Regulation of Hemolysin Expression and Virulence of <i>Staphylococcus aureus</i> by a Serine/Threonine Kinase and Phosphatase

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Abstract

Exotoxins, including the hemolysins known as the alpha (α) and beta (β) toxins, play an important role in the pathogenesis of <i>Staphylococcus aureus</i> infections. A random transposon library was screened for <i>S. aureus</i> mutants exhibiting altered hemolysin expression compared to wild type. Transposon insertions in 72 genes resulting in increased or decreased hemolysin expression were identified. Mutations inactivating a putative cyclic di-GMP synthetase and a serine/threonine phosphatase (Stp1) were found to reduce hemolysin expression, and mutations in genes encoding a two component regulator PhoR, LysR family transcriptional regulator, purine biosynthetic enzymes and a serine/threonine kinase (Stk1) increased expression. Transcription of the hla gene encoding α toxin was decreased in a <i>stpk1</i> mutant strain and increased in a Δ<i>stpk1</i> strain. Microarray analysis of a Δ<i>stpk1</i> mutant revealed increased transcription of additional exotoxins. A Δ<i>stpk1</i> strain is severely attenuated for virulence in mice and elicits less inflammation and IL-6 production than the Δ<i>stpk1</i> strain. In vivo phosphopeptide enrichment and mass spectrometric analysis revealed that threonine phosphorylated peptides corresponding to Stk1, DNA binding histone like protein (HU), serine-aspartate rich fibrinogen/bone sialoprotein binding protein (SdR) and a hypothetical protein (NWMN_1123) were present in the wild type and not in the Δ<i>stpk1</i> mutant. Collectively, these studies suggest that Stk1 mediated phosphorylation of HU, SdR and NWMN_1123 affects expression and virulence.

Introduction

Invasive bacterial infections remain a significant cause of morbidity and mortality in humans [1]. <i>Staphylococcus aureus</i> is among the most common human pathogens. Although 20% of the population are asymptomatically colonized with <i>S. aureus</i> in the skin, upper respiratory or gastrointestinal tracts, <i>S. aureus</i> is also the leading cause of invasive infections in both community and in healthcare settings [2,3,4]. Clinical manifestations of <i>S. aureus</i> range from superficial skin infections to severe or deep-seated infections such as pneumonia, bacteraemia, osteomyelitis, endocarditis and toxic shock [5].

A number of virulence factors that include hemolysins, exotoxins, leukocidins, superantigens, capsule and secreted enzymes allow <i>S. aureus</i> to overcome host defenses (for recent reviews, see [6,7]). <i>S. aureus</i> lysis of red blood cells is primarily mediated by the hemolysins known as alpha (α), beta (β) and delta (δ) toxins. The α toxin encoded by the hla gene is important for <i>S. aureus</i> pneumonia, sepsis, septic arthritis, brain abscess and corneal infections [8,9,10,11,12,13]. This 33kDa pore forming toxin is secreted by majority of <i>S. aureus</i> clinical isolates and is active against a wide range of mammalian cells, with especially marked activity against rabbit erythrocytes [14,15]. In addition to its pore forming ability, α toxin induces the release of cytokines and chemokines such as IL-6, IL-1β, IL-1α, IL-8, TNF-α, KC and MIP-2 [9,16,17,18,19,20]. Immunization with inactive α toxin was recently shown to protect mice against lethal <i>S. aureus</i> pneumonia [21,22,23]. These observations emphasize the importance of α in <i>S. aureus</i> infections.

Certain strains of <i>S. aureus</i> also secrete beta (β) toxin, a 35kDa sphingomyelinase encoded by the hlb gene [24,25,26]. In contrast to α toxin, β toxin is highly hemolytic for sheep but not for rabbit
erythrocytes [27]. Hemolytic activity of β toxin is enhanced after incubation at temperatures below 10°C, hence this toxin is often referred to as the ‘hot-cold’ hemolysin [24,27]. The importance of β toxin has been demonstrated in S. aureus infections of the human lung and cornea, and an ability to inhibit the ciliary activity of nasal epithelial cells has been described [28,29,30]. The presence of a cleavable signal sequence in the N-terminal region of α and β toxins suggests that they are secreted by the general secretory (sec) pathway (for a review, see [31]). Although not much is known about the Sec pathway in S. aureus, this pathway has been extensively characterized in B. subtilis and E. coli [32,33].

Delta (δ) hemolysin or toxin is a 26 amino acid peptide encoded by the hld gene [34,35]. This toxin is produced by 97% of S. aureus isolates and lyses erythrocytes, a variety of mammalian cells and sub-cellular structures such as membrane bound organelles, spheroplasts and protoplasts [36,37]. In contrast to the α and β toxins, δ toxin does not possess a cleavable signal sequence [27], and its mechanism of secretion is not completely understood. Because the structural gene for δ toxin is encoded within the RNA molecule (RNaseI) that activates transcription of a number of virulence factors such as α toxin, enterotoxins, and toxic shock syndrome toxin, and represses the transcription of cell surface proteins such as protein A, the precise contribution of δ toxin to S. aureus virulence is not known [38,39].

Despite the importance of S. aureus as a pathogen, our understanding of the molecular events that enable the organism to make the transition from a commensal state into an invasive pathogen is limited. Mechanisms that facilitate S. aureus infiltration into deeper tissues with subsequent dissemination and invasion of multiple organs remain to be completely understood. However, the diverse array of host niches encountered by S. aureus suggests that the pathogen efficiently adapts to changing environments to survive and establish stable infections. Pathogenic bacteria commonly utilize signaling systems to adapt, survive and invade a variety of host niches [40,41,42]. Signaling is primarily achieved through reversible phosphorylation of proteins, and these modifications are the most critical of the 200 different types of post-translational modifications [43,44]. A number of two-component systems (TCS) and one component transcriptional regulators regulate the transcription of S. aureus virulence genes [45,46]. These include the complex accessory gene regulatory system (agrABCD) that up-regulates the expression of superantigens, cytotoxins, and secreted enzymes, and represses the transcription of cell-wall proteins such as protein A and fibronectin binding proteins [38,45]. Other TCS known as SarR/SaeS and ArlR/ArlS also regulate the transcription of α (hlr) and β (hlb) toxins in S. aureus [47,48,49,50]. The staphylococcal accessory regulator known as SarA binds to conserved promoter regions known as ‘Sar boxes’ for transcriptional regulation. Sar homologues such as SarT and Rot are also described to repress transcription of hla [51,52,53,54,55].

In this study, we screened a random transposon library for mutants that showed either an increase or decrease in hemolysin expression compared to wild type (WT). Our studies identified 72 genes that affect hemolysin expression in S. aureus and include signaling enzymes commonly found in eukaryotes. We further demonstrate that these eukaryotic-like signaling enzymes affect α toxin (hla) transcription and S. aureus virulence.

