Multiple Small RNAs Alter Membrane Lipid Composition via Post-Transcriptional Regulation of Cyclopropane Fatty Acid Synthase

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Abstract

Microbial membranes are the first line of defense against environmental stress as well as the site of many metabolic processes. As such, maintenance of membrane integrity and homeostasis is key to cell survival. Altering membrane protein and lipid composition is an important strategy for maintaining membrane integrity in response to many environmental stresses. There are now numerous examples of small RNA (sRNA)-mediated regulation of membrane protein production, but less is known about how sRNAs regulate the types and relative proportions of different fatty acids in the membrane. The only sRNA known to regulate membrane fatty acid composition is RydC, which stabilizes *cfa* mRNA, encoding cyclopropane fatty acid (CFA) synthase, resulting in increased production of the synthase and higher levels of cyclopropane fatty acids (CFAs) in the cell membrane. Here, we report that three additional sRNAs, ArrS, CpxQ, and GadF also alter *cfa* translation and thus the amount of CFAs in cell membranes. RydC, ArrS, and GadF bind at sites that overlap a known RNase E cleavage site in the *cfa* mRNA 5'-untranslated region (UTR), resulting in increased *cfa* mRNA stability and translation. In contrast, CpxQ binds to a different site in the *cfa* mRNA 5'-UTR, and acts to reduce *cfa* translation. The physiological role of CFAs in membrane lipids is poorly understood, but CFAs have been shown to promote bacterial resistance to acid stress. We show that *cfa* translation increases in an sRNA-dependent manner when cells are subjected to mild acid stress. RydC is necessary for an increase in *cfa* translation at pH 5.0 and a *rydC* mutant is more sensitive to acid shock than the *rydC*$^+$ strain. Alteration of membrane lipid composition is a key mechanism for bacterial responses to many environmental stresses, including acid stress. This work suggests an important role for sRNAs in these responses through their regulation of *cfa* mRNA.

Introduction

Bacteria modify the biophysical properties of their membranes to adapt to changing environmental conditions, such as pH, temperature, and pressure fluctuations (1). Membrane properties can be altered by changing the type or abundance of proteins embedded in the membrane or by modifying the relative proportions of different types of phospholipids. Membrane fluidity is a crucial biophysical property as it affects membrane associated functions such as permeability to solutes, solute transport, and protein- protein interactions. Because the length and saturation of the acyl chains in the phospholipids determines the fluidity of the membrane, bacteria can adjust the ratio of saturated to unsaturated fatty acids (UFA) to adapt to their environment. For example, bacteria can increase resistance to toxic compounds, such as antimicrobial peptides produced by the immune system, by increasing the production of saturated fatty acids, which will form a tightly packed and less fluid membrane compared to a membrane rich in unsaturated fatty acids.

While *de novo* production of new fatty acids is an important adaptation mechanism, sometimes bacteria are subjected to abrupt environmental changes and require rapid modification of the fatty acids already incorporated in the membrane. One post-synthetic modification is the conversion of a pre-exisiting UFA to a cyclopropane fatty acid (CFA) by the enzyme cyclopropane fatty acid synthase (encoded by *cfa*). CFAs occur in the phospholipids of many species of bacteria and have been linked to pathogenesis (2-4). CFAs are formed by the addition of a methylene group, derived from S-adenosyl methionine, across the double bond of an UFA incorporated in a phospholipid (3, 5). Although widely studied, the physiological function of CFAs in phospholipids remains unclear. However, the conversion of UFAs to CFAs is energetically expensive, as it requires three ATP molecules to generate one S-adenosyl methionine (6). Given this energetic cost, it is expected that CFA formation, under some conditions, is advantageous. In fact, inactivation of a *cfa* homologue that introduces a cyclopropane ring in the major cell envelope component (α-mycolates) of *Mycobacterium*

tuberculosis leaves the bacteria unable to establish a persistent infection (4). One hypothesis is that the formation of CFAs may reduce membrane fluidity and permeability, thereby preventing undesirable molecules from entering the cell (3). Chang and Cronan identified the first phenotype for CFAs, which is protection from acid shock (7, 8). *Escherichia coli cfa* mutants were unable to survive a rapid shift from pH 7 to pH 3. CFAs in membranes have been shown to protect bacteria from acid shock because the conversion of UFA to CFA decreases permeability of the membrane to protons (9). CFAs are thought to be important for pathogenesis because an acid shock such as rapid shift from pH 7 to pH 3 occurs as an ingested pathogen goes through the stomach (2). In fact, it has been noted that pathogenic *E. coli* strains contain more CFAs and are more resistant to acid than nonpathogenic *E. coli* strains (8).

Production of CFAs is regulated in part by control of *cfa* transcription. In *E. coli* and *Salmonella*, the *cfa* gene has two promoters (10, 11). The distal promoter is σ^{70}-dependent and yields a longer *cfa* transcript with a 210-nt 5′-untranslated region (UTR). The proximal promoter is controlled by the general stress response σ factor (σ^{s}) encoded by *rpoS* and produces a shorter *cfa* transcript with a 33-nt 5′-UTR. The σ^{70}-dependent promoter is functional throughout growth, whereas transcription from the σ^{s} promoter occurs only during stationary phase. CFAs are produced during early stationary phase when typically all phospholipid UFAs are converted to CFAs (10).

Recently, *cfa* mRNA was also shown to be post-transcriptionally controlled by the sRNA RydC (12). RydC is 64-nucleotides (nt) long and binds to the RNA chaperone Hfq (13). The sequence of RydC is conserved in many enteric species, particularly at the 5' end, which contains nucleotides important for base pairing with *cfa* and other known target mRNAs (12). To date, RydC is known to regulate three mRNA targets, *cfa* (12), *yejABEF* (14), and *csgD* (15) mRNAs, though the conditions and signals stimulating RydC production and the physiological role of RydC in bacterial physiology remain unknown. RydC interaction with *cfa* mRNA stabilizes

cfa mRNA, and in turn increases production of cyclopropane fatty acid (CFA) synthase (12). RydC specifically base pairs in the 5'-UTR of the longer isoform of *cfa* mRNA and prevents RNase E-dependent decay of the mRNA. In *Salmonella,* RydC overexpression increased the abundance of CFA synthase about 11-fold and increased levels of CFAs in membrane lipids about 4-fold but the physiological importance of this has yet to be elucidated (12).

A recent study on global mapping of sRNA-RNA interactions using *in vivo* crosslinking and sequencing identified 3 additional sRNAs (ArrS, GadF and CpxQ) that interact with *cfa* mRNA (16). Interestingly, these sRNAs were previously linked to cell envelope stress or acid response. The sRNA CpxQ is part of the Cpx two-component system that responds to cell envelope stress (17). CpxQ is processed from the 3' UTR of *cpxP* mRNA, which encodes a chaperone of the Cpx system (17). Both the Cpx two-component system and the processing of CpxQ are induced when cell membrane potential is dissipated. Together with Hfq, CpxQ was shown to reduce translation of *nhaB* (a sodium-proton antiporter), *agp* (glucose-1-phosphatase), *fimA* (the major fimbrial subunit of type 1 pilus), and *skp* (a periplasmic chaperone) (17, 18). ArrS is an antisense sRNA that is encoded upstream of *gadE*, encoding the major acid resistance transcription factor, and is complementary to the 5'-UTR of the longest *gadE* transcript (19). ArrS expression is induced by low pH via σ^s and GadE. Overexpression of *arrS* leads to increased acid survival, compared to WT, in a GadE- dependent manner (20). GadF was identified as an sRNA located in the 3'-UTR of *gadE* mRNA (16). GadF has been detected as an sRNA via northern blot and is putatively processed from the *gadE* transcript by an RNase. To date, GadF has not been extensively studied.

Here, we sought to elucidate the mechanisms of regulation of *cfa* mRNA by these four sRNAs, RydC, ArrS, GadF, and CpxQ, and to gain insight into the physiological importance of sRNA-mediated regulation of membrane CFA composition in the model organism *E. coli.* Our results indicate that RydC, ArrS and GadF activate *cfa* translational fusions to varying degrees, but all three require a binding site in the 5'-untranslated region. RydC, ArrS and GadF all

regulate *cfa* translation in a positive manner, and all three bind at overlapping sites that correspond to a previously identified RNase E recognition site. Our results are consistent with the idea that all three activate *cfa* by a similar mechanism that was reported previously for RydC, involving protection of *cfa* mRNA from RNase E-dependent turnover. In contrast, CpxQ represses *cfa* translation by a mechanism requiring sequences upstream of the RNase E cleavage site. While the physiological role for regulation of *cfa* by each sRNA is still unclear, our data implicate RydC-mediated activation of *cfa* in protection from acid stress.

Results

Four sRNAs regulate CFA synthase mRNA in an isoform-specific manner

Previous studies have characterized two transcription start sites for the *cfa* gene, encoding cyclopropane fatty acid (CFA) synthase in *E. coli* (10). Transcription from the distal promoter is σ^{70}-dependent and yields a longer *cfa* transcript with a 212-nt 5'-UTR (Fig. 1A) that is subject to post-transcriptional regulation by sRNAs. The sRNA RydC binds to a site in the 5'-UTR and stabilizes *cfa* mRNA by inhibiting RNase E-dependent cleavage (12). A global study of sRNA-RNA interactions recently identified three additional sRNAs that bind to the 5'-UTR of the long *cfa* mRNA isoform: ArrS, GadF, and CpxQ (16). The base pairing interactions between the latter three sRNAs and *cfa* mRNA have not yet been characterized. To verify that these sRNAs alter *cfa* translation *in vivo*, we constructed two translational *cfa'-'lacZ* fusions under the control of the arabinose-inducible P_{BAD} promoter. One fusion has the full 212-nt 5'-UTR (called P_{BAD}-*cfa'-'lacZ*-Long, Fig. 1B), which contains the RydC binding site (12), while the other has only the 34-nt 5'-UTR (called P_{BAD}-*cfa'-'lacZ*-Short, Fig. 1C). Base pairing predictions from IntaRNA (22) suggested that ArrS, GadF and CpxQ would base pair in the same region of *cfa* mRNA as RydC (Fig. 1A, "RydC BS"), so we hypothesized that these sRNAs would alter the translation of the P_{BAD}-*cfa'-'lacZ*–Long but not the P_{BAD}-*cfa'-'lacZ*–Short. When the individual sRNAs were ectopically expressed from a plasmid, all sRNAs affected P_{BAD}-*cfa'-'lacZ*–Long activity (Fig. 1B)

147 and had no effect on P_{BAD}-*cfa'-'lacZ*–Short activity (Fig. 1C). RydC, ArrS, and GadF activated
148 the long fusion 18-, 10-, and 1.3-fold, respectively, while CpxQ repressed the long fusion ~3-
149 fold. These results indicate that *cfa* mRNA can be post-transcriptionally activated or repressed
150 by multiple sRNAs.
151
152 **Post-transcriptional regulation of *cfa* mRNA by sRNAs changes membrane lipid**
153 **composition**
154 CFA synthase forms CFAs by transferring a methylene group from S-adenosyl
155 methionine to the double bond of an unsaturated fatty acid (UFA) of a mature phospholipid that
156 is already incorporated in the membrane. Specifically, palmitoleic acid (C16:1) is converted into
157 methylene-hexadecanoic acid (C17CFA) and vaccenic acid (C18:1) is converted into
158 methylene-octadecanoic acid (C19CFA). To test how post-transcriptional regulation of *cfa* by
159 sRNAs alters the proportion of CFAs in membrane fatty acids, we conducted gas
160 chromatography on membrane lipids isolated from strains where each sRNA was ectopically
161 expressed (Figure 2, Table S1). The FA composition of all strains was similar except for CFA
162 and UFA content (Table S1). In strains carrying P_{lac}-*cfa,* there were reduced levels of 16:1 and
163 18:1 UFAs and ~6-fold increase in levels of C17CFA, compared to the strain carrying the vector
164 control. Similarly, ArrS-producing cells had ~5-fold higher levels of C17CFA and reduced 16:1
165 and 18:1 UFAs compared to vector control. RydC-producing cells likewise had reduced levels of
166 16:1 and 18:1 UFAs and ~8-fold increased levels of C17CFA compared to the control strain.
167 GadF production resulted in a slight increase in C17CFA (1.5-fold higher than vector control),
168 while CpxQ-producing strains had modestly reduced C17CFA (~2-fold reduced compared to
169 vector control). These data show that regulation of *cfa* mRNA translation by each sRNA is
170 correlated with changes in membrane CFA content, implying that the regulation by sRNAs could
171 contribute to meaningful changes in cell membrane structure and function.
172

Role of sRNAs in surviving acid shock

Because CFAs have been implicated in resistance to acidic pH (7, 8), we next investigated whether sRNAs promote survival after acid shock. Survival of wild-type, Δ*cfa,* Δ*rydC*, Δ*arrS,* Δ*gadF* and Δ*cpxQ* strains and complemented mutants was measured after rapid shift of cultures from pH 7 to pH 3 as described in Materials and Methods. Survival was determined by the ratio of CFUs recovered after acid shock compared to CFUs before acid shock (Fig. 3). Compared to the wild-type strain, the Δ*rydC,* and Δ*cfa* strains had lower survival rates after the acid shock. The Δ*cfa* mutant had the lowest survival rate of all the strains tested (Δ*cfa* 1.0 ± 0.3 compared to WT 4.3 ± 0.5). The Δ*rydC* strain had a survival rate intermediate between that of Δ*cfa* and wild-type (Δ*rydC,* 2.5 ± 0.4) suggesting that RydC might be important for activating *cfa* or other targets to promote resistance to acid shock. The Δ*arrS,* Δ*gadF* and Δ*cpxQ* strains showed the same survival rates as wild-type, implying that these three sRNAs do not play an important role in regulating *cfa* or other targets to promote acid shock resistance under these conditions.

