3. MATERIALS AND METHODS

3.1 Bacterial strains, plasmids, and culture conditions

All the cultures used in the study are listed in Table 3. *E. coli* cultures were grown in Luria Bertani medium and *S. aureus* in Todd Hewitt Broth from HiMedia Labs, India as per manufacturer's instructions. When required media was supplemented with ampicillin 100 µg/ml for *E. coli*, and erythomycin 10 µg/ml for *S. aureus* strains.

Table 3: Strains and plasmids used in this study

Strain or plasmid	Characteristic	Reference or source
S. aureus Newman	Wild type, MSSA[a]	Timothy Foster, Trinity College Dublin, Ireland
E. coli DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Lab stock
E. coli DC10B	*dam*$^+$, *dcm*$^-$ *hsd RMS endA1 recA1*	(Monk *et al*., 2012)
pBluescriptKS$^+$	Cloning vector ColE1 replicon, Amp^r	Lab stock
pCN40	*E. coli–S. aureus* shuttle vector, Ery^r	NARSA[b]
pMN*sprX*	pCN40 with *sprX*, Ery^r	(Kathirvel *et al*., 2016)
pCN*sprX*$_{KD}$[c]	pCN40 with *sprX* cloned in antisense orientation, Ery^r	This study
pBSKS*phrD*	pBSKS$^+$ with *phrD* gene, Amp^r	Lab stock

[a]Methicillin-susceptible *Staphylococcus aureus*, [b]Network on antimicrobial resistance in *Staphylococcus aureus (*https://www.beiresources.org/), [c]KD – knockdown; Amp^r-Ampicillin resistant; Ery^r-Erythromycin resistant

Unless otherwise specified, all the liquid cultures were grown in a 100 ml Erlenmeyer flask filled with 20 ml media (flask to medium ratio 5:1) with constant shaking at 150 rpm at 37°C. The growth of the culture was monitored as an increase in absorbance at 600 nm in Beckman Coulter DU® 720 UV/Vis Spectrophotometer.

3.2 Bioinformatics tools

3.2.1 IntaRNA

Online RNA-RNA interaction prediction tool IntaRNA (http://rna.informatik.uni-freiburg.de/IntaRNA) was used to determine the potential base pairing between target mRNA and sRNA SprX (Mann *et al*., 2017).

3.2.2 Mfold

Mfold Web Server (http://www.bioinfo.rpi.edu/applications/mfold) was used to analyze the secondary structure of sRNA SprX (Zuker, 2003).

3.2.3 Primer3

All the primers used in the study were designed using primer designing tool Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) (Untergasser *et al*., 2012).

3.2.4 BPROM

Putative promoter was predicted using promoter prediction tool BPROM (https://molbiol-tools.ca/Promoters.htm) (Solovyev and Salamov, 2013).

3.3 Molecular biology methods

3.3.1 Genomic DNA extraction

Genomic DNA was isolated from *S. aureus* Newman using the standard method. In brief, 1-3 ml of overnight grown culture was collected, centrifuged and resuspended into 1 ml of TE buffer (pH 8.0). Cells were lysed by incubating at 37°C for 1 h with the addition of 2.5 µl of lysostaphin (20 µg/ml) and treated with buffer containing 1% SDS, 50 mM EDTA and 20 µg/ml Proteinase-K for 1 h at 55°C. The DNA was extracted with phenol: chloroform: isoamyl alcohol (PCI) 25:24:1, followed by chloroform: isoamyl alcohol (24:1) and precipitated with $1/10^{th}$ volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of chilled absolute ethanol. The precipitated DNA was washed with 70% ethanol, dried, resuspended into 50 µl of TE buffer (pH 8.0) and stored at -20 °C.

