Identification of Single Nucleotide Polymorphisms Associated with Hyperproduction of Alpha-Toxin in Staphylococcus aureus

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Abstract

The virulence factor α-toxin (hla) is needed by Staphylococcus aureus in order to cause infections in both animals and humans. Although the complicated regulation of hla expression has been well studied in human S. aureus isolates, the mechanisms of hla regulation in bovine S. aureus isolates remain undefined. In this study, we found that many bovine S. aureus isolates, including the RF122 strain, generate dramatic amounts of α-toxin in vitro compared with human clinical S. aureus isolates, including MRSA WCUH29 and MRSA USA300. To elucidate potential regulatory mechanisms, we analyzed the hla promoter regions and identified predominant single nucleotide polymorphisms (SNPs) at positions −376, −483, and −484 from the start codon in α-toxin hyper-producing isolates. Using site-directed mutagenesis and hla promoter-gfp-luxABCDE dual reporter approaches, we demonstrated that the SNPs contribute to the differential control of hla expression among bovine and human S. aureus isolates. Using a DNA affinity assay, gel-shift assays and a null mutant, we identified and revealed that an hla positive regulator, SarZ, contributes to the involvement of the SNPs in mediating hla expression. In addition, we found that the bovine S. aureus isolate RF122 exhibits higher transcription levels of hla positive regulators, including agrA, saeR, arlR and sarZ, but a lower expression level of hla repressor rot compared to the human S. aureus isolate WCUH29. Our results indicate α-toxin hyperproduction in bovine S. aureus is a multifactorial process, influenced at both the genomic and transcriptional levels. Moreover, the identification of predominant SNPs in the hla promoter region may provide a novel method for genotyping the S. aureus isolates.

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Introduction

S. aureus is an important pathogen capable of causing both animal and human infections such as pneumonia, endocarditis, toxic shock syndrome and bovine mastitis. The continuous increase of infections associated with both hospital- and community-acquired methicillin resistant Staphylococcus aureus (HA-MRSA and CA-MRSA) has caused serious public health concerns [1]. The ability of this organism to cause a wide variety of infections partially depends on the coordinated and regulated expression of multiple cell- and surface-associated virulence factors [2,3] and exported proteins including various proteases and toxins [4].

Alpha-toxin plays a critical role in the modulation of S. aureus-induced cytotoxicity in Jurkat T-lymphocytes, human peripheral blood lymphocytes, monocytes [5] and epithelial cells [6], even though multiple virulence factors are required for the bacterium to induce apoptosis in endothelial cells [7]. Alpha-toxin can interact specifically with surface receptors of the host cells, form functional transmembrane pores, and selectively release ions, and/or leads to the activation of cell signaling pathways, thus inducing apoptosis and/or necrosis in various cell types [8–12]. Recently, we demonstrated that α-toxin interacts with αvβ1-integrin to interfere with S. aureus adhering to and internalizing into human lung epithelial cells [A549] [13]. The interaction of α-toxin with αvβ1-integrin contributes to the cytoxicity of α-toxin that is required for S. aureus to induce apoptosis and death of the epithelial cells [6]. However, the role played by α-toxin depends on the stage and/or type of infection and the quantities produced. It has been demonstrated that α-toxin is an important virulence factor in experimental brain abscesses and pneumonia [14–16] and intraperitoneal infection [17,18], whereas the overproduction of α-toxin significantly reduces virulence in experimental endocarditis [19].

Alpha-toxin expression is up-regulated in the stationary growth phase in vitro [20] and at later stages of animal infection [21,22]. The expression of α-toxin is simultaneously regulated by different regulators, which have been well documented and reviewed elsewhere [20,23]. The expression of α-toxin is positively regulated by various global regulators, including two-component signal transduction systems, such as the accessory gene regulator (Agr) [24,20], the staphylococcal accessory protein effector (SaeRS) [25,26], ArlRS [27,28] and transcriptional regulators Mgr [29] and SarZ [30–32]. In contrast, the homologues of staphylococcal accessory regulator (SarA), including Rot and SarT, repress the...
expression of α-toxin [33,34]. The role of SarA in modulating hla transcription is controversial: SarA affects hla expression in both an agr-dependent and agr-independent manner [22,35]. In addition, it has been revealed that hla transcription is affected by additional factors, including the alternative sigma B factor (σB) and environmental stimuli [36,37].

