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Review Article

Multifunctional Role and Regulation of RNAIII of the Agr Quorum Sensing System in *Staphylococcus aureus*

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Abstract

Advancement of genomics reveals a repertoire of small non-coding RNAs (sRNAs) which are involved in the regulation of many virulence factors of human pathogen such as *Staphylococcus aureus*. In the past two decades extensive studies on sRNAs in this bacterium confirm their direct or indirect involvement in complex regulatory network. RNAIII of the Agr quorum sensing system is the most extensive studied sRNA in *S. aureus*. It is the largest known regulatory sRNA of 514 nts in length with 14 structural stem loops with a half-life of more than 45 min, which suggest that it may, interacts with many targets. A majority of the clinical isolates produce a high level of RNAIII *in vitro* and *in vivo*. RNAIII has been shown to play a key role in virulence regulation by affecting many virulence factors. Previous studies suggest that RNAIII directly or indirectly affects many regulatory pathways, virulence factors and global regulators. However, their regulatory mechanisms and its role in regulatory circuits are still not fully understood. This review focus on in-depth analysis of known discoveries of RNAIII regulation, it's mode of action and future perspectives. The review will help readers to understand the multi factorial role and regulation of RNAIII in complex regulatory circuits of *S. aureus*.

Introduction

Small RNAs typically extend from 50 to 600 nts in length and present in order of hundreds in the genome of gram positive and gram negative bacteria. Majority of sRNAs in bacteria were discovered by computational method. They have well defined secondary structures which comprised helix and loop regions for direct and long distance interactions respectively [1,2]. Most of the sRNAs act by blocking the Ribosomal binding site (RBS) of the target mRNA and inhibit the translation but some also bind at the 5' and 3' untranslated regions of mRNAs and affect the stability and expression of mRNA [3-5]. Recent advancement in sequencing methods, particularly deep genome sequencing accelerated the small RNA research in bacteria in the last decade. It looks like the central dogma of biology which state the DNA as a master regulator stands to be modified because increasing knowledge of small RNAs suggest that RNA could also regulate DNA and proteins. *Staphylococcus aureus* is an important human and animal pathogen that causes superficial skin to deep tissue infections [6-8]. The capability of causing a variety of infections by this organism is due to the production of numerous virulence factors which are tightly regulated by transcriptional regulatory proteins, two-component systems and small regulatory RNAs [9-11]. Accessory gene regulator (Agr) is a well-defined quorum sensing system in *S. aureus*, which plays an important role in virulence gene regulation and affects numerous virulence factors such as capsule polysaccharide, biofilm formation and toxins production [11]. The effector molecule of this two-component system is the largest known small RNA known as RNAIII, which also encodes a small protein, delta hemolysin, at the 5' region [11]. In the past two decades RNAIII has been extensively studied and well established as a master player in virulence regulation of *S. aureus* in laboratory as well as clinical strains [12,13]. RNAIII acts via antisense mechanism by direct base pairing with its target mRNAs at post-transcriptional and translational level. It can also affect downstream genes indirectly by targeting transcriptional regulators and proteins [14-17]. This suggests that RNAIII may have numerous targets with diverse mechanisms of regulation in *S. aureus*. Currently, the known direct targets of RNAIII are *hla* (alpha hemolysin), *spa* (protein A), *sa1000* (fibrinogen binding protein), *sa2353* (coagulase precursor) and transcriptional regulators Rot and MgrA [15-17]. However, Rot and Agr transcriptomes only partially overlap and we have recently shown that RNAIII only affects part of MgrA regulon [17]. This advocates that our understanding of RNAIII mediated virulence regulation in *S. aureus* is not complete and more research with advance biochemical methods are needed.

RNAIII is a Member of Quorum Sensing System in *S. aureus*

The most well-defined key regulatory systems in *S. aureus* are Accessory gene regulator (Agr) and Staphylococcal accessory regulator (SarA). The Agr quorum sensing system plays a central role in virulence regulation and pathogenesis of *S. aureus*. Agr regulates the expression of several cell surface proteins, exotoxins, adhesion molecules and virulence factors [9-11]. Its role in pathogenesis has been demonstrated in several animal infection models such a subcutaneous abscess, arthritis and rabbit endocarditis [8-10]. The *agr* locus generates two primary transcripts, RNAII and RNAIII, from two divergent promoters, P2 and P3, respectively (Figure 1). The P2 operon is a sensory cascade that consist four genes *agrB, agrD, agrC* and *agrA*. AgrD

