Materials and Methods

Chapter-3

3. MATERIALS AND METHODS

3.1.Bacterial strains, plasmids and primers

3.1.1. Bacterial strains

The bacterial strains and plasmids used in this study are listed in Tables 5 and 6respectively. The restriction maps of the vectors are given in appendix I.

Table 5. List of bacterial strains used in this study.

Bacterial Strains	Description	Source
E. coli DH5α	(F^- Φ80d *lacZ*ΔM15 *Δ*(*lacZYA-argF*) U169 *endA*1 *recA*1 *hsdR*17 (rk^- mk^+)) *deoR thi*-1 *supE*44 λ^-*gyrA*96 *relA*1, host for DNA cloning	Lab source
E. coli DC10B	Derivative of *E. coli* KL16,*dam*$^+$ *dcm*$^-$ *hsdRMS endA1 recA1*	Timothy Foster, Trinity College, Dublin, Ireland
S. aureus strain Newman	Clinical isolate isolated from human infection, Wild type MSSA strain	Timothy Foster, Trinity College, Dublin, Ireland
S. aureus RN4220	Restriction deficient mutant strain derivative of RN450 (8325-4), *hsdR sauUSI*	Abraham L. Sonenshein, Tufts University, Boston
S. aureus pCN40	Restriction deficient mutant strain RN4220 containing *E. coli* - *S. aureus* shuttle vector with constitutive/inducible promoter *blaZ*, Em^r	Obtained from NARSA, USA (https://www.beiresources.org)
S. aureus Newman *sprX1::kan* disruption mutant	Isogenic mutant strain of Newman *sprX1::kan*, with one copy of SprX disrupted, Km^r	This work
S. aureus Newman *sprX1::kan*+pMNSprX	Complementation of pMNSprX in disrupted *sprX1* strain, Km^r, Em^r	This work

The name, description and source for each strain are given. The vector strain was obtained from the NARSA- Network on Antimicrobial Resistance in *Staphylococcus aureus*. Km - Kanamycin; Em - Erythromycin; r - resistant; MSSA - Methicillin Susceptible *Staphylococcus aureus*.

3.1.2. Plasmids

Table 6. List of plasmids used in this study.

Plasmid	Features	Size (in bp)	Source
pBluescriptKS+	Cloning vector for *E. coli*, Amp^r	2961	Lab source
pCN40	*E. coli* - *S. aureus* shuttle vector with constitutive/inducible promoter PblaZ in *S. aureus* RN4220 strain, Em^r, Amp^r	5829	Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) (https://www.beiresources.org)
pBSprX	$pBSKS^+$ encoding SprX1, Amp^r	3244	This work
pMNSprX	pCN40 encoding SprX1, Em^r	6145	This work
$pMNSprX_{AS}$	pCN40 encoding antisense SprX1, Em^r	6167	This work
pBSprB	$pBSKS^+$ encoding SprB, Amp^r	3101	This work
pMNSprB	pCN40 encoding SprB, Em^r	6008	This work
$pMNSprB_{AS}$	pCN40 encoding antisense SprB, Em^r	6023	This work
pBBR1MCS-2	Broad host range vector; originally derived from pBBR1 plasmid of *Bordetella bronchispetica*; Km^r	5144	Lab source
pIMAY	Temperature sensitive (Ts) shuttle vector derived from pIMC5, Cm^r	5743	Ian R. Monk, Trinity College Dublin, Ireland
pFsprX	Derivative of $pBSKS^+$ having flanking sequence of SprX1, Amp^r	4501	This work
pFsprXKan	Derivative of pFsprX1 having *sprX1::kan* gene disrupted cassette with kan (2.9 kb), Amp^r, Km^r	5925	This work
psprX1Kan	Derivative of Ts pIMAY having *sprX1::kan* gene disrupted cassette (2.9 kb), Cm^r, Km^r	8716	This work
pBSclfB	Interaction region of ClfB cloned in $pBluescriptKS^+$, Amp^r	3128	This work
pBShld	Interaction region of Hld cloned in $pBluescriptKS^+$, Amp^r	3139	This work
pBSPhrD	PhrD cloned in $pBluescriptKS^+$, Amp^r	3077	Lab source

Amp- Ampicillin; Cm- Chloramphenicol; Em- Erythromycin; Km- Kanamycin; r- resistant; Ts- Temperature sensitive.

3.1.3. Primers

Table 7. List of primers used in this study.