**Results**

**Identification of genes that regulate hemolytic activity of S. aureus**

Previous studies have established that hemolysins are important for infections caused by S. aureus. To identify novel factors that regulate hemolysin expression in S. aureus, we screened a transposon insertion library for mutants that showed either an increase or decrease in hemolytic activity compared to the wild type (WT) strain. The transposon library was obtained as a kind gift from Chiron Corporation/Novartis Vaccines (CMCC #51963, patent WO/2004/018624). This library consists of 6,725 individual, random transposon Tn5EZ mutants for which the insertion sites of the transposons in the S. aureus genome are known (see patent WO/2004/018624 entitled ‘Random transposon insertion in Staphylococcus aureus and use thereof to identify essential genes’ at http://www.wipo.int/ptdlib/en/text.jsp?WO=2004%2F018624&DISPLAY= DESC). The Tn5EZ mutants were derived from S. aureus RN2240, and all 6,725 mutants are contained on individual, 96-well microtiter plates. Although RN2420 has the genes encoding α (hla), β (hld), and δ (hld) toxins, an extra adenine residue in the 3' end of the gene encoding agrA decreases α and δ toxin expression (for details, see [56]). As expression of β toxin is not tightly regulated by agr [56], hemolytic activity due to this toxin is seen in RN2420. We hypothesized that screening the transposon library for mutants with altered hemolytic activity would enable us to identify genes that affect α and δ toxin expression despite decreased agr regulation, and also identify genes that regulate β toxin expression.

To identify mutants that showed altered hemolytic activity, we used a 48-well prong to transfer individual colonies onto sheep blood agar plates (SBA). These plates were incubated at 37°C overnight. Subsequently, the plates were transferred to 4°C overnight, and colonies that exhibited increased or decreased hemolytic activity after incubation at 37°C and 4°C were identified by visual screening (see Fig. 1, A and B for a representative plate). Analysis of the selected mutants on SBA was repeated to confirm the reproducibility of the hemolytic phenotype. Our studies indicate that transposon insertions in (a) 13 genes abolished hemolytic activity (No Hemolysis, NH), (b) 25 genes conferred decreased hemolysis (Less Hemolysis, LH), (c) 26 genes caused a moderate increase in hemolysis (Moderately hyper-hemolysis, MH), and (d) 8 genes conferred hyper-hemolysis (Hyper hemolysis, HH) when compared to the WT and most other Tn5EZ mutants on the same SBA plate (Table 1–4, Fig. 1 A and B).

As expected, transposon insertions in the hlb gene encoding β toxin, and a few known hemolysin activators such as agra [38,45], sarF [47,48] and cepA [57] decreased and/or abolished hemolytic activity in RN4220 (Table 1 and 2). Likewise, a Tn5EZ insertion in the previously described hemolysin repressor rot increased hemolytic activity (Table 3 and [55]). As the transposon library did not contain insertions in the structural genes for α toxin (hla), δ toxin (hld) and other known hemolysin regulators such as sarA, sarB and cepBC [45], these genes were not identified in the above analysis. A transposon insertion in the gene encoding acetate kinase down-regulated hemolysin expression; this may be due to decreased synthesis of acetyl phosphate, an intracellular signal for phosphorylation of two component response regulators (for a review, see [38]). Taken together, the above findings validate our screen for altered hemolysin expression in S. aureus. Importantly, our studies also identified a number of novel genes that regulate hemolysin expression of S. aureus (Table 1–4). Transposon insertions in the two component regulator PhoR, purine biosynthetic gene purF, a gene predicted to encode a transcriptional regulator of the LysR family (SA2555), and the serine/threonine kinase gene sktI increased the hemolytic phenotype (Table 3 and 4). As the increase in hemolytic activity of these mutants was observed at 37°C, we hypothesized that this may be due to an increase in α and/or δ toxin, as β toxin-mediated lysis of red blood cells is primarily seen after incubation at temperatures...
below 10°C [24,27]. Therefore, we compared hemolytic activity of these strains on rabbit blood agar (RBA), as α toxin demonstrates a >1000 fold increase in lysis of rabbit erythrocytes compared to sheep erythrocytes, whereas β toxin does not lyse rabbit erythrocytes [59]. Lysis of rabbit red blood cells (rRBC) was observed in strains with Tn5EZ insertions in SA2555, purF and stk1, in contrast to WT RN4220 (Fig. 1C), suggesting that these loci repress α toxin expression. As expected, the NH and LH strains did not demonstrate any difference in hemolysis on RBA compared to WT RN4220 (data not shown).

A serine/threonine kinase and phosphatase regulate hemolysin expression in *S. aureus*

The transposon screen indicated that an insertion in stk1 confers an increase in *S. aureus* hemolysin activity (Table 3). Previous studies from our laboratory have identified that homologues of stk1 regulate hemolysin expression in the gram-positive human pathogen group B streptococcus [60,61]. Therefore, we were interested in characterizing the role of Stk1 and its cognate serine/threonine phosphatase Stp1 in the regulation of hemolysin expression and virulence of *S. aureus*. To confirm that stk1 and

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**Figure 1. S. aureus transposon mutants showing increased or decreased hemolysis.** Hemolytic activity is represented by the zone of clearing around the colonies on the sheep blood agar plates. (A) and (B) represent the same sheep blood agar (SBA) plate. Forty-eight random TN5EZ mutants of *S. aureus* were stamped on a SBA plate and incubated O/N at 37°C. The plate was then incubated at 4°C O/N. (A) shows hemolysin activity after the 37°C incubation and (B) represents the same plate after the 4°C incubation. The wild type strain (WT, RN4220) was spotted as a control. Note that a few mutants showed hyper-hemolysis (HH), moderately hyper-hemolysis (MH) or lower hemolysis (LH) compared to WT in both A and B. These mutants were chosen for further analysis (Table 1–4). (C) Strains with transposon TN5EZ insertions in SA2555 (putative lysR family regulator), purF and stk1 show a marked increase in hemolytic activity compared to WT RN4220 on rabbit blood agar (RBA).

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### Table 1. TNSEZ insertions that abolished *S. aureus* hemolysin activity.

| # of mutants* | Hemolysis | Gene/Locus | Function or predicted function |
|---------------|-----------|------------|--------------------------------|
| 2             | NH        | SA0014     | Modified GGDEF guanylate cyclase/cyclic diGMP synthetase |
| 1             | NH        | SA0101     | Ornithine cyclodeaminase       |
| 2             | NH        | SA0164     | Gramicidin S synthetase 2 – Non-ribosomal peptide synthetase |
| 1             | NH        | SA0403     | Transcriptional antiterminator, BglG family |
| 4             | NH        | saeR       | Two-component regulator SaeR   |
| 1             | NH        | mnhE       | Na/H antiporter, MnhE component |
| 1             | NH        | fmt        | Methionyl-tRNA formyltransferase |
| 1             | NH        | infB       | Translation initiation factor IF-2 |
| 1             | NH        | ddpA       | Dihydropicolinate synthase - aspartate family |
| 2             | NH        | hlb        | Phospholipase C, Fatty acid and lipid metabolism |
| 1             | NH        | SA2072     | ATP-dependent RNA helicase, DEAD/DEAH box family |
| 1             | NH        | SA0403     | Transcriptional antiterminator, BglG family |

*NH represents no hemolysis compared to WT and most other TnSEZ mutants on sheep blood agar (also see Fig. 1). # of mutants represents the number of individual transposon insertions in the same gene or loci that alter hemolysin activity. doi:10.1371/journal.pone.0011071.t001

