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Regulation of biofilm formation by non-coding RNA in prokaryotes

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ABSTRACT

Biofilm refers to microbes that associate with each other or to a surface via self-synthesized exopolysaccharides and other surface-related structures. The presence of biofilms consisting of pathogenic microbes in the food and clinical environment can pose a threat to human health as microbes in biofilms are highly robust and are difficult to remove. Understanding the process of biofilm formation is crucial for the development of novel strategies to control or harness biofilm. The complex network of proteins, small RNA, and diverse molecules regulate biofilm formation at different steps in biofilm development, including triggering the switch from planktonic to sessile cells, maturation of biofilms, and eventual dispersion of microbes from the biofilms. Small non-coding RNAs are relatively small RNAs that are not translated into proteins and play diverse roles in metabolism, physiology, pathogenesis, and biofilm formation. In this review, we primarily focused on non-coding regulatory RNA that regulates biofilm formation in clinically relevant pathogens or threatens human health. Even though many ncRNA have recently been identified in Archaea, much characterization work remains. The mechanisms and regulatory processes controlled by ncRNA in prokaryotes are covered in this review.

1. Introduction

Biofilms are surface-associated microbes or microbes associated with each other via a self-synthesized matrix that can be attached to an abiotic or biotic surface (Flemming et al., 2016). This self-synthesized matrix assists microbes' survival during nutrient limitation, pH, and temperature fluctuations and offers protection against physical and chemical attacks and other environmental stresses. As a result, biofilms can be found in almost all habitats except oceans and influence biogeochemical cycles on earth (Flemming and Wuertz, 2019). Biofilms are resistant to harsh external conditions due to robust extracellular polysaccharides. As biofilms are robust, the removal of biofilms can be challenging. If left untreated, it can be a source of chronic infections, particularly in clinical settings and on patient accessories or devices such as on the surfaces of catheters and implants. Besides, biofilms can result in the spoilage of foods, and corrosion on industrial pipes and the hull of ships. Biofilm-associated conditions are more challenging to remove than planktonic cells by antibiotics. The tolerance of biofilms to antibiotics could be due to reduced penetration of antibiotics, reduction in growth, and presence of persister cells. A deeper understanding of the mechanism of biofilm formation is essential for developing strategies to combat microbial biofilms.

Biofilm formation depends on the species of microbes that make up the biofilm and the nutrient limitation or availability. The process starts with a reversible attachment of planktonic cells to a conditioned surface followed by irreversible attachment depending on the conditions that favor the sessile mode of subsequent growth. Free-floating planktonic cells switch to biofilm mode in a sequence of events from attachment followed by growth, maturation, and detachment. After irreversible attachment of microbial cells, a period of growth ensues, leading to formation of microcolonies with exopolysaccharide matrix. During this phase, cell-to-cell signaling and cell-surface interactions play a crucial role in biofilm development and assist the spread of microbes to a surface. When unfavorable conditions, nutrients become limiting, or toxic waste products develop, microbes detach from the biofilms and move to a new surface to start the process again.

The formation of biofilms depends on various regulatory signals, including small diffusible molecules called autoinducers via quorum sensing and second messengers such as cyclic di guanylate (c-di-GMP) two-component regulatory systems, alternative sigma factors, and small non-coding regulatory RNA (ncRNA). In many cases, these regulatory pathways or interactions can cross-talk or regulate each other. RNA

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regulators, including ncRNA, can guide the switch between free planktonic cells to biofilm mode. Small ncRNAs typically vary from 50 to four hundred base pairs that do not translate into proteins and play a diverse role in metabolism, stress response, pathogenesis, and biofilm formation. These ncRNAs can either activate or repress mRNAs or proteins. For those regulating mRNA, the ncRNA can be antisense RNA that binds to the target mRNA based on sequence complementarity, forms a duplex structure, and regulates gene expression and downstream targets in response to environmental triggers. Base pairing ncRNA can be either *cis* or *trans,* depending on the relative location of its target RNA. Non-coding RNA that are *cis*-acting can be found directly opposite their target mRNA.