Gene name			Sequence	
SprX1	F	5′	ATAATCTTTCTAGACGTATTCAAA	3′
	R	5′	CAGGCTATATAGTTCACTCCTACT	3′
SprB	F	5′	AAATGTAAAAATAAGACGACATGC	3′
	R	5′	TTTTATTTCAATTAGCGGAAAA	3′
FSprX1	F	5′	CGGGATCCCGGGTTTGGCAGACATTTCATAAC	3′
	R	5′	GGGGTACCCCGCCATGCTCAAAAGAGGTTT	3′
Kan	F	5′	TGCATGCATGCAACATGTGAGAGGCGGTTTGCGTATTG	3′
	R	5′	TGCATGCATGCAACATGTCCCGAAAAGTGCCACCTG	3′
$RsprX_{RT}$	F	5′	ACACATGCATCAACTATTTACA	3′
	R	5′	ACTACGGGGGAGTAGTATGA	3′
$RsprB_{RT}$	F	5′	TAAGACGACATGCGCGAAC	3′
	R	5′	AAAACCATTCCGCATTAAC	3′
clfB	F	5′	GGTGGAAGTGCTGATGGTG	3′
	R	5′	ATCTGGCGTTGGTTCTGGT	3′
clfA	F	5′	CGCTCTATGTCATGGGACAA	3′
	R	5′	TTTCACCAGGCTCATCAGG	3′
sbi	F	5′	GGCAGCAACAATTACGTTAGC	3′
	R	5′	TGTTTTGAGTTGTTTGGTGCT	3′
coa	F	5′	AAAATTCCACAGGGCACAA	3′
	R	5′	AATCGGGACCTTGAACGA	3′
sak	F	5′	GCGATGACGCGAGTTATTTT	3′
	R	5′	CGACATAATGAGGGGATAGCA	3′
hld/RNAIII	F	5′	TTAATTAAGGAAGGAGTGATTTC	3′
	R	5′	CCAAGGAAACTAACTCTACTAGC	3′
5S RNA	F	5′	GCAAGGAGGTCACACCTGTT	3′
	R	5′	GCCTGGCAACGTTCTACTCT	3′
$NclfB_{GMSA}$	F	5′	AGAAAACGCAAGCAAGATCA	3′
	R	5′	CCCAAGCTTGGGCCATACGTCATGACCCCTTT	3′
$SprX1_{GMSA}$	F	5′	**AATTAACCCTCACTAAAGGG**ACACATGCATCAACTATTTACATCT	3′
	R	5′	AAAAGCACCCCGTAAACTATTATAC	3′

The restriction enzymes are underlined. GGATCC - BamHI; GGTACC - KpnI; AAGCTT - HindIII; ATGCAT - NsiI; ACATGT - PciI; F- Forward primer, R- Reverse primer, Bold and underlined region is the T3 promoter sequence, GMSA- gel mobility shift assay, RT- Real time.

3.2. Media, chemicals, enzymes, biochemicals and kits

All enzymes and biochemicals for molecular biology were obtained from Bangalore Genei Pvt. Ltd, India; Sigma Aldrich Chemicals Pvt. Ltd., USA; New England Biolabs (NEB), USA; Thermo Fisher scientific (Fermentas), USA; Roche Applied Science,

Germany; Applied Biosystems, USA and Axygen Biosciences, USA. Media and general chemicals were obtained from HiMedia Laboratories and Sisco Research Laboratories, India.

3.3. Media and Culture conditions

All *E. coli* and *S. aureus* strains were cultured in Luria Bertani (LB)medium (HiMedia Laboratories, India) either in liquid with 120 rpm shaking or on agar 1.5 % (w/v) plates at 37°C. Mannitol salt agar (MSA) is a selective and differential growth medium used for *Staphylococcus aureus.* All the recombinants were grown in a media containing appropriate selection marker. Bacterial growth was measured on spectrophotometer at OD_{600}. For methicillin induction, *S.aureus* cultures were induced with 0.5 µg/ml of methicillin at the stationary phase OD_{600} of 6.0 for 1 h and 3 h. All the cultures were maintained either as slants or stabs at 4°C and glycerol stocks of 20% at -20°C.

Table 8. Concentration of antibiotics, IPTG and X-gal used in this study.

Antibiotics and others	Concentration of stock solution (mg/ml)	Final concentration (µg/ml)	
		E. coli	***S. aureus***
Ampicillin	**100**	**100**	**10**
Methicillin	**1**	**-**	**0.5**
Erythromycin	**10**	**-**	**10**
Kanamycin	**40**	**40**	**15**
Chloramphenicol	**10**	**25**	**10**
X-gal	**20**	**40**	**-**
IPTG	**20**	**20**	**-**
FITC	**1**	**-**	**5**

3.4. Bioinformatic tools used in this study:

3.4.1. Promoter prediction- BPROM Softberry is a bacterial sigma70 promoter recognition program. BPROM has accuracy and specificity of promoter prediction of about 80%. Link to BPROM http://linux1.softberry.com/berry.phtml topic=bprom& group= help&subgroup= gfindb.