### Table 2. TNSEZ insertions that reduced *S. aureus* hemolysin activity.

| # of mutants* | Hemolysis | Gene/Locus | Function or predicted function |
|---------------|-----------|------------|--------------------------------|
| 1             | LH        | SA0466     | Membrane protein, putative     |
| 1             | LH        | bioD       | Dethiobiotin synthase - Biosynthesis of cofactors |
| 2             | LH        | SA0555     | Peptidase, MA1 - Degradation of proteins, peptides, |
| 1             | LH        | SA0635     | Lipoate-protein ligase A - Protein modification |
| 1             | LH        | SA0762     | Hemolysin, putative -          |
| 3             | LH        | gcvH       | Glycine cleavage system H protein - energy metabolism |
| 1             | LH        | SA0958     | General stress protein 13      |
| 1             | LH        | SA1049     | 1,4-dihydroxy-2-naphthoate octaprenyltransferase, putative |
| 1             | LH        | SA1220     | Fibronectin-binding protein A-related |
| 2             | LH        | SA1294     | Metallo-beta-lactamase superfamly protein |
| 1             | LH        | glpD       | Aerobic glycerol-3-phosphate dehydrogenase |
| 1             | LH        | SA1563     | 2-oxoisovalerate dehydrogenase, lipoamide dehydrogenase, |
| 1             | LH        | secDF      | Protein-secretion, membrane protein, SecDF family |
| 1             | LH        | SA1751     | DHH family protein, putative   |
| 2             | LH        | ackA       | Acetate kinase - fermentation  |
| 1             | LH        | ccpA       | Catabolite control protein - DNA interactions |
| 1             | LH        | agrA       | Accessory gene regulator protein A |
| 1             | LH        | SA2027     | Hypothetical protein - not conserved |
| 9             | LH        | atpDGA     | ATP synthase F1, beta, gamma and alpha subunits - |
| 1             | LH        | SA2271     | Molybdenum ABC transporter, permease protein - cations |
| 1             | LH        | SA2375     | Transporter - unknown substrate |
| 1             | LH        | SA2534     | NAD(P)H-flavin oxidoreductase |
| 1             | LH        | SA2572     | Cation-transporting atpase, E1-E2 family |
| 1             | LH        | SA2624     | Acetyl-CoA synthetase, putative |
| 3             | LH        | SA2735     | Chromosome partitioning protein, ParB family |

*LH represents lower hemolysis compared to WT and most other TnSEZ mutants on sheep blood agar (also see Fig. 1). doi:10.1371/journal.pone.0011071.t002*
**Table 3.** TN5EZ insertions that increased *S. aureus* hemolysin activity.

| # of mutants* | Hemolysis | Gene/Locus | Function or predicted function |
|---------------|-----------|------------|--------------------------------|
| 2             | MH        | gyrA       | DNA gyrase, A subunit          |
| 9             | MH        | SA0007     | YjeF-related protein, C-terminus - unknown function |
| 3             | MH        | hutH       | Histidine ammonia-lyase        |
| 1             | MH        | SA0216     | Hypothetical protein           |
| 1             | MH        | SA0237     | Alcohol dehydrogenase, zinc-containing |
| 1             | MH        | SA0311     | Sodiumsolute symporter - Transport and binding proteins |
| 1             | MH        | geh        | Lipase precursor, interruption - disrupted reading frame |
| 6             | MH        | ksgA       | Dimethyladenosine transferase   |
| 1             | MH        | SA0713     | Hypothetical protein - not conserved |
| 1             | MH        | fruB       | 1-phosphofructokinase - Glycolysis/gluconeogenesis |
| 1             | MH        | SA1021     | Multidrug-efflux transporter, putative |
| 5             | MH        | purL       | Phosphoribosylformylglycinamidine synthase II |
| 1             | MH        | SA1096     | TRK system potassium uptake protein TrkA, putative - cations |
| 1             | MH        | pdhO       | Pyruvate dehydrogenase, E3 component, |
| 2             | MH        | stk1       | Protein kinase - regulatory functions - protein interactions |
| 1             | MH        | SA1309     | Pyruvate ferredoxin oxidoreductase, beta subunit, |
| 1             | MH        | SA1472     | Pathogenicity protein, putative |
| 2             | MH        | era        | GTP-binding protein Era - small molecule interactions |
| 1             | MH        | SA1740     | Two component DNA-binding regulator, PhoP |
| 1             | MH        | rot        | Virulence factor regulator protein |
| 3             | MH        | fumC       | Fumarate hydratase, class II - TCA cycle |
| 2             | MH        | pcrA       | PcrA protein - DNA replication, recombination, and repair |
| 1             | MH        | hutU       | Urocanate hydratase - Amino acids and amines |
| 1             | MH        | SA2308     | Transcriptional regulator, Rnt family |
| 9             | MH        | SA2737     | Glucose inhibited division protein A |
| 12            | MH        | thdF       | Thiophene and furan oxidation protein ThdF - detoxification |

MH represents moderately hyper-hemolysis compared to WT and other Tn5EZ mutants.

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*stp1* regulate α toxin expression, phage φ11 [62] was used to transduce the Tn5EZ insertions in *stk1* (Table 3) and *stp1* from the transposon library in RN4220 into the virulent strain of *S. aureus* known as Newman. *S. aureus* Newman is proficient in expression of α and δ toxin but deficient for β toxin due to the insertion of a prophage in *hlb* [63]. A comparison of hemolytic activity of these strains indicated that a Tn5EZ insertion in *stp1* (Newman 53E12) decreased hemolytic activity, whereas a Tn5EZ insertion in *stk1* (Newman 60C1) increased hemolytic activity compared to WT Newman (Fig. 2A).

To further establish the role of Stp1 and Stk1 on hemolysin expression and to examine their role in virulence of *S. aureus*, allelic exchange mutants (AEM) were constructed. To this end, the coding sequences of *stp1* and *stk1* were replaced with genes that

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**Table 4.** TN5EZ insertions that significantly increased *S. aureus* hemolysin activity.

| # of mutants* | Hemolysis | Gene/Locus | Function or predicted function |
|---------------|-----------|------------|--------------------------------|
| 3             | HH        | SA0704     | Iron compound ABC transporter, ATP-binding protein |
| 5             | HH        | purF       | Amidophosphoribosyltransferase - Purine biosynthesis |
| 2             | HH        | ptsl       | Phosphoenolpyruvate-protein phosphotransferase |
| 2             | HH        | pnp        | Polyribonucleotide nucleotidyltransferase - Degradation of RNA |
| 1             | HH        | SA2555     | Transcriptional regulator, LysR family - DNA interactions |
| 1             | HH        | SA2113     | Transcription termination factor Rho - transcription factors |
| 1             | HH        | SA2348     | QacA subfamily/drug transporter, putative |
| 1             | HH        | SA1281     | Membrane-associated zinc metalloprotease, |

*HH represents hyper-hemolysis compared to WT and most other Tn5EZ mutants on sheep blood agar (also see Fig. 1).

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conferred either chloramphenicol (Cm, [64]) or kanamycin resistance (Kn, [63]) in *S. aureus* RN4220 and subsequently transduced into *S. aureus* Newman (see Methods for details). We then compared the hemolytic activity of WT to isogenic Δstp1 and Δstk1 strains. Consistent with the transposon mutants (Fig. 2A), hemolytic activity was lower in a Δstp1 mutant constructed by allelic exchange and higher in the Δstk1 mutant, compared to WT Newman (Fig. 2B). Although changes in hemolysin expression are often observed in *S. aureus* due to spontaneous nonsense mutations in the *agr* regulatory system [56,66], a comparison of the DNA sequence of the *agr* locus (agr*ABCD*) between WT Newman and the isogenic Δstp1 and Δstk1 strains did not indicate the presence of any spontaneous *agr* mutations (data not shown). Similar changes in hemolysin expression were also observed in Δstk1 and Δstp1 mutants derived from WT *S. aureus* RN6390 (data not shown). Taken together, these data indicate that Stp1 and Stk1 oppositely regulate hemolytic activity of *S. aureus*.

