INTRODUCTION

Staphylococcus aureus, an opportunistic pathogen, produces a vast array of virulence factors responsible for adhesion, invasion, acquisition of nutrients, spreading in host tissues and for combating the host immune system (Novick 2003). Pathogenesis of *Staphylococcus aureus* relies upon its remarkable adaptation to diverse host environments, which involves regulated secretion of virulence factors, two-component systems, global regulatory proteins and small non-coding RNAs. Bacterial ncRNAs are 50-500 nucleotides long regulatory molecules that fine-tune various physiological processes, regulate virulence factors and influence pathogenicity. Non-coding RNAs control gene expression through a diverse mechanism, commonly at the post-transcriptional level by binding with target mRNA. These interactions positively or negatively regulate the mRNA translation and stability.

The recent genome-wide approaches in *Staphylococcus aureus* shows that around 10–20% of genes code for RNAs with important regulatory functions in adaptive processes. (Tomasini *et al*., 2014). *S. aureus* expresses around 750 ncRNAs most of which have unknown functions (Pabic *et al*., 2015). Several ncRNAs in *S. aureus* play a significant role in the regulation of surface and secreted toxins, biofilm formation, oxidative stress and antibiotic resistance (Romby *et al*.,2010; Kim *et al*.,2014; Eyraud *et al*.,2014). One of the well-characterized ncRNA is RNAIII, which is involved in the regulation of various virulence genes in *S.aureus*. RNAIII positively regulates the α-hemolysin (*hla*) expression by sequestering the anti-Shine and Dalgarno sequence hence activating hla translation, and negatively regulates the expression of a repressor of toxins (*rot*), protein A (*spa*), autolysin (*lytM*) and *sbi* mRNA by directly base-pairing and inhibits their translations. (Novick *et al*., 1993; Geisinger *et al.*, 2006; Chunhua *et al*., 2012; Chabelskaya *et al.*, 2014). Other examples of ncRNA involved in virulence gene regulation in *S. aureus* include SprD, which negatively regulates the expression of the immune-evasion molecule *sbi* (Chabelskaya *et al*., 2010), RsaA that regulates the mgrA and act as virulence suppressor (Romilly *et al*., 2014), and SprC which attenuates bacterial virulence and host cell phagocytosis (Pabic *et al*., 2015).

RATIONALE OF THE STUDY

Staphylococcus aureus exhibits resistant to multiple antibiotics and evolved a large number of strategies to specifically reprogram the expression of virulence genes for adaptation in various environmental conditions, also include the use of non-coding RNAs as an important regulatory molecule. Among the long list of expressed ncRNAs in *Staphylococcus aureus*, few have been functionally characterized. Identification of the targets and physiological functions each ncRNA is necessary for understanding their specific roles. The present study is focused on the small non-coding RNA SprX (Small pathogenicity island RNA X), encoded in the pathogenicity island control the expression of virulence genes such as delta hemolysin and clumping factor B in *Staphylococcus aureus* Newman (Kathirvel *et al*., 2016). SprX plays an important role in bacterial resistance to glycopeptide antibiotics, negatively regulates the expression of SpoVG and extracellular complement binding protein expression through the direct interaction with the ribosomal binding site (Eyraud *et al*.,2014; Ivain *et al*.,2017). Identification and control of potential target genes by SprX will permit a better understanding of the implication of SprX in *S. aureus* virulence network.

OBJECTIVES OF THE STUDY

I. Constuction of disruption and/or knockdown strain of SprX
II. Analysis of the influence of ncRNA SprX on the expression of secreted and cell-wall associated proteins of *S. aureus* and selection of differentially regulated proteins
III. Analysis of the influence of altered level of SprX on transcription of selected targets
IV. Assessment of direct/specific interaction of SprX with target mRNA

RESULTS

Procurement/construction of a modified strain of SprX

Here we have analyzed the functional role of ncRNA SprX in the regulation of virulence of *Staphylococcus aureus* strain Newman. A SprX overexpressing strain pMNSprX, previously generated in the lab was used to analyze the proteomic difference in *S. aureus*

Newman. SprX knockdown strain was constructed using pCN40 *S.aureus-E.coli* shuttle vector. Briefly, a 220bp fragment of SprX was obtained by PCR and cloned in the antisense orientation into pCN40 plasmid. Cloned knockdown SprX plasmid was transformed into *S.aureus* Newman. Attempts were also made for the construction of disruption strain of SprX using pMAD plasmid.

SprX differentially regulates the protein profile in *S. aureus* Newman.

Impact of the enhanced level of SprX was examined on extracellular and cell wall-associated proteins utilizing two-dimensional gel electrophoresis. A key down-regulated protein was identified as Immunodominant Staphylococcal Antigen A(IsaA). IsaA is a 29 kDa, profoundly antigenic protein that diminishes the biofilm formation in *S.aureus* (Lorenz *et al.,* 2000). It has a lytic trans-glycosylase domain that promotes lysis and regulates clumping of cells, thereby playing a crucial role in staphylococcal growth and survival (Sakata *et al.,* 2005).

Additionally, SprX was shown to directly or indirectly influence the activity of several autolysins in *S.aureus*. Effect of increased level of SprX on the cell wall degradation activity was analyzed using zymography of secreted proteins as well as its quantitative measurement that revealed 20% downregulation in cell wall degradation as compared to control in total autolysis assay. Altered expression of various proteins of ~101kDa,~82 kDa, ~65 kDa and ~29kDa was observed in zymography. Reduced level of ~29kDa protein indicates the IsaA activity and ~65 kDa protein may indicates AtlA activity. AtlA is the most prominent murein hydrolase expresses in *S. aureus,* involved in cell division and important in the initial stage of biofilm formation. IsaA, AtlA and LytM other major autolysin expresses in *S. aureus* are positively regulated by YycG/YycF (also known as WalKR), an essential two-component system. YycG/YycF (WalKR) is involved in *S. aureus* virulence and mainly control the cell wall metabolism (Dubrac and Msadek 2004, Dubrac *et al*., 2007, Dubrac *et al*.,2008).

SprX regulates the expression of the gene involved in cell wall metabolism.

Cell wall degrading activity and autolysis is regulated by YycFG in *S. aureus*. Among the YycG (essential histidine kinase) and YycF (the response regulator), YycF plays an important role by positively regulating staphylococcal autolysis (Zheng L., *et al* 2015). Effect of the modified level of SprX on *isaA, yycF, atlA,* and *lytM* mRNA expression was analyzed using qRT-PCR, exhibited more than 90% reduced expression in knockdown strain as compared to control, while no difference was observed in the strain expressing an increased level of SprX. Data indicates that in addition to regulation of IsaA, SprX is also involved in the regulation of autolytic activity in *S. aureus.*

Binding of SprX to the putative mRNA region of *isaA* and *yycF*

Putative RNA-RNA interaction between SprX and target mRNA (*isaA* and *yycF*) was predicted *in silico* using IntaRNA program. SprX showed binding at 3'region of *isaA* mRNA and at 5' region on *yycF* mRNA. The possible interaction and regulation of IsaA and YycF by SprX was analyzed by *in vitro* RNA-RNA interaction in gel mobility shift assay. Complex formation was observed between SprX and *yycF* mRNA in the gel mobility shift assay. Specificity of the complex was further analyzed by the competition of nonspecific RNA PhrD, which indicated SprX regulates the YycF expression by direct binding to the *yycF* mRNA.

WORK TO BE DONE

I. Analysis of autolytic activity of proteins under strain expressing reduced level of SprX
II. Elucidation of direct and specific interaction of SprX with *isaA* mRNA

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