HHS Public Access

Author manuscript

Mol Microbiol. Author manuscript; available in PMC 2018 July 01.

Published in final edited form as:

Mol Microbiol. 2017 July ; 105(2): 309–325. doi:10.1111/mmi.13702.

Alternative Pathways for *Escherichia coli* Biofilm Formation Revealed by sRNA Overproduction

Ashley Parker[1,2], **Suanur Cureoglu**[1,3], **Nicholas De Lay**[1,4], **Nadim Majdalani**[1], and **Susan Gottesman**[1,*]

[1]Laboratory of Molecular Biology, National Cancer Institute, Bethesda MD, 20892, USA

Abstract

Small regulatory RNAs have major roles in many regulatory circuits in *Escherichia coli* and other bacteria, including the transition from planktonic to biofilm growth. We tested Hfq-dependent sRNAs in *E. coli* for their ability, when overproduced, to inhibit or stimulate biofilm formation, in two different growth media. We identify two mutually exclusive pathways for biofilm formation. In LB, PgaA, encoding an adhesion export protein, played a critical role; biofilm was independent of the general stress factor RpoS or CsgD, regulator of curli and other biofilm genes. The PgaA-dependent pathway was stimulated upon overproduction of DsrA, via negative regulation of H-NS, or of GadY, likely by titration of CsrA. In YESCA (Yeast Extract Casamino acids) media, biofilm was dependent upon RpoS and CsgD, but independent of PgaA; RpoS appears to indirectly negatively regulate the PgaA-dependent pathway in YESCA medium. Deletions of most sRNAs had very little effect on biofilm, although deletion of *hfq*, encoding an RNA chaperone, was defective in both LB and YESCA. Deletion of ArcZ, a small RNA activator of RpoS, decreased biofilm in YESCA; only a portion of this defect could be bypassed by overproduction of RpoS. Overall, sRNAs highlight different pathways to biofilm formation.

Introduction

In response to various stress conditions and hostile environments, microorganisms can form communities of surface-adherent cells embedded in a matrix called biofilm. Biofilms are ubiquitous and are able to form on a variety of surfaces, contaminating food, water sources, and medical devices (Donlan & Costerton, 2002). A more complete understanding of the regulatory mechanisms involved in biofilm formation and how they change with growth conditions may lead to new strategies for successfully controlling its synthesis and treating biofilm-associated infections. Biofilm synthesis is a complex process involving a multitude of gene regulatory pathways. Flagella play a role in bringing cells to surfaces, and has been

[*]Corresponding author. Mailing address: National Institutes of Health, 37 Convent Dr. Bldg. 37 Room 5132 Bethesda, MD 20892, USA; Susan Gottesman, gottesms@helix.nih.gov.
[2]Current address: Office of the Director, NIH, Bethesda, MD. 20892, USA
[3]Current address: University of Gaziantep Medical School, Gaziantep, Turkey 27310
[4]Current address: University of Texas Health Science Center at Houston, Dept. of Microbiology and Molecular Genetics, Houston TX, 77030, USA

Author contributions

SC, ND, AP, NM and SG designed the study, SC, ND, AP and NM developed materials, carried out and analyzed the data, SG, AP, NM and SC wrote the manuscript.

implicated in other stages of biofilm formation as well; FlhDC acts as the master regulator for flagellar synthesis (Chevance & Hughes, 2008). Proteins, including curli amyloid fibers, and exopolysaccharides such as PGA [Polyβ-1,6-GlcNAc] contribute to the interaction of cells with surfaces, with each other, and contribute to formation of a matrix around the bacteria. The transcriptional regulator for curli synthesis is CsgD (Liu *et al.*, 2014). PGA synthesis is activated by the transcriptional regulator NhaR (Goller *et al.*, 2006) and translation is repressed by CsrA. CsrA is titrated by CsrB and CsrC, as well as the Hfq-dependent sRNA McaS (Wang *et al.*, 2005, Jorgensen *et al.*, 2013).

Small RNAs [sRNAs] are known to regulate genes involved in these networks and in the regulation of biofilm formation, with multiple sRNAs regulating expression of FlhDC and CsgD [reviewed in (Mika & Hengge, 2013, Mika & Hengge, 2014, Chambers & Sauer, 2013, Van Puyvelde *et al.*, 2013)]. There are at least 90 sRNAs detected in *Escherichia coli* (Raghavan *et al.*, 2011), and a large number of these require the chaperone protein Hfq (De Lay *et al.*, 2013). Here, we focus on Hfq-dependent sRNAs; these sRNAs regulate their mRNA targets at the post-transcriptional level by complementary base-pairing (Updegrove *et al.*, 2016).

Hfq has been shown to be required for biofilm formation (Monteiro *et al.*, 2012, Bak *et al.*, 2015), and is considered a master regulator of biofilm under various environmental conditions in *S.enterica* serovar Typhimurium (Monteiro *et al.*, 2012). In addition, many of the Hfq-dependent sRNAs play important roles during various stress responses and in regulation of motility (Zhao *et al.*, 2013, Chambers & Sauer, 2013, Thomason *et al.*, 2012, Van Puyvelde *et al.*, 2013) (De Lay & Gottesman, 2012) and curli synthesis (Boehm & Vogel, 2012), all pathways that are known to be important in the biofilm development process. Stress conditions such as changes in growth temperatures or pH, peroxide, high metal concentration, and biocides increase biofilm formation in *E. coli* and other enteric bacteria (Goller & Romeo, 2008); (Goller *et al.*, 2006, Zhang *et al.*, 2007). Three sRNAs, DsrA, ArcZ, and RprA play important roles in the induction of the general stress response controlled by the sigma factor RpoS (Battesti *et al.*, 2011), while at least two others, GadY and SdsR, are members of the RpoS regulon (Frohlich *et al.*, 2012, Opdyke *et al.*, 2004).

Many biofilm studies have been performed using rich (LB) medium; a smaller portion of these studies have used other media such as colonization factor antigen (CFA) medium and yeast extract-Casamino Acids (YESCA) to study specific regulated genes (Kikuchi *et al.*, 2005, Jorgensen *et al.*, 2013) (Hung *et al.*, 2013, Bordeau & Felden, 2014); (Thomason *et al.*, 2012). Here, we overproduced sRNAs to probe the pathways important for biofilm formation in *Escherichia coli* in both LB and YESCA media. In preliminary experiments, we found that RpoS was needed for biofilm in YESCA, but not in LB, suggesting that a comparison would be useful. Our work demonstrates the complexity of biofilm formation, with different sRNAs affecting this process in different media.

Results

Multiple sRNAs Regulate the Expression of Biofilm

Using a crystal violet assay for the development of biofilm by *E. coli* K12, we investigated whether overexpressed sRNAs can positively or negatively affect biofilm formation. sRNAs were overexpressed using a library of plasmids, each expressing a different Hfq-dependent sRNA under the control of an inducible P_{lac} promoter (Mandin & Gottesman, 2010). All of the Hfq-dependent sRNAs known at the time when this work was initiated were in the library, as was the Hfq-independent CsrB RNA. Strains harboring the vector control or sRNA plasmid were grown at 37°C, the plasmids were induced with IPTG, and cultures were incubated in microtiter plates at 25 °C with fresh LB-ampicillin or YESCA-ampicillin for 24–48 hrs. Growth was measured using the OD_{600} and biofilm levels were determined at OD_{550} by staining with 0.1% crystal violet. Those sRNAs with two-fold or greater effects, normalized to the level of biofilm in the plac vector control, are shaded in Fig. 1A and B.

Plasmids expressing sRNAs DsrA, GadY, and MicF increased biofilm formation in the library screen. Strains overexpressing MicF increased biofilm formation (five-fold) only in YESCA (Fig. 1B). Multicopy GadY increased biofilm 7-fold, only in LB (Fig. 1A). Overexpression of DsrA led to a 5-fold increase in the levels of biofilm formation using both types of media (Fig. 1A, B). Overall, overproduction of many (19/28) Hfq-dependent sRNAs had significant effects on biofilm formation in at least one of the two different types of media.

In the initial screen, strains overexpressing RydC, SdsR, and MicC reduced biofilm formation in both LB and YESCA media (Fig. 1A, B). A larger set of sRNAs reduced biofilm 2-fold or more in YESCA medium but not in LB, while RybD reduced biofilm in LB but not YESCA (Fig. 1, compare A and B).

McaS has previously been shown to increase motility and flagella synthesis via activation of *flhDC* (Thomason *et al.*, 2012). It directly represses translation of *csgD* but activates *pgaA*, causing increased biofilm formation in LB, CFA (colonization factor antigen), and YESCA media (Thomason *et al.*, 2012), (Jorgensen *et al.*, 2013). For reasons not currently understood, we did not observe this activation in either LB or YESCA; in fact, McaS repressed biofilm formation in YESCA (Fig. 1). Biofilm formation is, in part, activated via McaS interaction with CsrA, and the titration mechanism allows activation of *pgaA*, leading to biofilm production (Jorgensen *et al.*, 2013). Consistent with a negative role for CsrA in biofilm formation, overproduction of CsrB, which also titrates CsrA (Wang *et al.*, 2005, Weilbacher *et al.*, 2003), significantly increased biofilm formation in both LB and YESCA media (Fig. 1A, B). CsrB is not an Hfq-dependent sRNA and was not a focus of this work. Because the McaS plasmid did stimulate biofilm in preliminary experiments (not shown) and stimulated *pgaA* translation in a recent experiment (see below), it seems most likely that the plasmid used in Fig. 1 had acquired a mutation. We cannot rule out loss or mutation of other plasmids in this assay; thus the list of sRNAs affecting biofilm may be an underestimate.

Deletions of each of eleven of these Hfq-dependent sRNAs were tested for biofilm formation; also included was a deletion of *hfq* and a deletion of *csrB*, encoding a negative regulator of CsrA (Fig. 1C, D). The deleted sRNAs included all of those that activated biofilm when overproduced (DsrA, GadY and MicF), as well as some of those that showed strong negative effects when overproduced (ChiX, SdsR, RydC, ArcZ, DicF, and MicC). Finally, McaS, previously implicated in biofilm formation, and RprA known, with ArcZ and DsrA, to activate RpoS translation, were also included. In both LB and YESCA, the deletion of *hfq* had the greatest effect, essentially abolishing biofilm formation (Fig. 1C, D), consistent with a previous study carried out in LB (Bak *et al.*, 2015). Most of the other deletions had only modest effects, although deletion of *mcaS* reduced biofilm almost two-fold in LB, and deletion of *arcZ*, *dsrA* or *gadY* all reduced biofilm at least two-fold in YESCA. These differences between LB and YESCA reinforce the idea that different pathways are likely used in the different media.

Although a number of sRNAs had significantly decreased biofilm when overproduced (Fig. 1A, B), none of the deletions resulted in more biofilm under these conditions (RydC, ChiX and SdsR for instance, Fig. 1C, D). One explanation for this is that the effects on biofilm require significant overproduction, for instance to titrate Hfq. We note that ChiX is known to titrate Hfq (Ellis *et al.*, 2015, Moon & Gottesman, 2011, Santiago-Frangos *et al.*, 2016). If Hfq titration is the basis for the decrease in biofilm, we would not expect the chromosomal level of these sRNAs to significantly perturb Hfq availability. Alternatively, the target perturbed by overproduction of these sRNAs may not be rate-limiting for biofilm formation. For instance, if gene X is required for biofilm formation, but more X does not lead to more biofilm, overproduction of an sRNA that negatively regulates X may eliminate biofilm, but deletion of the gene for the same sRNA may not show a difference in biofilm levels. ArcZ, which negatively regulated biofilm formation when overproduced in YESCA (Fig. 1B), led to reduced biofilm when deleted, again only in YESCA medium (Fig. 1D). With the exception of ArcZ, discussed further below, the sRNAs that negatively regulated biofilm only when overproduced were not further investigated.

Here, we focused on four of these sRNAs, the three activators (DsrA, GadY, MicF) and one multicopy repressor, ArcZ. We start by focusing our attention on DsrA, which was a strong activator under both LB and YESCA conditions (Fig. 1).

