Cell PRESS

Molecular Cell
Article

The Base-Pairing RNA Spot 42 Participates in a Multioutput Feedforward Loop to Help Enact Catabolite Repression in *Escherichia coli*

Chase L. Beisel[1,*] and Gisela Storz[1,*]
[1]Cell Biology and Metabolism Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD 20892-5430, USA
*Correspondence: beiselcl@mail.nih.gov (C.L.B.), storz@helix.nih.gov (G.S.)
DOI 10.1016/j.molcel.2010.12.027

SUMMARY

Bacteria selectively consume some carbon sources over others through a regulatory mechanism termed catabolite repression. Here, we show that the base-pairing RNA Spot 42 plays a broad role in catabolite repression in *Escherichia coli* by directly repressing genes involved in central and secondary metabolism, redox balancing, and the consumption of diverse nonpreferred carbon sources. Many of the genes repressed by Spot 42 are transcriptionally activated by the global regulator CRP. Since CRP represses Spot 42, these regulators participate in a specific regulatory circuit called a multioutput feedforward loop. We found that this loop can reduce leaky expression of target genes in the presence of glucose and can maintain repression of target genes under changing nutrient conditions. Our results suggest that base-pairing RNAs in feedforward loops can help shape the steady-state levels and dynamics of gene expression.

INTRODUCTION

Bacteria sense and respond to changes in their environment through gene regulatory networks. These networks receive environmental signals and determine the strength and timing of the response. Efforts to study the structure of transcriptional regulatory networks have identified recurring patterns termed network motifs (Alon, 2007; Milo et al., 2002; Shen-Orr et al., 2002). These motifs have been implicated in signal processing and are thought to ensure an appropriate and accurate response to environmental changes.

One of the motifs prevalent in the transcriptional regulatory architecture of bacteria is the feedforward loop (reviewed in Alon, 2007). This motif is comprised of three genes (A, B, and C), where A and B coregulate C and A regulates B. When A and B coregulate multiple genes, the loop is defined as a multioutput feedforward loop. Each feedforward loop can be divided into two regulatory arms responsible for the regulation of C: a direct arm, where A regulates C, and an indirect arm, where A regulates C through B. When the regulatory arms work in opposition to activate or repress C, the loop is termed an incoherent feedforward loop. When the regulatory arms work together, the loop is termed a coherent feedforward loop. Coherent feedforward loops have been predicted to perform a number of functions, including processing multiple environmental signals and shaping the regulatory dynamics of gene expression (Kalir et al., 2005; Mangan and Alon, 2003; Mangan et al., 2003). While feedforward loops and other regulatory circuits comprised solely of transcription regulators have been studied in detail, we are only beginning to understand how regulators operating through other modes of gene regulation are incorporated into regulatory circuits (reviewed in Beisel and Storz, 2010).

A common class of posttranscriptional regulators in bacteria is the regulatory small RNAs (sRNAs) (reviewed in Waters and Storz, 2009). Bacterial sRNAs have been implicated in diverse cellular responses associated with environmental stresses, changes in nutrient conditions, and host invasion. sRNAs operate through multiple regulatory mechanisms, such as binding directly to regulatory proteins or base pairing with target mRNAs. Most base-pairing sRNAs rely on the RNA chaperone Hfq to form limited base-pairing interactions with target mRNAs. This base pairing affects mRNA stability and/or translational efficiency, resulting in either an increase or decrease in protein synthesis. While regulators and gene targets of many base-pairing sRNAs have been identified, little is known about how these sRNAs participate in regulatory networks to help cells respond to changes in the environment.

To better understand sRNA participation in gene regulatory networks, we explored how the Hfq-binding sRNA Spot 42 contributes to catabolite repression in *E. coli*. Catabolite repression is a prevalent regulatory mechanism that allows bacteria to selectively consume specific sugars in a mixture of carbon sources. Underlying catabolite repression are complex regulatory systems that sense the presence of preferred carbon sources and subsequently turn off the uptake and catabolism of nonpreferred carbon sources.

One of the central regulators in catabolite repression is the cyclic AMP (cAMP) receptor protein (CRP) (reviewed in Görke and Stülke, 2008). CRP is a transcription regulator capable of activating or repressing gene expression, and many of the genes activated by CRP are involved in the uptake and catabolism of nonpreferred carbon sources. CRP can regulate gene expression only when bound to the secondary messenger cyclic AMP. cAMP is synthesized by adenylate cyclase in response to different nutrient conditions, including the absence of glucose in *E. coli*.

