5 Summary

Staphylococcus aureus is a high-risk pathogen for immune-compromised individuals, in addition to the nosocomial and community-associated infections caused by these organisms. *S. aureus* is the causative agent of diverse human and animal infections, including abscesses, toxic shock syndrome, septicemia, and endocarditis. Infections of *S. aureus* are very difficult to eradicate due to several cell surface-associated and extracellular virulence factors, and increasing resistance to antibacterial agents. The expression of these virulence factors is regulated by global transcriptional regulators, regulatory proteins, and two component systems.

Small regulatory RNAs (sRNAs) have been recognized as an essential factor of virulence control and are identified as regulators of processes or pathways that are central to pathogenesis, metabolism, quorum sensing, stress adaptation, biofilm formation, or host-pathogen interactions, etc. sRNAs exert its regulatory effects by base pairing with target mRNAs affecting transcription, mRNA translation/stability, and by modifying the activity of proteins that act as translational repressors.

This study is aimed at uncovering the functional role of sRNA RsaF in the regulation of *S. aureus* virulence, amongst the other reported sRNAs. RsaF was analyzed *in silico* by Target RNA, IntaRNA and RNA predator programs to determine the putative mRNA targets. Potential virulence genes based on base-pairing interaction were selected for further studies. HysA, an extracellular enzyme capable of degrading hyaluronic acid present in the connective tissue, and SplD, a serine protease that acts on several olfactory receptors which are trans membrane proteins expressed in nares, the primary colonization niche of *S. aureus* were selected as the putative targets of RsaF.

Altered levels of RsaF were achieved by constructing *rsaF* overexpression and disruption strains to analyze the effect on target gene expression. RsaF was overexpressed from the pBla promoter in the *E. coli–S. aureus* shuttle vector pCN40. Two transcripts of RsaF were observed in the northern blots from overexpression strain, one each from the endogenous and the plasmid-borne promoter. Chromosomal disruption of RsaF was achieved by insertion of a kanamycin resistance gene by homologous recombination. Chromosomal disruption of *rsaF* was confirmed by southern blotting.

Overexpression of RsaF resulted in 2-4 fold increase in *hysA* mRNA levels as well as hyaluronate lyase enzymatic activity. While its disruption reduced *hysA* transcripts by 0.2 to 0.0002 fold and enzymatic activity by 0.2-0.1 fold. Disruption of *rsaF* displayed marked

down-regulation of *splD* transcripts by 0.8-0.005 fold and reduced activity of multiple proteases by zymography, whereas RsaF over expression did not show any significant difference when compared with the control.

The physiological influence of RsaF on biofilm formation was analyzed by crystal violet-stained microtiter plate assay and confocal microscopy. RsaF disruption strain led to a 45% increased production of biofilm compared to the control strain, in normal media and further increased by 93% when supplemented with HA. On the contrary, the overexpression of RsaF resulted in 20% decreased levels of biofilm formation under normal growth medium and decreased to 27% when supplemented with external HA. Microscopically, the disruption strain demonstrated complex biofilm composed of tightly packed cells in presence of exogenous HA.

The base pairing interaction of RsaF with *hysA* mRNA and *splD* mRNA was demonstrated by electrophoretic mobility shift assay. The specificity of the RsaF-HysA and RsaF-SplD mRNA interaction was established by cold competition in the presence of unlabeled RsaF. The strong interaction of RsaF with *hysA* and *splD* mRNA *in vitro* implies that RsaF influences the expression of HysA and SplD by direct interaction with its target mRNAs.

The stability of both *hysA* and *splD* mRNAs were enormously reduced in the absence of a functional RsaF. In a rifampicin mediated transcription inhibition, the *hysA* mRNA displayed a half-life of ~18 min in control, and was reduced to 1 min in *rsaF* disruption strain and the half-life of *splD* mRNA was ~10 min in the control which was decreased to less than 1 min in the RsaF mutant. Overexpression of RsaF leads to a 15 % -18 % increase in mRNA levels and half-life of 17 min for SplD, while it was less distinct for HysA, with a half-life of 21 min.

Overall this work establishes that RsaF positively regulates the virulence factors and the associated physiology of *S. aureus* Newman.