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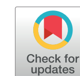
Prokaryotic ncRNAs: Master regulators of gene expression



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ABSTRACT

ncRNA plays a very pivotal role in various biological activities ranging from gene regulation to controlling important developmental networks. It is imperative to note that this small molecule is not only present in all three domains of cellular life, but is an important modulator of gene regulation too in all these domains. In this review, we discussed various aspects of ncRNA biology, especially their role in bacteria. The last two decades of scientific research have proved that this molecule plays an important role in the modulation of various regulatory pathways in bacteria including the adaptive immune system and gene regulation. It is also very surprising to note that this small molecule is also employed in various processes related to the pathogenicity of virulent microorganisms.

1. Introduction

Central Dogma is a very familiar term to all the researchers who predominantly work on molecular biology. Though this particular term has its significance and tells us about the basic theme of molecular biology, nowadays scientific research spanning the last few decades has also discovered other molecules playing significant roles in this particular basic theme. The established principle of central dogma is that DNA acts as a blueprint for the process of transcription of mRNA and consequently, this mRNA encodes the protein in a process called translation and proteins do all the functions in any biological system where gene regulation has a vital role to play in the energy conservation of any organism, where depending on the requirement, few proteins are overexpressed and few proteins are expressed at a very basal level at any given point of time (Saw et al., 2021).

As already mentioned, in the last two decades we have observed the discovery of a wide range of RNA molecules (especially non-coding RNAs; ncRNAs) having various physiological and biological functions starting from bacteria to human beings (Patil et al., 2014; Yao et al., 2019). This particular group of RNAs plays a very important role as a key regulator for the control of gene expression (Barbosa et al., 2020) through epigenetic regulation and their role is not only confined to higher eukaryotes or mammals, they are very much present in bacteria and as well as in Archaea. The discovery of a wide range of ncRNAs indeed shapes our understanding of molecular biology from a different perspective altogether. The protein is not the ultimate end product and gene regulation can also be at various levels where these molecules participate in various biological processes (Soltani-Fard et al., 2021).

The literal meaning of ncRNA is RNA which does not code for any protein (Panni et al., 2020; Makunin and Mattick, 2006), but, does not restrict its function in various physiological events, rather it is imperative to note that these molecules are of importance and can be classified into various groups of microRNAs and small nucleolar RNAs (snoRNAs) (Hombach and Kretz, 2016). Though the discovery of all these molecules is not yet done, it can be presumed that these molecules can be a potential part of the RNA regulatory network, and the gene expressions are regulated at various levels by the RNA regulatory networking pathways. Understanding these ncRNA molecules is important especially in the prokaryotes as this information will help us to decipher the molecular evolution of these molecules from prokaryotes to eukaryotes.

2. Strategies/approaches for identification of ncRNAs

Although ncRNAs cannot code for a protein, they perform a wide range of functions in Bacteria, Archaea, and Eukarya (Hüttenhofer and Vogel, 2006). They are mainly involved in the maintenance of chromosomes, regulation of transcription, processing of RNAs, translation, etc. (Yao et al., 2019). The ncRNAs identified in the past few decades are estimated approximately hundreds per bacterial genome as well as thousands per eukaryotic genome (Harris and Breaker, 2018). Presently,

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biocomputational prediction is employed in identifying ncRNAs in the genome of diverse model organisms (Wang et al., 2013). For instance, four different experimental strategies (Experimental RNomics) used for the identification of novel ncRNAs are (i) RNA sequencing, (ii) parallel cloning of non-coding RNAs through producing cDNA libraries (iii) microarray (iv) genomic SELEX (Hüttenhofer and Vogel, 2006; Zimmermann et al., 2010).

(i) RNA sequencing: The direct sequencing of ncRNAs is the most frequently used method for the identification of ncRNAs. In earlier days, it is used to identify tRNAs and rRNAs. It is widely employed to visualize and sequence the RNAs of different Gram-positive bacteria (Pichon and Felden, 2005; Trotochaud and Wassarman, 2005). The ncRNAs do not need to be reverse transcribed in this case. Previously, single ncRNA was identified based on their separation according to their size on denaturing gels. Distinct specific bands excised following visualization which represents single ncRNA (Hüttenhofer and Vogel, 2006). Therefore, ncRNAs must be present in high amounts which allowed their visibility as single specific bands. ncRNAs are labeled at their 5′ or 3′ ends before identification by sequencing. The 5′ end labeling is performed by the sequential removal of 5′phosphate groups with the aid of calf intestinal alkaline phosphatase followed by the addition of polynucleotide kinase and ATP gamma-^{32}P. The 3′ end-labeling of ncRNAs is done using T4 RNA ligase (Rio, 2014; Nilsen, 2014). In case of RNA extraction from the organism, the RNAs are sometimes labeled in vivo with orthophosphate which gets readily incorporated into the nucleic acids following their cellular uptake. Here, the extracted RNA is labeled randomly at any nucleotide which may be further used in RNA fingerprinting. 2D RNA fingerprinting as well as enzymatic or chemical sequencing of ncRNAs is used for sequence analysis of novel ncRNA after their extraction, separation, and elution from the gel. In the enzymatic sequencing method, the 5′or 3′ end-labeled ncRNAs are partially digested with adenine (A)-, guanine(G)-, cytosine(C)-, and uracil(U)-specific ribonucleases at a high temperature of 50–55 °C with 7M urea to prevent the interference of secondary and/or tertiary structure of RNA with their hydrolysis (Ramos and Laederach, 2014). In the chemical sequencing method, nitrogenous base-specific chemical reactions reveal the direct sequence of RNA terminally labeled with ^{32}P. Following the partial modification of RNA bases, aniline facilitates amine-catalyzed subsequent strand scission (Fleming et al., 2015) that ultimately reveals the position of nucleotides in the sequence. After the enzymatic or chemical sequencing method, the RNAs are fractioned with electrophoresis and then autoradiography helps in the identification of desired RNA sequence.

