3 MATERIALS AND METHODS

3.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Tables 7 and 8 respectively.

Table 7: List of bacterial strains used in the study

Strains	Characteristic	Reference or 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	Lab stock
E. coli DC10B	Derivative of *E. coli* KL16, *dam*+, *dcm*− *hsdRMS endA1 recA1*	Timothy Foster, Trinity College, Dublin, Ireland
S. aureus 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 Newman pCN40	Control strain of *S. aureus* Newman containing *E. coli* - *S. aureus* shuttle vector pCN40, Em^r	This work
S. aureus Newman *rsaF::kan* disruption mutant	Isogenic mutant strain of Newman *rsaF::kan*, Km^r	This work
S. aureus Newman pCN*rsaF*	Strain of *S. aureus* Newman containing pCN40 with *rsaF* gene, Em^r	This work

The name, description and source for each strain are given. Km - Kanamycin; Em - Erythromycin; r - resistant; MSSA - Methicillin Susceptible *Staphylococcus aureus*.

Table 8: List of plasmids used in the study

Plasmids	Features	Size (bp)	Reference or source
pBluescriptKS+	*E. coli* cloning vector, Amp^r	2961	Lab source
pCN40	*E. coli–S. aureus* shuttle vector, Ery^r	5829	NARSA[a]
pBSRsaF	$pBSKS^+$ encoding RsaF, Amp^r	3228	This work
pCNRsaF	pCN40 encoding RsaF, Em^r	6090	This work
pBBRMCS2	Broad host range vector; originally derived from pBBR1 plasmid of	5144	Lab source

	Bordetella bronchispetica; Km^r		
pMAD	*E. coli-S. aureus* temperature sensitive shuttle vector for gene replacement, Erm^r at 30°C, Em^r	9666	Christiane Wolz, University of Tubingen, Tubingen, Germany
pMRsaFGDC	pMAD with kanamycin disrupted RsaFGDC[b], Em^r	12466	This work

[a]Network on antimicrobial resistance in *Staphylococcus aureus* (http://www.beiresources.org), [b]RsaFGDC- RsaF gene disruption cassette. Amp- Ampicillin; Em- Erythromycin; Km- Kanamycin; r- resistant.

3.2 Media, chemicals, enzymes and kits

Media and general chemicals were purchased from HiMedia Laboratories, India and Sisco Research Labs, India. All molecular biology grade chemicals, enzymes and kits were procured from Merck, India; Roche, Switzerland; Sigma-Aldrich, USA; Thermo Fisher Scientific, USA; Bangalore Genei, India; New England Biolabs, USA.

3.3 Media and Growth conditions

E. coli strains were grown in Luria–Bertani medium and *S. aureus* strains were grown Brain Heart Infusion (BHI) medium either in liquid with constant shaking at 120 rpm or on 1.5% (w/v) agar plates at 37°C. Growth of the culture was measured as the absorbance at a wavelength of 600 nm in Beckman Coulter DU® 720 general purpose UV/Vis Spectrophotometer. Antibiotics, inducers and other biochemical were supplemented to the media as and when required in concentrations mentioned in Table 9.

Table 9: 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
Erythromycin	10	-	10
Kanamycin	40	40	15
X-gal	20	40	40
IPTG	20	20	-

3.4 Bioinformatic tools used in the study

3.4.1 Sequence retrieval

Sequence of RsaF sRNA (Geissmann et al., 2009) was retrieved from the NCBI database for *S. aureus* strain Newman (https://www.ncbi.nlm.nih.gov/nuccore/NC_009641.1).

3.4.2 Target prediction and secondary structure

Sequence of RsaF sRNA was retrieved from the NCBI database for *S. aureus* strain Newman. Online target prediction tools Target RNA (Tjaden, 2008), TargetRNA2 (Kery et al., 2014) (http://cs.wellesley.edu/~btjaden/TargetRNA2/), IntaRNA (Busch et al., 2008) (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp) , CopraRNA (Wright et al., 2014) (http://rna.informatik.uni-freiburg.de/CopraRNA/Input.jsp) and RNA predator (Eggenhofer et al., 2011) were used to predict the mRNA targets or to analyze base pair interactions for the sRNA RsaF. Potential virulent target mRNAs with base pairing of 9–25 nt having significant P value ≤ 0.01 were selected. The secondary structures of RsaF and mRNAs were analyzed by the *S*-fold web server (http://sfold.wadsworth.org/cgi-bin/index.pl) and RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

3.4.3 Primer design

Primers were designed using online tool Primer 3 (https://primer3.ut.ee/) and quality of primers with respect to self-annealing and hair-pin loop structures were checked by Oligo Calc: Oligo nucleotide properties calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html).