In contrast, *trans* ncRNA are encoded within the intergenic regions in the genome at different locations and exhibits limited similarity with their target mRNA. RNA chaperone Hfq is required for the interaction of *trans*-acting ncRNA with their target mRNA. Base pairing ncRNA affects the translation and stability of mRNA by changing accessibility to ribosome binding sites or facilitating degradation by ribonuclease, affecting the expression of target genes. Another group of ncRNA can bind to RNA binding proteins and inhibit or antagonize their actions. These ncRNAs mimic the sequence present in many mRNAs and prevent binding their target mRNA to their corresponding proteins. The third type of ncRNA regulates the maintenance of horizontally transferred nucleic acids mediated via bacteriophages or plasmids. Recent studies have shed light on the regulatory RNA from eukaryotic cells packaged in extracellular vesicles that can also regulate biofilm formation in bacteria and can be a strategy to control bacterial infections in eukaryotes (Stanton, 2021). Identifying and characterizing small non-coding RNA in controlling biofilm formation is crucial to developing novel strategies and therapeutics for many biofilm-forming pathogens. Fig. 1 represents various ways by which non-coding RNA regulates biofilm formation.

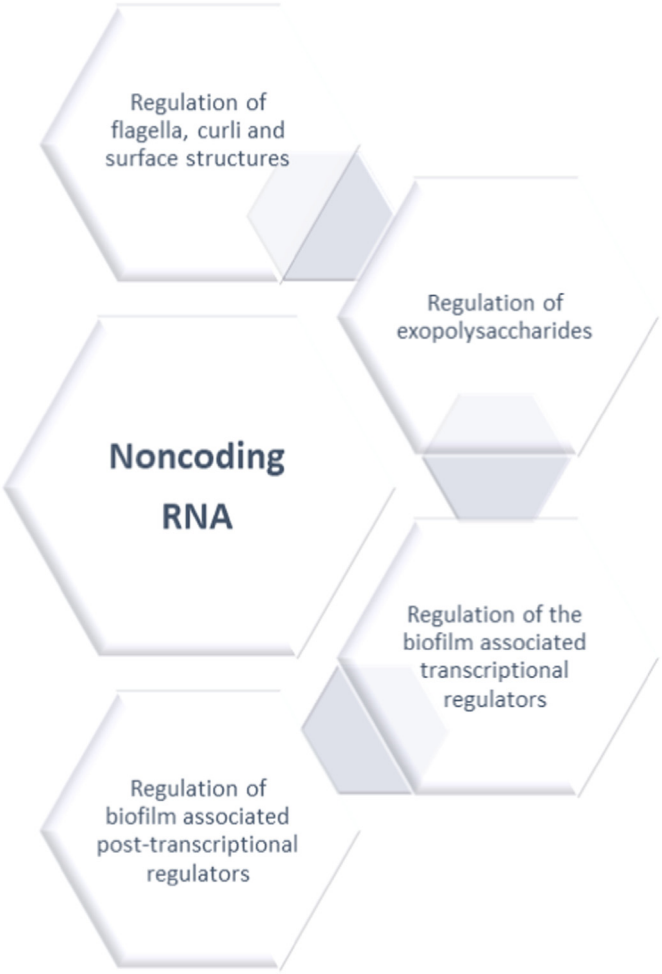

Fig. 1. Major cellular processes regulated by non-coding RNA in biofilm formation.

