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# Small RNAs in the control of RpoS, CsgD, and biofilm architecture of *Escherichia coli*

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**Keywords:** ArcZ, biofilm, c-di-GMP, cellulose, CsgD, curli, Hfq, McaS, RpoS, RprA

**Abbreviations:** cAMP, 3′, 5′-cyclic adenosine monophosphate; c-di-GMP, bis-(3′-5′)-cyclic di-guanosine monophosphate; DGC, diguanylate cyclase; PDE, c-di-GMP-specific phosphodiesterase; PGA, poly-β-1,6-D-N-acetylglucosamine; (p)ppGpp, guanosine-3,5-bis-pyrophosphate; RNAP, RNA polymerase; SD, Shine Dalgarno sequence; SL, stem loop; sRNA, small RNA; TF, transcription factor; UTR, untranslated region

Amyloid curli fibers and cellulose are extracellular matrix components produced in the stationary phase top layer of *E. coli* macrocolonies, which confer physical protection, strong cohesion, elasticity, and wrinkled morphology to these biofilms. Curli and cellulose synthesis is controlled by a three-level transcription factor (TF) cascade with the RpoS sigma subunit of RNA polymerase at the top, the MerR-like TF MlrA, and the biofilm regulator CsgD, with two c-di-GMP control modules acting as key switching devices. Additional signal input and fine-tuning is provided by an entire series of small RNAs—ArcZ, DsrA, RprA, McaS, OmrA/OmrB, GcvB, and RydC—that differentially control all three TF modules by direct mRNA interaction. This review not only summarizes the mechanisms of action of these sRNAs, but also addresses the question of how these sRNAs and the regulators they target contribute to building the intriguing three-dimensional microarchitecture and macromorphology of these biofilms.

## Introduction

Bacterial biofilms are multicellular communities in which cells are surrounded by a self-produced extracellular matrix of polymeric substances that can include exopolysaccharides, proteins, amyloid fibers, and exo-DNA. Matrix components allow cells to attach to each other and mediate adherence to biotic or abiotic surfaces. Bacteria inhabiting a biofilm are protected from physical stress, antimicrobials, and the host immune system, and thereby cause severe medical, environmental and technical problems.[1-3] Biofilm formation and architecture is controlled by complex regulatory networks that integrate multiple environmental signals at the transcriptional as well as post-transcriptional levels by using alternative sigma factors, two-component systems, small regulatory RNAs (sRNA), and second messengers (in particular c-di-GMP). In this review, we highlight the multiple roles of several sRNAs in controlling the expression of the stationary phase sigma factor RpoS ($\sigma^S$), the c-di-GMP generating diguanylate cyclase YdaM and the transcription factor CsgD, which are key factors in the regulatory network that controls biofilm matrix production in the model bacterium *Escherichia coli*. By focusing on highly structured macrocolony biofilms, we also address the question how these sRNAs and the regulators they target contribute to building the intriguing three-dimensional architecture and macromorphology of these biofilms (**Fig. 1**).

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## Biofilm Architecture and RpoS Control

### Matrix components, physiological heterogeneity, and microarchitecture of *E. coli* biofilms

When biofilms of *E. coli* K12 are grown at ambient temperature (below 30 °C), predominantly synthesized matrix components are amyloid curli fibers and cellulose. In addition, the exopolysaccharide colanic acid may be produced.[4,5] Besides these components, a variety of adhesins can mediate attachment of the bacterial cells to surfaces (for a review, see ref. 1). Flagella are likewise structural components of a biofilm and are involved in the initial attachment of the cells to a surface.[6-8] At 37 °C poly-*N*-acetylglucosamine (PGA) as well as sometimes curli and/or cellulose can form the extracellular matrix, while type I fimbriae or the adhesin Ag43 can mediate initial attachment of cells.[9-11] PGA has also been implicated in attachment to abiotic surfaces during growth below 37 °C.[10]

Depending on the genetic background of a strain, the nutrient supply, and the surface of the growth support, the actual biofilm composition and physical appearance are remarkably variable. *E. coli* biofilms grown as macrocolonies on an agar surface are highly structured and—following the nutrient gradient building up during growth of the biofilm—differentiate into two layers of morphologically and physiologically distinct cells[8,12] (**Fig. 1**). The air-exposed top layer features cells that are literally encased in basket-like networks formed by amyloid curli fibers only or by a curli–cellulose composite. These cells are non-dividing and of ovoid shape due to their stationary phase physiology. In the bottom layer and at the outer rim of a macrocolony, i.e., closer

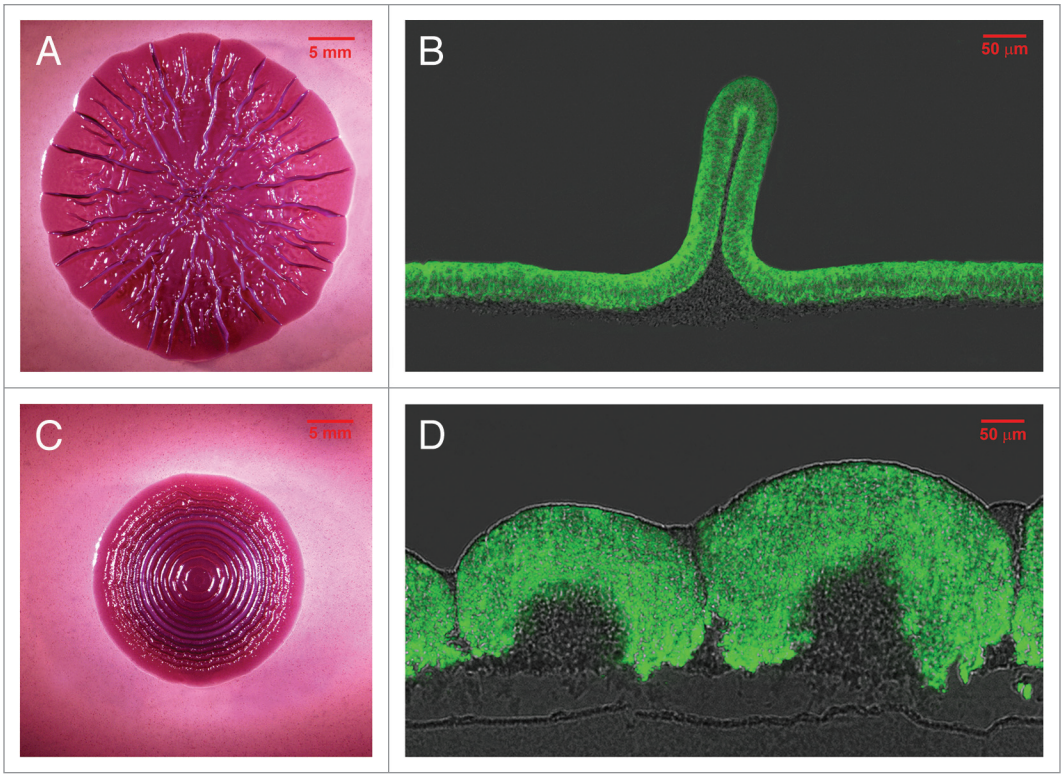


**Figure 1.** Morphology and physiological stratification of *E. coli* macrocolony biofilms. Macrocolonies of the curli- and cellulose-producing *E. coli* K-12 strain AR3110 (**A**) and the curli-producing, but cellulose-deficient original K-12 strain W3110 (**C**) were grown for 5 d at 28 °C on salt-free LB plates containing the amyloid dye Congo red. AR3110 is a direct derivative of W3110, in which a "domesticating" point mutation that generated a stop codon in the cellulose synthase operon was '"repaired."[12] Cryosections through macrocolonies of AR3110 (**B**) and W3110 (**D**) grown for 5 d at 28 °C on salt-free LB supplemented with thioflavin S (TS) are shown, with brightfield and fluorescence microscopic images merged. TS is an amyloid-staining dye that binds to both curli fibers and cellulose, which are produced under the control of RpoS in the upper layer of starving cells in the macrocolonies. Fluorescence images were false-colored green for TS. The figure illustrates previously described results,[12] but only (**D**) is a section of an image actually published in the previous study and is shown here with permission.