3.4.4 *in silico* restriction analysis

NEBcutterV2.0 (http://nc2.neb.com/NEBcutter2/) was used to generate restriction map of the given sequence and theoretical digests to determine the size of digested fragments.

3.5 Molecular biology techniques

3.5.1 Isolation of genomic DNA from *S. aureus*

Cells of overnight grown culture of *S. aureus* strain Newman were harvested by centrifugation and resuspended in 500 µl of TE buffer (pH 8.0). Cells were lysed with lysostaphin (20µg/ml) at 37°C for 90 min followed by treatment with buffer containing 1% SDS, 20mM Tris (pH 8.0), 2mM EDTA, and 2.5 µl of proteinase K (20 mg/ml) and incubated at 55°C for 1 h.

Chromosomal lysate was treated with phenol:chloroform:isoamyl alcohol and DNA was extracted.

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

Plasmid DNA from overnight grown cultures of *E. coli* was isolated by standard alkaline lysis method as described in (Green and Sambrook, 2012). For *S. aureus* cultures, cells were initially lysed with 20 µg/ml of lysostaphin followed by the standard alkaline lysis protocol.

3.5.3 Polymerase Chain Reaction (PCR)

Table 10: PCR conditions used in the present study

Gene	Initial denaturation 94°C	Denaturation temperature 94°C	Annealing temperature (°C), time (sec)	Extension temperature 72°C	Final extension 72°C
RsaF	4 min 45 sec	45 sec	55°C, 30 sec	30 sec	10 min
kan	4 min 45 sec	45 sec	55°C, 30 sec	50 sec	10 min
RsaF-up	4 min 45 sec	45 sec	57°C, 30 sec	45 sec	10 min
RsaF-Dn	4 min 45 sec	45 sec	60°C, 30 sec	50 sec	10 min
RsaF GDC fusion PCR	2 min 30 sec	First 8 cycles			
		30 sec	58°C, 20 sec	1 min	-
		24 cycles			
		30 sec	52°C, 25 sec	2 min 30 sec	15 min
HysA	4 min 45 sec	45 sec	58°C, 30 sec	30 sec	10 min
SplD	4 min 45 sec	45 sec	58°C, 30 sec	30 sec	10 min

Polymerase chain reactions were performed as per the standard guidelines mentioned in (Sambrook and Russell, 2001). Oligonucleotide sequences used in the current study were synthesized from Sigma Aldrich Chemicals Pvt. Ltd., MWG Biotech Pvt. Ltd or MWG Eurofins, Bengaluru. PCR amplifications were performed in Bio-rad thermal cycler. The PCR amplification reaction mixture contained 25-50 ng template, 1X Taq polymerase buffer with 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM of dNTPs and 1.5 units of High Fidelity Taq DNA polymerase. 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). A summary of PCR programs for different genes and a list of all the primers used in the present study is mentioned in the table 10 and 11 respectively.

Table 11: Primers used in the study

Primers	**Sequence (5'-3)**
Primers used for Cloning	
RsaF	F- TGCACTGCAGGTATTCAGACACGACGTTTT
	R- GACCTCGTACACCATAAATG
RsaFupF	CGGGATCCATTAACGCCAACTAGAATGA
RsaFupR-Km	CAGGTGGCACTTTTCGGGCAACTTCACTTCCCTCTTA
RsaFdnF-Km	CAATACGCAAACCGCCTCTGCACTTTTTATACATTAACGA
RsaFdnR	GCGTCGACGCTGAGTTACAAGGTCGTG
Kan	F- CCCGAAAAGTGCCACCTG
	R- GAGAGGCGGTTTGCGTATTG
Primers used for qRT-PCR	
RsaF	F- GTATTCAGACACGACGTTTTTTTATG
	R- TTAATCGTTAATGTATAAAAAGTGCAT
HysA	F- GGTGGTGAATTCAGAAGTGTTG
	R- CCATAGTTAACGTCCAGCCATG
SplD	F- ATCAATAACTGGTGTCGGCA
	R- CCCATCCATGTAACACCACT
5S	F- GCAAGGAGGTCACACCTGTT
	R- GCCTGGCAACGTTCTACTCT
Primers used to generate DNA template for *in vitro* transcription	
$RsaF_{T7}$	F- GCAATTAACCCTCACTAAAGGCCTTTGTTTAGCGTCGTGTATTC
	R- TTTTCGAATTTAACCAGTTCG
$HysA_{T3}$	F- TAATACGACTCACTATAGGGATTCTTTGTGGATTGTTTGACAG
	R- TGAACATTCGTATCAGCCACAG
$SplD_{T7}$	F- GCAATTAACCCTCACTAAAGGAGCACATTGAACAATAAACA
	R- TTTGGCTGTTTGTTGAATACC