DsrA Acts through H-NS to Promote Biofilm Formation

In *E. coli*, DsrA is a three stem-loop 87 nucleotide long sRNA that is known to regulate the translation of two global transcriptional regulators, H-NS and RpoS, by RNA-RNA interactions (Majdalani *et al.*, 1998) (Gottesman, 2004) (Lease & Belfort, 2000). DsrA stimulates translation of RpoS, the master regulator of the general stress response; the region of interaction with the *rpoS* mRNA is in the first stem loop of DsrA (bold type in Fig. 2A) (Majdalani *et al.*, 1998). Base pairing occurs upstream of the *rpoS* translational start site (nt −97 to −125 relative to the ATG; Fig. 2C). The global transcriptional repressor protein, H-NS is negatively regulated by DsrA, using the second stem loop (circled in Fig. 2A), with base pairing occurring just beyond the ATG in the coding region of *hns* (Fig. 2B) (Lease *et al.*, 1998, Lease & Belfort, 2000).

Because different regions of DsrA are involved in regulation of *rpoS* and *hns*, it is possible to distinguish which targets of DsrA are important for a given phenotype, using appropriate mutations (Fig. 2). DsrA*h (Fig. 2B) should disrupt pairing with *hns*, while DsrA*r disrupts pairing with *rpoS* (Fig. 2C). The specificity of these mutants was tested on appropriate translational reporter fusions (Fig. 2D). DsrA over-expression significantly reduced *hns-lacZ* expression levels, while the DsrA specificity mutant pDsrA*h lost the ability to repress (Fig. 2D, compare second and third bars for *hns-lacZ* fusion). The DsrA specificity mutant pDsrA*r, designed to prevent base pairing with *rpoS*, continued to strongly repress the *hns-lacZ* fusion (Fig. 2D, fourth bar). As expected, the effects of these mutations were reversed for regulation of the *rpoS-lacZ* fusion, as expected, with DsrA*h still able to activate this fusion while DsrA*r was unable to (Fig. 2D, *rpoS-lacZ* fusion). These results confirm that DsrA regulation of *hns* and *rpoS* is independent, as previously seen (Majdalani *et al.*, 1998), and provide tools for asking which of these effects contribute to DsrA regulation of biofilm.

Overexpression of wild-type DsrA transformed in the MG1655 background increased biofilm formation by 5-fold in LB (Fig. 3). A plasmid overexpressing DsrA*h did not stimulate biofilm formation, while expression of DsrA*r had an effect similar to expression of wild-type DsrA (Fig. 3). This result suggests that negative regulation of H-NS by DsrA may be sufficient for increased biofilm, and that stimulation of RpoS by DsrA is not necessary.

To confirm that it is DsrA pairing with *hns* that leads to increased biofilm, rather than another target of DsrA that pairs with this same region, we constructed a compensatory mutant, *hns**, first tested it in the *hns*-lacZ fusion (Fig. 4) and then introduced this mutation into the chromosomal copy of *hns*. As seen in Fig. 4, right panel, wild-type DsrA and DsrA*r are unable to regulate *hns**-lacZ; DsrA*h, which can pair with *hns**, can repress it. Note that the basal level of expression of the *hns*-lac* fusion is reduced significantly, suggesting that this mutation (in the second to fourth codons of *hns*, Fig. 2B) reduces translation.

If DsrA is working only through its repression of *hns*, the plasmid overexpressing DsrA*h should stimulate biofilm formation only when it is able to pair with *hns* (in the *hns** strain). This was what was observed (Fig. 3, right side); DsrA*h increased biofilm formation in the *hns** strain relative to the plac vector control, while $DsrA^+$ and DsrA*r (unable to regulate the *hns** strain) did not. This experiment confirms that the increase in biofilm formation is specifically due to DsrA negative regulation of *hns* and not another target. The reduced HNS in the *hns** strain did not have a significant effect on biofilm levels (compare level of biofilm in plac (vector control) lanes in MG1655 and *hns** strain). This suggests that chromosomally-encoded DsrA regulation of *hns* is not important for the biofilm levels observed in wild-type cells, since the *hns** allele should be resistant to endogenous DsrA. The lack of an effect of endogenous DsrA on biofilm in LB was confirmed by deletion of *dsrA* (Fig. 1C). In addition, the lower level of expression of the *hns*-lacZ* fusion suggests that reducing the levels of *hns* translation two-fold is not sufficient to increase biofilm.

A prediction of these results is that deletion of *hns* should also increase biofilm. This was confirmed for growth in LB (Fig. 5). Under these conditions, deletion of *hns* mimicked the

effect of DsrA overproduction, consistent with H-NS negatively regulating biofilm. An *rpoS* deletion mutant was also included in the biofilm assay and no changes in biofilm were observed in LB (Fig. 5). Therefore, under our LB biofilm assay conditions, neither more RpoS (activation by DsrA) nor absence of RpoS affects biofilm levels. We return to the role of RpoS in YESCA medium later in the manuscript.

DsrA and H-NS Targets and Biofilm

H-NS is known to repress multiple genes in *E. coli* (Bertin *et al.*, 1994, Soutourina *et al.*, 1999) (Chib & Mahadevan, 2012, Donato *et al.*, 1997). We carried out epistasis experiments to try to identify possible critical H-NS targets for biofilm formation. A selected set of genes, each important for specific pathways implicated in biofilm formation, were deleted and tested for biofilm formation in the presence or absence of H-NS or in the cells overexpressing DsrA (Fig. 6). In LB, deletion of *flhD*, the regulator of flagellar synthesis, reduced the basal level of biofilm in *hns*$^+$ cells (Fig. 6A, compare lane 4 to lane 1), while deletion of *rpoS*, *csgD* (regulator of curli synthesis and other genes associated with biofilm formation) or *pgaA*, encoding the first gene in the *pgaABCD* operon for synthesis and export of PGA adhesin, had no effect (Fig. 6A, lanes 2, 3, and 5). However, the increased level of biofilm in either Δ*hns* or pDsrA cells was reduced to or below the level of WT cells by deletion of *pgaA* (Fig. 6A, compare lane 7 to lane 6 and lane 12 to lane 11). These results show that the increased biofilm levels observed when H-NS is either deleted or down-regulated by DsrA is dependent upon PGA.

In *Actinobacillus pleuropneumoniae*, H-NS represses the *pgaA* operon and thus regulates biofilm formation (Bosse *et al.*, 2010). The regulation of *pgaA* by H-NS was examined in *E. coli*, initially using a fusion under the control of the *pgaA* promoters, that measures both transcription and translation of *pgaA*. In this strain, deletion of *hns* increased expression (Fig. S1C, compare lane 4 to lane 1), and also increased activity on Lactose MacConkey indicator plates (Fig. S1B, compare plac quadrant to plac in Fig. S1A). However, multicopy DsrA had a less dramatic effect, although it showed some induction, consistent with down-regulation of H-NS (Fig. S1A, S1C). DsrA specificity mutants tested on Lactose MacConkey indicator plates (Fig. S1A) were consistent with DsrA acting via repression of H-NS synthesis; pDsrA*h had no effect, and pDsrA*r was similar to wild-type DsrA (Fig. S1A). Therefore, *pgaA* is a direct or indirect target of H-NS, but it seems likely from these results that additional H-NS targets contribute to the higher biofilm formation in Δ*hns* cells. We suggest that in *hns*$^+$ cells, redundant pathways, possibly including PGA, contribute to the basal biofilm formation so that deletion of *pgaA* had little or no effect. This is consistent with reports that wild-type MG1655 does not produce much PGA (Itoh *et al.*, 2008).

As noted above, RpoS was not needed for the basal biofilm levels in LB (Fig. 5, Fig. 6). If anything, lack of RpoS appeared to modestly promote biofilm levels in cells deleted for *hns* or expressing multicopy DsrA (Fig. 6A, compare lane 10 to lane 6 and lane 15 to lane 11). This is consistent with work suggesting that RpoS can repress biofilm formation in cells assayed in LB media (Ferrieres *et al.*, 2009).

In two cases, the deletion of *hns* and overproduction of DsrA gave somewhat different results; we suggest that these reflect differences in the timing and contribution of motility to

biofilm development. First, deletion of *flhD* reduced biofilm in the Δ*hns* strain, but not in the strain overproducing DsrA. The ability of DsrA to overcome the deletion of *flhD* was still dependent on the ability to pair with *hns* (Fig. S2A). Second, multicopy DsrA, in strains deleted for *pgaA*, led to a total loss of the basal level of biofilm; this reduction of the basal level was not seen in cells deleted for *hns* (Fig. 6, compare lanes 2, 7, and 12). This result suggested that DsrA either negatively affects a target important for the basal level of biofilm or positively regulates a negative regulator of biofilm formation. DsrA is known to stimulate RpoS synthesis, so we asked if RpoS was (indirectly) interfering with biofilm formation in the Δ*pgaA* cells. We examined the ability of DsrA to repress biofilm in cells deleted for *rpoS*, *pgaA*, or a double mutant of *pgaA* and *rpoS* (Fig. S2B). DsrA suppressed biofilm in the Δ*pgaA* cells, as seen in Fig. 6; deletion of *rpoS* did not relieve this repression.

If biofilm levels were measured after 48 hours (rather than 24 hours) in LB, the dependence on *flhD* disappeared, and the repression by DsrA in cells deleted for *pgaA* also was lost (Fig. S2C). Note that the basal level of biofilm was somewhat lower at 48 hrs in LB (compare Fig. 6, lane 1 to Fig. S2C, lane 1). Deletion of *flhD*, encoding a master regulator for motility, decreased biofilm at 24 hours (Fig. 6, compare lane 4 to lane 1). However, after 48 hours, not only was the dependence on *flhDC* lost, but the basal level of biofilm was increased (Fig. S2C, compare lane 9 to lane 1). The low level of biofilm at 48 hr was no longer repressed by DsrA in the absence of *pgaA* (Fig. S2C, compare lane 20 to lane 17). This suggests that DsrA negatively regulates a process needed early but not late in biofilm formation, presumably slowing the process. One candidate would be motility. Therefore, it is possible that DsrA overproduction negatively regulates motility or something else under FlhDC control; this process would be critical, as is FlhDC, at the early stages of biofilm formation, but would, if anything, be detrimental at later times. We would also suggest that the ability of DsrA to stimulate PGA synthesis changes the pathway or kinetics of biofilm formation sufficiently to overcome the dependence on FlhDC. Differences in the kinetics of biofilm formation and thus the dependence on motility genes in cells in which H-NS is totally lost (Δ*hns*), compared to that in cells in which H-NS is down-regulated by DsrA may explain why cells deleted for *hns* had different behavior (Fig. 6).

Overall, we conclude that, in LB, loss of H-NS, via deletion or via expression of DsrA, increases biofilm in a process that is fully dependent upon *pgaA*. This pathway of biofilm production is independent of RpoS and CsgD, and conditionally dependent upon FlhDC.

Biofilm Production in YESCA

Some sRNAs had effects on biofilm in LB but not in YESCA (GadY, in particular, stimulated only in LB) or in YESCA but not LB (MicF stimulated in YESCA but not in LB) (Fig. 1A, B). In addition, in previous studies, we had noted a requirement for RpoS for biofilm formation in YESCA (Parker & Gottesman, 2016), suggesting that different pathways might be responsible for biofilm formation in this media. Therefore, parallel experiments were done in YESCA.

Most strikingly, and consistent with our previous observations, deletion of *rpoS* or *csgD*, neither of which were required in LB, significantly reduced basal levels of biofilm formation in the MG1655 strain grown in YESCA (Fig. 6B, lanes 3 and 5). The defect in *csgD* cells

could not be overcome by deletion of *hns*, or by overproduction of DsrA (Fig. 6B, lanes 8 and 13). In general, deletion of *hns* or overproduction of DsrA had much less effect in increasing biofilm in YESCA than in LB (compare Fig. 6B to Fig. 6A, lane 11 vs. lane 1). The increase in biofilm in cells deleted for *hns* was, as in LB, dependent upon *pgaA* (compare Fig. 6B, lanes 6 and 7). Deletion of *flhD* reduced biofilm, but not as much as in LB (compare lanes 4 to 1 in Fig. 6A and 6B). Interestingly, the deletion of *hns* fully suppressed the deletion of *rpoS* in YESCA, consistent with H-NS repression of important downstream targets of RpoS, and with a possible inhibitory effect of RpoS in the absence of H-NS.

Thus, when *E. coli* K12 grows in YESCA, biofilm formation switches from the RpoS-independent, PGA-dependent pathway seen in LB overexpressing DsrA to an RpoS-dependent, CsgD-dependent pathway.