(ii) **Shotgun cloning of small ncRNAs through producing cDNA libraries:** In contrast to the direct sequencing method, this method involved reverse transcription of ncRNA. In case of conventional mRNA cloning, reverse transcription of mRNA with oligo (dT) primer is followed by 2nd strand synthesis to produce a cDNA library of protein-coding transcripts (Head et al., 2014). mRNAs are usually comprised of >500 nucleotides but ncRNAs are smaller in size and therefore can be isolated from total RNA by denaturing PAGE. Besides this, immunoprecipitation can be used to isolate the total ncRNAs with the help of a specific RNA-binding protein or antibody. The following methods were used to reverse transcribe ncRNAs into cDNAs:

C-tailing method- Most ncRNAs are lacking poly-adenylated tails. Therefore, oligo(C) or oligo (A) tails are first added to the RNA with the aid of poly(A) polymerase using ATP or CTP followed by reverse transcription using DNA polymerase I, RNase H, and the addition of DNA linkers to produce cDNA followed by cloning into a vector to generate cDNA library (Jalkanen et al., 2014).

C-tailing and linker addition - In this case, followed by C-tailing on the 3′ positions, at the 5′ terminals of ncRNA, an RNA or DNA oligonucleotide linker needs to be added with the help of T4 RNA ligase. During RT-PCR, oligo (dC) or oligo (dT) is needed along with a 5′ primer which can bind to the sequence at the 5′ ends (Head et al., 2014).

Linker addition - RNA oligonucleotides are added to both 5′ and 3′ ends by T4 RNA ligase. The 5′ end lacks phosphorylation and the 3′ ends are blocked to mitigate the problem of multimerization of linker sequences (Hüttenhofer and Vogel, 2006; Head et al., 2014).

Each of these methods to synthesize cDNAs is followed by cloning of the cDNAs into suitable vector systems.

(iii) **Microarray analysis:** Microarray analysis is a well-documented method based on nucleic acid hybridization of labeled RNA targets with their complementary probes. In the past years, microarray analysis was used for protein-coding mRNAs, however, nowadays it is also used for screening ncRNAs. Single-stranded DNA oligonucleotides (25–70 nucleotides in length), as well as double-stranded PCR products, serves as the most commonly used DNA probes. The probes are usually tagged with Cy3 or Cy5 (Stoughton, 2005). Samples for microarray analysis are RNA isolated from an organism or cDNA. The sample is to be mixed with a hybridization buffer before its application on the glass slide. Following hybridization, the fluorescence of the hybridization spots is measured by the scanner. The more the quantity of the transcript, the more the color intensity.

(iv) **Genomic SELEX:** The aforementioned methods allowed the identification of ncRNAs from the cellular RNA pools by direct sequencing, cloning, and microarray analysis. In comparison to the cDNA cloning method which demands the isolation of ncRNAs from an organism or cell, genomic SELEX is independent of isolating RNA from the organism. Genomic SELEX can produce ncRNAs that can form ribonucleoproteins that promote ncRNA folding into active conformation and also facilitates their protection from nucleases. The tight association between ncRNA and protein indicating its biological function is required for genomic SELEX (Zimmermann et al., 2010).

All the experimental methods discussed above are expensive as well as time-consuming. To overcome this limitation, three major computational methods are developed for the identification of ncRNAs namely (a) homology-based methods, (b) de novo method, and (c) transcriptomics.

a) **Homology-based methods:** In this method, the unknown RNA sequence is compared with known ncRNAs from databases depending on their sequence or structural alignment. BLAST and BLAT are the two well-known sequence-based methods (Zhang et al., 2017; Wang et al., 2013). The structure-based method is very fast and also used the secondary structure of RNA to determine the similarity. QRNA and RNAz are the two most popular structure-based methods for ncRNA identification (Zhang et al., 2017). Besides this, sequence and structural information can be used together to determine RNA similarity in the hybrid method (Zhang et al., 2017).
b) De novo **method:** In contrast to the homology-based methods that rely on the homology of the RNA sequences, the de novo method does not require that. The de novo method is also classified into sequence-based (nucleotide sequence), structure-based, and hybrid methods. The De novo method is more sensitive as compared to the homology-based method (Zhang et al., 2017; Wang et al., 2013).
c) **Transcriptomics and sequence assembly methods:** Unlike the homology-based methods and de novo method, here direct sequencing of coding and non-coding RNA transcripts is also possible (Zhang et al., 2017).
d) **RNA Family specific method:** There are also some computational methods have been discovered specifically for the miRNA and lncRNA RNA family (Zhang et al., 2017).

3. Non-coding RNAs (ncRNAs): the multipurpose master regulators

Very recently, researchers put considerable attention to the non-coding RNAs (ncRNAs) as they play a pivotal role as multipurpose master regulators of several biological functions both in prokaryotes (Desgranges et al., 2019), as well as in eukaryotes (Zhou et al., 2018; Youness and Gad, 2019; Aliperti et al., 2021; Chen et al., 2022). In brief, ncRNAs are those RNA molecules that do not undergo translation to form protein molecules. There are several types of ncRNAs [e.g. tRNAs (transfer RNAs), rRNAs (ribosomal RNAs), other small RNAs molecules like microRNAs, piRNAs, siRNAs, snoRNAs, exRNAs, snRNAs, and the long ncRNAs)] present which modulates complex molecular and cellular processes. Several cutting-edge strategies like deep-sequencing as well as next-generation sequencing (NGS) have been employed to unravel their structure functions in cell biology (Zhang et al., 2019; Aliperti et al., 2021; Chen et al., 2022). It has already been mentioned earlier that, ncRNA plays an important role in bacteria and helps them to modify their physiology to combat various environmental changes (Repoila and Darfeuille, 2009). In bacteria, sRNA, antisense RNA, and riboswitches are the key players that control the expression of many genes which encode metabolic proteins or virulence factors (Desgranges et al., 2019; Mahendran et al., 2022). In addition, bacterial sRNAs are small non-coding RNAs (40–500 nucleotides) that regulate gene expression by binding to mRNA or their protein target. The binding of sRNA with their specific target may lead to positive regulation or negative regulation. In the case of positive regulation, the secondary structure of target mRNA is modified by the sRNA causing the unveiling of the ribosome binding site and finally leading to translation. In negative regulation, the interaction between sRNA and target mRNA leads to the degradation of the mRNA by destabilizing it. Negative regulation may also be achieved by inhibiting translation through direct binding of sRNA to RBS (ribosome binding site) or by binding and altering the function of the post-transcriptionally regulatory protein (Filip et al., 2016).