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

3.5.4 Restriction enzyme digestion

DNA was mixed with 5 units of restriction enzyme per microgram of DNA, 1X supplied buffer with or without BSA to a final concentration of 100µg/ml and incubated at 27/37°C for 3 h. In the case of double digestion, either a compatible buffer was used; or sequential digestion was done.

3.5.5 Agarose gel electrophoresis

DNA was mixed with 6 X bromophenol blue gel loading dye (0.25% bromophenol blue, 40% sucrose in water) to make it 1 X and loaded in 0.8-1.5% agarose gel. The gel was run through 0.5 X TBE (Tris-borate EDTA buffer, pH 8.0) at 15 Volts/cm for 45 min to 2 h and stained in 0.5 µg/ml Ethidium Bromide solution for 20 min. The gel image was documented in Bio-Rad's Gel Doc™ EZ Imager after staining.

3.5.6 Elution of DNA from agarose gels

DNA was resolved on 0.8% low melting agarose and the required band was cut out of the gel into a pre-weighed microfuge tube. DNA was eluted from the agarose gel by freeze thaw method of elution as described in Green and Sambrook, 2012. Occasionally, gel elution kit with spin columns (Roche Diagnostics) was used to elute DNA as per the manufacturer's instructions. Small amount of the purified DNA was subjected to gel electrophoresis to confirm the purity and quantity.

3.5.7 Ligation

Ligations were carried out with T4 DNA ligase (Fermentas, USA) as per the manufacturer's instructions at a concentration of 5 units of ligase per 50 ng of plasmid DNA. The molar ratio of vector to insert DNA was usually 1:5 for cohesive end ligations, and 1:10 for blunt end ligations at an optimized temperature of 22°C for 1 h.

3.5.8 Transformation in *E. coli* and *S. aureus*

Competent cells were prepared from mid log phase at 0.3-0.4 OD_{600} for *E. coli* and 0.5-0.6 OD_{600} for *S. aureus.* Ligation mixtures or plasmid DNA was transformed into competent *E. coli* cells prepared by $CaCl_2$ method (Green and Sambrook, 2012) or into electro competent *S. aureus* by electroporation as per the manufacturer's instructions (BTX Harvard ECM 630).

Electro-competent cells were prepared using logarithmic grown culture of *S. aureus* of OD_{600} 0.5-0.6 according to the protocol described in (Monk et al., 2012) with some modifications. 100 ml of *S. aureus* culture of 0.5 - 0.7 OD_{600} was washed with equal volume of cold 0.5 M sucrose at 4°C. Two more wash of 0.5 M sucrose were given with half and one fourth volume and finally resuspended in 1 ml of 0.5 M sucrose containing 10% glycerol and stored at -20°C. 3 - 5 µg of purified plasmid DNA was mixed with 200 µl of ice thawed electro competent *S. aureus* cells and electroporated at 2.3kV, 25µF, and 100ohms. The cells were immediately resuspended in 1 ml of LB containing 0.5 M sucrose with shaking for 2 h at 30°C/37°C and plated onto the appropriate antibiotic containing medium and incubated at

30°C/37°C for 24-48 h. The clones were confirmed by restriction digestion and PCRs of the plasmid isolated from transformants.

3.5.9 Cloning of RsaF in multicopy plasmid

All the cloning procedures were carried out as described (Sambrook and Russell, 2001). The *rsaF* gene without its endogenous promoter amplified from the genomic DNA of *S. aureus* Newman was cloned in the *E. coli–S. aureus* shuttle vector pCN40 at PstI/BamHI sites under P*bla* promoter using RsaF-F and RsaF-R primers (Table 11). The recombinant plasmid was electroporated into *S. aureus* strain Newman after passing through intermediate host *S. aureus* RN4220. The clones were confirmed by restriction digestion and also by DNA sequencing at MWG Eurofins Ltd., Bangaluru.

