RESEARCH ARTICLE

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Isobaric tags for relative and absolute quantitation proteomics analysis of gene regulation by SprC in *Staphylococcus aureus*

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Aim: To explore the complete gene networks regulated by small RNA SprC and its targets in *Staphylococcus aureus*. **Materials & methods:** The isobaric tags for relative and absolute quantitation and bioinformatic methods were utilized to identify and analyze the target proteins affected by SprC in *S. aureus* N315. **Results:** Proteomic analysis showed that the expression of 44 proteins was modulated by SprC. Further, bioinformatic analysis displayed that these affected proteins mainly associated with metabolic and cellular process, biological regulation and catalytic activity. **Conclusion:** Our data provide a rich resource of SprC targets in *S. aureus*, although the mechanism of regulation by SprC is yet to be elucidated.

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Staphylococcus aureus is a ubiquitous pathogen that is the causative agent of community- and hospital-acquired infections [1]. The pathogenicity of *S. aureus* is, to a large extent, determined by its rapid adaption to diverse host microenvironments and its toxin repertoire, which involves the coordinated action of complex regulatory circuits influenced by the two-component systems, global regulatory proteins and small regulatory RNAs (sRNAs) [2,3].

sRNAs are typically between 50 and 300 nucleotides in length, and most of them regulate the translation and decay of genes by binding to the target mRNAs [3,4]. In *S. aureus*, about 730 sRNAs have been verified, most of which currently have unknown functional, structural and mechanistic details [3,5–8]. Small pathogenicity island RNAs of *S. aureus* are expressed from genomic pathogenicity islands that present virulence and antimicrobial resistance [9]. At present, at least eight small pathogenicity island RNAs (SprA–G and X) are found [9,10], and some of them have been investigated widely. SprA_{AS} occludes translation initiation signals through base pairing to repress the translation of the SprA1-encoded cytolytic peptide [11]. SprD was identified as critical to the expression reduction of the Sbi immune-evasion molecule and the virulence increase of *S. aureus* [12]. SprX (alias RsaOR) was reported to possess a major role in glycopeptides resistance and virulence gene expression [6,10]. SprC negatively modulates the expression of the staphylococcal autolysin, reduces *S. aureus* phagocytosis by human leucocyte and decreases the virulence of bacteria, however, it is adverse for *S. aureus* resistance to oxidative stress [13].

KEYWORDS

• iTRAQ • proteomics • SprC
• sRNA • *Staphylococcus aureus*

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In view of the different roles of SprC, in this study we applied isobaric tags for relative and absolute quantitation (iTRAQ) to explore the global view of the changes in protein expression and intercommunication in *sprC* deletion mutant of *S. aureus* N315 and find more targets of SprC.

Materials & methods

• Strains & plasmids

The bacterial strains and plasmids are listed in Table 1. *S. aureus* strains were cultured at 37°C in Tryptic Soy Broth (TSB; Oxoid, Basingstoke, UK) or on Tryptic Soy Agar (Oxoid); when necessary, the media were supplemented with either chloramphenicol (10 µg/ml) or anhydrotetracycline hydrochloride (50 ng/ml). *Escherichia coli* strains were grown at 37°C in Luria–Bertani liquid medium or on Luria–Bertani agar containing a suitable antibiotic: ampicillin or kanamycin (50 µg/ml), as appropriate.

Genomic DNA was extracted by TIANamp Bacterial DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China). Plasmids from *E. coli* were extracted using a plasmid purification kit (Promega, WI, USA); for plasmid extraction of *S. aureus*, the cells were predigested with a digestion buffer with 40 U/ml lysostaphin, 10 mg/ml lysozyme and 10% (v/v) glycerol.

• Construction of the isogenic *sprC* deletion mutant strains

To inactivate the *sprC* gene, DNA fragments of 940-bp upstream and 930-bp downstream of *sprC* were amplified from *S. aureus* strain N315 chromosomal DNA, and then cloned into the BamHI-EcoRI and EcoRI-HindIII sites of plasmid pET28a, respectively. The resulting plasmid pET28aΔ*sprC* was aimed to provide DNA templates containing the upstream and downstream of *sprC* gene. Using primers *sprC*-att1 and *sprC*-att2, another PCR reaction was performed with the plasmid pET28aΔ*sprC* as a template. The PCR products with attB sites at both ends were used for recombination with temperature-sensitive plasmid pKOR1, generating plasmid pKOR1Δ*sprC*. The pKOR1Δ*sprC* was transformed first into *S. aureus* strain RN4220, and then into *S. aureus* N315 through electroporation. The procedure for homologous recombination was performed as previously described [16]. The deletion of *sprC* was confirmed by PCR and sequencing. For restoring the SprC activity, primers *sprC*-up-EcoRI and *sprC*-down-BamHI were designed to amplify a 236-bp fragment which contained the *sprC* gene and its own putative promoter region. Then the PCR products were digested and

Table 1. Bacteria and plasmids used.		
Strain or plasmid	Genotypes or characteristics	Ref.
<i>Staphylococcus aureus</i>		
RN4220	Restriction-negative strain that accepts foreign DNA, 8325-4 derivative	[14]
N315	MRSA strain isolated from the pharyngeal smear of a Japanese patient	[15]
N315Δ <i>sprC</i>	Isogenic <i>sprC</i> deletion mutant of N315	This study
<i>Escherichia coli</i>		
DH5α	A host strain for cloning, <i>supE44 ΔlacU169</i> (80d <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Tiangen company
Plasmids		
pKOR1	Temperature-sensitive vector for allelic replacement via λ recombination and <i>ccdB</i> selection	[16]
pKOR1Δ <i>sprC</i>	pKOR1 derivative for allelic replacement of <i>sprC</i>	This study
pET28a	Bacterial expression vector with T7lac promoter	Novagen company
pET28aΔ <i>sprC</i>	pET28a vector containing the 940-bp upstream genomic sequence of <i>sprC</i> and its 930-bp downstream sequence, excluding the <i>sprC</i> gene sequence	This study
pOS1	<i>Escherichia coli</i> – <i>staphylococcal</i> shuttle cloning plasmid	[17]
pOS1 <i>sprC</i>	pOS1 with <i>sprC</i> under the control of the putative natural <i>sprC</i> promoter	This study

cloned into plasmid pOS1. Primers used for cloning were listed in **Table 2**.