3.4.2. Rho independent transcription terminators- Erpin (Gautheret & Lambert, 2001) and ARNold (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php) program were used to predict the possible stem loop formation in a given sequence. These programs

work on the principle based on training set which contains structure of annotated terminator sequences from model organisms and finds score of unknown sequence.

3.4.3. Staphylococcal Regulatory RNA database (SRD)

Staphylococcal Regulatory RNA database, (SRD) is a storehouse for the staphylococcal sRNAs (http://srd.genouest.org/) dedicated to the staphylococcus genus were used to retrieve the sequence, copy number and location of staphylococcal regulatory RNAs (srns) in *S. aureus* strain Newman. The SR database also allows the user to predict the sRNA structure with Mfold and provides link to the blast server and target prediction program to predict the targets (Sassi *et al.,* 2015).

3.4.4. Target predictions- Online web server tools that were used for putative mRNA targets prediction implemented in this study are TargetRNA (http://snowwhite. wellesley.edu/targetRNA), TargetRNA2 (http://cs.wellesley.edu/~btjaden /TargetRNA2/advanced.html), RNApredator (http://rna.tbi.univie.ac.at/RNApredator2/ target_search.cgi)**,** IntaRNA (http:// rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp) and RNAup (http://www.tbi. univie. ac.at/~ulim/RNAup/).

3.4.5. Sequence Retrieval - Genomic sequence of *S. aureus* strain Newman was retrieved from NCBI database (nucleotide acc no. NC_009641) http://www.ncbi.nlm.nih.gov/ nuccore /NC_009641. The homology and similarity of the obtained sequence was compared with other staphylococcal strains and species by using BLASTN (nucleotide BLAST) program https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=Blast Search&LINKLOC=blasthome.

3.4.6. Primer design -Free online tool Primer3 Program http://primer3.ut.ee/, was used to design and analyze primers for PCR, oligos for hybridization probes and primers for real time PCR experiments. The quality of the designed primers with respect to the self-annealing and hairpin structures due to the presence of inter/intramolecular complementarities and GC percentage was approved using Oligo Calc: Oligonucleotide Properties Calculator http://www.basic.northwestern.edu/biotools/oligocalc.html. Oligonucleotide sequences used in the current study were routinely synthesized from Sigma Aldrich Chemicals Pvt. Ltd., MWG Biotech Pvt. Ltd.

3.4.7. *in silico* restriction analysis- NEBcutterV2.0 was used to generate a variety of outputs to the given sequence of interest including restriction enzyme maps, theoretical

digests to determine the size of digested fragments and links into the restriction enzyme database. It also highlights all sites that are affected by DNA methylation (*dam, dcm* etc.,)http://nc2.neb.com/NEBcutter2/.

3.4.8. Multiple sequence alignment- ClustalW2 program was used for alignment of multiple sequences of DNA http://www.ebi.ac.uk/Tools/msa/clustalw2/, to study the relationship of the studied sequences and pair wise alignmenthttp://www.ebi.ac.uk/Tools/psa/ that aligns the two given sequences, was used to identify regions of similarity that may help to understand the relationships between two biological sequences.

3.4.9. RNA secondary structure prediction- *Sfold*, Software for statistical folding of nucleic acids and studies of regulatory RNAs http://sfold.wadsworth.org/cgi-bin/srna.pl and *mfold***,** oldest web server for computational molecular biology were used for prediction of secondary structures of sRNAs http://mfold.rna.albany.edu/?q=mfold/rna-folding-form.

3.5. Molecular biology techniques

3.5.1. Isolation of genomic DNA from *Staphylococcus aureus*

Chromosomal DNA was isolated from *Staphylococcus aureus* strain Newman cultured on Luria Broth at 37°C for 24 h, using the modified protocol with lysostaphin (Sigma Aldrich Chemicals Pvt. Ltd., USA). Cells from overnight grown culture of *Staphylococcus aureus* strain Newman at 37°C under vigorous shaking were recovered by centrifugation and resuspended in 500 µl of TE buffer (pH 8.0). Cell suspensions were lysed with 2.5 µl of lysostaphin (20 µg/ml) by incubating at 37°C in a water bath for 90 min and treated with the buffer containing 1% SDS, 20 mM Tris (pH 8.0), 2 mM EDTA and 2.5 µl of proteinase K (20 µg/ml) followed by incubation at 50°C for 1 h. The mixture was extracted with Phenol: Chloroform: Isoamyl alcohol (PCI), precipitated with $1/10^{th}$ volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of ice cold absolute ethanol. The DNA thread was washed with 70% ethanol, dried and dissolved in autoclaved distilled water and stored at -20°C.