Complementation of the *cfa, rydC* and *arrS* mutations by expression of the corresponding gene from a plasmid resulted in enhanced survival of acid shock compared to the wild-type strain. Enhanced acid resistance for ArrS-producing strains was observed previously and was attributed to ArrS positively regulating *gadE* expression (20). Our data suggest that ArrS-dependent activation of *cfa* might also contribute to enhanced acid resistance of ArrS-producing cells. The complemented *gadF* and *cpxQ* strains were not different from wild-type (Fig. 3). We have shown so far that ArrS, GadF, and RydC all activate *cfa* translation and increase the CFA content in cell membranes but only deletion of *rydC* renders otherwise wild-type cells more susceptible to acid shock. These results suggest that at least RydC-mediated activation of *cfa* translation promotes cell survival during an acid shock.

Small RNA-dependent regulation of *cfa* at acidic pH

To further investigate sRNA-dependent regulation during acid stress, we examined sRNA-dependent regulation of *cfa* translational fusions at neutral and acidic pH. Cells carrying P_{BAD}-*cfa'-'lacZ*-Long (Fig. 4A), P_{BAD}-*cfa'-'lacZ*-Short (Fig. 4B), or a fusion containing a deletion of the 5' region of the *cfa* 5'-UTR containing the sRNA binding sites (P_{BAD}-*cfa'-'lacZ*-ΔsRNABS, Fig. 4C) were grown in TB medium pH 7.0 to early exponential phase then subcultured into TB medium at either pH 7 or at pH 5. Samples were harvested 120 minutes later and assayed for β-galactosidase activity. For P_{BAD}-*cfa'-'lacZ*-Long, activity was higher at pH 5 compared to pH 7 (Fig. 4A). Activity of P_{BAD}-*cfa'-'lacZ*-Short was similar at both pH 5 and pH 7 (Fig. 4B). Likewise, P_{BAD}-*cfa'-'lacZ*-ΔsRNABS had similar activity at pH 5 and pH 7 (Fig. 4C). These observations indicate that activation of *cfa* mRNA in response to acidic pH occurs post-transcriptionally, and the loss of regulation of a fusion lacking the sRNA binding sites suggested that one or more sRNAs could be responsible for regulation under these conditions.

To further probe the effects of sRNA regulation of *cfa* mRNA at acidic pH, the experiment was performed in wild-type background and strains with mutations in each sRNA (Fig. 5). Activity of P_{BAD}-*cfa'-'lacZ*-Short was not affected by changes in pH or by deletion of any of the sRNAs (Fig. S1). For P_{BAD}-*cfa'-'lacZ*-Long, wild-type, Δ*arrS*, and Δ*gadF* strains all had similar activities at pH 7 and pH 5, with clearly increased activity at pH 5 (Fig. 5A). The Δ*cpxQ* mutant had higher *cfa'-'lacZ* activity at pH 7 compared to wild-type (Fig. 5A). Since ectopic expression of *cpxQ* repressed *cfa* translation (Fig. 1B), the increased levels of *cfa'-'lacZ* activity in the Δ*cpxQ* mutant suggests that at neutral pH, CpxQ is produced at sufficient levels to repress *cfa*. The Δ*cpxQ* mutation had no effect on P_{BAD}-*cfa'-'lacZ*-Long at pH 5, where levels were similar to wild-type. The Δ*rydC* mutant had lower *cfa'-'lacZ* activity at both pH 7 and pH 5 compared to wild-type, suggesting that RydC may be produced at sufficient levels at both neutral and acidic pH to have an activating effect on *cfa* translation under both conditions. We have shown that ArrS, GadF, and RydC all activate *cfa* translation and increase the CFA content in cell membranes but only deletion of *rydC* affects P_{BAD}-*cfa'-'lacZ*-Long activity at pH

5.0. This observation fits with our previous result that deletion of *rydC* renders otherwise wild-type (*cfa*$^{+}$) cells more susceptible to acid shock (Fig. 3).

To further investigate the interplay of the sRNAs on *cfa* mRNA translation at different pH values, we deleted *rydC* in combination with every other sRNA in the P_{BAD}-*cfa'-'lacZ*-Long strain (Fig. 5B) and P_{BAD}-*cfa'-'lacZ*-Short (Fig. S1B) and measured β-galactosidase activity of the reporter fusions at pH 7 and pH 5. Deletions of each sRNA in the Δ*rydC* background had no effect on P_{BAD}-*cfa'-'lacZ*-Short activity (Fig. S1B). Deletion of *arrS* or *gadF* in the Δ*rydC* background had no effect on P_{BAD}-*cfa'-'lacZ*-Long activity compared to the Δ*rydC* parent (Fig. 5B), suggesting that neither ArrS nor GadF play a role in regulation of *cfa* translation under these conditions.

Compared with the Δ*rydC* parent, the Δ*cpxQ* Δ*rydC* strain had slightly higher P_{BAD}-*cfa'-'lacZ*-Long activity at pH 7 (Fig. 5B). In contrast, at pH 5, the Δ*rydC* parent and Δ*cpxQ* Δ*rydC* strain had similar levels of P_{BAD}-*cfa'-'lacZ*-Long activity (Fig. 5B). Altogether, the data are consistent with the hypothesis that RydC exerts a positive effect on *cfa* mRNA at both neutral and acidic pH under our growth conditions. In contrast, CpxQ has a mild repressive effect on *cfa* mRNA only at neutral pH.

RpoS is not responsible for observed differences in *cfa* mRNA regulation

The σ^{s}-dependent *cfa* promoter was previously implicated in increased CFA levels when cells were grown at pH 5 compared to pH 7 (7). P_{BAD}-*cfa'-'lacZ*-Long contains region encompassing the σ^{s} dependent promoter, so it was possible that RpoS could be regulating *cfa* transcription in the context of the P_{BAD}-*cfa'-'lacZ*-Long during mild acid stress. To determine if RpoS impacts regulation of *cfa* under our conditions, we measured P_{BAD}-*cfa'-'lacZ*–Long activity at pH 5 and 7 in a Δ*rpoS* background (Fig. 6A). Deletion of *rpoS* had no effect on P_{BAD}-*cfa'-'lacZ*-Long activity at pH 5 or 7 and we observed the same increase in activity in response to pH

5 in both wild-type and Δ*rpoS* backgrounds, indicating that RpoS does not impact the observed activity of the P_{BAD}-*cfa'-'lacZ*-Long fusion.

We showed that ectopic production of each sRNA, RydC, ArrS, GadF, and CpxQ can regulate P_{BAD}-*cfa'-'lacZ*-Long (Fig. 1B). While the experiment above suggests that pH-dependent regulatory effects are not mediated by RpoS, we wanted to further test whether sRNAs could be acting indirectly on *cfa* via post-transcriptional regulation of *rpoS*. We ectopically expressed CpxQ, RydC, ArrS, or GadF in wild-type and Δ*rpoS* P_{BAD}-*cfa'-'lacZ*-Long strains and did not observe any differences in sRNA-dependent regulation between wild-type and Δ*rpoS* (Fig. 6B), indicating that the sRNA-dependent regulation is not mediated indirectly through RpoS. These data further support the idea that the regulation of *cfa* by these sRNAs is direct.

Activating and repressing sRNAs bind distinct sites on *cfa* mRNA

RydC, ArrS, GadF, and CpxQ all regulated the *cfa* translational fusion containing the *cfa* 212-nt 5'-UTR but not the *cfa* translational fusion containing only the 34-nt 5'-UTR suggesting that each sRNA base pairs within the -212 to -34 (relative to the start of translation) region of *cfa* mRNA. The mechanism of RydC regulation of *cfa* mRNA was shown to be stabilization via pairing with an RNAse E recognition site to prevent cleavage (12). To determine the mechanism of regulation by ArrS, GadF, and CpxQ, we used previously published data paired with computational methods to predict each sRNA binding site (Fig. 7A). To genetically test these base pairing predictions, we first constructed a translational fusion with a 133-nt deletion from the beginning of the 212-nt 5'-UTR, which contains predicted binding sites for all sRNAs (P_{BAD}-*cfa'-'lacZ*-ΔsRNABS, Fig. 7B), including the RNase E recognition site that RydC protects. Of note, the P_{BAD}-*cfa'-'lacZ*-ΔsRNABS fusion had a higher basal level of activity compared to P_{BAD}-*cfa'-'lacZ*-Long (Fig. 7C compared to Fig. 1B), presumably because the P_{BAD}-*cfa'-'lacZ*-

ΔsRNABS fusion lacks the known RNase E cleavage site at position 109-nt (12). When each individual sRNA was ectopically expressed from a plasmid, none of the sRNAs affected P_{BAD}-*cfa'-'lacZ*-ΔsRNABS activity (Fig. 7C), suggesting each sRNA binds at a site upstream of -79.

To test base-pairing predictions with each sRNA more specifically, we did a mutational analysis. RydC, GadF, and ArrS are all predicted to bind the same region of *cfa* mRNA (Fig. 2A, Fig. 3A-C). To investigate the importance of these interactions, we introduced three point mutations in each sRNA that were predicted to disrupt base pairing to P_{BAD}-*cfa'-'lacZ*-Long (C7G, G8C, A9U for *arrS1* (Fig. 8A), C26G, G28C, A29U for *gadF1* (Fig. 8B), C5G, G6C, A7U for *rydC*6 (Fig. 8C)). These mutated sRNA variants, *arrS1*, *gadF1* and *rydC6*, could no longer activate P_{BAD}-*cfa'-'lacZ*-Long (Fig. 8D). We made the compensatory mutations (U103A, C102G, G101C) to create P_{BAD}-*cfa'-'lacZ*-LongAGC, to restore putative base pairing interactions with the mutant sRNAs (Fig. 8A-C, E). Wild-type ArrS, GadF, and RydC could not activate the mutant P_{BAD}-*cfa'-'lacZ* -LongAGC fusion (Fig. 8E), consistent with the idea that these residues in the *cfa* 5'-UTR are critical for interactions with all three sRNAs. In contrast, all three mutants: RydC6, GadF1, and ArrS1, activated the P_{BAD}-*cfa'-'lacZ*-LongAGC fusion (Fig. 8E), with fold-activation restored to levels similar to the wild-type mRNA-sRNA pairs (Figs. 1B, 8D).

To further characterize CpxQ-mediated regulation of *cfa* mRNA, we deleted the first 22-nt of the 212-nt 5'-UTR (-212 to -190, called P_{BAD}-*cfa'-'lacZ*-ΔCpxQBS, Fig. 9A) which includes the putative CpxQ binding site (Fig. 7A). This fusion was insensitive to CpxQ, suggesting that CpxQ-dependent repression requires a site within the -212 to -190 region of *cfa* 5'-UTR. Interestingly, P_{BAD}-*cfa'-'lacZ*-ΔCpxQBS had lower basal activity compared to P_{BAD}-*cfa'-'lacZ*-Long, suggesting that the -212 to -190 region of the 5′ UTR *cfa* mRNA may play a role in *cfa* mRNA structure and stability. Based on these data, we hypothesize that ArrS and GadF regulate *cfa* by the same mechanism as RydC, stabilizing the *cfa* mRNA transcript by impairing RNAse E-mediated decay (12). CpxQ-mediated repression must occur via a different mechanism that has yet to be determined.