3.5.10 Construction of *rsaF* disruption strain

rsaF gene was disrupted by Kanamycin resistance (Km^r) marker, cloned into plasmid pMAD and passed through an intermediate strain *E. coli* DC10B followed by electroporation into *S. aureus* Newman, to achieve disruption of chromosomal *rsaF* by homologous recombination. A 1.4kb long (Km^r) gene was amplified with KanF and KanR primer pair from plasmid pBBRMCS2. The 679bp long upstream and 811bp long downstream regions of *rsaF* were amplified using primer pair RsaFupF-RsaFupR-Km and RsaFdnF-Km-RsaFdnR respectively (Table 11). The reverse and forward primers from the above pair shared an 18 bp homology with the kanamycin marker. An overlap extension PCR was carried out to fuse the amplified fragments. The resulting *rsaF* gene disruption cassette (*rsaF*GDC) was cloned into temperature-sensitive shuttle vector pMAD. The recombinant selection was done as described (Arnaud *et al.,* 2004). Briefly, recombinants were selected at 30˚C on TSA plates containing erythromycin and X-gal. Single blue colony containing recombinant plasmid was streaked on the same medium and incubated at 30˚C. In the next step, a single blue colony was cultured in TSB without any antibiotic and incubated with shaking at 30˚C for 2h, followed by 6h at 42˚C. Serial dilutions of this culture were plated on TSA plates containing X-gal and kanamycin followed by incubation at 42˚C overnight. Amongst majority of light blue clones which represents the integration of the vector by a single crossover event, a white colony represents a candidate clone resulting from double cross over event and loss of the vector. Kanamycin resistant and erythromycin sensitive white colony was selected and analyzed for *rsaF* chromosomal disruption by PCR and southern blot.

3.5.11 DNA/RNA hybridization studies

3.5.11.1 Southern blotting

Approximately 10 µg of genomic DNA of *S. aureus* was digested with restriction enzymes such as HindIII and PstI and separated on 0.7% agarose gel. Electrophoresis of DNA was done at low voltage for 4-5 h and the gel was processed as described in Sambrook and Russell, 2001. The gel was denatured with denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 45 min at room temperature under constant gentle agitation followed by a wash with double distilled water for 5 min. The gel was treated with neutralization buffer (1 M Tris (pH 7.4), 1.5 M NaCl) twice for 15 min at room temperature. Nylon membrane immersed in transfer buffer for 10 min was placed on the gel and DNA was transferred on the membrane by upward capillary transfer. The transfer was carried out for 18 h / overnight with 10X SSC buffer to obtain acceptable transfer of DNA from agarose gel. The transferred DNA was UV-cross linked and processed for hybridization as described below (in 3.5.11.4).

3.5.11.2 Total RNA extraction

Total RNA was extracted from *S. aureus* cultures by acid guanidinium isothiocyanate phenol chloroform extraction method (Chomczynski & Sacchi, 2006). Cells were lysed with lysostaphin (20 µg/ml) for 15 min. Cell lysate (1×10^7 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 β-mercaptoethanol for 20 min. To 1 ml of lysate, 0.1 ml 2 M sodium acetate (pH 4.0), 1 ml water saturated phenol (pH 4.0) and 0.2 ml chloroform / isoamyl alcohol (49:1) were added sequentially and mixed. Samples were 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 separated carefully and precipitated with equal volume of isopropanol. The RNA was precipitated overnight at -20°C followed by centrifugation. The pellet was washed with 70% ethanol, 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. Before electrophoresis, RNA was mixed with equal volume of 2X Formamide gel loading dye (95% de-ionized Formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 5 mM EDTA pH 8.0, 0.025% SDS) and denatured by heating at 65°C for 10 min followed by snap cooling.