(i) Regulation of CRISPR/Cas bacterial adaptive immune system

Very recently, CRISPR/Cas9 system was discovered in prokaryotes by which bacteria protect themselves from phage attacks (Barrangou et al., 2007). In brief, when bacteria is attacked for the first time by phage, it incorporates short sections of phage genetic material within the bacterial chromosome. In the future, when bacteria will again be encountered by the same type of phage virus, this system will recognize it with the help of transcribed RNA sequences. Next, one nuclease enzyme is then directed to cleave that DNA at a particular sequence. CRISPR consists of a small repetitive sequence of DNA that is flanked by short sections of nucleotides called spacer DNA. The 'Cas' genes are associated with CRISPR repeats and subsequently code for nuclease enzymes with an important function in cutting and unwinding target DNA. The CRISPR system functions by integrating phage or plasmid DNA sequence into its genome (Jinek et al., 2014). To date, researchers have discovered 2 classes and 6 sub-classes of CRISPR/Cas systems in bacteria. However, different pathways that regulate the CRISPR/Cas system and their activity are not well understood and warrant further investigation (Campa et al., 2021). Recently, scientists discovered that several bacteria are also capable of controlling CRISPR/Cas by quorum sensing (QS) to increase defense against phage attack e.g. in *Serratia*, *P. aeruginosa*, *Burkholderia glumae*, *Pectobacterium atrosepticum* (Bowden et al., 2013; Gao et al., 2015; Patterson et al., 2016, 2017). Hence, it is expected that the ncRNAs associated with the bacterial QS system might play a pivotal role in the regulation/control of CRISPR/Cas loci (Shao et al., 2013; Feng et al., 2015; Patterson et al., 2016; Shivram et al., 2021; Pu et al., 2022).

The CRISPRi (CRISPR interference) technology has been found very effective in studying functional genomics in bacteria and can also be employed for mapping phenotypes to ncRNAs. Interestingly, the gene knockout libraries derived from transposon insertion (known as Tn-seq) have been used successfully by researchers for quantitative gene-phenotype mapping, but there were certain limitations for which the versatile CRISPR/Cas9 gene-editing technologies have been exploited for microbial functional genomics research (Van Opijnen et al., 2009; Wang et al., 2018).

The CRISPR/Cas9 system revolutionized the gene-editing technology in prokaryotes and has recently gained importance in ncRNA-based cancer therapy. However, the advancement in CRISPR-based techniques brought a breakthrough in gene manipulation in prokaryotes as well as ex-vivo gene editing strategy (Song, 2017; Li et al., 2020). Before the introduction of the CRISPR/Cas9 genome editing technique to the whole researchers' fraternity, they used to employ RNAi technology where the unusual expression of different protein-coding genes needs to be suppressed. Later on, it was found that the RNAi technique was not substantially effective while dealing with the interference of ncRNAs (Yang et al., 2018). In the next section, we have tried to summarize the ncRNA targeting by CRISPR-Cas gene editing in humans.

(ii) Targeting ncRNA through CRISPR-Cas precise genome editing in unraveling novel molecular targets

As essential key regulators in gene regulation networks, ncRNAs have been found to interact and work with other essential biomolecules, including DNA, coding RNAs, and proteins (Zhang et al., 2019). Moreover, scientists across the globe have identified many ncRNAs involved in the diagnostics, development of therapeutic targets of several aberrant human physiologies, genetic-epigenetic studies, oncology research, and other diseases as reported in Bhatti G et al., 2021. Interestingly, researchers have discovered that the recognition and the role of ncRNAs in human diseases might be linked to the unusual existence and expression of the profile of aberrant miRNA (microRNA) in human oncology (Esteller, 2011; Liz and Esteller, 2016; Adams et al., 2014; Chen et al., 2019; Anastasiadou et al., 2018). To date, the CRISPR/Cas 9 system had been employed to knock out a few ncRNAs, which include miRNAs, snoRNAs, and lncRNAs (Zhao et al., 2014; Ho et al., 2015; Yang et al., 2018). ncRNA-based several therapeutic approaches have been depicted in Fig. 1.

Although there are several advantages of CRISPR/Cas9 precise gene-editing technology over ZFNs, TALENs, and RNAi for the alteration of gene expression at the transcript level, however, CRISPR/Cas system is still associated with certain limitations which researchers need to overcome in near future: (i) sometimes, the system is not able to execute functional knockouts in non-coding genes due to a lack of specific ORF (open-reading-frame) as the CRISPR/Cas9 system often introduces small InDels, and (ii) during CRISPR/Cas-based gene editing, if the small guide RNA (sgRNA) binds to the off-target region, that can cause certain unexpected genetic alterations, leading to other complex human diseases.