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is a precursor for auto inducing signaling molecule (AIP) which is processed and transported by AgrB. AgrC is a sensor histidine kinase and AgrA is a response regulator. AgrC binds to extracellularly accumulated AIP and activates AgrA which binds to the promoters P2 and P3 and mediates RNAII and RNAIII transcriptions. RNAIII is a regulatory RNA but also encodes a small toxin called alpha-hemolysin. This positive feedback mechanism of quorum sensing system fine-tunes the regulation of virulence genes at specific cell density [11]. The agr quorum sensing system has two modes of gene regulation. RNAIII independent gene regulation mediated through AgrA which includes metabolic and *psm* genes, and RNAIII dependent gene regulation which include toxins, proteases, transcription factors and regulatory proteins [18]. RNAIII regulates many genes by direct base pairing with target mRNAs such as *hla, spa, fnb, map* [12-15] or indirectly through targeting global transcription factors such as Rot and MgrA [12,16,17].

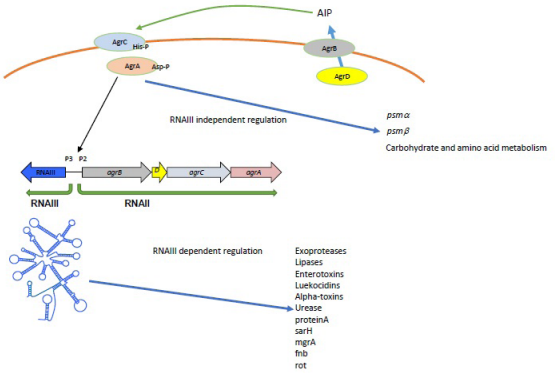

Figure 1: Model of agr quorum sensing system and RNAIII regulation. The RNAIII structure is adapted from [19].

RNAIII is a Multi-Structural/Multi-Functional sRNA in *S. aureus*

Comparative genome analysis of *S. aureus* strains suggest the presence of 12 sRNAs, of which 7 are present in pathogenicity island (SprA,B,C,D,E,F,G) and 5 in the genome [20]. Another computational approach found 47 novel sRNAs in *S. aureus* [21,22]. Most of these sRNAs have conserved c-rich motifs which are required for binding at ribosomal binding site and inhibit translation. However, RNAIII which is known as the largest small RNA in *S. aureus* comprised both short distance and long distance interacting regions (Figure 2). RNAIII is 514 nts in length which consist 14 stem loops and two long distance motif structures [19]. The 5'domain of RNAIII (helix H2 and H3) interacts with alpha-hemolysin mRNA to activate its expression [23]. The helix H7, H13 and H14 has c-rich motifs which interact with target mRNA of *rot* (repression of toxin), *coa* (coagulase) and *spa* (protein A) by antisense mechanism and repress the synthesis of these virulence factors [19] (Figure 2). Rot is a pleiotropic transcription factor which regulates toxins, cell surface proteins, proteases and transporters [24]. RNAIII directly interacts with Rot mRNA post transcriptionally to inhibit protein synthesis [14,15]. Several exoproteases and toxins are repressed by Rot but activated by RNAIII such as serine proteases (*splA/F*), cysteine proteases (*sspA/B*), lipase (geh), hemolysins (*hla* and *hld*) [15,25]. However, cell surface proteins and virulence factors such as clumping factor B (*clfB*) and protein A (*spa*) have been shown to be activated by Rot but repressed by RNAIII [24,26]. Hence, RNAIII and Rot work as antagonist and most of the toxins and cell surface proteins are indirectly regulated by RNAIII through repression of Rot protein [24]. However, transcriptomes of Rot and Agr only partially overlap [12,24]. Several exonucleases such as RexA, DnaQ and cell surface proteins such as clumping factor A, iron regulated cell surface proteins and extracellular matrix and plasma binding proteins are differentially expressed in Δ*agr* but not affected by Rot in our recent RNA-seq analysis (unpublished data). This suggests that RNAIII might interact with other targets that have not been identified. Recently, we reported that RNAIII activates global transcription factor MgrA, which is known to affect more than 300 genes in *S. aureus* [17,27]. RNAIII directly binds to the 5' untranslated region of *mgrA* mRNA and increases its stability to mediate the effect on virulence factors such as capsule polysaccharide, spontaneous autolysis and alpha hemolysin (*hla*) [17]. However, we found that only part of *mgrA* regulon are affected through this mechanism. Furthermore, we compared the transcriptomes of Rot, MgrA and Agr and found 64 genes which are differentially expressed in Δ*agr* but not affected by Rot or MgrA [12,24,27]. This emphasizes that our knowledge of RNAIII regulation in *S. aureus* is still incomplete. Previous studies have linked the Agr system with several two-component systems such as ArlRS, SaeRS, SrrAB [28,29], indicating that RNAIII could interact with these regulators to affect its downstream genes. It is interesting that RNAIII potentially base-pairs with SarT in silico [14], another member of SarA family which repressed the alpha hemolysin [30]. sRNA regulation is majorly through direct RNA-RNA interactions however there are several studies which suggest the indirect regulation. The indirect regulation is mainly mediated through proteins or other RNAs. Several *in vitro* biochemical methods have been attempted to identify the interacting proteins of a known RNA/sRNA in bacteria [31,32,33]. Recently, an *in vitro* study using streptavidin aptamer based pull-down assay was used to identify interacting proteins with RNAIII [34]. However, this study lacks the *in vivo* environment of bacterium which is important for proper folding and secondary structure of RNA and its interactions. RNAIII has been shown to directly interact with a transcriptional regulatory protein WalR [34] and WalR directly bind to the 5' untranslated region of a major autolysin, *lytM* in *S. aureus* [35]. Moreover, microarray study showed that *agr* repressed the expression of *lytM* [19]. Thus, RNAIII may indirectly affect the expression of *lytM* through direct interaction with WalR protein. There are several important regulatory proteins which have been shown to interact with RNAIII in *in vitro* pull-down assay such as Clp proteases ClpX and ClpC, transcriptional regulator MgrA, WalR and cell division protein FtsZ [34]. These studies suggest that RNAIII interact directly and indirectly with diverse targets including sRNAs, two-component systems, transcriptional regulators and proteins to mediate its regulatory functions.