2. Methods to identify sRNA and their targets

Computational methods have been used earlier to predict non-coding RNA. While robust programs such as QRNA employs comparative genomics analysis, other programs such as RNAz 2.0 indicates the thermodynamic stability of RNA. Newer programs such as SPOT use existing computational tools to predict ncRNA and filter the data based on experimental data (King et al., 2019). Besides, microarray-based approaches have been used for identification of ncRNA globally. Some ncRNA has been co-purified with RNA binding proteins such as Hfq and CsrA. Other ncRNA prediction tools such as sRNAPredict2 evaluates the information based on RHO-independent terminators. Another online platform, PresRAT uses attributes of sRNA sequence to identify sRNA (Kumar et al., 2021). The RNA-seq (RNA sequencing) method, which employs cDNA sequencing has been extensively used to identify non-coding RNA in bacteria. RNA-seq has been used to identify novel small RNA molecules that act differentially in response to differential environmental conditions typically in biofilm vs. planktonic mode (Amador et al., 2018). Targets of the ncRNA have also been studied using several methods. Induction of the sRNA and analysis of mRNA levels was another useful method for identifying ncRNA targets. Other strategies include ribosome profiling, proteomics, regulatory complex purification, affinity purification, and RNA sequencing technologies that are increasingly used to delineate complex interactions among ncRNA and their targets. Some investigators have used RIL-seq (RNA interaction by ligation and sequencing) to identify sRNA targets and their networks (Matera et al., 2022). Fig. 2 represents predictive secondary structure of SrbA, a noncoding RNA that regulates biofilm formation in *Pseudomonas aeruginosa* (see Fig. 2).

3. Biofilm regulation by non-coding RNA in gram-negative bacteria

Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* are the causative agents of several health-associated enteric infections which can lead to diarrhea, fever, morbidity, and mortality. Of these enteric diseases, many are mediated in a biofilm-dependent manner. Much work on small ncRNA has been carried out in *Salmonella typhimurium* and *Escherichia coli*. Table 1 shows representative ncRNA that is associated with biofilm formation in Gram-negative bacteria.

Pathogenic strains of *Escherichia coli* are the causative agents of a wide range of diseases such as diarrhea, systemic infections, urinary tract infections, and catheter-associated infections. In many of these cases, biofilm formation is associated with expression of virulence factors and attributes. Hfq, the RNA chaperone, plays a critical role in forming biofilms in *Escherichia coli*. Deletion of *hfq* gene leads to a defective biofilm formation when grown in LB or Yeast Extract Casamino Acid medium (YESCA) (Parker et al., 2017). Furthermore, significant change in biofilm formation was observed in the small RNA deletion mutants of *arcZ*, *dsrA* and *gadY* only in YESCA medium but not in LB. CsrA, the global carbon storage regulator, is also a regulator of biofilm formation, which is controlled at the level of transcription and post-transcriptionally, both by indirect and direct mechanisms (Jackson et al., 2002; Mitra et al., 2013). Two small ncRNA, namely, CsrB and CsrC, bind to CsrA and sequester its activity (Carzaniga et al., 2021).

Salmonella typhi, the causative agent of Typhoid, is transmitted via contaminated food and or water. Typhoid is common in resource-constrained regions, where proper sanitation or hygiene might be missing, and increases the burden of infectious diseases in those regions. Using molecular genetics, AsfD, a cis antisense RNA, has been identified from *S. typhi*. AsfD has been shown to interact with *flHDC* operon, and