4. Bacterial sRNAs in gene regulation

Small non-coding RNA molecules are mostly derived from intergenic regions and vary from 50 to 200 bp in length. (Gottesman, 2004; Storz et al., 2011). ncRNA employs a multitude of molecular strategies to regulate bacterial gene expression exerting either positive or negative effects at every level of gene regulation of biological events like cell proliferation, differentiation, development, metabolism, apoptosis, stress response as well as signal transduction (Johansson and Cossart, 2003; Ebert and Sharp, 2012; Waters and Storz, 2009). As a positive regulator, ncRNA can either network with 5′-UTR of the transcript followed by modifying the secondary structure in making the ribosome-binding site available enabling translation; otherwise, it can stabilize the transcript by binding with the 3′-UTR and therefore enhance gene expression (Guillier and Gottesman, 2008; Papenfort et al., 2013). Alternatively, some ncRNAs can bind with mRNA 5′-UTR to exert inhibitory effects, resulting in reduced stability and transcript degradation, blocking of RBS, and translation inhibition (Aiba, 2007). The key mechanisms of bacterial

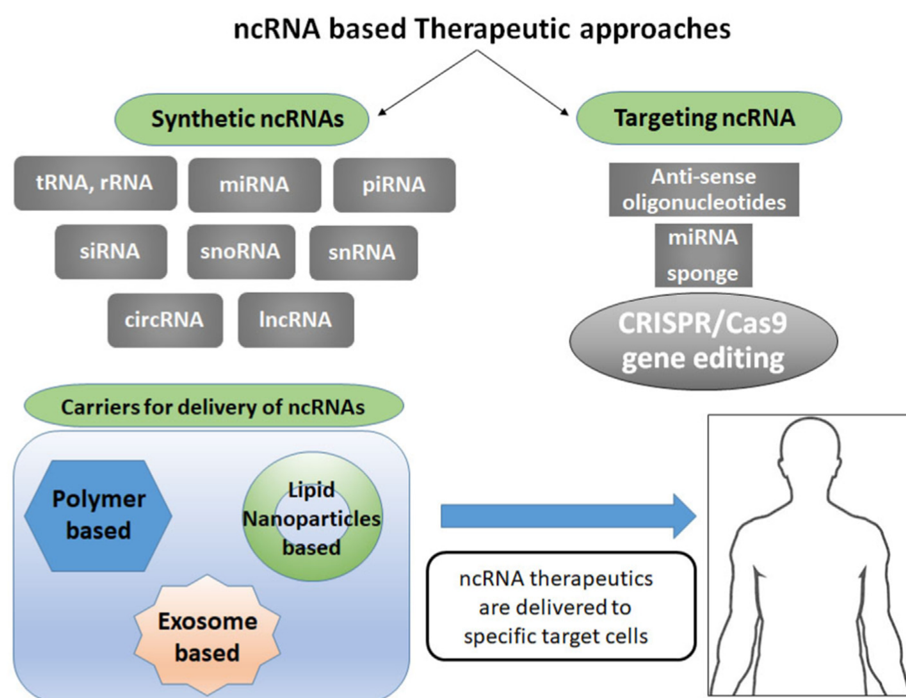

Fig. 1. The ncRNA-based therapeutic approaches. Various ncRNAs (miRNAs, lncRNAs, rRNAs, tRNAs, piRNAs, snoRNAs, and circRNAs) have been identified to date. Besides antisense oligonucleotides (asRNA) and miRNA sponges, most recently, the CRISPR/Cas has been considered a promising approach that acts as an antagonist to ncRNA and inhibits the target ncRNAs expression. In brief, the CRISPR/Cas9 complexes enter the cell and target ncRNAs by changing their DNA sequence. As a therapeutic approach, several carrier components (exosome, lipid nanoparticle, and synthetic polymer-based) are available for the delivery of ncRNAs to specific target cells.

ncRNAs in gene regulation are as follows:

(i) Cis-encoded ncRNAs on the other hand have complementary target sequences in the same location of the genome wherefrom they are encoded and therefore are denoted as anti-sense RNAs. Their size is highly variable with their location within the UTRs of corresponding genes to form a stable RNA-RNA hybrid with the fully complementary sense RNA sequence (involving 100 bp or more) which in turn interferes with either ribosome binding, protein translation, transcript stability, and termination events (Wagner, 2013).

For antisense ncRNAs of both chromosomal and extra-chromosomal origin, multi-step interactions are of significance in base pairing with bacterial transcripts (Brantl, 2007; Wagner et al., 2002). The target transcript is recognized by them initially by a fast and high-affinity interaction via a few nucleotides which are exposed in the stem-loop regions either of the regulator, the target, or both. This is followed by additional base pairing that involves rearrangements in RNA secondary structure (Han et al., 2010) (Fig. 2).

The cis-acting 5′ sRNA is typically found in the 5′ position of an mRNA and its expression is controlled by the ncRNA. Conformational change in the ncRNA occurs either (i) binding to riboswitches or (ii) alteration of temperature (known as thermoregulators) or pH (known as pH sensors) and these changes influence the operon by altering the level of RNA synthesis or protein synthesis of the downstream gene or genes contributing to biological processes like genomic imprinting, cardiac gene regulation, circadian rhythm, and antigen receptor genes recombination.

According to their orientation and degree of overlapping, they are classified into 3 types: a) head-to-head (5′ to 5′), b) tail-to-tail (3′ to 3′), and c) fully overlapping. Among them, tail-to-tail orientation is the most prevalent form (Saberi et al., 2016). Overlapping transcripts may encompass two protein-encoding genes. Either one protein-encoding and one non-encoding gene, or two non-encoding transcripts (Lapidot and Pilpel, 2006).

(ii) Trans-encoded ncRNA is generally encoded on the genome at a discrete location from their target sites with which they share partial complementarity. They target the Shine-Dalgarno sequence of candidate transcripts to sequester the RBS (Storz et al., 2011). RNase activity coupled with these non-coding RNAs contributes to RNA turnover. Often RNA–binding protein Hfq augments ncRNA-mediated regulation of gene expression, particularly in Gram-negative bacteria by enhancing the stability or functionality of the target small RNA (Saramago et al., 2014). In *Staphylococcus aureus* ncRNAs like RNAIII, SprD, and RsaE use conserved stretches of sequence rich in Cytosine located within the nearby loop regions (Chabelskaya et al., 2010; Geissmann T et al., 2009). As in enteric bacteria, many other trans-encoded ncRNAs for pairing require regions that are included within single-stranded stretches implicating that secondary structures are not essential for the RNA-hybrid formation. (Peer and Margalit, 2011) (Fig, 2).