Future Perspectives

This review provides a comprehensive overview of RNAIII roles and regulation in *S. aureus* virulence regulation. We anticipate that availability of super resolution microscopy would help in the real time 3-D structure of RNAIII and its target interactions. High throughput global approaches to identify direct and indirect target molecules will leads to the development of new therapeutic approach against this human and animal pathogen. Our knowledge to establish sRNA as a major cellular component is strengthening day by day by the evident

role of sRNAs in cellular processes, regulatory functions and pathogenesis. However, how they fit in the known cellular architecture remain largely unknown. They could function as a mediator of pathway or independently generated and regulate the cellular processes. In present time, antibiotic resistance is a major problem for the available drugs against gram positive as well as gram negative bacteria, therefore development of alternative therapeutic approach is much needed. We believe targeting sRNA could be very useful for drug resistance strains and development of alternative approach to treat infections. It is interesting that sRNA regulation is by transient interaction with its counterpart whether mRNA or protein, hence create less selective pressure on bacteria that suggest targeting sRNA will create less resistance development in bacteria instead of targeting DNA or protein. Now, it is well established that sRNA play significant role in almost all cellular physiology but how bacteria respond to diverse environment and various extracellular and intracellular environment and how these sRNAs integrated in the cellular response will require further studies. Whole genome sequencing and advance computational method will help in further understanding of sRNA regulation in diverse bacterial species.

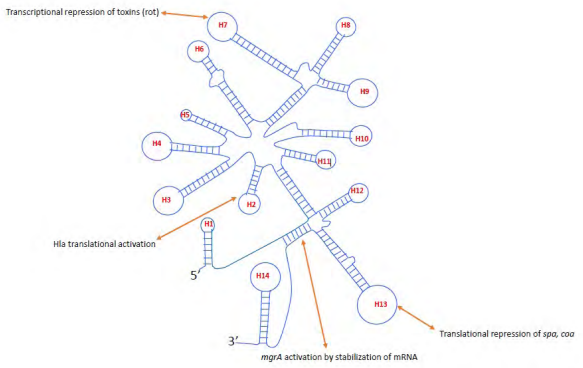

Figure 2: RNAIII structure and its interacting regions. The RNAIII structure is adapted from [19].

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