Alpha-toxin also serves as a virulence factor in S. aureus-induced mastitis [38,39]. It has been found that significant increases in milk antibodies to α- and β-toxins are present in cows with chronic staphylococcal mastitis [40], and that many S. aureus isolates from the mammary gland of dairy cows produce α-toxin [41]. The comparative genomic analysis of human and bovine S. aureus isolates suggests that a unique mechanism may be involved in the pathogenicity of bovine S. aureus isolates [42] and that some bovine S. aureus isolates generate dramatic amounts of α-toxin [43]. However, the mechanism of hyperproduction of α-toxin in some bovine S. aureus isolates remains undefined.

In the present study, we compared the expression profiles of exported proteins between bovine S. aureus isolates and human clinical S. aureus isolates. We found hyperproduction of α-toxin in many bovine S. aureus isolates and investigated potential mechanisms of up-regulation of hla using site-directed mutagenesis, transcriptional promoter-reporter fusion, and quantitative RT-PCR approaches. Our results indicate α-toxin hyperproduction in bovine S. aureus is a multifactorial process, influenced at both the genomic and transcriptional levels.

Results

Hyperproduction of α-toxin in bovine mastitis S. aureus isolates, including RF122

The comparative genomic analysis of human and bovine S. aureus isolates suggested that a unique mechanism may be involved in the pathogenicity of bovine S. aureus isolates [42]. Numerous studies have demonstrated that staphylococcal exported proteins, especially toxins, are important virulence factors in bovine mastitis [40,41]. To examine whether human and bovine S. aureus isolates produce distinct levels of toxins, we conducted hemolytic assays using a sheep blood agar plate. The supernatants from the overnight cultures of human isolates, WCUH29 and COL strains, had dramatically less hemolytic activity compared to that of bovine isolate RF122 (Fig. 1A). To further determine whether there are different exported protein profiles between human and bovine S. aureus isolates, we chose human clinical isolates, including WCUH29, different strains of MRSA and twelve bovine S. aureus isolates, including the RF122 strain, to compare their exported proteins using SDS-PAGE. None of the human isolates produced dramatic amounts of α-toxin (Fig. 1B). In contrast, seven bovine strains, including RF122, exhibited extremely high levels of α-toxin production (Fig. 1C), although five bovine strains produced α-toxin at levels similar to the human isolates (Fig. 1D). To further confirm the identity of the over-expressed protein, we conducted a MALDI mass spectrometry assay and identified that the highly
expressed protein is α-toxin (data not shown). Furthermore, qPCR analysis identified that RF122 has a 7-fold higher level of hla transcript compared to WCUH29, suggesting the up-regulation of α-toxin is at the transcription level (Table 1).

Identification of single nucleotide polymorphisms (SNPs) in the hla promoter region

To elucidate the potential mechanisms involved in up-regulating hla expression in the bovine S. aureus isolate RF122, we performed alignment analyses of hla promoter region based upon the published S. aureus genomes in the NCBI genome database. We found that the DNA sequences of hla promoter region are almost identical among the human S. aureus hla region led us to hypothesize that the SNPs may be associated with hla predominant SNPs in the RF122 promoter region (Fig. 2A). However, several nucleotides of the hla promoter region of bovine RF122 in the positions −484, −483 and −376 from the start codon are different from those of human isolates (Fig. 2A). To further investigate whether these differences exist in other human and bovine isolates, we isolated the chromosomal DNA from these isolates and the hla promoter region was amplified by PCR for sequencing. The DNA sequencing results showed that among seven α-toxin over-expressing S. aureus isolates, six isolates possess the same hla promoter DNA sequence as RF122 (Fig. 2B), indicating that these bovine isolates have predominant SNPs in the hla promoter region. In contrast, the hla promoter sequences of the five α-toxin hypoproduction strains were identical to the human S. aureus isolates (Fig. 2B). In addition, the DNA sequences of hla promoter region from eight human isolates were identical to the published human isolates (data not shown).

The SNPs in the hla promoter region affects hla transcription

The identification of predominant SNPs in the hla promoter region led us to hypothesize that the SNPs may be associated with the modulation of hla transcription. To test this possibility, we created both the human and bovine S. aureus hla promoter-gfp-luxABCDE dual reporter constructs in the human isolate WCUH29 and determined the hla expression levels by measuring bioluminescence intensity. The dual reporter construct carrying the RF122 hla promoter showed a significantly higher level of hla transcription than the dual reporter construct carrying the WCUH29 hla promoter (Fig. 3). This data suggests that the predominant SNPs in the RF122 hla promoter region are likely involved in the transcriptional modulation of hla expression.