The 3' end secondary structure with poly (U) tail protects the ncRNA against exonuclease activity and promotes Rho-independent termination of RNA synthesis. Moreover, the chaperone protein Hfq is also indispensable for the function and stabilization of many of these ncRNAs. Another strategy employed by trans-encoded ncRNAs is similar to eukaryotic microRNAs where they interact with the target mRNAs using a conserved seed sequence pairing mechanism as demonstrated in the case of target recognition by *Salmonella* RybB or MicC (Papenfort et al., 2010; Pfeiffer et al., 2009). Pairing location for target transcripts can be as variable as sequence overlapping or adjacent to ribosome binding site or can be present 70 nucleotides upstream or 15 nucleotides downstream of the start codon as reported in *Salmonella* and *E. coli*. Again small RNAs that play role in translational activation can recognize a target sequence that is located at a greater distance where they prevent the development of an inhibitory secondary structure by an anti-antisense mechanism reported in Fröhlich and Vogel (2009). Transcript stability can be governed by ncRNA recognition sites which are located within the target mRNA coding sequence (Pfeiffer et al., 2009).

(iii) ncRNA can also interact with proteins with regulatory properties altering their activities by molecular mimicry and therefore competing with the respective nucleic acid target. This interaction is best exemplified by CsrA/RsmA family regulators in virulent bacteria where the expression of ncRNAs is regulated by the two-component system in the immediate proximity of the RBS

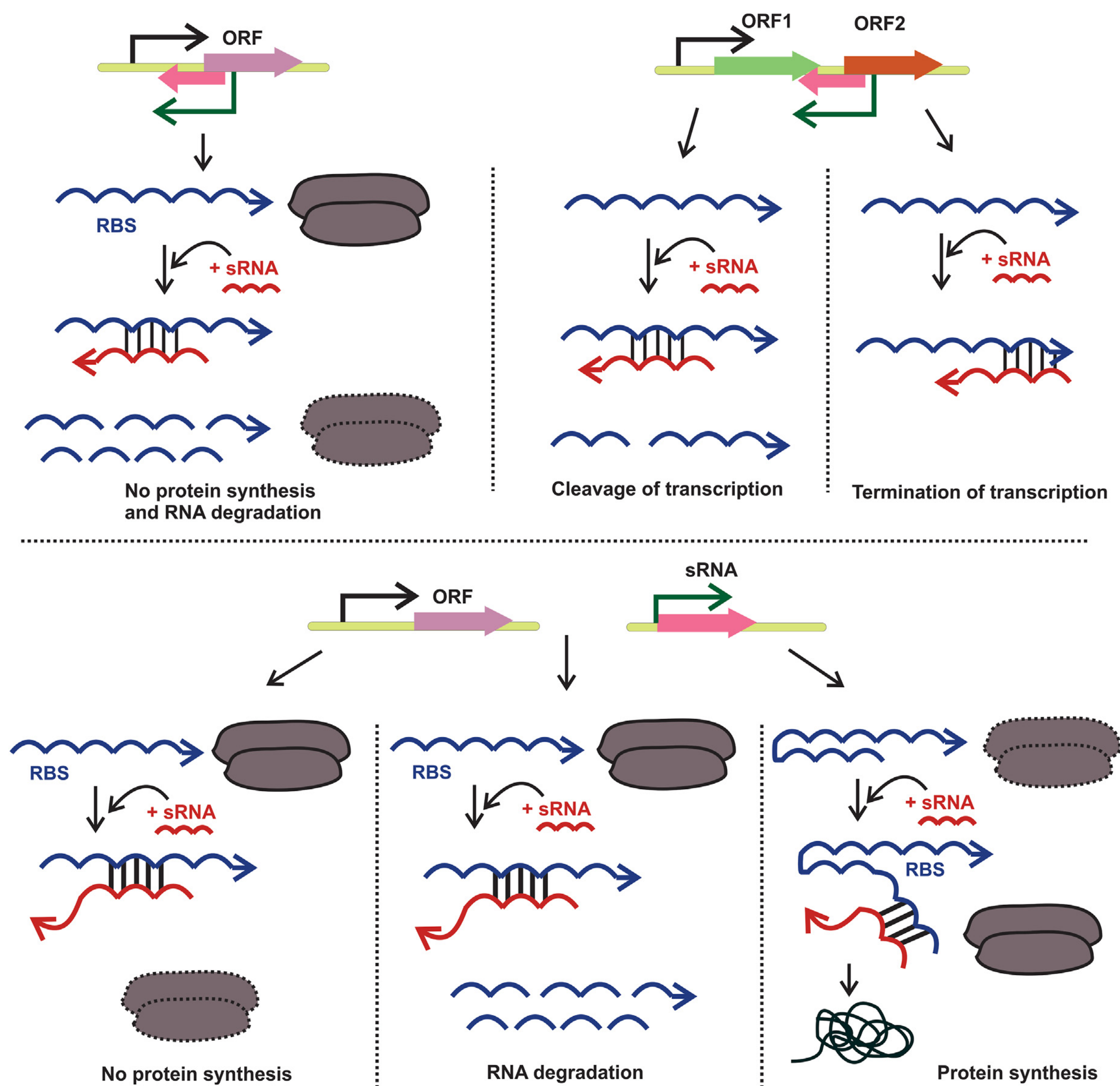

Fig. 2. Regulatory functions of sRNAs through base pairing.

sequestering CsrA. This phenomenon enhances the translation of the transcripts which were previously blocked (Duss et al., 2014). For instance, CsrB and 6 S RNAs of *Escherichia coli* bind CsrA protein with greater specificity than target mRNA.