3.3.2 Plasmid DNA extraction

Plasmid DNA from *E. coli* was extracted using a standard alkaline lysis method (Sambrook and Russell, 2001). Briefly, 1.5 ml of overnight grown culture was centrifuged at 10,000 rpm for 10 min. Culture supernatant was removed and pelleted cells were resuspended into 100 µl of ice-cold alkaline lysis solution I by vortexing vigorously. Followed by this 200 µl of freshly prepared alkaline lysis solution II was added to the bacterial suspension, combined by inverting the tubes and stored on ice for 5 min. 150 µl

of ice-cold alkaline lysis III was added in tubes and dispersed by inverting the tubes several times and stored on ice for 5 min. The tubes were centrifuged at maximum speed for 10 min and the supernatant was transferred in a fresh tube. The plasmid DNA was recovered by precipitating with 2 volumes of ethanol at room temperature followed by centrifugation at maximum speed for 10 min. The supernatant was removed, and the pellet was washed with 1 ml of 70 % ethanol, air-dried, and resuspended into TE buffer (pH 8.0).

3.3.3 Restriction enzyme digestion

For cloning, DNA was digested with restriction enzyme (RE) (5 units/ µg of DNA) by mixing with 1X buffer supplied by manufacturer and incubated at 37°C for 3 h.

3.3.4 Polymerase chain reaction

Polymerase Chain Reactions (PCR) were performed as per standard protocol mentioned in (Sambrook and Russell, 2001). *S. aureus* genomic DNA was used as a template for the amplification of all genes used in this examination. Primers used in this study were designed using the Primer3 program and listed in Table 4. The amplification reaction mixture comprises 50 ng of DNA template, dNTPs (0.2 mM), forward and reverse primers (0.5 µM each), 1.5-3 U Polymerase [Taq polymerase or XT-5 Polymerase (Genei)], with 1X concentration of respective polymerase buffer in 20 µl system. The standard PCR program used for the amplification includes the denaturation of template DNA at 95 ℃, annealing in the range of 55-60 ℃ depending upon the Tm of the respective primers, and extension at 72 ℃ (Table 5).

3.3.5 Agarose gel electrophoresis and DNA elution

DNA was loaded on 0.8-1.2 % agarose gel by mixing with 6X DNA gel loading dye (0.25% bromophenol blue, 40% sucrose) to make the final concentration 1X and run in 0.5X TBE (Tris-borate EDTA buffer pH 8.0) at 15 Volts/cm for 45 min to 2 h. DNA was visualized by staining the gel in 0.5X TBE solution containing Ethidium Bromide (0.5 µg/ml) and image was documented in Bio-Rad's Gel Doc™ EZ Imager. The correct sized DNA was purified by resolving on 0.8% low melting agarose gel and eluted with GeneJET gel extraction kit (Thermo scientific) as per the manufacturer's instruction. The purity and quantity of eluted DNA was confirmed by subjecting to agarose gel electrophoresis.

Table 4: Oligonucleotides used in this study

Primers	**Sequence (5′-3′)**
Cloning	
$SprX_{KD}$	F - CGG**GGTACC**ACACATGCATCAACTATTTACATCT
	R - CGC**GGATCC**ACAGGCTATATAGTTCACTCCTACT
qRT-PCR	
SprX	F - ATAATCTTTCTAGACGTATTCAAA
	R - CAGGCTATATAGTTCACTCCTACT
WalR	F - CAAATGGCTAGAAAAGTTGTTGTAG
	R - CAGTAAGCATTATTATTGGCATTTCG
AtlA	F - AACAGCACCAACGGATTAC
	R - CATAGTCAGCATAGTTATTCATTG
IsaA	F - GCAGGTGCTACTGGTTCATCAG
	R - GATTCACGAGCGATGATTGC
LytM	F - CAGCAACAGCAGGAGATAAC
	R - ATAATTGACCTTTCCATTACCATC
5S	F - GCAAGGAGGTCACACCTGTT
	R - GCCTGGCAACGTTCTACTCT
DNA template for *in vitro* transcription	
SprXT3	F - **AATTAACCCTCACTAAAGGG**ACACATGCATCAACTATTTACATCT
	R - AAAAGCACCCCGTAAACTATTATAC
WalRT3	F - **AATTAACCCTCACTAAAGGG**CAAATGGCTAGAAAAGTTGTTGTAG
	R - CAGTAAGCATTATTATTGGCATTTCG
IsaAT7	F -**TAATACGACTCACTATAGGG**CATCAGGTGCTTCAGGTTTATTCC
	R - AAACTCTCCCCAATTTCTATGGGAA