To confirm this finding, we first performed site-directed mutations in the hla promoter region using the RF122 hla promoter-gfp-luxABCDE dual reporter construct. The site mutations were confirmed by DNA sequencing. Then, we determined the impact of the nucleotide mutations on growth and hla expression by monitoring lux expression levels in the WCUH29 strain. The mutations in the hla promoter region did not affect growth (data not shown). However, the nucleotide mutations in either −376 (T→G) or −484 and −483 (TC→AT) in the RF122 hla promoter region significantly decreased the reporter gene expression level compared to that of the parental promoter (Fig. 3). Furthermore, the double mutations (T→G/TC→AT) in the RF122 hla promoter region dramatically diminished the reporter gene expression, which was comparable to the construct carrying the WCUH29 hla promoter (Fig. 3).

To further confirm the role of the SNPs of the hla promoter region in controlling the hla over-expression in RF122 strain, we electroporated the above hla promoter-gfp-lux dual reporter constructs into the RF122 strain and examined the impact of the site-directed mutations in the hla promoter region on reporter gene expression. Surprisingly, no bioluminescence signal was detectable in the cultures of these strains. However, we found that the introduction of either the RF122 hla promoter-gfp-lux dual reporter or either of the single (T→G, TC→AT) mutated hla promoter-gfp-lux dual reporters into the RF122 strain either eliminated or severely reduced endogenous hla expression, respectively, indicating that dominant-negative effects occurred in the strains (Fig. 4A, lane 2, 3 and 4). In contrast, the introduction of the WCUH29 hla promoter-gfp-lux reporter and the double mutated (T→G/TC→AT) RF122 hla promoter-gfp-lux reporter did not have an appreciable effect on endogenous hla expression (Fig. 4A, lane 5 and 6). In addition, we determined the impact of the SNPs of hla promoter region on reporter gfp expression using a Western blotting assay. An intense band of Gfp was exhibited in the whole cell lysates of the reporter construct carrying the wild-type RF122 hla promoter; contrastingly, either no band or a weak band of Gfp was detected in the whole cell lysates of constructs carrying either the WCUH29 hla promoter or the double (T→G/TC→AT) mutated RF122 hla promoter (Fig. 4B). Both the T→G and the TC→AT mutations in the RF122 hla promoter led to a 2- to 3-fold decrease of gfp expression (Fig. 4B). Taken together, the above data demonstrate that the SNPs in the hla promoter region participate in the modulation of hla expression in the bovine S. aureus RF122 isolate.

SarZ is associated with the SNPs in the regulation of hyperproduction of α-toxin

In order to identify regulators that are involved in the hla regulation, we employed DNA affinity purification using a biotinylated RF122 hla promoter region oligonucleotide bound to Dynabeads M-280 Streptavidin coated paramagnetic beads. The cytoplasmic proteins of RF122 specifically binding to the hla promoter region oligonucleotide were eluted from the beads and separated by SDS-PAGE (data not shown). To determine the identity of hla promoter region binding proteins, we employed MALDI mass spectrometry assays and revealed SarZ and Mgr bound to the hla promoter region of RF122 (data not shown).

To confirm whether SarZ protein is associated with the SNPs in the modulation of hla expression genetically, we constructed a sarZ null mutant in the RF122 strain by phage transduction. The sarZ null mutant, BSasarZ, was confirmed by using diagnostic PCR and DNA sequencing. The five individual hla promoter-gfp-lux report vectors were introduced into BSasarZ by electroporation. First, we examined how the loss of SarZ affected the endogenous hla expression by using a Sheep Blood Agar hemolysis assay and

Table 1. Comparison of gene expression by qPCR analysis between RF122 and WCUH29 strain.

| ORF (N315) | Gene | Fold change (RF122/WCUH29) |
|-----------|------|---------------------------|
| Sa1007    | hla  | +7±0.1***                 |
| Sa2174    | sarZ | +2±0.6                    |
| Sa1246    | arlS | +2±0.1***                 |
| Sa0660    | saeS | +5                        |
| Sa1844    | agra | +26±0.1**                 |
| Sa1583    | rot  | −2±0.1*                   |
| 16S rRNA  |      | 0                         |

*P≤0.05;  
**P≤0.01.  
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SNPs and α-Toxin Hyperproduction

A

ATGTAATGGCAAAATTTATTTCCCGACGAAATTTCAACATATAATTACCCCTTTTTTCCT

MW2
H315
Mu3
Mu50
JH1
JH9
MSSA476
COL
Newman
NCT8325
sabac-123
USA3000FPR3757
USA3000TCH1516
RF122

TCT

MW2
H315
Mu3
Mu50
JH1
JH9
MSSA476
COL
Newman
NCT8325
sabac-123
USA3000FPR3757
USA3000TCH1516
RF122

