S5 File).

Protein collection and processing
Bacteria (10 A_600 units) were harvested after 2 and 6 h cultivation by centrifugation and suspended in 1 mL of lysis buffer containing 50 mM tris-HCL, 8 M urea, and 1.5 mM phenylmethylsulfonyl fluoride. The bacterial suspensions were lysed for 40 s at a setting of 6 m/s in a FastPrep instrument (MP biomedical) and the lysate was clarified by centrifugation for 5 min at 20,800 × g at 4 °C. Cell-free lysates were stored at -80 °C until use. Protein concentrations were determined using a Modified Lowry Protein Assay Kit (Fisher Scientific) and diluted to a
final concentration of 235 μg/mL in ultrapure water. Protein samples were mixed with a 3X volume of cold acetone and incubated at -20 °C for 30 min and centrifuged for 2 min at 15,000 x g to pellet the precipitated proteins. Protein pellets were washed twice with cold acetone, dried 15 min in a speed vacuum, and stored at -80 °C. Protein samples were solubilized in 20 μL denaturing buffer (25 mM ammonium bicarbonate, pH 8.0; 10 mM tris (2-carboxyethyl) phosphine hydrochloride (TCEP); 5% sodium deoxycholate) and incubated for 10 min at 60 °C. Thiol alkylation was achieved by adding 5 μl alkylation buffer (100 mM iodoacetamide in water) and incubating the samples for 1 h at room temperature. Following alkylation, samples were diluted with 275 μL dilution buffer (25 mM ammonium bicarbonate, pH 8), 2 μL trypsin solution (1 mg/mL) was added, and incubated overnight at 37 °C. To stop the reaction, 10 μL of 10% trifluoroacetic acid (TFA) was added, incubated for 30 min, and centrifuged at 15,000 x g. Supernatants were transferred to microfuge tubes and used for LC-MS analysis.

**LC-MS analysis**

All analyses were carried out using a Waters nanoAcquity UPLC System coupled to a Waters Synapt G2 mass spectrometer. The mobile phases were composed of Solvent A (0.1% formic acid in H₂O) and Solvent B (0.1% formic acid in acetonitrile). Injection volume was 2 μL. Following injection, the peptides were concentrated on a Trap C-18 enrichment column (0.3 x 1 mm, Waters) and washed at 10 μL/min with Solvent A for 3 min. The enrichment column was then switched into the nanoflow path (500 nL/min) and further separated on a C-18 reversed phase nanocolumn (0.075 x 250 mm; Waters) coupled with the nanoelectrospray ionization (nESI) source of Synapt G2 mass spectrometer. Separation of peptides was achieved at the following gradient: T = 0 min: 5% B; T = 95 min: 50% B; T = 96 min: 85% B; T = 97 min: 85% B; T = 98 min: 5% B; T = 99 min: 5% B; T = 100 min: 85%; B; T = 101 min: 85% B T = 102 min: 5% B and T = 120 min: 5% B (column re-equilibration). MS data were collected in positive, Data Independent Acquisition (MS²) mode under a capillary voltage of 2,900 V. The source temperature was set at 70 °C. Cone gas flow was maintained 6 L/min. Acquisition range was 50–2,000 m/z. MS² data was collected with alternating low (4 eV) and elevated (ramp from 17 to 42 eV) energy over a 100–1500 m/z range. Spectra and peptide identification statistical analysis were carried out using Progenesis software (Waters Corporation, MA) and was searched against all S. aureus Mu50 predicted proteins (NCBI database).

**Power analysis**

Experimental variability was assessed using a pilot proteome study of strains SA564 and SA564-ΔhptRS cultivated in TSB. A power analysis was conducted to determine the number of biological replicates needed to achieve the probability of detecting a true difference between strains during the exponential growth phase (2 h) and post-exponential growth phase (6 h) when one exists. It was determined that 5 biological replicates of each strain, growth phase (hour), and cultivation condition (media) combination would achieve a minimum of 74% power assuming dispersion (variability) of 0.05 and 99% power assuming dispersion of 0.01 (Table A in S2 File). The power analysis was conducted using SAS software, Version 9.4 of the SAS System for Windows (Copyright 2002–2012, SAS Institute Inc.). SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA. The supporting information file 1 contains examples of the SAS code corresponding to the power analysis (S1 File).
Statistical analysis of the total cytosolic proteome

The relative normalized abundance of each peptide was extracted as a .txt file from Progenesis for statistical analysis. Statistical analysis was performed using R 3.3 software, R Core Team (2017) [48]. The supporting information file 1 contains examples of the R code corresponding to the proteomic analysis (S1 File). Final data sets were exported into Microsoft Excel files for final organization and interpretation.

Peptide data were visualized in a histogram and the relative normalized peptide abundance had a unimodal, right skewed, distribution with responses greater than or equal to 0. For this reason, data were modeled using a generalized linear model that assumed the relative normalized peptide abundance followed a gamma distribution with strain (s), growth phase (h), and media (m) as fixed effects and the relative normalized abundance as the response variable [49]. Peptides with a 0 abundance value, due to an abundance not included in the gamma distribution, were assigned a value of 0.0001 to facilitate modeling and statistical analysis. Peptide abundance and variability were combined and protein abundance was modeled and analyzed for interactions between variables of strain (s), media (m), and/or growth phase (h) (Tables B-J in S2 File). A significant interaction would indicate that the effect of one variable depends on the magnitude of change of another variable. For any significant interaction (\( p \leq 0.2 \)) the simple effects, the effect of a single variable given the level of another variable on protein abundance, were assessed (\( p \leq 0.05 \); Figures F-I in S6 File; Tables A-D in S3 File). For all proteins showing no significant interaction between variables, the main effect of each variable was analyzed by protein (\( p \leq 0.2 \); Figures J-L in S6 File; Tables E-G in S3 File). A main effect is the effect of a single variable on protein abundance, averaged over all other levels of variables. For any protein with a main effect of strain, the abundance of each protein was analyzed for significant differences between strains SA564, SA564-ΔhptRS, SA564-ΔccpA and SA564-ΔhptRS/ΔccpA (\( p \leq 0.05 \); Figure L in S6 File; Table G in S3 File). In order to quantify any differences between SA564, SA564-ΔhptRS, SA564-ΔccpA and SA564-ΔhptRS/ΔccpA the relative fold-change between strains was computed and exported for proteins with a significant interaction or main effect that included strain. All proteins that were identified as significantly (\( p \leq 0.05 \)) different between the strains were contained within the 3-way interaction of variables strain, media, and growth phase (s x m x h), two-way interaction of strain and growth phase averaged over media (s x h | m), and the main effect of strain (Figures F, H, L in S6 File; Tables A-C in S4 File). Only proteins, with at least two peptides identified within the LC-MS\(^E\) analysis, a significant interaction or a main effect (\( p \leq 0.2 \)), and with a significant difference (\( p \leq 0.05 \)) between the strains were taken into account for subsequent interpretation and transcriptional analysis (Table 3; Tables D, E in S4 File).

Results and discussion

Deletion of hptRS does not alter growth or sensitivity to stressors

To assess the growth and viability of strain SA564-ΔhptRS relative to the isogenic wild-type strain SA564, the growth and pH profiles were determined (Fig 1). The growth of strains SA564 and SA564-ΔhptRS were equivalent when cultivated in TSB and TSB-dex (Fig 1A and 1B). Similarly, strains SA564 and SA564-ΔhptRS had equivalent growth when cultivated anaerobically or in iron-limited TSB (Figure A in S6 File). Strains SA564-ΔhptRS and SA564 responded comparably when cultivated in TSB with oxidative stress-inducing compounds (i.e., streptonigrin, hydrogen peroxide, diamide, and paraquat) or membrane de-couplers (i.e., carbonyl cyanide 3-chlorophenylhydrazon E and nigericin) (Figures B, D in S6 File). In addition, the antibiotic resistance profiles were equivalent when compared using disk diffusion.
Table 3. Significant protein differences in SA564-ΔccpA relative to SA564-ΔhptRS/ΔccpA.