Discussion

Small RNAs (sRNAs) are important post-transcriptional regulators that play key roles in the response to environmental stress. We are interested in how sRNAs can contribute to membrane modification in response to stressors, particularly how sRNAs can lead to altered phospholipid composition. In the present work, we investigate the roles of multiple sRNAs in regulating the translation of *cfa* mRNA, encoding a key enzyme used to alter lipid composition of bacterial membranes. We show that the *cfa* gene can be post-transcriptionally regulated by four sRNAs, all of which act on the same long isoform of *cfa* mRNA that results from transcription from the constitutive σ^{70}-dependent promoter. The sRNAs RydC, ArrS, and GadF activate *cfa* translation resulting in increased membrane CFA levels, while the sRNA CpxQ represses *cfa* translation leading to reduced membrane CFA levels. Our data suggest that one of the physiological functions of sRNA-mediated regulation of *cfa* mRNA is protection of cells from acid stress. Loss of *rydC* made *cfa*$^{+}$ cells less resistant to acid shock (Fig. 3), which was consistent with the observation that loss of RydC reduced *cfa* translation at both neutral and acid pH (Fig. 5A,B). Acid stress phenotypes were also apparent for strains ectopically expressing ArrS (Fig. 3), which may be due to both ArrS-mediated activation of *cfa* and *gadE*.

Previous studies determined that the RydC-*cfa* mRNA base pairing prevents RNase E-mediated decay and stabilizes the mRNA to allow increased translation (12). We hypothesize, because ArrS and GadF base pair with the same region of *cfa* mRNA as RydC, that ArrS and GadF would regulate *cfa* mRNA stability via a similar mechanism. However, CpxQ must be repressing *cfa* translation by a different mechanism. CpxQ has been shown to repress other mRNA targets using one of two conserved seed regions (17). One of these seed regions matches the region of CpxQ predicted to base pair with *cfa* mRNA. CpxQ represses its other targets by base pairing near the Shine-Dalgarno sequence to prevent ribosome binding and

directly inhibit translation initiation, or by base pairing within the coding region and stimulating mRNA decay by RNase E (17). Neither of these mechanisms is analogous to regulation of *cfa* by CpxQ. We observed that when we deleted the putative CpxQ binding site from the P_{BAD}-*cfa'-'lacZ*-Long fusion, the basal level of activity of the fusion decreased. This may indicate that the CpxQ base pairing site is required for *cfa* mRNA stability, leading to increased turnover of *cfa* mRNA when the site is deleted. We propose that CpxQ base pairing with *cfa* mRNA may also stimulate mRNA turnover. The known RNase E recognition site protected by RydC far downstream of the putative CpxQ binding site (Fig. 7A). It is possible that CpxQ binding upstream promotes a structural rearrangement that makes that site more sensitive to RNase E. Future work will analyze the full structure of the *cfa* 5'-UTR in the presence and absence of CpxQ, and determine whether CpxQ-mediated regulation of *cfa* depends on RNase E.

Regulation of *cfa* mRNA by multiple sRNAs suggests that under some condition(s) these sRNAs influence membrane lipid composition and membrane integrity to promote fitness or stress resistance. Towards elucidating the physiological roles of RydC, ArrS, GadF, and CpxQ in the regulation of membrane composition, we investigated the role of each sRNA in acid stress, a condition where CFA synthase is known to play a role (7, 8). We determined that *cfa* translation was higher when cells were exposed to pH 5 compared to pH 7. When *rydC* was deleted, *cfa* translation could not reach maximum wild-type levels at pH 5 and Δ*rydC* mutants were more susceptible to acid shock compared to wild-type, giving us the first phenotype associated with RydC. However, it is not currently known if activation of *cfa* at acidic pH is the major physiological role of RydC, as the conditions and signals stimulating RydC production are not yet known.

While ectopic expression of *arrS* increased *cfa* translation and promoted higher levels of acid resistance, deletion of *arrS* did not affect *cfa* translation or acid shock survival, indicating that ArrS may not play a role acid stress under the conditions tested. ArrS has been characterized as an activator of the transcriptional activator GadE and ectopic expression of

ArrS increases acid resistance though its activation of *gadE* but a deletion phenotype has not been reported (19, 20). We propose that ArrS may activate *cfa* under another condition where CFAs in the membrane contribute to resistance, such as heat or pressure. Currently, the only know stimulus of ArrS transcription is acid (19).

Expression of GadF only slightly increased *cfa* translation and a *gadF* deletion did not affect *cfa* translation or acid shock survival, indicating that GadF also does not play a role in acid stress under the conditions tested. GadF is relatively uncharacterized but was shown to activate *acrB* 12-fold compared to the reported 2-fold activation of *cfa* (16). AcrB is an efflux pump that uses proton motive force to pump compounds, such as antibiotics, organic solvents, and detergents, out of the cell (23). GadF regulation of *cfa* mRNA may be relevant under these conditions, as CFA membrane content has been shown to promote resistance to organic solvents (24). Understanding the regulation of GadF itself would provide insight into the importance of its regulation of *cfa* mRNA, however, it is not currently known how *gadE* mRNA (from which GadF is derived) is processed or in response to what stimuli.

CpxQ is the only sRNA of the four we have characterized that represses *cfa* translation. Deletion of *cpxQ* resulted in higher *cfa* translation at pH 7 compared to wild-type, but did not affect *cfa* translation at pH 5. Responding to acid stress is likely not the relevant physiological role for CpxQ, since deletion of *cpxQ* did not affect acid shock survival. However, our observation that CpxQ represses *cfa* translation at neutral pH combined with what is known about CpxQ may provide some insight. CpxQ is part of the Cpx two-component system that responds to cell envelope stress (17, 18). Both the Cpx two-component system and the processing of CpxQ are induced when cell membrane potential is dissipated (17). CpxQ was shown to reduce translation of four inner membrane proteins including *nhaB*, a sodium-proton antiporter (17). When the cytoplasm becomes basic, NhaB can pump in $3H^+$ in exchange for $2Na^+$ out. However, if NhaB pumps in too many H^+, the inner membrane potential is dissipated and CpxQ is induced to repress *nhaB* translation (17). We predict that CpxQ would also

repress *cfa* translation under this condition because CFAs in the inner membrane are known the decrease membrane permeability to H^+ and in a situation where H+ concentration is too high in the cytoplasm, H^+ could passively cross the inner membrane to get out of the cytoplasm. Determining how and under what conditions CpxQ differentially regulates its regulon would elucidate the link between inner membrane stress and acid stress.

The UFA to CFA conversion by CFA synthase is energetically expensive, so it is not surprising that CFA synthase production is tightly controlled at the transcriptional level and at the post-transcriptional level by multiple sRNAs. Regulation of *cfa* mRNA by multiple sRNAs may have many advantages. There are a few examples of multiple sRNAs acting on the same target. Post-transcriptional control of the important transcription factor RpoS (σ^s) by multiple sRNAs to mediate responses to diverse conditions is well documented (25). The *rpoS* gene is post-transcriptionally regulated by at least four sRNAs in response to different conditions. sRNA regulation of *rpoS* highlights the potential importance of regulation of the same mRNA target by multiple sRNAs: synthesis of the sRNAs are induced by different stresses which allows RpoS in turn to properly respond to these various stress signals. The stimulus that causes synthesis of ArrS, CpxQ, GadF, and RydC would give more insight into how and why *cfa* mRNA is regulated by multiple sRNAs. It is known that transcription of *arrS* is induced by acid stress and that CpxQ is processed in response to cell membrane potential dissipation, but how that relates to their regulation of *cfa* is not understood. The regulation of *rydC* itself has not been elucidated, and it is not currently known how *gadE* is processed or in response to what stimuli.

Regulation of *cfa* mRNA by multiple sRNAs may provide an additional mechanism to deal with acid stress beyond that of RpoS and the σ^S-dependent promoter of *cfa*. The σ^{70}-dependent promoter, which yields the longer *cfa* transcript, is functional throughout growth, whereas transcription from the σ^s promoter occurs only during stationary phase (10). Thus, the *cfa* transcript from the σ^{70}-dependent promoter would potentially be in the cell available to be stabilized by RydC if the pH becomes acidic. Consistent with this hypothesis, we found that

404 transcription from the σ^{70} dependent promoter does not increase upon exposure to mild acid
405 (data not shown) but that an sRNA produced under those conditions can stabilize the already
406 present transcripts.

407 Previous work has extensively shown that sRNAs play an integral role in changing
408 membrane protein composition in response to changing environments but how sRNAs influence
409 membrane fatty acid composition is not well studied. Here, we build on the first example of an
410 sRNA altering fatty acid composition by showing regulation of *cfa* by four sRNAs. Currently, the
411 sRNAs studied here are the only examples of sRNAs altering the fatty acid composition of
412 membranes, which can consequently alter the stability and permeability of the membrane.
413

414 **Materials and Methods**

415 **Strain and plasmid construction**

416 Strains and plasmids used in this study are listed in Table S2. All strains used in this
417 study are derivatives of *E. coli* K-12 DJ624 (D. Jin, National Cancer Institute). Oligonucleotide
418 primers used in this study are listed in Table S3. Integrated DNA Technologies synthesized the
419 primers. Δ*rpoS* and Δ*cfa* mutations were made via P1 *vir* transduction from the Keio collection
420 (26). All other chromosomal mutations were made using λ Red recombination (27, 28) and
421 marked alleles were moved between strains by P1 *vir* transduction (29). Kanamycin markers
422 were removed using pcp20 (27).

423 P_{BAD}- *cfa'-'lacZ* -Long, P_{BAD}- *cfa'-'lacZ* -Short, P_{BAD}- *cfa'-'lacZ* -ΔsRNABS, and P_{BAD}- *cfa'-*
424 *'lacZ* -ΔCpxQBS translational *lacZ* reporter fusions under the control of the P_{BAD} promoter were
425 constructed by PCR amplifying the *cfa* 5'UTR fragment of interest using primers containing 5'
426 homologies to P_{BAD} and *lacZ* (Table S3). P_{BAD}- *cfa'-'lacZ* -Long and P_{BAD}- *cfa'-'lacZ* -Short were
427 made by Alisa King (King et al. 2018). P_{BAD}- *cfa'-'lacZ* -LongAGC was made using Gibson
428 Assembly (30). pBR322 was amplified using primers with 5' homologies to the *cfa* 5'UTR
429 containing the three point mutations. Gibson reaction was performed using NEBuilder HiFi DNA

Assembly Master Mix (New England Biolabs) according to the manufacture's protocol. The Gibson product was transformed into XL10 competent cells and the plasmid was purified. The mutated *cfa* 5'UTR fragment of interest was PCR amplified from this plasmid using primers containing 5' homologies to P_{BAD} and *lacZ* (Table S3). PCR products were recombined into PM1205 using λ Red homologous recombination and counter-selection against *sacB* as previously described (31).

Plasmids containing CpxQ, ArrS, RydC, GadF, Cfa under the control of the P_{LlacO} promoter were constructed by PCR amplifying each gene from *E. coli* MG1655 chromosomal DNA using oligonucleotides containing HindIII and BamHI restriction sites (Table S3). PCR products and vector pBRCS12 (32) were digested with HindIII and BamHI (New England Biolabs) restriction endonucleases. Digestion products were ligated using DNA Ligase (New England Biolabs) to produce the plasmids.

P_{LlacO}-*arrS1*, P_{LlacO}-*gadF1*, and P_{LlacO}-*rydC6* were created using QuikChange mutagenesis (Agilent Technologies) using primers in Table S3.

Media and growth conditions

Bacteria were cultured in Luria–Bertani (LB) broth medium or on LB agar plates at 37°C, unless stated otherwise. When necessary, media were supplemented with antibiotics at following concentrations: 100 μg ml^{-1} ampicillin (Amp) or 25 μg ml^{-1} kanamycin (Kan). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used at 0.1 mM for induction of expression from $P_{LlacO-1}$ promoter.