T

B

ATGTAATGGCAAAATTTATTTCCCGACGAAATTTCAACATATAATTACCCCTTTTTTCCT

RF122
BSa39
BSa55
BSa60
BSa67
BSa68
BSa97
BSa74
BSa12
BSa22
BSa28
BSa83
BSa110
WCUH29

C

BSa39
BSa55
BSa60
BSa67
BSa68
BSa97
BSa74
BSa12
BSa22
BSa28
BSa83
BSa110
WCUH29

C

BSa39
BSa55
BSa60
BSa67
BSa68
BSa97
BSa74
BSa12
BSa22
BSa28
BSa83
BSa110
WCUH29

A

BSa39
BSa55
BSa60
BSa67
BSa68
BSa97
BSa74
BSa12
BSa22
BSa28
BSa83
BSa110
WCUH29

G

BSa12
BSa22
BSa28
BSa83
BSa110
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WCUH29

G
Involvement of agr, arlRS, saeRS and rot in the modulation of hyperproduction of α-toxin in the bovine S. aureus isolate RF122

It is well known that different regulators, including two-component signal regulators, such as Agr, SarRS, and AirlRS systems, and transcriptional regulators such as SarA family proteins, such as Rot, coordinately regulate hla expression [20,23]. Building off of our previous finding that RF122 has a higher sarZ transcript level, we hypothesized that the hyper-production of α-toxin may also be attributable to differential expression of these regulators between the bovine S. aureus RF122 and the human S. aureus WCUH29. To address this question, we examined the transcriptional levels of selected hla regulators using qPCR. The real-time RT-PCR results are shown in Table 1. In the bovine S. aureus RF122 strain, the transcriptional levels of hla positive regulator genes agr, saeRS and arlRS were higher than those in the human WCUH29 strain, whereas the transcriptional level of hla repressor gene rot was lower than that in the human WCUH29 strain (Table 1). This data suggests that the over-transcription of the hla positive regulator genes agr, arlRS, and saeRS and decreased transcription of the hla negative regulator gene rot also, at least in part, contributes to the α-toxin hyper-production phenotype of RF122.

Discussion

Our studies revealed that the hla promoter region possesses predominant SNPs that contribute to the modulation of hla overexpression in some bovine S. aureus isolates. Although no specific SNP is identified in the hla promoter region of the human S. aureus isolates tested, we cannot exclude the possibility that SNPs may exist in the hla promoter region in other human S. aureus isolates. Notably, we identified that a known hla transcriptional regulator, SarZ, likely has a higher binding affinity for the SNPs region and contributes to the involvement of SNPs in regulating hla expression. Moreover, we revealed that the transcription level of sarZ is higher in the bovine S. aureus RF122 strain than in the human WCUH29 strain. This suggests that the higher transcription level of sarZ is, at least in part, involved in the α-toxin hyper-production phenotype of the RF122 strain. Importantly, we also demonstrated that the SNPs contribute to the regulation of hla regardless of the genetic background, WCUH29 or RF122, in that the RF122 hla promoter-gfp-lux dual reporter construct had higher levels of expression in both backgrounds compared to the WCUH29 hla promoter-gfp-lux dual reporter construct. Additionally, the site-directed mutation of the RF122 promoter’s SNPs progressively decreased reporter expression in both backgrounds (Fig. 3 and 4B). Our findings are partially supported by a previous report that ST151 strains, including the RF122 strain, have elevated levels of hla and RNAIII expression [43]. Taken together, these findings indicate that unique mechanisms may be involved in the up-regulation of hla expression in bovine S. aureus isolates, which in turn may partially contribute to the pathogenicity in bovine mastitis.

DNA sequencing analysis indicates that there are predominant SNPs in the hla promoter region of bovine S. aureus isolates, which occur at positions −376, −483 and −484 upstream, of the hla start codon. In the α-toxin hyperproduction isolates, there are

SDS-PAGE and found that the sarZ null mutation abrogates the α-toxin hyper-production phenotype of RF122 (Fig. 4C, lanes 1 and 2; and E). Furthermore, the sarZ mutation eliminated the dominant-negative effects of the RF122 hla promoter-gfp-lux dual reporter (Fig. 4C, lanes 3 to 7) compared to the wild-type strains (Fig. 4A). Next, we determined the impact of SarZ on reporter expression using a Western blotting assay. In contrast to the RF122 wild-type strain (Fig. 4B), no obvious difference of expression was revealed in the whole cell lysates of sarZ mutants carrying the WCUH29 hla promoter, the single (T→G or TC→AT) mutated RF122 hla promoter, the double (T→G/TC→AT) mutated RF122 hla promoter, or the wild-type RF122 hla promoter (Fig. 4D).

