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Small RNAs and their role in biofilm formation

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Abstract

The formation of biofilms is initiated by bacteria transitioning from the planktonic to the surface associated mode of growth. Several regulatory systems have been described to govern the initiation and subsequent formation of biofilms. Recent evidence suggests that regulatory networks governing the decision of bacteria whether to attach and form biofilms or remain as planktonic cells, are further subject to regulation by small non-coding RNAs (sRNAs). This is accomplished by sRNAs finetuning regulatory networks enabling concentration-specific responses, by sequestering, antagonizing or activating regulatory proteins in response to environmental cues, or by directly affecting the synthesis of proteins promoting/disfavoring the formation of biofilms. This review is aimed at giving an overview of the contribution of sRNAs in regulating the switch from the planktonic to the sessile bacterial lifestyle by highlighting how sRNAs converge with known regulatory systems required for biofilm formation.

Keywords

Small RNAs; Biofilm; Quorum Sensing

The converging worlds of small RNAs and biofilms

Since the first description in 1981 of a 108 bp long RNA involved in blocking replication of the ColE1 plasmid, an increasing number of non-coding, small RNAs (sRNA) primarily ranging in length 25–500 nucleotides have been discovered to play regulatory roles in both prokaryotes and eukaryotes. sRNAs regulate gene expression by binding mRNA or proteins, resulting in the modulation of translation primarily through altering target stability, affecting protein-DNA binding, or inducing conformational changes within the mRNA via direct antisense base-pairing between the sRNA and mRNA [1]. Targets of sRNA regulation tend to be regulatory genes themselves, thus enabling additional levels of control in regulatory networks. In eukaryotes, sRNA regulation has been implicated in processes such as differentiation in late development and programmed cell death. While sRNA regulation in bacteria was initially described as being predominantly involved in coordinating stress responses, recent evidence suggests that, similar to eukaryotic cells, bacteria also use sRNAs as key elements in the control of developmental processes as well as multi-cellular behavior [2]. The formation of biofilms is one such process. Biofilms are surface-associated multicellular communities encased in a self-produced extracellular matrix composed of proteins, polysaccharides, and DNA [3, 4]. Biofilms likely represent the prevalent microbial mode of existence in nature, with estimates suggesting that more than 90% of bacteria exist within biofilms. Existence in a biofilm affords bacteria many advantages over a planktonic existence including improved adaptation to nutrient deprivation, and increased resistance to predation and antimicrobial agents, characteristics that render biofilms extremely difficult to

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control in medical, industrial and agricultural settings [5, 6]. Biofilm-associated microorganisms have been shown to colonize a wide variety of man-made and medical devices and have been implicated in over 80% of chronic inflammatory and infectious diseases of soft tissues and chronic infections of humans with underlying predispositions [5]. The formation of biofilms is initiated with surface attachment by planktonic (single cell) bacteria that, once attached, grow into a complex community characterized by the presence of differentiated, mushroom- or pillar-like structures or microcolonies interspersed with fluid-filled channels [7]. The developmental progression leading to mature biofilms not only coincides with observable phenotypic changes but also requires cell density dependent (quorum sensing, QS) and independent regulated gene expression, many of which are governed by multiple regulatory networks. Following the initial discovery by Romeo and colleagues of the RNA-binding protein CsrA and two sRNAs (CsrB, CsrC) playing a role in *E. coli* biofilm formation [8, 9], multiple sRNAs have been identified that modulate the expression or activity of transcriptional regulators and components of regulatory networks important for attachment and biofilm formation (Table 1).

The function of sRNAs in the regulation of biofilm formation occurs via two general mechanisms, (i) sRNAs acting by basepairing with other RNAs and (ii) protein binding. Protein-binding sRNAs antagonize and sequester their cognate regulatory proteins by mimicking the protein binding sequences found in several mRNAs. Basepairing sRNAs are categorized as *cis* or *trans* based on their location within the bacterial genome relative to their mRNA targets. sRNAs transcribed from the DNA strand directly opposite of their mRNA targets are designated *cis*-encoded sRNAs and in general, share extensive complementarity to their targets. In contrast, *trans*-encoded sRNAs are located elsewhere on the genome, function in *trans* as diffusible molecules, and share only limited (10-25 bp) complementarity in their base-pairing interactions [10]. Trans-encoded sRNAs often rely on the RNA chaperone Hfq to form limited base-pairing interactions with target mRNAs. The importance of Hfq in trans-encoded sRNA-mediated regulation pathways likely accounts for the pleiotropic phenotypes observed in *hfq* mutant strains, including reduced virulence and biofilm formation [11-13] (Table 1). Basepairing between sRNAs and the target mRNAs leads to changes in mRNA translation and stability by altering the accessibility to ribosome binding sites (RBS) or enhancing ribonuclease-mediated degradation, thereby influencing target gene expression [14].

Considering the growing interest in biofilms and appreciation for the need to prevent and control biofilms in the medical setting and beyond, this review will highlight the role of sRNAs in biofilm developmental processes by focusing on well-characterized regulatory systems governing the transition from the planktonic to the surface associated mode of growth. To that end, the roles of sRNAs that affect surface attachment and motility, QS, stress response, and modulation of adhesiveness will be addressed with a particular emphasis on their contribution to the underlying regulatory mechanisms in these processes.