to the nutrient-providing agar surface, cells are rod-shaped and they divide and produce flagella. These cells are in a mode of restricted growth corresponding to the post-exponential phase with many genes—as for instance flagellar genes—being activated by cAMP-CRP. Interestingly, in the bottom zone of the macrocolony, flagella form a tight mesh tying cells together.[8,12] In biofilms also containing cellulose, flagella can serve as scaffolds for the assembly of cellulose.[12,13]

Overall, the temporal succession of the post-exponential and stationary phases in the growth cycle of a planktonic culture thus translates into a spatial pattern of physiological differentiation, i.e., stratification of the macrocolony biofilm.[14] As a consequence, we have to envision all the regulatory networks that shape this biofilm as differentially operating both in time and in the three-dimensional space of a structured community of billions of cells. This means that the "curli/cellulose control cascade"[15] operates in the stationary phase top layer, whereas the "flagellar control cascade"[16] is active in the post-exponentially growing bottom layer. In between there is a transition zone, in which the switching mechanisms operate that make these two transcription factor cascades mutually exclusive.[14,17] It should also be noted that until recently the inverse regulation of flagella and curli by numerous regulatory factors has been interpreted as a "motile/planktonic-vs.-biofilm" dichotomy. However, the recent studies that have revealed the physiological two-layer structure of macrocolony biofilms demonstrate this interpretion to be misleading—flagella expression also occurs in the growing zones of biofilms, and curli expression has long been known to also occur in stationary phase planktonic cells.[8,12,18] Thus, the inverse regulation of flagella and curli/cellulose is embedded in the growth-to-stationary phase transition, which occurs in planktonic cultures as well as in biofilms.

High production of curli fibers and cellulose is also required to generate the amazing macrocolony morphology of wild-type enteric bacteria that summarily has been termed "wrinkled," "rugose," or "rdar" (for red, dry, and rough on plates containing the amyloid dye Congo red).[11,19] While the concomitant production of curli fibers and cellulose generates strong cohesion and elasticity that allows macrocolonies to buckle up into long and very high ridges and elaborate wrinkles, production of a curli-only matrix results in concentric breaks, and a macroscopic ring pattern of the colonies[8,12] (**Fig. 1**). Also, the network of entangled flagella at the bottom of the colony contributes to this

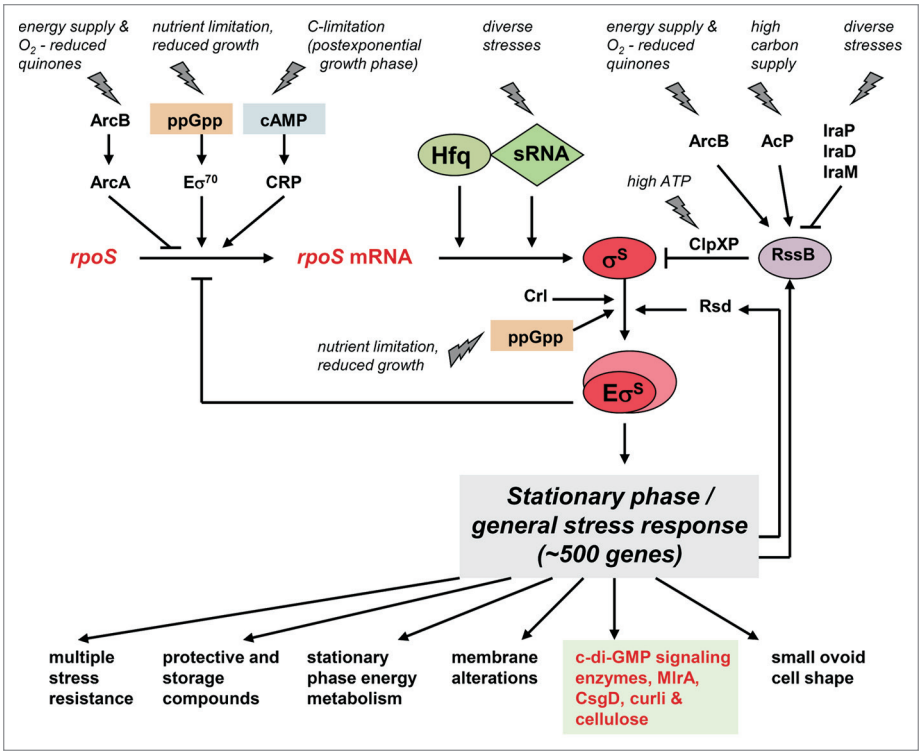


**Figure 2.** Signal integration via transcription factors, sRNAs, and modulators of protein turnover in the control of RpoS and RpoS-dependent functions in *E. coli*. At least four levels of control can be distinguished, i.e., *rpoS* transcription, *rpoS* mRNA translation, RpoS ($\sigma^S$) activation by binding to RNAP core enzyme, and RpoS proteolysis. Numerous environmental signals are integrated at all these levels, with several key signaling systems—such as the stress alarmone (p)ppGpp or the ArcB/ArcA+RssB phosphorelay system—affecting more than one regulatory level (for reviews, see references 20, 25, and 132. By controlling approximately 500 genes,[21] RpoS-containing RNAP globally changes cellular physiology as indicated. Via sigma factor competition for RNAP core enzyme, RpoS also provides for negative feedback on its own $\sigma^{70}$-dependent expression. In addition, the RpoS-dependent proteins RssB and Rsd generate negative and positive feedback loops by promoting RpoS proteolysis and RpoS binding to RNAP, respectively.

morphology, since strains defective in flagellin or the flagella motor protein MotA form less structured macrocolonies.[8]

**RpoS as a key regulator of the general stress response and biofilm architecture**

As a major sigma subunit of RNAP, RpoS is the master regulator of stationary phase gene expression in *E. coli*. Moreover, even growing cells can rapidly accumulate high-RpoS levels when suddenly exposed to diverse stress conditions, which makes RpoS also the general stress sigma factor (for a comprehensive review, see ref. 20). RpoS can activate approximately 10% of all *E. coli* K-12 genes,[21-24] which profoundly changes cellular physiology and even cellular morphology. Hallmarks of cells under the "RpoS regime" are small and ovoid shape, a pronounced multiple stress resistance, altered energy metabolism, and the production of the biofilm matrix components curli and cellulose[20] (**Fig. 2**).

RpoS induction during the transition into stationary phase is highly regulated at the transcriptional, translational, and post-translational levels (**Fig. 2**; comprehensively described in ref. 20). Transcription of *rpoS* is stimulated by high levels of cAMP and (p)ppGpp, which accumulate when growth is slowing down because nutrients become more and more limiting. Turnover, and above all, translation of *rpoS* mRNA are equally highly controlled processes, as described in detail below. Furthermore, intricate multicomponent regulatory networks control the proteolysis of RpoS as well as its binding to RNAP core enzyme in competion with other sigma factors (**Fig. 2**), but for these levels of regulation, the reader is referred to comprehensive reviews in references 20, 25 and 26.