Thus, when glucose is absent in the environment, cAMP activates CRP, which in turn upregulates the expression of genes involved in the consumption of nonpreferred carbon sources.

One of the many targets of CRP is the *spf* gene encoding the base-pairing sRNA Spot 42. This sRNA was originally identified over 30 years ago in *E. coli* as a cellular RNA that is highly abundant in the presence of glucose (Sahagan and Dahlberg, 1979a, 1979b). Subsequent work showed that transcription of *spf* is repressed by CRP (Polayes et al., 1988). In addition, overexpression of Spot 42 was shown to hamper growth on succinate and introduce a lag in growth following the transfer of cells from minimal glucose media to Luria-Bertani broth (LB) or to minimal glucose media containing casamino acids (Rice and Dahlberg, 1982). Most recently, Spot 42 was shown to function as an Hfq-binding sRNA that base pairs with the *galK* mRNA to mediate discoordinate expression of the *gal* operon (Møller et al., 2002a, 2002b). Although *galK* was the only known target of Spot 42, the reported growth phenotypes following Spot 42 overexpression suggested that this sRNA plays a much broader role in cellular metabolism.

In this study we report that Spot 42 regulates at least 14 additional operons involved in multiple aspects of cellular metabolism, including the uptake and catabolism of diverse nonpreferred carbon sources. Many of these operons are coregulated by CRP and Spot 42, which participate in a coherent feedforward loop. We found that, within this loop, Spot 42 can expand the dynamic range of gene regulation and influence the dynamics of the transition into and out of catabolite repression. Our results suggest that feedforward loops containing proteins and sRNAs display different regulatory properties than do feedforward loops containing only protein regulators, allowing sRNAs to fill unique regulatory roles in cells.

RESULTS

Spot 42 Represses Genes Linked to Catabolite Repression

We first carried out microarray analysis to determine whether the Spot 42 RNA acts on transcripts in addition to the *galK* mRNA. Relative mRNA levels were measured from *E. coli* cells after transient overexpression of Spot 42. These analyses revealed 16 different genes—5 singly encoded and 11 encoded in 10 operons—whose levels were consistently increased or decreased at least 2-fold relative to the empty vector control across three independent experiments (Table 1). mRNAs for some of the genes were enriched following Hfq coimmunoprecipitation in *E. coli* and *Salmonella* and showed altered levels in a *Salmonella hfq* deletion strain (Table S2) (Ansong et al., 2009; Sittka et al., 2008; Zhang et al., 2003). One of the 16 genes was the known Spot 42 target *galK*, supporting the validity of the newly identified targets. Although a previous study suggested that Spot 42 represses *galK* only at the level of translation, our microarray analysis suggests that overexpression of Spot 42 can destabilize the *galK* mRNA.

Most of the downregulated genes identified by microarray analysis are involved in metabolic processes. The gene *gltA* encodes a citrate synthase that is part of the citric acid cycle, while *maeA* encodes an NADH-dependent malate dehydrogenase involved in gluconeogenesis. Another gene, *sthA*, encodes a pyridine nucleotide transhydrogenase that uses NAD^+ to oxidize NADPH. Most of the remaining genes are involved in the uptake and catabolism of diverse carbon sources, including L-fucose (*fucI*, *fucK*), D-xylose (*xylF*), D-sorbitol (*srlA*), N-acetylneuraminic acid (*nanC*, *nanT*), and L-lactic acid (*lldP*). The utilization of many of these carbon sources is suppressed under catabolite repression (Table 1), in line with Spot 42-mediated repression of the corresponding genes in the presence of glucose. The two remaining downregulated genes, *yjiA* and *ytfJ*, are uncharacterized but may encode regulatory proteins.

One of the upregulated genes, *dppB*, encodes part of a dipeptide transporter that imports exogenous peptides as a source of amino acids, which may reduce the metabolic burden of amino acid biosynthesis. The other upregulated gene, *gsp*, encodes a glutathionylspermidine amidase/synthetase. Recent evidence suggests that glutathionylspermidine is synthesized under oxidizing conditions in *E. coli* and acts as an antioxidant, which may protect glucose-fed cells from oxidative damage during increased metabolic activity (Chiang et al., 2010).