Should I stay or should I go – CsgD as a key regulator in the switch between the planktonic and sessile mode of growth

Adhesins such as pili and flagella have been demonstrated in many bacterial species to contribute to initial contact with a surface, with attachment triggering alterations in gene expression that allow bacteria to develop a more permanent association with the surface via surface associated motility (e.g. twitching) and expopolysaccharide biosynthesis [15]. Such a transition to the surface-associated lifestyle requires reprogramming of gene expression profiles. In *E. coli* and *Salmonella* strains this shift relies on control cascades that inhibit flagellar expression and activate the synthesis of adhesive curli fimbriae [16-18]. The transcriptional regulator CsgD, a key player in the complex regulatory circuit that decides

whether *E. coli* or *Salmonella* strains form biofilms, has been shown to be required for attachment and subsequent biofilm formation by activating the production and export of curli fimbriae while repressing the expression of several flagellar biosynthesis genes [16-18]. CsgD also transcriptionally activates *adrA* (previously known as *yaiC*) encoding a diguanylate cyclase, which synthesizes bis-(3'-5')-cyclic-diguanosine monophosphate (c-di-GMP), the second messenger that allosterically stimulates the production of cellulose, an extracellular polymeric substance (EPS). However, different *E. coli* isolates synthesize various EPS including cellulose, lipopolysaccharides, K antigen, colanic acid, and the cell-bound poly- β -1,6-*N*-acetyl-D-glucosamine (PGA) [19]. Thus, CsgD acts as a switch between the planktonic and sessile lifestyle by inversely coordinating the expression of genes involved in flagellar/motility control and adhesiveness. Given that CsgD lies at the heart of the complex switch from a motile to a sessile lifestyle, it comes as no surprise that its expression is regulated by a multitude of cellular cues (Figure 1). No less than a dozen different transcription factors, as well the signaling molecule c-di-GMP produced by diguanylate cyclases such as YdaM, are known to activate the *csgD* promoter. In addition to transcriptional regulation, expression of *csgD* is regulated at the mRNA level by no less than five Hfq-dependent sRNAs (McaS, RprA, OmrA/OmrB, GcvB) in response to environmental cues (Figure 1, Table 1). All five trans-encoded sRNAs act as repressors by basepairing with the 5' untranslated region (5'UTR, ~150 bp long) of the *E. coli csgD* mRNA, probably by occluding the ribosome binding site (RBS) and interfering with translational initiation. Yet, each of them belongs to a different regulon, and is expressed under different growth conditions. For example, expression of OmrA/B is regulated in response to high osmolarity via the two-component regulatory system (TCS) EnvZ-OmpR. Overexpression of these two redundant sRNAs results in curli deficiency, likely due to downregulation of *csgD*, and conditions favoring the planktonic mode of growth [20]. The sRNA GcvB is a global post-transcriptional regulator of amino acid transport and synthesis genes that also represses *csgD* in response to amino acid availability and is in turn regulated by GcvA and GcvR, the two primary transcription factors involved in regulation of the glycine cleavage system [21, 22].

The sRNAs not only serve as input modules for diverse environmental cues through their own transcriptional regulators but also cross-link individual branches of the CsgD network (Figure 1). Expression of the *rprA* sRNA is stimulated by the Rcs proteins, a multi-component phosphorelay system that responds to cell envelope stress. RprA is a post-transcriptional activator of RpoS, the general stress response sigma factor, with RpoS in turn being a major regulator/activator of *csgD* expression and curli biosynthesis upon entry into stationary phase [23-25]. RprA has also been reported to inhibit the synthesis of the diguanylate cyclase YdaM by binding downstream of the translation initiation region of the *ydaM* mRNA, thus preventing/reducing *csgD* transcription via YdaM and c-di-GMP [26]. The findings indicate that RprA not only interferes with *csgD* expression directly by hampering its translation but also indirectly by modulating optimal *csgD* transcription. Moreover, the Rcs system activates the expression of yet another biofilm matrix component, the exopolysaccharide colanic acid, and the Rcs controlled *csgD* repressor RprA may serve the additional function of preventing undesired expression of colonic acid with curli/EPS [27] or alternatively, may function in balancing the expression of both matrix components, cellulose and colanic acid, in response to environmental cues. Similar to RprA, McaS (a 95 bp sRNA, expression is induced by non-preferred carbon sources and entry into stationary phase [28]) not only represses the synthesis of CsgD directly but also activates the expression of genes that further promote the planktonic mode of growth (thus disfavoring biofilm formation) by directly inducing genes involved in flagellar biosynthesis [22, 28, 29].

In *Salmonella enterica* serovar Typhimurium, CsgD is equally required as a switch between the planktonic and biofilm mode of growth in response to environmental cues and is

responsible for the rdar (red, dry, and rough) morphotype, a biofilm behavior characterized by the presence of curli fimbriae and the production of cellulose [17, 30, 31]. However, although *E. coli*'s close sibling, *Salmonella* not only lacks the McaS sRNA, but also shows sequence deviation in the 5' UTR of *csgD* suggesting a different set of sRNAs repress *csgD* translation. The findings furthermore suggest that while CsgD in *Salmonella* and *E. coli* are homologous systems, CsgD is subject to substantially different regulation in these bacterial species. Recent findings by Monteiro *et al.* [32] implicated a different set of Hfq-dependent sRNAs, ArcZ and SdsR, in CsgD-mediated biofilm formation of *Salmonella*. SdsR was found to be required for maintaining steady state levels of *csgD*, while ArcZ was shown to repress *fliC*, a gene encoding a core component of the flagellar machinery [32, 33]. While neither sRNA has been shown to interact directly with *csgD* transcripts, both either regulate RpoS [33] or are directly regulated by RpoS [34].