Embedded in global stress physiology, RpoS is also a master regulator for biofilm formation, in particular, for macrocolony biofilms at ambient temperature. As the master regulator of the "curli/cellulose control cascade," RpoS is essential for the production of these matrix components in the upper stationary phase layer of macrocolonies, which, together with RpoS-dependent multiple stress resistance, affords strong protection of biofilm-resident cells. The "backbone" of the regulatory network that controls curli and cellulose formation is a three-level transcription factor (TF) cascade with RpoS at the top, the MerR-like TF MlrA (with YdaM acting as a directly interacting co-activator) and the TF CsgD, a key biofilm regulator (TF modules I, II, and III, underlied in yellow in **Fig. 3**). Whether TF module II activates expression of CsgD, depends on a c-di-GMP-dependent switch device (c-di-GMP module A, underlied in light red in **Fig. 3**). This switching module includes the "trigger enzyme" YciR, a bifunctional protein that acts by direct protein–protein interactions that are modulated by its c-di-GMP-degrading phosphodiesterase (PDE) activity, and the c-di-GMP-generating diguanylate cyclases (DGC) YegE and

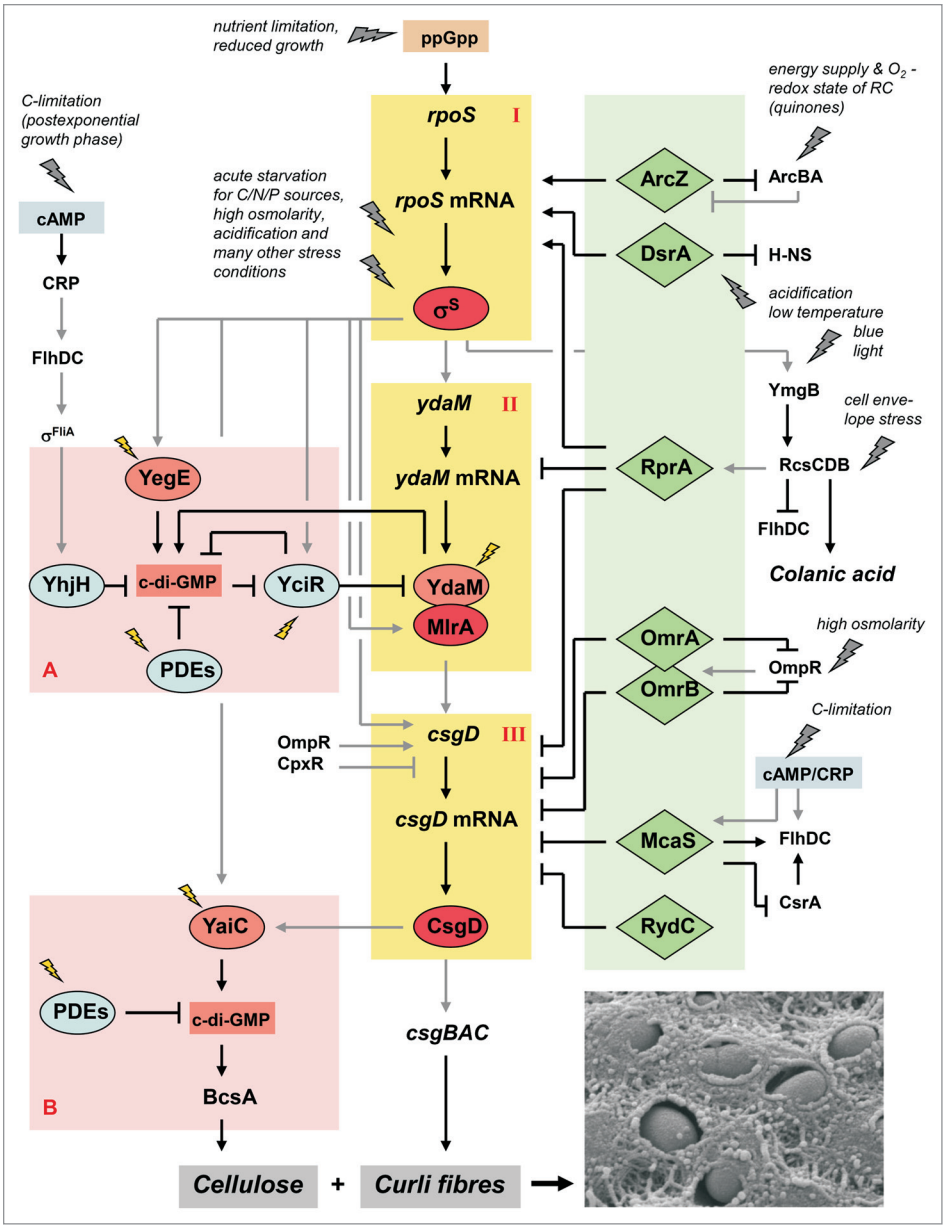


**Figure 3.** Signal integration in the RpoS-dependent network that regulates the synthesis of extracellular the matrix components curli and cellulose. The "backbone" of the control network is a TF cascade consisting of three modules (I, RpoS as the master regulator; II, YdaM/MlrA; III, CsgD; highlighted by yellow boxes). A c-di-GMP-dependent control module (A; with DGCs and PDEs highlighted by light red and light blue ovals, respectively) with the PDE YciR acting as a trigger enzyme provides for an essential switch between TF modules I and II. A second c-di-GMP control module (**B**) allows for specific signal input into cellulose production without affecting curli expression. sRNAs (highlighted in green) provide for environmental input along the entire TF cascade, with *rpoS* mRNA and *csgD* mRNA acting as major hubs for signal integration. Only sRNAs are included whose direct binding at the respective mRNAs was demonstrated by compensatory basepair exchanges. For simplicity, the reverse action is not included in the figure, i.e., that strong production of these mRNA, e.g., during entry into stationary phase, can scavenge the sRNAs, and thereby, affect their other regulatory effects. Activation of gene expression by TFs is depicted by gray arrows, all other regulatory effects are symbolized by black arrows or lines. For mechanistic details, see main text. The photographic inset shows a scanning electronmicroscopic image of the surface of a macrocolony of the curli and cellulose-producing strain AR3110 (as the one shown in **Fig. 1A**), with the extracellular matrix material consisting of a curli-cellulose composite.[12]

YdaM, which control the activity of MlrA (TF module II). In still-growing cells, which contain undetectable c-di-GMP levels (mainly due to the PDE YhjH, which is under control of the flagellar regulatory cascade, **Fig. 3**),[27] YciR inhibits the DGC YdaM and the TF MlrA by direct interaction. However, this inhibition is relieved when YciR begins to operate as a PDE—in other words, when the RpoS-induced DGC YegE drives up the cellular c-di-GMP level. This state is stabilized by a positive feedback loop since the now 'de-inhibited' YdaM can also produce c-di-GMP. Moreover, YdaM now activates MlrA by direct interaction.[17,18,28] Again, together with RpoS-containing RNAP, MlrA then initiates transcription of *csgD*. In addition, several other TFs, e.g., OmpR and CpxR, can positively or negatively modulate transcription at the *csgD* promoter.[29-31] As a central regulator for biofilm formation, CsgD then activates the transcription of the curli structural operon *csgBAC* and, again together with RpoS-RNAP, the DGC gene *yaiC*.[18,30,32-35] c-di-GMP production by YaiC is crucial for activating cellulose synthase via its subunit BcsA[36] (c-di-GMP module B in **Fig. 3**).

At low temperature RpoS also contributes to the expression of another biofilm matrix component, the exopolysaccharide colanic acid. This requires the 19 gene *wca* operon that is under positive control of the RcsC/RcsD/RcsB phosphorelay system.[37] The positive role of RpoS lies in transcriptional activation of a small protein, YmgB, which can stimulate the activity of the Rcs system in a not-yet-clarified manner[38] (**Fig. 3**). The Rcs system, which responds to cell envelope cues, negatively controls flagella expression, it promotes biofilm maturation, and was recently shown to be involved in determining cell shape.[39-42] Most important for the topic of this review, the Rcs system also drives expression of RprA, which is the only sRNA that targets all three TF modules in the curli/cellulose control cascade.