Expression of two of the identified genes, *maeA* and *dppB*, is known to be regulated by other base-pairing sRNAs. *maeA* is repressed by the sRNA FnrS (Durand and Storz, 2010), and *dppA*, the gene upstream of *dppB* in the same operon, is repressed by the sRNA GcvB (Urbanowski et al., 2000). This observation adds to the growing number of examples of genes and operons whose expression is regulated by multiple sRNAs (see Beisel and Storz, 2010; Vogel, 2009).

To help confirm that Spot 42 affects the expression of the identified targets, we measured mRNA levels for *gltA*, *maeA*, *sthA*, and *srlA* following Spot 42 overexpression (Figure 1). To increase the mRNA levels of *srlA*, strains were supplemented with D-sorbitol, the natural inducer of the *srl* operon (Yamada and Saier, 1988). Northern blot analysis demonstrated that overexpression of Spot 42 reduced *gltA*, *maeA*, and *sthA* mRNA levels (Figure 1A). The analysis also revealed that the addition of the inducer IPTG rapidly increased *srlA* mRNA levels, which was counteracted by overexpression of Spot 42 (Figure 1B). The increase in *srlA* mRNA levels following the addition of IPTG was confirmed using four different oligo probes with a plasmid-free strain (data not shown). Overall, these results suggest that Spot 42 represses the levels of the *gltA*, *maeA*, *sthA*, and *srlA* mRNAs.

Spot 42 Base Pairs Directly with Target Genes via Three Separate Regions

As an independent assay of gene regulation by Spot 42, we constructed *lacZ* translational reporter fusions under the control of the pBAD promoter for eight of the repressed genes: *gltA*, *maeA*, *sthA*, *srlA*, *nanC*, *xylF*, *fucI*, and *fucK*. The *lacZ* fusions were constructed with regions from each gene, spanning from either the transcriptional start site (*gltA*, *maeA*, *sthA*, *srlA*, *nanC*, *xylF*) or ~100 nts upstream of the start codon (*fucI*, *fucK*) to 9–15 codons into the coding region (Mandin and Gottesman, 2009). Assays of these fusions showed that overexpression of wild-type Spot 42 reduced β-galactosidase levels for almost all of the tested fusions (Figure 2). The extent of repression varied from 2-fold for *gltA* to 47-fold for *nanC*. The one exception was the *fucK* fusion, which showed no effect of Spot 42

Table 1. Microarray Analysis of Genes Modulated at Least 2-Fold following Spot 42 Overexpression across Three Independent Experiments

Gene	Operon	Assigned Genetic Function of Regulated Gene[a]	Evidence for CRP Regulation?[b]	Evidence for Hfq Binding and Regulation?[c]	Ratio[d]
Central/Secondary Metabolism					
gltA	*gltA*	Citrate synthase	Y	Y	−3.0 ± 0.5
maeA[e]	*maeA*	Malate dehydrogenase	–	Y	−2.4 ± 0.2
Redox Balancing					
sthA	*sthA*	Pyridine nucleotide transhydrogenase	–	Y	−4.2 ± 0.5
Sugar Transport					
dppB[f]	*dppABCDF*	Subunit of dipeptide ABC transporter	–	Y	2.9 ± 0.8
lldP	*lldPRD*	Lactate transporter	–	Y	−4.1 ± 3.3
nanC	*nanCM*	N-acetylneuraminic acid outer membrane channel	Y	–	−3.5 ± 1.8
nanT	*nanATEK-yhcH*	Sialic acid MFS transporter	Y	Y	−4.3 ± 1.7
srlA	*srlAEBD-gutM- srlR-gutQ*	Glucitol/sorbitol-specific enzyme IIC	Y	Y	−12.6 ± 15.9
xylF	*xylFGHR*	Subunit of xylose ABC transporter	Y	–	−3.3 ± 0.9
Sugar Catabolism					
ebgC	*ebgAC*	Cryptic gene (evolved β-galactosidase subunit)	–	–	−2.8 ± 1.0
fucI	*fucPIKUR*	L-fucose isomerase	Y	–	−3.6 ± 1.9
fucK	*fucPIKUR*	L-fuculose kinase	Y	–	−3.2 ± 1.4
galK	*galETKM*	Galactokinase	Y	Y	−2.7 ± 0.5
Antioxidant Biosynthesis					
gsp	*gsp*	Fused glutathionylspermidine amidase and synthetase	–	Y	4.4 ± 0.5
Unknown					
yjiA	*yjiAX*	P-loop guanosine triphosphatase	–	Y	−5.0 ± 0.6
ytfJ	*ytfJ*	Predicted protein	–	Y	−5.6 ± 0.7