Considering the large number of regulators acting upon CsgD, it is not surprising that *csgD* mRNA is considered a hub for signal integration via multiple sRNAs and a key player in the complex regulatory circuit that decides whether *E. coli* or *Salmonella* spp. form biofilms [35]. One is left to wonder, however, why CsgD is such a hotspot for regulation. Considering that bacteria must cope with fluctuations in nutrient availability and stress conditions, sRNAs may provide feedback regulation to titrate *csgD* synthesis under physiological conditions (stationary phase, biofilms) that call for motility or in response to environmental cues that are unfavorable for biofilm formation. This is further supported by sRNAs not only modulating *csgD* synthesis but also by directly influencing the decision to make flagella with ArcZ, OmrA and OmrB negatively and McaS positively regulating motility [36].

Stress Management

CsgD is a module within the general stress response, for which the general stress response sigma factor RpoS acts as the master regulator [23, 24]. The accumulation of RpoS is regulated at multiple levels, including posttranscriptionally by small regulatory RNAs (sRNAs) (Table 1). In *E. coli*, the alternative sigma factor RpoS responds to multiple stresses and is strongly up-regulated during entry into stationary phase. RpoS activates a large number of genes that allow bacteria to adapt to changing environmental conditions and correlates with downregulation of motility and activating the expression of YdaM (Figure 1), all of which are essential to activate transcription of *csgD* [24]. Given its connection to CsgD and that biofilm formation has been generally linked to slow growth and stressful growth conditions, it is not surprising that inactivation of *rpoS* results in reduced *E. coli* biofilm formation and increased expression of genes involved in flagella synthesis [37, 38]. The accumulation of RpoS in *E. coli* and *Salmonella* sp. is regulated at multiple levels, including the regulation of its translation by four Hfq-dependent sRNAs, OxyS, ArcZ, DsrA, and RprA (Figure 1) [39]. OxyS is the only repressor of *rpoS* translation while ArcZ, DsrA, and RprA activate translation. In the absence of activation factors, translation of *rpoS* mRNA is hindered by a stem-loop structure that sequesters the Shine-Dalgarno (SD) ribosome-binding site. Basepairing of ArcZ, DsrA, and RprA with one strand of the stem loop releases the occluded SD site for effective ribosome binding [40, 41]. Similar to that of CsgD, posttranscriptional regulation of RpoS is linked to environmental cues and physiological conditions. OxyS is induced upon sensing oxidative stress, ArcZ is under the control of the aerobic/anaerobic-sensing TCS ArcA/B and only expressed under aerobic conditions, while DsrA is induced at low temperatures [41]. In addition to regulating RpoS, DsrA, itself regulated by the *E. coli* AI2-based QS system, also functions as a regulator of both capsular polysaccharide biosynthesis and multidrug efflux pumps [42-44]. Nishino *et al.* [44] identified DsrA in an *E. coli* mutant screen to identify regulatory elements involved in the expression of other multidrug resistance systems. Inactivation of *dsrA* correlated with

decreased drug susceptibility to oxacillin while overexpression of *dsrA* conferred resistance to oxacillin, erythromycin, rhodamine 6G and novobiocin in an *E. coli* lacking a functional *arcB* (ArcB is a component of the nodulation–cell division efflux pump). While no detailed mechanism was elucidated, DsrA expression coincided with increased polysaccharide synthesis and *rpoS* expression, and increased expression of *mdtE*, a component of the MdtEF multidrug efflux pump in *E. coli* [44].

The global regulatory activity of RpoS in relation to biofilm growth is also present in various other bacteria. In *P. aeruginosa* PAO1, where the prevalence of a slow-growing population within these communities has been repeatedly demonstrated under different conditions, *rpoS* expression is increased in biofilms compared to stationary phase [45, 46] with inactivation resulting in altered biofilm architecture compared to the wild type [47]. However, RpoS plays slightly different roles in biofilm development in *E. coli* and *P. aeruginosa* as evidenced by the recent findings of RpoS being a positive regulator of *psl* gene expression in *P. aeruginosa* PAO1 [48]. Psl not only contributes to attachment but is also considered to be the primary structural polysaccharide essential for biofilm maturation [49]. In the plant pathogen *Serratia* sp. ATCC 39006, the Hfq-binding sRNA RprA directly increases *rpoS* translation leading to decreased antibiotic production [50]. Although the importance of biofilm formation remains to be studied in this organism, RpoS-mediated biofilm growth is important in the closely related rhizospheric bacterium *Serratia plymuthica* IC1270 with inactivation resulting in reduction of biofilm formation [51]. Direct regulation of *rpoS* translation upon sensing stress conditions plays a large role in biofilm formation. However, while RpoS is a key player in biofilm formation, the above cited work highlights that the regulatory function of RpoS is interconnected with multiple regulatory systems governing the transition from the planktonic to the biofilm mode of growth.