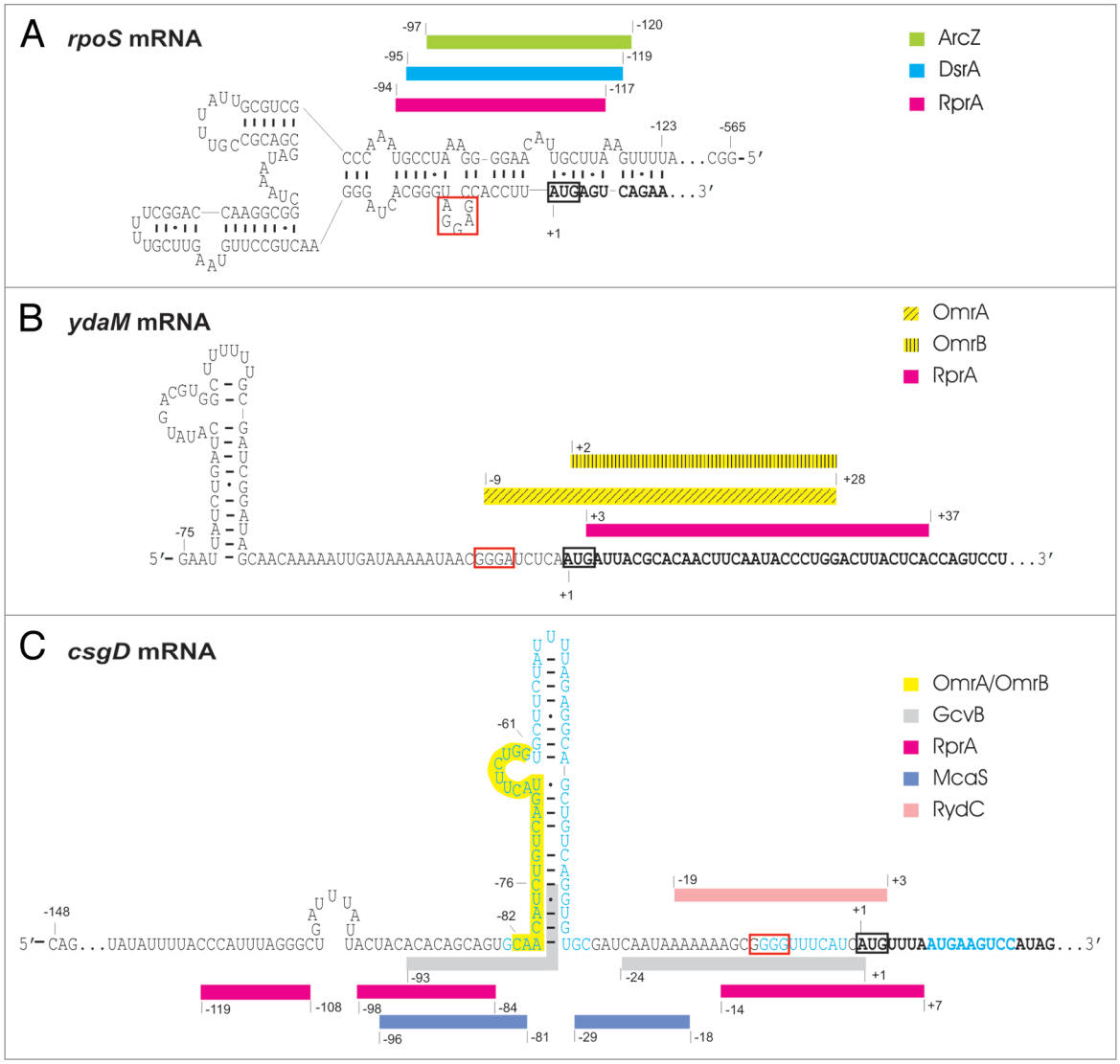


**Figure 4.** 5′-UTR parts of the mRNAs of *rpoS*, *ydaM*, and *csgD* with relevant secondary structures and sRNA binding regions. The images for *rpoS* and *csgD* mRNAs with relevant references have been published before[46] and are used here with permission. The binding region of RydC on *csgD* mRNA is according to reference 118. The *ydaM* mRNA secondary structure is based on computational prediction. The site of RprA interaction with *ydaM* mRNA was demonstrated by compensatory basepair exchanges.[93] OmrA/OmrB were found to downregulate *ydaM* expression, with their putative binding regions on *ydaM* mRNA being predicted (Mika F and Hengge R, unpublished).

Overall, the RpoS-dependent biofilm matrix-controlling network is a combination of three TF modules (I, II, and III in **Fig. 3**) arranged in a cascade with two c-di-GMP switching modules (A and B in **Fig. 3**) that link the output of the cascade, i.e., curli and cellulose production, to various cellular and environmental signals. Furthermore, all three TF modules in the cascade are targeted by sRNAs (underlied in green in **Fig. 3**) that integrate even more stress signals into this network.

## sRNAs and the Integration of Diverse Environmental Signals in RpoS Control

Bacteria have to adapt to stressful environmental conditions no matter whether they grow planktonically or in a biofilm. While in a structured biofilm, the environmental microconditions are somewhat more predictable than for free-living cells, bacteria in a crowded biofilm are also exposed to self-generated stresses such as intense nutrient competition, harmful waste products (e.g., low pH generated by mixed acid fermentation in low-oxygen zones), or even toxins generated by subpopulations. Not surprisingly, RpoS and CsgD, i.e., the two TFs that control large regulons involved in biofilm formation, therefore serve as regulatory hubs for multiple environmental signal integration with a series of sRNAs providing distinct inputs. Together, with the RNA chaperone Hfq and several RNases, these sRNAs directly target *rpoS*, *ydaM*, and *csgD* mRNAs (**Fig. 4**), and thereby, affect cellular levels as well as translation of these mRNAs.[43-46]