Three sRNAs, DsrA, ArcZ, and RprA, are known to each activate translation of RpoS [reviewed in (Battesti *et al.*, 2011)]. Consistent with the dependence of biofilm formation in YESCA on RpoS (Fig. 6B and 7B, lane 5 compared to lane 1), we note that deletion of *arcZ* or *dsrA* reduced biofilm formation specifically in YESCA medium (Fig. 1D). ArcZ negatively regulates multiple targets, in addition to activating RpoS (Mandin & Gottesman, 2010, Monteiro *et al.*, 2012, Papenfort *et al.*, 2009). Overproduced RpoS was fully able to complement the deletion of *rpoS* for biofilm formation (Fig. S3, compare *rpoS::kan* pRpoS, lane 10 to lane 6), but only partially suppressed deletion of *arcZ* (compare *arcZ::zeo*/pRpoS, lane 11 to lanes 5 and 9). While these other ArcZ targets important for biofilm development have not been identified, ArcZ has also been shown to be essential for curli-dependent biofilm formation in *Salmonella*, and this effect is not entirely due to effects on RpoS (Monteiro *et al.*, 2012). ArcZ also negatively regulates the master regulator of the flagellar genes (De Lay & Gottesman, 2012). Therefore, ArcZ regulates multiple targets that contribute to biofilm formation in YESCA medium, one of which is likely RpoS.

Small RNAs Regulate Multiple Biofilm Targets

As noted above, different pathways are important for biofilm formation in LB and YESCA (Fig. 6), and different sRNAs stimulate biofilm in LB and YESCA (Fig. 1A, B). We compared the role of DsrA, discussed above, to two other sRNAs that were observed to increase biofilm, GadY, and MicF, in strains deleted for the various pathways (Fig. 7).

Consistent with the results in Fig. 6, DsrA stimulated biofilm formation in LB in a manner that was fully dependent upon *pgaA*, but was unaffected by *csgD* or *rpoS* (Fig. 7A, compare lanes 4, 8 and 16). Note that, as discussed for Fig. 6, DsrA led to loss of the basal level of biofilm in cells deleted for *pgaA* (Fig. 7A, compare lane 20 to lane 17).

GadY, like DsrA, stimulated biofilm formation in LB, dependent upon *pgaA* (Fig. 7A, lane 18 compared to lane 2), but was independent of *rpoS* and *csgD* (Fig. 7A, compare lane 6 and 14 to lane 2). Unlike DsrA, GadY did not repress biofilm formation in the absence of *pgaA* (Fig. 7A, compare the effect of DsrA, lane 20 to the effect of GadY, lane 18). GadY also activates expression of a *pgaA-lacZ* fusion, more than DsrA (Fig. S1A, C). However, GadY

does not stimulate *pgaA* via repression of H-NS, since the stimulation by the GadY plasmid was seen even in cells deleted for *hns* (Fig. S1B and S1C).

We further examined how GadY might act on *pgaA* expression. McaS, an Hfq-dependent sRNA that regulates some targets by direct pairing, was found to positively regulate *pgaA* by titrating the translational repressor CsrA (Jorgensen *et al.*, 2013). This induction was shown to be dependent on GGA sites within McaS and on CsrA binding sites close to the ribosome binding site within the *pgaA* leader (Jorgensen *et al.*, 2013). We asked if GadY might act in a similar fashion, based on the presence of two GGA sites in this sRNA (underlined in Fig. 8A). First, GadY, activated translation of *pgaA* in two fusions, both of them driven by a pBAD promoter, and both of them activated by McaS as well (Fig. 8B). One of these fusions contains the full 234 nt leader for *pgaA*; the other contains only 30 nt of the leader. The fusion with the 30 nt leader has been shown to still be subject to CsrA repression (Jorgensen *et al.*, 2013). Therefore, GadY activates translation, and at least some of the critical sites are close to the ribosome binding site. Second, mutations were made in each or both of the two GGA sites, changing the A to T. Both mutants were still able to repress a target that pairs with GadY, although with somewhat lower efficiency (Fig. S1D). Mutation of A34 to T reduced activation of a transcriptional and translational fusion (Fig. 8C) and of a translational fusion under control of the pBAD promoter (Fig. 8D). Inactivation of *csrA* (right hand panel in Fig. 8D) increased the expression of the fusion, as expected; in these cells, GadY stimulation was reduced from the 2.5× seen in the *csrA*$^{+}$ host to less than fold; the A34T mutant was not significantly less effective (Fig. 8D, compare left, *csrA*$^{+}$, and right, csrA^{-}, panels). We note that this *csrA* mutant is not a complete null, and that both CsrB and McaS have been shown to show some, but reduced activation in this csrA- strain (Jorgensen *et al.*, 2013). Therefore, we suggest that GadY, like McaS, activates biofilm formation via increased *pgaA* expression, and that this is due at least in part to the ability of a GGA site to titrate CsrA.

In YESCA medium, overexpression of GadY did not significantly stimulate biofilm formation (Fig. 7B, compare lane 2 to lane 1), consistent with it acting on the *pgaA*-dependent pathway that is not used in YESCA. Strikingly, however, GadY overexpression fully suppressed the need for RpoS, giving biofilm levels that were significantly higher than in the Δ*rpoS* host (Fig. 7B, compare lane 5 to lane 6). It seemed likely that biofilm in this case, in the absence of RpoS, was again *pgaA*-dependent. This was tested by measuring the ability of GadY to stimulate biofilm formation in YESCA in cells deleted for both *rpoS* and *pgaA* (Fig. S4). Basal levels of biofilm were again decreased in the *rpoS::tet* mutant, and were similarly low in the *pgaA::kan rpoS::tet* double mutant. However, while multicopy DsrA or GadY could stimulate biofilm in the *rpoS* mutant, they did not in the *pgaA rpoS* double mutant. These results suggest that normally in YESCA, biofilm is independent of PgaA and dependent upon RpoS, and that conditions that might be expected to increase the PgaA-dependent pathway are somehow blocked by RpoS. Thus, there is no additive effect, at least in our crystal violet staining assay, of expressing GadY in an *rpoS*$^{+}$ *csgD*$^{+}$ strain, and in some experiments in which the PGA pathway should be activated in YESCA, for instance in the absence of H-NS, there is more biofilm in the absence of RpoS than with it (Fig. 6B, compare lane 6 to 10). This suggests that one or more products of the RpoS regulon are responsible for blocking the PGA pathway. A similar, but less striking, stimulation of

biofilm is seen in YESCA in cells deleted for *csgD* (Fig. 7B, compare lanes 14 and 16 to lane 13), but not for cells deleted for *flhD* (Fig. 7B, compare lanes 10 and 12 to lane 9).

MicF stimulated biofilm formation only in a wild-type strain in YESCA (Fig. 1A, B, Fig. 7, compare lane 3 to lane 1 in A and B), suggesting it is working via the CsgD/RpoS pathway. It behaved similarly to the vector control in all of the epistasis experiments in Fig. 7. Therefore, unlike DsrA or GadY, MicF does not change the pathway for biofilm formation but instead may increase the efficiency with which these cells use the RpoS and CsgD pathway for biofilm formation. MicF represses the major outer membrane porin OmpF, as well as the Lrp and CpxR regulators in *E. coli* (Mizuno *et al.*, 1984, Delihas & Forst, 2001) (Holmqvist *et al.*, 2012) (Lee & Gottesman, 2016). In *Salmonella*, MicF overexpression decreased expression of the mRNA for *bssS*, a negative regulator of biofilm (Domka *et al.*, 2006) (Corcoran *et al.*, 2012). All of these genes are possible relevant targets of MicF in our experiments; we did not further investigate what the downstream target(s) of MicF might be.

Discussion

There are many genes, including those encoding sRNAs, that regulate biofilm formation; in this study we have examined the ability of overproduced sRNAs to affect biofilm formation under both LB and YESCA growth conditions and found that they were significantly different. When sRNAs were overproduced, both positive and negative regulators of biofilm were identified. We focused on the subset of Hfq-dependent sRNAs that increased biofilm formation, as measured by crystal violet staining, when overproduced. These were then tested in epistasis experiments to identify the pathways they acted on. Consistent with a role for sRNAs, deletion of *hfq* abolished biofilm formation in both LB and YESCA (Fig. 1C, D), as previously found by others using cells grown in LB (Bak *et al.*, 2015). The defect in *hfq* mutants was more severe than effects of deleting any single one of the sRNAs studied, likely suggesting that combinations of sRNAs are involved. In addition, *hfq* mutants have global changes in gene expression and slow growth rates that may lead to more complex effects on biofilm formation.

Our results show two of the alternative pathways that *E. coli* K12 can use in establishing biofilms, and how changing the expression of an sRNA regulator can shift the bacteria from one pathway to another. While deletion of the sRNAs had only modest effects under our laboratory conditions (Fig. 1C, D), overproduction of sRNAs allowed us to highlight these alternative pathways, and emphasized the complex cross talk between sRNAs and transcriptional regulators. This is outlined in Fig. 9 and discussed further below.

Basal Levels of Biofilm Differ in LB and YESCA

Requirements for the basal level of biofilm were defined by effects of mutants in various pathways implicated in biofilm formation, in the absence of the sRNA. By this definition, FlhD, the master regulator of flagellar synthesis, is needed in both LB and YESCA (Fig. 6 and 7) for the full level of biofilm. None of the other tested mutants decreased basal levels of biofilm in LB, but mutants in both *rpoS*, encoding the stationary/stress sigma factor, and *csgD,* encoding the master regulator for curli synthesis and other genes involved in biofilm formation, abolished biofilm in YESCA (Fig. 6B, compare lanes 3 and 5 to lane 1; Fig. 7B,

compare lanes 5 and 13 to lane 1). Therefore, we suggest that in LB the low basal level of biofilm may use multiple redundant pathways. In YESCA medium, CsgD and RpoS played important roles for the basal level of biofilm, while PgaA did not (Fig. 6, 7), consistent with previous reports for a minor role of PGA in wild-type *E. coli* K12 (Itoh *et al.*, 2008) (Fig. 9).

sRNAs stimulate a PGA-dependent pathway of biofilm formation in LB

In LB, increased biofilm was seen in the presence of multicopy DsrA or multicopy GadY or in the absence of H-NS; this increased biofilm was dependent upon *pgaA*, encoding the polysaccharide beta-1, 6 N-acetyl-D-glucosamine outer membrane porin (Fig. 6, 7). This pathway, which was independent of both *rpoS* and *csgD*, is shown as Pathway 1 in Fig. 9.

Our results show that DsrA stimulates biofilm specifically by negatively regulating *hns* translation (Fig. 9). DsrA stimulates biofilm only when it can pair with *hns* (Fig. 3), and deletion of *hns* mimicked the effect of overproducing DsrA in most experiments (Fig. 6). H-NS repression of *pgaA* transcription is likely to contribute to this increase (Fig. S1), but the *pga* operon is likely not the only target of H-NS. For instance, H-NS has complex effects on motility, including effects on cyclic-di-GMP regulation (Kim & Blair, 2015). We do not currently know if H-NS repression of *pgaA* transcription is direct and/or indirect, for instance, via repression of an activator of *pgaA*, such as NhaR (Dover *et al.*, 1996); (Goller *et al.*, 2006). Finally, under the growth conditions used here (LB, 25°C), chromosomally-encoded DsrA did not contribute significantly to biofilm formation (Fig. 1C, Fig. 3), although it seems possible that there are growth conditions under which DsrA repression of H-NS may be important. We note that deletion of *dsrA* did have a biofilm-deficient phenotype in YESCA (Fig. 1D), likely due to the role of DsrA in stimulating RpoS translation.

A previous study demonstrated increased adhesion, a critical process for biofilm formation, upon inactivation of *hns* (Landini & Zehnder, 2002), although the role of PGA in adhesion was not investigated in that paper. Studies in *A. pleuropneumoniae* reported that *hns* mutants led to increased biofilm formation (Dalai *et al.*, 2009, Bosse *et al.*, 2010), consistent with our findings in *E. coli*, and that H-NS specifically repressed the *pga* operon and the expression of the PGA polysaccharide matrix (Bosse *et al.*, 2010). Our results support a similar pathway for biofilm in *E. coli* K12, at least in rich (LB) medium, shown both by increased biofilm (Fig. 5, 6) and increased expression of a *pgaA-lacZ* fusion in the absence of H-NS (Fig. S1B, C). Still to be explained, however, is why multicopy DsrA was less effective than deletion of *hns* in increasing expression of this fusion (Fig. S1). Possibly the promoter for *pgaA* is a particularly sensitive target for H-NS, and residual H-NS present when DsrA is overexpressed was still sufficient for some *pgaA* repression. Intriguingly, microarray analysis of an *hns* mutant in *A. pleuropneumoniae* suggested that, unlike the large number of genes regulated by H-NS in *E. coli* (Hommais *et al.*, 2001), only the *pgaA* operon was up-regulated in an *hns* mutant (Bosse *et al.*, 2010).