### Three Way Interaction (s x m x h)

| Protein     | CcpA Regulated | E.C. | Protein Function                  | Peptides/Protein | FC TSB 2h | FC TSB 6h | FC TSB-dex 2h | FC TSB-dex 6h |
|-------------|----------------|------|-----------------------------------|------------------|-----------|-----------|----------------|----------------|
| CLPL_STAAM  |                |      | ATP-dependent Clp protease        | 60               | 0.998     | 1.396     | 0.938          | 1.651          |
| DHA1_STAAM  | *              | EC:1.4.1.1 | Alanine dehydrogenase          | 7               | 0.944     | 1.276     | 1.096          | 2.550          |
| LDH2_STAAM  |                | EC:1.1.1.27 | L-lactate dehydrogenase 2      | 42              | 1.114     | 2.427     | 1.206          | 1.478          |
| OTC_STAAM   | ***            | EC:2.1.3.3 | Ornithine transcarbamoylase     | 5               | 1.024     | 1.568     | 1.128          | 2.291          |
| PFLB_STAAM  | ***            | EC:2.3.1.54 | Formate C-acetyltransferase     | 146             | 1.049     | 2.765     | 1.136          | 1.908          |
| IDH_STAAM   | ***            | EC:1.1.1.42 | Isocitrate dehydrogenase        | 64              | 0.693     | 0.635     | 0.749          | 0.869          |
| SYS_STAAM   |                | EC:6.1.1.11 | Seryl-tRNA synthetase           | 69              | 1.019     | 1.011     | 1.119          | 0.703          |

### Two Way Interaction (s x h)

| Protein     | CcpA Regulated | E.C. | Protein Function                  | Peptides/Protein | FC 2h | FC 6h |
|-------------|----------------|------|-----------------------------------|------------------|-------|-------|
| Y840_STAAM  |                |      | Uncharacterized protein SAV0840    | 32               | 1.328 | 1.712 |
| ADH_STAAM   | *              | EC:1.1.1.1 | Alcohol dehydrogenase            | 41               | 1.009 | 2.241 |
| ASP23_STAAM |                |      | Alkaline shock protein 23         | 52               | 1.416 | 1.723 |
| DAPH_STAAM  |                | EC:2.3.1.89 | Tetrahydrodipicolinate N-acetyltransferase | 2 | 1.026 | 1.897 |
| SBI_STAAM   | *              |      | Immunoglobulin-binding protein    | 9                | 1.002 | 1.532 |
| OHRL_STAAM  |                |      | Organic hydroperoxide resistance protein-like | 3   | 1.158 | 2.595 |
| ACKA_STAAM  | *              | EC:2.7.2.1 | Acetate kinase                    | 73               | 1.172 | 1.489 |
| Y1710_STAAM |                |      | Putative universal stress protein SAV1710 | 30   | 1.102 | 1.617 |
| Y1625_STAAM |                |      | Uncharacterized protein SAV1625    | 20               | 1.717 | 2.095 |
| SYG_STAAM   |                | EC:6.1.1.14 | Glycyl-tRNA synthetase           | 52               | 0.967 | 0.769 |
| PCKA_STAAM  | ***            | EC:4.1.1.49 | Phosphoenolpyruvate carboxykinase | 73               | 1.008 | 0.778 |
| TKT_STAAM   |                | EC:2.2.1.1 | Transketolase                    | 84               | 0.821 | 0.697 |
| FTHS_STAAM  | ***            | EC:6.3.4.3 | Formyltetrahydrofolate synthetase | 87   | 0.857 | 0.672 |
| FOLD_STAAM  | *              | EC:1.5.1.5 | Methylenetetrahydrofolate dehydrogenase | 48   | 0.936 | 0.790 |
| ENO_STAAM   | *              | EC:4.2.1.11 | 2-phospho-D-glycerate hydro-lyase | 78   | 0.806 | 0.740 |
| TPX_STAAM   |                | EC:1.11.1.15 | Probable thiol peroxidase        | 27               | 0.794 | 0.553 |
| G6PI_STAAM  |                | EC:5.3.1.9 | Glucose-6-phosphate isomerase     | 77               | 0.896 | 0.738 |

### Main Effect (s)

| Protein     | CcpA Regulated | E.C. | Protein Function                  | Peptides/Protein | FC   |
|-------------|----------------|------|-----------------------------------|------------------|------|
| DNAK_STAAM  |                |      | Chaperone protein                  | 151              | 1.216 |
| EFG_STAAM   |                |      | Elongation factor G                | 173              | 1.111 |
| IMDH_STAAM  | *              | EC:1.1.1.205 | Inosine-5’-monophosphate dehydrogenase | 125   | 1.197 |
| MQO2_STAAM  |                | EC:1.1.5.4 | Probable malate-quinone oxidoreductase 2 | 125 | 1.159 |
| QOX2_STAAM  |                | EC:1.10.3.12 | Probable quinol oxidase subunit 2 | 45               | 1.238 |
| RSBW_STAAM  | *              | EC:2.7.11.1 | Serine-protein kinase RsbW        | 2                | 1.227 |
| SARS_STAAM  | *              |      | HTH-type transcriptional regulator | 6                | 1.640 |
| SODM2_STAAM | *              | EC:1.15.1.1 | Superoxide dismutase              | 10               | 1.660 |
| ALDA_STAAM  | ***            | EC:1.2.1.3 | Putative aldehyde dehydrogenase   | 88               | 0.784 |
| CRTN_STAAM  |                | EC:1.3.8.2 | Dehydrodsqualene desaturase       | 2                | 0.684 |
| CYSK_STAAM  |                | EC:2.5.1.47 | Cysteine synthase                 | 66               | 0.798 |
| DBH_STAAM   |                |      | DNA-binding protein HU            | 46               | 0.714 |
| DEOC2_STAAM |                | EC:4.1.2.4 | Deoxyribose-phosphate aldolase 2  | 41               | 0.824 |
| DNLJ_STAAM  |                | EC:6.5.1.2 | DNA ligase                        | 7                | 0.450 |

(Continued)
assays (i.e., ampicillin, penicillin, tetracycline, kanamycin, neomycin, doxycycline, chloramphenicol, vancomycin, minocycline, and oxytetracycline) (Figure C in S6 File). The lack of clear phenotypic differences between strains SA564 and SA564-ΔhptRS indicated that this two-component system was inactive under the conditions tested, was non-functional, or that there was overlapping regulation that masks the loss of the hptRS two-component system. To determine if and/or when hptRS is transcribed, the mRNA abundances of hptR and hptS were assessed during the exponential (2 h) and post-exponential (6 h) growth phases in strain SA564 during aerobic and anaerobic cultivation (Figure E in S6 File). Transcription of hptS was reduced 2.42-fold in the post-exponential growth phase during aerobic cultivation (Figure E in S6 File; \( p \leq 0.05 \)). In contrast, transcription of hptR (1.8-fold) and hptS (1.5-fold) was greater in the post-exponential growth phase during anaerobic cultivation (Figure E in S6 File; \( p \leq 0.05 \)). These data demonstrate that hptS and hptR are temporally regulated, and indicate that overlapping regulation may mask the effects of hptRS deletion.