To further confirm the specific effect of SarZ and identify binding affinity differences, we performed gel-shift assays. The gel-shift assays showed that with the addition of as little as 100 ng of SarZ protein a weak shifted band appeared for the RF122 hla promoter region, whereas no such band was detected for the WCUH29 hla promoter region (data not shown). These results suggest that the RF122 hla promoter binding affinity of SarZ is likely higher than the WCUH29 hla promoter region.

To examine whether the overproduction of α-toxin is also attributable to different levels of SarZ between bovine and bovine isolates, we performed real-time RT-PCR. We found that in the RF122 strain the transcriptional level of sarZ was 2-fold higher than that of the WCUH29 strain (Table 1). Taken together, the above results suggest that the SNPs’ regulation of hla expression may function through SarZ directly. Further-

Figure 3. Influence of SNPs hla promoter-luxABABCDE reporters on bioluminescence intensity of S. aureus WCUH29. The maximal light intensity values are given as relative light units (RLU). The symbol *” indicates a significant difference (P<0.05) between SaWH1207 and all other strains.

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apparent nucleotide shifts from G to T and T to C at positions −376 and −483, respectively, compared with the α-toxin hypoproduction isolates. These nucleotide shifts lead to a decrease in the binding affinity of SarZ, which is consistent with the result of the hla promoter-gfp reporter assay. It has been reported that the DNA-binding protein, SarZ, regulates hla expression by binding to the hla promoter region [32,31], and is associated with the pathogenicity of S. aureus [31]. Furthermore, the introduction of RF122 hla promoter-reporter fusion into the RF122 strain led to a dominant-negative effect on endogenous hla expression; this effect was eliminated in sarZ null background. Taken together, the above data indicate that the SarZ protein has a higher binding affinity for the RF122 hla promoter and is directly involved in differential regulation of hla expression through SNPs, which in turn may contribute to virulence of bovine S. aureus. Our results are similar to previous findings that a single nucleotide (T→C) mutation at position −215 bp in the promoter region of the nitrate reductase operon narGHJI in Mycobacterium tuberculosis and Mycobacterium bovis leads to differential activity of reductase and altered virulence capacity [44]; and that in Vibrio cholerae, nucleotide differences at positions −65 and −66 bp in the tcpPH encoding a toxin-coregulated pilus transcriptional activator pair, TcpPH, promoter region is not only responsible for determining the classical and EL Tor biotypes, but also contributes to differential regulation of virulence gene expression through a tcpPH regulator, AphB [45]. Although we found that the DNA binding motif of Mgr is not located in the SNPs, we cannot exclude the possibility that Mgr may contribute to the hyperproduction of α-toxin, because sarZ gene is transcriptionally regulated by Mgr [32].

It was reported that the ET3 clone is predominant in bovine mastitis S. aureus isolates [46] and that different subtypes exhibit different expression levels of α-toxin in the ET3 clone [43]. The RF122 strain belongs to the ET3 clone ST151 subtype [43]; therefore, it is necessary to determine whether bovine S. aureus isolates that possess the predominant SNPs in the hla promoter region belong to the common bovine S. aureus clone (ET3) and/or the same subtype within the ET3 clone. The results would allow us to evaluate whether the identified predominant SNPs of the hla promoter region would be useful as a target for molecular diagnosis of S. aureus isolates that posses an α-toxin hyperproductive phenotype and may cause severe bovine mastitis.

The agr locus in S. aureus has been examined for its role in the expression of exotoxins and cell surface proteins [47]. It has been reported that variations in α-toxin production of S. aureus isolates from humans and bovines were due to variations of the RNA III transcript in the agr locus [8,48]. Our data also revealed that in the RF122 strain the transcriptional levels of hla positive regulator genes agr, sarR and arlR are higher in the bovine S. aureus RF122 strain than in the human WCUH29 isolate in the same culture medium; however, the transcription level of the hla represor gene rot is lower.
in the RF122 strain. Although the SNPs are located outside of the hla promoter DNA-binding regions (GEEA\textsubscript{4N}GTAA from −405 to −390 or TTTA\textsubscript{3N}GTAA from −195 to −175) of phosphorylated SaeR [49] and the DNA-binding motif of Mgr [30], to define the mechanisms of their regulation is beyond the scope of this study. In addition, we found that the impact of SNPs on hla expression in the sigma B deficient strain RN4220 (Figure S1) is the same as in the sigma B positive strains WCUH29 and RF122. Thus, we can exclude the potential effect of sigma B factor on the SNPs’ involvement in regulating hla expression, although sigma B factor is associated with mediating hla expression [36,37].