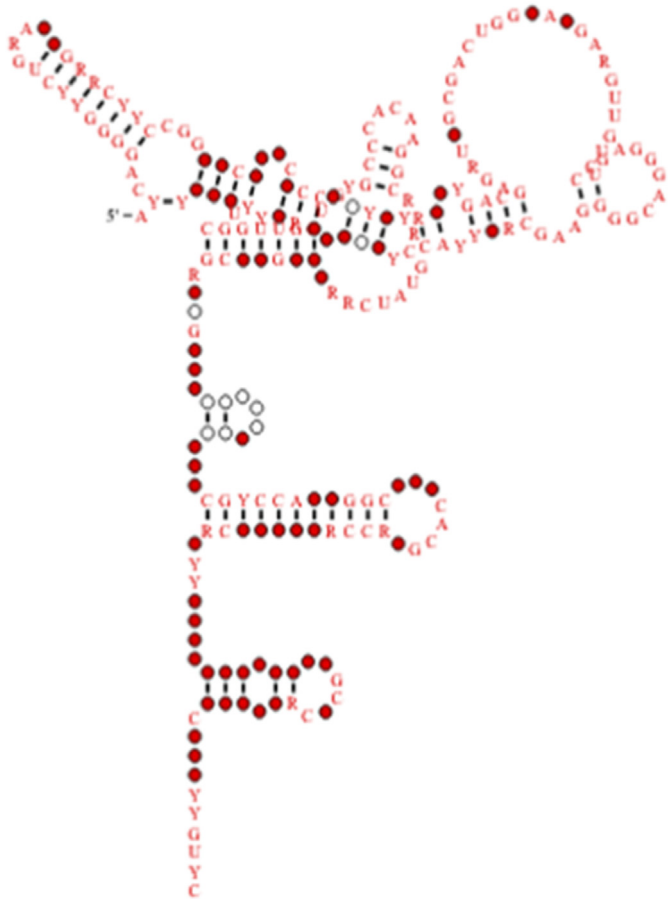

Fig. 2. Secondary structure of SrbA, a non-coding regulatory RNA present in *Pseudomonas aeruginosa* (The image is predicted in Rfam, a database of RNA molecules).

Table 1
Biofilm-associated non-coding regulatory RNA in Gram-negative bacteria.

Gram-negative bacteria	Non-coding RNA	Role/Function	References
Escherichia coli	RydC	Reduces curli production and biofilm production	Bordeau and Felden (2014)
Escherichia coli	McaS	flagellar transcriptional activator	Jorgensen et al. (2013)
Escherichia coli	CsrB and CsrC	Sequesters activity of CsrA by binding to it	
Salmonella typhi	AsfD	Upregulates *flhDC* operon	Chen et al. (2020)
Salmonella typhimurium	MicA	regulates biofilm formation independent of *luxS*	Kint et al. (2010)
Pseudomonas aeruginosa	SrbA	activates biofilm formation via transcriptional activation of *amrZ*	Taylor et al. (2017)
Pseudomonas aeruginosa	ErsA	activates biofilm formation via transcriptional activation of *amrZ*	Falcone et al. (2018)
Vibrio cholerae	VqmR	regulates *vpsT*, a biofilm regulator	Papenfort et al. (2015)
Vibrio cholerae	Qrr1-4 sRNA	four quorum regulatory RNA that stimulates the translation of *aphA* mRNA, which in turn facilitates biofilm formation	Weber et al. (2011)
Erwinia carotovora	RprA	exopolysaccharide synthesis, biofilm dispersal	Peng et al. (2021)

stimulates the expression of *flhDC*, upregulates motility and enhances biofilm formation (Chen et al., 2020). In the case of *Salmonella typhimurium*, Hfq also acts as a master regulator of biofilm formation. Another

Table 2
Biofilm-associated non-coding regulatory RNA in Gram-positive bacteria.

Gram-positive bacteria	Non-coding RNA	Role/Function	References
Staphylococcus aureus	RsaA	enhances biofilm formation by repression of MgrA	Tomasini et al. (2017)
Staphylococcus aureus	RNAIII	stimulates Agr quorum sensing mediated pathway	Tomasini et al. (2017)
Staphylococcus epidermidis	IcaZ	represses icaR mRNA translation	Lerch et al. (2019)
Bacillus subtilis	RsaE	regulates PIA mediated biofilms	Schoenfelder et al. (2019)
Streptococcus mutans	FasX	inhibits collagen-binding pilus and biofilm	(Liu et al., 2012; Danger et al., 2015)
Streptococcus mutans	sRNA0426	regulates exopolysaccharide biosynthesis	Yin et al. (2020)
Streptococcus mutans	ASvicR	negatively regulates biofilm biomass	Lei et al. (2018)
Streptococcus sanguinis	csRNA	negatively regulates biofilm formation by repression of pilT mRNA	Ota et al. (2018)
Streptococcus suis	rss04	represses capsular polysaccharides and enhance biofilm formation	Xiao et al. (2017)
Enterococcus faecalis	AswalR	reduces exopolysaccharide synthesis and biofilm formation	Wu et al. (2021a)

RIL-seq study identified OppX, which acts as an RNA sponge of MicF, another small ncRNA that represses porin proteins. The presence of OppX, causes derepression of porin proteins and facilitates nutrient uptake (Matera et al., 2022). MicF has been shown to increase biofilm formation in *Escherichia coli* when grown in YESCA (Parker et al., 2017).

