REGULATION OF *STAPHYLOCOCCUS AUREUS* VIRULENCE FACTOR(S) BY NON-CODING RNA

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INTRODUCTION

Staphylococcus aureus is a yellow pigmented, gram-positive bacteria which causes range of illness from minor skin infections such as impetigo, cellulitis, pimples, boils, folliculitis to life-threatening diseases such as meningitis, endocarditis, pneumonia, or septic shock. The high morbidity and mortality in *S.aureus* pathogenesis is mediated by the production of virulence factors which includes secreted toxins, capsule, extra cellular enzymes and several surface proteins contributing to host adhesion. The expression of these virulence factors are controlled by transcriptional factors, two component systems and regulatory RNAs [10].

Non-coding RNAs are bacterial small untranslated regulatory RNAs typically 50-500 nucleotides in length. They are usually expressed from Intergenic Region (IGR) or genomic islands under specific stress responses or during virulence, iron uptake, quorum sensing, biofilm formation and pathogenicity. These ncRNAs exert their regulatory functions by base pairing with mRNA or by interacting with proteins thereby affecting mRNA translation and the activity of specific proteins [2]. An increasing number of non-coding RNAs (ncRNAs) have been described in different pathogenic bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Vibrio cholerae* and *Pseudomonas aeruginosa* during the last few years.

Several ncRNAs are known to regulate virulence genes in *S.aureus.* RNAIII is the first regulatory sRNA of *S.aureus* to be discovered that regulates the levels of alpha hemolysin, protein A and *Rot* by direct base pairing with their respective mRNAs [2]. SprD, sRNA of *S.aureus* down regulates *sbi* (IgG binding protein) mRNA by sequestering the ribosomal binding site of the *sbi* [5]. SprX ncRNA in *S.aureus* enhances the levels of delta haemolysin and Down regulates spoVG which contributes to *S.aureus* glycopeptide resistance [8]. RsaA, a staphylococcal conserved non-coding RNA, directly regulates the expression of master regulatory protein MgrA at the post-transcriptional level. Through MgrA regulation, RsaA activates biofilm formation and inhibits capsule synthesis.

RATIONALE OF THE STUDY

Staphylococcus aureus is known to have approximately 500 noncoding RNAs reported in SRD (Staphylococcal Regulatory Database) database to date of which a few are functionally characterized. The current study aims at the functional characterization of noncoding RNA RsaF of *S.aureus*. Initial investigation on ncRNAs of *S.aureus* leads to selection of ncRNA RsaF which showed a potent virulence factor HysA as its putative target. By elucidating the regulatory effect of the ncRNA RsaF on the virulence of *S.aureus* would provide advanced knowledge on the regulation of pathogenicity of *Staphylococcus aureus* in human.

OBJECTIVES OF THE STUDY

1. Construction of strains to achieve altered level of expression of RsaF ncRNA in *S.aureus* Newman by its over-expression and Disruption.
2. Analysis of the influence of altered expression of RsaF on target gene expression. (at mRNA level by qRT-PCR and at translational level by physiology assay)
3. Assessment of direct interaction of RsaF with the target by Electro Mobility Shift Assay.

RESULTS

Non-coding RNAs (RsaA-K) reported by Geissmann *et al. 2010,* were analyzed bioinformatically. Bioinformatics analysis of these ncRNAs was carried out to determine their potential regulatory role on candidate virulence genes as targets. Among these, ncRNA RsaF, having pathogenicity genes as putative targets was selected for further study. RsaF is a 104 nt long ncRNA flanked by two hypothetical proteins. Putative targets for RsaF were identified using online programs, RNA predator and Target RNA. RsaF shows *in-silico* interaction with HysA (Hyaluronate lyase) and splD (Serine protease like protein) as its putative target.

Hyaluronate Lyase, a potential virulence determinant of *S.aureus* is an extra-cellular enzyme capable of degrading acidic mucopolysachharide of the connective tissue. HysA is

known as the ‘spreading factor’ and is involved in the early stages of infection. HysA is known to express at early to mid exponential phase of growth in *S.aureus*.

SplD, another putative target of RsaF, is one of the six genes in Spl operon (Spl- Serine protease-like genes (splA-F)). Staphylococcal serine proteases encoded in the spl operon are the secreted proteolytic enzymes. The operon is located on a pathogenicity island vSaß and is adjacent to the genes encoding the enterotoxins and leukocidins, the well-characterized virulence factors located on pathogenicity island vsaβ. This operon is expressed at early stationary phase and is positively regulated by Agr (Accessory gene regulator).

RsaF over-expression and Disruption construct

RsaF over-expression strain was constructed by amplifying RsaF gene from *S.aureus* Newman genomic DNA using RsaF specific primers followed by cloning under the p*bla* promoter in *Escherichia-Staphylococcal* shuttle vector pCN40. The construct was transformed in *S.aureus* Newman (pCNRsaF) and the clone was confirmed by PCR and restriction digestion. Subsequently the expression of pCNRsaF was analyzed by northern blotting.

A gene disruption cassette was generated by inserting a kanamycin marker to disrupt the chromosomal copy of RsaF by homologous recombination. RsaF gene disruption Cassette was cloned into pMAD temperature sensitive *E.coli-S.aureus* shuttle vector followed by transformation in *S.aureus* Newman. Transformant was selected on X-gal plates and further confirmation was done by PCR with different primer pairs.

Disruption of RsaF by CRISPR-Cas9 genome editing system is also underway.

Expression of HysA under RsaF over-expression

Expression of HysA was analyzed by qRT-PCR under RsaF over-expression. HysA was found to be up-regulated by 20 folds in pCNRsaF at mid exponential phase.

Hyaluronate lyase (HL) in the culture supernatant was assayed by HL activity assay to analyze the HysA expression at translational level. This HL activity assay indicate that expression of HysA is up-regulated by 2.21 fold under RsaF over-expression at mid exponential phase.

The results at transcriptional level as well as in physiology indicate that the expression of HysA is positively regulated by RsaF.

Interaction of RsaF with splD

The interaction of RsaF with splD mRNA was analyzed by Electro Mobility Shift Assay (EMSA). RsaF and splD mRNA was transcribed in-vitro where one labeled RNA was allowed to react with the increasing concentration of other RNA under standard conditions. RsaF-*splD* mRNA complex formation was seen in a form of shifted band in EMSA, thus proving splD is the direct target of RsaF.

WORK TO BE DONE :

- Selection of RsaF disruption construct by shifting the culture at higher temperature to achieve the homologous recombination.
- Expression analysis of HysA (by qRT-PCR and enzyme activity assay) using RsaF disruption strain.
- Analysis of direct interaction of RsaF with HysA mRNA by Electro Mobility Shift Assay.
- Analysis of relative expression of splD (by qRT-PCR) and physiology (by Zymography) under altered levels of RsaF.

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