It was previously reported that the RF122 strain belongs to ST151 (a subtype of ET3 clone) [43,42]. Our unpublished data showed that the supernatant of RF122 culture is more toxic than the supernatant of WCUH29 using MAC-T cells [50]. This is consistent with recent findings that among ET3 subtypes, the ST151 strain is more virulent in an intramammary gland infection [43]. Previous studies have also demonstrated that most S. aureus isolates from the mammary gland of dairy cows produce α-toxin [41]. Significantly higher amounts of antibodies against both α- and β-toxins are exhibited in milk isolated from cows with chronic staphylococcal mastitis [40], and immunization of animals with attenuated α-toxin protects from S. aureus-induced mastitis [51]. Taken together, the above data suggest that the hyperproduction of α-toxin is a probable key factor for S. aureus to cause severe cow mastitis, although we cannot rule out the importance of other virulence factors in the pathogenesis of bovine mastitis [52].

In conclusion, we are the first to identify SNPs in the hla promoter region that results in the hyperproduction of α-toxin in many bovine S. aureus isolates. Importantly, we have identified and demonstrated that the DNA-binding protein SarZ contributes to the involvement of SNPs in differential regulation of hla expression. In addition, we found that the over-expression of agrA, arlR and sarZ, and the down-regulation of rot, may be partially attributed to the hyperproduction of α-toxin in the RF122 strain.

Materials and Methods

Bacterial strains, plasmids and growth media

The bacterial strains and plasmids used in this study are listed in Table 2. The human S. aureus strains were obtained from the Network on Antimicrobial Resistance in Staphylococcus aureus or references indicated. The bovine S. aureus isolates were obtained from geographically diverse animals that received care at the University of Minnesota’s Veterinary Diagnostics Laboratory. The S. aureus cells were cultured in Trypticase soy broth (TSB) at 37°C with shaking. E. coli strains were grown in Luria-Bertani (LB) medium. Transformants containing recombinant plasmids were selected on LB agar containing ampicillin (100 μg/ml) for E. coli and TSA containing chloramphenicol (10 μg/ml) for S. aureus.

SDS-PAGE analysis of exported proteins

The supernatants were collected from the overnight cultures of S. aureus isolates in TSB medium by centrifugation at 3900 × g. The exported proteins were precipitated from an equal volume of supernatant using ethanol as described [17]. The exported protein profiles were detected by a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining.

Statistical analysis

Data are the means ± standard errors of the means from three experiments. The symbol “*” indicates a significant difference (P<0.05) using an unpaired t test.

Construction and detection of promoter-gfp-lux dual reporter fusions

In order to further confirm the transcriptional regulation of hla expression, we created hla promoter-gfp-lux dual reporter constructs as previously described [25]. The gfp-lux dual reporter fusion system was provided courtesy of Philip Hill [55]. An approximate 1 kb upstream region of hla was amplified from both WCUH29 chromosomal DNA and RF122 chromosomal DNA by PCR, respectively, using the primers listed in Table 3, digested with EcoRI and XhoI, and ligated into the upstream region of the promoterless gfp-lux of pCY1006, which was digested with the same enzymes. The resulting recombinant plasmids pXLI1107 and pXLI1207 were electroporated into S. aureus RN4220, then into both WCUH29 and RF122, resulting in S. aureus strains SaRN1107, SaRN1207, SaWH1107, SaWH1207, SaRF1107 and SaRF1207. The lux expression was monitored until early stationary phase in TSB with an appropriate antibiotic at 37°C with a Chiron luminometer. The relative light units (RLU) were calculated (bioluminescence intensity/optical density at 600 nm). For western blot analysis of Gfp expression in different hla promoter-gfp fusions in RF122, a 1:100 dilution of an overnight culture was grown and the same number of bacterial cells was harvested from cultures at an optical density of 1 at 600 nm by centrifugation. The whole cell lysates were prepared, and the same volume of lysate was loaded on 12% SDS-PAGE and probed by rabbit anti-Gfp antiserum using western blot assay. The density of the reaction band in equal area was scanned and calculated using ImageJ software.