***rpoS* mRNA turnover and translation: Roles for RNases, codon usage, and the RNA chaperone Hfq**

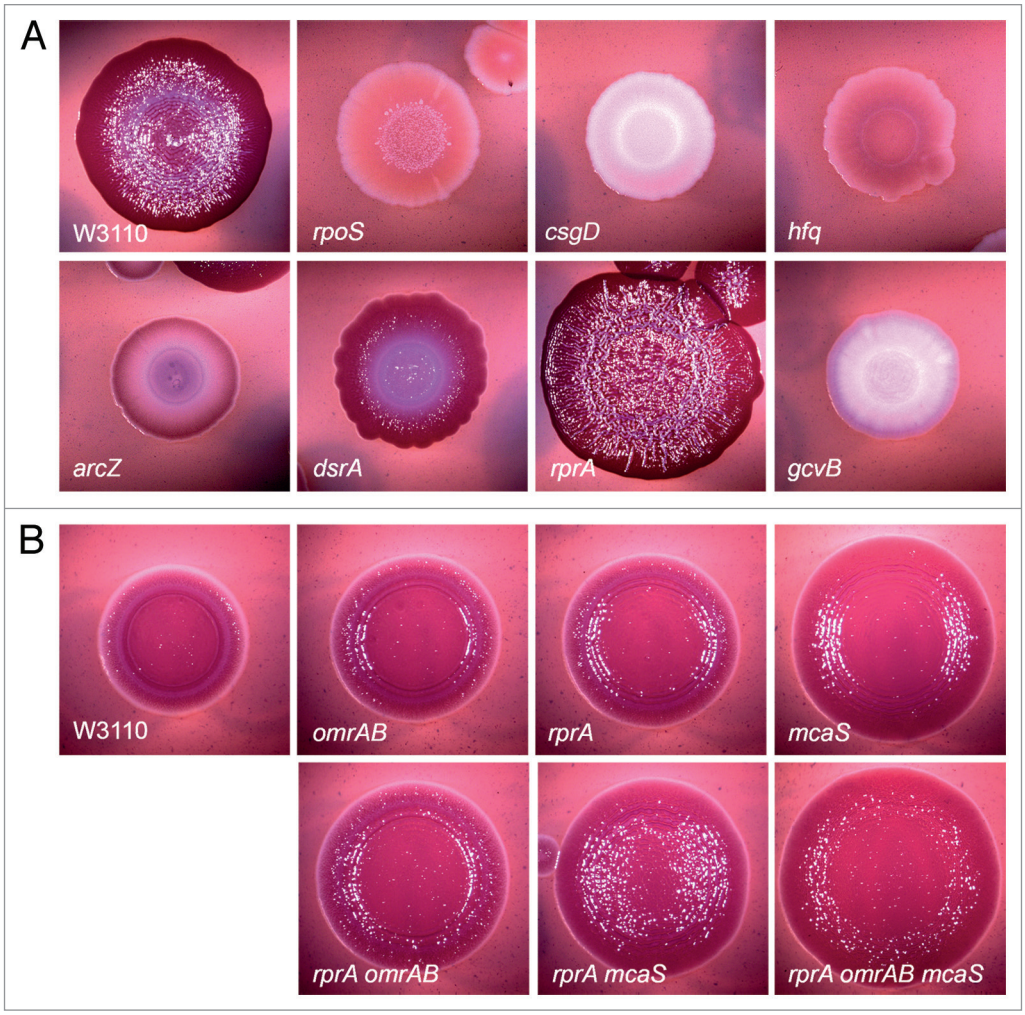


**Figure 5.** sRNA-knockout mutations affect macrocolony morphology of *E. coli* K-12. (**A**) Derivatives of strain W3110 with mutations in *rpoS, csgD, hfq*, and in sRNA genes affecting RpoS and/or CsgD expression were grown for 5 d at 28 °C on Congo Red agar plates,[8] which were supplemented with 5 g/l NaCl to mildly reduce colony structure (compare with **Fig. 1C**), which allows to detect not only inactivating but also activating effects of mutations. (**B**) Derivatives of strain W3110 harboring mutations of the indicated sRNAs that negatively affect CsgD expression were grown as in (**A**), but for 2 d only.

*rpoS* mRNA half-life is modulated by two major RNases in *E. coli*. Binding of trans-encoded sRNAs (DsrA and RprA) to the 5′-UTR of *rpoS* mRNA prevents degradation of the message by RNase E.[47] RNase III is likewise implicated in *rpoS* mRNA turnover, but in general its activity on *rpoS* mRNA does not seem to be changed by the presence of sRNAs.[47,48] Nevertheless, binding of the sRNA DsrA restructures the *rpoS* 5′-UTR and redirects the RNase III cleavage site to a different position.[49]

Translation of *rpoS* mRNA is affected by an intriguing codon usage. In contrast to the transcript that encodes its close homolog RpoD, i.e., the vegetative $\sigma^{70}$, the *rpoS* mRNA contains a high proportion of rare codons. This results in mRNA coverage by a higher density of frequently pausing ribosomes, which protects against mRNA degradation, and therefore, leads to higher RpoS expression.[50] A similar discrepancy in the use of rare codons in the transcripts of other isoenzymes that are expressed in exponential and stationary phase, respectively, suggests that this may be a more widespread mechanism.[50] In addition, MiaA-catalyzed tRNA modification contributes to RpoS expression, since *rpoS* mRNA is enriched for *leu* codons that are decoded by these modified tRNAs.[51]

Translation of *rpoS* mRNA per se is inefficient as the Shine-Dalgarno (SD) sequence and the AUG start codon are buried in a stem-loop structure, that prevents ribosome entry.[52-54] Three sRNAs, i.e., ArcZ, DsrA, and RprA have extensive complementarity and can thus engage in an alternative base-pairing to the upstream sequence that base-pairs to the SD region (**Fig. 4A**). As a consequence, they can activate translation of *rpoS* mRNA by enabling ribosome entry to the SD sequence.[55-58] Overall, this structural rearrangement of *rpoS* mRNA secondary structure has become a paradigm of translational activation by small RNAs.[44]

An important player in this *rpoS* mRNA translational activation is the RNA chaperone Hfq.[54,59] This homohexameric

protein adopts a doughnut-like structure with conserved Sm motifs.[60] mRNA binding via A-rich sequences occurs at the distal binding face,[61-63] wheras U-rich tails and internal U-rich stretches close to stem-loop structures of sRNAs are bound at the proximal face.[64,65] Also, lateral sites of the Hfq hexamer are capable of sRNA binding and a recent model proposes 3′-end U binding in the proximal pore and sRNA body and seed binding to the lateral binding sites.[66] By promoting sRNA–mRNA heteroduplex formation, Hfq can support positive or negative regulation of mRNA translation by sRNAs, depending on whether sRNA binding releases or blocks accessability of the ribosomal binding site of the mRNA.[43,67] In addition, Hfq binding and heteroduplex formation, e.g., also in the coding sequence of the mRNA, can recruit RNaseE, and thereby, trigger turnover of both RNAs.[68-70]

On the *rpoS* mRNA, Hfq recognizes $(AAN)_4$ sites in the 5′-leader region far upstream of the sRNA interaction site.[44,63,71] These sites are essential for positive regulation of *rpoS* translation by the sRNAS RprA and ArcZ, but of only partial importance for regulation by DsrA.[44,72] Although the Hfq binding motifs are placed far away from the sRNA interaction sites, it is thought that a flexible linker between the inhibitory stem domain and the upstream A-rich domain in the *rpoS* 5′-UTR allows optimal positioning of Hfq toward the sRNA binding sites.[72] In addition, Hfq is involved in structural rearrangements of the *rpoS* 5′-UTR that improve sRNA binding as well as ribosome entry.[60,73,74] Whether a differential binding mode to the two faces of Hfq contributes to the ability of sRNAs to target *rpoS* mRNA is not yet clear.[71,75] For the annealing between DsrA and *rpoS*, a conserved arginine patch at the outer rim of Hfq is crucial.[76]

Mechanistically, Hfq thus stabilizes sRNA/*rpoS* mRNA duplex formation, which releases the SD sequence, thereby allows ribosome entry and stimulates RpoS synthesis.[44] Although this is only one of many processes in RpoS control,[20] it is clearly of crucial importance for RpoS expression and biofilm formation, since *hfq* mutants have strongly reduced RpoS levels,[54] low curli fiber production, and therefore, grow as unstructured macrocolonies (**Fig. 5**).

**ArcZ: A sRNA that reflects the redox state of the respiratory chain**

During aerobic growth, ArcZ (*arc*-associated sRNA Z) is expressed especially in stationary phase, i.e., under conditions of energy limitation. Under conditions of low oxygen availability, its expression is repressed by the ArcB/ArcA two-component system.[57] By acting as a cis-located 3′-end antisense RNA for *arcB*, ArcZ also interferes with ArcB expression,[57] which sets up a switch based on mutual negative regulation of ArcB/ArcA and ArcZ. ArcZ is processed into three forms, with only the shortest form of 56 nt being able—together with Hfq—to bind to *rpoS* mRNA.[44]

Under conditions of aerobic growth and transition into stationary phase, ArcZ seems to be the most important of the three sRNAs that stimulate *rpoS* mRNA translation, since deletion of *arcZ* leads to lower RpoS levels than observed in *dsrA* and *rprA* mutants.[57] With this strong positive effect on RpoS expression, ArcZ also opposes the negative role of ArcB, which phosphorylates two response regulators, i.e., ArcA, a repressor of *rpoS* transcription, and RssB, the proteolytic targeting factor which delivers RpoS at the ClpXP protease[77] (**Fig. 2**). Thus, cells switch between a low-RpoS state established by high ArcB/ArcA/RssB activity (and thus, low ArcZ expression) and a high-RpoS state enabled by low ArcB/ArcA/RssB activity and boosted by high ArcZ levels. The crucial signal input that can throw this switch is the redox state of the respiratory chain, since oxidized quinones inactivate ArcB.[78] In this way, the balance between energy and oxygen supplies, i.e., the rates of entry and exit of electrons in the respiratory chain, strongly contributes to the control of RpoS levels.[77]