Cholera, caused by *Vibrio cholerae*, is endemic in many resource-constrained regions of the world and causes acute infections inside the human intestine. *V. cholerae* forms biofilms in a quorum sensing-dependent manner. It has four quorum-regulatory small RNAs, Qrr-1 to Qrr-4, that are transcriptionally activated by phosphorylated LuxO under low-cell density (Svenningsen et al., 2009). These small regulatory RNAs have different mechanisms of action and are responsible for biofilm formation and virulence at low cell density (Feng et al., 2015). At low cell density, it can form biofilms and turn on virulence genes to make the host weak or sick but at high cell density, it gets detached from the host by activation of protease only to infect the next host. At high cell density, however, LuxO is dephosphorylated, and no transcription of these small RNA occurs, and consequently, no biofilm formation occurs. A RNA-seq study has identified VqmR, a small ncRNA to regulate VpsT, required for the formation of biofilms (Papenfort et al., 2015). In *Vibrio alginolyticus*, quorum sensing dependent sRNA, Qrr, has been shown to regulate biofilm formation along with growth, virulence, swarming motility, and EPS production (Liu et al., 2020). *Vibrio alginolyticus*, an opportunistic pathogen, causes sepsis, soft tissue infections, and other extraintestinal infections due to consumption of undercooked or raw seafood or water activity. Vvrr1, a ncRNA, regulates the adhesion ability of *V. alginolyticus* as overexpression of Vvr1 reduced the adhesion ability of the pathogen. *Erwinia amylovora* is the causative agent of fire blight in plants such as apples and pears. Infection of the pathogen begins at the leaves, following which biofilm formation occurs within xylem vessels, restricting the flow of water and causing wilting. Further migration of *E. amylovora* through the xylem vessels can also result in systemic infection. A small ncRNA, RprA controls the shift from biofilm to planktonic mode and regulates exopolysaccharide production (Peng et al., 2021). ArcZ, another ncRNA and Hfq, is thought to promote biofilm development in the pathogen *Erwinia carotovara,* which causes soft rot disease in plants (Kharadi and Sundin, 2021).

Pseudomonas aeruginosa, another Gram-negative bacterium, infects both plants and humans. ErsA, a ncRNA, has been shown to regulate biofilm formation in *P. aeruginosa*. A deletion mutant of ErsA forms flat biofilms compared to the mushroom-shaped biofilms in isogenic wild type. ErsA also interacts with AmrZ transcriptional regulators to regulate biofilm formation and motility (Falcone et al., 2018). Another small RNA, SrbA, regulates biofilm formation as a loss of SrbA expression has reduced biofilm biomass and virulence in the *C. elegans* model (Taylor et al., 2017). Another regulatory RNA, CrcZ in *P. aeruginosa*, competes with Hfq-mediated riboregulation (Sonnleitner et al., 2017). An RNA-seq study identified RsmW, which is differentially regulated in biofilm and planktonic states. RsmW has been demonstrated to bind RsmA, the global posttranscriptional regulator, and improves biofilm formation (Miller et al., 2016).

Bartonella henselae, a zoonotic Gram-negative pathogen, causes cat scratch disease, which can be transmitted from cats to cats or humans. Forming biofilms can help in the persistence of the bacteria in the human host and cat flea vector. BadA adhesin of *B. henselae* is required for biofilm formation and was shown to be regulated by a non-coding RNA, Brt, via control of a DNA binding protein (Okaro et al., 2020). *Acinetobacter baumanii*, an opportunistic human pathogen, shows resistance to conventional antibiotics and is often detected as a cause of pneumonia in hospital-acquired infections. A global transcriptomic analysis identified sRNA differentially expressed in planktonic and biofilm mode. The study has seen a novel RNA, sRNA 13573, which is highly described in biofilm formation and attachment to human alveolar cells (Alvarez-Fraga et al., 2017). Yet another study identified a plasmid-encoded sRNA in New Delhi Metallo-beta-lactamase (NDM-1) strain, a carbapenemase-producing bacteria known to hydrolyze carbapenem, used in the treatment caused by antibiotic-resistant bacteria. Expression of the plasmid, pNDM-HN380, in *Escherichia coli,* downregulates flagellar and chemotaxis genes during the exponential phase. Characterization of the plasmid identified a small RNA, IGR plas2, which, when knocked down, resulted in the downregulation of biofilm formation (Huang et al., 2020).