β-Galactosidase assays

Ectopic expression of sRNAs:

Bacterial strains harboring translational *lacZ* reporter fusions carrying an empty, P_{lac}- *arrS,* P_{lac}- *cpxQ,* P_{lac}- *gadF,* or P_{lac}- *rydC* plasmid were cultured overnight in Terrific Broth (TB) with Amp and 0.002% L-arabinose then subcultured 1:100 to fresh TB medium containing Amp and 0.002% L-arabinose. Strains were grown at 37°C with shaking to early exponential phase then

0.1 mM IPTG was added to induce sRNA expression. Samples were harvested 60 minutes later and β-galactosidase assays were then performed as previously described (29).

Acid:

Bacterial strains harboring translational *lacZ* reporter fusions were cultured overnight in TB medium pH 7.0 with 0.002% L-arabinose then subcultured 1:100 to fresh TB medium pH 7.0 containing 0.002% L-arabinose. Strains were grown at 37°C with shaking for 2 hours then strains were subcultured 1:100 again into TB medium with 0.002% L-arabinose at pH 7.0 or pH 5.0. Samples were harvested 120 minutes later and β-galactosidase assays were then performed as previously described (29).

Analysis of fatty acids

Cells were cultured overnight in LB containing Amp and subcultured 1:100 to fresh LB containing Amp. Cells were grown to an OD_{600} of 0.1 then plasmids were induced with 0.1M IPTG for one hour. Cells were then spun down and the cell pellet was washed twice with MilliQ water. Fatty acids were extracted according to a previously described protocol, method 3.1 (33). Pentadecanoic acid methyl ester was used as an internal standard. The bacterial sample was spiked with 500 µg of the internal standard, pentadecanoic acid, and then the sample was transesterified with 2 mL of 0.5M sodium methoxide for 1 min at room temperature. Fatty acid methyl esters (FAMEs) were then extracted using 2 mL of hexane. The solution was centrifugation at 2000 rpm for 5 min and the organic upper phase was removed and dried under nitrogen. FAMEs were treated with trimethylsilyldiazomethane in methanol to ensure complete methylation. FAMEs were identified on an Agilent 6890N GC with a 5973 MS and a Zebron WAX column (30m x 0.25mm x 0.25µm).

Acid Shock Assay

Strains were grown overnight in LB with Amp then subcultured 1:100 into LB with Amp at pH 7.0 and grown at 37°C with shaking until OD_{600}= 0.1. Plasmids were induced with 0.1mM IPTG.

When strains reached an OD_{600} of 0.2, cultures were diluted 10x into LB pH 3.0 and incubated at 37°C with no shaking for 60 minutes. Survival was determined by the ratio of CFUs on the LB plates after acid shock to CFUs on the LB plate before acid shock.

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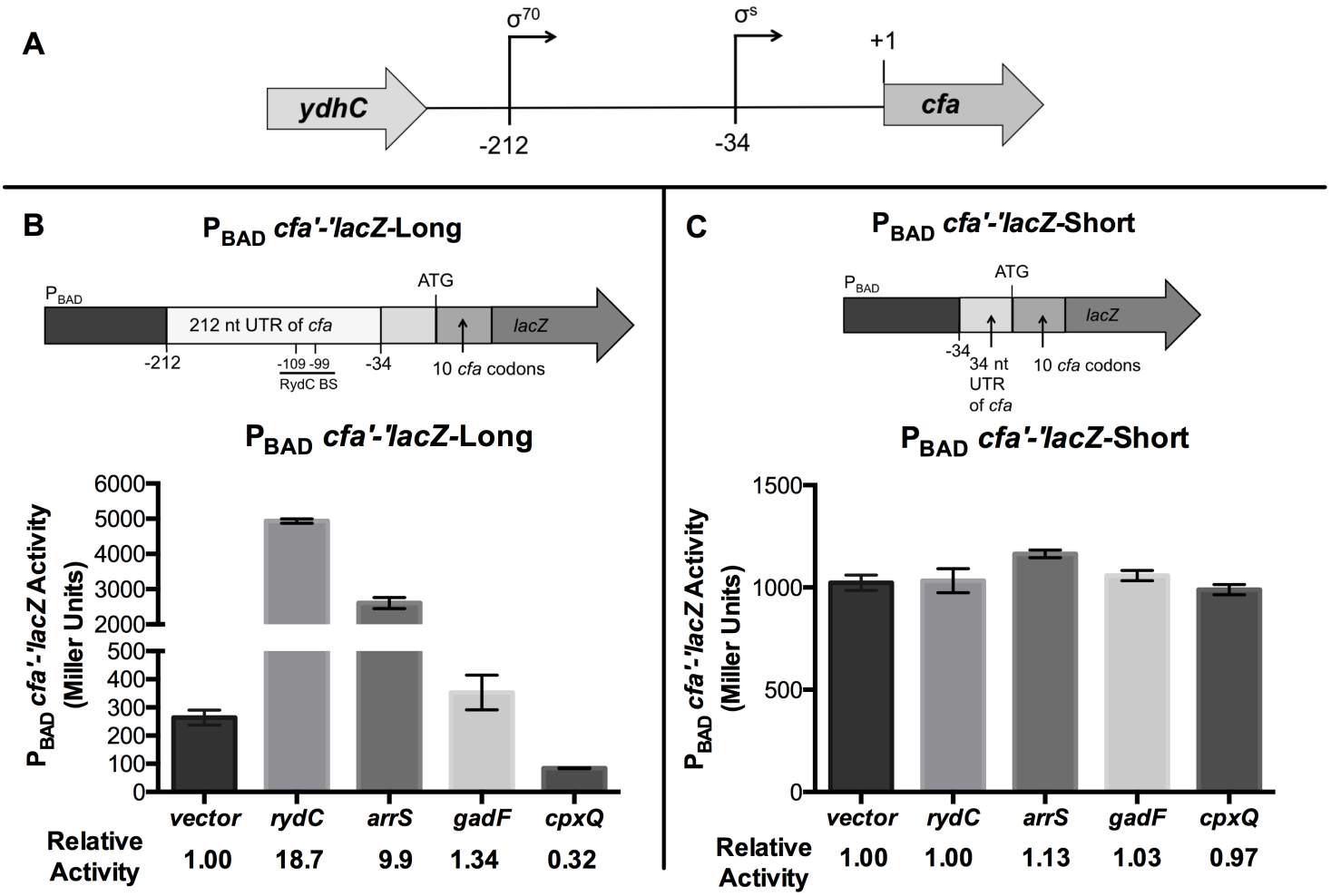


Figure 1: *cfa* translation is controlled by multiple sRNAs. (A) *cfa* has two promoters. Transcription from the distal promoter is σ^{70}-dependent and yields a longer *cfa* transcript with a 212-nt 5′ UTR. Transcription from the proximal promoter is controlled by σ^{s} and produces a shorter *cfa* transcript with a 34-nt 5′ UTR. (B-C Top) Two *cfa* translational fusions to *lacZ* (controlled by P_{BAD} promoter) were constructed. The P_{BAD}-*cfa'-'lacZ*-Long fusion (B) is from the distal σ^{70} dependent promoter which contains a 212-nt 5′ UTR that includes the RydC binding site (indicated by bar labeled "RydC BS") and the sites predicted for the ArrS, CpxQ, and GadF. The P_{BAD}-*cfa'-'lacZ* Short fusion (C) contains only proximal the σ^{s} dependent promoter and consequently not the predicted sRNA binding sites. (B-C Bottom) P_{BAD}- *cfa*'-'*lacZ*-Long (B) and P_{BAD}- *cfa*'-'*lacZ*-Short (C) carrying an empty, P_{lac}- *arrS,* P_{lac}- *cpxQ,* P_{lac}- *gadF,* or P_{lac}- *rydC* plasmid were grown in TB medium with 0.002% L-arabinose to early exponential phase then 0.1 mM IPTG was added to induce sRNA expression. Samples were harvested 60 minutes later and assayed for β-galactosidase activity of the reporter fusion. Error bars represent standard deviation for three biological replicates.

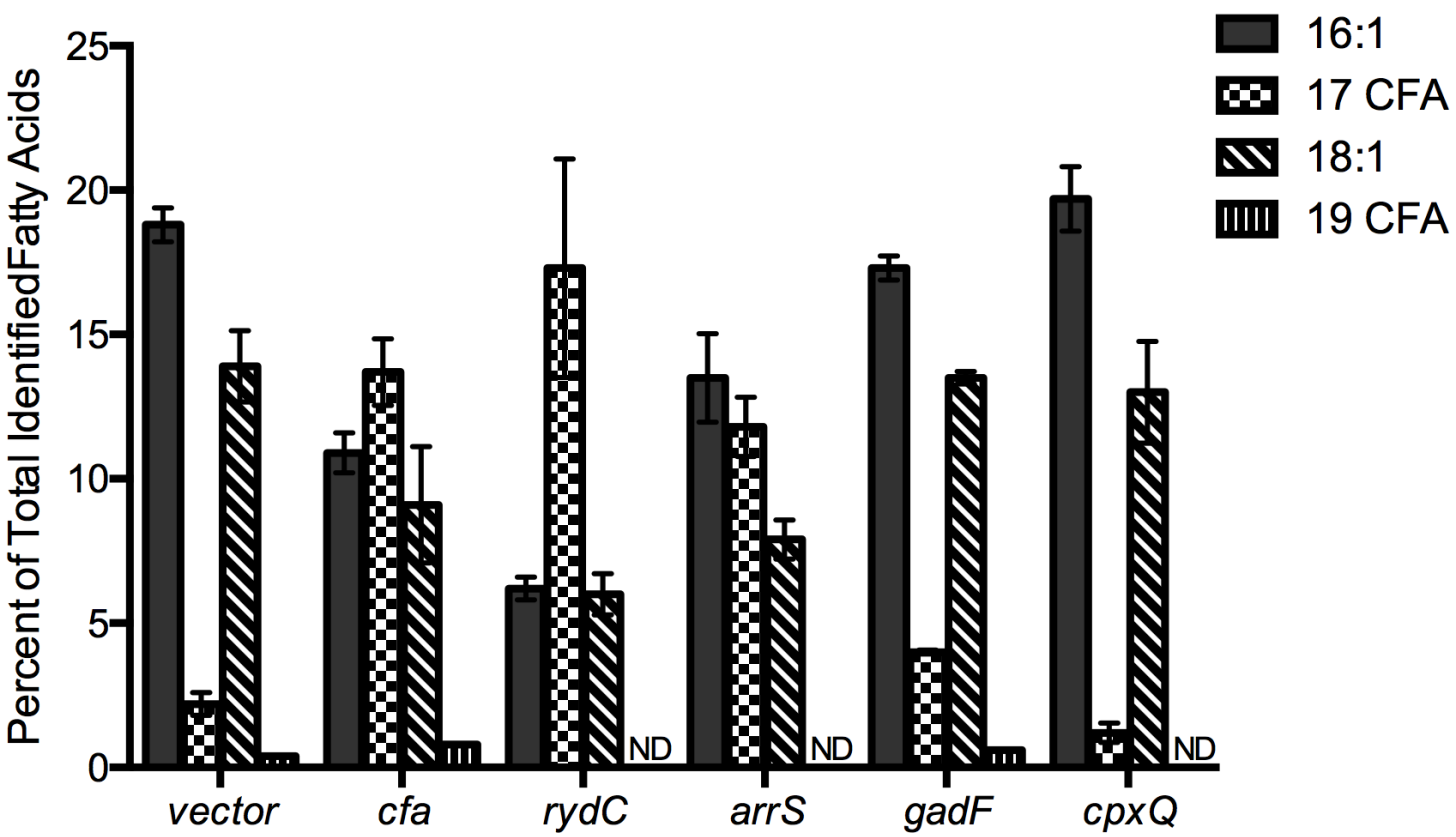


Figure 2: Expression of sRNAs can alter fatty acid composition: Relative qualification of fatty acids in *E. coli* in response to ectopic expression of vector control, *cfa*, *rydC*, *arrS*, *gadF*, or *cpxQ*. Fatty acids are presented as a percent of total identified fatty acids. Error represents average ± standard deviation, n=3 or *n=2. ND: not detected