• Quantitative real-time PCR

Overnight cultures were diluted 1:100 into 50 ml fresh TSB and incubated at 37°C with agitation and stopped at various phases of growth. Cells were then collected by centrifugation at 10,000 × *g* for 10 min, resuspended in 150-μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 10-μg lysostaphin and 200-μg proteinase K and incubated for 30 min at 37°C. The subsequent procedure for RNA extraction was carried out by a MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The purified RNA was quantified using a NanoDrop spectrometer (Thermo Fisher Scientific, MA, USA). The primer pairs used in quantitative real-time PCR were listed in **Table 2**. Total RNA was reverse transcribed into cDNA using the PrimeScript™RT reagent Kit with gDNA Eraser (TaKaRa), according to the manufacturer's protocol. The resulting cDNAs were stored at -20°C until they were required. The quantitative real-time PCR was carried out in 20-μl reaction mixture containing 10-μl 2× SYBR Premix Ex Taq (Tli RNaseH Plus, TaKaRa), 0.4-μl PCR forward primer (10 μM), 0.4-μl PCR reverse primer (10 μM), 0.4-μl ROX Reference Dye II (50×), 2-μl cDNA and 6.8-μl dH₂O (TaKaRa). The cycling parameters were incubated at 95°C for 30 s, and then 40 cycles consisting of 95°C for 5 s and 60°C for 34 s, followed by a dissociation step of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s on Applied Biosystems 7500

Real Time PCR System (Applied Biosystems, CA, USA). The housekeeping gene 16S rRNA was selected as the internal control for normalization. The primer efficiency of the amplification for each gene was assessed by melting curve analysis.

• Isobaric tags for relative & absolute quantitation

Sample preparation

S. aureus strains N315 and N315Δ*sprC* were maintained in TSB and grown to late logarithmic growth phase at 37°C with constant agitation at 150 r.p.m. under aerobic conditions. The cells were collected by centrifugation and washed twice with phosphate-buffered saline. SDT lysis buffer (4% SDS, 1 mM DL-Dithiothreitol, 100 mM Tris-HCl, pH 7.6) was added to the samples. The cells were then ultrasonically disrupted ten-times for 10 s intervals with 15 s rest periods, followed by boiling water bath for 15 min. The supernatant was filtered with 0.22 μm filter. And the BCA Protein Assay Kit (Bio-Rad, CA, USA) was used for the quantification of filtrate.

Enzyme digestion & iTRAQ labeling

A total of 200 μg of proteins for each sample were taken and combined with DL-Dithiothreitol to a final concentration of 100 mM. The mixtures were put in a boiling water bath for 5 min, and cooled to room temperature. Then 200-μl urea lysis buffer (8 M urea, 150 mM Tris-HCl, pH 8.0) was added for removing the detergent. The mixtures were transferred into an ultrafiltration device (Microcon units, 10 kD) followed by centrifugation at 14,000 × *g* for

Table 2. Oligonucleotides used in this study.

Primer name	Sequence
For isogenic <i>sprC</i> deletion mutant	
<i>sprC</i> -up-BamHI	CGCggatccATTACCTCCAATGTTATATCC
<i>sprC</i> -up-EcoRI	CCGgaattcATTTTAAAGTGAAATTTAGTTC
<i>sprC</i> -down-EcoRI	CCGgaattcCGTTGACTTATTATATTATAAT
<i>sprC</i> -down-HindIII	CCCaaagcttTCTAAACCAAATATAGTATAGC
<i>sprC</i> -att1	ggggacaagtttgtacaaaaagcaggctATTACCTCCAATGTTATATCC
<i>sprC</i> -att2	ggggaccacttgtacaagaaagctgggtTCTAAACCAAATATAGTATAGC
For <i>sprC</i> complementation	
<i>sprCr</i> -up-EcoRI	CCGgaattcAAGTATTGAAAAATAAAATATTT
<i>sprCr</i> -down-BamHI	CGCggattcAACATATATATTTACTATGAAC
For qRT-PCR	
<i>sprC</i> -F	CCATGCGTGGACAGTAAAACG
<i>sprC</i> -R	ACTGAAGCTTCTACTCTCATGGC
qRT-PCR: Quantitative real-time PCR.	

15 min. The precipitates were resuspended in 200- μ l urea lysis buffer and the ultrafiltration step was repeated. Then 100- μ l iodoacetamide (100 mM iodoacetamide in urea lysis buffer) was added to the samples and the mixtures were incubated for 30 min in darkness. After centrifugation, the precipitates were washed two-times with 100 μ l of urea lysis buffer, and then 100 μ l of dissolution buffer (DS buffer) twice. Finally, the precipitates were reconstituted in 40- μ l trypsin buffer (4- μ g trypsin in 40- μ l dissolution buffer), agitated at 600 r.p.m. for 1 min and then incubated overnight at 37°C. The filtrates were collected in fresh collection tubes after centrifugation. The peptides of each sample were desalted on C18 Cartridges (Sigma-Aldrich, MO, USA), concentrated by vacuum centrifugation and reconstituted in 40 μ l of 0.1% (v/v) formic acid. Peptide content was estimated by measuring the optical density at 280 nm. After quantification, 100 μ g of peptide mixture of each sample was labeled using an iTRAQ 8-plex Multiplex kit (Applied Biosystems) according to the manufacturer's instructions. All the experiments for each sample were performed with three biological replicates. iTRAQ reagents 113, 114 and 115 were used for wild-type N315 strain and reagents 116, 117 and 118 were applied for N315 Δ *sprC* mutant strain.