[a] Descriptions taken from Ecocyc (http://www.ecocyc.org).
[b] See Table S1 for a summary of the evidence for catabolite repression and CRP regulation. Y, evidence available; –, no evidence available.
[c] See Table S2 for a summary of the evidence for Hfq binding and regulation. Y, evidence available; -, no evidence available.
[d] Ratio of mRNA levels for pBRplac and pSpot42 samples. Values reflect the average and standard deviation from three independent experiments. Positive and negative values reflect induction and repression, respectively.
[e] *maeA* is directly regulated by the base-pairing sRNA FnrS (Durand and Storz, 2010).
[f] *dppA*, the gene encoded upstream of *dppB* in the same operon, is directly regulated by the base-pairing sRNA GcvB (Urbanowski et al., 2000).

overexpression. The lack of regulation suggests that Spot 42 base pairs with *fucK* outside of the region included in the *lacZ* fusion, or Spot 42 indirectly regulates *fucK* mRNA levels by targeting *fucI* in the same operon.

We used the folding algorithm NUPACK to predict whether Spot 42 can base pair directly to the genes used to construct the *lacZ* fusions (Zadeh et al., 2011). NUPACK predicted that Spot 42 base pairs to these mRNAs through three single-stranded regions found in the sRNA (I–III) (Figures 3A and 3C) (Møller et al., 2002b). In contrast, NUPACK predicted no base-pairing interactions between Spot 42 and other known sRNA targets, including *ompC*, *ompF*, *rpoS*, and *sodB*. Base pairing with the Spot 42 target genes is predicted to occur primarily near the ribosome binding site, although pairing was also predicted upstream of the ribosome binding site (*gltA*) and within the coding region (*sthA*) (Figure 3C). The one exception was *maeA*, for which no base-pairing interactions with Spot 42 were predicted. Consistent with the prediction that much of the sRNA is involved in base pairing with target mRNAs, the entire length of Spot 42 is highly conserved in enterobacteria (Møller et al., 2002b). Although most base-pairing sRNAs are predicted to act via one single-stranded region, the FnrS sRNA has been shown to regulate target genes through two single-stranded regions (Durand and Storz, 2010).

The *lacZ* translational fusions were used to investigate whether Spot 42 directly regulates the expression of identified genes

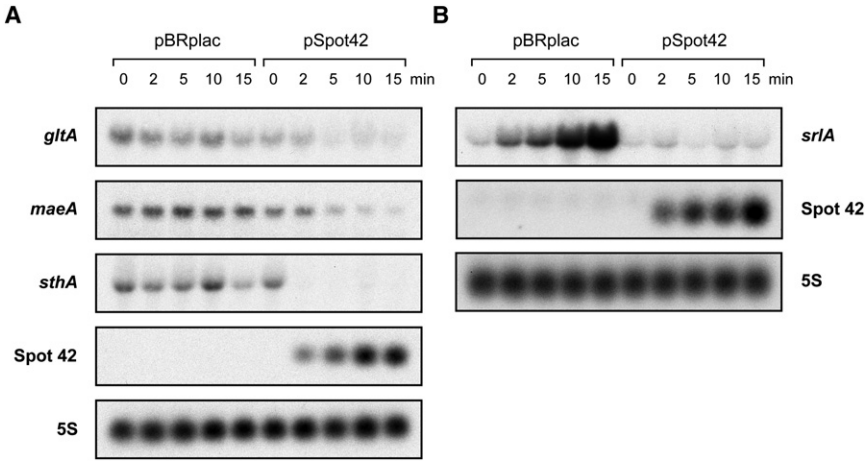

Figure 1. Repression of *gltA*, *maeA*, *sthA*, and *srlA* following Spot 42 Overexpression
(A and B) The Spot 42 gene *spf* was cloned downstream of an IPTG-inducible promoter in the plasmid pBRplac, yielding pSpot42. NM525 Δ*spf::kanR* (GSO433) cells transformed with pBRplac or pSpot42 were grown in LB (A) or LB containing 0.2% D-sorbitol (B) to an OD_{600} of ~0.3 and treated with 1 mM IPTG. After different periods of time, total RNA was isolated and subjected to northern blot analysis with probes for *gltA*, *maeA*, *sthA* (A), or *srlA* (B). 5S RNA served as a loading control. Results are representative of two independent experiments.