Deletion of ccpA alters the accumulation and/or depletion of ammonia, acetate, and glucose in the culture media

When cultivated in TSB, inactivation of ccpA slightly reduced the growth rates (\( \mu \)) of strains SA564-ΔccpA (\( \mu = 1.432 \) h\(^{-1} \)) and SA564-ΔhptRS/ccpA (\( \mu = 1.474 \) h\(^{-1} \)) relative to strain SA564 (\( \mu = 1.673 \) h\(^{-1} \); Fig 1A). Concomitant with the reduced growth rates in the ccpA inactivated strains, ammonia accumulation was slightly increased and the rate of glucose consumption was slightly decreased during the exponential phase (Fig 2A and 2E), indicating that the reduced growth rate is accompanied by reduced carbon flow through glycolysis and increased amino acid catabolism (Fig 3). Interestingly, while acetate accumulation in strains SA564, SA564-ΔhptRS and SA564-ΔccpA cultivated in TSB were similar, strain SA564-ΔhptRS/ccpA had a significantly altered acetate accumulation and depletion profile (Fig 2C). Similarly, ammonia accumulation was significantly different when strains SA564-ΔccpA and SA564-ΔhptRS/ccpA were compared (Fig 2A and 2B). The absence of a growth phenotype in strain SA564-ΔhptRS, but the presence of a growth phenotype in strain SA564-ΔhptRS/ccpA that is

### Table 3. (Continued)

| Genbank Accession | EC | Gene Product | Fold Change |
|-------------------|----|--------------|-------------|
| FEMA_STAAM        | EC:2.3.2.17 | Aminoa cyltransferase | 2 | 0.654 |
| GLYA_STAAM        | EC:2.1.2.1 | Serine hydroxymethyltransferase | 58 | 0.839 |
| ISPD2_STAAM       | EC:2.7.7.40 | 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase 2 | 18 | 0.817 |
| MURA2_STAAM       | EC:2.5.1.7 | UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2 | 11 | 0.784 |
| RS1_STAAM         | 30S ribosomal protein S1 | 58 | 0.746 |
| THIO_STAAM        | Thioredoxin | 46 | 0.777 |
| THLA_STAAM        | Probable acetyl-CoA acyltransferase | 32 | 0.818 |
| Y2578_STAAM       | HTH-type transcriptional regulator | 2 | 0.753 |
| Y968_STAAM        | Uncharacterized protein SAV0968 | 44 | 0.791 |

***—Bioinformatically Identified and Experimentally Verified as CcpA regulated [8, 18]
**—Bioinformatically Identified as CcpA regulated through Regprecise database [18]
'—Experimentally Identified as CcpA regulated [8]

Cells with diagonal line backgrounds = Not Significant
Cells with bold borders = Significant (\( p \leq 0.05 \))
FC—Fold Change

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different from strain SA564-ΔccpA, indicates there is an antagonistic relationship between HptRS and CcpA. As expected, these differences decreased when the strains were cultivated in TSB-dex (Figs 1B, 1D, 2B and 2D). While these observations are consistent with previous reports on CcpA, they establish a previously unobserved linkage between HptRS and CcpA co-

Fig 1. The growth and pH profile of SA564-ΔhptRS/ccpA is moderately different from SA564, SA564-ΔhptRS, and SA564-ΔccpA during the post-exponential phase. The A_{600} (A, B) and pH (C, D) were measured every hour for 12 hours. S. aureus strains SA564 (black symbols), SA564-ΔhptRS (red symbols), SA564-ΔccpA (green symbols), and SA564-ΔhptRS/ccpA (blue symbols) were cultivated in TSB (A, C) or TSB-dex (B, D). Data are representative of the mean of experiments performed in biological triplicate, with error bars representing the standard error of the mean. A statistically significant difference (p ≤ 0.05) between SA564-ΔccpA and SA564-ΔhptRS/ccpA is represented with an (*).

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Fig 2. Metabolism of strain SA564-ΔhptRS/ccpA is different from strains SA564, SA564-ΔhptRS, and SA564-ΔccpA. Ammonia accumulation (A, B), acetate accumulation and depletion (C, D), and glucose depletion (E) in the culture media for S. aureus strains SA564 (black symbols), SA564-ΔhptRS (red symbols), SA564-ΔccpA (green symbols), and SA564-ΔhptRS/ccpA (blue symbols) cultivated in TSB (A, C, E) and TSB-dex (B, D) are depicted. Data are representative of the mean metabolite concentrations plotted as a function of cell density ($A_{600}$) for experiments performed in biological triplicate, with error bars representing the standard error of the mean for absorbance ($A_{600}$) and [NH$_3$] or [Acetate] or [Glucose].

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CcpA and HptRS co-regulation of transcription

regulation [6, 8, 40, 50, 51]. To determine the extent of the regulatory linkage between CcpA and HptRS, total cytosolic proteomes were analyzed by mass spectroscopy during the exponential (2 h) and post-exponential (6 h) growth phases in the presence or absence of glucose.

Proteomics overview (experimental design and statistical analysis)

Cytosolic protein fractions were harvested for five biological replicates for each strain, cultivated in TSB and TSB-dex, during the exponential (2 h) and post-exponential (6 h) growth phases for a total of 80 samples, and analyzed by LC-MS. Using LC-MS, a total of 33,026 spectral peaks were collected and analyzed. The raw data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD008708 [52]. The spectra were compared against S. aureus strain Mu50 predicted proteins (NCBI database) using Progenesis QI, and yielded 11,971 identifiable spectral peaks, with sequences that were assigned to 501 proteins. Comparative quantitative analysis was completed for each...
protein incorporating the relative normalized abundance for each peptide, yielding a relative abundance for each protein. Proteins identified by two or more peptides (n = 438 proteins) were analyzed for interactions between experimental variables \([i.e., \text{strain (s)}, \text{media (m)}, \text{and growth phase (h)}]; \text{Tables B-F in S2 File}]. Several groups of proteins emerged within this data set based on the interaction between the variables: (i) Proteins with an abundance that was altered between bacterial strains, cultivation media, and growth phases were defined as having a 3-way interaction \((s \times m \times h)\) (Table C in S2 File). (ii) Proteins with an abundance that was altered between bacterial strains and cultivation media, irrespective of the growth phase, were defined as having a 2-way interaction averaged over the growth phase \((s \times m \mid h)\) (Table D in S2 File). (iii) Proteins with an abundance that was altered between bacterial strains and within the two growth phases, regardless of the cultivation media were defined as having a 2-way interaction averaged over media \((s \times h \mid m)\) (Table E in S2 File). (iv) Proteins with an abundance that was altered between different cultivation media and within the growth phases, irrespective of the strain, were defined as having a 2-way interaction averaged over strain \((m \times h \mid s)\) (Table F in S2 File). (v) Proteins with an abundance that was altered between the strains but unaffected by growth phase or cultivation media, were defined as having a main effect of strain and no other interactions (Table I in S2 File). A \(p\)-value cutoff of 0.2, representing that there is 80% confidence in rejecting the null hypothesis that protein abundance was equal between variables, was utilized to determine which proteins would be further analyzed. Using this cutoff, 190 proteins were selected for further analysis. Of these proteins, 105 had designated Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations and enzyme commission (EC) numbers (Table J in S2 File). All proteins that were selected for further analysis were input into the Protein ANnotation THrough Evolutionary Relationship (PANTHER) classification system and sorted by molecular function (Fig 4) [53]. Of the 105 proteins input into PANTHER, the majority were predicted to have catalytic activity (Fig 4) [8, 51, 54].