By activating RpoS expression, ArcZ indirectly stimulates the expression of biofilm matrix components curli and cellulose. In *Salmonella*, ArcZ also seems to promote biofilm formation via a not resolved activation of CsgD expression that appears independent of its action on RpoS.[79] Overall, *arcZ* mutants of *E. coli* are completely devoid of curli and cellulose production, and therefore, generate unstructured macrocolonies (**Fig. 5**). All these findings imply that ArcZ is active in the upper layer of the macrocolony biofilm, in which RpoS is active and matrix components are expressed, but no flagella are produced. Repression of flagella expression is also supported by ArcZ, which can bind to the 5′-UTR of the mRNA of the master regulator of the flagella regulatory cascade, FlhDC.[80]

**DsrA: A sRNA responding to cold stress**

The sRNA DsrA (downstream of *rcsA*) shows enhanced expression at low temperature (25 °C) and contributes to increased levels of RpoS under these conditions.[81] How a temperature signal is integrated in DsrA expression is not entirely clear, but it affects *dsrA* promoter activity as well as stability of this sRNA.[82,83] Nevertheless, DsrA partially acts on *rpoS* translation also at 37 °C.[57] Furthermore, it was observed that DsrA as well as RprA (see below) are also induced by low pH with both contributing to the RpoS-mediated acid stress response.[84,85] Mechanistically, DsrA is the sRNA that is least dependent on Hfq for binding to *rpoS* mRNA.[44] Yet, this heteroduplex formation does not seem sufficient to allow access of ribosomes to the translation initiation region, but Hfq itself—acting as a RNA chaperone—also contributes to converting *rpoS* mRNA into a translationally competent conformation.[74] At low temperature, not only DsrA but also the DEAD box helicase CsdA are required for efficient *rpoS* mRNA translation.[86]

Another direct, but negatively regulated target of DsrA is the global transcriptional repressor H-NS.[55,87] Therefore, DsrA can also act indirectly by affecting H-NS targets. This may again apply to RpoS, since H-NS reduces *rpoS* translation and promotes RpoS proteolysis,[88-90] as well as to flagella expression, where H-NS plays a positive role by downregulating HdfR, a repressor for *flhDC*,[91] i.e., the gene encoding the flagellar master regulator.

**RprA: A sRNA triggered by cell envelope stress**

Using a multicopy plasmid library, overexpression of the sRNA RprA (RpoS regulator A) was found to stimulate RpoS expression by directly affecting *rpoS* mRNA structure.[44,56,92] The physiological role of RprA in *rpoS* regulation is still not clear, since *rprA*-knockout mutations have little effect on cellular RpoS

levels.[93] Expression of RprA is activated by the RcsC/RcsD/RcsB phosphorelay system in response to various kinds of cell envelope stress.[40,56] In addition, RprA turnover by RNase E and RNase III is counteracted by binding to Hfq and *rpoS* mRNA and seems subject to complex control, which for instance, contributes to its regulation by osmolarity.[47,75,94] Also, *rpoS* translation is strongly stimulated by shift to high osmolarity.[95,96] While RprA was reported to play a role in this regulation in a *dsrA* mutant background,[92] neither RprA nor any of the other sRNAs known to bind to *rpoS* mRNA are essential or sufficient for this regulation (Kolmsee T and Hengge R, unpublished results). Similarly, RprA is induced by acidification[84,85] and so is RpoS, but again, RprA is not essential for this regulation of RpoS, which occurs at the levels of both translation and proteolysis[97] (Heuveling J and Hengge R, unpublished results).

A function of the Rcs system in biofilm formation was suggested early on since it stimulates the synthesis of the exopolysaccharide colanic acid.[37,39,98] By negatively regulating the RpoS-dependent biofilm regulator CsgD, and therefore, the production of the biofilm matrix components curli and cellulose, RprA also contributes to biofilm control[93,99] (and see below). The opposite regulation of RpoS and CsgD by RprA may be interpreted as RprA being important under some severe cell envelope stress conditions, where the RpoS-mediated general stress response is required to repair and prevent damage, but where continued production and extrusion of curli fibers and cellulose through the cell envelope may become deleterious.

**OxyS: An oxidative stress regulator**

Transcription of OxyS (OxyR-regulated sRNA) is triggered by OxyR, which is activated by hydrogen peroxide.[100] In contrast to ArcZ, DsrA, and RprA, this fourth sRNA regulator of *rpoS* acts as a negative regulator, probably by affecting Hfq function.[100,101] Mechanistically, this effect has remained unclear. Possibly, OxyS competes with other sRNAs for Hfq binding. Thus, overexpression of OxyS has been observed to titrate Hfq resulting in lower levels of DsrA and ArcZ as these are not stabilized against degradation by Hfq binding.[102] Likewise, high-level production of other sRNAs or Hfq-bound mRNAs has the potential to interfere with efficient *rpoS* translation.[102,103] OxyS binding may also disturb the direct mRNA chaperoning function of Hfq, which in addition to sRNA-*rpoS* mRNA heteroduplex formation seems to play a role in activating *rpoS* translation.[74] Finally, Hfq binding leads to a conformational change of OxyS that modulates its degradation by various RNases in ways that differ during growth and stationary phase and may co-modulate *rpoS* mRNA turnover.[48,75]

A direct and more conventionally regulated target of OxyS is the operon encoding the master regulator of the flagellar cascade, *flhDC*.[80] In a macrocolony biofilm, the deletion of *oxyS* has no effect on colony morphology, emphasizing its role as a highly specific stress regulator[100] (Mika F and Hengge R, unpublished).

## sRNAs in the Control of Biofilm Matrix Synthesis

By modulating RpoS levels, DsrA, ArcZ, and RprA indirectly control the production of the biofilm matrix components curli and cellulose. In addition, six sRNA, i.e., RprA, McaS, OmrA, OmrB, GcvB, and RydC, regulate the expression of downstream components of the "curli control cascade" more directly (recently summarized by refs. 45 and 46). These sRNAs can directly interact with *csgD* mRNA, and thereby, reduce the expression of CsgD, which is strongly induced during entry into stationary phase, and therefore, in the upper layer of macrocolony biofilms grown at 28 °C. Three of these sRNAs, i.e., RprA and OmrA/OmrB, also target the mRNA of the DGC and transcriptional co-activator YdaM, which together with MlrA, activates *csgD* transcription (**Fig. 4**).

**Control of CsgD and YdaM by the sRNAs RprA, McaS, OmrA/B, GcvB, and RydC**

The *csgD* 5′-UTR has a length of 148 nt and forms two hairpin structures designated as stem-loop SL1 (nt -83 to -28) and stem-loop SL2 (nt -10 to +16), with the latter also including the SD sequence.[46,93,99,104] As negatively acting CsgD regulators, the six sRNAs bind to partially overlapping regions in the 5′-UTR of *csgD* mRNA with mechanistically different consequences (**Fig. 4C**). In addition, the sRNAs require Hfq for relative stability and their function as *csgD* regulators.[48,99,104,105]

*ydaM* mRNA has a 5′-UTR of 75 nt that is predicted to form a stem-loop structure close to the 5′-end. On *ydaM* mRNA, RprA binding occurs right downstream of the AUG[93] in a region, where sRNAs can efficiently interfere with ribosome binding[106] (**Fig. 4B**). OmrA and OmrB, which can also downregulate *ydaM* expression, also have regions complementary to the translation initiation region on *ydaM* mRNA.