In contrast to our findings, in another study screening many sRNAs in *E. coli*, overexpression of DsrA reduced biofilm levels rather than increasing them, and deletion of *dsrA* modestly increased biofilm (Bak *et al.*, 2015). The biofilm assays in that study were performed using a 12 hour incubation period, at 30°C (Bak *et al.*, 2015), compared to the 24

hours at 25°C that we used. Although we have not fully explored this difference, we note that these investigators also saw a decrease in motility when DsrA was overproduced. It seems likely that with these short incubation periods, inhibition of motility may be sufficient to explain their observations. As discussed above, at 24 hours, deletion of *flhD* (master regulator for flagellar synthesis) lowered basal levels of biofilm significantly (Fig. 6, compare lane 1 to lane 4, Fig. 7, compare lane 9 to lane 1, Fig. S2A), while after 48 hours the same deletion increased, rather than decreased biofilm levels (Fig. S2C, compare lane 9 to lane 1). Bak et al detected little effect of DsrA on *csgD*-lacZ, *flhD′-'lacZ* or *pgaA'-'lacZ* translational fusions (Bak *et al.*, 2015), consistent with our findings that DsrA is likely to work primarily via silencing of H-NS, a transcriptional repressor. However, when *pgaA* was absent, we observed DsrA repression of biofilm in LB (Fig. 6, 7, Fig. S2B); this effect disappeared after 48 hours (Fig. S2C). We suggest that this reflects negative regulation of motility by DsrA, and this may also explain the repression by DsrA noted by Bak et al (Bak *et al.*, 2015).

GadY increased PgaA-dependent biofilm (Fig. 6, 7); this can be explained by its ability to increase expression of a *pgaA-lacZ* fusion (Fig. S1). While this fusion reflects both transcriptional and translational regulation of *pgaA*, our further work suggests that GadY stimulates *pgaA* translation (Fig. 8B), and may do this by titrating CsrA, a negative regulator of *pgaA* (Wang *et al.*, 2005). The stimulation of *pgaA* is dependent on a GGA sequence, contained in a short hairpin, within GadY, can stimulate *pgaA* translation even when most of the leader is deleted, and is partially suppressed by a *csrA* mutant (Fig. 8). This pattern is similar to that observed for McaS, another Hfq-dependent sRNA that has been shown to activate *pgaA* via CsrA titration (Jorgensen *et al.*, 2013). Therefore, there may be more bifunctional sRNAs that link the Hfq and CsrA regulons than first thought. Bak et al, who found inhibition of biofilm with DsrA overproduction (discussed above), did not find any effect of GadY on biofilm levels, although they did report that GadY repressed swarming and activated translation of *pgaA* (Bak *et al.*, 2015). Possibly these two effects balance out in their experiments. Our findings, in addition to others, suggest that GadY activation of biofilm in LB is primarily via translational activation of *pgaA*.

RpoS stimulates CsgD dependent biofilm in YESCA medium

Pathway 2 in Fig. 9 depicts the dependence of biofilm in YESCA on both RpoS and CsgD. CsgD, known to be involved in adhesion via production of curli and biofilm formation, and is a modulator of a subset of genes in the *rpoS* regulon (Gualdi *et al.*, 2007). *csgD* transcription is directly controlled by RpoS (Battesti *et al.*, 2011, Ogasawara *et al.*, 2010). The requirement for RpoS in YESCA (Fig. 6B, lane 5 vs. lane 1, Fig. 7B, lane 5 vs. lane 1) is likely at least in part due to its role in regulating *csgD* expression (Hammar *et al.*, 1995). Deletions of ArcZ or DsrA, both positive regulators of RpoS translation, reduced biofilm formation in YESCA, but not in LB (Fig. 1C, D), consistent with their effect on RpoS levels, although ArcZ probably has other targets in this pathway (Fig. S3).

Consistent with our findings, others have found a role for curli in YESCA, but not in LB. Uropathogenic *E. coli* (UPEC) strain UT189 was shown to produce a curli and cellulose dependent morphology on YESCA agar and form curli-dependent biofilms when grown in

YESCA medium (Lim *et al.*, 2012, DePas *et al.*, 2013); other studies have shown that biofilms developed in LB are not curli dependent (Cegelski *et al.*, 2009).

When MicF was overexpressed, biofilm increased, but unlike the effects of GadY and DsrA, this biofilm was still fully dependent upon RpoS and CsgD (Fig. 7B) and is only seen in YESCA (Fig. 1B, Fig. 7B). Therefore, it seems likely that MicF, by an as yet undefined pathway, specifically stimulates Pathway 2.

Interference between Pathway 1 and Pathway 2

Pathway 1 and 2 appear to be mutually exclusive. In our experiments, RpoS interferes with PgaA-dependent (Pathway 1) biofilm production in YESCA (Fig. 9). This conclusion is based on the inability of GadY or DsrA to stimulate biofilm in YESCA unless cells also carry a deletion of *rpoS* (Fig. 7B, lanes 5–8); that biofilm was dependent upon *pgaA* (Fig. S4). The mechanism of this inhibition remains to be determined. A negative effect of RpoS on biofilm formation was previously reported, with different strains and under somewhat different growth conditions (Corona-Izquierdo & Membrillo-Hernandez, 2002).

Other work supports an “either-or” regulation of these two pathways. For instance, the sRNA McaS stimulates the PgaA pathway by titrating CsrA (Jorgensen *et al.*, 2013), but represses *csgD* (Jorgensen *et al.*, 2012). We would predict that further work will uncover further mechanisms for down-regulating one pathway while up-regulating the other.

Negative Regulation of Biofilm by sRNA Overproduction

While we did not investigate the basis for negative regulation by many sRNAs (Fig. 1A, B), we can imagine a number of ways for sRNAs to repress biofilm. CsgD, clearly important for biofilm formation in YESCA, has been reported to be negatively regulated by six sRNAs (Thomason *et al.*, 2012) (Bordeau & Felden, 2014), four of which (McaS, RydC, RprA, and OmrB) we saw as biofilm repressors in Fig. 1B. However, RydC is likely to have another important biofilm target, since it also inhibited biofilm in LB, which is not dependent on *csgD* (Fig. 1A). We note that there was not a good correlation of sRNAs that inhibited motility in TB agar (De Lay & Gottesman, 2012) with those that inhibited biofilm (Fig. 1A, B), suggesting that this is not the major pathway for inhibiting biofilm. ChiX, a negative regulator of biofilm in YESCA (Fig. 1B), is known to effectively titrate Hfq (Moon & Gottesman, 2011, Ellis *et al.*, 2015) and thus overproducing this sRNA may partially mimic the effect of deleting Hfq. It is interesting that ChiX negative regulation was much stronger in YESCA than in LB, suggesting a particularly sensitive Hfq-dependent target for biofilm formation in YESCA, possibly translation of RpoS.

In conclusion, several Hfq-dependent sRNAs are capable of perturbing biofilm synthesis pathways. The variation between different media highlights the redundancy between pathways and the ways in which the cell coordinates alternative regulatory circuits. Our work reinforces previous findings suggesting that positive and negative regulation by RpoS has a role in which pathway cells use.

Experimental Procedures

Growth conditions

Strains were grown aerobically at 37°C in LB (10g tryptone, 5g yeast extract, 10g NaCl per liter) overnight or until stationary phase. Biofilm formation was performed in 96-well microtiter plates using LB or YESCA (10g casamino acids and 1g yeast extract per liter) media (Epstein *et al.*, 2009). Ampicillin (100μg/ml), Kanamycin (25μg/ml), Arabinose (.01%), isopropyl-β-D-thogalactopyranoside (IPTG) (100μM), and 5-bromo- 4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (20 μg/ml) were used where needed.

Strains and Plasmids

The strains used in this study are listed in Table S1. Translational fusions in this study were engineered using lambda red recombination (Yu *et al.*, 2000). The *hns**-lacZ fusion was designed by moving the hns-mut1 DNA oligonucleotide into PM1205 (Supplementary Table S1 and S2) and selecting on minimal glycerol X-gal plates containing sucrose plates to create strain SC71. Chromosomal *hns** (SC144) was designed by first PCR amplifying the ccdB-kan cassette from NM570 genomic DNA, using primers Kn-hnsmut-ccdBfor and Kn-hnsmut-ccdBrev. Lambda red recombination was used to recombine the PCR fragment into MG1655 containing mini-lambda::tet (NM1100) (Yu *et al.*, 2000) (Court *et al.*, 2003), and the recombinants were selected using 1% glucose-kanamycin agar plates at 32°C. This *hns::kan ccdB* chromosomal deletion, in which the toxin CcdB is expressed from a P_{BAD} promoter (Tripathi *et al.*, 2012) was confirmed using primers (ab05- Δhns::km forward and ab06- Δhns::km reverse). In a second step, the *hns::kan ccdB* cassette was further replaced by the hns-mut1 oligonucleotide (*hns**) as described (Court *et al.*, 2003). The *hns** strain was counter-selected using 1% arabinose agar plates and confirmed by loss of kan^R and sequencing.

The *pgaA-lacZ* fusion present in NM750 was constructed using a standard PCR (ABI) reaction using pgaA_trnsltnl.F and pgaA_trnsltnl.R primers and MG1655 genomic DNA as template; the region of interest was designed to include the promoter, upstream elements of the promoter, as well as the 5′ UTR, to the ATG. This region extended from −502 relative to ATG or −268 relative to the start of transcription to the ATG. This PCR fragment also contained 40 extra nucleotides on either end that are homologous to the region of insertion in NM580, namely to the zeo^R marker on the 5′ end and to the *lacZ* ORF on the 3′end. This PCR fragment was recombineered into electrocompetent λ-Red proficient NM580 cells, recovered overnight on the bench in LB-1% glucose and plated on LB-1% arabinose for counter-selection. Recombinants were screened for Kan^S and checked by PCR for the correct insertion and sequenced to confirm. The resulting strain was named NM750. This strain was then transduced with P1 to *hns*::kan to yield NM751 (Table S1).

Deletion-substitutions of *dsrA* (NM607) and *arcZ* (NM665) were created by introducing PCR products from NM1201 with the appropriate primers (dsrA-zeo.F and dsrA-zeo.F for Δ*dsrA*7::*zeo* and arcZ-zeo.F and arcZ-zeo.R for arcZ::zeo) into NM1100 by recombineering, selecting for zeomycin resistance.

The plasmids used in this study are derived from the pBR-plac vector (Guillier & Gottesman, 2006) (Table S1). Mutations in the pDsrA plasmid vector were constructed using Quik Change Lightning Site-Directed Mutagenesis Kit (Stratagene). The pDsrA plasmid was used to generate mutations in the region important for pairing with the *rpoS* 5′ UTR (pDsrA*^{r}) using primers DsrA*rmut2for and DsrA*rmut2rev; mutations in the pairing regions important for *hns* (pDsrA*^{h}) was previously constructed (Lee & Gottesman, 2016) (Table S1). Small RNA plasmids were introduced into strains using TSS transformation (Chung & Miller, 1988) and deletion mutants were constructed in the chromosome using P1 transduction.

Mutagenesis of pGadY was performed using the QuickChange kit from Agilent (Santa Clara, CA) according to the manufacturer's specifications. Primer sets used for these reactions were gadY_a34t/gadY_a34t-as and gadY_a60t/gadY_a60t-as. Plasmids were sequenced to confirm the mutations.