Suck it up – sequestration as a transition switch towards surface associated growth

Early attempts to understand the underlying mechanisms of RpoS as a central activator of general stress responses and stationary-phase gene expression [39] led to the recognition that CsrA, like an ‘evil twin’ of RpoS, counters the activity of RpoS by repressing stationary-phase gene expression and activating genes needed for growth [52]. Originally identified as regulating glycogen biosynthesis in *E. coli*, the RNA-binding protein CsrA regulates primary and secondary metabolic pathways, motility, virulence circuitry of pathogens, quorum sensing and genes involved in stress response [9, 52, 53]. Considering its RpoS opposing activity, it is not surprising that CsrA represses biofilm formation [8], by activating translation of genes involved in the planktonic lifestyle while repressing the synthesis of genes associated with the sessile lifestyle. This is accomplished by CsrA dimers binding to GGA motifs in the 5′ UTR of target mRNAs, thereby altering their translation and/or turnover. For instance, the polysaccharide PGA is required for attachment, cell–cell adherence, and stabilization of the biofilm structure. The *pgaABCD* operon is required for PGA synthesis (PgaC and PgaD) and secretion (PgaA and PgaB) [54]. CsrA inhibits PGA synthesis and biofilm formation by cooperatively binding to six sites in the *pgaABCD* mRNA leader, competing with the ribosome for binding and repressing translation of *pgaA* [55]. Recent evidence suggests that *pgaABCD* translation is further regulated by McaS, potentially by acting as an antagonist of CsrA [28], as high levels of McaS resulted in increased PGA-dependent biofilm formation while a strain lacking McaS exhibited reduced biofilm formation. In the case of *flhDC* mRNA (encoding DNA-binding protein that initiates a regulatory cascade for the expression of genes required for motility and chemotaxis), CsrA binding stabilizes the respective mRNA, with overexpression of *csrA* resulting in increased motility [56].

CsrA activity is counteracted by two sRNAs, CsrB and CsrC. Both sRNAs contain multiple CsrA binding sites, which mimic the GGA binding sites of CsrA target mRNAs. CsrB is a 366-nucleotide sRNA containing 18 CsrA binding sites, while CsrC is 245-bp long containing only 9 CsrA binding sites, with these sites capable of sequestering CsrA and thereby inhibiting the protein's regulatory activity [57]. Thus, when CsrB and CsrC levels increase, the sRNAs effectively sequester the CsrA protein away from target mRNA leaders (Figure 2a). The ability of these two sRNAs to negatively regulate CsrA activity impacts the ability of *E. coli* to form and maintain biofilms, as both depletion of CsrA and overexpression of CsrB/C lead to cellular autoaggregation and enhanced biofilm formation [57]. Transcription of the *csrB* and *csrC* genes is induced by the BarA-UvrB two-component regulators when cells encounter nutrient poor growth conditions, oxidative stress, weak acids (formate and acetate), and perturbations in the levels of Krebs cycle intermediates [58]. The CsrB and CsrC RNAs also are regulated at the level of stability through the CsrD protein which is not a ribonuclease but instead recruits the ribonuclease RNase E to degrade the sRNAs [59] (Figure 2b). Interestingly, CsrD protein contains GGDEF and EAL domains and while both domains are required for CsrD activity, the regulation of CsrB/C decay does not involve cyclic di-GMP metabolism [59]. The Csr system contains multiple layers of regulation by making use of non-coding RNAs that sequester multiple copies of CsrA to provide precise control of CsrA levels and activity and, thus, of the transition to the surface associated lifestyle. Negative feedback loops of the Csr system exists with CsrA repressing *csrD* expression in *E. coli* as well as *Salmonella* [59, 60].

Csr homologous systems

CsrA homologs are highly conserved among many pathogenic bacteria including *P. aeruginosa*, *Salmonella enterica*, *Helicobacter pylori*, *Erwinia* sp., *Legionella pneumophila* and *Vibrio cholerae* and posttranscriptionally modulate diverse transcripts in order to control virulence mechanisms and group behaviors. In *Yersinia pseudotuberculosis*, sequestration of CsrA by CsrB/C results in a loss of motility, likely through decreased CsrA activation of flagella biosynthesis genes [61]. Unlike in *E. coli*, however, CsrB and CsrC expression decreases rather than increases following activation of the BarA/UvrY homologs. Moreover, CsrB and CsrC are differentially expressed depending on environmental conditions and nutrient availability, with CsrC expression increasing during growth in rich complex medium and CsrB expression increasing during late stationary phase [61]. *S. typhimurium* also possesses a CsrA homologue with deletion of both CsrB and CsrC resulting in increased motility but decreased biofilm formation due to increased CsrA activity [62].