Bold region indicates the restriction enzyme site in the primer, *KpnI*-GGTACC and *BamHI*-GGATCC. Bold and underlined region indicates the T3 and T7 promoter sequence, F-forward, R-reverse

3.3.6 Ligation

T4 DNA ligase from Thermo Scientific was used for the ligation reaction. The RE digested purified plasmid DNA (25 ng) was incubated with 5 units of ligase in the reaction buffer with 20:1 insert to vector molar ratio and ligation was performed as per manufacturer's instruction.

Table 5: PCR condition used in the study

Genes	Initial Denaturation (Temperature and Time)	Denaturation (Temperature and Time)	Annealing (Temperature and Time)	Extension (Temperature and Time)	Final extension (Temperature and Time)
	Step-1	Step-2	Step-3	Step-4	Step-5
$SprX_{KD}$	95 °C 4 min 30 sec	95 °C 45 sec	54 °C, 30 sec	72 °C 30 sec	72 °C 10 min
SprX			58 °C, 30 sec		
WalR			55 °C, 30 sec		
AtlA			55 °C, 30 sec		
IsaA			58 °C, 30 sec		
LytM			55 °C, 30 sec		
5S			58 °C, 30 sec		
SprXT3			60 °C, 30 sec		
WalRT3			54 °C, 30 sec		
IsaAT7			55 °C, 30 sec		
		Step-2 to Step-4 repeated for 30 cycle			

3.3.7 Transformation in *E. coli*

E. coli DH5α was transformed with plasmids and ligation mixtures as per the standard $CaCl_2$ method (Sambrook and Russell, 2001). Briefly, competent cells were prepared by collecting the logarithmically growing cells (OD_{600} ~ 0.3), washed with ice-cold $MgCl_2$ (80 mM) - $CaCl_2$ (20 mM) solution and resuspended into ice-cold 0.1 M $CaCl_2$ containing 20 % glycerol. The competent cells were aliquoted and transformed with the ligation mixture. The transformants were selected on respective antibiotics.

3.3.8 Transformation of *S. aureus*

The plasmid DNA was introduced into *S. aureus* by electroporation as reported earlier with a slight modification (Kathirvel *et al.*, 2016). Electrocompetent cells were prepared by diluting the overnight grown culture into a fresh medium and cells were collected at OD_{600} ~ 0.3- 0.4. Cells were washed twice with 0.5 M sucrose, resuspended into 0.5 M sucrose containing 10 % glycerol and electroporated at 2.5 kV, 150 Ω and 50 µF. The cells were directly resuspended into a growth medium containing 0.5M sucrose and transformants were selected on respective antibiotics.

3.4 Construction of *sprX* knockdown strain

sprX knockdown plasmid was constructed in *S. aureus-E. coli* shuttle vector pCN40. *sprX* containing an inherent antisense promoter at the 3′ end, recognized by BPROM (https://molbiol-tools.ca/Promoters.htm) (Solovyev and Salamov, 2013) was PCR amplified using primers listed in Table 4 and cloned in the antisense orientation into pCN40 plasmid at BamHI/KpnI sites. The resulting knockdown plasmid pCN*sprX*$_{KD}$ was passed through the methylation defective mutant *E. coli* DC10B and electroporated into *S. aureus* Newman.

3.5 Proteomic studies

3.5.1 Preparation of proteins for two-dimensional gel electrophoresis

The overnight grown culture was diluted to an OD_{600} of 0.05 in fresh THB medium and harvested at early stationary phase for extracellular proteins. Cells were centrifuged at 10,000 rpm for 10 min, supernatant was filtered and collected. Protein from the culture supernatant was precipitated using the standard chloroform-methanol precipitation method. Precipitated proteins were dissolved in 6 M Urea containing 0.1 % Triton X-100 and protein concentration was determined by the Folin Lowry method.