3.5.2. Isolation of plasmid DNA from *E. coli* and *S. aureus*

Plasmid DNA from *E. coli* was isolated from overnight grown cultures by standard alkaline lysis/ boiling lysis method. For *S. aureus*, cells were initially lysed with 20 µg/ml of lysostaphin followed by the standard protocol as described in Sambrook and Russell, 2001.

3.5.3. Polymerase Chain Reaction (PCR) conditions

The PCR reaction set up was based on the guidelines given in Sambrook and Russell, 2001. PCR amplifications were performed in Eppendorf master cycler personal or Eppendorf master cycler gradient thermal cycler. The reaction system and temperature profiles used are described in Table 9.

Table 9. PCR conditions used in the present study.

PCR reaction system							
PCR reaction mixture	**SprB**	**SprX1**	**Hld**	**NClfB**	**FSprX 1**	**Kanamycin**	**FSprX Kan**
DNA template	50 ng gDNA of Newman					10 ng of pBBRMCS2	10 ng of pFsprXKan
10x buffer (µl)	2	2	2	2	2	2	2
Forward primer (µM)	0.4	0.4	0.4	0.4	0.2	0.5	0.3
Reverse primer(µM)	0.4	0.4	0.4	0.4	0.2	0.5	0.3
dNTPs (µM)	200	200	200	200	200	200	200
$MgCl_2$ (mM)	1.5	1.5	1.5	1.5	1.5	1.5	1.5
X-Taq DNA polymerase (U/ µl)	1.0	1.0	1.0	1.0	1.5	1.5	1.5
Autoclaved double distilled water : To make up the volume to 20 µl							
PCR program used for amplification							
94°C (Initial denaturation)	4’ 30”	4’ 30”	4’ 30”	4’ 30”	4’ 30”	4’ 30”	4’ 30”
94°C (Denaturation)	45”	45”	45”	45”	45”	45”	45”
Annealing temperature	54˚C, 30”	59˚C, 30”	54˚C, 30”	58˚C, 30”	61˚C, 30”	58˚C, 30”	61˚C, 30”

72°C (Extension)	30”	30”	30”	30”	1’ 50”	1’ 40”	3”
Cycles: 30							
72°C (Final extension)	10’	10’	10’	10’	15’	15’	15’

All PCRs were standardized with Taq DNA polymerase and High fidelity X- Taq DNA polymerase enzyme (Bangalore Genei Pvt. Ltd, India; Sigma Aldrich Chemicals Pvt. Ltd., USA).

3.5.4. Restriction enzyme digestion

Restriction enzymes (RE) used in this study was obtained from different companies mentioned in 3.2. Each reaction was performed with optimised 1x reaction buffer with and without the addition of BSA to a final concentration of 100 µg/ml.

3.5.5. Ligation

The DNA to be cloned was purified from agarose gel by freeze thaw method as described in Sambrook and Russell, 2001. Occasionally, AxyPrep DNA gel extraction kit (Axygen Biosciences, USA) was used to elute DNA bands from 0.7% LMP agarose according to manufacturer’s instructions. The most commonly used enzyme is T4 DNA Ligase (Fermentas, USA) with the molar ratio of insert to vector DNA of 10:1 for blunt end and 3:1 for cohesive end at an optimized temperature of 22°C for 55 min.

3.5.6. DNA transformation in *E. coli* and *S. aureus*

Competent cells were prepared from mid log phase of 0.4 OD_{600} for *E. coli* and 0.3 OD_{600} for *S. aureus* and chemical transformation in *E. coli* was carried out as described in Sambrook and Russell, 2001.Plasmid DNA was introduced into *S. aureus* by electroporation. The electrocompetent cells were prepared as per the manufacturer’s instructions (BTX Harvard apparatus, USA) with modifications from Monk *et al.*, 2012. 100 ml of *S. aureus* culture of 0.3 OD_{600} was washed with equal volume of 0.5 M sucrose at 4°C followed by a second wash with half the volume of 0.5 M sucrose and finally resuspended in 1.0 ml of buffer containing 0.5 M sucrose with 10% glycerol and stored at -20°C. 500 ng - 3 µg of purified plasmid DNA was mixed with 100-200 µl of ice thawed electrocompetent *S. aureus* cells and electroporated with range of parameters as follows: voltage 2.0-2.3 kV; capacitance 25-50 µF; resistance 25-100 ohms. The cells were immediately suspended in 500 µl of LB containing 0.5 M sucrose after electroporation at 37°C for 2 h and plated onto the respective antibiotic containing

medium and incubated at 37°C for 24-48 h. Transformants were analyzed by isolating plasmid from *S. aureus* and the clones were confirmed by restriction digestion and PCRs.