Among the Csr homologous systems, however, few have received more attention than the *P. aeruginosa* LadS/RetS/Gac/Rsm signal transduction network [63, 64] (Table 1). This intricate signaling system has been implicated as a switch between planktonic and biofilm modes of growth as well as acute and chronic infections by reciprocally regulating gene expression associated with type III and VI secretion and exopolysaccharide production via the CsrA homolog RsmA (regulator of stationary phase metabolites) [65] (Figure 2c). Similarly to CsrA, RsmA controls gene expression by binding to leader segments of target mRNAs, affecting their translation and stability. For instance, Irie *et al.* [48] demonstrated binding of RsmA to the 5' UTR of *psI* mRNA, thus preventing ribosome access and protein translation. In *P. aeruginosa*, RsmA function is antagonized by the sRNAs RsmZ and RsmY whose expression is directly controlled by GacA–GacS, the TCS homolog of the *E. coli* BarA/UvrY system. GacA–GacS function is in turn inversely controlled by the TCS hybrids RetS and LadS [64, 66, 67]. RetS negatively controls RsmYZ gene expression and inactivation of *retS* results in hyperattachment with elevated Psl exopolysaccharide gene expression and suppression of the type III secretion system (TTSS), with the phenotype abolished by a secondary mutation in *gacS* [64]. LadS positively controls sRNA levels, and

ladS inactivation results in decreased attachment, reduced Psl production, and elevated TTSS expression, suggesting that LadS may function to counteract RetS. While the mode of LadS activity remains uncharacterized, RetS reduces sRNA expression by interfering with GacS autophosphorylation through the formation of RetS–GacS heterodimers [68].

Recent evidence suggests that the Rsm signal transduction network does not simply function as a switch to enable the transition from the planktonic to the sessile mode of growth. This is based on the findings that while increased expression of RsmYZ results in enhanced initial attachment to abiotic surfaces, subsequent surface-attached growth and biofilm development is hampered by high levels of these sRNAs, in particular RsmZ [69, 70]. Moreover, the histidine phosphotransfer protein B (HptB) and SagS contribute to sRNA modulation. SagS is a sensor-regulator hybrid that participates in a phospho-transfer event with HptB [71, 72] and, similarly to HptB, is involved in the regulation of attachment and biofilm formation [70]. SagS represses sRNA levels predominantly under planktonic growth conditions, with inactivation of *sagS* resulting in a temporary enhancement of attachment but a defect in the later stages of biofilm formation [70]. Considering that the Δ *sagS* phenotype furthermore superseded those of both *gacA* and *rsmYZ* mutants, the findings suggested that SagS represents a novel level of Gac/Rsm regulation of attachment [70]. The findings further indicated a requirement for tight modulation of RsmYZ levels as the bacterial population progresses through the different phases of biofilm growth [69] (Figure 2c).

While not sharing sequence similarity or binding specificities with CsrA, the mRNA-binding protein Crc (catabolite repression control) of *Pseudomonads* is likewise a global regulator of carbon metabolism and other processes whose activity is governed by binding to antagonistic sRNAs [73]. In *P. putida*, Crc is controlled by the functionally redundant sRNAs CrcZ and CrcY [73]. Catabolite repression conditions correlated with low CrcZY levels and inactivation of both sRNAs led to constitutive catabolite repression that compromised growth on some carbon sources. While the authors did not link their findings to biofilm formation, it is likely that at least CrcZ (CrcY is present in *Pseudomonas fluorescens*, *P. putida*, and *Pseudomonas syringae*, but absent in *Pseudomonas aeruginosa* [73]) is linked to biofilm formation as a *crc* mutant has been characterized by impaired type IV pili production, decreased synthesis of exopolysaccharide, and reduced biofilm formation [74, 75].

Regulation of stickiness in bacterial lifestyle switching

Another key player governing the transition to the surface associated mode of growth by inversely controlling motility and curli/EPS expression is the signaling molecule c-di-GMP. C-di-GMP, predicted to be present in 85% of all bacteria, controls the switch between biofilm formation and motility depending on its intracellular concentration, with high levels favoring the sessile lifestyle and thus, the stickiness or adhesiveness of the bacterial community [76-78]. Likewise, the transition of biofilm bacteria to the planktonic growth state, a process called dispersion, has been linked to modulation of c-di-GMP levels [79]. C-di-GMP is synthesized from two GTP molecules by diguanylate cyclase (DGC) enzymes containing GGDEF domains consisting of approximately 170 amino acids, and is degraded by phosphodiesterase (PDE) enzymes containing EAL or HD-GYP domains that are approximately 250 amino acids in length. As a key player in the decision between the motile planktonic and sedentary biofilm-associated bacterial 'lifestyles', c-di-GMP binds to an unprecedented range of effector components and controls diverse outputs, including transcription, the activities of enzymes and larger cellular structures [78].

Recent evidence suggests that the modulation of c-di-GMP levels is subject to regulation by sRNAs, both directly and indirectly. In *E. coli*, indirect regulation of c-di-GMP levels has

been linked to the sRNA-controlled biofilm regulator CsgD. For instance, CsgD activates the DGC AdrA. Expression of *adrA* has been indirectly correlated (via increased levels of c-di-GMP and CsgD) with inhibition of flagellum production and rotation, and increased biofilm formation [16]. *adrA* expression has also been linked to increased cellulose synthesis [80]. Moreover, CsgD is itself subject to regulation by c-di-GMP via the DGC YdaM which is required for the expression of the biofilm-associated curli fimbriae [80] (Figures 1, 2b). Both CsgD and YdaM are negatively controlled by the sRNA RprA [26]. Thus, RprA creates a negative feedforward loop, directly resulting in the downregulation of CsgD- and YdaM-regulated genes and ensuring c-di-GMP levels to remain too low to activate *csgD* expression.