5. Regulatory activities of bacterial ncRNAs

Regulatory ncRNAs achieve a variety of physiological effects in bacteria through diverse mechanisms, including alterations in RNA conformation, binding of proteins and DNA, and can base pair with other RNAs.

a) sRNAs modulating Protein Activity

Protein-binding small RNAs have diverse physiological effects. They may exhibit intrinsic activity or impart crucial functions to an RNase P and 4.5S respectively. Otherwise, they can antagonize the activities of target proteins through structural mimicry with other nucleic acids. In *Escherichia coli* the RNA binding protein CsrA is crucial for controlling carbon usage and bacterial motility in stationary phase or other adverse situations. In turn, the activity of the CsrA is regulated by ncRNAs CsrB and CsrC (Babitzke and Romeo, 2007). CsrA influences the stability and translation of candidate transcripts in dimeric form binding to the GGA motif in the 5′UTR of the latter. Transcripts of CsrB and CsrC at an elevated level with multiple binding sites (GGA motif) for CsrA can sequester it from target mRNAs. The levels of CsrB and CsrC can further be regulated in the nutrient-scarce situation by BarA-UvrB two-component system or RNase E activity by CsrD protein (Suzuki et al., 2006). Homologous counterparts of these two ncRNAs, namely, RsmY and RsmZ, can inhibit corresponding CsrA homologs in various bacteria like *Salmonella* sp, *Erwinia* sp, *Pseudomonas* sp, and *Vibrio* sp and therefore affect attributes of quorum sensing, epithelial cell invasion, and secondary metabolism and hence, their pathogenicity (Lapouge et al., 2008; Lucchetti-Miganeh et al., 2008). In the stationary phase of *E. coli*, the elevation of ncRNA 6 S RNA allows it to interact with and sequester σ^{70}-associated RNA polymerase in an open promoter inhibiting transcription there. On the contrary, similar interaction of 6 S RNA with σ^{S} regulated promoters elevates transcription from there (Wassarman, 2007; Trotochaud and Wassarman, 2005). In *Escherichia coli*, using an independent mechanism two sRNAs GlmZ and GlmY can interact with protein GlmS glucosamine-6-phosphate synthase and promote its accumulation. By an anti-sense mechanism, GlmZ directly triggers glms mRNA translation with Hqf. GlmY acts upstream of GlmZ by upregulating *glmS* through antagonizing GlmZ RNA inactivation (Urban and Vogel, 2008).

b) Cis-*encoded* sRNAs Base Pairing

There are two categories in regulation: one group form extensive base pairing with target mRNA while the second group exhibit limited complementarity. The cis-encoded ncRNAs share extended complementary areas of 75 or more base pairs long with their candidate RNAs in the same region but opposite orientation. Some of them can also function as diffusible molecules as trans-encoded ncRNAs (Brantl, 2007). Most of the extra-chromosomal cis-encoded ncRNAs maintain their copy number either through strategies like inhibition of transposase translation or replication primer formation. Another category of cis-encoded ncRNAs antagonizes toxic protein translation that destroys cells wherefrom the mobile element has been lost (Wagner et al., 2002; Brantl, 2007). A subpopulation of chromosomally encoded ncRNAs (for instance, IstR and OhsC in *Escherichia coli*) can degrade or suppress the translation of proteins that are toxic at elevated levels and thus have antitoxic activities (Brantl, 2007). Otherwise, they can also target gene expression in an operon where they are encoded in a region complementary to the intervening region between ORFs. In *gadBC* operon of *Escherichia coli* to combat acid stress, GadY ncRNA targets *gadXW* mRNA and influences the normal functioning of the acid fitness island of the bacteria (Tramontiet al., 2008).

c) Trans-encoded sRNAs Base Pairing

The trans-encoded ncRNAs of prokaryotes are functionally equivalent to eukaryotic microRNAs, exhibiting only limited complementarity with their candidate transcripts and mostly downregulating mRNA stability and/or protein translation (Aiba, 2007; Gottesman, 2005). They primarily bind to the 5' UTR of mRNA, blocking either the ribosome binding site or interacting from a greater distance from the start codon of the dysregulated gene. Thus either protein level is reduced by inhibiting ribosome binding or by degradation of the target transcript. Microbial iron uptake is negatively regulated by Fur protein which further is downregulated by ncRNA RyhB at the level of translation (Večerek et al., 2007). The inhibitory secondary structure is usually disrupted by an anti-antisense mechanism that otherwise seals the ribosome binding site (Hammer and Bassler, 2007; Urban and Vogel, 2008). For instance, *Escherichia coli glmS* mRNA, coding for an essential enzyme in amino-sugar metabolism is activated by ncRNAs GlmY and GlmZ (Urban and Vogel, 2008). Every trans-encoded ncRNA can target multiple mRNAs because of transient binding with target transcripts in discontinuous stretches (~10–25 bps) instead of extended stretches of perfect complementarity (Gottesman, 2005; Prévost et al., 2007). There are several instances where Hfq facilitates RNA-RNA interaction in trans-encoded ncRNA-mediated gene expression regulation. Hfq helps in RNA structure remodeling by melting inhibitory secondary structures or by enhancing complementarity by increasing the local concentration of ncRNAs and mRNAs (Aiba, 2007). Many of the cis-encoded ncRNAs are expressed constitutively while most of the trans-encoded ncRNAs are generated under specific growth conditions. For instance, in *E. coli*, these antisense regulatory RNAs can be induced by Fur-repressed RyhB, OxyR-activated OxyS, σ^E-induced MicA, and RybB, GcvA-induced GcvB, and several other factors (De Lay and Gottesman, 2009; Johansen et al., 2008).

d) Twin Functional RNAs

Often the differentiation between some categories of regulatory RNAs is not well defined. For example, some of the trans-encoded ncRNAs are protein-encoding apart from base pairing with target transcripts. The *S. aureus* RNAIII not only encodes a δ-hemolysin peptide but also targets the virulence factors encoding transcript and also a transcription factor (Boisset et al., 2007). It is also mention-worthy that few cis-encoded antisense sRNAs, apart from regulating their cognate sense mRNA, may base-pair with other mRNAs via partial complementarity or, can bind other proteins independently affecting their functions (Waters and Storz, 2009).