3.5.7. DNA sequencing

The recombinant plasmid was confirmed by DNA sequencing (Xcelris Labs Ltd, Ahmedabad or from Bangalore Genei, India). The DNA sequences were determined by single pass analysis.

3.5.8. DNA/RNA Hybridization studies

3.5.8.1. DNA/RNA Probe labeling

About 1 µg of PCR generated DNA template was eluted from the gel by AxyPrep DNA gel extraction kit (Axygen Biosciences, USA) and suspended in 16 µl of sterile water. The purified DNA was then denatured in a boiling water bath for 10 min and then quickly chilled on ice. The denatured DNA was mixed with 4 µl of DIG-High Prime labelling mix, containing random hexamers, nucleotides, DIG- dUTP, klenow enzyme and buffer and incubated for 20 h at 37°C. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0). The DIG labelled DNA probe was purified using High pure PCR product purification kit (Roche diagnostics, Germany) and stored at -20°C. Labelled RNA probe was generated from the PCR generated DNA template by *in vitro* transcription using T3/T7 RNA polymerase (Roche applied science, Germany). The labelled probes were quantified using detection kit (Roche diagnostics, Germany) to determine the probe efficiency for hybridization and subsequent detection by enzyme immunoassay.

3.5.8.2. RNA isolation

The RNA was isolated based on acid guanidinium isothiocyanate phenol chloroform extraction method (Chomczynski & Sacchi, 2006). Cells were harvested at two different growth phases of *S. aureus*, mid log (OD_{600}- 3.0) and stationary (OD_{600}- 6.0) and lysed with lysostaphin (20 µg/ml) for 10-15 min. Cell lysate (1 x 10^7 cells) was resuspended in 1 ml of denaturing solution (solution D) containing 4 M guanidine thiocyanate, 25 mM sodium citrate, (pH 7.0), 0.5% (wt/vol) *N*- laurosylsarcosine (Sarkosyl) and 0.1 M 2-mercaptoethanol for 20-30 min. The mixture was treated with sequential addition of 0.1 ml of 2 M sodium acetate (pH 4.0), 1ml of water saturated phenol (pH 4.0) and 0.2 ml of

chloroform/isoamyl alcohol (49:1) and cooled on ice for 15 min followed by centrifugation at 10,000 rpm for 20 min at 4°C. The upper aqueous phase containing RNA was precipitated with 1 ml of isopropanol at -20°C. The RNA pellet was washed with 0.5 ml of 75% ethanol, air dried and dissolved in 25 µl of DEPC treated water containing formamide at 60°C for 10-15 min and stored at either -20°C or -80°C.

3.5.8.3. Transfer of RNA to membrane

15-20 µg of total RNA isolated from *S. aureus* was separated on denaturing 6% Urea polyacrylamide gel (PAGE) at 200 V, 15 mA for 4-5 h. The RNA on the PAGE gel was electro transferred onto positively charged nylon membranes at 5 V, 220 mA for 2 h in 1x TBE buffer in electrotransfer apparatus.

Table 10. Composition of RNA-PAGE (as described in Sambrook and Russell, 2001).

Reagents	Stacking gel (for 5ml)	Resolving gel (for 30ml)
Acrylamide/Bisacrylamide (45% w/v) (ml)	6.05	3.99
7 M Urea (g)	2.1	12.6
10x TBE (ml)	0.5	3.0
10% APS (µl)	35.0	210
TEMED (µl)	2.5	15
Nuclease free water (ml)	2.07	12.42

3.5.8.4. Transfer of DNA to membrane

Approximately 10 µg genomic DNA of *S. aureus* was digested completely with enzymes such as HindIII and EcoRV and fractionated on 0.7% agarose gel for 5-7 h at slow voltage and the gel was stained with EtBr (0.5 µg/ml) to verify the complete digestion before processing of gel as described in Sambrook and Russell, 2001. The gel was submerged in 0.1 M HCl for 10 min, with shaking at room temperature for mild depurination. The gel was then rinsed thoroughly with double distilled water and treated with denaturation solution for 15 min twice at room temperature under gentle shaking to denature the DNA into single stranded and increase the accessibility of the probe. After a wash with double distilled water the gel was treated with neutralization solution for 15 min twice at room temperature. Nylon membrane immersed in transfer buffer for 15 min was placed on the gel. Upward capillary transfer was carried out with a high salt 20x

SSC buffer for 18 h to obtain acceptable transfer of DNA from a 5 mm thick 0.7% agarose gel.