Biofilm assay

Strains used for biofilm experiments were grown in LB (with Ampicillin at 100 μg/ml where appropriate) at 37°C overnight or until stationary phase, using well-aerated tubes in a roller drum rotating at 250rpm. Cultures were diluted to a final OD600 concentration of ~0.05 in fresh LB or YESCA, LB+amp (100μg/ml)+IPTG (100μM), or YESCA+amp (100μg/ml) +IPTG (100μM). 200μl of each culture was aliquoted into separate wells in a 96-well plate. The wells on the edge of the 96-well plate were avoided as they generate more variance. LB or YESCA media was used as negative controls. Microtiter plates were carefully wrapped using parafilm and placed in a 25°C incubator without shaking. After 24hrs or 48hrs, the plates were removed and the planktonic cell growth (OD600) was measured using a SpectraMax Plus microplate reader. Planktonic cells were removed using a multi-channel pipette without disturbing the biofilm area and individual wells were washed twice using 200μl of dH_2O. To stain for biofilm, 220μl of 0.1% crystal violet solution was added to each well for 10 min and then washed twice with 200μl of dH_2O and once with 300μl of dH_2O. The plates were allowed to dry for 10 min at 37°C prior to dissolving the stained biofilm with 230μl of 20% Acetone/80% Ethanol solution for 10 minutes at room temperature. Biofilm levels were measured using the OD_{570} normalized by the OD_{600} (planktonic cell growth). This assay does not provide insight into the structure of the biofilm and it is possible that staining with crystal violet of cells in the two pathways studied here is not equivalent. Because cell growth was very slow in the YESCA medium, we report the 48 hr rather than the 24 hr results for YESCA. For LB, the amount of biofilm was seen to decrease at 48 hr (compare WT plac cells in Fig. S2C to Fig. S2A and B or Fig. 7A) and the dependence upon *flhD* disappeared (Fig. S2C).

β-Galactosidase assays

Overnight cultures of *hns-lacZ* (HL1061), *hns*-lacZ* (SC71), and *rpoS-lacZ* (PM1409) containing plac, pDsrA, pDsrA*h, pDsrA*r plasmids were diluted 200-fold into fresh LB medium containing IPTG (100μM), ampicillin (100μM), and arabinose (0.001%), and incubated at 37°C in a roller drum for 6 hours. The cultures were then removed, and a β-galactosidase assay was performed as described by Miller (Miller, 1992). P*gaA-lacZ*

(NM750) and Δ*hns pgaA-lacZ* (NM751) transcriptional/translational fusions were grown overnight in LB and diluted 1:1000 in LB medium or in LB ampicillin (100μM) for strains containing plasmids. GS0568, GS0641, GS0644 and GS0645 were grown and assayed similarly, except that 0.2% arabinose was added to the culture medium after dilution to induce the pBAD promoter. Cultures were grown in a shaking water bath at 37°C and strains with plasmids were induced at OD_{600} = 0.3 using 100μM IPTG. Samples were taken at OD_{600} 1.5–1.8 and assayed for activity using the Miller assay (Miller, 1992).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Gottesman and Storz labs for comments on the work and the manuscript. We thank G. Storz (NICHD) for strains used in this work. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. The authors do not have any conflicts of interest to report.

References

Bak G, Lee J, Suk S, Kim D, Young Lee J, Kim KS, Choi BS, Lee Y. Identification of novel sRNAs involved in biofilm formation, motility, and fimbriae formation in *Escherichia coli*. Scientific reports. 2015; 5:15287. [PubMed: 26469694]

Battesti A, Majdalani N, Gottesman S. The RpoS-mediated general stress response in *Escherichia coli*. Annual review of microbiology. 2011; 65:189–213.

Bertin P, Terao E, Lee EH, Lejeune P, Colson C, Danchin A, Collatz E. The H-NS protein is involved in the biogenesis of flagella in Escherichia coli. Journal of bacteriology. 1994; 176:5537–5540. [PubMed: 8071234]

Boehm A, Vogel J. The csgD mRNA as a hub for signal integration via multiple small RNAs. Molecular microbiology. 2012; 84:1–5. [PubMed: 22414234]

Bordeau V, Felden B. Curli synthesis and biofilm formation in enteric bacteria are controlled by a dynamic small RNA module made up of a pseudoknot assisted by an RNA chaperone. Nucleic acids research. 2014; 42:4682–4696. [PubMed: 24489123]

Bosse JT, Sinha S, Li MS, O'Dwyer CA, Nash JH, Rycroft AN, Kroll JS, Langford PR. Regulation of pga operon expression and biofilm formation in Actinobacillus pleuropneumoniae by sigmaE and H-NS. Journal of bacteriology. 2010; 192:2414–2423. [PubMed: 20207760]

Cegelski L, Pinkner JS, Hammer ND, Cusumano CK, Hung CS, Chorell E, Aberg V, Walker JN, Seed PC, Almqvist F, Chapman MR, Hultgren SJ. Small-molecule inhibitors target Escherichia coli amyloid biogenesis and biofilm formation. Nature chemical biology. 2009; 5:913–919. [PubMed: 19915538]

Chambers JR, Sauer K. Small RNAs and their role in biofilm formation. Trends in microbiology. 2013; 21:39–49. [PubMed: 23178000]

Chevance FFV, Hughes KT. Coordinating assembly of a bacterial macromolecular machine. Nat Rev Microbiol. 2008; 6:455–465. [PubMed: 18483484]

Chib S, Mahadevan S. Involvement of the global regulator H-NS in the survival of Escherichia coli in stationary phase. Journal of bacteriology. 2012; 194:5285–5293. [PubMed: 22843842]

Chung CT, Miller RH. A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic acids research. 1988; 16:3580. [PubMed: 3287331]

Corcoran CP, Pdkaminski D, Papenfort K, Urban JH, Hinton JCD, Vogel J. Superfolder GFP reporters validate diverse new mRNA targets of the classic porin regulator, MicF RNA. Molecular microbiology. 2012; 84:428–445. [PubMed: 22458297]

Corona-Izquierdo FP, Membrillo-Hernandez J. A mutation in *rpoS* enhances biofilm formation in *Escherichia coli* during exponential phase of growth. FEMS microbiology letters. 2002; 211:105–110. [PubMed: 12052558]

Court DL, Swaminathan S, Yu D, Wilson H, Baker T, Bubunenko M, Sawitzke J, Sharan SK. Mini-lambda: a tractable system for chromosome and BAC engineering. Gene. 2003; 315:63–69. [PubMed: 14557065]

Dalai B, Zhou R, Wan Y, Kang M, Li L, Li T, Zhang S, Chen H. Histone-like protein H-NS regulates biofilm formation and virulence of Actinobacillus pleuropneumoniae. Microbial pathogenesis. 2009; 46:128–134. [PubMed: 19095055]

De Lay N, Gottesman S. A complex network of small non-coding RNAs regulate motility in Escherichia coli. Molecular microbiology. 2012; 86:524–538. [PubMed: 22925049]

De Lay N, Schu DJ, Gottesman S. Bacterial small RNA-based negative regulation: Hfq and its accomplices. The Journal of biological chemistry. 2013; 288:7996–8003. [PubMed: 23362267]

Delihas N, Forst S. MicF: an antisense RNA gene involved in response of Escherichia coli to global stress factors. Journal of molecular biology. 2001; 313:1–12. [PubMed: 11601842]

DePas WH, Hufnagel DA, Lee JS, Blanco LP, Bernstein HC, Fisher ST, James GA, Stewart PS, Chapman MR. Iron induces bimodal population development by Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110:2629–2634. [PubMed: 23359678]

Domka J, Lee J, Wood TK. YliH (BssR) and YceP (BssS) regulate *Escherichia coli* K-12 biofilm formation by influencing cell signaling. Applied and environmental microbiology. 2006; 72:2449–2459. [PubMed: 16597943]

Donato GM, Lelivelt MJ, Kawula TH. Promoter-specific repression of fimB expression by the Escherichia coli nucleoid-associated protein H-NS. Journal of bacteriology. 1997; 179:6618–6625. [PubMed: 9352908]

Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical microbiology reviews. 2002; 15:167–193. [PubMed: 11932229]

Dover N, Higgins CF, Carmel O, Rimon A, Pinner E, Padan E. Na+-induced transcription of *nhaA*, which encodes an Na+/H+ antiporter in *Escherichia coli*, is positively regulated by *nhaR* and affected by *hns*. J Bacteriol. 1996; 178:6508–6517. [PubMed: 8932307]

Ellis MJ, Trussler RS, Haniford DB. Hfq binds directly to the ribosome-binding site of IS10 transposase mRNA to inhibit translation. Molecular microbiology. 2015; 96:633–650. [PubMed: 25649688]

Epstein EA, Reizian MA, Chapman MR. Spatial clustering of the curlin secretion lipoprotein requires curli fiber assembly. Journal of bacteriology. 2009; 191:608–615. [PubMed: 19011034]

Ferrieres L, Thompson A, Clarke DJ. Elevated levels of sigma S inhibit biofilm formation in Escherichia coli: a role for the Rcs phosphorelay. Microbiology. 2009; 155:3544–3553. [PubMed: 19696107]

Frohlich KS, Papenfort K, Berger AA, Vogel J. A conserved RpoS-dependent small RNA controls the synthesis of major porin OmpD. Nucleic acids research. 2012; 40:3623–3640. [PubMed: 22180532]

Goller C, Wang X, Itoh Y, Romeo T. The cation-responsive protein NhaR of Escherichia coli activates pgaABCD transcripiton, required for the production of the biofilm adhesin poly-beta-1,6-N-acetyl-D-glucosamine. Journal of bacteriology. 2006; 188:8022–8032. [PubMed: 16997959]

Goller CC, Romeo T. Environmental influences on biofilm development. Curr Top Microbiol Immunol. 2008; 322:37–66. [PubMed: 18453271]

Gottesman S. The small RNA regulators of Escherichia coli: roles and mechanisms*. Annual review of microbiology. 2004; 58:303–328.

Gualdi L, Tagliabue L, Landini P. Biofilm formation-gene expression relay system in Escherichia coli: modulation of sigmaS-dependent gene expression by the CsgD regulatory protein via sigmaS protein stabilization. Journal of bacteriology. 2007; 189:8034–8043. [PubMed: 17873038]

Guillier M, Gottesman S. Remodelling of the Escherichia coli outer membrane by two small regulatory RNAs. Molecular microbiology. 2006; 59:231–247. [PubMed: 16359331]

Hammar M, Arnqvist A, Bian Z, Olsen A, Normark S. Expression of two csg operons is required for production of fibronectin- and congo red-binding curli polymers in *Escherichia coli* K-12. Molecular microbiology. 1995; 18:661–670. [PubMed: 8817489]

Holmqvist E, Unoson C, Reimegard J, Wagner EGH. A mixed double negative feedback loop between the sRNA MicF and the global regulator Lrp. Molecular microbiology. 2012; 84:414–427. [PubMed: 22324810]

Hommais F, Krin E, Laurent-Winter C, Soutourina O, Malpertuy A, Le Caer JP, Danchin A, Bertin P. Large-scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein, H-NS. Molecular microbiology. 2001; 40:20–36. [PubMed: 11298273]

Hung C, Zhou Y, Pinkner JS, Dodson KW, Crowley JR, Heuser J, Chapman MR, Hadjifrangiskou M, Henderson JP, Hultgren SJ. Escherichia coli biofilms have an organized and complex extracellular matrix structure. mBio. 2013; 4:e00645–00613. [PubMed: 24023384]

Itoh Y, Rice JD, Goller C, Pannuri A, Taylor J, Meisner J, Beveridge TJ, Preston JF 3rd, Romeo T. Roles of *pgaABCD* genes in synthesis, modification, and export of the *Escherichia coli* biofilm adhesin poly-beta-1,6-N-acetyl-D-glucosamine. J Bacteriol. 2008; 190:3670–3680. [PubMed: 18359807]

Jorgensen MG, Nielsen JS, Boysen A, Franch T, Moller-Jensen J, Valentin-Hansen P. Small regulatory RNAs control the multi-cellular adhesive lifestyle of Escherichia coli. Molecular microbiology. 2012; 84:36–50. [PubMed: 22250746]

Jorgensen MG, Thomason MK, Havelund J, Valentin-Hansen P, Storz G. Dual function of the McaS small RNA in controlling biofilm formation. Genes & development. 2013; 27:1132–1145. [PubMed: 23666921]

Kikuchi T, Mizunoe Y, Takade A, Naito S, Yoshida S. Curli fibers are required for development of biofilm architecture in Escherichia coli K-12 and enhance bacterial adherence to human uroepithelial cells. Microbiology and immunology. 2005; 49:875–884. [PubMed: 16172544]