6. Contribution of ncRNA in pathogenicity of virulent microorganisms

A large number of trans-encoded regulatory ncRNAs play a pivotal role in the regulation of virulence expression and stress responses and thus contribute to adaptation of pathogenic bacteria (vacuolar as well as cytosolic) in intracellular environment niches in the host body evading the host defense mechanism (Chao and Vogel, 2010; Hör et al., 2020). Using transient base pairing with different target transcripts these ncRNAs direct the expression of genes controlling multiple facets of bacterial physiology like biofilm formation and pathogenicity post-transcriptionally (Nitzan et al., 2017; Sy and Tree, 2021).

CsrA/RsmA proteins constitute the most common yet complex post-transcriptional regulatory network driving virulence factor expression of several proteobacteria (predominantly gamma-proteobacteria) that are intracellular pathogens (Vakulskas et al., 2015; Pourciau et al., 2020). The network is functional at the level of protein synthesis, turnover, and elongation of mRNA (Park et al., 2015). CsrA activity can be controlled by ncRNAs with manifold *tar* sites for CsrA, which enable them to sequester manifold CsrA homodimers away from the transcripts targeted (Vakulskas et al., 2015; Romeo and Babitzke, 2018). In *Escherichia coli* CsrA regulates the expression of traits associated with motility, biofilm formation, and T3SS all of which contribute to its virulence. Here the CsrA activity in return is regulated by Csr sRNAs, namely, CsrB, CsrC, and McaS. The CsrB RNA derived from *Escherichia coli*, for instance, is 369 bp long, with 18–22 sites for binding CsrA. In this coliform, the stability of ncRNAs inhibiting CsrA is under the firm control of CsrD through RNase-dependent turnover (Pourciau et al., 2020). During the stationary growth phase of *Escherichia coli* the small ncRNA fimR2 antagonizing activity of CsrA contributes to the survival of the bacteria under the condition of nutrient depletion through biofilm formation and alteration of outer membrane architecture (Raad et al., 2022). In enterohaemorrhagic *E. coli* another two ncRNAs GlmY and GlmZ induce T3SS effector EspFu and reduce other T3SS effector expressions which in turn contributes to the generation of A/E lesions (Gruber et al., 2014). Effect of Type 1 pili (T1P) in uropathogenic *E. coli* is dysregulated via down-regulation of target genes fimA or fimB directly by ncRNAs RybB and MicA (Chao and Vogel, 2016.)

Similarly in *Salmonella typhimurium,* CsrA activity driving the expression of components involved in motility, biofilm formation, SPI1 T3SS is further under the control of ncRNAs CsrB, CsrC, and *fimAICDHF* (Raad et al., 2022). Infection by *Salmonella enterica* is triggered by the fimR2 counterpart of this enteric pathogen by targeting effector protein secretion by T3SS (Coburn et al., 2007). In *Salmonella* sp*,* ncRNA PinT synchronizes the expression of SPI1 and SPI2 T3SS virulence factors permitting the pathogen to undergo the transition from its invasive mode to its persistent infective form. CpxQ ncRNA of *Salmonella* sp down-regulates fimA expression and therefore under membrane stress conditions monitors T1P expression (Westermann et al., 2016; Chao and Vogel, 2016). The *Salmonella* variant of ncRNA fimR2S aggravates the invasiveness of *S. enterica*, through induction of a T3SS-chaperone expression (Raad et al., 2022). In *S. enterica* serovar, Typhimurium transcription of virulence regulatory genes located in *Salmonella* pathogenicity islands is under the control of CsrA (Lou et al., 2019). Two RybB homologous ncRNAs in *Salmonella enterica*, namely RfrA and RfrB play a crucial role in regulating the expression of genes associated with O_2 stress, pH tolerance, and iron homeostasis within host cells facilitating the intra-macrophage replication of the pathogen (Calderón I et al., 2014). Fur as a suppressor of RybB ncRNA is also vital for the internalization and intracellular persistence of *Salmonella* in human macrophages (Ahmed et al., 2016). In *S. typhimurium* the ABC transport system is deregulated at the transcript level by the ncRNA GcvBs (Sharma et al., 2011). In *Salmonella* Pathogenicity Island the non-coding RNAs IsrJ and IsrM play a

significant role in the regulation of invasion of intestinal epithelial cells by a virulent form of the bacteria (Gong et al., 2011). IsrM controls the expression of major virulence genes *Salmonella* outer protein SopA and the transcription factor *HilE*, which are vital for bacteria to remain viable in the host macrophage overpowering the host immune system (Padalon-Brauch et al., 2008). Intra-macrophage survival and virulence of *Salmonella typhimurium* are further strengthened by MgtC virulence protein which in return is regulated by AmgR, an antisense RNA (Lee and Groisman, 2010).

In *Pseudomonas aeruginosa,* the transition between acute and chronic infection of the respiratory tract is marked by significant modifications of gene expression in virulence factors. RsmA triggers modification in the expression of T3SS-associated genes and type IV pili that are positive regulators of acute infection as well as factors involved in biofilm formation and T6SS that are negative regulators of chronic infection. *Pseudomonas* ncRNAs (RsmY and RsmZ) antagonize the activity of RsmA protein. The stability of these ncRNAs is significantly higher than their *E. coli* counterparts with a lesser number of target binding sites as the mechanism of CsrD mediated turnover of ncRNAs is absent in *Pseudomonas* (Suzuki et al., 2006; Reimmann et al., 2005).