3.5.8.5. Hybridization and detection

Hybridization was carried out as described in the DIG labeling kit manual (Roche diagnostics, Germany) with slight modifications. The UV cross linked membrane was prehybridized with DIG Easy Hyb granules (10 ml/100 cm^2 membrane), followed by hybridization with denatured DIG-labelled DNA/RNA probe (25 ng/ml) at 37°C for 16h under gentle shaking. Nonspecific binding of probe was removed by washing the membrane with 2x SSC and 0.1% SDS for 10 min, followed by 0.1x SSC and 0.1% SDS at 65°C for 15 min twice under constant agitation. The membrane was blocked with 1x blocking solution for 30 min to prevent non specific binding of antibody and immunodetected with Anti-digoxigenin antibody-AP (75 mU/ml) for 30 min. The nonspecific binding of antibody was removed by using washing solution (pH 7.5) twice for 15 min and the blot was finally submerged in 10 ml of detection buffer (pH 9.5). The membrane was treated with 0.5 ml of chemiluminescence substrate CSPD and exposed to X-ray film (Kodak/Fuji film) for the range of 10 min to overnight until a clear image was obtained.

3.5.9. RT- PCR

3.5.9.1. Semi quantitative Reverse Transcriptase PCR

cDNAs were prepared from the RNA, isolated from High Pure RNA isolation kit (Roche Applied Science, Germany). An amount of 1 µg of total RNA (DNase I treated) was used to generate cDNA using GeNei^{TM} AMV RT-PCR Kit (Bangalore Genei, India). First strand cDNA was synthesized by incubating RNA with 1 µl of Random primer at 65°C for 10 min and at room temperature for 2 min, followed by addition of 1 µl of RNase inhibitor, 1 µl of 0.1 M DTT, 4 µl of 5x RT buffer, 2 µl of 0.2 mM dNTPs, 0.5 µl of Reverse transcriptase (10 U/µl) to the reaction system of 20 µl with nuclease free water at 42°C for 1 h. The cDNA quality was checked by amplification with gene specific primers by PCR. PCR conditions were standardized with 25-50 ng of cDNA as template DNA, primer concentration of 0.5-0.9 µM and at various annealing temperatures as follows: Initial denaturation at 94°C for 4 min 30 s; denaturation at 94°C

for 45 s, annealing 55°- 62°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 10 min. 5S rRNA was used as endogenous control.

3.5.10.2. Real time PCR

For real time PCR, amplification was performed with SYBR Green Master Mix (Thermo Scientific, USA) in a Step One Thermal Cycler (Applied Biosystems, USA). The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceed background level). The reaction system and program used for real time PCR is given in the table 11.

Table 11. Real time PCR reaction system and its program.

Assay system used		**Temperature profile**	
Sterile nuclease free water	7.0 µl	Initial denaturation	94°C for 5 min
SYBR Green I master mix 2x	10.0 µl	Denaturation	94°C for 45 s
Forward Primer (0.3-0.9 µM)	1.0 µl	Annealing	58°C for 30 s
Reverse Primer (0.3-0.9 µM)	1.0 µl	Extension	72°C for 30 s
Template cDNA (25 ng/µl)	1.0 µl	For 40 cycles	
		Melt curve	80°C for 10 min
Total System	20 µl	Final extension	72°C for 10 min

Ct value < 29 is indicative of abundant target RNA in the sample, a Ct value of 30-37 indicated reasonable amounts of target RNA whereas a Ct value of 38-40 was considered as minimal or no presence of RNA. The excitation and emission maxima of SYBR Green I is at λ_{494} nm and λ_{521} nm respectively. ROX passive reference dye included in the master mix served as an internal reference for normalization of the SYBR Green I fluorescent signal. No template control (NTC) was included which contained all components except template cDNA to assess any reagent contamination or primer dimers. Each sample was tested in triplicates and the fold change was calculated using the comparative Ct method using the formula $2^{-\Delta\Delta Ct}$. 5S rRNA was used as an internal control in real time PCRs. The primers used are listed in Table 7.

3.6. Estimation of proteins by Folin Lowry method

Proteins were estimated by using Folin Lowry method as described in Lowry *et al.*, 1951.

3.7. Physiological assays

Growth rates were measured at OD_{600}. All experimental data were normalized for mg/ml cell protein to account for the difference in growth rates.