To determine if proteins with significant interactions or main effects contained statistically significant differences \((p \leq 0.05)\) between the strains, growth phases, growth medium, or a combination of these variables, multiple comparisons were calculated and \(p\)-values generated for the simple effects (combination of variables) or main effect of each variable (Tables A-G in S3 File). For a given protein, if a significant 3-way interaction was observed, then the effects of each variable were considered \((p \leq 0.05; \text{3-way multiple comparison})\); if no significant 3-way interaction was observed but a significant 2-way interaction was observed, then the effects of each variable within the 2-way analysis was considered \((p \leq 0.05)\) averaging over the third variable (2-way multiple comparison); and if no significant 3-way or 2-way interactions were observed, then the effect of strain was considered \((p \leq 0.05)\) averaged over media and growth phase (main effect comparison). A \(p\)-value cutoff of 0.05 was used to determine if there was a statistically significant difference between the effects of strain \((s)\), media \((m)\) and growth phase \((h)\). With this cutoff, it was determined that all proteins identified as being significantly altered were contained within the 3-way interaction \((s \times m \times h)\), 2-way interaction \((s \times h \mid m)\), and the main effect of strain \((s)\), hence an emphasis was placed on the simple effects or main effect within these categories for comparisons between strains (Table J in S2 File; Tables A, C, G in S3 File). To quantify specific differences in protein abundance between strains SA564, SA564-\(\Delta hptRS\), SA564-\(\Delta ccpA\) and SA564-\(\Delta hptRS/\Delta ccpA\) fold changes \((i.e., \text{SA564/mutant or mutant/mutant})\) were computed for each protein with a significant interaction or main effect (Table 3; Tables A-E in S4 File). In total, 13 proteins were identified as having significant 3-way interactions, 53 proteins had significant 2-way interactions between strain and growth phase, and 124 proteins were identified for further analysis due to the main effect of strain, providing a high probability of identifying specific proteomic differences within and between strains SA564, SA564-\(\Delta hptRS\), SA564-\(\Delta ccpA\) and SA564-\(\Delta hptRS/\Delta ccpA\) (Table J in S2 File).
Media and temporal changes in strain SA564

*S. aureus* adaptations to glucose-rich and -limited environments have been studied [8, 51, 55]. Cultivation of *S. aureus* in glucose-rich media increases the accumulation of proteins involved in glycolysis and protein synthesis. In contrast, cultivation in glucose-limited media increased the abundance of proteins involved in the tricarboxylic acid cycle, amino acid catabolism, and gluconeogenesis. Although less well-studied, temporal protein changes during cultivation in medium containing glucose have also been examined [55]. Temporal changes occur when glucose is depleted and incompletely oxidized carboxylic acids become the primary carbon sources (Fig 3). In response to changing carbon sources, the proteome of *S. aureus* must adapt to supply energy and metabolic precursors for growth, which causes a large temporal rearrangement in protein accumulation. Many of these temporal changes are mediated in part by CcpA [8, 12, 51]; hence, several of the significant proteins found in the 3-way and 2-way
interactions are translated from genes that are known or predicted to be regulated by CcpA (Tables A-D in S3 File; Table D in S4 File). As examples: (i) Glycolytic/gluconeogenic proteins triosephosphate isomerase (EC:5.3.1.1) and glyceraldehyde 3-phosphate dehydrogenase (EC:1.2.1.12) (s x h | m, m x h | s; p ≤ 0.2) are significantly decreased in strain SA564 during the post-exponential growth phase in TSB and increased during the post-exponential phase in TSB-dex (p ≤ 0.05); Phosphoglycerate kinase (EC:2.7.2.3) (s x h | m, m x h | s, m x s | h; p ≤ 0.2) is increased in strain SA564 cultivated in TSB-dex relative to TSB and increased during the post-exponential phase in TSB-dex (p ≤ 0.05); and pyruvate kinase (EC:2.7.1.40) (s x m x h, p ≤ 0.2) is significantly decreased in strain SA564 during the post-exponential growth phase in TSB relative to TSB-dex (p ≤ 0.05). (ii) TCA cycle proteins aconitate hydratase (EC:4.2.1.3) (s x h | m, m x h | s; p ≤ 0.2) and isocitrate dehydrogenase (EC:1.1.1.42) (s x m x h; p ≤ 0.2) are significantly increased in both TSB and TSB-dex during the post-exponential growth phase in strain SA564 (p ≤ 0.05) relative to the exponential growth phase, and succinyl-coA synthase subunit A (EC:6.2.1.5) (s x h | m, m x h | s, m x s | h; p ≤ 0.2) and succinyl-coA synthase subunit B (EC:6.2.1.5) are significantly increased in strain SA564 during the post-exponential phase in TSB (p ≤ 0.05) relative to TSB-dex. (iii) The amino acid catabolic protein alanine dehydrogenase 1 (EC:1.4.1.1) (s x m x h; p ≤ 0.2) is increased during the post-exponential growth phase in TSB-dex (p ≤ 0.05) relative to TSB in strain SA564, while glutamate dehydrogenase (EC:1.4.1.2) (s x h | m, m x h | s; p ≤ 0.2) is increased during the post-exponential growth phase in TSB and TSB-dex (p ≤ 0.05) in strain SA564 relative to the exponential growth phase. (iv) The gluconeogenic protein phosphoenolpyruvate carboxykinase (EC:4.1.1.49) (s x h | m, m x h | s; p ≤ 0.2) is significantly increased during the post-exponential growth phase in strain SA564 cultivated in TSB and TSB-dex relative to the exponential phase (p ≤ 0.05). The media and temporal differences in strain SA564, and previous observations on cultivation media and temporal differences in other S. aureus strains [8, 55], establish an important baseline for understanding the proteomic changes associated with CcpA and HptRS transcriptional regulation.

**Protein abundances are similar between strains SA564 and SA564-ΔhptRS**

Consistent with the growth and pH profiles, deletion of hptRS did not significantly (p ≥ 0.05) alter cytosolic protein abundance in response to changing growth phases or media composition in strain SA564-ΔhptRS relative to strain SA564 for proteins identified as having significant 3-way (s x m x h, p ≤ 0.2) or 2-way (s x h | m, p ≤ 0.2) interactions, or main effect of strain (s, p ≤ 0.2) (Tables A-C in S4 File). These results indicated that HptRS may be inactive under these conditions or the genes regulated by HptRS are also under the control of another glucose-dependent transcriptional regulator, such as CcpA. To determine if CcpA masks the effect of hptRS genetic inactivation, the proteomes of strains SA564-ΔccpA and SA564-ΔhptRS/ΔccpA were analyzed.

**Inactivation of ccpA decreases the accumulation of glycolytic proteins, and increases TCA cycle and amino acid catabolic proteins**

For proteins identified as having significant 3-way (s x m x h, p ≤ 0.2), 2-way (s x h | m, p ≤ 0.2), or a main effect of strain (s, p ≤ 0.2), a total of 56 were significantly different between strains SA564-ΔccpA and SA564 (p ≤ 0.05), (Table D in S4 File). Of these proteins, 69.6% have been experimentally identified or predicted by promoter sequence to be regulated by CcpA [8, 12, 18, 19, 51]. These include proteins associated with glycolysis, such as fructose bisphosphate aldolase (EC:4.1.2.13), glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase (EC:4.2.1.11), and pyruvate kinase, which were decreased in strain SA564-ΔccpA
relative to strain SA564, primarily during the exponential growth phase \( (p \leq 0.05) \). In contrast, the TCA cycle proteins aconitate hydratase, 2-oxoglutarate dehydrogenase (EC: 2.3.1.61), succinyl-CoA complex proteins, and fumarate hydratase (EC: 4.2.1.2) were increased by \( \text{ccpA} \) inactivation relative to strain SA564 during the exponential growth phase. Amino acid metabolic proteins such as alanine dehydrogenase, threonine deaminase (EC: 4.3.1.19), aminomethyltransferase protein (EC: 2.1.2.10), and glutamate dehydrogenase were also increased during the exponential growth phase in strain SA564-\( \Delta \text{ccpA} \) relative to strain SA564. As expected, fewer proteins were significantly different between SA564-\( \Delta \text{ccpA} \) and SA564 during the post-exponential growth phase when glucose was depleted; however, \( \text{CcpA} \) regulated several proteins even in the absence of glucose, such as pyruvate kinase, 1-pyrroline-5-carboxylate dehydrogenase (EC:1.2.1.88), and succinyl-CoA synthetase subunit beta [8, 55]. These data indicate that \( \text{CcpA} \)-mediated changes in protein abundance were both glucose-dependent and -independent, suggesting factors other than glycolytic intermediates and HPr (e.g., Stk1), antagonize \( \text{CcpA} \) transcriptional regulation [20] (Table D in S4 File).