4. Biofilm regulation by non-coding RNA in gram-positive bacteria

Hfq facilitates the interaction between non-coding RNA and its target mRNA. Small ncRNA-mediated regulation has not been studied as extensively in Gram-positive organisms as in Gram-negative organisms, possibly due to the lack of presence of RNA chaperone in many Gram-positive microorganisms. Consequently, a lack of Hfq can be considered a lack of the interaction between ncRNA and mRNA, and investigating such organisms is thought to be futile. However, many Gram-positive pathogens may be examined further because regulatory RNA can act in a non-Hfq-dependent manner and can influence metabolism, virulence, and biofilm formation.

Group A streptococci (GAS) causes a wide range of infections varying from mild conditions such as pharyngitis to invasive diseases such as necrotizing fasciitis (Castro and Dorfmueller, 2021; Graham et al., 2001). Regulatory RNAs have been shown to play a critical role in GAS infections. A 205 nt sRNA, FasX binds to the *cpa* mRNA, which encodes a pilus biosynthesis protein. The GAS pilus binds to collagen, enabling attachment to host cells and facilitating biofilm formation. FasX inhibits the translation of *cpa* mRNA by preventing access to RBS, and negatively regulates collagen binding pilus in GAS (Liu et al., 2012). FasX also inhibits adhesin promoting PrtF1 and PrtF2 by interacting with 5′- UTR of prtF1 and prtF2 and preventing access to the ribosome binding site (Danger et al., 2015). Interestingly, FasX also stimulates the activity of the thrombolytic agent, streptokinase, which helps spread microbe. Thus, FasX is thought to be a riboswitch that triggers a switch from biofilm to planktonic mode of bacterial physiological process. *Streptococcus sanguinis* harbors a small RNA, cia-dependent RNA, or csRNA, which inhibits the expression of Type IV pilus and retards biofilm formation (Ota et al., 2018). Interestingly, a recent work investigated the role of Aronia melanocarpa juice in the degradation of extracellular RNA, which can control biofilm formation by streptococcus in the oral cavity (Lee et al., 2020).

Enterococcus faecalis is a Gram-positive bacterium responsible for nosocomial infections, particularly urinary tract infections. Apart from clinical settings, they are often thought to cause food-borne infections and dental diseases (Elghaieb et al., 2020; Anderson et al., 2015). *E. faecalis* can form biofilms in the oral cavity, which may act as a mechanism for horizontal gene transfer and the spread of antibiotic resistance. Many strains are resistant to antimicrobial agents, which makes their treatment difficult, partly due to the formation of biofilms in hospital environments. As*walR*, has been shown to repress walR RNA as determined by expression studies and reduce exopolysaccharide synthesis and biofilm formation. Furthermore, overexpression of AswalR was demonstrated to reduce virulence in vivo (Wu et al., 2021a). RibS, another non coding RNA in *S. typhi*, synthesized from 3′-UTR of RibE which also enhances biofilm formation. RibS is also thought to enhance biofilm formation by activation of cyclopropane fatty acid synthase, cfa (Zhao et al., 2018). MicA was demonstrated to regulate biofilm formation in a *luxS* deficient strain (Kint et al., 2010).