Kim EA, Blair DF. Function of the histone-Like Protein H-NS in motility of *Escherichia coli*: Multiple regulatory roles rather than direct action at the flagellar motor. J Bacteriol. 2015; 197:3110–3120. [PubMed: 26195595]

Landini P, Zehnder AJ. The global regulatory hns gene negatively affects adhesion to solid surfaces by anaerobically grown Escherichia coli by modulating expression of flagellar genes and lipopolysaccharide production. Journal of bacteriology. 2002; 184:1522–1529. [PubMed: 11872702]

Lease RA, Belfort M. A trans-acting RNA as a control switch in Escherichia coli: DsrA modulates function by forming alternative structures. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97:9919–9924. [PubMed: 10954740]

Lease RA, Cusick ME, Belfort M. Riboregulation in Escherichia coli: DsrA RNA acts by RNA:RNA interactions at multiple loci. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95:12456–12461. [PubMed: 9770507]

Lee HJ, Gottesman S. sRNA roles in regulating transcriptional regulators: Lrp and SoxS regulation by sRNAs. Nucleic acids research. 2016

Lim JY, May JM, Cegelski L. Dimethyl sulfoxide and ethanol elicit increased amyloid biogenesis and amyloid-integrated biofilm formation in Escherichia coli. Applied and environmental microbiology. 2012; 78:3369–3378. [PubMed: 22389366]

Liu Z, Niu H, Wu S, Huang R. CsgD regulatory network in a bacterial train-altering biofilm formation. Emerg Microbes Infect. 2014; 3

Majdalani N, Cunning C, Sledjeski D, Elliott T, Gottesman S. DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. Proceedings of the National Academy of Sciences of the United States of America. 1998; 95:12462–12467. [PubMed: 9770508]

Mandin P, Gottesman S. Integrating anaerobic/aerobic sensing and the general stress response through the ArcZ small RNA. The EMBO journal. 2010; 29:3094–3107. [PubMed: 20683441]

Mika F, Hengge R. Small regulatory RNAs in the control of motility and biofilm formation in *E. coli* and *Salmonella*. Int, J Mol Sci. 2013; 14:4560–4579. [PubMed: 23443158]

Mika F, Hengge R. Small RNAs in the control of RpoS, CsgD, and biofilm architecture of Escherichia coli. RNA biology. 2014; 11:494–507. [PubMed: 25028968]

Miller, JH. A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria. Cold Spring Harbor Laboratory Press; Plainview, NY: 1992.

Mizuno T, Chen MY, Inouye M. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proceedings of the National Academy of Sciences of the United States of America. 1984; 81:1966–1970. [PubMed: 6201848]

Monteiro C, Papenfort K, Hentrich K, Ahmad I, Le Guyon S, Reimann R, Grantcharova N, Romling U. Hfq and Hfq-dependent small RNAs are major contributors to multicellular development in Salmonella enterica serovar Typhimurium. RNA biology. 2012; 9:489–502. [PubMed: 22336758]

Moon K, Gottesman S. Competition among Hfq-binding small RNAs in Escherichia coli. Molecular microbiology. 2011; 82:1545–1562. [PubMed: 22040174]

Ogasawara H, Yamada K, Kori A, Yamamoto K, Ishihama A. Regulation of the Escherichia coli csgD promoter: interplay between five transcription factors. Microbiology. 2010; 156:2470–2483. [PubMed: 20466769]

Opdyke JA, Kang JG, Storz G. GadY, a small-RNA regulator of acid response genes in Escherichia coli. Journal of bacteriology. 2004; 186:6698–6705. [PubMed: 15466020]

Papenfort K, Said N, Welsink T, Lucchini S, Hinton JCD, Vogel J. Specific and pleiotropic patterns of mRNA regulation by ArcZ, a conserved, Hfq-dependent small RNA. Molecular microbiology. 2009; 74:139–158. [PubMed: 19732340]

Parker A, Gottesman S. Small RNA Regulation of TolC, the Outer Membrane Component of Bacterial Multidrug Transporters. Journal of bacteriology. 2016; 198:1101–1113. [PubMed: 26811318]

Raghavan R, Groisman EA, Ochman H. Genome-wide detection of novel regulatory RNAs in E. coli. Genome research. 2011; 21:1487–1497. [PubMed: 21665928]

Santiago-Frangos A, Kavita K, Schu DJ, Gottesman S, Woodson SA. C-terminal domain of the RNA chaperone Hfq drives sRNA competition and release of target RNA. Proceedings of the National Academy of Sciences of the United States of America. 2016; 113:E6089–E6096. [PubMed: 27681631]

Soutourina O, Kolb A, Krin E, Laurent-Winter C, Rimsky S, Danchin A, Bertin P. Multiple control of flagellum biosynthesis in Escherichia coli: role of H-NS protein and the cyclic AMP-catabolite activator protein complex in transcription of the flhDC master operon. Journal of bacteriology. 1999; 181:7500–7508. [PubMed: 10601207]

Thomason MK, Fontaine F, De Lay N, Storz G. A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in Escherichia coli. Molecular microbiology. 2012; 84:17–35. [PubMed: 22289118]

Tripathi A, Dewan PC, Barua B, Varadarajan R. Additional role for the ccd operon of F-plasmid as a transmissible persistence factor. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:12497–12502. [PubMed: 22802647]

Updegrove TB, Zhang A, Storz G. Hfq: the flexible RNA matchmaker. Curr Opin Microbiol. 2016; 30:133–138. [PubMed: 26907610]

Van Puyvelde S, Steenackers HP, Vanderleyden J. Small RNAs regulating biofilm formation and outer membrane homeostasis. RNA biology. 2013; 10:185–191. [PubMed: 23324602]

Wang X, Dubey AK, Suzuki K, Baker CS, Babitzke P, Romeo T. CsrA post-transcriptionally represses pgaABCD, responsible for synthesis of a biofilm polysaccharide adhesin of Escherichia coli. Molecular microbiology. 2005; 56:1648–1663. [PubMed: 15916613]

Weilbacher T, Suzuki K, Dubey AK, Wang X, Gudapaty S, Morozov I, Baker CS, Georgellis D, Babitzke P, Romeo T. A novel sRNA component of the carbon storage regulatory system of Escherichia coli. Molecular microbiology. 2003; 48:657–670. [PubMed: 12694612]

Yu D, Ellis HM, Lee EC, Jenkins NA, Copeland NG, Court DL. An efficient recombination system for chromosome engineering in Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97:5978–5983. [PubMed: 10811905]

Zhang XS, Garcia-Contreras R, Wood TK. YcfR (BhsA) influences Escherichia coli biofilm formation through stress response and surface hydrophobicity. Journal of bacteriology. 2007; 189:3051–3062. [PubMed: 17293424]

Zhao X, Koestler BJ, Waters CM, Hammer BK. Post-transcriptional activation of a diguanylate cyclase by quorum sensing small RNAs promotes biofilm formation in Vibrio cholerae. Molecular microbiology. 2013; 89:989–1002. [PubMed: 23841714]

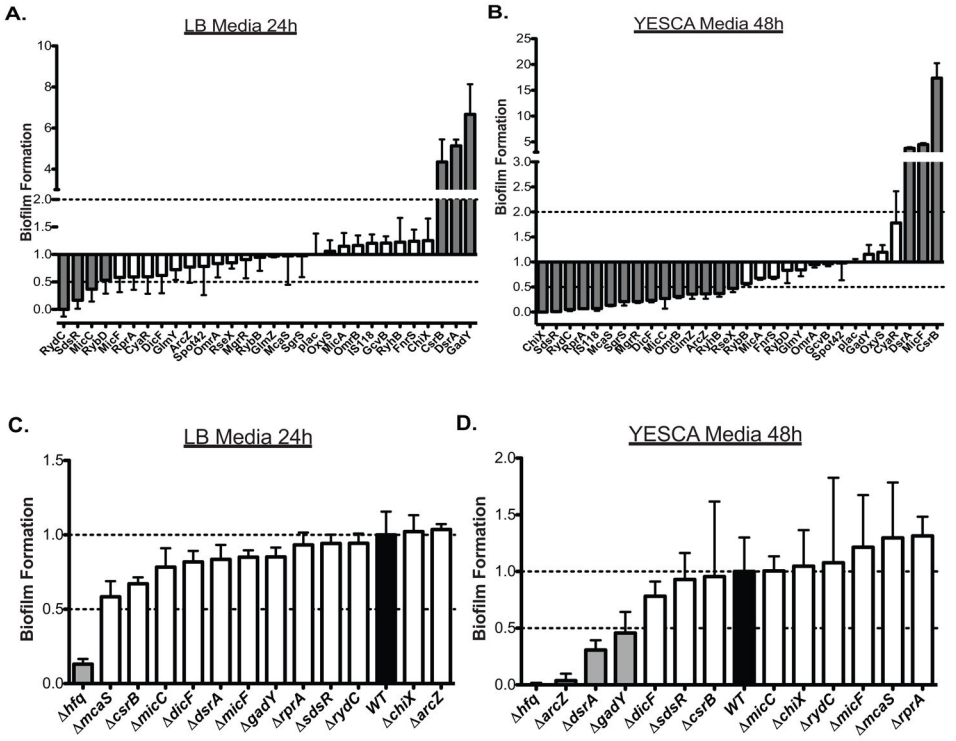

Figure 1. Effects of overexpressed sRNAs and sRNA deletions on Biofilm A, B: Effect of sRNAs overexpressed from a plasmid library

Overnight cultures of WT MG1655 harboring pBR-plac (vector) or a derivative expressing one of 29 different sRNAs from a lac-based promoter were grown at 25°C in microtiter dishes and assayed for biofilm formation as described in Materials and Methods. Cells were grown for (A) 24 hours of incubation in LB-Amp or (B) 48 hours in YESCA-Amp. The fold change relative to the strain harboring the vector control was calculated. The results are an average of three independent experiments, with error bars representing standard deviation. The shaded grey bars indicate over-expressed sRNAs that increased or decreased biofilm formation two-fold or more.

C, D: Effect of sRNA deletions. Strains containing sRNA and *hfq* deletions were grown overnight in LB media and tested for biofilm formation after (C) 24 hours incubation in LB or (D) 48 hours in YESCA at 25°C. Biofilm was measured and the fold change relative to the wild-type MG1655 control (black filled bars), set to 1, was calculated. The results are an average of three independent experiments, with error bars representing standard deviation. The grey bars represent a significant decrease in biofilm formation (of two-fold or greater). Strain names are listed in Table S1.

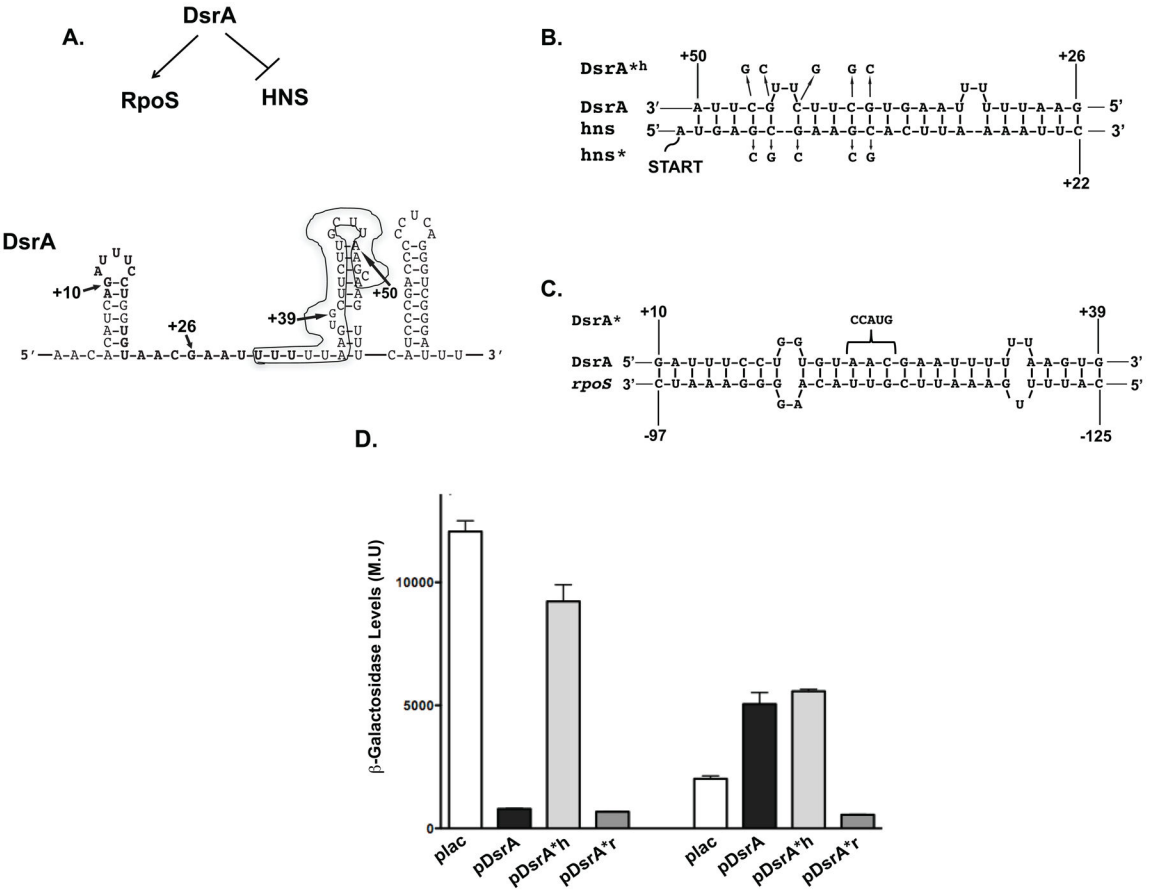

Figure 2. Interaction of DsrA with downstream targets *hns* and *rpoS*

(A) DsrA sequences necessary for interaction with *hns* are circled and for interaction with *rpoS* are shown in bold.