Cell wall associated proteins were extracted by lysostaphin treatment under isotonic conditions as described earlier with some modifications (Taverna *et al.*, 2007). Overnight grown cultures were diluted to an OD_{600} of 0.05 in fresh THB medium and harvested at mid-log phase. After harvesting, the cells were washed twice with ice-cold PBS and once with the digestion buffer (10 mM Tris-Cl, pH 7.6, 1 mM EDTA, 5 mM $MgCl_2$). Cells were suspended in digestion buffer containing 35% raffinose, 1 mM Iodoacetamide, 2 U of lysostaphin and incubated at 37 ºC for 30 min. Protoplasts were sedimented by low speed centrifugation (6000 x g) for 30 min at 4 ºC. Cell wall associated proteins from the supernatant were further precipitated using chloroform-methanol precipitation method, dissolved in 6 M urea and protein concentration was determined by the Folin Lowry method.

3.5.2 Isoelectric focusing (IEF), 2D-PAGE and protein identification

Isoelectric focusing was performed by separating the proteins on a 7 cm immobilized pH gradient (IPG) strip with a range of 4-7 pH (Ready Strip IPG strips, Bio-Rad). The IPG strips were rehydrated for 16 h in the rehydration buffer [7 M Urea, 2 M thiourea, 1 % (w/v) ASB-14, 40 mM Tris-Cl and 0.001 % bromophenol blue] containing 25 µg of

proteins. After rehydration proteins were separated according to their pI in the IEF apparatus and focusing was performed as mentioned in Table 6 at a default chamber temperature of 25 ℃.

The IPG strips were subjected to a two-step equilibration following IEF. The proteins were first reduced by incubating the IPG strip in equilibration buffer (50 mM Tris-Cl/pH 8.8, 6 M urea, 30 % glycerol and 2 % SDS) containing 1 % 1,4-Dithiothreitol (DTT) followed by alkylation in equilibration buffer containing 2.5 % iodoacetamide for 15 min each. The equilibrated IPG strips were immersed in 1X SDS gel running buffer, and mounted on 10 % SDS-PAGE.

The second dimension was performed on vertical SDS-PAGE using Mini-PROTEAN SYSTEM (Bio-Rad, CA, USA) at a constant voltage of 100 V for 2 h. Protein spots were visualized using the silver staining method. Differentially regulated protein spots were excised from the gels and subjected to LC-MS analysis (C-CAMP, Bangalore, India).

Table 6: IEF run parameters used for 7 cm IPG strip

Focusing steps	Voltage	Time	Volt-Hrs	Ramp
Step 1	250	30 min	-	Linear
Step 2	4000	2 h 30 min	-	Linear
Step 3	4000	-	10,000 V-h	Rapid
Total		~6 h	14,000 V-h	

3.5.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated according to their molecular weight using the standard SDS-PAGE (Sambrook and Russell, 2001). The required amount of proteins was mixed with SDS gel loading buffer (Appendix III Table A1), boiled at 95℃ for 5 min and loaded on SDS – Polyacrylamide gel containing 5% stacking gel and 10% resolving gel (Appendix III Table A2). Gels were run at 20 mA constant current in 1X SDS running buffer (25 mM Tris Base, 192 mM Glycine, 0.1 % SDS) and proteins were visualized by silver staining or Colloidal Coomassie staining method.

3.5.4 Colloidal Coomassie staining with CBB G-250

The sensitive colloidal Coomassie staining method was performed as reported previously (Dyballa and Metzger, 2009). Briefly after the electrophoresis, gels were rinsed thrice with MilliQ water, covered with Coomassie solution {0.02% (w/v) CBB G-250, 5 % (w/v) aluminum sulfate-(14-18)-hydrate, 10 % (v/v) ethanol (96%) and 2 % (v/v) orthophosphoric acid (85 %)} and incubated for 4-12 h with shaking. After staining, gels were rinsed with MilliQ water and incubated in the de-staining solution [10 % (v/v) ethanol (96 %), 2 % (v/v) orthophosphoric acid (85 %)] until the visualization of clear bands.