**Many differentially regulated proteins in strain SA564-\( \Delta \text{hptRS/ccpA} \) are common to the CcpA regulon**

For proteins having significant 3-way \( (s \times m \times h, p \leq 0.2) \) or 2-way \( (s \times h \mid m, p \leq 0.2) \) interactions, or a main effect of strain \( (s, p \leq 0.2) \), 98 proteins were identified as significantly different between SA564-\( \Delta \text{hptRS/ccpA} \) and SA564 \( (p \leq 0.05) \) (Table E in S4 File). Of these proteins, 46.9% have been experimentally determined or predicted to be regulated by \( \text{CcpA} \) [8, 18]. The majority \( (n = 48) \) of significantly different proteins found in SA564-\( \Delta \text{ccpA} \) \( (n = 56 \text{ proteins}) \) were also found in SA564-\( \Delta \text{hptRS/ccpA} \) relative to SA564 (Tables D, E in S4 File). Interestingly, while \( \text{CcpA} \)-regulated proteins were common to both SA564-\( \Delta \text{hptRS/ccpA} \) and SA564-\( \Delta \text{ccpA} \), several proteins \( (n = 12) \) had different fold changes in protein abundance relative to SA564. As examples, pyruvate formate lyase (EC: 2.3.1.54) and alcohol dehydrogenase (EC: 1.1.1.1) were present in greater abundance in strain SA564-\( \Delta \text{ccpA} \) relative to SA564, yet lower in abundance in strain SA564-\( \Delta \text{hptRS/ccpA} \) relative to SA564. These data suggest an antagonistic regulatory relationship between HptRS and \( \text{CcpA} \) and that certain enzymatic/protein differences caused by inactivation of \( \text{HptRS} \) can only be visualized by removing \( \text{CcpA} \)-regulated proteins from the proteome of strain SA564-\( \Delta \text{hptRS/ccpA} \). To do this, significant differences in protein abundance in strain SA564-\( \Delta \text{hptRS/ccpA} \) relative to SA564-\( \Delta \text{ccpA} \) were analyzed.

**HptRS antagonizes CcpA-dependent regulation**

A total of 47 proteins, identified as having significant 3-way \( (s \times m \times h, p \leq 0.2) \), 2-way \( (s \times h \mid m, p \leq 0.2) \), or main effect of strain \( (s, p \leq 0.2) \), were identified as having significantly altered abundance in strain SA564-\( \Delta \text{ccpA} \) relative to SA564-\( \Delta \text{hptRS/ccpA} \) \( (p \leq 0.05) \); Table 3). Of these proteins, 34% are predicted to be regulated by \( \text{CcpA} \) [8, 18]. Specifically, genes encoding enzymes pyruvate formate lyase B, alanine dehydrogenase, ornithine transcarbamoylase (EC:2.1.3.3), acetate kinase (EC: 2.7.2.1), and alcohol dehydrogenase, have predicted \( \text{cre} \) sites in their promoter regions, and were significantly increased during the post-exponential growth phase in strain SA564-\( \Delta \text{ccpA} \) relative to SA564-\( \Delta \text{hptRS/ccpA} \) [8, 15, 18] (Table 3). These results suggest that the regulons of \( \text{CcpA} \) and HptRS likely overlap, particularly during the post-exponential growth phase.

Significant differences were also observed within pyruvate, amino acid metabolic, and TCA cycle enzymes during the post-exponential phase \( (p \leq 0.05) \); Table 3). As examples, pyruvate formate lyase B, and acetate kinase had significantly increased protein abundances in strain SA564-\( \Delta \text{ccpA} \) relative to SA564-\( \Delta \text{hptRS/ccpA} \). Amino acid metabolic enzymes such as alanine...
dehydrogenase, tetrahydrodipicolinate acetyltransferase (EC: 2.3.1.89), and ornithine transcarbamoylase had significantly increased protein abundances in strain SA564-ΔccpA relative to strain SA564-ΔhptRS/ccpA. These differences in pyruvate metabolic and amino acid catabolic protein abundance are consistent with the physiological differences in acetate and ammonia accumulation between strains SA564-ΔccpA and SA564-ΔhptRS/ccpA (Figs 1 and 2; Table 3). Additionally, the abundance of the TCA cycle enzyme isocitrate dehydrogenase was significantly decreased in strain SA564-ΔccpA relative to strain SA564-ΔhptRS/ccpA (p ≤ 0.05; Table 3). Taken together, these data suggest that HptR functions to antagonize transcription of a subset of CcpA-regulated genes, which give rise to alterations in protein accumulation. To assess this possibility, the mRNA abundance of select genes was determined to gauge transcriptional regulation of genes encoding proteins that were significantly different in the proteome.

**CcpA and HptRS regulate genes encoding for proteins identified as being significantly different in proteomic analysis**

Proteomic analysis demonstrated that genetic inactivation of the genes coding for the transcriptional regulators ccpA alone or in combination with hptRS resulted in differential protein abundances. The most likely explanation for these results is that transcription of the genes coding for the differentially-regulated proteins was altered. To assess this possibility, mRNA transcript levels were determined for select genes using RT-qPCR. Genes for pyruvate formate lyase B (pflB; sav0226) and alanine dehydrogenase (dha1; sav1439) were selected for analysis due to their significant 3-way interaction (s x m x h; p ≤ 0.2), and having a protein abundance fold-change greater than 1.5 (FC > 1.5) in strain SA564-ΔccpA relative to SA564-ΔhptRS/ccpA (Table 3). Alcohol dehydrogenase (adh; sav0605) was assessed due to its significant 2-way interaction (s x h | m; p ≤ 0.2) and having a protein abundance fold-change greater than 1.5 (FC > 1.5) in strain SA564-ΔccpA relative to SA564-ΔhptRS/ccpA (Table 3). Lastly, the 1-pyrroline-5-carboxylate dehydrogenase gene (roca; sav2554) was assessed due to its significant 3-way interaction (s x m x h; p ≤ 0.2), and also having a protein abundance fold-change above 1.5 (FC > 1.5) in strains SA564-ΔccpA and SA564-ΔhptRS/ccpA relative to SA564 (Tables D, E in S4 File). In addition, roca was chosen as a control due to the lack of a difference in protein abundance between strains SA564-ΔccpA and SA564-ΔhptRS/ccpA, which indicated this effect was caused by CcpA alone. An additional consideration for choosing these genes was that pflB, dha1, adh, and roca have been previously identified or predicted to be transcriptionally regulated by CcpA [18, 51, 56].

The mRNA abundances of pflB, dha1, and adh in SA564-ΔccpA were significantly decreased (FC ≥ 1.5; p ≤ 0.05) during the exponential growth phase (Fig 5A and 5B) and significantly increased (FC ≥ 1.5; p ≤ 0.05) during the post-exponential growth phase (Fig 5C and 5D) relative to SA564 in either TSB or TSB-dex (Tables A, B in S5 File). CcpA is a transcriptional repressor of pflB, adh, and dha1, therefore ccpA inactivation should increase mRNA abundance [8]. Interestingly, an increase mRNA abundance was only observed during the post-exponential growth phase for strain SA564-ΔccpA in TSB and TSB-dex (Fig 5C and 5D). As expected, post-exponential growth phase transcription of pflB, adh, and dha1 was significantly increased (p ≤ 0.05) in strain SA564-ΔccpA cultivated in TSB relative to that cultivated in TSB-dex (Table D in S5 File). Together, these data suggest there are one or more transcriptional activators whose activity is repressed by glucose, or more appropriately glucose depletion, and that activates post-exponential growth phase transcription of pflB, adh, and dha1 when CcpA repression is relieved.
Consistent with there being an activator of \( \text{pflB} \), \( \text{dha1} \), and \( \text{adh} \) transcription, a significant reduction (FC \( \geq 1.5; p \leq 0.05 \)) in mRNA abundance of \( \text{pflB} \), \( \text{dha1} \), and \( \text{adh} \) in strain SA564-\( \Delta \text{hptRS/ ccpA} \) relative to strain SA564-\( \Delta \text{ccpA} \), irrespective of media or growth phase, (Fig 5; Table C in S5 File) was observed, indicating that HptRS activated transcription of these genes upon CcpA de-repression or inactivation. During the post-exponential growth phase, when cultivated in TSB, strain SA564-\( \Delta \text{hptRS/ ccpA} \) produced transcripts for \( \text{pflB} \), \( \text{dha1} \), and \( \text{adh} \), indicating that other regulators are also involved in glucose-dependent transcription of these genes (Fig 5C). In contrast to \( \text{pflB} \), \( \text{dha1} \), and \( \text{adh} \) mRNA levels, significant exponential growth phase increases (\( p \leq 0.05 \)) in mRNA abundance of \( \text{rocA} \) were observed in \( \text{ccpA} \) inactivated
strains relative to SA564, irrespective of hptRS inactivation (Fig 5A and 5B; Tables A, B in S5 File). During the post-exponential growth phase, there were only small differences in rocA mRNA levels between strains SA564-ΔccpA and SA564-ΔhptRS/ccpA (Fig 5C and 5D), confirming that rocA is primarily regulated by CcpA. Taken together, these data demonstrate that transcription of pflB, dha1, and adh is co-regulated by CcpA and HptRS, while rocA is largely regulated by CcpA. These data also demonstrate the CcpA and HptR regulons intersect and that CcpA functions as a transcriptional repressor, while HptR functions as a transcriptional activator.