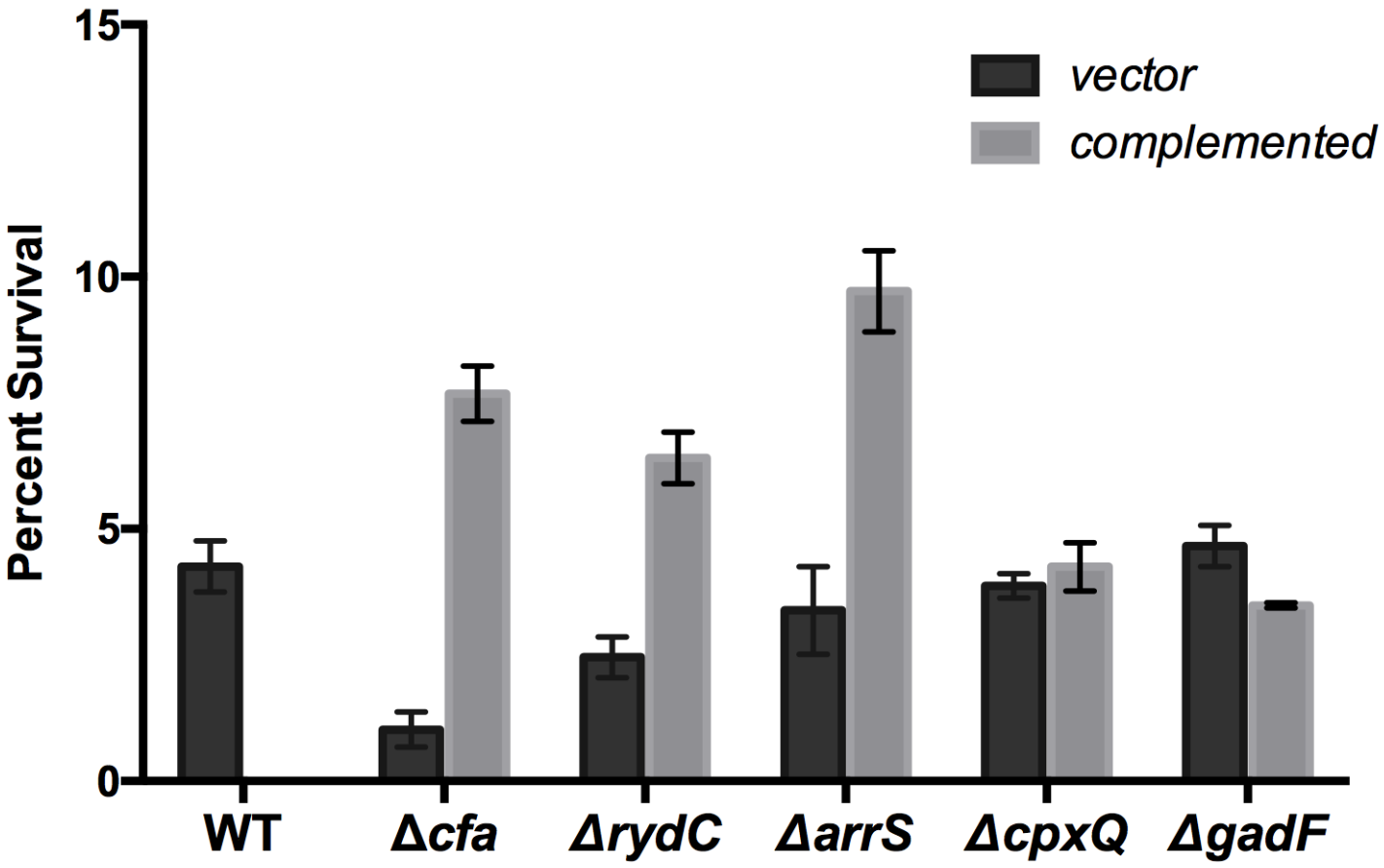


Figure 3. Survival during an acid challenge. Strains were grown overnight in LB then subcultured into LB at pH 7.0 and grown at 37°C with shaking. Plasmids were induced with 0.1mM IPTG. When strains reached an OD_{600} of 0.2, cultures were diluted 10x into LB pH 3.0 and incubated at 37°C with no shaking for 60 minutes. Survival was determined by the ratio of CFUs on the LB plates after acid shock to CFUs on the LB plate before acid shock. Error bars represent average ± standard deviation of 3 technical replicates.

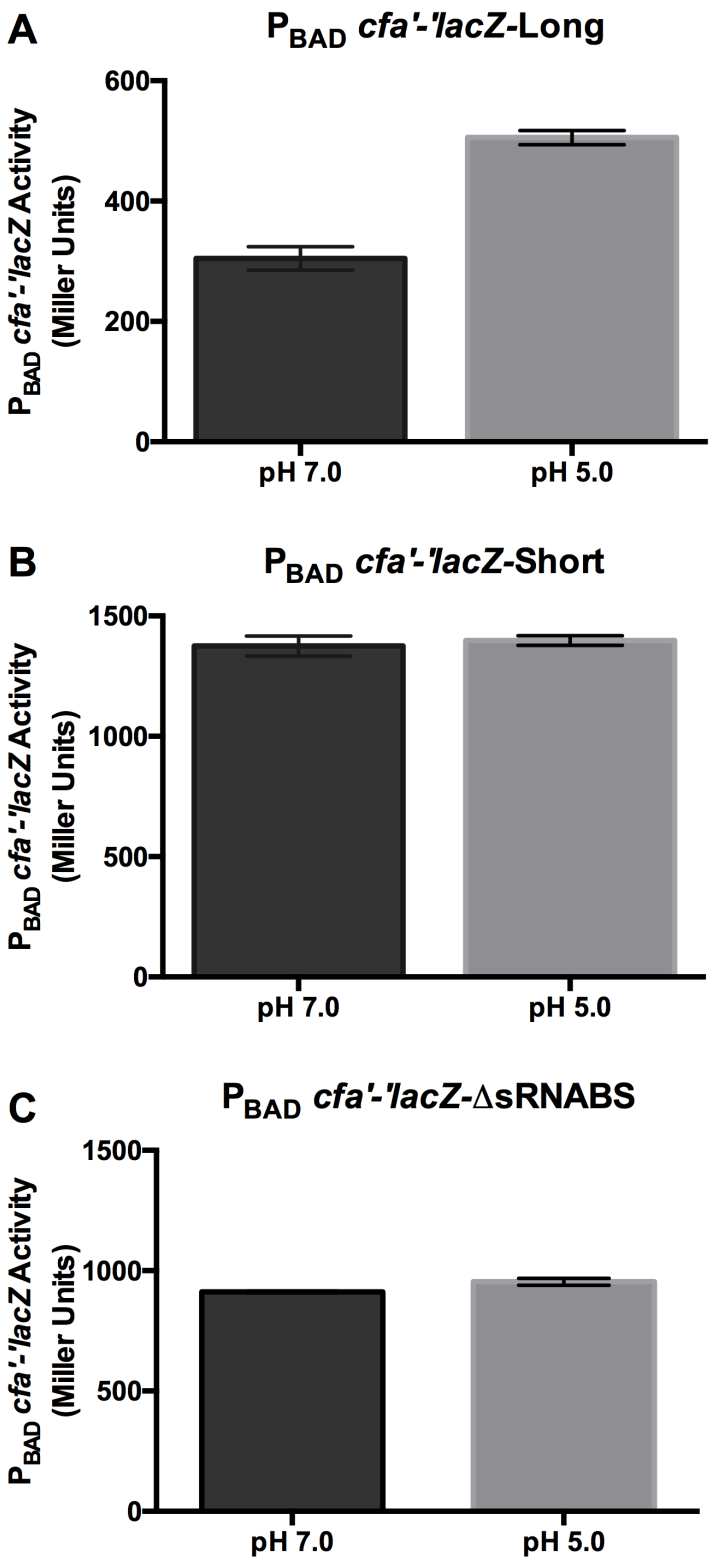


Figure 4: *cfa* translation is induced at acidic pH. Cells carrying (A) P_{BAD}- *cfa*'-'*lacZ*-Long, (B) P_{BAD}- *cfa*'-'*lacZ*-Short, or (C) P_{BAD}-*cfa'-'lacZ*-ΔsRNABS were grown in TB medium pH 7.0 with 0.002% L-arabinose to early exponential phase then cells were subcultured into TB medium with 0.002% L-arabinose at either pH 7.0 (pH 7.0) or pH 5.0 (pH 5.0). Samples were harvested 120 minutes later and assayed for β-galactosidase activity of the reporter fusion. Data was analyzed as described in Figure 1.

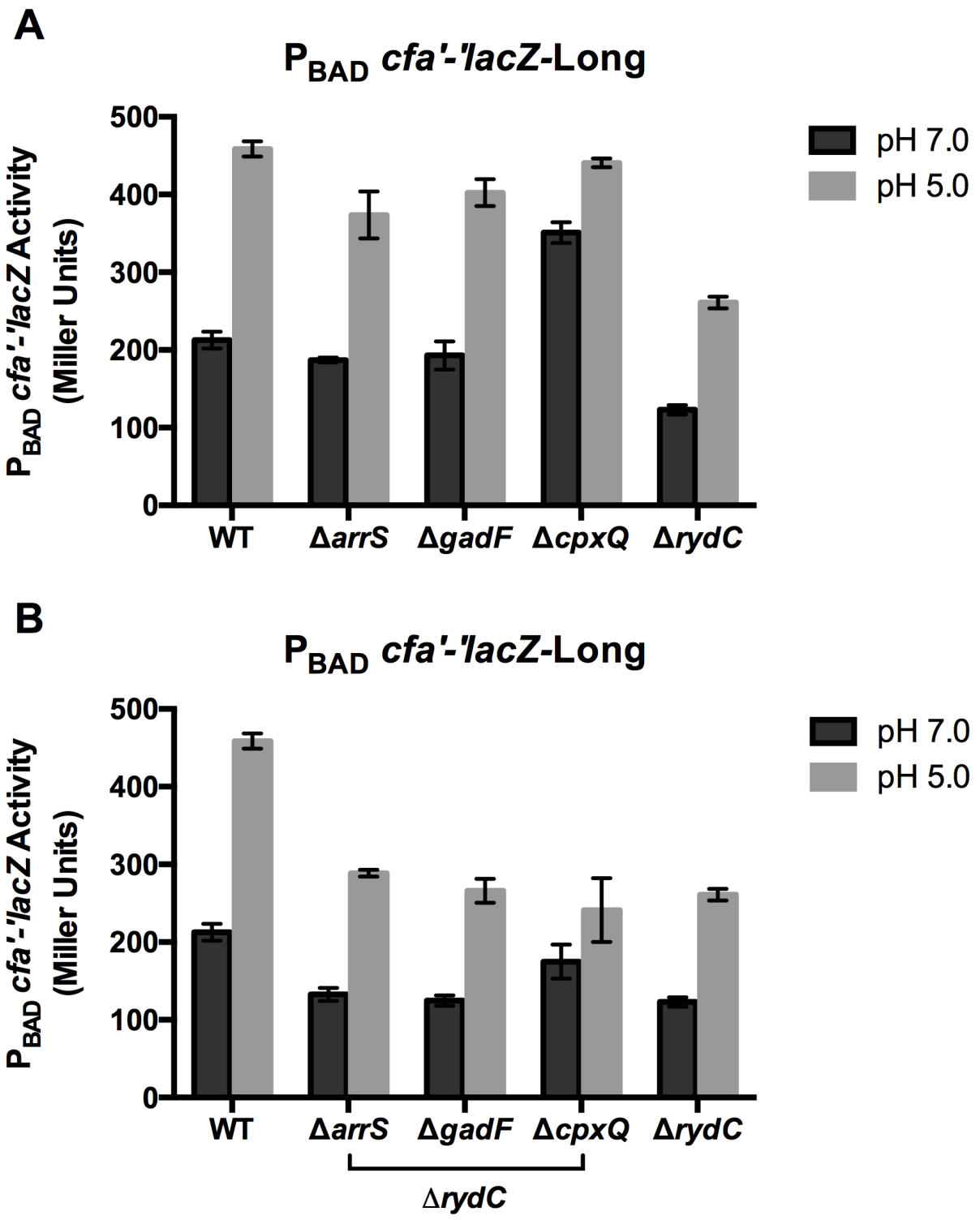


Figure 5: Deletion of sRNAs affect *cfa* translation and the response to acidic pH. (A) Cells carrying P_{BAD}- *cfa*'-'*lacZ*-Long in either WT background or a background where one sRNA is deleted were grown as described in Figure 4. (B) Cells carrying P_{BAD}- *cfa*'-'*lacZ*-Long (in either WT background or a background where *rydC* and one other sRNA are deleted) were grown as described in Figure 4. *ΔrydC* single mutant is included for reference. Error bars represent standard deviation for three biological replicates.

