Summary

SUMMARY

Staphylococcus aureus, a versatile human pathogen is equipped with a large variety of pathogenicity determinants which are responsible for adhesion, invasion, and dissemination in host tissues, acquisition of nutrients and combating the host immune system. This multitude of gene expression is governed by several global regulatory systems such as *agr, sarA, rot, mgrA, sae,* sigma factor B and small noncoding RNAs (ncRNAs). Small regulatory ncRNAs in bacteria have emerged as one of the most important elements in regulatory hierarchy of gene expression, mediating diverse biological events including bacterial quorum sensing, biofilm formation, metabolism, oxidative stress, antibiotic resistance and expression of various virulence factors. The mechanism of regulation of ncRNA is linked with the modulation of transcription, mRNA stability, mRNA decay and translational activation or repression by base pairing with multiple mRNAs as targets or proteins. In last 10 years, a promising list of ncRNAs has been identified in *S. aureus* which play a major regulatory role in maintaining the balance of staphylococcal pathogenicity and adaptive processes.

This work is focused on elucidating the impact of two noncoding RNAs SprX and SprB encoded in the pathogenicity island of Methicillin Susceptible *Staphylococcus aureus*(MSSA) strain Newman, a clinical isolate, in the regulation of several virulence genes.

Computational characterization of reported ncRNAs namely SprA/B/C/D/E/F/G/X which are expressed from the pathogenicity island in Methicillin resistant *Staphylococcus aureus* Strain N315 (MRSA) were used to identify their homologues in MSSA strain Newman. The location of putative orphan promoter and rho-independent transcriptional terminator of the ncRNAs present in the intergenic region of *S. aureus* Newman were analyzed using bioinformatic tools Softberry and Erpin/Arnold respectively.

Several ncRNAs were screened for potential interaction with multiple virulent factors as mRNA targets using *in silico* target prediction tools TargetRNA1, TargetRNA2, RNApredator, IntaRNA and RNAup. Two ncRNAs were selected namely SprX and SprB both of which are highly conserved among the staphylococcal strains. SprX is

present in three copies; SprX1, SprX2, SprX3 at distant locations, whereas SprB is present in a single copy in the chromosome of *S. aureus* Newman.

Potential mRNA targets with continuous stretch of base pairing with SprX include delta hemolysin (*hld*), clumping factor B (*clfB*), staphylokinase (*sak*), staphylocoagulase (*coa*), immunoglobulin binding protein (*sbi*).Targets of SprB consists of clumping factor A (*clfA*), clumping factor B (*clfB*) and staphylocoagulase (*coa*). These targets which are essential for establishing pathogenicity of *S. aureus* infection were selected for further studies. SprX2 and SprX3 showed weak base pairing interaction with targets of SprX1 in bioinformatic analysis as both the copies differ from SprX1 by 6 nucleotides.

The ncRNAs SprX1and SprB along with their predicted promoters were cloned in the *E. coli*-staphylococcal shuttle vector pCN40, under the constitutive/methicillin inducible beta-lactamase promoter (*blaZ*) in both the sense and antisense orientation to achieve overexpression and knockdown constructs respectively.

sprX1 disruption in *S. aureus* strain Newman was achieved by homologous recombination of temperature sensitive, shuttle recombinant plasmid pIMAY containing the gene disruption cassette *sprX1::kan*. Disruption mutant was confirmed by PCR, Northern and Southern blot analysis.

The overexpressing strain of SprX1 expressed two different transcripts in northern blots, one from the cloned native endogenous promoter and vector borne promoter. Although, SprX1 overexpression from methicillin inducible blaZ promoter was low, higher expression of the intact RNA from the endogenous promoter in the multi-copy clone resulted in sufficient fold increase to make comparisons.

Overexpression and disruption of SprX1 resulted in 1.5-fold and 0.3- fold expression of delta hemolysin (*hld*) transcripts respectively in the quantitative real time PCRs. The increased expression of *hld* also indicated increased RNAIII synthesis, since *hld* is encoded within RNAIII. The alpha hemolysis activity was also tested, since alpha hemolysin (*hla*) is one of the well known targets of RNAIII. Cells overexpressing SprX1 exhibited increased delta and alpha hemolysis activity by 72% and 32% respectively as compared to the vector control in hemolysis assay of human and rabbit RBCs, suggesting that this sRNA may have a major influence in the host infection.