3.7.1. Quantitative biofilm production by microtiter plate assay

Biofilm production by *S. aureus* modified strains grown in Luria broth (LB) was determined using a semi quantitative adherence assay in 96-well microtiter plate with slight modifications (Zmantar *et al.*, 2010). Briefly, an equal number of cells of overnight grown culture were induced with 2% glucose and 200 µl of the cell suspension was seeded in a 96-well microtiter plate. The plates were incubated aerobically at 37°C for 48 h. The plates were then washed thrice with 200 µl of phosphate buffered saline to remove planktonic cells, adherent cells were stained with 200 µl of 1% crystal violet for 45 min and unbound crystal violet was removed by washing with sterile distilled water. The adhered cells were air dried and suspended in 200 µl of 33% glacial acetic acid. The absorbance of each well was measured at OD_{570}. Each strain was tested in four replicates.

3.7.2. Confocal microscopy of biofilm formation and clumping of cells

Cultures were grown on a glass cover slip of 18 by 18 mm submerged in LB supplemented with 2% glucose for 24 h. Planktonic cells were removed and washed with PBS, adhered cells were then fixed using a 5% glutaraldehyde solution for 1 h at 50°C. Cells were washed in 2 ml PBS, followed by labelling with FITC (0.001%) and the plate was incubated without shaking for 1 h at room temperature. Cells were washed twice with PBS. Biofilms or adhered cells on the cover glass were inverted and mounted onto a clean glass slide and imaged using excitation wavelength of 488 nm and emission of 505 to 530 nm (Hochbaum *et al.*, 2011). Confocal microscopy images were obtained on a Carl Zeiss LSM confocal inverted microscope.

Clumping of cells in the cell suspensions was studied by growing *S. aureus* cells harbouring the sRNA constructs overnight in LB induced with 2% glucose. Equal number of cells (100 µl) corresponding to an OD_{600} of 1.0 were washed with PBS and labelled with FITC (0.001%). The labelled cells were washed thoroughly with PBS thrice. 5 µl of cells were placed on glass slide, covered with cover slip and mounted and analysed under a confocal microscope.

3.7.3. Delta hemolysis assay

1 ml of blood collected in EDTA containing vial was centrifuged for 5 min at 3000 rpm to remove the serum fraction. The blood was washed thrice with freshly prepared 150 mM NaCl and finally resuspended in 1 ml of phosphate buffered saline (pH 7.4). Washed RBC cells were diluted with phosphate buffer 1:10 to yield suspension of ~5 x 10^6 RBC/ml. Culture supernatants (400 µl) collected from the late exponential phase were subjected to heat treatment at 100°C for 45 s, to eliminate other hemolysins and incubated with equal number of washed RBCs and the reaction was made up to 1 ml with phosphate buffer. Samples were incubated in water bath at 37°C for 1 h. PBS serve as negative control whereas RBC incubated with 1% Triton X-100 was used as positive control, since it caused 100% lysis of RBC membranes. Samples were then centrifuged at 10,000 rpm for 5 min. The released hemoglobin in the supernatant was measured at OD_{541} with PBS as blank (Wiseman, 1975; Black *et al.,* 2003).

3.7.4. Alpha hemolysis assay

The hemolytic effect of alpha-hemolysin on rabbit erythrocytes was quantified by a method described by Bayer *et al.,* 1997. Culture supernatants from *S. aureus* strains grown at 37°C in LB with respective antibiotics were collected at the stationary phase (OD- 6.0). 400 µl aliquots of equal concentration were incubated with 1% suspension of washed rabbit RBCs in 0.01 M phosphate buffered saline (PBS; pH 7.2) containing 0.1% bovine serum albumin at 37°C for 1 h. Samples were then centrifuged at 10,000 rpm for 5 min and the supernatant were measured at OD_{416} with PBS as blank.

3.7.5. Extraction of carotenoid pigment

Staphyloxanthin pigment produced by the modified strains was extracted using methanol as described in Mishra *et al.,* 2011 with slight modifications. Briefly, cultures were streaked on Luria agar (LA) plates supplemented with erythromycin (10 µg/ml) to retain the introduced plasmids and incubated at 37°C for 24 h followed by incubation at cold temperature of 4°C for different time intervals. The cells from the LA plate were scratched out using the nichrome loop and suspended in 2 ml of PBS (pH 7.4). Equal number of cells per mg protein were then harvested by centrifugation at 10,000 rpmfor1 min and washed with water. The cells were then suspended in 200 µl of methanol and heated at 55°C for 3 min. Cell debris were removed by centrifugation at 10,000 rpm for 1 min. The extraction was repeated once again. The extracts were collected in one tube

and methanol was added to make up the final volume to 1 ml. Absorbance of the methanol extracts was measured in the spectrophotometer at OD_{465}.