**Predicted membrane-associated proteins are also regulated by CcpA and HptRS**

Histidine kinase HptS, the protein of unknown function HptA, and UhpT are predicted to be membrane-associated and similar to homologous proteins found in *E. coli*, hence they are unlikely to be present in the cytosolic fraction used for proteomic analysis [32, 57]. To determine if CcpA regulates transcription of hptS, hptA, and uhptT, mRNA abundance was assessed in the exponential and post-exponential growth phases from bacteria cultivated in TSB or TSB-dex (Fig 5). Post-exponential growth phase transcription of hptS and hpta was significantly (*p* ≤ 0.05) increased in strain SA564-ΔccpA relative to strain SA564 cultivated in TSB (Fig 5C; Table A in S5 File). As expected, the magnitude of this effect was lessened when the bacteria were cultivated in TSB-dex (Fig 5D, Table B in S5 File). These results are consistent with a hypothesis put forth previously, whereby CcpA can mediate repression even during cultivation in the absence of glucose [8]. Transcription of uhptT was significantly increased (*p* ≤ 0.05) in strain SA564-ΔccpA relative to strain SA564 during the exponential and post-exponential growth phases (Fig 5; Tables A, B in S5 File). Importantly, this transcriptional activation was abolished in strains SA564-ΔhptRS and SA564-ΔhptRS/ccpA, demonstrating the HptRS two-component system activates uhptT transcription. In addition, the post-exponential growth phase mRNA level of uhptT was increased (FC = 2.92) in strain SA564 cultivated in TSB-dex relative to TSB, suggesting that HptRS transcriptional activation can occur without exogenous glucose (Table D in S5 File). These data reveal a complex regulation of uhptT transcription involving CcpA repression and HptRS activation, partially in response to changes in the availability of glucose or glucose-derived metabolites. The significance of these observations relate to the importance of UhpT in susceptibility to the antibiotic fosfomycin [36]. To investigate if genetic inactivation of ccpA or hptRS altered antibiotic susceptibility through their coordinated regulation of transcription, the antibiotic susceptibility of each strain was analyzed using broth microdilution and disk diffusion assays.

**Inactivation of ccpA and/or hptRS alters antibiotic susceptibility**

Previously, susceptibility to fosfomycin was studied in Mueller Hinton medium with added glucose-6-phosphate, which can alter activity of CcpA and subsequent transcription of hptRS and uhptT [31, 58–60]. To determine if CcpA and/or HptRS mediate susceptibility to fosfomycin and other antibiotics without the addition of glucose-6-phosphate, broth micro-dilution and disc diffusion assays were utilized. As expected, strain SA564-ΔccpA (MIC = 4 μg/mL) was significantly (*p* ≤ 0.05) more susceptible to fosfomycin when compared to strains SA564 (MIC = 64 μg/mL) and SA564-ΔhptRS (MIC = 64 μg/mL) (Fig 6A). Interestingly the increase in fosfomycin susceptibility in strain SA564-ΔccpA is similar to other reports where glucose-6-phosphate is added to the culture medium, indicating the addition of exogenous glucose-6-phosphate alters CcpA activity, causing changes in hptRS and uhptT transcript abundance [58, 60]. Importantly, inactivation of hptRS in the ccpA mutant strain, SA564-ΔhptRS/ccpA
Fig 6. Transcriptional regulation by CcpA and HptRS alter fosfomycin and ampicillin susceptibility. Antibiotic susceptibility to fosfomycin (A) and ampicillin (B) was investigated using broth microdilution. Data are the mean and standard error of the mean for 3 biological replicates, each with 6 technical replicates. Significant differences ($p \leq 0.05$) between SA564-ΔccpA and SA564-ΔhptRS/ccpA are represented with an (*).

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(MIC = 16 μg/mL), significantly (p ≤ 0.05) reduced susceptibility to fosfomycin (Fig 6A). These data demonstrate that fosfomycin susceptibility is at least partially dependent upon transcriptional regulation by CcpA and HptRS.

As reported, CcpA mutants also have increased susceptibility to beta-lactam antibiotics [40, 61]. Consistent with this observation, inactivation of ccpA increased susceptibility to penicillin and ampicillin, but also to kanamycin, neomycin, and chloramphenicol relative to strains SA564, SA564-ΔhptRS, and SA564-ΔhptRS/ΔccpA (p ≤ 0.05; Figs 6B and 7). Inactivation of hptRS in the ccpA mutant background restored the resistance profile to a level similar to the

![Graph showing antibiotic susceptibility](https://doi.org/10.1371/journal.pone.0207161.g007)

**Fig 7. Transcriptional regulation by CcpA and HptRS mediate antibiotic susceptibility.** Antibiotic susceptibility was investigated using antibiotic disks on TSA. Data represent the mean and standard error of the mean of the zone of inhibition (edge of the disk to the first colony) for 3 biological replicates. Significant differences (p ≤ 0.05) between SA564-ΔccpA and SA564-ΔhptRS/ΔccpA are represented with an (*).
wild-type strain, suggesting that HptRS antagonizes CcpA-mediated transcriptional regulation of genes that are involved in antibiotic susceptibility (Figs 6 and 7).

Conclusions

Gene regulation by multiple transcriptional regulators is common and overlapping regulation impedes the identification of genes controlled by individual regulators. On this latter point, deletion of genes coding for the sensor histidine kinase (hptS) and the response regulator (hptR) produced no observable phenotypic effects on growth, metabolism, or protein abundance. In contrast, inactivation of both ccpA and hptRS caused significant changes in pyruvate metabolism, amino acid metabolism, and TCA cycle protein abundance, which affected growth and metabolism relative to the single mutants. By combining rigorous statistical analyses with exhaustive proteomic data, it was possible to determine that HptRS and CcpA function antagonistically to co-regulate transcription of a subset of CcpA-regulated genes (Fig 8). This approach led to the identification of a small group of proteins altered by inactivation of hptRS. These data provided focus to determine which protein alterations were the result of transcriptional changes due to hptRS inactivation. Lastly, these data provide important

![Diagram](https://doi.org/10.1371/journal.pone.0207161.g008)
information about the complex regulation of antibiotic susceptibility and how "standard" susceptibility assay conditions can influence the outcomes of these assays.