3.5.5 Silver Staining

The gels were washed twice with MilliQ water and incubated overnight in the fixing solution [methanol: glacial acetic acid: water (30:10:60)] with constant shaking. The gels were then incubated in 5 volumes of 30 % methanol for 30 min, washed thrice with MilliQ water and covered with freshly prepared 0.02 % sodium thiosulphate solution for 1 min. The gels were again rinsed with MilliQ water and submerged into 0.2 % silver nitrate solution for 30 min. Before developing, gels were rinsed with MilliQ water and then incubated into the developing solution (2.2 % Na_2CO_3, 0.05 % formaldehyde) until desired contrast was obtained. The reaction was stopped by incubating the gels in 1 % acetic acid solution.

3.6 Preparation of extracellular and cell wall associated autolysins

The overnight grown culture was diluted to OD_{600} 0.05 in fresh THB medium and allowed to grow at 37°C until the stationary phase. For extraction of extracellular autolysins, filtered cell free supernatant was collected, and precipitated with chloroform-methanol precipitation method. Cell wall associated autolysins were extracted with 4 % sodium dodecyl sulfate with slight modification (Antignac *et al*., 2007). Cells were harvested at mid-exponential phase, washed with 50 mM Tris-Cl (pH 7.5), extracted into 4 % SDS at 37 ℃ for 30 min with stirring and then heated at 95℃ for 3 min. The supernatant containing autolysins was collected after centrifugation. Protein concentration was estimated by the standard Folin Lowry method.

3.6.1 Zymography

Cell wall was prepared as a substrate for zymography of autolysins, as reported earlier (Liu *et al*., 2011). Briefly, *S. aureus* Newman culture was grown until the mid-logarithmic phase, cells were harvested and resuspended into 8 % SDS followed by boiling for 30 min. The cell wall substrate was washed with distilled water several times to remove the SDS, mechanically disrupted, and lyophilized. Cell wall hydrolyzing autolytic activity was investigated by separating extracellular and cell wall associated proteins on 10 % SDS-PAGE containing lyophilized *S. aureus* cell wall (1 mg dry weight/ml) as substrate. Following electrophoresis, the gel was washed in distilled water to remove SDS, allowed to renature in a buffer (50 mM Tris-Cl pH 7.5, 0.1 % Triton X-100, 10 mM $CaCl_2$ and 10 mM $MgCl_2$) at 37°C and stained with methylene blue. Autolytic activity appeared as zones of clearing in the blue background (Jayaswal *et al*., 1990).

3.7 RNA isolation and quantitative real time PCR

Overnight grown culture was diluted to OD_{600} of 0.05 in fresh THB medium and allowed to grow at 37°C. Total RNAs were extracted at different time points using Aurum total RNA isolation kit (Bio-Rad) as per the manufacturer's instructions. First-strand cDNAs were synthesized with Verso cDNA synthesis kit (Thermo Scientific) and qRT-PCR was performed with Maxima SYBR Green/RoxqPCR Master Mix 2X (Thermo Scientific) using Step-One real-time PCR system (Applied Biosystem). Fold change in the expression was calculated as per the standard $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) after normalization with 5S rRNA. *S. aureus* Newman WT (pCN40) was used as a control to compare the gene expression in *sprX* overexpression (pMN*sprX*) and *sprX* knockdown (pCN$sprX_{KD}$) strains.

3.8 Triton X-100-induced autolysis assay

Triton X-100-induced autolysis assay was performed as specified previously (Bose *et al*., 2012) with slight modification. Overnight grown culture, diluted to OD_{600} of 0.05 in fresh THB medium was allowed to grow at 37°C till OD_{600} of 1. Cells were harvested, washed, and resuspended into the autolysis buffer (50 mM Tris-Cl pH 7.2, 0.1 % Triton X-100) and the cell suspension was incubated at 30°C with shaking at 250 rpm. A reduction in the optical density was measured at the wavelength of 580 nm up to 5 h and the data reported as the percent of the initial optical density for each sample.