Similar to CsgD, the Csr system has been linked to c-di-GMP modulation. Jonas *et al.* [60] demonstrated that inactivation of *csrA* resulted in increased expression of two genes encoding cyclases, *ycdT* and *ydeH*, and led to modestly increased levels of c-di-GMP. Overexpression of *ycdT* and *ydeH*, however, increased the intracellular c-di-GMP levels 20-fold [60]. It is of interest to note that *ycdT* and *ydeH* are among a handful of target mRNAs which have been shown to be directly regulated by CsrA at the post-transcriptional level [81]. Conversely, activation of CsrA in *Salmonella* spp. lead to repression of DGC activity but stimulation of PDE activity causing c-di-GMP levels to decrease. Decreased c-di-GMP levels coincided with increased expression of motility-associated genes, while increased c-di-GMP levels favored the switch to a sessile lifestyle [60, 82]. Moreover, CsrA was found to regulate the expression of five additional GGDEF/EAL proteins [60]. An additional level of c-di-GMP-related regulation exists via CsrD, which is essential for the RNase E-mediated decay of the CsrA antagonistic sRNAs CsrB/C in *E. coli* [59] (Figure 2b). Interestingly, although CsrD, a member of the GGDEF-EAL domain family, neither produces nor degrades c-di-GMP, CsrD activity requires the presence of both GGDEF and EAL domains [59]. Altogether, these data demonstrate a global role for CsrA in the regulation of c-di-GMP metabolism by regulating the expression of GGDEF/EAL proteins at the post-transcriptional level. Moreover, by tightly modulating c-di-GMP levels, sRNAs are capable of altering global gene expression responsible for transitioning between the motile and sessile lifestyles.

Fine-tuning bacterial communication

Quorum sensing (QS) enables bacteria to communicate using extracellular signaling molecules called autoinducers (AIs) and is achieved by the synthesis, secretion, and detection of these molecules that accumulate in proportion to increasing cell density [83]. This process ensures that bacteria behave as individuals at low cell density but exhibit group behaviors at high cell density due to the coordination of gene expression on a population-wide scale [83]. The QS circuits identified in Gram-negative bacteria resemble the canonical quorum sensing circuit of the symbiotic bacterium *Vibrio fischeri* and contain, at a minimum, homologues of two *V. fischeri* regulatory proteins called LuxI and LuxR. LuxI homologues are responsible for the biosynthesis of AIs (acylated homoserine lactones, AHLs) while LuxR-type proteins are transcriptional regulators that bind cognate AIs. Given the close proximity and density of cells within biofilms, it is not surprising that QS plays an important role in biofilm formation. This was first demonstrated by Davies *et al.* [84] by showing that QS in *P. aeruginosa* is crucial for proper biofilm formation. Specifically, a *P. aeruginosa* Δ *lasI* mutant formed only flat, undifferentiated biofilms (monolayers) unlike wild-type biofilms which are characterized by a structured and differentiated architecture. Exogenous addition of the AI synthesized by LasI restored biofilm formation to wild-type levels indicating that the defect was a result of AI absence. *P. aeruginosa* possesses two hierarchically organized QS circuits [Las, Rhl] that are activated by two types of AHLs that control the expression of more than 300 genes. Another type of bacterial signal molecule is

the *Pseudomonas* quinolone signal (PQS), which positively regulates a subset of QS-dependent genes and biofilm formation [85]. PQS is regulated by the transcription factor PqsR [86] which in turn is posttranscriptionally activated by the *cis*-acting sRNA PhrS that base-pairs to a short open reading frame located directly upstream of the translationally coupled *pqsR* gene [87]. PhrS binding resolves an inhibitory secondary structure, resulting in the unmasking of the ribosome binding site.

sRNAs have also been demonstrated to play a role in QS and biofilm formation by *Vibrio harveyi*. This QS circuit possesses features reminiscent of both Gram-negative and Gram-positive quorum sensing systems. Similar to other Gram-negative bacteria, *V. harveyi* produces and responds to AIs of the AHL class, while also utilizing a TCS circuit containing a phosphorelay cascade comparable to the QS system present in Gram-positive bacteria for QS signal transduction. In the absence of AI (low cell density), the membrane bound histidine kinase and a response regulator hybrid LuxO is phosphorylated and indirectly activates the translation of the low cell density master regulator AphA. AphA controls ~ 300 low cell density target genes [88] and inhibits translation of the high cell density master regulator LuxR. In contrast, when LuxO is dephosphorylated at high cell densities, AphA translation stops and LuxR translation occurs, with LuxR controlling ~ 700 high cell density target genes (Figure 3) [89]. Reciprocal control of LuxR and AphA by LuxO is achieved through five Hfq-dependent sRNAs called Qrr1–5 (quorum regulatory RNA) whose expression is activated by phosphorylated LuxO. Once expressed, Qrr sRNAs activate translation of AphA while simultaneously repressing translation of LuxR (Figure 3) [89]. Shao and Bassler [89] demonstrated that Qrr1 is less effective than Qrr2–5 in activating *aphA*, as Qrr1 lacks one of two required pairing regions. Qrr1, however, was found to be as effective as the other Qrr sRNAs at controlling targets like *luxR*. Moreover, the Qrr sRNAs are not redundant but act additively, resulting in the production of a LuxR protein concentration gradient that differentially affects expression of target genes including those involved in virulence, pilus and flagellar synthesis, and biofilm formation [89, 90]. Additionally, Qrr sRNAs negatively regulate their own expression by base-pairing with and inhibiting the expression of *luxO*, which is responsible for the activation of the *qrr* genes, further emphasizing that the sRNAs are integrated into regulatory QS circuits. Thus, in addition to fine tuning the QS output, Qrr sRNAs participate in regulatory or feedback loops by not only regulating the translation of LuxR and HapR but also their own expression.