(B) DsrA and *hns* base pairing shows the DsrA pairing region with *hns* (Lease *et al.*, 1998). The nucleotides in DsrA mutated to create DsrA*h and in *hns* to create *hns**, designed to study the role of this interaction in biofilm formation, are shown above or below the sequences.

(C) Base pairing of DsrA upstream of the *rpoS* translational start is required to positively regulate *rpoS*. The nucleotides mutated in DsrA to create DsrA*r are shown above the line.

(D) Specificity of regulation in DsrA mutants.

Beta-galactosidase assays of *hns* and *rpoS* translational fusions (HL1061 and PM1409, see Table S1) were used to confirm the specificity of pDsrA mutations. pDsrA*r should interfere with pairing to *rpoS* and pDsrA*h should disrupt pairing with *hns*. WT pDsrA and each of the mutant plasmids were introduced into the fusion strains. Overnight cultures of each strain grown in LB ampicillin media were diluted 200-fold into fresh LB medium containing IPTG (100μM), ampicillin (100μg/ml), and arabinose (0.001%), and incubated at 37°C for 6 hours. The activity of the *hns* and *rpoS* fusions were determined using the Miller assay (Miller, 1992). The error bars are a representation of the standard deviation of three independent experiments.

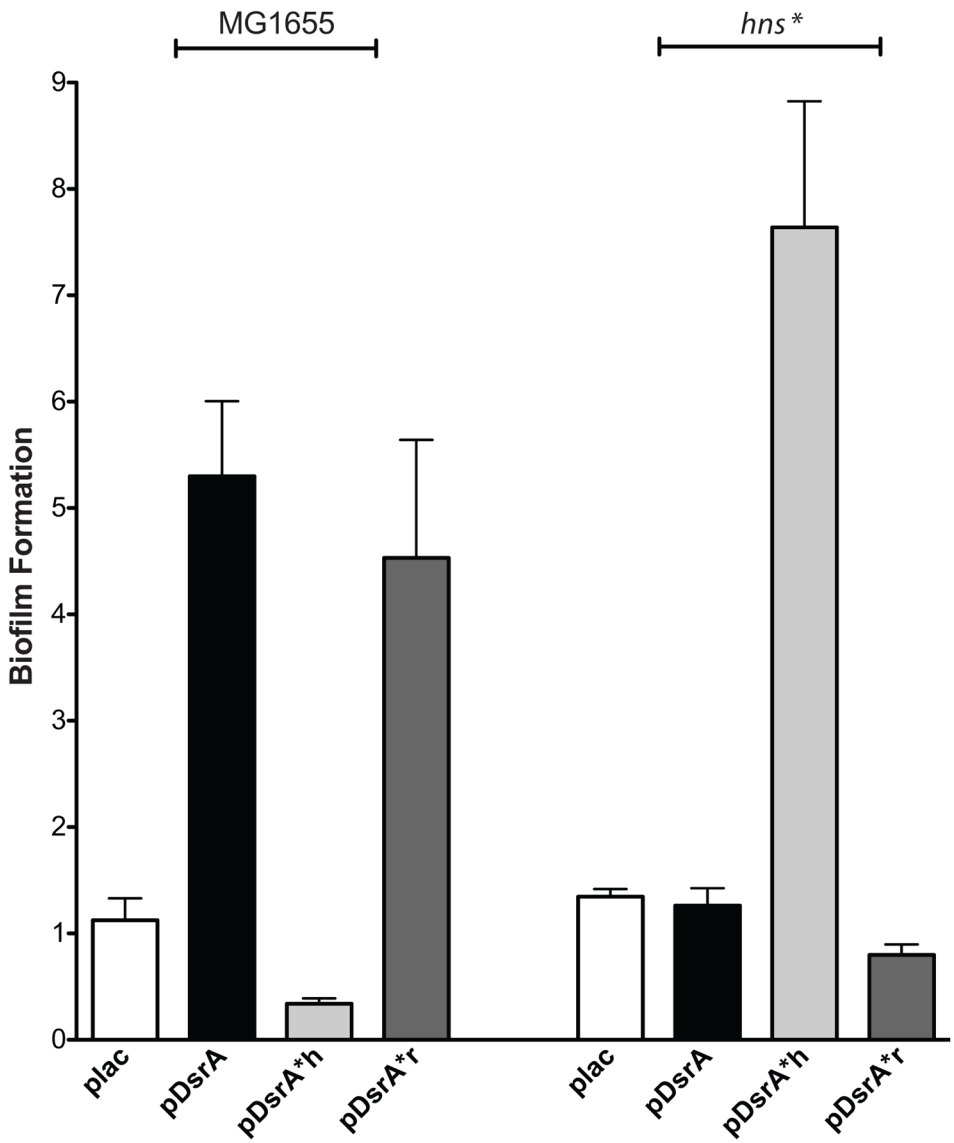

Figure 3. DsrA regulation of biofilm formation through *hns* pairing

DsrA multi-copy plasmids were used to transform MG1655 and the chromosomal *hns** derivative (SC144) to test the specificity of pDsrA*h on biofilm formation. Overnight cultures were diluted to an OD_{600} of ~0.05 in fresh LB containing ampicillin (100μg/ml) and IPTG (100μM). These strains were assayed for biofilm as measured by crystal violet staining after 24 hours incubation at 25°C. Biofilm values for each strain were normalized to the plac vector control. Error bars are a representation of the standard deviation of three independent experiments.

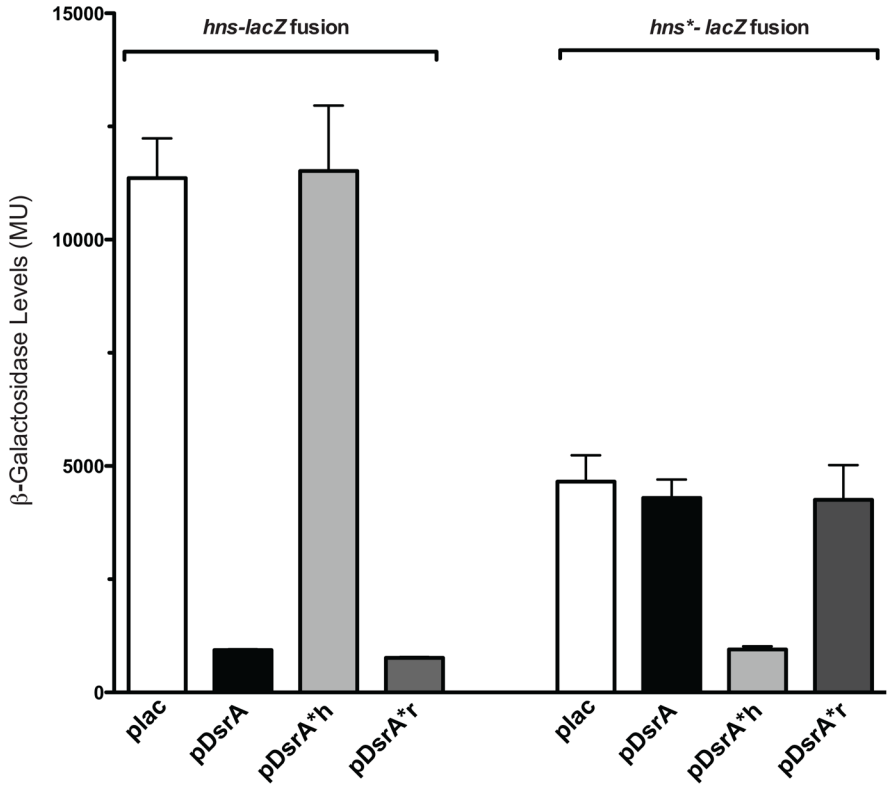

Figure 4. Multi-copy DsrA post-transcriptional regulation of *hns* by direct base- pairing

Mutations in the *hns* translational fusion (*hns**-lacZ, SC71) were tested for regulation by pDsrA, pDsrA*h, and pDsrA*r. Overnight cultures of each strain were diluted 200-fold into fresh LB medium containing IPTG (100μM), ampicillin (100μg/ml), arabinose (0.001%), and cultures were incubated at 37°C for 6 hours. The activity of the *hns* and *hns** fusions were determined as described using the Miller assay (Miller, 1992). Error bars are a representation of the standard deviation of three independent experiments.

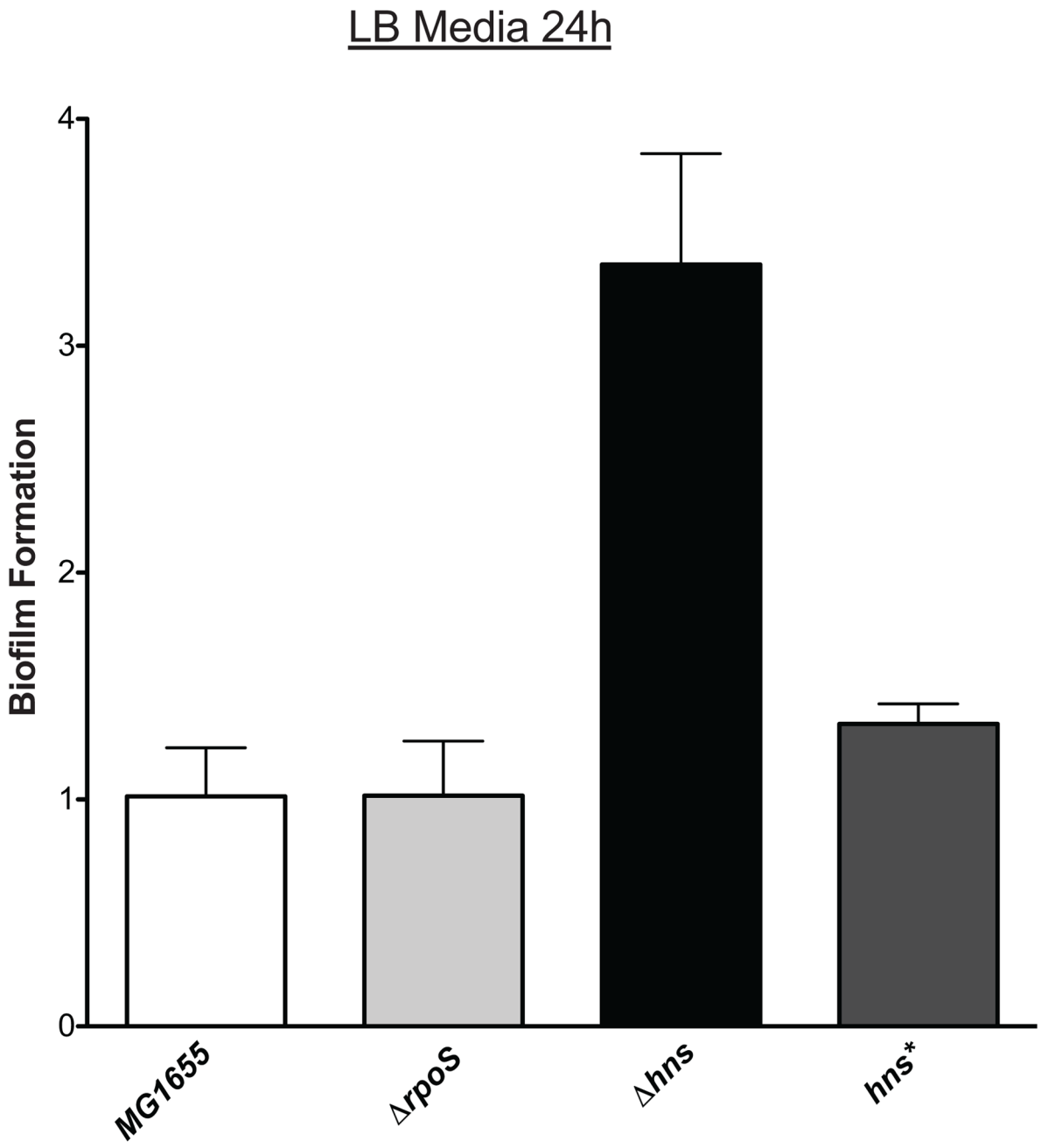

Figure 5. *hns* chromosomal deletion mutant increases biofilm formation

MG1655, an *rpoS* deletion mutant (SC124), an *hns* deletion mutant (SC110), and the *hns** mutant (SC144) were grown overnight and tested for biofilm formation in LB after 24 hours incubation at 25°C. Biofilm formation was measured as OD_{570}/OD_{600} expressed as the fold change relative to the MG1655 control. The results are an average of three independent experiments, with error bars representing standard deviation.