Peptide fractionation with strong cation exchange chromatography

iTRAQ-labeled peptides were fractionated by strong cation exchange chromatography using the AKTA Purifier 100 (GE Healthcare, Little Chalfont, UK). The dried-labeled samples were resuspended and acidified with buffer A (10 mM KH_2PO_4 and 25% acetonitrile, pH 3.0) and then loaded onto a 4.6 $\times$ 100 mm PolySulfoethyl column (5 μ m, 200 Å; PolyLC, MD, USA). During the strong cation exchange separation, the peptides were eluted at a flow rate of 1 ml/min with a gradient of 0–8% buffer B (500 mM KCl, 10 mM KH_2PO_4 and 25% acetonitrile, pH 3.0) for 22 min, 8–52% during 22–47 min, 52–100% during 47–50 min, 100% during 50–58 min and 0% after 58 min. The fractions were collected every 1 min and the elution was monitored by absorbance at 214 nm. The collected fractions were lyophilized prior to desalination in a C18 Cartridge (Sigma-Aldrich) and concentrated by vacuum centrifugation for subsequent liquid chromatography–tandem mass spectrometry analysis.

Liquid chromatography–tandem mass spectrometry

An Easy nLC system (Thermo Fisher Scientific) was used for liquid separation of each fraction. The peptide mixtures were loaded onto a reverse phase trap column (2 cm $\times$ 100 μ m, 5 μ m) connected to the C18-reversed phase analytical column (75 μ m $\times$ 10 cm, 3 μ m). Buffer A (0.1% formic acid) was used for equilibration of chromatographic columns and buffer B (84% acetonitrile and 0.1% formic acid), with a linear gradient at a flow rate of 300 nl/min, was utilized for mixture separation. The peptides were subjected to nano-electrospray ionization followed by tandem mass spectrometry (MS/MS) in a Q-Exactive mass spectrometer (Thermo Fisher Scientific) coupled to Easy nLC. The mass spectrometer was operated in positive-ion detection mode, and the most abundant precursor ions from the scanning range of 300–1800 m/z were chosen to obtain MS data. Automatic gain control target was set to 3e6 and a first-order maximum injection time was 10 ms. The dynamic exclusion duration was 40 s. Survey scans were obtained at a resolution of 70,000 at m/z 200 and resolution for higher-energy collisional dissociation (HCD) spectra was set to 17,500 at m/z 200. Other parameters were as follows: the isolation window was 2 m/z, the second-order maximum injection time was 60 ms, normalized collision energy was 30 eV and the underfill ratio was defined as 0.1%.

Data analysis

Database searches and quantitative analyses were carried out using Mascot engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4 (Thermo Fisher Scientific). The proteins sequence of *S. aureus* 2926 in the UniProt knowledge base [18] was selected as a reference. For proteins identification, a peptide mass tolerance of ± 20 p.p.m. and a fragment mass tolerance of 0.1 Da were permitted with allowance for two missed cleavage in the trypsin digests. Fixed modifications included carbamidomethyl (C), iTRAQ 4/8plex (N-term) and iTRAQ 4/8plex (K), while oxidation (M) and iTRAQ 4/8plex (Y) were tested as potential variable modifications. Only proteins that appeared in more than two biological replicates were included in the analysis. The quantitative protein ratios were normalized by the median ratio in Mascot and the median protein ratio should be 1 after the normalization.

Proteins with a 1.2-fold change and a two-tailed p -value < 0.05 were considered significant. The BLAST2GO [19] was used to extract gene ontology (GO) terms and add function annotations to the target proteins. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server [20] was utilized to identify the metabolic pathways and functional groups and STRING (version 10.0) was applied to explicitly show the differentially expressed proteins in the context of protein–protein interaction networks. The subcellular localization distribution of proteins was predicted with PSORTb v3.0.2.

Statistical analyses

Statistically significant differences were determined by Student's t -test using SAS 9.3 for Windows software (SAS Institute Inc., NC, USA) and all tests were considered to be statistically significant when $p < 0.05$.

Results

• SprC expression during growth

The growth curves of wild-type N315 and N315 Δ sprC mutant strains were nearly the same in TSB broth (Figure 1A). These results indicated the knockout of *sprC* did not affect *S. aureus* proliferation. The data of the SprC expression during the growth of N315 displayed that SprC was already expressed during the early exponential phase and decreased slightly at the middle exponential phase. Subsequently, the gradual increasing transcription

of SprC was monitored at late exponential and stationary growth phases (Figure 1B).