Legionella sp uses CsrA to govern the functioning of effector molecules that modify a host-cell function and create an intracellular replicative niche. Here CsrA targets T4SS to modify ER–Golgi vesicular trafficking of VipA, RalF, and YlfA, with the inclusion of ncRNA-binding protein (Nevo O et al., 2014). Here also CsrB, CsrC and CsrD functions as regulatory ncRNAs. Apart from these, ncRNAs, namely RsmY and RsmZ, drives RsmA protein expression to influence *Legionella* replication in macrophage, which directly aim at T4SS regulatory genes to enable endurance of the pathogen intracellularly (Kulkarni et al., 2006). AbcR1 and AbcR2 are two homologous ncRNA in *Brucella*, that collectively play a substantial role in their intracellular growth in macrophages and therefore in virulence and chronic infection establishment (Caswell et al., 2012).

In *Listeria monocytogenes* overexpression of ncRNA Rli31, and Rli33-1 help in the intracellular survival of the pathogen in the macrophage by targeting genes *pgd*A, and *pbp*X. By some unknown mechanism similar effect is also shown by two other ncRNAs Rli50, and Rli112 in their normal cellular concentration (Mraheil et al., 2011). In *Legionella pneumophila* SsrS (6 S) is necessary for targeting the activity of RNA polymerase aiding the intra-macrophage survival of the pathogen (Faucher et al., 2010). Again in *Chlamydia thrachomatis* and *Chlamydia pneumoniae* upregulation of IhtA causes alteration in the activity of histone homolog Hc1and thus affecting the differentiation of actively metabolizing reticulate bodies to metabolically inactive elementary bodies (Grieshaber et al., 2006). In *Streptococcus pyogenes*, the regulatory RNAs RivX and FasX are reported to be involved in virulence gene regulation through CovR/S two-component system and interactions with host cells, respectively (Roberts and Scott, 2007; Kreikemeyer et al., 2001). Adhesion is one of the major virulence factors of UA159 and other clinical strains of *Streptococcus mutans,* which is regulated post-transcriptionally by several ncRNAs. A study by Zhu et al. revealed several novel putative adhesion-related ncRNAs (sRNA0593, sRNA0329, sRNA0330, sRNA0656, sRNA0679, sRNA0187, and sRNA0698) in *S. mutans* associated with regulation of adhesion in *S. mutans* at mRNA level (Zhu et al., 2018). A member of normal microflora, *Staphylococcus aureus* is also a principal contributor to nosocomial infections globally. In the pathogenic form, a multifaceted regulatory RNA, RNAIII, couples quorum sensing with virulence by targeting the *agr* system (sensor of population density). Through the *agr* system RNAIII targets a multitude of factors like exotoxins, cell wall-associated proteins, multiple dual-component systems, and master regulators of biofilm formation, cell wall biosynthesis, etc. – all of which contribute to its pathogenicity (Felden et al., 2011). Another two virulence genes *spa* gene encoding for surface protein A and *coa* gene encoding staphylocoagulase in *S. aureus* are also regulated by RNAIII (Huntzinger et al., 2005). A study of whole-genome transcriptional sequencing has identified 89 putative ncRNAs with a definitive role in the virulence of *Streptococcus pneumonia*.

The information available regarding the contribution of bacterial non-coding RNAs in the regulation of pathogenicity remains undefined. For the future study, the critical aspects that can be addressed are (i) the identification of ncRNAs that are the master regulator of infection; (ii) the phase of infection at which the ncRNAs are functional; (iii) the mode of activating/deactivating these regulatory circuits by the pathogens; and (iv) contribution of ncRNA activity in the process of infectivity.

7. sRNAs/ncRNAs as a regulator of immune response

There are many reports that bacterial sRNA can modulate their gene expression which eventually leads to bacterial pathogenesis (Waters and Storz, 2009). Nonetheless, recent reports have highlighted the role of bacterial/viral sRNA indirectly interacting with the host and modulating host response. Virus-associated sRNA, VA1, and VA2 of Adeno-associated virus and sRNA of Epstein-Barr virus, EBR1, and EBR2 can suppress host translation (Bhat and Thimmappaya, 1983; Andersson et al., 2005). Similarly, HIV sRNA, $tRNA^{Lys3,}$ and $tRNA^{Lys5a}$ slow down the host protein synthesis by acting as a primer for HIV RT polymerase (Schopman et al., 2012). There are reports of cross-kingdom gene silencing in animals and plants both. It has been reported that bacterial sRNAs, DsrA, and OxyS could regulate genes of *Caenorhabditis elegans* (Liu et al., 2012). sRNA of different pathogens have been reported in the body fluids of infected individuals. For example, sRNAs from *Trypanosoma cruzi*, the protozoa that cause Chagas disease has been seen in the host.

These recent studies have opened a new aspect of sRNA, where sRNA can regulate host gene expression. Studies on the role of sRNA in modulating the host immune system are yet to be fully understood. It will be interesting to see if the sRNA of disease-causing pathogens can modulate the genes involved in the innate and adaptive immunity of the host in their favor. In that case, strategies targeting such sRNAs may become a new approach to antimicrobial therapy.

8. Conclusion

The present review catalogs various aspects of ncRNA, their occurrences, especially their biological roles focusing mostly on prokaryotes. We have tried to elaborate on various important aspects of ncRNAs like different strategies and approaches for identification of ncRNAs, their role as master regulators in gene regulation, targeting ncRNA through CRISPR-Cas precise genome editing in unraveling novel molecular targets, the crosstalk between various ncRNAs, and their substantial contribution of them in pathogenicity of virulent microorganisms. It is worth mentioning here that, though ncRNAs do not undergo transcriptional bursting, they function in controlling several signaling pathways. We have written this review intending to draw the attention of the broader scientific community to such recent discoveries on ncRNAs and their known as well as putative physiological roles to initiate further research activities in this important field.

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RPS and AS conceived the study and participated in its design, analysis, and coordination. RM, SG, AD, MKS, and SS contributed to writing the manuscript. RM, SG, RPS, and AS supervised the work. All authors contributed to the interpretation of the data and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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