The quorum sensing circuit of the closely related pathogenic bacterium *V. cholerae* resembles that of *V. harveyi*, but *V. cholerae* only has Qrr1–4, which are largely redundant [91]. Moreover, in contrast to other bacterial pathogens that induce virulence factor production and/or biofilm formation at high cell density in the presence of AIs, *V. cholerae* represses these behaviors at high cell density (Figure 3). Hammer and Bassler [92] demonstrated that inactivation of the LuxR homolog *hapR* ‘locked’ *V. cholerae* in a state mimicking low density while enhancing attachment. Enhanced attachment correlated with increased expression of genes involved in the synthesis of the VPS exopolysaccharide required for *V. cholerae* biofilm development in freshwater environments [92, 93]. In contrast, high cell density conditions achieved by inactivation of *luxO* impaired attachment. An additional layer of sRNA-mediated regulation of HapR and QS-dependent gene expression exists in *V. cholerae* via the CsrA protein and BarA–UvrY homologs VarS–VarA [94]. VarS–VarA controls transcription of CsrBCD, three redundant sRNAs that, similarly to the sRNAs CsrBC of *E. coli*, bind and sequester the global regulatory protein CsrA. When active, CsrA acts through LuxO to increase expression of Qrr sRNAs with activation of CsrB being required for full HapR activity [94, 95]. Thus, expression of Qrrs, in addition to being activated by phosphorylated LuxO and repressed by Qrrs, is subject to regulation by the Csr system. Consistent with the regulatory network described above, Jang *et al.* [96] determined that inactivation of *varS* resulted in decreased expression of *csrBC* and *hapR*,

and increased attachment by *V. cholerae* 2740-80. Likewise, biofilm formation is repressed upon *agr* QS activation in *Staphylococcus epidermidis* and *S. aureus* [97, 98]. To our knowledge, the only sRNA described so far to play a role in staphylococcal biofilm formation is the multifunctional regulatory RNA called RNAIII, an effector molecule of the *agr* QS system which primarily acts as a repressor of translation [99].

Concluding remarks

This review was aimed at giving an overview of the role of sRNAs involved in biofilm development, a research field still in its infancy. The overview is by no means complete and may occasionally present conflicting information. Nevertheless, it is apparent from the above-cited studies that sRNAs are important players in regulatory networks controlling the transition to the surface attached lifestyle. Not only are sRNAs regulatory components that enable fine-tuning with respect to induction or repression of gene expression in a dose-dependent manner, as in the case of Qrr sRNAs or the Csr system, they also enable feedback or feedforward loops and mediate cross-talk between global regulatory networks. An example of the latter is the role of the *V. cholerae* Qrr1 in repressing QS as well as activating translation of *vca0939* encoding a GGDEF domain harboring protein involved in the synthesis of cyclic di-GMP [100]. Moreover, several studies only inferred the role of sRNAs in biofilm formation based on their effect on components of major regulatory networks previously described to either enhance or impair attachment and biofilm formation. Studies lacking such associations were not included with one exception, as the study provided a possible link between sRNAs and drug tolerance, a hallmark of biofilms involving enhanced resistance to antimicrobial compounds [5]. While no detailed mechanism was elucidated nor a role of DsrA in biofilm drug tolerance determined, the findings by Nishino *et al.* [44] underscore the need for determining sRNA function in relation to biofilm developmental aspects, in particular when considering that biofilm formation requires regulatory cascades that control the temporal and spatial expression of genes. A combination of genetic and molecular techniques used in combination with microscopy will be required to fully elucidate these complex pathways, including the identification and characterization of novel sRNAs. Obtaining a complete picture of sRNA regulation during biofilm development will require moving beyond studies focusing on attachment into the full lifecycle of biofilm growth, maturation, and eventual dispersion. Given their abundance in a wide variety of cellular processes including initial biofilm formation, it is very likely that additional sRNAs involved in biofilm growth await discovery.

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Figure 1. Regulatory network controlling *CsgD* and effect on biofilm formation and motility *csgD* expression is controlled at the transcriptional level by signal integration by the sigma factor RpoS, the TCS OmpR and indirectly by the cyclase YdaM via the modulation of c-di-GMP levels. In addition, *csgD* expression is controlled posttranscriptionally via several antisense sRNAs whose expression is affected by a wide range of environmental stimuli. Cell surface stress activates RprA (via RcsB), GcvB is activated depending on the glycine status of the cell (via GcvA), osmotic imbalance results in the activation of OmrA/B via OmpR, and quality of carbon source/growth rate results in the activation of McaS gene expression (transcription factor(s) unknown). Activation of *CsgD* represses genes associated with the planktonic lifestyle (motility, e.g. *flhDC*) and activates genes associated with surface attached mode of growth including curli fimbriae and polysaccharide gene expression (e.g. for PGA and cellulose). In *E. coli*, RpoS is regulated by several sRNAs in response to environmental stimuli including low temperature (DsrA; transcription factor(s) unknown), and oxygen tension (ArcA-ArcB represses ArcZ under anaerobic conditions) which result in activation of RpoS. RpoS is repressed upon sensing oxidative stress (OxyR activates OxyS). Adapted with permission from Boehm and Vogel [35].