3.5.11.3 Probe labeling

DIG High Prime labeling and detection kit from Roche Diagnostics was used that uses Digoxygenin (DIG), a steroid hapten for DNA probe labeling. Approximately 1 µg of PCR amplified gel purified DNA template in 16 µl was denatured by heating in a boiling water bath for 10 min followed by snap cooling. It was then mixed with 4 µl of 5X DIG-High Prime labeling mixture containing optimal concentrations of random primers, nucleotides, dUTP (alkali-labile), Klenow enzyme, and buffer components and incubated for 16 h at 37°C. The reaction was stopped by either adding 2 µl of 0.2 M EDTA (pH 8.0) or heating at 65°C for 10 min. The DIG labeled DNA probe was purified using High pure PCR product purification kit (Roche diagnostics, Germany). The purified labeled DNA probe was then quantified by the dot blot method described in the same kit and stored at -20°C until further use.

3.5.11.4 Hybridization and detection

Total RNA was separated on a 6% polyacrylamide gel containing 6 M urea in 0.5X TBE at 200 V, 15mA for 4 – 5 h or till bromophenol blue dye front reached the bottom of the gel. RNA on the PAGE gel was then electro transferred on neutral Hybond nylon membrane at 300 mA for 2 h in 1X TBE. The RNA was UV cross-linked to the membrane and processed for hybridization.

Table 12: Composition of 6% polyacrylamide 6 M Urea gel

Components	**Volume**
30% acrylamide:bisacrylamide mix	7 ml
Urea	12.6 gm
10X TBE	3.5 ml
Water	Make up the volume to 35 ml
10% ammonium persulfate	350 µl
TEMED	35 µl
Total	35 ml

Hybridization procedure was carried out as described in the DIG labeling kit manual (Roche diagnostics, Germany) with some 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 probe (25 ng/ml) at 37°C for 16-18h 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 treated with 1x blocking solution for 30

min to prevent non specific binding of antibody and immuno-detected with Anti-digoxigenin antibody-AP (75mU/ml) for 30 min. The nonspecific antibody binding was removed by washing the membrane with washing buffer (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.

3.6 Quantitative Real time PCR (qRT-PCR) assays

Real time PCR assays were performed using total RNA isolated at different time points from cultures grown in BHI. First-strand cDNAs were synthesized using Verso cDNA synthesis kit (ThermoFisher). Quantitative real-time PCR was performed using gene-specific primers (Table 11) with Maxima SYBR Green/ Rox qPCR Master Mix (2X) (Thermo Scientific, Massachusetts, USA) using Step-one real-time PCR system (Applied Biosystem). The first time point of the WT strain consisting of empty vector pCN40 was used as the calibrator and fold change in expression was calculated after normalization with 5S rRNA by 2^-ΔΔCt method (Livak and Schmittgen, 2001).

3.7 Hyaluronate lyase activity measurements

Hyaluronate lyase enzyme activity in the culture supernatant was assayed spectrophotometrically by measuring the absorbance of the liberated N-acetyl glucosamine (NAG) along growth as described (Makris et al., 2004), with few modifications. Culture supernatant was mixed with 0.6% HA solution [200 mM NaCl, 1% (w/v) sodium azide, 0.6% (w/v) HA (sigma)] and allowed to react at 37°C for 15 min. 20µl of potassium tetraborate (0.8 M, pH 9.1) was added and the samples were boiled exactly for 3 min followed by snap cooling. The color production was initiated by adding 600µl of 0.1 X DMAB reagent [10% (w/v) *p*-dimethylaminobenzaldehyde, 12.5% 10M HCl, 87.5% (v/v) glacial acetic acid] and the resulting solutions were incubated at 37°C for 20 min. The absorbance was measured at 544nm in a Beckman Coulter DU® 720 spectrophotometer and HL activity was plotted against the standard curve of NAG. The specific activity of hyaluronate lyase was expressed as NAG released min^{-1} ml^{-1} mg $protein^{-1}$.

3.8 Assessment of biofilm formation

3.8.1 Quantitative biofilm assay

Biofilm production by *S. aureus* modified strains was measured semi quantitatively by microtitre plate method as described (Zmantar et al., 2010). Overnight grown cultures were

diluted to an OD_{600} 0.05 into fresh BHI + 2% glucose with or without 2 mg/ml HA. 200 µl of diluted cells were seeded into 96-well microtiter plates and incubated at 37 °C for 24 h. The plates were washed thrice with 200 µl of phosphate buffer saline (PBS) to remove the non-adherent cells. Adherent cells were stained with 1% crystal violet for 45 min, washed with distilled water to remove excess strain, dried and solubilized in 200 µl of 33% glacial acetic acid. Biofilm formation was quantitated by the absorbance at OD_{570}. Each strain was tested in triplicates and data reported as the percentage compared to the control.