• Differentially expressed proteins between N315 & N315 Δ sprC mutant strains

Proteins extracted from N315 and N315 Δ sprC mutant strains during the late exponential phase were analyzed and three repetitions were set for each strain. A total of 1051 proteins were identified from the original mass spectrometry data. Among these proteins, 44, meeting the criteria as depicted above under the 'Data analysis', were revealed to be differentially expressed (Table 3). Further analysis showed that eight proteins were higher in N315 Δ sprC mutant strains compared with the parent strain N315 with the fold change ranging from 1.21 to 2.93 (Table 3). Of note, two proteins presented at the highest concentration in *sprC* mutant were LukeE (A0A0E1VG99) and LukD (A0A0E1VQ50), which had fold change values of 2.75 and 2.93, respectively. In contrast, 36 proteins were measured at decreased levels (Table 3), with fold values ranging from -1.2 to -3.16, of which three (DNA-binding helix-turn-helix protein, a SNARE-like domain protein and an EDD domain [common to three different folds: mannose transporter EIIA domain, dihydroxyacetone kinase, and DegV-like protein fold] protein of DegV family) were observed to have the greatest degree of fold change (-3.16, -2.69 and -2.28, respectively; Table 3). The MS/MS spectra of the representative peptides of

Figure 1. SprC RNA expression profile in *Staphylococcus aureus* N315 strain. (A) The growth curves of N315 and N315 Δ sprC mutant. **(B)** The expression of SprC of *S. aureus* N315 at four time points representing early exponential phase, middle exponential phase, late exponential phase and stationary growth phase.

OD: Optical density.

Table 3. Proteins showing alteration in abundance in isobaric tags for relative and absolute quantitation analyses of *Staphylococcus aureus* SprC mutant compared with wild-type strain.

ORFID [†]	Locus tag [‡]	Accession number [§]	Protein/description	AAs [¶]	MW (kDa)	Calc. pI	Fold difference	t-test p-value	Localization	Pathway	Biological process	Molecular function
SA2092	SAOUHSC_02570	A0A0E1VYJ4 [#]	Irac, transcriptional regulator, AraC family	651	75.99	8.15	1.34	0.003	Unknown		Metabolic process, cellular process, biological regulation	Nucleic acid binding transcription factor activity, binding
SA1132	SAOUHSC_01267	A0A0E1VHV2	2-oxoglutarate ferredoxin oxidoreductase subunit beta	288	31.34	5.48	1.21	0.005	Cytoplasmic	Carbon metabolism, glycolysis, gluconeogenesis, TCA cycle, carbon fixation pathways in prokaryotes, pyruvate metabolism, butanoate metabolism	Metabolic process, cellular process	Catalytic activity, binding
SA1637	SAOUHSC_01954	A0A0E1VQ50	Leukotoxin LukD, β-channel forming cytolsin	327	36.92	9.22	2.93	0.003	Extracellular	<i>Staphylococcus aureus</i> infection	Cellular process, biological regulation, multi-organism process, cell killing	
SA1638	SAOUHSC_01955	A0A0E1VG99	Leukotoxin LukE, β-channel forming cytolsin	311	34.85	9.50	2.75	0.005	Extracellular	<i>Staphylococcus aureus</i> infection	Cellular process, biological regulation, multi-organism process, cell killing	
SA1361	SAOUHSC_01627	A0A0E1VIY6	Uncharacterized protein	193	21.46	9.20	1.24	0.006	Unknown			
SA2105	SAOUHSC_02585	A0A0E1VNW7 ^{††}	HTH domain protein (fragment)	229	26.51	7.15	1.50	0.006	Cytoplasmic membrane		Metabolic process, cellular process, biological regulation	Nucleic acid binding transcription factor activity

[†]ORFID of *Staphylococcus aureus* subsp. *aureus* N315 DNA, complete genome. GenBank no. BA000018.3.

[‡]Locus tag of *Staphylococcus aureus* subsp. *aureus* NCTC8325 chromosome. GenBank no. NC_008895.1.

[§]The protein accession number in UniProt database [18].

[¶]The number of amino acids.

[#]Proteins present at the prime cluster of STRING map (Figure 4).

AA: Amino acid; EDD: Common to three different folds (mannose transporter EIIA domain, dihydroxyacetone kinase, and DegV-like protein fold); GNAT: Gcn5-related N-acetyltransferase; HTH: Helix-turn-helix; MW: Molecular weight; ORFID: Open reading frame identity; pI: Isoelectric point; TCA: Tricarboxylic acid cycle.