3.9 Observation of cell morphology and analysis of cell clustering

Bacterial morphology and cell clustering were visually studied by both phase contrast and by scanning electron microscopy (SEM) and quantitated by sedimentation assay. Primarily, cell clustering was observed under phase-contrast microscopy on agarose coated slides. Statically grown cultures (5 µl) were directly spotted on 1% agarose pad, covered with a cover slip and clustering was observed under phase-contrast, at 1000X magnification.

For SEM analysis statically grown cells were harvested, washed and mounted on a coverslip. Cells were sputter-coated with gold and examined under JEOL JSM- 6380 LV scanning electron microscope at accelerating voltage of 20 kV.

Bacterial sedimentation as a result of cell clustering was measured as reported earlier with slight modification (Trunk *et al*., 2018). Overnight grown culture was diluted to OD_{600} of 0.8 in fresh medium, incubated statically and decrease in optical density from the top of the tube was measured at a wavelength of 600 nm up to 8 h. Data was reported as the percentage of the initial density of each sample.

3.10 Biofilm assay

Biofilm formation was measured as described (Zmantar *et al.*, 2010). The overnight grown culture was diluted to OD_{600} of 0.05 in fresh THB medium supplemented with 2% glucose. 200 µl of diluted cells were seeded into 96 well microtiter plate and incubated at 37 ℃ for 24 h. Biofilm formation was analyzed by staining the adherent cells with crystal violet and the absorbance of each well was measured at a wavelength of 570 nm. Data reported are the percentage of each strain compared to the control.

3.11 *In vitro* transcription and RNA labeling

DNA templates for transcription of desired genes were generated by PCR amplification using primers containing the T3 or T7 promoter in the forward primer. In the case of PhrD, the template was generated, by digesting the plasmid pBSKS*phrD* containing a cloned PhrD. RNAs were *in vitro* transcribed from purified DNA templates using T3 or T7 Polymerase (Roche), rNTPs (Roche) as per the manufacturer's instructions. SprX was labeled by *in vitro* transcription using Digoxygenin (DIG) labeling mix (Roche) containing Digoxygenin-11-UTP. *In vitro* transcribed RNAs were separated on 6 M Urea/6 % PAGE at 100 V in 0.5 X TBE buffer (Table 7). RNA transcripts of the correct size were eluted from gel as reported previously (Nilsen, 2013). The gel piece was cut

and placed in a tube containing elution buffer (20 mM Tris-Cl pH 7.5, 250 mM sodium acetate without pH adjusted, 1 mM EDTA pH 8.0, and 0.25 % SDS) and frozen for 15 min. RNAs were allowed to diffuse from the gel during overnight incubation at RT. Tubes were centrifuged at 10,000 rpm for 10 min at RT and supernatant containing RNA was precipitated and stored at -20 °C.

Table 7: Urea-PAGE composition for RNA analysis

Stock solution component	6 % gel
6 M Urea	3.6 gm
40 % Acrylamide:bisacrylamide solution (19:1)	1.5 ml
10 X TBE	1 ml
10 %(APS)	0.1ml
TEMED	0.005ml
Total volume	10 ml

3.12 Electrophoretic Mobility Shift Assay (EMSA)

DIG-labeled *in vitro* transcribed SprX (0.1 pmol) was incubated with increasing molar concentrations (10X-400X) of unlabeled putative target mRNAs in 10 mM Tris-Cl (pH 7.0), 100 mM KCl, 10 mM $MgCl_2$ at 37°C for 30 min followed by separation on 6% native polyacrylamide gel (Table 8) (Vockenhuber and Suess, 2012). RNAs were then electro-blotted on to the nylon membrane, UV cross-linked and detected by chemiluminescence. Specific interactions between RNAs were observed by shift in the electrophoretic mobility. Specificity of base-pair interaction was assessed by competition assay in the presence of an excess molar concentration of nonspecific RNA PhrD or unlabeled SprX (cold SprX).

Table 8: Native-PAGE composition used for RNA-RNA interaction analysis

Stock solution component	6 % gel
DEPC treated MilliQ water	29.5 ml
40 % Acrylamide:bisacrylamide solution (19:1)	6.0 ml
10 X TB without EDTA	4.0 ml
10 %(APS)	0.4ml
TEMED	0.02 ml
Total volume	40 ml