In biofilm control, RprA is unique as it positively controls RpoS, but negatively controls YdaM and CsgD in a complex feedforward pattern, i.e., CsgD is controlled both directly and indirectly via YdaM (**Fig. 3**). *rpoS* and *csgD* mRNAs respond to RprA with different sensitivities as only strong overexpression of RprA leads to elevated RpoS levels, whereas CsgD downregulation already occurs with a moderate increase in RprA levels.[93] In *csgD* mRNA, RprA binds to two sites that are located upstream and downstream of the major hairpin (SL1) in the 5′-UTR. The second binding site overlaps with the SD sequence, and thus, RprA interferes with *csgD* translation (**Fig. 4C**). In addition, RprA binding downregulates *csgD* mRNA levels.[93,99] This may occur by degradation (although RNase III and RNase E were shown not to be required) and/or premature termination of *csgD* transcription.[93] RprA also indirectly downregulates transcription initiation at the *csgD* promoter since it interferes with translation of *ydaM* mRNA.[93]

McaS (multi-cellular adhesive RNA) expression is activated by cAMP-CRP in response to carbon limitation and seems to be indirectly negatively regulated by RpoS in stationary phase,[105] i.e., it shows the typical expression pattern of a post-exponentially expressed gene. By interfering with CsgD expression while promoting FlhDC expression at the level of *flhDC* mRNA, McaS contributes to inverse regulation of the flagellar and curli/cellulose cascades with actually giving the former priority over the latter.[46,80,105] McaS binding sites in the 5′-UTR of *csgD* mRNA are located immediately up and downstream of the central stem-loop structure (SL1) and do not overlap with the ribosomal binding

site (**Fig. 4C**). Since McaS seems to be co-degraded with *csgD mRNA*, its direct role is apparently to promote degradation rather than to inhibit ribosome binding to *csgD* mRNA, although McaS binding can interfere with 30S ribosome binding in an in vitro assay.[99,105] McaS also binds and blocks CsrA, a mRNA-binding protein that positively controls FlhDC expression and interferes with the production of the exopolysaccharide PGA.[107,108] Via CsrA, McaS has the potential to indirectly negatively regulate FlhDC expression—which is in contrast to its direct positive effect by binding to *flhDC* mRNA (see above)—and to activate PGA production. Thus, during growth at 37 °C—where PGA is made but usually not curli/cellulose—McaS may again contribute to an inverse regulation of flagella expression and biofilm matrix production, but now in favor of the latter.

OmrA and OmrB (OmpR regulated sRNAs A and B) are activated by the EnvZ/OmpR two-component system in response to high osmolarity.[109] These two highly similar sRNAs, which are encoded by adjacent genes that probably arose by gene duplication, recognize the same set of mRNAs encoding outer membrane porins and also their own regulator OmpR.[110,111] More recently, they were also found to directly bind to *csgD* mRNA. The mapped binding site resides within the 5′-half of the major hairpin SL1[104] (**Fig. 4C**). Upon binding, this stem-loop structure is partially opened with binding of the sRNA possibly blocking a ribosome loading site located upstream of the SD sequence.[104] In contrast to McaS and RprA, OmrA/OmrB do not seem to reduce *csgD* mRNA levels.[104] Like RprA, OmrA/OmrB can also indirectly downregulate *csgD* transcription by affecting the expression of YdaM. The predicted interaction site in *ydaM* mRNA overlaps with the RprA binding site (**Fig. 4B**) and overexpression of OmrA or OmrB downregulated a *ydaM*::*gfp* fusion (Mika F and Hengge R unpublished results). Furthermore, OmrA/OmrB may indirectly affect *csgD* transcription by inhibiting expression of OmpR,[110] which is also involved in *csgD* transcription. Recently, OmrA/OmrB were shown to also interfere with flagella expression by binding in the 5′-UTR of *flhDC* mRNA at a site overlapping the SD sequence.[80] OmrA/OmrB-mediated downregulation of both CsgD and FlhDC may play a role under acute osmostress conditions, where large "construction sites" for the assembly of curli fibers and flagella in the cell envelope may become deleterious.

GcvB (GcvA-regulated sRNA B) also reduces *csgD* expression and its binding sites in the *csgD* 5′-UTR are predicted to overlap with the RprA binding sites[93,99] (**Fig. 4C**). Transcription of GcvB is induced by the regulator of the glycine cleavage system GcvA in response to high amino acid supply.[112] GcvB has multiple targets in amino acid metabolism and transport in a manner opposite to the role of the global regulator Lrp, with which GcvB is engaged in mutually negative regulation.[113] Furthermore, GcvB has been implicated in the RpoS-dependent acid stress response of *E. coli,* where it seems to activate *rpoS* expression by an uncharacterized mechanism,[114] which is consistent with a probably indirect activation of transcription of the RpoS-dependent *hdeAB* operon—encoding two periplasmic chaperones important for acid resistance—by a mechanism that involves Hfq.[115] Overall, GcvB is expressed in growing cells,[116] and under conditions of acidification, may contribute to RpoS expression, but at the same time may prevent RpoS-dependent CsgD expression, and therefore, inadequate curli/cellulose expression. Other studies, however, did not detect a significant positive effect of GcvB on RpoS.[57,117] This discrepancy may have to do with different growth conditions. In macrocolonies, knocking out *gcvB* eliminates any morphological structure just as mutations in *rpoS* and *csgD* do (**Fig. 5A**). This would be consistent with strongly reduced RpoS expression or activity, but may also indicate yet another unidentified GcvB target among the many factors that control CsgD expression (**Fig. 3**).

Very recently, a sixth sRNA, RydC, was reported to downregulate CsgD expression by directly base-pairing to the translational initiation region of *csgD* mRNA.[118] RydC forms a pseudoknot, which has to be resolved with the help of the RNA chaperone Hfq in order to allow interaction with *csgD* mRNA. RydC, which also controls expression of a peptide transport system and cyclopropane fatty acid synthase in a positive manner, is expressed in growing cells,[118,119] and thus, may contribute to fine-tuning CsgD expression, when the latter is just induced during the transition into stationary phase.

**sRNA-mediated control of RpoS and CsgD is reflected in macrocolony morphology**

The morphology of macrocolony biofilms is a highly complex phenotype that requires strong production and extracellular assembly of curli fibers and/or cellulose.[8,12,19] The *E. coli* K-12 laboratory strains do not produce cellulose, but strain W3110 produces high levels of curli, which—on agar plates supplemented with the amyloid dye Congo red—generates a dark red macrocolony pattern of concentric rings (**Figs. 1C and 5A**). If curli is not expressed during growth at 37 °C or due to knockout mutations in *rpoS, csgD*, or *csgB*, macrocolonies appear pale red to white and unstructured (**Fig. 5A** and ref. 8). Deletion of *hfq* was described to have very pleiotropic effects.[120,121] Given its crucial activating role in *rpoS* expression, it is not surprising that macrocolonies of *hfq* mutants look similar as those of *rpoS* mutants (**Fig. 5A**).

While sRNA-knockout mutations are often notorious for the absence of clear phenotypes reflecting their physiological roles, macrocolony morphology has turned out to provide such a phenotype for several of the sRNAs involved in RpoS and CsgD control (**Fig. 5A**). As can be expected, knocking out ArcZ or DsrA—the two sRNAs that activate RpoS expression but do not affect CsgD expression in *E. coli*—eliminates or reduces macroscopic colony structure, respectively. The similar phenotype of the *gcvB* mutant indicates a positive role of GcvB in RpoS or CsgD expression. The macrocolony phenotype of the *rprA*-knockout mutation, i.e., even slightly increased wrinkling, is consistent with little or no effect on RpoS and the negative role of RprA in CsgD expression being dominant. In younger macrocolonies (grown for 2 d only; **Fig. 5B**), the structure-promoting effect of knockout mutations in *rprA* as well as in *omrA/omrB* and *mcaS* becomes even clearer. Here, increased CsgD and curli production translates into an earlier appearance

of the curli-dependent concentric ring morphology. Combining these sRNA mutations further exacerbates this effect and also leads to somewhat flatter and larger macrocolonies (**Fig. 5B**) indicating that RprA, OmrA/OmrB, and McaS act additively in the control of CsgD and curli fiber production.