Table 3. Proteins showing alteration in abundance in isobaric tags for relative and absolute quantitation analyses of <i>Staphylococcus aureus</i> SprC mutant compared with wild-type strain (cont.).												
ORFID [†]	Locus tag [‡]	Accession number [§]	Protein/description	AA [§]	MW (kDa)	Calc. pI	Fold difference	t-test p-value	Localization	Pathway	Biological process	Molecular function
SA1291	SAOUHSC_01475	A0A0E1VHE5 [#]	N-acetyl-alpha-D-glucosaminyl L-malate synthase BshA	384	43.24	5.91	1.28	0.033	Cytoplasmic		Metabolic process, cellular process	Catalytic activity
SA0527	SAOUHSC_00552	A0A0E1VTS2 [#]	NagB, glucosamine-6-phosphate deaminase	252	28.45	5.74	1.27	0.045	Cytoplasmic	Amino sugar and nucleotide sugar metabolism	Metabolic process, cellular process	Catalytic activity
SA1019	SAOUHSC_01138	A0A0E1VSB7	Uncharacterized N-acetyltransferase HMPREF0776_2177	146	16.99	5.00	-1.23	0.001	Unknown		Metabolic process, cellular process	Catalytic activity
SA1252	SAOUHSC_01425	A0A0E1VHI0	Acetyltransferase, GNAT family	169	19.83	4.63	-1.35	0.002	Cytoplasmic		Metabolic process, cellular process	Catalytic activity
SA0997	SAOUHSC_01106	A0A0E1VKF5 [#]	MurI, glutamate racemase	266	29.68	7.55	-1.24	0.002	Unknown	D-glutamine and D-glutamate metabolism	Single-organism process, metabolic process, cellular process, developmental process, cellular component organization or biogenesis, biological regulation	Catalytic activity
SA0681	SAOUHSC_00736	A0A0E1VLK0 [#]	Lipid kinase, YegS/Rv2252/BmrU family	305	33.61	4.94	-1.61	0.002	Unknown		Signaling, single-organism process, metabolic process, cellular process, response to stimulus, biological regulation	Catalytic activity, binding
[†] ORFID of <i>Staphylococcus aureus</i> subsp. <i>aureus</i> N315 DNA, complete genome. GenBank no. BA000018.3.												
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SA1288	SAOUHSC_01472	A0A0E1VHA4 [#]	DinG, putative DnaQ family exonuclease/DinG family helicase	897	–	6.84	-1.72	0.003	Cytoplasmic		Single-organism process, metabolic process, cellular process, response to stimulus	Catalytic activity, binding
SA2126	SAOUHSC_02611	A0A0E1VXW4	LyrA, CAAX amino terminal protease family protein	419	46.76	5.03	-1.31	0.004	Cytoplasmic membrane		Metabolic process	Catalytic activity
SA1693	SAOUHSC_02090	A0A0E1VZ60 [#]	YfkB-like domain protein	394	45.63	7.23	-1.56	0.004	Cytoplasmic		Metabolic process	Catalytic activity, binding
SA0704	SAOUHSC_00764	A0A0E1VJ18	EDD domain protein, DegV family	288	31.98	5.06	-2.28	0.004	Cytoplasmic			
SA1440	SAOUHSC_01716	A0A0E1VR30	Peptidase, U32 family	422	47.62	4.86	-1.71	0.004	Cytoplasmic	Epithelial cell signaling in <i>Helicobacter pylori</i> infection	Metabolic process	Catalytic activity
SA0949	SAOUHSC_01045	A0A0E1VKH8	DNA-binding helix-turn-helix protein	179	20.73	4.77	-3.16	0.005	Cytoplasmic			Binding
SA2318	SAOUHSC_02839	A0A0E1VPU1	SdaAA, L-serine dehydratase, iron-sulfur-dependent, alpha subunit	299	30.95	4.81	-2.02	0.007	Cytoplasmic	Carbon metabolism, biosynthesis of amino acids, glycine, serine and threonine metabolism, cysteine and methionine metabolism	Metabolic process, cellular process	Catalytic activity
SA2111	SAOUHSC_02594	A0A0E1VN77 [#]	HAD hydrolase, family 1A, variant 3	211	23.55	5.00	-1.39	0.007	Cytoplasmic		Metabolic process, cellular process	Catalytic activity
SA0816	SAOUHSC_00892	A0A0E1VLD1 [#]	S1 RNA binding domain protein	114	13.42	8.31	-1.47	0.008	Cytoplasmic			Binding

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[‡]Locus tag of *Staphylococcus aureus* subsp. *aureus* NCTC8325 chromosome. GenBank no. NC_008895.1.

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SA1939	SAOUHSC_02379	A0A0E1VKF3 [#]	Putative deoxyribose-phosphate aldolase (fragment)	135	14.74	4.94	-1.28	0.013	Cytoplasmic	Pentose phosphate pathway	Metabolic process, cellular process	Catalytic activity
SA1027	SAOUHSC_01148	A0A0E1VKB5 [†]	Cell division protein DivIB	439	50.18	7.53	-1.43	0.018	Cell wall	Cell cycle – <i>Caulobacter</i>	Single-organism process, metabolic process, cellular reproduction, cellular component organization or biogenesis	Structural molecule activity, binding
SA1103	SAOUHSC_01237	A0A0E1VHS8 [‡]	Upps, undecaprenyl pyrophosphate synthetase	267	31.06	8.41	-1.22	0.019	Cytoplasmic	Terpenoid backbone biosynthesis	Metabolic process, cellular process	Catalytic activity, binding
SA1494	SAOUHSC_01774	A0A0E1VGT1 [‡]	HemC, porphobilinogen deaminase	308	34.33	5.40	-1.29	0.019	Cytoplasmic	Porphyrin and chlorophyll metabolism	Metabolic process, cellular process	Catalytic activity
SA2147	SAOUHSC_02636	A0A0E1VNB1	Transcriptional regulator, MarR family	151	17.51	9.11	-1.43	0.019	Cytoplasmic		Metabolic process, cellular process, biological regulation	Nucleic acid binding transcription factor activity, binding
SA0781	SAOUHSC_00855	A0A0E1VL90 [†]	Oxidoreductase, 2-nitropropane dioxygenase family protein	355	38.54	6.42	-1.69	0.023	Cytoplasmic	Nitrogen metabolism	Metabolic process, cellular process	Catalytic activity
SA2026	SAOUHSC_02489	A0A0E1VYD4 [#]	InfA, translation initiation factor IF-1	72	8.27	7.42	-1.50	0.024	Cytoplasmic		Metabolic process, cellular process, biological regulation	Binding

[†]ORFID of *Staphylococcus aureus* subsp. *aureus* N315 DNA, complete genome. GenBank no. BA000018.3.

[‡]Locus tag of *Staphylococcus aureus* subsp. *aureus* NCTC8325 chromosome. GenBank no. NC_008895.1.

[§]The protein accession number in UniProt database [18].

[¶]The number of amino acids.

[#]Proteins present at the prime cluster of STRING map (Figure 4).

AA: Amino acid; EDD: Common to three different folds (mannose transporter EIA domain, dihydroxyacetone kinase, and DegV-like protein fold); GNAT: Gcn5-related N-acetyltransferase; HTH: Helix-turn-helix; MW: Molecular weight; ORFID: Open reading frame identity; pI: Isoelectric point; TCA: Tricarboxylic acid cycle.

Table 3. Proteins showing alteration in abundance in isobaric tags for relative and absolute quantitation analyses of *Staphylococcus aureus* SprC mutant compared with wild-type strain (cont.).