A

B

Figure 2. CsrA and CsrA homolog RsmA and effect of sequestration by sRNA

(A) Gene expression is controlled by CsrA binding to leader segments of target mRNAs (e.g. *pgaABCD* involved in PGA biosynthesis and export) affecting their translation and stability. CsrA activity is repressed via sequestration of CsrA by sRNAs CsrB/C, thereby inhibiting CsrA regulatory activity. (B) CsrA activity is enhanced by CsrD as well as the sRNA McaS, a component of the CsgD network which represses CsgD. Repression by CsrA on *pga* mRNA translation is negated by NhaR, an activator of PGA biosynthesis. (C) Schematic overview of the signaling cascade that converges on the sRNAs RsmY and RsmZ, which act by sequestering the translational repressor RsmA. RsmA reciprocally regulates factors involved in the planktonic/sessile switch indicated by activation of genes involved in motility or by repressing genes required for biofilm formation (Pel, Psl polysaccharides). Dashed lines indicate that the connection has not been fully demonstrated or is not understood at the molecular level. Adapted with permission from Mikkelsen *et al.* [101].

Figure 3. Model for Qrr sRNA regulation of QS in *Vibrio* sp

At low cell density (green arrows), phospho-LuxO activates expression of the *qrr* genes encoding the Qrr sRNAs. The Qrr sRNAs promote translation of the low cell density master regulator AphA and inhibit translation of the high cell density master regulator LuxR/HapR. At high cell density (red arrows), Qrr sRNA production ceases because dephosphorylated LuxO is inactive. AphA translation stops and LuxR/HapR translation occurs. LuxO production is repressed by the Qrr sRNAs in a negative feedback loop. AphA and LuxR repress each other at the transcriptional level. LuxO activity is furthermore regulated via CsrA and sRNAs CsrB/C (blue arrows). Adapted with permission from Shao and Bassler [89].

Table 1
Summary of known sRNAs, their target regulatory circuits and role in attachment and biofilm formation

Targets	sRNA	Organism	Regulation of target mRNA	Phenotype related to sRNA levels	Refs.
Hfq		<i>E. coli</i>		Deletion reduced attachment	[13]
		<i>S. typhimurium</i>		Deletion reduced <i>rdar</i> phenotype (includes reduced attachment and decreased EPS-component expression)	[32]
CsgD	OmrA, OmrB	<i>E. coli</i>	Repression	Overexpression decreased curli and cellulose production	[20]
	GcvB	<i>E. coli</i>	Repression	Basal expression decreased curli production	[22]
	RprA	<i>E. coli</i>	Repression	Overexpression decreased CsgD synthesis	[26]
	McaS	<i>E. coli</i>	Repression	Overexpression decreased curli formation	[28]
	ArcZ	<i>S. typhimurium, E. coli</i>	Activation	Overexpression decreased motility and increased biofilm formation. Deletion reduced <i>rdar</i> phenotype	[32, 33]
PgaA	SdsR	<i>S. typhimurium</i>	Activation	Deletion reduced <i>rdar</i> phenotype	[32]
	McaS	<i>E. coli</i>	Activation	Overexpression increased curli-independent attachment (Potentially via CsrA sequestration)	[22]
CsrA	CsrB, CsrC	<i>E. coli, Y. pseudotuberculosis</i>	Repression by sequestration	Deletion reduced biofilm formation	[8, 57, 61]
YdaM	RprA	<i>E. coli</i>	Repression	Direct binding reduced YdaM expression (likely decreasing c-di-GMP levels)	[26]
Crc	CrcZ	<i>Pseudomonas spp.</i>	Repression by sequestration	Phenotype related to biofilms has not been reported	[73]
RsmA	RsmY, RsmZ	<i>P. aeruginosa</i>	Repression by sequestration	Biofilm development	[101]
AphA	Qrr1-4	<i>V. cholerae</i>	Activation	Overexpression increased AphA (likely resulting in increased biofilm formation)	[89]
	Qrr1-5	<i>V. harveyi</i>	Activation	Overexpression increased AphA (likely resulting in decreased biofilm formation)	[89]
HapR (LuxR homolog)	Qrr1-4	<i>V. cholerae</i>	Repression	Overexpression decreased HapR (likely resulting in increased biofilm formation)	[89]
LuxR	Qrr1-5	<i>V. harveyi</i>	Repression	Overexpression decreased LuxR (likely resulting in decreased biofilm formation)	[89]
PqsR	PhrS	<i>P. aeruginosa</i>	Activation	Phenotype related to biofilms has not been reported	[87]
RpoS	ArcZ	<i>S. typhimurium, E. coli</i>	Activation	Overexpression increased RpoS reporter	[32,41]

Targets	sRNA	Organism	Regulation of target mRNA	Phenotype related to sRNA levels	Refs.
	RprA	<i>E. coli</i>	Activation	activity Overexpression activates RpoS synthesis	[25]
	OxyS	<i>E. coli</i>	Repression	Overexpression decreased RpoS reporter activity	[41]
	DsrA	<i>E. coli</i>	Activation	Overexpression increased RpoS reporter activity	[41]