**Role of Stp1 and Stk1 in transcription of hla**

We next examined whether the absence of Stp1 or Stk1 affected the transcription of the gene encoding α toxin. To test this possibility, RNA was isolated from three independent biological replicates of WT *S. aureus* Newman and isogenic Δstp1 and Δstk1 strains grown in tryptic soy broth (TSB) to an optical density (OD_{600}) of 0.6 (exponential phase) or ~3.0 (post-exponential phase), and quantitative real time PCR (qRT-PCR) was performed as described [67]. The housekeeping gene *spa* was used to normalize transcription of *hla* and *hld* by the comparative C_T method [68]. In these studies, we also compared transcription of the gene encoding β toxin (*hld*), the *Agr*-repressed gene encoding Protein Λ (*spa*) and *agrA*. We observed that transcription of *hla* was approximately 2-fold lower in the Δstp1 mutant and significantly higher (6.4 fold) in the Δstk1 mutant compared to WT, but only during post-exponential phase growth (Table 5, P<0.05, student’s t test). Under these conditions, transcription of *hld*, *spa* and *agrA* were not significantly different between WT, Δstp1 and Δstk1 mutants (Table 5). Given that *agrA* positively regulates transcription of *hla* and *hld*, and negatively regulates *spa* transcription during post-exponential phase growth of *S. aureus*, our results suggest that Stp1 and Stk1 regulation of α toxin transcription is independent of global *agr* regulation. To confirm that the change in *hla* transcription in Δstp1 and Δstk1 correlated with altered α toxin protein levels, we performed quantitative western blots as described ([21], also see Methods). Four independent experiments indicated that extracellular or secreted α toxin was 1.2–2 fold higher in the Δstk1 strain compared to WT *S. aureus* Newman (Fig. 3A). Decreased α toxin levels were observed in the Δstp1 strain compared to WT Newman (Fig. 3B). However, intracellular α toxin levels in Δstp1 and Δstk1 mutants were not consistently different from that of WT (data not shown).

To confirm that the change in hemolytic activity of the Δstp1 and Δstk1 strains was due to the absence of these genes, we performed complementation studies. The genes encoding *stp1* and *stk1* were PCR-amplified and cloned into the complementation vector pDCerm to generate pStp1 and pStk1, respectively (Table S1). The complementing plasmids pStp1 and pStk1, and the vector pDCerm, were electroporated into the RN4220 Δstp1 and Δstk1 strains. Subsequently, using Φ-11, these plasmids were transduced into Newman Δstp1 and Δstk1 strains. qRT-PCR and quantitative western blots confirmed that α toxin was restored to WT levels in both the Δstp1 and Δstk1 strains containing the complementing plasmids (Table 5, Fig. 3A & B). Hemolytic activity was restored to WT levels in both the Δstp1 and Δstk1 strains containing the complementing plasmids (see Δstp1/pStp1 and Δstk1/pStk1 in Fig. 3C), in contrast to those containing only the vector pDCerm. These data confirm that Stp1 and Stk1 regulate expression of α toxin in *S. aureus*.

![Figure 2](https://example.com/figure2.png)

**Figure 2. A serine/threonine phosphatase and kinase oppositely regulate hemolytic activity of *S. aureus*. (A) A transposon (TnSE5) insertion in stp1 (53E12) decreased hemolytic activity whereas the strain with the TnSE5 insertion in stk1 (60C1) increased hemolytic activity compared to the isogenic WT Newman. (B) Shows hemolytic activity of Newman WT, Δstp1 and Δstk1 strains on sheep blood agar. Note that hemolytic activity is decreased in the Δstp1 strain and increased in a Δstk1 mutant compared to WT Newman.**

| Table 5: Transcription of *hla* is decreased in *S. aureus* Δstp1 and increased in Δstk1 mutants. |
|---------------------------------------------------------------|
| **Relative Gene Expression**                        | **hla (α toxin)** | **hld (β toxin)** | **agrA** | **spa** |
|---------------------------------------------------------------|
| Δstp1                                                      | 0.31±0.15*       | 0.78±0.53         | 0.55±0.18 | 2.0±1.92 |
| Δstk1                                                      | 6.42±1.98*       | 1.33±0.09         | 1.19±0.32 | 1.39±0.47 |
| Δstp1/pStp1                                                | 1.5±0.5          | NT                | NT        | NT       |
| Δstk1/pStk1                                                | 2.0±0.5          | NT                | NT        | NT       |

*QRT-PCR was performed as described in Methods. Gene expression is denoted as fold difference relative to the WT *S. aureus* strain Newman. Standard deviation is indicated. NT indicates not tested.
*indicates statistically significant, P values <0.05, 2 tailed t test.

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Microarray analysis

The change in hla transcription observed in the Δstp1 and the Δstk1 strains (Table 5) prompted us to examine global changes in gene expression during post-exponential phase growth (OD₆₀₀~3.0). Transcriptional profiling analysis was performed on RNA isolated from WT Newman and isogenic Δstp1 and Δstk1 mutants, and the results are shown in Table S2. These results confirmed that stk1 expression was similar to WT in the Δstp1 strain but was 200-fold lower in the Δstk1 mutant (Table S2). Similarly, sip1 expression was 100-fold decreased in the Δstp1 strain but similar to WT in the Δstk1 mutant (Table S2). A comparison of gene expression between WT and Δstp1 or Δstk1 indicated that 32 genes were up-regulated and 43 genes were down-regulated in the Δstk1 strain, whereas 84 genes were
up-regulated and 55 genes were down-regulated in the Δstp1 strain (Table S2). As expected, transcription of α toxin was decreased in the Δstp1 mutant and increased in the Δstk1 mutant. Interestingly, transcription of a number of other exotoxins including LukF and LukS were increased in a Δstk1 background (2.0–4.9 fold, Table S2) whereas transcription levels of these genes was similar to the WT in the Δstp1 strain. In contrast, transcription of genes encoding capsular polysaccharide biosynthetic enzymes was 2.0–2.5 fold increased in the Δstp1 mutant and decreased in the Δstk1 mutant. A comparison of genes that regulate hemolysin expression (Table 1–4) to those observed in the microarray (Table S2) indicated that genes important for hemolysin activity, such as FruB, SACOL0495 and SACOL2072 (see ** in Table S2), showed decreased expression in the Δstp1 mutant. Conversely, Δapd4 encoding dihydrodipicolinate synthase, that is also important for hemolysin expression (Table 1), showed a modest increase in transcription only in the Δstp1 mutant (see * in Table S2).

Surprisingly, decreased transcription of genes important for hemolytic activity, i.e. FruB and the transcriptional anti-terminator SACOL0403 (Table 3), were observed in the Δstk1 mutant (see # in Table S2). Whether altered transcription of these genes contributes to the change in hemolytic activity in Δstp1 and Δstk1 (Fig. 2) mutant strains has yet to be established.