ORFID [†]	Locus tag [‡]	Accession number [§]	Protein/description	AAs [¶]	MW (kDa)	Calc. pI	Fold difference	t-test p-value	Localization	Pathway	Biological process	Molecular function
SA1345	SAOUHSC_01610	A0A0E1VHB8 [†]	Uncharacterized protein	149	16.70	4.78	-1.27	0.024	Cytoplasmic		Metabolic process	Catalytic activity
SA2472	SAOUHSC_03015	A0A0E1VM88	HisZ, ATP phosphoribosyl-transferase regulatory subunit	272	31.63	5.86	-1.34	0.025	Unknown		Metabolic process, cellular process	Catalytic activity
SA0243	SAOUHSC_00222	A0A0E1VP02 [‡]	CDP-glycerol:poly (glycerophosphate) glycerophosphotransferase	564	66.06	6.33	-1.23	0.027	Cytoplasmic membrane			Catalytic activity
SA0672	SAOUHSC_00726	A0A0E1VU81 [†]	Uncharacterized protein	236	27.11	6.73	-1.35	0.029	Unknown		Single-organism process, metabolic process, cellular process, response to stimulus	Catalytic activity
SA0610	SAOUHSC_00661	A0A0E1VJT9 [‡]	Hydrolase, α/β domain protein	347	39.81	8.73	-1.25	0.029	Unknown		Metabolic process, cellular process	Catalytic activity, binding
SA2301	SAOUHSC_02816	A0A0E1VPV9	SNARE-like domain protein	203	23.42	###	-2.69	0.029	Cytoplasmic membrane		Single-organism process, metabolic process, cellular process, response to stimulus	Catalytic activity
SA1400	SAOUHSC_01673	A0A0E1VJ23 [‡]	KH domain protein	315	34.89	6.98	-1.20	0.031	Cytoplasmic			Binding
SA1355	SAOUHSC_01621	A0A0E1VGW9 [‡]	NusB, N utilization substance protein B homolog	129	15.05	5.20	-1.21	0.031	Unknown		Metabolic process, cellular process, biological regulation	Binding
SA1727	SAOUHSC_02131	A0A0E1VZ09	UPF0316 protein HMPREF0776_0072	200	22.94	8.09	-1.29	0.034	Cytoplasmic membrane			

[†]ORFID of *Staphylococcus aureus* subsp. *aureus* N315 DNA, complete genome. GenBank no. BA000018.3.

[‡]Locus tag of *Staphylococcus aureus* subsp. *aureus* NCTC8325 chromosome. GenBank no. NC_008895.1.

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^{**}Proteins present at the prime cluster of STRING map (Figure 4).

AA: Amino acid; EDD: Common to three different folds (mannose transporter EIA domain, dihydroxyacetone kinase, and DegV-like protein fold); GNAT: Gcn5-related N-acetyltransferase; HTH: Helix-turn-helix; MW: Molecular weight; ORFID: Open reading frame identity; pI: isoelectric point; TCA: Tricarboxylic acid cycle.

Table 3. Proteins showing alteration in abundance in isobaric tags for relative and absolute quantitation analyses of *Staphylococcus aureus* SprC mutant compared with wild-type strain (cont.).

ORFID [†]	Locus tag [‡]	Accession number [§]	Protein/description	AAs [¶]	MW (kDa)	Calc. pI	Fold difference	t-test p-value	Localization	Pathway	Biological process	Molecular function
SA0777	SAOUHSC_00850	A0A0E1VIX9	SUF system FeS assembly protein, NifU family	154	17.01	4.82	-1.26	0.036	Cytoplasmic		Metabolic process, cellular component organization or biogenesis	Binding
SA1687	SAOUHSC_02008	A0A0E1VP96	Uncharacterized protein	278	31.91	7.85	-1.22	0.036	Cytoplasmic membrane	ABC transporters	Localization	Transporter activity, catalytic activity, binding
SA1083	SAOUHSC_01210	A0A0E1VK98 [#]	TrmD, tRNA (guanine-N(1)-)-methyltransferase	245	28.10	5.63	-1.22	0.036	Cytoplasmic		Metabolic process, cellular process	Catalytic activity
SA0601	SAOUHSC_00651	A0A0E1VLV5	Uncharacterized protein	277	29.90	8.63	-1.27	0.040	Cytoplasmic membrane			
SA1119	SAOUHSC_01253	A0A0E1VJZ9 [#]	FtsK/SpoIIIE family protein	792	88.54	8.75	-1.24	0.045	Cytoplasmic membrane		Single-organism process, cellular process	Catalytic activity, binding
SA1379	SAOUHSC_01649	A0A0E1VQW7 [#]	Peptidase, S54 family	487	56.82	9.11	-1.23	0.048	Cytoplasmic membrane		Metabolic process	Catalytic activity, binding
SA0529	SAOUHSC_00554	A0A0E1VLM7 [#]	HxIB, 6-phospho 3-hexuloisomerase	182	19.55	5.15	-1.44	0.049	Unknown	Carbon metabolism, biosynthesis of amino acids, pentose phosphate pathway, methane metabolism		

[†]ORFID of *Staphylococcus aureus* subsp. *aureus* N315 DNA, complete genome. GenBank no. BA000018.3.

[‡]Locus tag of *Staphylococcus aureus* subsp. *aureus* NCTC8325 chromosome. GenBank no. NC_008895.1.

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A0A0E1VYJ4 (transcriptional regulator, AraC family) and A0A0E1VKB5 (cell division protein DivIB) are shown in **Figure 2**.