Supporting information
S1 File. Code for power and peptide analysis.
(PDF)
S2 File. Power analysis and study of interactions.
(XLSX)
S3 File. Analysis of significantly different proteins.
(XLSX)
S4 File. Proteomics summary tables.
(XLSX)
S5 File. Analysis of transcript abundance.
(XLSX)
S6 File. Phenotypic analysis, anaerobic mRNA abundance, and protein abundance figures.
(PDF)

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References
1. van Belkum A, Verkaik NJ, de Vogel CP, Boelens HA, Verver E, Nouwen JL, et al. Reclassification of Staphylococcus aureus nasal carriage types. J Infect Dis. 2009; 199: 1820–1826. https://doi.org/10.1086/599119 PMID: 19419332
2. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis. 2005; 5: 751–762. https://doi.org/10.1016/S1473-3099(05)70295-4 PMID: 16310147
3. Erikson NH, Espersen F, Rosdahl VT, Jensen K. Carriage of *Staphylococcus aureus* among 104 healthy persons during a 19-month period. Epidemiol Infect. 1995; 115: 51–60. PMID: 7641838

4. Klein E, Smith DL, Laxminarayan R. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999–2005. Emerg Infect Dis. 2007; 13: 1840–1846. https://doi.org/10.3201/eid1312.070629 PMID: 18258033

5. Brown SA, Palmer KL, Whiteley M. Revisiting the host as a growth medium. Nat Rev Microbiol. 2008; 6: 657–666. https://doi.org/10.1038/nrmicro1955 PMID: 18679171

6. Somerville GA, Proctor RA. At the crossroads of bacterial metabolism and virulence factor synthesis in *Staphylococcus*. Microbiol Mol Biol Rev. 2009; 73: 233–248. https://doi.org/10.1128/MMBR.00005-09 PMID: 19487727

7. Somerville GA, Beres SB, Fitzgerald JR, DeLeo FR, Cole RL, Hoff JS, et al. *In vitro* serial passage of *Staphylococcus aureus*: Changes in physiology, virulence factor production, and agr nucleotide sequence. J Bacteriol. 2002; 184: 1430–1437. https://doi.org/10.1128/ JB.184.5.1430-1437.2002 PMID: 11844774

8. Seidl K, Müller S, François P, Kriebitzsch C, Schrenzel J, Engelmann S, et al. Effect of a glucose impulse on the CcpA regulon in *Staphylococcus aureus*. BMC Microbiol. 2009; 9: 95. https://doi.org/10.1186/1471-2180-9-95 PMID: 19450265

9. Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K. Two-component system VraSR positively regulates the cell-wall biosynthesis pathway in *Staphylococcus aureus*. Mol Microbiol. 2004; 49: 807–821.

10. Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol. 2003; 48: 1429–1444. PMID: 12791129

11. Stock AM, Robinson VL, Goudreau PN. Two-Component signal transduction. Annu Rev Biochem. 2000; 69: 183–215. https://doi.org/10.1146/annurev.biochem.69.1.183 PMID: 10966457

12. Nuxoll AS, Halouska SM, Sadykov MR, Hanke ML, Bayles KW, Kielian T, et al. CcpA regulates arginine biosynthesis in *Staphylococcus aureus* through repression of proline catabolism. PLoS Pathog. 2012; 8: e1003033. https://doi.org/10.1371/journal.ppat.1003033 PMID: 23209408

13. Görke B, Stülke J. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat Rev Microbiol. 2008; 6: 613–624. https://doi.org/10.1038/nrmicro1932 PMID: 18628769

14. Morrissey JA, Cockayne A, Brummell K, Williams P. The staphylococcal ferritins are differentially regulated in response to iron and manganese and via PerR and Fur. Infect Immun. 2004; 72: 972–979. https://doi.org/10.1128/IAI.72.3.972-979.2004 PMID: 14742543

15. Pagels M, Fuchs S, Pané-Farré J, Kohler C, Menschner L, Hecker M, et al. Redox sensing by a Rfx-family repressor is involved in the regulation of anaerobic gene expression in *Staphylococcus aureus*. Mol Microbiol. 2010; 76: 1142–1161. https://doi.org/10.1111/j.1365-2958.2010.07105.x PMID: 20374494

16. Geissmann T, Chevalier C, Cros M-J, Boisset S, Fechter P, Noirot C, et al. A search for small noncoding RNAs in *Staphylococcus aureus* reveals a conserved sequence motif for regulation. Nucleic Acids Res. 2009; 37: 7239–7257. https://doi.org/10.1093/nar/gkp668 PMID: 19786493

17. Balasubramanian D, Vanderpool CK. New developments in post-transcriptional regulation of operons by small RNAs. RNA Biol. 2013; 10: 337–341. https://doi.org/10.4161/rna.23696 PMID: 23932245

18. Novichkov PS, Kazakov AE, Ravcheev DA, Levyn SA, Kovaleva GY, Sutormin RA, et al. RegPrecise 3.0—a resource for genome-scale exploration of transcriptional regulation in bacteria. BMC Genomics. 2013; 14: 745. https://doi.org/10.1186/1471-2164-14-745 PMID: 24175918

19. Li C, Sun F, Cho H, Yelavarthi V, Sohn C, He C, et al. CcpA mediates proline auxotrophy and is required for *Staphylococcus aureus* pathogenesis. J Bacteriol. 2010; 192: 3883–3892. https://doi.org/10.1128/JB.00237-10 PMID: 20525824

20. Leiba J, Hartmann T, Cluzel ME, Cohen-Gonsaud M, Delolme F, Bischoff M, et al. A novel mode of regulation of the *Staphylococcus aureus* catabolite control protein A (CcpA) mediated by Stk1 protein phosphorylation. J Biol Chem. 2012; 287: 43607–43619. https://doi.org/10.1074/jbc.M112.418913 PMID: 23132867

21. Mijakovic I, Poncet S, Galianer A, Monedero V, Fieulaine S, Janin J, et al. Pyrophosphate-producing protein dephosphorylation by HPr kinase/phosphorylase: A relic of early life? Proc Natl Acad Sci. 2002; 99: 13442–13447. https://doi.org/10.1073/pnas.212410399 PMID: 12359880

22. Mason PW, Carbone DP, Cushman RA, Waggoner AS. The importance of inorganic phosphate in regulation of energy metabolism of *Streptococcus lactis*. J Biol Chem. 1981; 256: 1861–1866. PMID: 6780554

23. Pané-Farré J, Jonas B, Förster K, Engelmann S, Hecker M. The cbb regulon in *Staphylococcus aureus* and its regulation. Int J Med Microbiol. 2006; 296: 237–258. https://doi.org/10.1016/j.ijmm.2005.11.011 PMID: 16644280
24. Guledimann C, Boor KJ, Wiedmann M, Guariglia-Oropesa V. Resilience in the face of uncertainty: Sigma factor B fine-tunes gene expression to support homeostasis in gram-positive bacteria. Appl Environ Microbiol. 2016; 82: 4456–4469. https://doi.org/10.1128/AEM.00714-16 PMID: 27208112

25. Schramke H, Laermann R, Vegettmeyer HE, Brachmann A, Jung K, Altendorf K. Revisiting regulation of potassium homeostasis in Escherichia coli: the connection to phosphate limitation. Microbiolopen. 2017; 6: e00438 https://doi.org/10.1002/mbo3.438 PMID: 28097817

26. Price-Whelan A, Poon CK, Benson MA, Eidem TT, Roux CM, Boyd JM, et al. Transcriptional profiling of Staphylococcus aureus during growth in 2 M NaCl leads to clarification of physiological roles for Kdp and Ktr K+ uptake systems. MBio. 2013; 4: e00407–13. https://doi.org/10.1128/mBio.00407-13 PMID: 23963175

27. Heermann R, Jung K. The complexity of the ‘simple’ two-component system KdpD/KdpE in Escherichia coli. FEMS Microbiol Lett. 2010; 304: 97–106. https://doi.org/10.1111/j.1574-6968.2010.01906.x PMID: 20146748

28. Freeman ZN, Dorus S, Waterfield NR. The KdpD/KdpE two-component system: Integrating K+ homeostasis and virulence. PLoS Pathog. 2013; 9; e1003201. https://doi.org/10.1371/journal.ppat.1003201 PMID: 23555240