### Stp1 is essential for *S. aureus* virulence

The virulence of Δstp1 and Δstk1 mutants was compared with WT using the mouse sepsis model of infection described previously [69]. Six week-old female C57BL/6J mice (n = 10) were inoculated intravenously with 2.5 × 10^7 colony-forming units (CFU) of WT, Δapd4 or Δstk1 strains as described [69]. Kaplan-Meier survival curves shown in Fig. 4A indicate that 100% of mice infected with WT *S. aureus* Newman succumbed to infection, whereas 90% of the mice infected with a Δapd4 mutant survived the infection (P value < 0.0001, log rank test). Similar results was also observed with the Newman apd4–Tn5EZ strain (data not shown). Although the mice infected with a Δstk1 mutant did not show a significant difference in survival compared to the WT (P value = 0.44), a large number of abscesses were observed in the kidneys of mice infected with this strain (Fig. 4B). In contrast, the kidneys obtained from mice infected with a Δapd4 mutant showed no visible abscesses (Fig. 4B). Histological examination of infected kidneys at five days post-infection indicated the presence of massive abscesses and necrotic tissue surrounding bacterial microcolonies in mice infected with WT or a Δstk1 mutant, but not in those infected with a Δapd4 mutant (Fig. 4C). These data emphasize the importance of Stp1 and Stk1 for *S. aureus* virulence and abscess formation.

The differences in survival and renal abscesses observed in the mice infected with the Δapd4 and Δstk1 strains prompted us to compare bacterial spread and cytokine responses during infection. Six week-old female C57BL/6J mice (n = 5) were inoculated intravenously with 1 × 10^7 colony-forming units (CFU), and kidneys, spleen and brain were harvested at 5 days post-infection as described [69]. We observed that the bacterial CFU in the kidneys, spleen and brain of mice infected with the WT, Δapd4 or Δstk1 strains were not significantly different at 5 days post-infection (Fig. 5, A–C). These results indicate that the attenuated virulence of Δapd4 is not due to decreased survival or dissemination within the host. A comparison of the host inflammatory response to infection revealed a two-fold decrease in IL-6 production in the kidneys of mice infected with the Δapd4 strain compared to kidneys of mice infected with the Δstk1 strain (Fig. 6A). We did not observe significant changes in KC (the murine functional homologue of IL-8) and IL-1β production. A two-fold decrease in IL-6 expression was also observed when bone marrow-derived macrophages (BMMΦ) of C57BL/6 mice were stimulated with the Δapd4 mutant, compared to the Δstk1 strain (Fig. 6B). These studies indicate that the staphylococcal serine/threonine kinase and phosphatase affect the IL-6 response to *S. aureus* infection.

### Phosphopeptide enrichment analysis

To identify potential substrates of Stk1, we performed in *vivo* phosphopeptide enrichment and mass spectrometric analysis on total proteins isolated from WT Newman and the isogenic Δstk1 strain as described previously [70] with a few modifications (see Methods). Phosphopeptides (i.e. peptides that showed neutral loss of phosphate during collision induced dissociation, CID) corresponding to Stk1, DNA binding histone like protein (Hu), serine-aspartate rich fibrinogen/bone sialoprotein binding protein (SdrE) and a hypothetical protein (NWMN_1123) were identified in the WT and not in the Δstk1 mutant (Table 6, Fig. 7A–C). These data suggest that Stk1 phosphorylates Hu, SdrE and NWMN_1123 in *S. aureus*. As phosphopeptides corresponding to a NAD-specific glutamate dehydrogenase (GudB), pyruvate kinase (Pyk), anti-sigma B factor antagonist (RsbV), threonyl-tRNA synthetase (ThrS) and an acetyltransferase (NWMN_2273) were identified in both WT and the Δstk1 mutant (Table 6), phosphorylation of these proteins are likely to be independent of Stk1. Collectively, these studies suggest that post-translational regulation of Hu, SdrE and NWMN_1123 by Stk1 and Stp1 affects *S. aureus* gene expression and virulence.

### Cell morphology, antibiotic resistance or growth in minimal media

Recently Beltramini et al. reported that an stk1 mutant derivative of *S. aureus* N315 was more susceptible to cell wall-acting antibiotics and showed morphological changes such as incomplete septa and irregular cell size, whereas an apd4 mutant had increased peptidoglycan [PG, [71]]. In contrast to these findings, we observed no significant differences in the sensitivity of WT Newman, Δstk1 and Δapd4 strains to the cell wall-acting antibiotics imipenem, ceftriaxone and the fluoroquinolone ciprofloxacin (MIC < 0.047, 4 and 0.125 µg/mL, respectively). Furthermore, cross-sectional TEM did not reveal any irregularities in PG thickness or cell shape of these strains (Fig. S1).

Other studies have suggested that Stk1 phosphorylates PurA (adenylsuccinate synthase) in *S. aureus* strain 8325 to regulate purine biosynthesis [72]. To determine whether Stk1 and Stp1 are important for purine biosynthesis in *S. aureus* Newman, we compared the ability of WT, Δstk1 and Δapd4 strains to grow in chemically defined media (CDM) with or without exogenous purines and/or pyrimidines as described [73]. The Δstk1 and Δapd4 mutants did not exhibit any significant differences during growth in chemically defined media (CDM) supplemented with purines and pyrimidines, CDM supplemented only with either purines or pyrimidines, and CDM lacking both purines and pyrimidines (see Fig. S2). As expected, the control strain with the transposon Tn5EZ insertion in purC was unable to grow in the absence of exogenous purines (Fig. S2) and was also avirulent in the mouse sepsis model of infection (data not shown). The apparent differences in the roles of Stk1 and Stp1 may be due to differences in target substrates in *S. aureus* strains.

### Discussion

In recent years, invasive infections due to *S. aureus* have escalated in hospital and community settings. The importance of hemolysins such as α toxin to *S. aureus* virulence has been
Figure 4. Stp1 is important for virulence of *S. aureus*. (A) Ten, six week-old female C57BL/6J mice were intravenously injected with 2–5 $\times$ $10^7$ CFU of *S. aureus* WT, Δstp1 or Δstk1 strains. As a control, ten mice were inoculated in parallel with PBS. Kaplan-Meier survival curves show the percent
survival of mice after the infection. Note that 100% of mice infected with the WT and Δstk1 strains succumbed to infection, in contrast to only 10% of mice infected with the Δstp1 mutant. Survival of mice infected with the Δstp1 mutant was significantly different from that of the WT and the Δstk1 mutant (P value < 0.0001, log rank test). However, mice infected with the Δstk1 mutant did not show a significant difference in survival compared to the WT (P value = 0.44, log rank test). (B) Kidneys harvested from the mice that were moribund or at the end of the experiment. Arrows represent abscesses that were observed on the infected kidneys. Note that a large number of abscesses are seen in the kidneys of mice infected with the Δstk1 mutant in comparison to those infected with the Δstp1 strain. One representative from ten infected kidney pairs after inoculation with the S. aureus strain is shown. (C) Histological examination of infected kidney tissue was performed at 5 days post-infection. Kidneys were fixed in formalin, embedded in paraffin, thin-sectioned, stained with hematoxylin and eosin (H&E), and examined by microscopy as described previously [69,97]. Abscesses and necrotic tissue are observed in kidneys of mice infected with WT and Δstk1 strains but not with the Δstp1 mutant.