• Global functional classification of differentially expressed proteins

The functions of 44 affected proteins were categorized according to the GO annotation based on the biological functional hierarchy. GO analysis revealed that affected proteins were associated with 12 biological processes, which mainly included metabolic process (GO: 0008152, 33.7%), cellular process (GO: 0009987, 30.6%) and biological regulation (GO: 0065007, 10.2%; **Figure 3A**). The GO classification of identified proteins according to cellular components indicated that 46.9% of differentially expressed proteins are involved in cell (GO: 0005623), 21.9% in both membrane (GO: 0016020) and macromolecular complex (GO: 0032991) and 9.4% in extracellular region (GO: 0005576; **Figure 3B**). Functional classification according to molecular functions indicated

that 56.6% are involved in catalytic activity (GO: 0003824), 34% in binding (GO: 0005488), 5.7% in nucleic acid binding transcription factor activity (GO: 0001071) and 1.9% in both structural molecule activity (GO: 0005198) and transporter activity (GO: 0005215; **Figure 3C**). All of the above signified the multifunctional role of SprC.

• Subcellular distribution of the differentially expressed proteins

As shown in **Figure 4** and **Table 3**, 1 (2.3%), 22 (50.0%), 9 (20.5%), 2 (4.5%) and 10 (22.7%) proteins were classified as cell wall, cytoplasmic, cytoplasmic membrane, extracellular and unknown proteins, respectively. The affected proteins are especially enriched with cytoplasmic and cytoplasmic membrane proteins, suggesting the SprC's significant role in the formation of cytoplasm.

• KEGG pathway enrichment analysis

By mapping the differentially expressed genes to KEGG pathways, a total of 21 signal/metabolic

Figure 2. Representative tandem mass spectrometry spectra of isobaric tags for relative and absolute quantitation labeling peptides. (A) Tandem mass spectrometry spectra of the peptide A0A0E1VYJ4 (transcriptional regulator, AraC family); **(B)** Tandem mass spectrometry spectra of the peptide A0A0E1VKB5 (cell division protein DivIB).

FTMS: Fourier transform mass spectrometer; HCD: Higher-energy collisional dissociation.

Figure 3. Classification of the differentially expressed proteins according to biological functions (see previous page). The biological processes, cellular components and molecular functions of the identified significant proteins influenced by SprC were classified by the Gene Ontology database. GO: Gene ontology.

pathways related to 21 differentially expressed proteins were extracted. A large number of proteins were concerned with carbon metabolism (ko01200), biosynthesis of amino acids (ko01230), pentose phosphate pathway (ko00030) and *S. aureus* infection (ko05150). Each remaining pathway was related to only one affected protein (Figure 5 & Table 3).

• Protein–protein interaction network analysis using STRING

Interaction knowledge from STRING suggested that SprC may have the potential to interact with distinct genes. The STRING map of the proteins affected by SprC showed one prime cluster including 26 proteins, while the remaining proteins presented sporadic relations. The proteins clustered are mainly involved in metabolic process (80.8%, 21/26), cellular process (73.1%, 19/26) and catalytic activity (69.2%, 18/26). Moreover, cytoplasm was the leading subcellular location of proteins belonging to this cluster (53.8%, 14/26; Figure 6 & Table 3).

Figure 4. Localization distribution of differentially expressed proteins predicted by PSORTb v3.0.2.

Discussion

It is generally known that staphylococcal pathogenicity is determined by a variety of virulence factors rather than a single toxin. The virulence factors, including surface and extracellular molecules, enable bacteria to adhere to host cells, damage tissue, evade the immune system and consequently, cause acute infections. In recent years, various proteomic techniques have become powerful scientific tools to explore the interactions among virulence factors responsible for the complex pathogenicity of bacteria. iTRAQ is an excellent experimental technique for quantitative mass spectrometry in proteome research and provides a strategy to allow the identification and comparison of protein expression in different strains [21]. In the present study, 44 differentially expressed proteins, primarily impacting bacterial functions, were identified by the iTRAQ method and bioinformatic analysis uncovered that the proteins influenced by SprC were principally involved with metabolic and cellular process, biological regulation and catalytic activity.

So far, *S. aureus* sRNAs have been shown to be able to regulate bacterial pathogenesis, antibiotic resistance and host–pathogen interactions in a dynamic manner [3,22]. *sprC*, situated next to Leucocidin ED (LukED), shows large variations in expression among different *S. aureus* strains. For example, its expression is abundant in strain N315, whereas it is almost switched off in strains MRSA252 and 502A [9], suggesting that SprC may be involved in the regulation of staphylococcal virulence. Therefore, it is necessary to identify comprehensive gene networks modulated by SprC. Bacterial growth is most active in logarithmic phase that is involved with DNA synthesis, coupling with transcription and translation and synthesizing indispensable biological macromolecules [23]. In the present study, we chose the late exponential growth phase bacteria (cultured for 6 h) for analysis of proteomics, because SprC reached the maximum amount at this period compared with early and middle exponential growth phases (Figure 1). As expected, the proteomics data provided important clues and the basis for further analysis to uncover new targets of SprC.

Figure 5. Pathways associated with the differentially expressed proteins determined by Kyoto Encyclopedia of Genes and Genomes analysis. The differentially expressed proteins regulated by SprC have relatively main influence on the pathways related to carbon metabolism, biosynthesis of amino acids, glucose metabolism (pentose phosphate pathway) and *Staphylococcus aureus* infection. ABC: ATP-binding cassette; TCA: Tricarboxylic acid cycle.