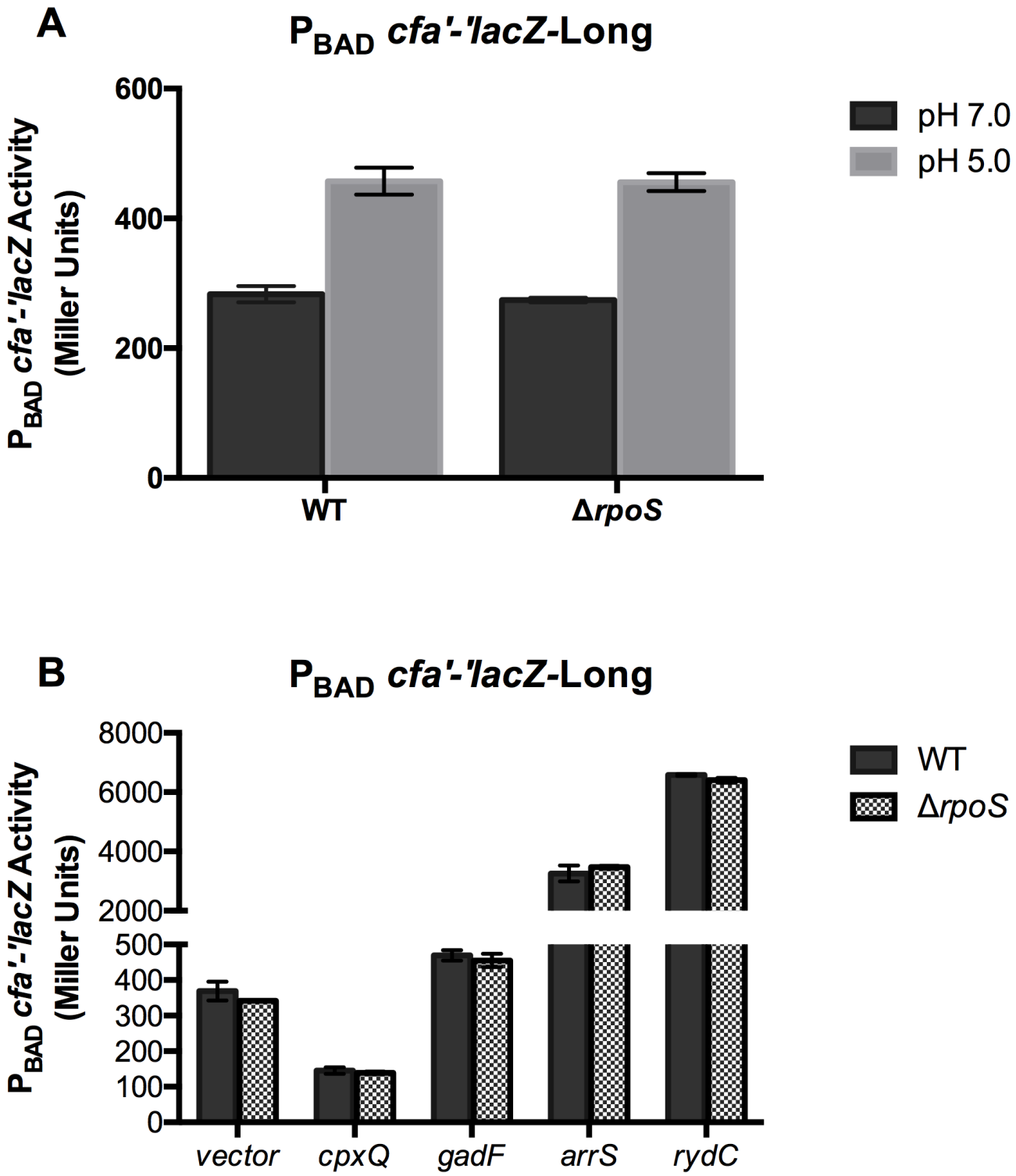
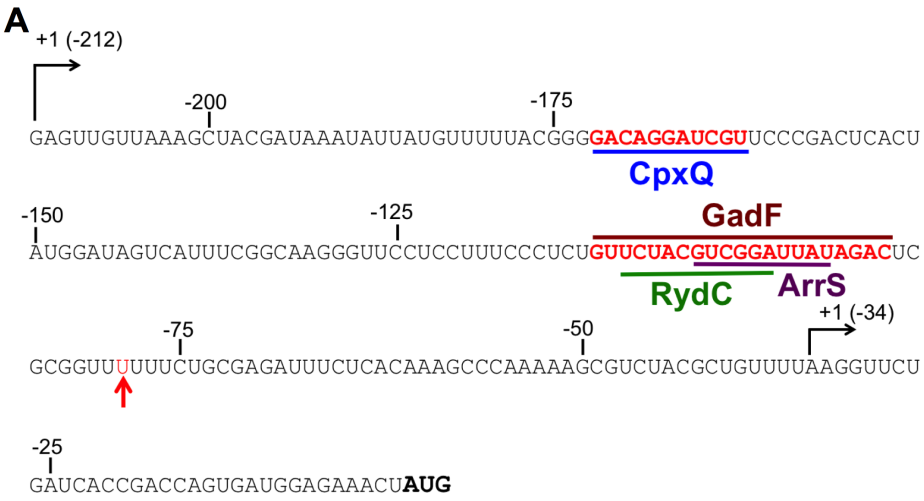
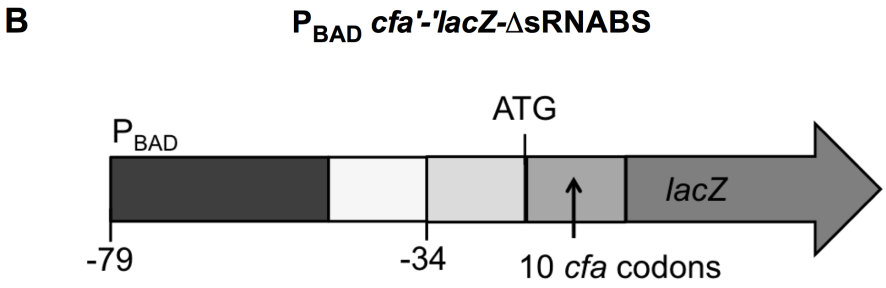


Figure 6: Deletion of *rpoS* does not affect P_{BAD}- *cfa*'-'*lacZ* -Long activity. (A) Cells carrying P_{BAD}- *cfa*'-'*lacZ*-Long in a WT or Δ*rpoS* background were grown as described in Figure 4. (B) P_{BAD}- *cfa*'-'*lacZ*-Long in a WT or Δ*rpoS* background carrying an empty, P_{lac}- *arrS,* P_{lac}- *cpxQ,* P_{lac}- *gadF,* or P_{lac}- *rydC* plasmid were grown as described in Figure 1.





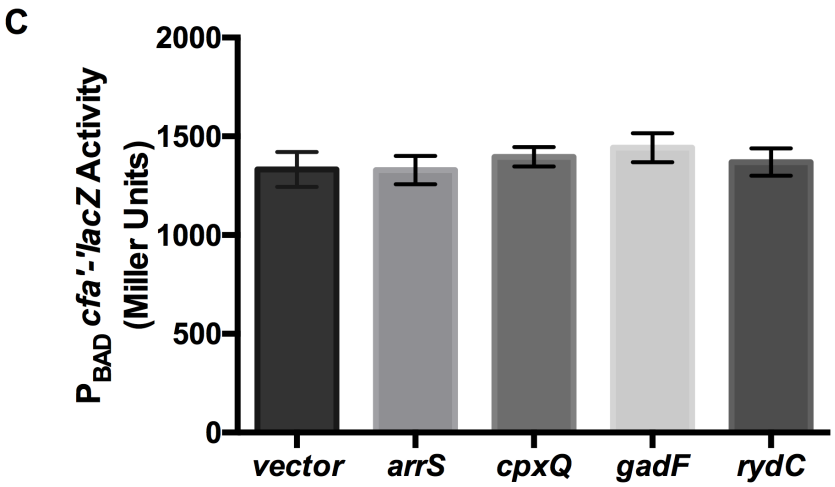


Figure 7: Deletion of the putative sRNA binding site removes regulation of *cfa* translation by ArrS, CpxQ, GadF, and RydC (A) 5′ UTR of *cfa* gene. Arrows mark transcriptional start sites, sRNA binding sites are underlined, and -79 nt is marked by a red arrow. (B) A *cfa* translational fusion to *lacZ* (P_{BAD}-*cfa'-'lacZ*-ΔsRNABS) that begins immediately downstream of the predicted sRNA binding site was constructed. This fusion contains the proximal σ^s dependent promoter and 79-nt upstream of this promoter. (B) P_{BAD}-*cfa'-'lacZ*-ΔsRNABS cells carrying an empty, P_{lac}- *arrS,* P_{lac}- *cpxQ,* P_{lac}- *gadF,* or P_{lac}-*rydC* plasmid were grown as described in Figure 1. Data was analyzed as described in Figure 1.

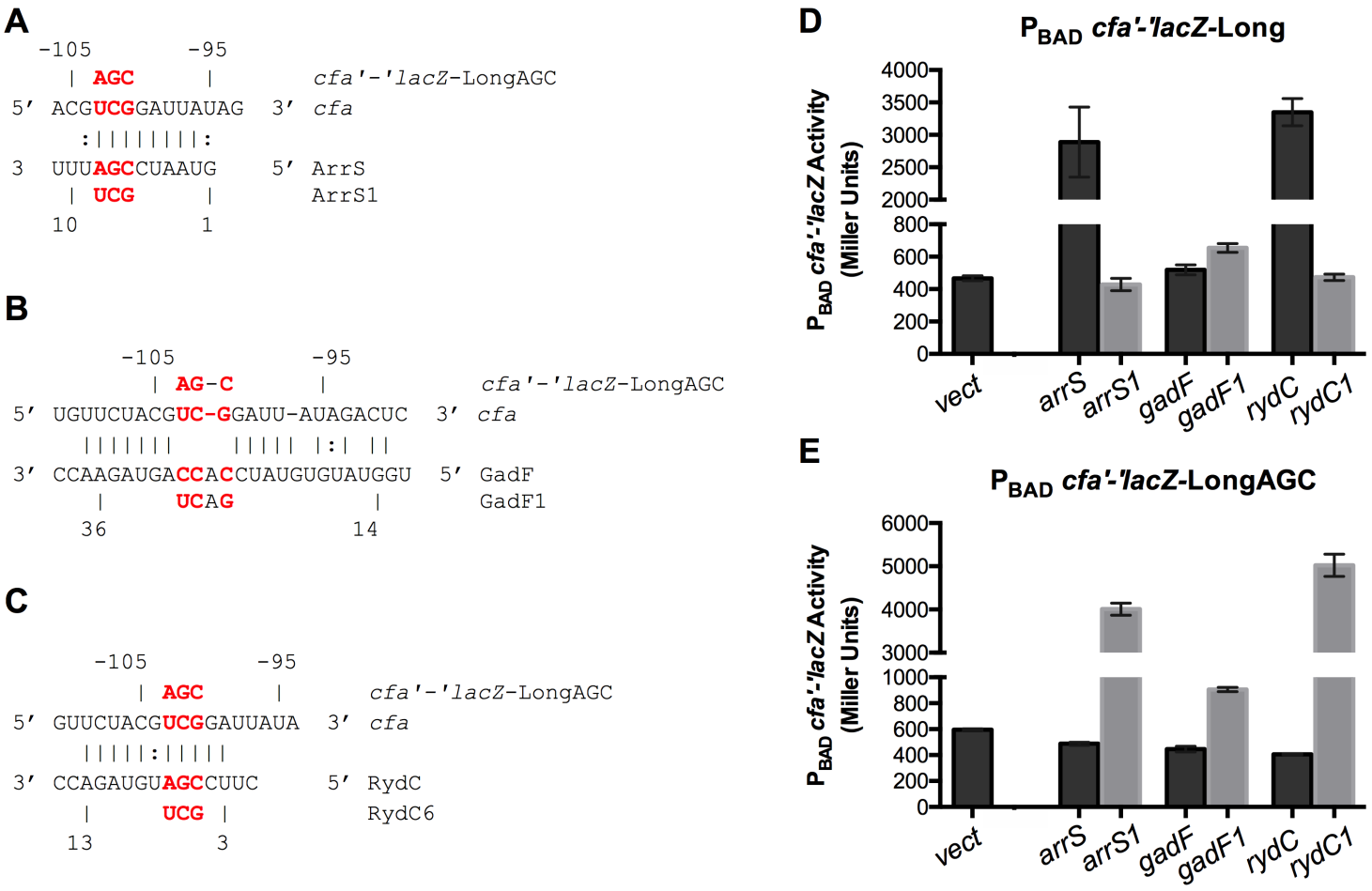


Figure 8: ArrS, GadF, and RydC base pair with the same site on *cfa* mRNA. (A-C) Predicted base pairing between *cfa* mRNA and (A) ArrS, (B) GadF, (C) RydC. The red nucleotides were mutated to test base pairing. (D) Mutant sRNAs (ArrS1, GadF1, RydC6) and WT sRNAs were tested for activation of WT P_{BAD}-*cfa'-'lacZ*-Long as described in Fig. 1B. (E) Three point mutations (U103A, C102G, G101C) were made in P_{BAD}-*cfa'-'lacZ*-Long (called P_{BAD}-*cfa'-'lacZ*-LongAGC). Mutant sRNAs (ArrS1, GadF1, RydC6) and WT sRNAs were tested for activation of P_{BAD}-*cfa'-'lacZ*-LongAGC as described in Fig. 1B.