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extensively characterized. Given that S. aureus commonly resides as a commensal in the nasal passages and gastrointestinal tract but is also capable of invading deeper tissues, the pathogen must appropriately express hemolysins and other virulence factors in response to its external environment. To identify novel regulators of the hemolysins, we screened a transposon insertion library for mutants that showed altered toxin expression. Our studies identified 72 genes that affect the hemolytic activity of S. aureus. While some of the identified genes include previously established regulators such as agr, sae, rot, and ccppA, we also identified novel genes that repress or activate the expression of S. aureus hemolytic toxins. Notably, insertions in a putative cyclic di-GMP synthetase decreased hemolysin expression, whereas insertions in purine biosynthetic enzymes, a LysR family transcriptional regulator and a serine/threonine kinase enhanced hemolysin expression. Given that spontaneous mutations in the agr locus have been associated with changes in hemolysin expression in S. aureus, we confirmed that agr mutations are not responsible for the hemolysin phenotypes of the Nemwam Δstp1 and Δstk1 mutants. Although similar studies need to be performed with the other genes identified in this study, a few loci that were identified as regulators of toxin expression such as lipodeoxyheangase (SA1563), dihydrodipicolinate synthase (dpdA) and phosphoribosylformylglycinamidine synthase (purL) were previously identified in screens for S. aureus mutants that showed virulence attenuation [74,75,76,77].

Signal transduction in bacteria classically involves the reversible phosphorylation of histidine residues, whereas phosphorylation of serine/threonine residues was originally described in eukaryotes. However, examples of eukaryotic-type serine/threonine phosphorylation are increasingly encountered in bacteria. Previous studies from our laboratory have indicated that a serine/threonine kinase regulates hemolysin expression in Group B Streptococcus [60,61]. Therefore, we further characterized the role of the kinase Stk1 and its cognate phosphatase Stp1 in the regulation of S. aureus hemolysin expression and virulence. As the S. aureus strain RN4220 used to construct the transposon library is not virulent, we evaluated the effect of transposon insertions in stk1 and stp1 in a virulent WT S. aureus strain. A comparison of hemolysin expression in the mutant strains revealed that Stk1 negatively affects transcription of α toxin whereas the phosphatase Stp1 promotes α toxin expression in virulent S. aureus Newman. The identification of a phosphopeptide corresponding to the DNA binding histone like protein (HU) suggests that S. aureus modulates the function of HU for regulation of gene expression similar to histone phosphorylation in eukaryotes [78,79]. It has also been suggested that HU binding introduces structural changes to the DNA which can facilitate or inhibit the binding of regulatory proteins to their specific sites. Recently in E. coli, HU was described to regulate the expression of ~8% of the genome [80]. As hla transcription is decreased in Δscp1 and increased in Δstk1, we predict that HU may regulate hla transcription in S. aureus. Likewise, changes in gene expression observed in the Δscp1 and Δstk1 strains could also be attributed to altered HU function. In S. aureus, HU is encoded by the gene hsa and is essential for growth [81]. Further studies to identify all genes that are directly regulated by HU are being pursued and will reveal the role of post translational regulation of HU in S. aureus.

Given the importance of S. aureus adherence to extracellular matrix components, we hypothesize that the increase and decrease in kidney abscesses observed with the Δstk1 and Δscp1 strains may, in part, be linked to reversible phosphorylation of SdrE. SdrE is a member of the family of surface proteins with serine (S)–aspartate (D) repeats (R), and also includes SdrC, SdrD and clumping factors (Cfa and Cfb). These proteins belong to a sub-family of adhesins that specifically bind extracellular matrix molecules and are also known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs, [82,83]). Although an exact molecular function for SdrE is not known, it is proposed to bind host extracellular matrix components and has been shown to contribute to platelet aggregation [84,85]. Immunization with the cell surface proteins SdrD and SdrE along with two other antigens generated significant protective immunity [84]. Furthermore, S. aureus with an sdeE+positive gene profile are associated with bone infections [83]. Further studies to understand the role of phosphorylation on SdrE function and S. aureus abscess formation and virulence are being pursued. Likewise, similar studies to understand the role of the hypothetical protein NWMN_1123 are also being investigated to establish the role of phosphorylation of the Stk1 targets in regulation of gene expression and S. aureus virulence.

In contrast to our findings, Beltrami et al recently concluded that Stp1 and Stk1 do not regulate hemolysin expression in the methicillin resistant S. aureus (MRSA) strain N315 [71]. However, the role of either Stp1 or Stk1 in the virulence of strain N315 was not examined [71]. Recently, stk1 mutations generated in S. aureus strain SH1000 showed attenuated virulence which was attributed to purine auxotrophy [72,86]. In contrast, we observed that Stp1 and Stk1 are dispensable for purine biosynthesis in S. aureus Newman (Fig S2), and a Δstk1 mutation does not attenuate virulence in this strain background (Fig. 4). Collectively, these observations suggest that Stk1 and Sttp1 may mediate the phosphorylation of different targets in S. aureus strains Newman, N315 and SH1000. Identification of the in vivo Stk1 substrates in N315 and SH1000 will elucidate the role of serine/threonine phosphorylation in these S. aureus strains. Although the strain Newman has been extensively used to study S. aureus pathogenesis, recent studies have indicated the presence of a missense mutation in the sensor histidine kinase SaeS that affects downstream gene regulation [87,88]. Consequently, evaluation of the role of Stk1 and Sttp1 in hemolysin expression and S. aureus virulence in clinical isolates such as those of the USA300 lineage, are in progress in our laboratory and, will provide further significance to our observations in S. aureus Newman.

In this study, we have also identified a number of novel genes encoding a putative LysR family transcription factor and a putative cyclic di-GMP synthetase that regulate hemolysin expression in S. aureus. Further characterization of these loci will establish novel mechanisms by which the expression of exotoxins and other S. aureus virulence genes are regulated.
Methods

Ethics Statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of Seattle Children’s Research Institute (IACUC protocol # 08-0515) and performed using accepted veterinary standards.

Bacterial strains and growth conditions

*S. aureus* RN420 random transposon library containing 6,725 individual Tn5EZ mutants and the location of the these insertions were kindly provided by Novartis Vaccines and Diagnostics (CMCC #51963, see patent WO/2004/018624 entitled ‘Random transposon insertion in *Staphylococcus aureus* and use thereof to identify essential genes’ at http://www.wipo.int/pctdb/en/wo.jsp?WO = 2004%2F018624&IA = WO2004%2F018624&DISPLAY = DESC). All other strains, plasmids and primers used in this study are listed in Table S1. *S. aureus* strains were cultured in Tryptic Soy Broth (Difco Laboratories, Detroit, MI) at 37°C unless indicated otherwise. Cell growth was monitored by reading optical density at 600 nm. Antibiotics were added at the

Figure 5. Stp1 and Stk1 do not regulate in vivo proliferation of *S. aureus*. Five, six week old female C57Black6 mice were intravenously injected with 1×10⁸ CFU of WT, Δstp1 or Δstk1 strains. At approximately 5 days post-infection, kidneys, spleen, and brains were harvested from infected mice and bacterial CFU enumerated. Note that the CFU of WT, Δstp1 or Δstk1 *S. aureus* were not significantly different in the kidneys (A), spleens (B) or brains (C) of infected mice. doi:10.1371/journal.pone.0011071.g005