Site-directed mutagenesis

To determine whether hla transcription is modulated by single nucleotide polymorphisms (SNPs) in the RF122 hla promoter, site-directed mutations were generated by PCR using the pXLI1207 as a template and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions except that primer extension was allowed to continue for 8 min. The primers used for site-directed mutagenesis are listed in Table 3. The mutations were confirmed by DNA sequencing of the region containing the mutation. The reformed plasmids were designated pXLI1307, pXLI1407 and pXLI1507, and electroporated into RN4220, then into WCUH29 and RF122. The resulting S. aureus strains were named SaRN1307, SaRN1407, SaRN1507, SaWH1307, SaWH1407, SaWH1507, SaRF1307, SaRF1407 and SaRF1507.

The chromosomal DNA from each bovine mastitis S. aureus isolate listed in Table 2 was purified and the promoter region of hla from each isolate was obtained by PCR using the same primer listed in Table 3. The PCR products were purified and sequenced; the DNA sequences were deposited in GenBank (accession #HQ923430 to HQ923496).

DNA affinity purification of hla promoter region binding proteins

In order to identify regulators associated with SNPs, we utilized Dynabeads M-280 streptavidin coated paramagnetic beads (Invitrogen, Carlsbad, CA) to identify DNA-binding proteins according to the manufacturer’s protocol. A 266 bp PCR fragment spanning the RF122 hla promoter SNP region was PCR amplified using the primers RF122-phla217for and phlaRev482 listed in Table 3 and purified using a PCR cleanup kit (Promega, Madison, WI). Approximately 9 μg of purified biotinylated hla promoter region was mixed with the beads. The mixtures were incubated at room temperature for 30 minutes with occasional gentle mixing. The beads were washed and resus-
pended in Protein Binding Buffer (10 mM Tris–HCl pH 7.5, 50 mM NaCl, and 1 mM DTT), then mixed with 500 mg of total cytoplasmic protein of RF122. The reaction mixtures were incubated at room temperature for 30 minutes with gentle, occasional swirling. The extra protein supernatant was removed and the beads were washed with Protein Binding Buffer to remove nonspecific binding proteins before being resuspended in 28 μl of Elution Buffer (10 mM Tris–HCl pH 7.5, 10% glycerol, 1 M NaCl, and 1 mM DTT) and incubated at room temperature for 30 minutes with occasional vortexing. A portion of the cytoplasmic protein fraction, washes, and elutes from the beads were detected by SDS-PAGE and visualized by Coomassie Blue staining.

Construction of sarZ null mutant in RF122 strain

Construction of sarZ null mutant in RF122 isolate was performed by plaque transduction as described [32]. Dr. Adhar Manna provided us with a sarZ mutant of RN6390 (AM1090). A phage Φ602lyste of AM1090 was prepared to infect bovine S. aureus isolate RF122 to create a sarZ null mutant of RF122, which was confirmed by diagnostic PCR and DNA sequencing of the flanking regions of sarZ. This null mutant was designated as BSasarZ. The different hla-promoter-gfp-lux reporter fusions pXL1107, pXL1207, pXL1307, pXL1407, and pXL1507, were each electroporated into BSasarZ. The resulting S. aureus strains were named BSasarZ1107, BSasarZ1207, BSasarZ1307, BSasarZ1407, and BSasarZ1507.

Cloning, expression, and purification of recombinant SarZ protein

The sarZ coding region was obtained by PCR using sarZ specific primers (sarZNdeIfor1 and sarZXhoIrev) listed in Table 3 from S. aureus and cloned into NdeI and XhoI sites of the E. coli expression Table 2. Strains and plasmids used in this study.