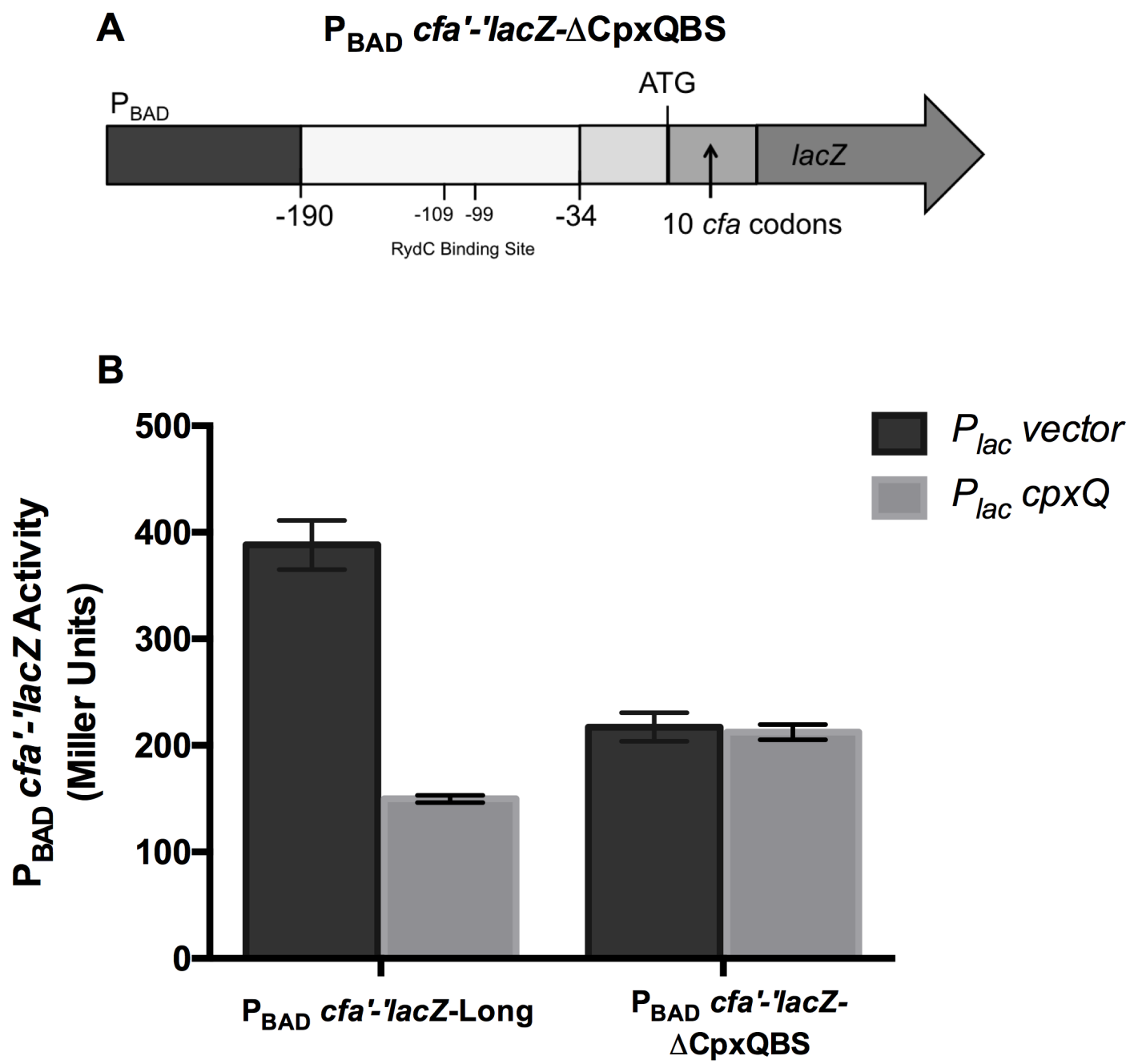


Figure 9: CpxQ cannot repress *cfa* translation when putative CpxQ binding site is deleted. (A) The last 20 nt of the -212-5′ UTR (-210 to -190) which includes the putative CpxQ binding site, was deleted in P_{BAD}-*cfa'-'lacZ*-Long fusion (called P_{BAD}-*cfa'-'lacZ*-ΔCpxQBS). (B) WT P_{BAD}-*cfa'-'lacZ*-Long or P_{BAD}-*cfa'-'lacZ*-ΔCpxQBS carrying an empty or P_{lac}- *cpxQ* plasmid were grown as described in Figure 1. Data was analyzed as described in Figure 1.

Supplemental:

Table S1: Expression of sRNAs can alter fatty acid composition: Relative qualification of fatty acids in *E. coli* in response to ectopic expression of *arrS*, *cfa*, *gadF*, *cpxQ*, *rydC* or vector control. Fatty acids are presented as a percent of total identified fatty acids. Error represents average ± standard deviation, n=3 or *n=2. ND: not detected

	p_{lac}					
Fatty Acid	*arrS*	*cfa**	*gadF**	*cpxQ*	*rydC**	*vector*
C12:0	2.5 ± 1.1	1.8 ± 0.7	1.28 ± 0.09	1.0 ± 0.1	5.2 ± 0.5	1.29 ± 0.08
C14:0	7.4 ± 1.5	6.0 ± 1.0	3.42 ± 0.05	3.1 ± 0.1	12.6 ± 3.8	3.6 ± 0.4
C14:1	ND	ND	0.3 ± 0.3	0.18 ± 0.07	ND	ND
C16:0	43.9 ± 1.1	43.3 ± 4.0	37.1 ± 0.46	30.7 ± 1.6	42.8 ± 0.3	33.6 ± 1.7
C16:0 methyl	0.8 ± 0.6	0.48 ± 0.08	1.13 ± 0.05	0.8 ± 0.2	ND	0.9 ± 0.2
C16:1	13.5 ± 1.5	10.9 ± 0.7	17.6 ± 0.3	19.9 ± 1.1	6.2 ± 0.4	18.9 ± 0.7
C17:0 CFA	11.8 ± 1.0	13.7 ± 1.2	3.99 ± 0.06	1.2 ± 0.3	17.3 ± 3.8	2.2 ± 0.4
C18:0	12.2 ± 1.6	14.4 ± 2.1	18.9 ± 0.1	25.4 ± 3.6	9.8 ± 2.0	21.7 ±0.6
C18:1	7.9 ± 0.7	9.1 ± 2.0	14.4 ± 0.5	13.8 ± 1.7	6.0 ±0.7	14.9 ± 0.9
C18:2	ND	ND	ND	ND	ND	ND
C19:0 CFA	ND	0.9 ± 0.3	0.57 ± 0.01	ND	ND	ND
C20:0	ND	ND	0.49 ± 0.08	1.1 ± 0.3	ND	0.9 ± 0.1
C22:0	ND	ND	0.33 ± 0.04	1.1 ± 0.3	ND	0.7 ± 0.2
C24:0	ND	ND	0.36 ± 0.04	1.1 ± 0.3	ND	0.8 ±0.2
C26:0	ND	ND	0.100 ± 0.008	0.6 ± 0.3	ND	0.3 ± 0.2

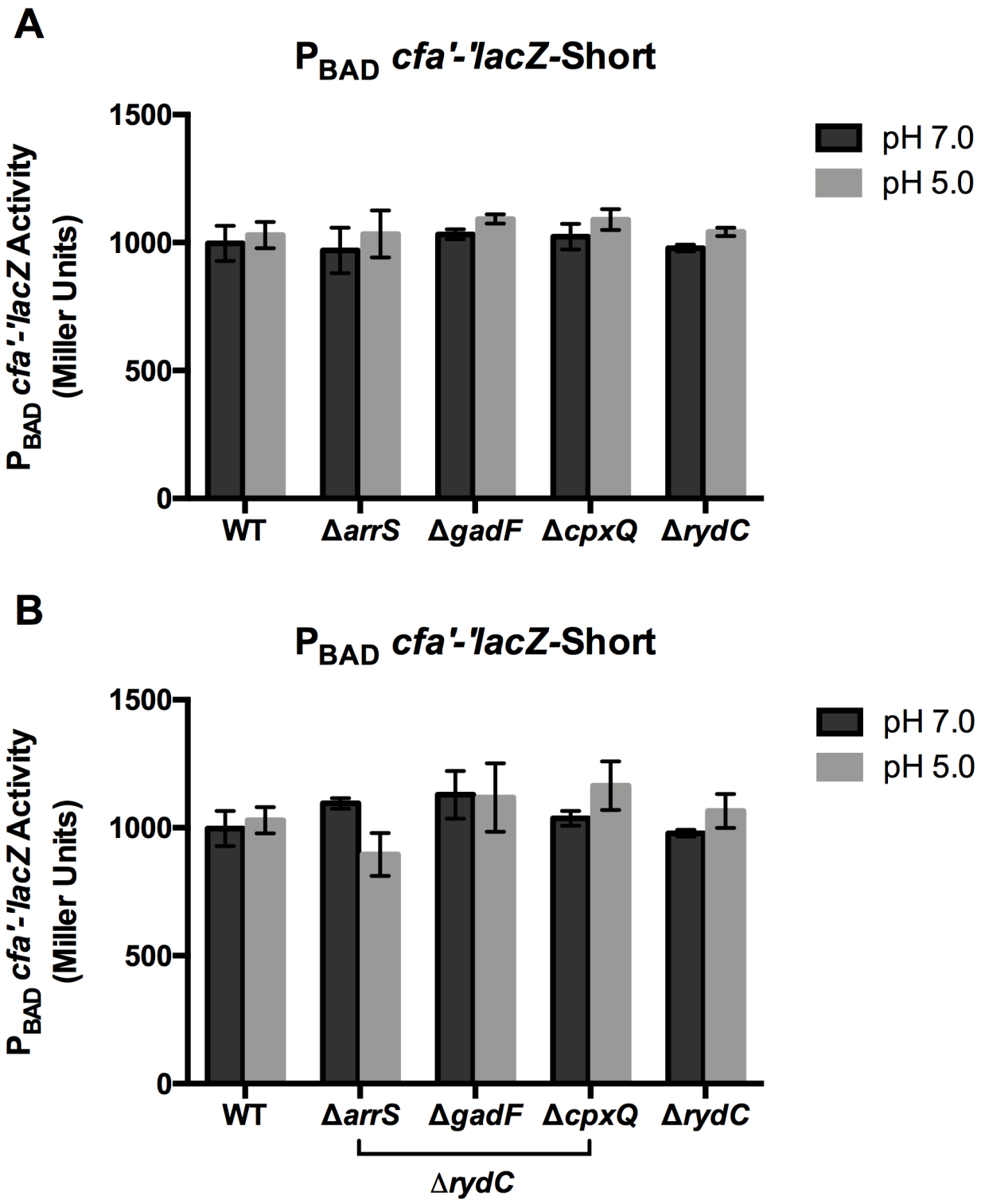


Figure S1: Deletion of sRNAs do not affect P_{BAD}- *cfa*'-'*lacZ* -Short activity. (A) Cells carrying P_{BAD}- *cfa*'-'*lacZ* -Short, as described in Figure 1C, in either a WT background or a background where one sRNA is deleted were grown as described in Figure 4. (B) Cells carrying P_{BAD}- *cfa*'-'*lacZ*-Short (in either a WT background or a background where *rydC* and one other sRNA are deleted) were grown as described in Figure 4. *ΔrydC* single mutant is included for reference.