3.8.2 Confocal microscopy of biofilm formation

Microscopically, biofilm formation was observed by confocal laser scanning microscope (CLSM), as described (Hochbaum et al., 2011). Cells were grown adherent for 24 h on a glass cover slip immersed in 3 ml culture (OD600 0.05) in a Petri dish supplemented with BHI + 2% glucose with or without 2 mg/ml HA . The biofilms were washed with PBS and adhered cells were fixed with 5% gluteraldehyde for 1 h at 50°C. The cells were washed with PBS and labeled with FITC (0.001 %) for 30 min at room temperature. Biofilms on the glass cover slip were inverted and mounted on a clear glass slide and imaged on a Carl Zeiss LSM confocal inverted microscope.

3.9 Detection of Protease activity by zymography

The proteins were extracted by precipitation from an 8 hour grown culture supernatant by chloroform methanol extraction precipitation, by mixing supernatant : methanol : chloroform : water in the ratio of 1:4:1:3. Equal amount of proteins were taken to analyze for the protease activity in modified strains of *S. aureus* Newman.

The protease activity was detected by zymography using 0.1% gelatin as a substrate as described (Madhu et al., 2020). 12% SDS-PAGE gel electrophoresis was carried out at 100V in 1X SDS gel running buffer. The gel was washed twice with distilled water and incubated in renaturing buffer (2.5% Triton-X 100, 50mM Tris-Cl, 5mM $CaCl_2$, 1µM $ZnCl_2$) for 1 hour to remove SDS, followed by overnight incubation at 37˚C with gentle agitation. The gel was stained overnight with 0.5% Coomassie Brilliant Blue R 250 (Himedia Laboratories, India) and de-stained with Methanol: Water: Acetic acid (4:5:1) (V/V) solution till the zone of clearance was observed. The protease activity was observed as the bands of substrate degradation against the blue background.

3.10 RNA/RNA interaction

3.10.1 *In vitro* transcription, elution and labeling of RNA

DNA templates for the transcription of desired genes were generated by PCR amplification with the primer pair harboring T7 or T3 promoter in the forward primer using *S. aureus* genomic DNA. RNAs were *in vitro* transcribed from the purified DNA templates using T3 or T7 RNA polymerase (Roche, Germany) and rNTPs (Roche) as per manufacturer's instructions. RsaF was labeled by *in vitro* transcription using DIG labeling mix (Roche) containing Dig-11-UTP. After separation on 6M Urea/6% PAGE, RNA transcripts of the correct size were eluted and stored at -20˚C (Nilsen, 2013).

3.10.2 Electrophoretic mobility shift assay

The transcripts were denatured at 65°C for 10 min before using for binding assays. DIG-labeled RsaF transcript at a fixed concentration was incubated with increasing molar concentrations of unlabelled putative target mRNAs (*hysA* and *splD*) in RNA binding buffer (10mM Tris-Cl (pH 7.0), 100mM KCl, 10mM $MgCl_2$) at 37°C for 30 min followed by separation on 6% native polyacrylamide gel (Vockenhuber and Suess, 2012). Cold competition was done by adding unlabeled RsaF transcript to demonstrate the binding specificity. RNAs were then electro blotted on to the nylon membrane, UV-cross linked and visualized by chemiluminescence. RNA-RNA specific interactions were observed by a shift in electrophoretic mobility.

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

Reagents	Concentration
DIG-labeled RsaF	0.01 pmol
hysA / splD mRNA	1.0/2.0/3.0/4.0 pmol
10x RNA binding buffer	1 x
Cold RsaF	20, 100 fold excess
Nuclease free water (µl)	Up to total volume
Total volume	10.0 µl

3.11 Assay for mRNA stability

The mRNA stability within cells were estimated by analyzing the mRNA half-life following transcription inhibition by rifampicin, where changes in mRNA levels solely reflect mRNA degradation. *S. aureus* strains were grown till exponential phase (OD_{600} ~4.0) at 37 °C with shaking. Rifampicin (200 µg/mL) was added to the cultures to arrest transcription. Cultures

were harvested at 0 min (before adding the rifampicin), 1, 3, 5, 10, 20, 30 and 60 min post transcriptional arrest and processed for RNA isolation and qRT-PCR. 0 min was used as the calibrator and fold change in expression was determined after normalization with 5S rRNA by 2^-ΔΔCt method.