| Strain /plasmid | Description | Reference or Resource |
|-----------------|-------------|-----------------------|
| RN4220          | laboratory S. aureus strain (rsbU−) | [53]                 |
| WCUP29          | human clinical MRSA isolate | NCIMB40771           |
| COL             | human clinical MRSA isolate | NARS 10105-10384       |
| NRS105-10384    | human MRSA isolates            | NARS 10105-10384       |
| MW2 and USA300   | human MRSA isolates            | [54]                 |
| RF122           | bovine mastitis S. aureus isolate | [42]              |
| BSasarZ         | sarZ mutant of RF122 with sarZ::ermC | This study |
| BSa12-Bsa110     | bovine mastitis S. aureus isolates | CVM Dia. Lab. |
| SaRN1107        | RN4220 carrying pXL1107        | This study |
| SaRN1207        | RN4220 carrying pXL1207        | This study |
| SaRN1307        | RN4220 carrying pXL1307        | This study |
| SaRN1407        | RN4220 carrying pXL1407        | This study |
| SaRN1507        | RN4220 carrying pXL1507        | This study |
| SaWH1107        | WCUP29 carrying pXL1107        | This study |
| SaWH1207        | WCUP29 carrying pXL1207        | This study |
| SaWH1307        | WCUP29 carrying pXL1307        | This study |
| SaWH1407        | WCUP29 carrying pXL1407        | This study |
| SaWH1507        | WCUP29 carrying pXL1507        | This study |
| SaRF1107        | RF122 carrying pXL1107         | This study |
| SaRF1207        | RF122 carrying pXL1207         | This study |
| SaRF1307        | RF122 carrying pXL1307         | This study |
| SaRF1407        | RF122 carrying pXL1407         | This study |
| SaRF1507        | RF122 carrying pXL1507         | This study |
| BSasarZ1107     | BSasarZ carrying pXL1107       | This study |
| BSasarZ1207     | BSasarZ carrying pXL1207       | This study |
| BSasarZ1307     | BSasarZ carrying pXL1307       | This study |
| BSasarZ1407     | BSasarZ carrying pXL1407       | This study |
| BSasarZ1507     | BSasarZ carrying pXL1507       | This study |
| pCY1006         | shuttle vector, derives from pSB2019, CmR, AmpR | [25] |
| pXL1107         | WCUP29 hla promoter-gfp-lux reporter, CmR, AmpR | This study |
| pXL1207         | RF122 hla promoter-gfp-lux reporter, CmR, AmpR | This study |
| pXL1307         | RF122 (T→G) hla promoter-gfp-lux reporter, CmR, AmpR | This study |
| pXL1407         | RF122 (TC→AT) hla promoter-gfp-lux reporter, CmR, AmpR | This study |
| pXL1507         | RF122 (T→G/TC→AT) hla promoter-gfp-lux reporter, CmR, AmpR | This study |

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vector pET24b. The recombinant DNA (pETsarZ) was confirmed by using PCR and DNA sequencing and transformed into E. coli strain BL21. The transformants were incubated until mid-log phase of growth (OD600 nm = 0.4); followed by induction of sarZ expression by adding IPTG (final concentration 1 mM). The His-tagged SarZ protein expression and purification were conducted as described [56]. The purity of purified His-tagged SarZ protein was evaluated in a 12% SDS-PAGE followed by DIG-labeled DNA, 1 mg of poly-(dl-dC), 25 mM NaH2PO4 (pH 8.0), 50 mM NaCl, 2 mM MgCl2, 1 mM DTT, 10% glycerol and increasing amount of SarZ protein. An unlabeled DNA fragment of the promoter region as a specific competitor was added into the reaction with 100-fold excess to the labeled probe. After incubation at 25 °C for 20 min, the reaction mixtures were analyzed by 5% native PAGE.

RNA purification and quantitative RT-PCR analysis (qPCR)
Overnight cultures of S. aureus (WCUH29 and RF122) were incubated in TSB medium and grown to the mid-exponential phase of growth (OD600 nm = 0.5) at 37 °C with shaking. Cells were harvested by centrifugation at 3900 × g, and the RNA was isolated by the RNAPrep Kit [Promega, Madison, WI] according to the manufacturer’s instructions. Contaminating DNA was removed with the TURBO DNA-free Kit [Ambion, Austin, TX] and the RNA yield was determined spectrophotometrically at 260 nm. The integrity of the purified RNA was analyzed by electrophoresis in 1.2% agarose-0.66 M formaldehyde gels. The 23S and 16S rRNA bands were clear without any obvious smearing patterns.

The first strand cDNA was synthesized using reverse transcriptase SuperScript III [Invitrogen, Carlsbad, CA]. PCR reactions were set up in duplicate by using the SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA); there were also controls using purified total RNA as the templates. Real-time sequence-specific detection and relative quantification were performed with the Stratagene Mx3000P Real Time PCR System. Relative quantification of the product was calculated using the Comparative CT method, as described for the Stratagene Mx3000P system. The housekeeping gene 16s rRNA was used as an endogenous control [25]. All samples were analyzed in duplicate, normalized against 16s rRNA gene expression, and statistically analyzed by Student’s t test, using Microsoft Excel software. P values of ≤0.05 were considered significant.

Supporting Information

Figure S1 Influence of SNPs hla promoter-luxABCDE reporters on bioluminescence intensity of S. aureus RN4220. The maximal light intensity values are given as relative light units (RLU). The symbol “*” indicates a significant difference (P ≤ 0.05) between SaRN1207 and all other strains.

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Author Contributions
Conceived and designed the experiments: XL JWH YJ. Performed the experiments: XL JWH JY MY KD. Analyzed the data: XL JWH JY RB YJ. Wrote the paper: XL JWH JY MY KH RB YJ.

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