Table S2: Plasmids and Strains used in this study

Plasmid	Vector	Genotype	Source or Reference
pBRCS12	pHDB3	vector control	Wadler *et al.*, 2009
pDK13			
pBRCB1	pBRCS12	P_{lac}-*cfa*	This study
pBRCB2	pBRCS12	P_{lac}-*arrS*	This study
pBRCB3	pBRCS12	P_{lac}-*cpxQ*	This study
pBRCB4	pBRCS12	P_{lac}-*gadF*	This study
pBRCB5	pBRCS12	P_{lac}-*rydC*	This study
pBRCB6	pBRCS12	P_{lac}-*arrS1*	This study
pBRCB7	pBRCS12	P_{lac}-*gadF1*	This study
pBRCB8	pBRCS12	P_{lac}-*rydC6*	This study

Strain	Background	Genotype	Source or Reference
DJ480	MG1655	ΔlacX74	D. Jin, NCI*
PM1205	PM1203	lacI':: P_{BAD}-*cat-sacB-lacZ*, miniλtet^R, Δ*araBAD araC*+, *mal::lacI*^{q}	Mandin et al., 2009
CB 438	DJ480	λattB::lacIq	This study
CB 546	CB 438	lacIq λatt ΔArrS plac vector	This study
CB 547	CB 438	lacIq λatt ΔArrS plac ArrS	This study
CB 548	CB 438	lacIq λatt ΔCpxQ plac vector	This study
CB 549	CB 438	lacIq λatt ΔCpxQ plac CpxQ	This study
CB 550	CB 438	lacIq λatt ΔGadF plac vector	This study
CB 551	CB 438	lacIq λatt ΔGadF plac GadF	This study
CB 552	CB 438	lacIq λatt ΔRydC plac vector	This study
CB 553	CB 438	lacIq λatt ΔRydC plac RydC	This study
CB 554	CB 438	lacIq λatt plac vector	This study
CB 567	CB 438	lacIq λatt Δcfa plac vector	This study
CB 568	CB 438	lacIq λatt Δcfa plac cfa	This study
AK 27	PM1205	P_{BAD}-*cfa'-'lacZ*-Long	This study (King et al. 2018)
AK 28	PM1205	P_{BAD}-*cfa'-'lacZ*-Short	This study (King et al. 2018)
CB 582	AK 28	P_{BAD}-cfa'-'lacZ-Short ΔcpxQ	This study
CB 583	AK 27	P_{BAD} -*cfa'-'lacZ-Long* ΔcpxQ	This study
CB 588	AK 27	P_{BAD} -*cfa'-'lacZ-Long* ΔrydC	This study
CB 589	AK 28	P_{BAD}-cfa'-'lacZ-Short ΔrydC	This study
CB 590	AK 27	P_{BAD} -*cfa'-'lacZ-Long* ΔgadF	This study
CB 591	AK 28	P_{BAD}-cfa'-'lacZ-Short ΔgadF	This study
CB 594	AK 27	P_{BAD} -*cfa'-'lacZ-Long* ΔarrS	This study
CB 595	AK 28	P_{BAD}-cfa'-'lacZ-Short ΔarrS	This study
CB 600	AK 27	P_{BAD} -*cfa'-'lacZ-Long* ΔrydC ΔcpxQ	This study
CB 601	AK 28	P_{BAD}-cfa'-'lacZ-Short ΔrydC ΔcpxQ	This study
CB 602	AK 27	P_{BAD} -*cfa'-'lacZ-Long* ΔrydC ΔgadF	This study
CB 603	AK 28	P_{BAD}-cfa'-'lacZ-Short ΔrydC ΔgadF	This study
CB 611	AK 27	P_{BAD} -*cfa'-'lacZ-Long* ΔrydC ΔarrS	This study
CB 612	AK 28	P_{BAD}-cfa'-'lacZ-Short ΔrydC ΔarrS	This study
CB 615	AK 27	P_{BAD} -*cfa'-'lacZ-Long* Δ*rpoS*	This study
CB 617	AK 27	P_{BAD} -*cfa'-'lacZ-Long* Δ*rpoS Plac-arrS*	This study
CB 618	AK 27	P_{BAD} -*cfa'-'lacZ-Long* Δ*rpoS Plac-cpxQ*	This study
CB 619	AK 27	P_{BAD} -*cfa'-'lacZ-Long* Δ*rpoS Plac-gadF*	This study
CB 620	AK 27	P_{BAD} -*cfa'-'lacZ-Long* Δ*rpoS Plac-rydC*	This study
CB 621	AK 27	P_{BAD} -*cfa'-'lacZ-Long* Δ*rpoS Plac-vector*	This study
CB 622	PM 1205	P_{BAD} -*cfa'-'lacZ-Long* Δ*sRNABS*	This study
CB 623	PM 1205	P_{BAD} -*cfa'-'lacZ-Long* Δ*sRNABS Plac-cpxQ*	This study
CB 624	PM 1205	P_{BAD} -*cfa'-'lacZ-Long* Δ*sRNABS Plac-gadF*	This study
CB 625	PM 1205	P_{BAD} -*cfa'-'lacZ-Long* Δ*sRNABS Plac-rydC*	This study
CB 626	PM 1205	P_{BAD} -*cfa'-'lacZ-Long* Δ*sRNABS Plac-vector*	This study
CB 627	PM 1205	P_{BAD} -*cfa'-'lacZ-Long* Δ*sRNABS Plac-arrS*	This study
CB 639	PM 1205	P_{BAD} -*cfa'-'lacZ-Long* Δ*CpxQBS*	This study
CB 736	PM 1205	P_{BAD} -*cfa'-'lacZ-Long*AGC	This study

*NCI, National Cancer Institute, Frederick, MD

Table S3: Oligos used in this study

Oligo	Description	Seqience 5'-3'
CB128	Forward primer for FRT-kanR-FRT casette insertion in ArrS	CCAGTTTGTGATCTCTGAAGAATATTACTAAAGTTAAAATATTCCGGGGATCCGTCGACC
CB131	Reverse primer for FRT-kanR-FRT casette insertion in ArrS	ACATGAATGCGTTATTTACTCAGGTAATTTCAATGCGTTATGTAGGCTGGAGCTGCTTCG
CB132	Forward primer for FRT-kanR-FRT casette insertion in RydC	ATTATGGTTTTATTTATCATACAAATAAATATAATAGGCGATTCCGGGGATCCGTCGACC
CB133	Reverse primer for FRT-kanR-FRT casette insertion in RydC	CTACGCATGATGCCGCGTAAACGTTCCTGAAGGATATTTATGTAGGCTGGAGCTGCTTCG
CB134	Forward primer for FRT-kanR-FRT casette insertion in GadF	ATTACCCCCGGTGATTACTAAAGGAGAGGCTAAAACGAATTCCGGGGATCCGTCGACC
CB135	Reverse primer for FRT-kanR-FRT casette insertion in GadF	GATACAGGCACAGTGATCGACATGGTGAGGTCAACGACTGTAGGCTGGAGCTGCTTCG
CB136	Forward primer for FRT-kanR-FRT casette insertion in CpxQ	GTTGAAGCTATTGAGTAGTAGCAACTCACGTTCCCAGtagATTCCGGGGATCCGTCGACC
CB137	Reverse primer for FRT-kanR-FRT casette insertion in CpxQ	GCAAATTGAGGATAAAAAAAACCCCCACAGCATGTGGGGGTGTAGGCTGGAGCTGCTTCG
CB91	Forward confirmation primer for FRT-kanR-FRT casette cloning in ArrS	GCGGATTACTGCCCAAGAATAAG
CB92	Reverse confirmation primer for FRT-kanR-FRT casette cloning in ArrS	CCGTGTCTCCAGACGCTATATA
	Forward confirmation primer for FRT-kanR-FRT casette cloning in RydC	
	Reverse confirmation primer for FRT-kanR-FRT casette cloning in RydC	
CB109	Forward confirmation primer for FRT-kanR-FRT casette cloning in GadF	AGGACATAAGCAACTGAAATTGATG
CB110	Reverse confirmation primer for FRT-kanR-FRT casette cloning in GadF	CGAGGAGAGATATGAGGGATT
CB113	Forward confirmation primer for FRT-kanR-FRT casette cloning in CpxQ	GCAATGGCAAAAAAGTTCATCG
CB114	Reverse confirmation primer for FRT-kanR-FRT casette cloning in CpxQ	GACCCGGTATACCACCAT
CB124	Forward primer for cloning *E. coli ArrS* into pBRCS12 contains Bam site	CCCCCCCCGGATCCGTAATCCGATTTAAATATCGAGTCTC
CB98	Reverse primer for cloning *E. coli ArrS* into pBRCS12 contains HindIII site	CCCCCCCCAAGCTTCGTTATTTACTCAGGTAATTTCAATGCG
CB125	Forward primer for cloning *E. coli RydC* into pBRCS12 contains Bam site	CCCCCCCCGGATCCCTTCCGATGTAGACCCGTATTCTT
CB100	Reverse primer for cloning *E. coli RydC* into pBRCS12 contains HindIII site	CCCCCCCCAAGCTTCCGCGTAAACGTTCCTGAAGGATAT
CB126	Forward primer for cloning *E. coli GadF* into pBRCS12 contains Bam site	CCCCCCCCGGATCCCTTTATTCCCCTGGTATGTGTATCC
CB102	Reverse primer for cloning *E. coli GadF* into pBRCS12 contains HindIII site	CCCCCCCCAAGCTTGATCGACATGGTGAGGTCAAC
CB127	Forward primer for cloning *E. coli CpxQ* into pBRCS12 contains Bam site	CCCCCCCCGGATCCTTTTCCTTGCCATAGACACCATCCC
CB106	Reverse primer for cloning *E. coli CpxQ* into pBRCS12 contains HindIII site	CCCCCCCCAAGCTTTGACGCTAGTATAACGGAAGC
CB175	Forward primer for cloning *cfa* 5'UTR into PM1205 to make PBAD -cfa'-'lacZ-Long ΔsRNABS	ACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATTTCTGCGAGATTTCTCACAA
CB176	Reverse primer for cloning *cfa* 5'UTR into PM1205 to make PBAD -cfa'-'lacZ-Long ΔsRNABS	TAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACCGGGGCAGAACCGTTAATGG
CB202	Forward primer for cloning *cfa* 5'UTR using Gibson to make PBAD -cfa'-'lacZ-LongAGC	AAAGCCCAAAAAGCGTCTACGCTGTTTTAAGGTTCTGATCACCGACCAGTGATGGAGAAACTATGAGTTCA
CB203	Reverse primer for cloning *cfa* 5'UTR using Gibson to make PBAD -cfa'-'lacZ-LongAGC	GTAGACGCTTTTTGGGCTTTGTGAGAAATCTCGCAGAAAAAACCGCGAGTCTATAATCGCTCGTAGAACAC
CB204	Forward confirmation primer for PBAD -cfa'-'lacZ-LongAGC	AAGCATTTATCAGGGTTATTGTCTCATGAGC
CB205	Reverse confirmation primer for PBAD -cfa'-'lacZ-LongAGC	AAACAACTGGCGGTATGGATGC
CBQ1	Forward primer for QuikChange mutagenesis of *ArrS to make ArrS1*	cgaaacaaggagactcgatatttaaaagcgattacggatccagtatcttgttatc
CBQ2	Reverse primer for QuikChange mutagenesis of *ArrS* to make *ArrS1*	gataacaagatactggatccgtaatcgcttttaaatatcgagtctccttgtttcg
CBQ3	Forward primer for QuikChange mutagenesis of *GadF to make GadF1*	caacgaagggttctactagtcgatacacataccagggg
CBQ4	Reverse primer for QuikChange mutagenesis of *GadF* to make *GadF1*	cccctggtatgtgtatcgactagtagaacccttcgttg
CBQ5	Forward primer for QuikChange mutagenesis of *RydC to make RydC6*	gcgaagaatacgggtctacaagcgaagggatccagtatcttgt
CBQ6	Reverse primer for QuikChange mutagenesis of *RydC* to make *RydC6*	acaagatactggatcccttcgcttgtagacccgtattcttcgc
	Primer for cpxQ BS	
	Primer for Ak27 and 28	