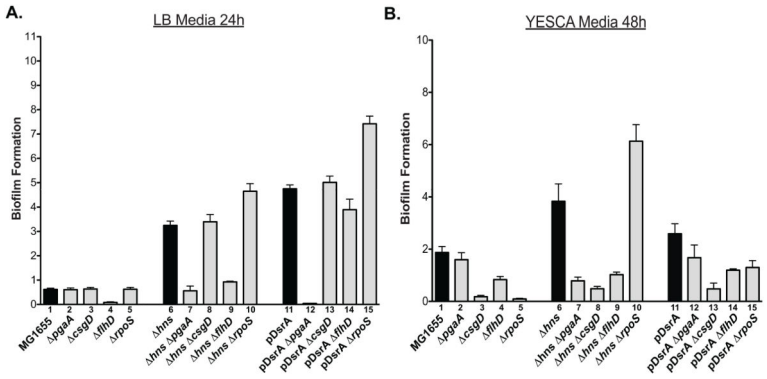

Figure 6. H-NS regulation of biofilm

Deletion mutants of known biofilm targets, either alone (SC135: Δ*pgaA*::kan; SC137: Δ*csgD*::cat; SC138: Δ*flhD*::kan; SC124: Δ*rpoS*::tet), in Δ*hns* strains (SC134: Δ*pgaA* Δ*hns*::kan; SC133: Δ*csgD*::cat *hns*::kan; SC136: Δ*flhD hns*::kan; SC122: Δ*rpoS*::tet Δ*hns*::kan) or in the presence of an induced multicopy DsrA plasmid were assayed for biofilm formation. (A) Overnight cultures were grown and diluted in LB or, for cultures containing plasmids, were diluted to an OD_{600} concentration of ~0.05 in fresh LB containing ampicillin (100μg/ml) and IPTG (100μM), incubated in microtiter wells for 24 hours at 25°C or (B) in YESCA medium with IPTG and ampicillin as appropriate for 48 hours at 25°C, and then measured for biofilm by crystal violet staining as described in Materials and Methods. Biofilm formation was determined as OD_{570}/OD_{600} and the error bars are a representation of the standard deviation of triplicate experiments.

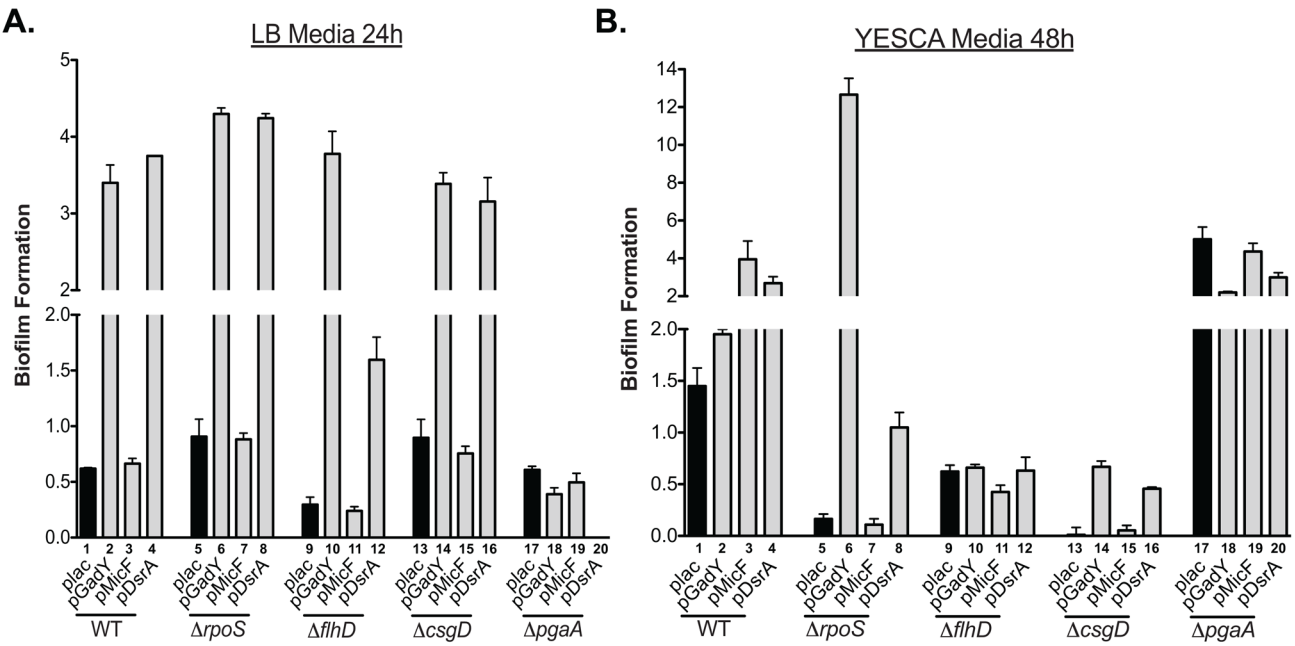

Figure 7. Alternative biofilm pathways regulated by sRNAs

Single deletion mutants of known biofilm targets (WT: MG1655; Δ*rpoS*: SC124; Δ*flhD*: SC138; Δ*csgD*: SC137; Δ*pgaA*: SC135) were transformed with the vector control plac and multicopy plasmids pGadY, pMicF, and pDsrA. Overnight cultures were diluted to an OD_{600} ~0.05 in fresh LB (A) or YESCA (B) containing ampicillin (100μg/ml) and IPTG (100μM). Biofilm assays were performed after (A) 24 hours incubation in LB or (B) 48 hours in YESCA media at 25°C. Biofilm formation was determined as OD_{570}/OD_{600} and the error bars are a representation of the standard deviation of triplicate experiments.

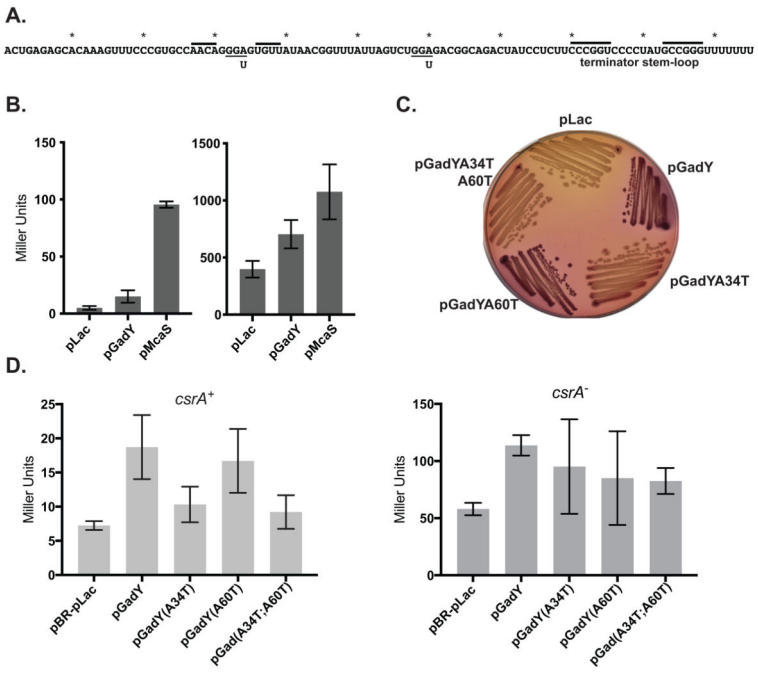

Figure 8. GadY activation of *pgaA* translation

A. Sequence of GadY with mutations of GGA sequences (underlined) tested shown below the line. The terminator stem is overlined, as is the short stem around the first GGA sequence. B. Strains carrying *pgaA-lacZ* translational fusions, each driven by a pBAD promoter, with either the full leader of 234 nt (GS0568; left panel) or truncated to a 30 nt leader (GS0641; right panel) were transformed with a vector, pGadY or pMcaS plasmids, grown to an OD_{600} ~ 0.2 in LB/amp at 37C before addition of 0.1M IPTG and 0.2% Ara; assayed in triplicate. Note the difference in the y axis for the two fusions. C. Lactose MacConkey indicator plate with NM750, carrying a *pgaA-lacZ* transcriptional and translational fusion, transformed with vector, GadY, and mutant derivatives of GadY. D. Isogenic *csrA*$^+$ (GS0644) and *csrA*::kan (GS0645) strains, each carrying the pBAD-*pgaA-lacZ* translational fusion with a full leader were transformed with vector control, pGadY, and GadY mutant plasmids, grown and assayed as in Fig. 8B. Note the different y-axis scales for these two strains.

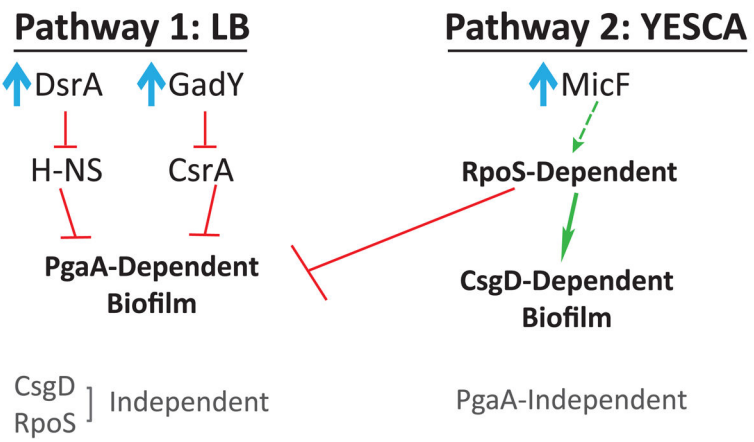

Figure 9. Alternative pathways for Biofilm formation

Two pathways for biofilm formation were defined in this paper. Pathway 1, seen here with cells grown on LB, is dependent on PgaA and independent of RpoS or CsgD. Pathway 2, seen here with cells grown on YESCA, is dependent upon RpoS and CsgD but is independent of PgaA. Increased biofilm in LB in the presence of high levels of DsrA or GadY (blue arrows) is via Pathway 1. DsrA stimulates biofilm by down-regulation of H-NS; H-NS negatively regulates *pgaA* expression. GadY stimulates translation of the *pgaA* operon by titrating CsrA, a known negative regulator of *pgaA*. Both H-NS and GadY may have other targets that contribute to Pathway 1 function expression. In YESCA media, biofilm via Pathway 2 is dependent upon RpoS and CsgD and not on PgaA. High levels of MicF increase biofilm expression using this pathway; the direct MicF targets are not yet known. High levels of DsrA or GadY are only able to significantly stimulate PgaA-dependent biofilm formation in YESCA in the absence of RpoS (see text), suggesting that RpoS or something dependent upon RpoS inhibits this pathway (red bar from RpoS to PgaA-dependent biofilm).