3.7.6. Antibiotic susceptibility Test

Antibiotic susceptibility testing (AST) was carried out by Kirby-Bauer disc diffusion method. 10^9 cells corresponding to 1 OD culture from *S. aureus* Newman strains overexpressing SprB and vector pCN40 control in 0.8% soft agar were seeded separately onto the bottom agar, over which antibiotic octadisc containing fixed concentrations of different antibiotics was placed with a sterile forceps. Antibiotic discs of fixed concentration (HiMedia) include: Amyoxicillin (Amx) - 10 µg, Tetracycline (T) - 10 µg, Penicillin V (PV) - 3 µg, Cloxacillin (Cox) - 5 µg, Penicillin (P) - 2 µg, Cefalexin (CN) - 30 µg, Erythromycin (E) - 15 µg, Co-Trimaxazole (COT) - 25 µg, Ampicillin (AMP) - 10 µg, Clindamycin (CD) - 2 µg, Oxacillin (OX) - 1 µg, Cephalothrin (CEP) - 30 µg, Erythromycin (E) - 15 µg, Vancomycin (VA) - 30 µg, Chloramphenicol (C) - 30 µg, Gentamycin (GEN) - 10 µg. The plates were incubated for 24 h at 37°C and diameter of zone of inhibition was measured.

3.8. *in vitro* transcription of RNA

in vitro transcription was carried out using PCR generated templates carrying T3 promoter for $SprX1_{150}$ and recombinant plasmids carrying target constructs. Recombinant plasmids carrying ClfB, Hld targets and PhrD were linearized with HindIII and transcribed using T7/T3 RNA polymerase and ribonucleotide (RNTP) mix to produce run-off RNA transcripts. The transcripts were generated using the protocol based on the manufacturer's (Roche, Germany) instructions as mentioned in Table 12:

Table 12. Reaction system for *in vitro* transcription of RNA.

Reagents	Volume
Template DNA (1 µg)	~6.0 µl
Nuclease free water	7.0 µl
10x RNTP mix (25 mM)	2.0 µl
RNase inhibitor	1.0 µl
10x transcription buffer	2.0 µl
T3/T7 RNA polymerase (20 U/µl)	2.0 µl
Total volume	20.0 µl

The length and integrity of the RNA transcript was checked on a 6% native polyacrylamide gel at 5 V/cm for 1 h and stained with 0.5 µg/ml of EtBr solution.

3.9. Electrophoretic gel mobility shift assay

RNA gel mobility shift assays were carried out as described in Fender *et al.*, 2010. The transcripts were denatured at 65°C for 10 min before using for binding assays. DIG-labelled SprX1 (0.5 pmol) of fixed concentration was incubated with increasing concentrations of unlabelled mRNAs ranging from 0.5, 1.0, 1.5, 2.5 and 3.0 pmol in 1x TMN RNA binding buffer containing 20 mM Tris acetate (pH 7.6), 5 mM magnesium acetate, 100 mM sodium acetate to a final system of 15 µl.

Table 13. Reaction system for *in vitro* RNA-RNA interaction.

Reagents	concentration
sRNA SprX (pmol)	0.5
mRNA (pmol)	0.5/1.0/1.5/2.5/3.0
10x RNA binding buffer	1 x
PhrD (competitor RNA)	5- fold excess
Nuclease free water (µl)	Up to total volume
Total volume	15.0 µl

RNA-RNA duplex formation was allowed by incubating the transcripts at 37°C for 30 min. Samples were loaded onto a native 6% polyacrylamide gel at 4°C for 4-5 h and the gel was electro blotted onto the nylon membrane with 1x TBE at 250 V, 6 mA for 2 h. The membrane was UV- cross linked and treated with anti-digoxygenin (75 mU/ml) and CSPD substrate and the image was captured on the X-ray film.

3.10. Animal infection studies

Groups of three female BALB/c mice of eight weeks old were infected with different constructs of *S. aureus* Newman by intravenous injection of 10^9 cells for sepsis model of infection (Chabelskaya *et al.,* 2010). Uninfected mice, injected with phosphate buffered saline (PBS), served as negative controls. The mice were maintained in an aseptic isolated area. Mice were killed after 7 days of infection and assessed by clinical signs, measurement of body weight and biochemical markers such as blood urea nitrogen (BUN), creatinine, serum glutamic oxaloacetic acid transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) (Jyot Pathology Lab, Vadodara). Kidneys of the infected mice were excised, homogenized with sterile PBS with 0.1% Triton X-100 and

bacterial loads were enumerated by plating serial dilutions on mannitol salt agar. The results are represented as dot blots and statistical analysis was done using one/two-way ANOVA to interpret the data.