Figure 6. Stp1 and Stk1 regulate the host immune response to *S. aureus* Newman. (A) Cytokine IL-6 levels were measured in kidney homogenates obtained from infected mice at 5 days post-inoculation. IL-6 levels were significantly lower in kidneys obtained from Δstp1 mutant-infected mice compared to Δstk1 mutant-infected mice (P value = 0.003). (B) Bone marrow derived macrophages (BMMφ) were stimulated with WT, Δstp1 or Δstk1 *S. aureus* cells and cytokine IL-6 levels measured as described in the Methods. Secretion of IL-6 was increased in response to *S. aureus* Δstk1 in comparison to Δstp1 or WT (P value = 0.04). The experiment was repeated three times and similar results obtained. Data shown represent the mean and SD of one experiment. doi:10.1371/journal.pone.0011071.g006
following concentrations when necessary: for E. coli, ampicillin 100 μg/ml, kanamycin 50 μg/ml, erythromycin 300 μg/ml, chloramphenicol 10 μg/ml for S. aureus, kanamycin 50–100 μg/ml, erythromycin 1–5 μg/ml, and chloramphenicol 10 μg/ml. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and molecular biology reagents were purchased from New England Biolabs or Promega Corporation, USA.

Construction of S. aureus allelic exchange mutants

Approximately 1 kb of DNA flanking the 5’ and 3’ ends of stp1 or stk1 were PCR-amplified from S. aureus Newman using High fidelity PCR (Invitrogen CA, USA). The primer pairs used to amplify 1 kb regions located upstream and downstream of stp1 were SaStpBamHIR and SaStpEcoRIF for stp1, the gene conferring chloramphenicol (cm) resistance was amplified from the plasmid pDC123 [64] using the primers SA-CatF and SA-CatR, respectively. Likewise, the primer pairs used to amplify 1 kb regions located upstream and downstream of stk1 were PSAF3 and SAsptnewKanR, and PSAF2+ and PSAR4, respectively. For allelic replacement of stp1, the gene conferring chloramphenicol (cm) resistance was amplified from the plasmid pDC123 [64] using the primers SA-CatF and SA-CatR. For allelic replacement of stk1, the gene conferring kanamycin (kn) resistance was amplified from pGIV2 [65] using the primers SA-KanF and SA-KanR. Subsequently, SOEing PCR [91] was performed to introduce the antibiotic resistance gene (cm or kn) between the flanking regions of stp1 or stk1. The PCR fragments were then ligated into the temperature sensitive vector pHY304, [92] and the resulting plasmids pKB8 (∆stp1::cm) and pKB1 (∆stk1::kn) were electroporated into S. aureus strain RN4220 using methods described previously [69]. Selection for single crossovers and screening for the double crossovers was performed as described [60,69]. PCR was used to verify the presence of the allelic replacement cassette and the absence of the gene of interest. Subsequently, the above mutations were transduced into the virulent S. aureus wild type (WT) strain Newman using phage phi1 (φ11) as described [62]. PCR was used to verify the presence of the allelic replacement cassette in the region of interest in S. aureus Newman. Complemented strains were constructed using the vector pDCerm [93]. The genes encoding stp1 and stk1 were amplified using the primers SaStpBamHIR and SaStpEcoRIF for stp1, and pDCSaStkBamHIR and pDCSaStkEcoRIF for stk1, and ligated into pDCerm to generate pStp1 and pStk1, respectively (Table S1).

Quantitative western blots

S. aureus WT Newman and isogenic mutants were grown in TSB to post-exponential phase (OD600~3.0). The cells were centrifuged and secreted proteins were precipitated from cell-free supernatants using 25% TCA as described previously [21]. Cell pellets were disrupted using the Fastprep FP101 bead beater (Bio 101) and centrifuged to remove unlysed cells and cell debris. Intracellular or cytoplasmic proteins were quantified in the supernatants using the Bradford protein assay [94]. Equal amounts of secreted or cytoplasmic proteins (40 μg) from each strain were subjected to 4–12% SDS-PAGE. One μg of purified α toxin (Sigma Chemical Co., USA) was included as a positive control. Following electrophoresis, the proteins were transferred to a PVDF membrane as described [94]. The membrane was blocked in Odyssey buffer (Licor, USA) containing 5% normal goat serum. Subsequently, α toxin antibody (Sigma Chemical Co., USA) was added at a 1:10,000 dilution followed by secondary Alexa Fluor goat anti-rabbit antibody (Invitrogen) at 1:10,000. Washes and imaging were performed as per manufacturer’s instructions for the Odyssey Licor infrared imager.

Isolation and purification of total RNA

Total RNA was isolated using the RNeasy Mini kit (QIAGEN, Inc., Valencia, CA). In brief, S. aureus strains were grown to an OD600 of 0.6 or ~3.0, centrifuged, and washed in 1:1 mixed RNA Protect and TE buffer (QIAGEN, Inc., Valencia, CA). The cells were then resuspended in RLT buffer (QIAGEN, Inc., Valencia, CA) and lysed using a FastPrep FP101 bead beater (Bio 101) with 3 × 30 s bursts at a power setting of 6, followed by centrifugation.

Table 6. S. aureus phosphopeptides identified using in vivo phosphopeptide enrichment and mass spectrometry.

| Locus  | Phosphopeptide | Protein Description | Strain     |
|--------|----------------|---------------------|------------|
|        |                |                     | WT | astk1   |
| NWMN_1123 | PLMTKSFST*NSK | Hypothetical protein | ✓ | ×       |
| HU     | INVAEQADLT*K  | DNA-binding protein HU | ✓ | ×       |
| SdrF   | GGLKDGETYT*VK | Ser-Asp rich fibrinogen/bone sialoprotein-binding protein | ✓ | ×       |
| SdrF   | TENNS*TNP     | Ser-Asp rich fibrinogen/bone sialoprotein-binding protein | ✓ | ×       |
| Stk1   | ALSETSLGT*NHVLGTQVQFSPEQAK | Serine/threonine kinase | ✓ | ×       |

Peptides listed above showed neutral loss of phosphoric acid in the LC/MS analysis. The serine or threonine residue that is phosphorylated is indicated as S* or T*, respectively. A ✓ indicates that the phosphopeptide was identified in the mass spectrometric analysis from the corresponding strain. A ‘×’ indicates that the phosphopeptide was not identified in the mass spectrometric analysis from the corresponding strain. NWMN numbers correspond to the ORF of the gene in the genome sequence.

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RNA was isolated from the supernatants using the RNeasy Mini kit as described by the manufacturer (QIAGEN, Inc., Valencia, CA). RNA concentration and integrity was determined using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and a NanoDrop 1000 (NanoDrop, Wilmington, DE) for subsequent use in qRT-PCR and microarray.

Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

RNA isolated from three independent biological replicates for each strain was purified as described above. qRT-PCR was performed using a two-step QuantiTect SYBR Green RT-PCR kit (QIAGEN, Inc., Valencia, CA) following the manufacturer’s instructions for a total reaction volume of 20 µL (iCycler thermocycler; Bio-Rad, Hercules, CA). cDNA synthesis was achieved using 50 ng of total RNA, and PCR amplification was performed as recommended by the manufacturer (Qiagen, USA). All runs were immediately followed by a melting curve analysis to evaluate PCR specificity and showed single primer-specific melting temperatures. All assays were performed with at least three independent biological replicates and three technical replicates for each gene. All primers were calculated using Primer3 services (http://frodo.wi.mit.edu/cgi-bin/primer3_www.cgi), purchased from Integrated DNA Technologies (Coralville, IA) or Sigma Chemical Company, and are listed in Table S1. The housekeeping gene rpoD was used to normalize transcript measurements, and relative gene expression calculated using the comparative CT method [68]. Statistical significance was determined using students t test as described previously [61].

Microarray

For microarray analysis, RNA was isolated from two independent biological replicates of each strain as described above. Purified RNA was sent to NimbleGen Systems, Inc. (Madison, WI) for full expression services. NimbleGen performed: cDNA synthesis, labeling of the cDNA, and hybridization of the labeled cDNA to the Staphylococcus aureus COL custom chip (009327_Saur_COL_SACOL1232.Expr_X4; NimbleGen Systems, Inc.)
experiments were repeated three times in triplicate. All assays. IL-6 ELISA assays were performed using the DuoSet kit as described by the manufacturer (R&D Systems, USA). Bone marrow derived macrophages (BMMΦ) were derived as described previously [95] with some modifications. Briefly, for each experiment, femurs were flushed, washed and resuspended using complete medium containing 20% fetal calf serum (RPMI 1640) and stimulated with WT, Δstk1, ΔΔkl and Δkl strains, 1×10⁷ CFU of each S. aureus strain was injected via the tail vein into 6 week-old female C57BL/6J mice (n = 10) purchased from Jackson Laboratories (Bar Harbor, ME). Infected mice were monitored every 12 hrs for signs of morbidity. Kidneys were obtained from mice that were moribund or at the end of an experiment, visually examined for abscess formation, and homogenized for CFU enumeration. To compare CFU between WT, Δspf1 and ΔΔkl strains, 1×10⁷ CFU of each S. aureus strain was injected via the tail vein into 6 week-old female C57BL/6J mice (n = 10), and spleen, kidneys and brain were harvested from infected mice (n = 10 per group) 5 days after infection. Bacterial CFU were enumerated from the homogenized tissue of five mice by plating serial 10-fold dilutions on TSB agar. Kidneys from the remaining mice (n = 5) were fixed in formalin, embedded in paraffin, thin-sectioned, stained with hematoxylin and eosin (H&E) and examined by microscopy as described previously [69].

Host immune response

IL-6 ELISA assays were performed on kidney homogenates from above using the DuoSet® kit as described by the manufacturer (R&D Systems, USA). Bone marrow derived macrophages (BMMΦ) were derived as described previously [95] with some modifications. Briefly, for each experiment, femurs were excised from three C57BL/6J mice (n = 10) purchased from Jackson Laboratories (Bar Harbor, ME), and spleen, kidneys and brain were harvested from infected mice (n = 10 per group) 5 days after infection. Bacterial CFU were enumerated from the homogenized tissue of five mice by plating serial 10-fold dilutions on TSB agar. Kidneys from the remaining mice (n = 5) were fixed in formalin, embedded in paraffin, thin-sectioned, stained with hematoxylin and eosin (H&E) and examined by microscopy as described previously [69].

Statistical analysis

The Mann-Whitney test was used to evaluate differences between cytokine levels and CFU/organ between S. aureus strains. Survival analyses were performed using a log rank test. These tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Phosphopeptide enrichment and Mass spectrometry

Total protein was isolated from WT Newman and ΔΔkl mutant as described previously [70] with a few modifications. Briefly, each sample was normalized to contain equal amount of protein and the proteins were denatured and reduced in 10mM DTT containing 0.1% Rapigest (Waters, USA) at 50°C for 30 min. Subsequently, the reduced cysteines were alkylated with 30mM iodoacetamide for 1hr in the dark and the samples were digested overnight at 37°C using sequencing grade trypsin (1:100, trypsin:total protein). Rapigest was removed and samples were desalted using Sep-Pak C-18 columns according to manufacturer’s instructions (Waters, USA) and dried using a SpeedVac. From the peptide samples of each strain (500µg), phosphopeptides were enriched and captured using soluble nanopolymer (PolyMAC) as described previously [96]. Unbound non-phosphopeptides were washed and phosphopeptides were eluted as described previously [96]. The samples were analyzed by capillary liquid chromatography-nanoelectrospray tandem mass spectrometry (μLC-nanoESI-MS/MS) using a high resolution hybrid linear ion trap orbitrap (LTQ-Orbitrap Velos, Thermo Fisher) coupled with Eksigent Ultra2D nanoflow HPLC and methods described previously [70,96]. Data were searched using Proteome Discoverer™ software with Sequest™ algorithm at 10 ppm precursor mass accuracy cutoff. The searches included static modification on Cys residues (+57.0214), and variable modifications on methionine (+15.9949) and Ser and Thr residues (+79.997) to identify phosphorylation as described previously [70,96]. Spectra were searched against the S. aureus strain Newman database (NC_009641) with a 5% FDR cutoff based on the reverse database decay search using methods described [96]. The experiment was performed using two independent biological replicates of each strain.

Supporting Information

Figure S1 Cell morphology of stp1 and stk1 mutants is similar to the WT S. aureus Newman. Cross sectional transmission electron micrographs shown are at a magnification of 100,000. Arrows show regions of PG that are marked with a line of the same size across the three panels. Significant differences in cell morphology or thickness of peptidoglycan are not apparent between WT Newman and isogenic stp1 and stk1 mutants. Found at: doi:10.1371/journal.pone.0011071.s001 (0.91 MB DOC)

Figure S2 Stp1 and Stk1 are dispensable for purine and pyrimidine biosynthesis of S. aureus Newman. S. aureus strains were grown in chemically defined media (CDM) at 37°C and cell growth was monitored every half hour. The OD600 of the strains after 5 hrs of growth is shown. CDM was prepared as described (Richardson et al., 2006, Molecular Microbiology 61: 927–939). ‘Complete CDM (cCDM)’ represents CDM containing exogenous purines (adenine, guanine and xanthine) and the pyrimidine (uracil). ‘Deleted CDM (dCDM)’ represents CDM lacking both purines and pyrimidines. ‘cCDM + Purines’ represents CDM that had the purines (adenine, guanine and xanthine) but did not contain pyrimidines. ‘dCDM + Pyrimidines’ represents CDM that was supplemented only with uracil and lacked purines. Note that the control strain with a transposon insertion in purA showed no growth in media lacking purines. Growth and doubling time of the stp1 and stk1 mutants was comparable to WT S. aureus Newman. Found at: doi:10.1371/journal.pone.0011071.s002 (0.03 MB DOC)

Table S1 Strains, Plasmids, and Primers.
Table S2  Genes with altered expression in stpl and stkl mutants at post-exponential phase.

| Genes       | Expression Change |
|-------------|-------------------|
| stpl        | Increased         |
| stkl        | Decreased         |

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Author Contributions

Conceived and designed the experiments: KB AL MeBR AI NTB JEC WJL BZS ARR. Analyzed the data: KB AL MeBR AI NTB JEC WJL BZS ARR. Contributed reagents/materials/analysis tools: ARR FCF WAT LR. Wrote the paper: KB ARR FCF WAT LR.

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