A complex family of virulence determinants of *S. aureus* is the bi-component pore-forming leukocidins which target leukocytes [24]. LukED, one toxin of this family, is mainly responsible for killing leukocyte cells by inducing Ca^{2+} -activated transmembrane pores, thus plays a significant role in the ability of *S. aureus* to avoid host defenses [25–27]. Studies showed that LukED is associated with bloodstream infections, bullous impetigo and postantibiotic diarrhea [28]. Therefore, as shown in this

study, reduced expression of the LukED toxin in *S. aureus*, in the presence of *sprC*, is expected to confer a clinical benefit. However, the specific relationship between SprC and the expression of LukED needs to be investigated further.

Due to the clinical importance of *S. aureus*, identifying the virulence factors, along with their regulatory networks, associated with *S. aureus* pathogenesis has been facilitated by an enormous amount of effort [29]. The expression of virulence genes is usually coordinately regulated by

Figure 6. Functional cluster analysis of SprC affected proteins by STRING (version 10.0). The STRING map of the proteins affected by SprC showed one prime cluster which contained 26 proteins, while the remaining proteins exhibited sporadic relations. The interactions and functions were established using minimum required interaction score of 0.15.

two-component systems and transcription regulators, among which the accessory gene regulator (*agr*) system and staphylococcal accessory regulator (SarA) are representatives, respectively [30–32]. In this study, four differentially expressed proteins (A0A0E1VYJ4, A0A0E1VNW7, A0A0E1VKH8 and A0A0E1VNB1), possibly possessing the functions of DNA binding and transcriptional regulation according to the prediction and analysis of bioinformatics approach, were found. This result indicated that altered expression of the regulatory factors, under the control of SprC, is likely to be the cause of changes of other gene expression in our study.

In the process of infection, the organism faces metabolic and environmental stresses from the host such as reactive oxygen species and lipid peroxides [33,34]. If the bacterium has an ineffective response to the injury generated by oxidative stress, it is in danger of amassing harmful mutations, membrane breakdown and cell death [35]. Generation of low-molecular-weight (LMW) thiols is a wise strategy applied by bacteria to maintain its cellular redox balance and soften the effects of reactive oxygen species [34]. In eukaryotes and Gram-negative bacteria, LMW thiol glutathione (GSH) is prevalent [36,37], while in *S. aureus*, bacillithiol (BSH) is the major LMW thiol [38]. The retaining glycosyltransferase (BshA) catalyzes the chemical synthesis of N-acetylglucosaminylmalate (GlcNAc-mal), which is the first committed step in the BSH biosynthesis pathway [38,39]. Our proteomics data showed that SprC downregulated the expression of *bshA*, suggesting that this sRNA may be involved in the oxidative stress response of *S. aureus*. This has been verified in the *S. aureus* Newman strain [13].

By regulating expression of DivIB and SpoIIIE, SprC is involved in *S. aureus* cell division which is a fundamental process required for successful completion of the bacterial cell cycle. SpoIIIE, a homolog of the *Bacillus subtilis* (45.9% identity) along with FtsK, operates in independent pathways for correcting chromosome segregation during cell division [40,41]. DivIB is necessary for a molecular checkpoint to guarantee the correct assembly of the bacterial divisome which is a macromolecular machine formed by a variety of proteins [42]. Notably, the growth curve of N315Δ*sprC* mutant did not obviously change when compared with that of wild-type N315 strain, indicating that the effects of DivIB and SpoIIIE's differential expression in

this study were inadequate for influencing the proliferation of *S. aureus*.

NagB (glucosamine-6-phosphate deaminase) has a critical role in the glycolysis pathway and cell-wall biosynthesis [43]. In *Streptococcus mutans*, the alteration of NagB expression can affect the virulence of the bacteria [44], but the corresponding role in *S. aureus* remains unclear. Another protein affected by SprC is undecaprenyl diphosphate synthase, which is crucial for peptidoglycan biosynthesis and consequently influences bacterial cell growth. Because of this characteristic, undecaprenyl diphosphate synthase has become a target for developing new antibiotics [45]. Therefore, SprC may play a significant role in cell-wall biosynthesis by regulating the target proteins mentioned above.

Among the differentially expressed proteins, LyrA (A0A0E1VXW4) has been revealed to be associated with resisting lysostaphin that is secreted by *Staphylococcus simulans* with bacteriolytic activity against staphylococci [46]. This potentially extends the impacts of SprC in *S. aureus*.

Many proteins in bacteria including *S. aureus* are expressed as polycistron. In the present study, some of the genes for differentially expressed proteins were clustered as operon; however, other member proteins of the same operon were not identified. For instance, in the operon expressing proteins SA1119, SA1120, SA1121, SA1122 and SA1123, only SA1119 (FtsK/SpoIII E family protein) was detected in proteomics study. This phenomenon has also been found in previous studies [47,48]. The potential explanations may be diverse modification in transcriptional and translational level, some intracellular chemical reaction and/or the presence of protease affecting the amount of protein.

Conclusion

SprC could be related to the expression of a variety of proteins with a direct or indirect mechanism based on our research. However, these affected proteins were rarely enriched in a few concentrated pathways, biological processes and molecular functions, which indicated that SprC regulated the various aspects of gene expression through base-pairing interactions, and sRNAs exerted complicated and significant roles in the regulatory networks of *S. aureus*. Further studies will be necessary to explore the underlying regulatory mechanisms to confirm the key role of SprC in metabolism and pathogenesis of *S. aureus*.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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SUMMARY POINTS

- SprC shows variations of expression at different growth phases of *Staphylococcus aureus* N315, among which the amount of SprC reaches the highest level in the stationary phase bacteria.
- A total of 44 proteins are found to be significantly affected by SprC, including eight upregulated and 36 downregulated molecules.
- Bioinformatic analysis shows that the proteins affected by SprC are mainly related to metabolic and cellular process, biological regulation and catalytic activity of *S. aureus*.

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