## RpoS-Dependent sRNAs: SdsR, GadY, and SraL

As many sRNAs have higher expression levels in stationary phase than in growing cells,[111,116,122] it seemed likely that some are part of the RpoS regulon, and thereby contribute to RpoS-driven cellular physiology. To date, three RpoS-dependent sRNAs have been characterized in *E. coli* or *Salmonella*: SdsR, GadY, and SraL.[123-125]

SdsR (sigmaS-dependent sRNA) was reported to be involved in the control of biofilm formation in *Salmonella* by activating *csgD* and curli expression by a non-characterized mechanism.[79] In addition, SdsR downregulates the porin OmpD in *Salmonella* by directly binding to the coding sequence of *ompD* mRNA.[123] In *E. coli*, SdsR is involved in RpoS-dependent increased mutagenesis by directly downregulating expression of the mismatch repair protein MutS.[126] SdsR also inhibits FlhDC expression and could contribute to inverse regulation of flagella and biofilm matrix components, but the mode of action is still unclear.[80]

GadY (*gad* gene-related sRNA Y) is part of the acid stress response pathway in *E. coli* and requires RpoS for expression.[124] It is a *cis*-encoded small anti-sense RNA, that is transcribed from the opposite DNA strand between the *gadX* and *gadW* genes, actually overlapping the 3′-UTR of the *gadX* mRNA. GadX and GadW are transcriptional regulators, with GadX playing a major role in acid resistance.[127] GadY acts by basepairing to the *gadXW* dicistronic mRNA, thereby mediating RNase III cleavage between *gadX* and *gadW*, which results in stabilization of both monocistronic derivatives of the *gadXW* mRNA.[124,128,129] GadY may be relevant for coping with acidification by mixed acid fermentation in low-oxygen regions of macrocolony biofilms.

SraL (small RNA L) was predicted and mapped already in 2001 and shown to be strongly induced in stationary phase.[111,116] Recently, it was demostrated that SraL is indeed transcribed by RpoS-containing RNAP and the first SraL target has been characterized in *Salmonella*, which is *tig* mRNA.[125] The *tig* gene codes for trigger factor, a ribosome-associated protein folding chaperone.[130] SraL interacts with the 5′-UTR upstream of the SD sequence of *tig* mRNA and probably interferes with ribosome binding.[125] By upregulating SraL, RpoS may adjust the expression of trigger factor to reduced ribosome and protein synthesis during entry into stationary phase.

## Conclusions and Perspectives

In *E. coli* macrocolony biofilms, amyloid curli fibers and cellulose are the matrix components that confer physical protection to cells and generate strong cohesion, elasticity, and the striking macroscopic morphology of these biofilms (**Fig. 1**). Their production in the stationary phase top layer of macrocolonies is controlled by a cascade of three transcription factors (TF; **Fig. 3**), i.e., RpoS (I), YdaM/MlrA (II), and CsgD (III). This cascade is complemented by two c-di-GMP switch modules (A and B in **Fig. 3**), which trigger signal propagation between TF modules I and II and differential control of curli and cellulose production at the very end of the cascade, respectively (**Fig. 3**). As detailed in this review, at least eight sRNAs have a crucial input into all three TF modules, and thereby, connect these modules to a wide range of environmental signals. A question remaining for future research is whether even more sRNAs may have an impact in this intriguing network.

Even though most of what we currently know about the regulation of and by sRNAs is based on research with liquid or planktonic cultures, this knowledge has implications for biofilm growth and morphogenesis that deserve further studies, in particular, in situ in macrocolonies. ArcZ and McaS expression responds to signals that in fact reflect the long range gradients of oxygen, energy, and carbon sources in a macrocolony and—by inversely regulating FlhDC and RpoS/CsgD—are highly likely to contribute to setting up the two physiologically distinct layers with flagella-producing growing cells in the bottom layer and outer colony edges and matrix-producing stationary phase cells in the top layer.[14] Other sRNAs integrate stress signals such as cell envelope imbalances, increased osmolarity, low temperature, acidification, or oxidative stress that further shape macrocolony biofilms, but also contribute to sudden acute stress reactions which may be more relevant for "free-living" planktonic cells. Thus, RprA and OmrA/OmrB could be key regulators in making the decision whether to get immobilized in a biofilm matrix or staying potentially motile, because in the curli/cellulose control cascade, they can still downregulate CsgD-driven matrix production under stationary phase conditions, where RpoS is already fully active and the YciR-controlled c-di-GMP switch has been thrown to "ON" (see **Fig. 3**). By interfering with both flagella and curli production, OmrA/OmrB and OxyS may actually act as stress-induced "emergency" sRNAs, which in general inhibit production of large cellular appendices, and thereby, allow cells to focus their resources on coping with acute stress effects (for a more detailed discussion of these complex regulatory patterns and their physiological implications, see ref. 46).

The set of sRNAs in the curli/cellulose control cascade also provides an ideal opportunity to study the sometimes-surprising behavior of RNA-based networks, in which an sRNA can bind to multiple mRNAs, which in turn, bind additional sRNAs (**Fig. 3**). In contrast to classical hierarchical and amplificatory transcription factor networks, such a sRNA/mRNA network operates in a non-hierarchical and stoichiometric mode in which sRNAs "target" mRNAs as well as vice versa. The output of this network crucially depends on competition and titration reactions that are determined by the rates of synthesis and cellular levels of sRNAs, mRNAs, and Hfq, and the affinities between all these players, which generates threshold-linear responses and priorities in cross-talk within the sRNA/mRNA network.[46,131] In the curli/cellulose control cascade, this means that *csgD* mRNA is not only "targeted" by several sRNAs (which bind with different affinities

to overlapping sites on *csgD* mRNA), but that strong accumulation of *csgD* mRNA—as e.g., during entry into stationary phase—can also scavenge sRNAs, and thereby, indirectly affect the function of their other target mRNAs.[93] Thus, effects of dynamic changes in sRNAs as well as distinct "hub" mRNAs can "horizontally" propagate through the network, which can be a challenge for the interpretation of genetic analyses, which in principle rely on hierarchical "one-way" causal relationships.

Another point of interest for future analysis is the heterogenous expression of CsgD in the transition zone between the two physiological layers of a macrocolony biofilm, which features matrix-surrounded $CsgD^{ON}$ cells right next to "naked" $CsgD^{OFF}$ cells.[8] As these cells are exposed to the same environmental conditions, this heterogeneity may reflect bistability in the control of CsgD. The underlying control network (**Fig. 3**) indeed features several double-negative feedback loops, i.e., a potentially bistability-generating motif. One is the above-mentioned non-hierarchical mutual inhibition of *csgD* mRNA and sRNAs. The other double negative feedback loops surround the trigger enzyme and PDE YciR, i.e., the key switch protein in the c-di-GMP module (**Fig. 3**). It will be a challenge for future work to sort out, how these motifs contribute to the apparent bistable expression of CsgD, and therefore, to heterogenous matrix production in distinct zones of the macrocolony biofilm. Finally, the intricate interplay between c-di-GMP-mediated control and the sRNA network in curli/cellulose regulation suggests that searching for additional regulatory connections between nucleotide second messenger signaling and sRNAs may yield interesting new insights.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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