Staphylococcus aureus is an opportunistic pathogen and can be found in diverse environments. Even though the organism may be present as commensals, it can cause various disease conditions, varying from bacteremia and invasive endocarditis to toxic shock syndrome. Many strains of *S. aureus* are resistant to antibiotics such as Methicillin or Vancomycin, making treatment of these ailments challenging. Transcriptional regulator, MgrA has been shown to negatively regulate biofilm formation by interaction with psm promoter regions and repress its expression (Jiang et al., 2018). RsaA sRNA has been shown to negatively regulate mgrA mRNA by inhibiting translation initiation. Consequently, RsaA enhances biofilm formation and reduces capsule production (Tomasini et al., 2017). In contrast, RNAIII, a significant regulator of Agr quorum sensing, interacts with *mgrA* mRNA, enhances MgrA production, and consequently upregulates biofilm formation. RsaI a small ncRNA, regulates gene expression under glucose limiting conditions via catabolite control protein A in *Staphylococcus aureus* (Bronesky et al., 2019). SprX, a ncRNA in *S. aureus* interacts with walR mRNA, encoding an autolysin regulator, and promote adhesion and biofilm formation (Buchad and Nair, 2021). Another study has shown the role of antisense yycF RNA in reducing biofilm formation and downregulation of biofilm related genes, enhancement in vancomycin sensitivity and reduced invasion in rat model of infection in methicillin resistant *Staphylococcus aureus* (Wu et al., 2021b). *Staphylococcus epidermidis* is a representative commensal in human skin and mucosa. A long non-coding RNA molecule, IcaZ has been shown to interact with the 5′-UTR of the *icaR mRNA* and interfere with translation of IcaR, relieving the repression of the polysaccharide intercellular adhesin. This adhesin is a significant regulator of biofilm formation, and consequently, IcaZ enhances biofilm formation (Lerch et al., 2019). Table 2 highlights representative non-coding RNA that is associated with gram-positive bacteria (see Table 2).

5. Biofilm regulation by non-coding RNA in acid-fast bacteria and archaea

Not many studies have explored the regulatory role of non-coding RNA in biofilm formation in acid-fast bacteria and archaea. B11 sRNA was initially identified in *Mycobacterium tuberculosis,* and found to be conserved in the genus of *Mycobacterium.* Homologs of B11 have been identified in the genus of *Streptomyces* and *Corynebacterium*. This small RNA has been shown to repress the expression of target mRNA. Transposon mutagenesis studies in *Mycobacterium kansasii*, an opportunistic pathogen, have shown that B11 sRNA mutant exhibited a defect in growth and biofilm formation (Budell et al., 2020). RNA-seq studies have identified many non-coding RNA in archaea, even though they have not been studied as extensively as bacteria or eukarya. In archaea, *cis*-acting

RNA is more common than that *trans*-acting RNA and is typically between 50 and 500 nucleotides in length. An RNA-seq study identified a double-stranded non-coding RNA molecule, RrrR (RNase R resistant RNA), that regulates biofilm development in a hyperthermophile, *Sulfolobus acidocaldarius*. A total of 29 ncRNA have been identified that are differentially regulated in cells that form biofilms. Loss of RrrR resulted in a reduction in biofilm formation. Interestingly, RrrR+, the plus transcript, can base pair with its antisense, RrrR-, which can potentially control the activity of the plus transcript. While deletion in the plus strand transcript, RrrR + strand decreases biofilm development, overexpression of RrrR + leads to improved biofilms (Orell et al., 2018).

6. Conclusions

Biofilm infections are extremely difficult to treat because of resistance to chemicals and antibiotics. The understanding of the regulation of biofilms is evolving, and it will continue due to insights on how small non-coding RNA in microbes regulate biofilm formation at the gene expression level or via sequestration of target proteins and other novel mechanisms. Non-coding RNA control gene expression at the post-transcriptional level for adaptation in a dynamic environment and also maintains whether a planktonic or biofilm mode of existence is preferable. Furthermore, ncRNA regulates whether microbes in biofilms persist in biofilm mode or disperse from the biofilms based on environmental cues. In the context of microbial pathogenesis, regulatory roles of biofilm formation and development shed critical insights into microbial adaptation, pathogenesis, and perhaps ways to control microbes to prevent health-associated infections in the clinical and food environment. Non-coding RNAs are being continuously screened and evaluated for their regulatory roles in diverse cellular processes, including biofilm formation and pathogenesis.

CRediT authorship contribution statement

Arindam Mitra: Conceptualization, Writing – review & editing. **Suman Mukhopadhyay:** Writing – review & editing.

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.

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