Clumping factor B, an important colonization factor present on the surface for biofilm formation in *S. aureus* was shown a target of SprX1with potential base pairing of 14 nucleotides within the 3' coding region of *clfB* mRNA. Upregulation of *clfB* by 2.24-fold and downregulation by 0.7- fold was observed in the SprX1overexpression and *sprX1* disruption respectively, in real time PCRs. Cells overexpressing SprX1 revealed increased adherence of biofilm by 40% in microtiter plate assay and on glass cover slip in confocal microscopic analysis. This marked increase in biofilm phenotype could also be attributed to increased levels of RNAIII, since RNAIII is reported to have influence on biofilm structuring and dissemination.

Complementation of overexpression plasmid pMNSprX in *sprX1* disruption mutant strain resulted in the restoration of delta and alpha hemolysis activities and biofilm formation.

The expression of other bioinformatically predicted targets, immunoglobulin binding protein (*sbi*), staphylocoagulase (*coa*) and staphylokinase (*sak*) did not show correlation in their expression with respect to SprX1 levels in real time PCRs. However, the downregulation of *coa* and *sbi* was observed under the disruption of *sprX1* suggesting that these may not be the direct targets for SprX1. In contrast, upregulation of *sak* mRNA levels by 3.6- and 8.2- fold was observed in both the SprX1 overexpression and disruption strains respectively as compared to the vector control.

The interaction of SprX1 on *clfB* and *hld* mRNA was assessed by *in vitro* interaction studies in the presence and absence of non specific competitor. Transcripts corresponding to the interaction region of ClfB and Hld were generated by PCR, cloning and *in vitro* transcription. Higher molecular weight complex of SprX1:Hld was observed best at the maximum ratio of 1:5 pmol and at 1:3 pmol for SprX1:ClfB complex. The duplex formation of either mRNAs with SprX1 was not affected when nonspecific competitor RNA was added.

SprX1 enhanced the virulence of *S. aureus* Newman in mice model of infection. The strain overexpressing SprX1 was found to be more pathogenic in comparison to *S. aureus* Newman containing vector control, knockdown and *sprX1::kan* mutant strains by intravenous infection. SprX1 overexpression exhibited an increase in the levels of pathophysiological markers-blood urea nitrogen (BUN), creatinine kinase(CK), serum transaminases (glutamic oxaloacetic and glutamic pyruvic acid) - SGOT and SGPT

which are used for assessing kidney, liver and heart function and also displayed organ disintegration, multiple abscesses in kidneys and lungs and high bacteria load in the infected kidneys.

Overexpression of another ncRNA SprB which is also encoded within the pathogenicity island of *S. aureus* Newman showed maximum expression from the endogenous promoter at the log phase which produced the transcript of 119 nt in Northern blot. Overexpression of SprB significantly downregulated the transcription of clumping factor (*clfA/B*) and staphylocoagulase (*coa*) along with the reduction in the biofilm formation even in the presence of the inducing agent glucose.

Production of golden carotenoid pigment staphyloxanthin was reduced to 32.4% in strains overexpressing SprB, over the phase of 14 days. This decrease was observed notably after incubation at cold temperature 4°C. SprB overexpression also resulted in marked increase in resistance to four β-lactam antibiotics namelyamyoxicillin, penicillin, penicillin V and ampicillin and increased sensitivity to aminoglycoside antibiotic gentamycin and β-lactam antibiotic cloxacillin.

SprB did not show much influence on the pathogenicity of *S. aureus* in the mice model of infection as there was no significant difference in the level of biochemical markers, morphological features and bacterial load in the infected organs of mice infected with strain overexpressing SprB and the vector control.

Thus in this study, SprX and SprB revealed a significant role in the expression of virulent factors and regulation of physiology of *S. aureus* Newman in influencing pathogenicity.

Precise knowledge on regulatory mechanisms of noncoding RNAs in controlling diverse virulence gene expression to establish pathogenicity would open up new perspectives for combating *S. aureus* infections using small RNAs as therapeutic agents in future.