29. Xue T, You Y, Hong D, Sun H, Sun B. The Staphylococcus aureus KdpDE two-component system couples extracellular K+ sensing and agr signaling to infection programming. Infect Immun. 2011; 79: 2154–2167. https://doi.org/10.1128/IAI.01180-10 PMID: 21422185

30. Kuroda M, Ohta, T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, et al. Whole genome sequencing of meticillin-resistant Staphylococcus aureus. Lancet. 2001; 357: 1225–1240. PMID: 11418146

31. Park JY, Kim JW, Moon BY, Lee J, Fortin YJ, Austin FW, et al. Characterization of a novel two-component regulatory system, HptRS, the regulator for the hexose phosphate transport system in Staphylococcus aureus. Infect Immun. 2015; 83: 1620–1628. https://doi.org/10.1128/IAI.03109-14 PMID: 25644013

32. Yang Y, Sun H, Liu X, Wang M, Xue T, Sun B. Regulatory mechanism of the three-component system HptRSA in glucose-6-phosphate uptake in Staphylococcus aureus. Med Microbiol Immunol. 2016; 205: 241–253. https://doi.org/10.1007/s00430-015-0446-6 PMID: 26711212

33. Kadar RJ. Genetic control of the transport of hexose phosphates in Escherichia coli: Mapping of the uhp locus. J Bacteriol. 1973; 116: 764–770. PMID: 16559166

34. Fann MC, Maloney PC. Functional symmetry of UhpT, the sugar phosphate transporter of Escherichia coli. J Biol Chem. 1998; 273: 33735–33740. PMID: 9837961

35. Winkler HH. Distribution of an inducible hexose-phosphate transport system among various bacteria. J Bacteriol. 1973; 116: 1079–1081. PMID: 4583235

36. Xu S, Fu Z, Zhou Y, Liu Y, Xu X, Wang M. Mutations of the transporter proteins Glpt and Uhpt confer fosfomycin resistance in Staphylococcus aureus. Front Microbiol. 2017; 8: 914. https://doi.org/10.3389/fmicb.2017.00914 PMID: 28579984

37. Nakano MM, Zuber P, Sonenshein AL. Anaerobic regulation of Bacillus subtilis Krebs cycle genes. J Bacteriol. 1998; 180: 3304–3311. PMID: 9642180

38. Fuchs S, Panè-Farré J, Kohler C, Hecker M, Engelmann S. Anaerobic gene expression in Staphylococcus aureus. J Bacteriol. 2007; 189: 4275–4289. https://doi.org/10.1128/JB.00081-07 PMID: 17384184

39. Novick RP. Genetic systems in staphylococci. Methods Enzymol. 1991; 204: 587–636. PMID: 16585722

40. Seidl K, Stucki M, Ruegg M, Goerke C, Wolz C, Harris L, et al. Staphylococcus aureus CcpA affects virulence determinant production and antibiotic resistance. Antimicrob Agents Chemother. 2006; 50: 1183–1194. https://doi.org/10.1128/AAC.41.4.1183-1194.2006 PMID: 16569828

41. Kasatiya SS, Baldwin JN. Nature of the determinant of tetracycline resistance in Staphylococcus aureus. Can J Microbiol. 1967; 13: 1079–1086. PMID: 6049594

42. Brückner R. Gene replacement in Staphylococcus carnosus and Staphylococcus xylosus. FEMS Microbiol Lett. 1997; 151: 1–8. PMID: 9196277

43. Cockeill R, Willek M, Alder J, Dudley MN, Ellopoulos GM, Ferraro MJ, et al. CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. In: CLSI document M07-A9, 9th ed. Clinical and Laboratory Standards Institute, Wayne, PA, 2012. pp.1-69.

44. Horton RM, Cai ZL, Ho SN, Pease LR. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques. 1990; 8: 528–535. PMID: 23573735

45. Foster TJ. Molecular genetic analysis of staphylococcal virulence. Methods Microbiol. 1998; 27: 433–454.

46. Ledala N, Zhang B, Seravalli J, Powers R, Somerville GA. Influence of iron and aeration on Staphylococcus aureus growth, metabolism, and transcription. J Bacteriol. 2014; 196: 2178–2189. https://doi.org/10.1128/JB.01475-14 PMID: 24706736
47. Bustin SA, Benes V, Garson JA, Huggett J, Kubista M, et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem. 2009; 55: 611–622. https://doi.org/10.1373/clinchem.2008.112797 PMID: 19246619

48. R Core Team. R: A Language and Environment for Statistical Computing; 2017 [cited 2018 Jul 31]. Database: R Foundation for Statistical Computing, Vienna, Austria [Internet]. https://www.R-project.org.

49. Cai L, Friedman N, Xie XS. Stochastic protein expression in individual cells at the single molecule level. Nature. 2006; 440: 358–362. https://doi.org/10.1038/nature04599 PMID: 16541077

50. Richardson AR, Somerville GA, Sonenshein AL. Regulating the intersection of metabolism and pathogenesis in gram-positive bacteria. Microbiol Spectr. 2015; 3: https://doi.org/10.1128/microbiolspec.MBP-0004-2014 PMID: 26185086

51. Halsey CR, Lei S, Wax JK, Lehman MK, Nuxoll AS, Steinke L, et al. Amino acid catabolism in Staphylococcus aureus and the function of carbon catabolite repression. MBio. 2017; 8: e01434–16. https://doi.org/10.1128/mBio.01434-16 PMID: 28196956

52. Vizcaí no JA, Csordas A, Del-Toro N, Dianes JA, Griss J, Lavidas I, et al. 2016 update of the PRIDE database and its related tools. Nucleic Acids Res. 2016; 44: D447–D456. https://doi.org/10.1093/nar/gkv1145 PMID: 26528881

53. Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, et al. PANTHER: A library of protein families and subfamilies indexed by function. Genome Res. 2003; 13: 2129–2141. https://doi.org/10.1101/gr.772403 PMID: 12952881

54. Sadykov MR, Hartmann T, Mattes TA, Hiatt M, Jann NJ, Zhu Y, et al. CcpA coordinates central metabolism and biofilm formation in Staphylococcus epidermidis. Microbiology. 2011; 157: 3458–3468. https://doi.org/10.1099/mic.0.051243-0 PMID: 21964732

55. Liebeke M, Dörries K, Zühle D, Bernhardt J, Fuchs S, Pané-Farré J, et al. A metabolomics and proteomics study of the adaptation of Staphylococcus aureus to glucose starvation. Mol Biosyst. 2011; 7: 1241–1253. https://doi.org/10.1039/c0mb00315h PMID: 21327190

56. Caspi R, Altman T, Billington R, Dreher K, Foerster H, Fulcher CA, et al. The MetaCyc Database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. Nucleic Acids Res. 2014; 42: D459–D471. https://doi.org/10.1093/nar/gkt1103 PMID: 24225315

57. Island MD, Wei BY, Kadner RJ. Structure and function of the uhp genes for the sugar phosphate transport system in Escherichia coli and Salmonella typhimurium. J Bacteriol. 1992; 174: 2754–2762. PMID: 1569007

58. Grimm H. In vitro investigations with fosfomycin on Mueller-Hinton agar with and without glucose-6-phosphate. Infection. 1979; 7: 256–259. PMID: 389806

59. Forsgren A, Walder M. Antimicrobial activity of fosfomycin in vitro. J Antimicrob Chemother. 1983; 11: 467–471. PMID: 6874631

60. Dette GA, Knothe H, Schönenbach B, Plage G. Comparative study of fosfomycin activity in Mueller-Hinton media and in tissues. J Antimicrob Chemother. 1983; 11: 517–524. PMID: 6885678

61. Bizzini A, Entenza JM, Moreillon P. Loss of penicillin tolerance by inactivating the carbon catabolite repression determinant CcpA in Streptococcus gordonii. J Antimicrob Chemother. 2007; 59: 607–615. https://doi.org/10.1093/jac/dkm021 PMID: 17327292