through the predicted regions of base pairing. We generated three variants of Spot 42, containing mutations in each single-stranded region, which we designed to disrupt the predicted base-pairing interactions (Figure 3A). Northern blot analysis showed that the size and stability of the Spot 42 variants were similar to that of the wild-type Spot 42 (Figure 3B). For almost all of the tested fusions, mutations in the single-stranded region of Spot 42 predicted to be involved in base pairing showed the greatest defect in repression by Spot 42 (Figures 2A and 3C). The mutation in region I impaired the repression of the *gltA*, *xylF*, and *nanC* fusions; the mutation in region II impaired the repression of the *srlA* and *fucI* fusions; and the mutation in region III impaired the repression of the *sthA* fusion. Note that NUPACK did not predict base pairing between *fucI* and the mutated nucleotides in region II of Spot 42, suggesting that the NUPACK predictions for *fucI* are incorrect. None of the mutations substantially impaired repression of the *maeA* fusion, suggesting that this fusion is regulated through base-pairing interactions unaffected by the tested mutations or that the effects of Spot 42 are indirect. The *maeA* and *srlA* fusions were more strongly repressed by Spot 42 variant I than by Spot 42, which may be attributed to formation of novel base-pairing interactions between this variant and each fusion as predicted by NUPACK (Figure S1). Together, these results suggest that the three single-stranded regions of Spot 42 are involved in direct base-pairing interactions with the *gltA*, *nanC*, *xylF*, *srlA*, and *sthA* mRNAs.

To validate base pairing through the three single-stranded regions of Spot 42, we made compensatory mutations in the *nanC*, *srlA*, and *sthA* fusions (Figures 3A and 3C). The compensatory mutations restored regulation by the corresponding variant of Spot 42 and disrupted regulation by the other variants (Figure 2B). The only exception was repression of the mutated *srlA* fusion by Spot 42 variant I, which also was observed for the *srlA* fusion (Figure 2A). These results confirm that three single-stranded regions of Spot 42 are involved in base pairing with target mRNAs.

Overexpression of Spot 42 Limits Growth on Specific Nonpreferred Carbon Sources

Previous studies showed that cells overexpressing Spot 42 grow slowly on succinate as the sole carbon source (Rice and Dahlberg, 1982). Our analysis identified genes regulated by Spot 42 involved in the consumption of other carbon sources, including N-acetylneuraminic acid (*nanC*, *nanT*), D-xylose (*xylF*), D-sorbitol (*srlA*), L-fucose (*fucI*), and L-lactic acid (*lldP*). We hypothesized that overexpression of Spot 42 would hamper growth on these carbon sources, as observed for succinate. To test this hypothesis, we examined growth for cells overexpressing Spot 42 in different types of media (Figure 4). Overexpression of Spot 42 in cells grown in nutrient-rich LB or in casamino acid-enriched M9 containing glycerol slightly reduced growth as cells approached stationary phase. In contrast, overexpression of Spot 42 in cells grown in casamino acid-enriched M9 containing L-fucose, D-sorbitol, D-xylose, N-acetylneuraminic acid, and L-lactic acid reduced growth as early as 2 hr after Spot 42 induction. The observed growth phenotypes were similar to the previously reported growth defect on succinate (Figure 4) (Rice and Dahlberg, 1982). Removal of casamino acids had a negligible impact on the growth defect for glycerol and strengthened the growth defect for L-fucose and D-xylose (Figure S2A). We also measured the growth defect for Δ*fucI* cells grown in L-fucose and Δ*xylF* cells grown in D-xylose. The growth defect for both deletion strains was weaker than the growth defect in Spot 42-overexpressing cells, suggesting that Spot 42 regulates multiple genes involved in L-fucose and D-xylose catabolism (Figure S2B). The additional mRNA targets and the numerous growth phenotypes suggest that Spot 42 plays a much broader role in cellular metabolism than previously realized.

CRP and Spot 42 Are Components of a Multioutput Feedforward Loop

To begin exploring how sRNAs participate in regulatory networks, we investigated how the confirmed targets of Spot 42 are regulated at the transcriptional level. We found evidence suggesting that at least 8 out of the 16 identified genes are transcriptionally activated by CRP (Table 1). The evidence includes the presence of potential CRP binding sites, in vitro binding assays, and the requirement for CRP for transcription initiation (Table S1). Integrating the fact that CRP represses the transcription of Spot 42 (Polayes et al., 1988; Sahagan and Dahlberg, 1979b), CRP and Spot 42 appear to participate in a feedforward

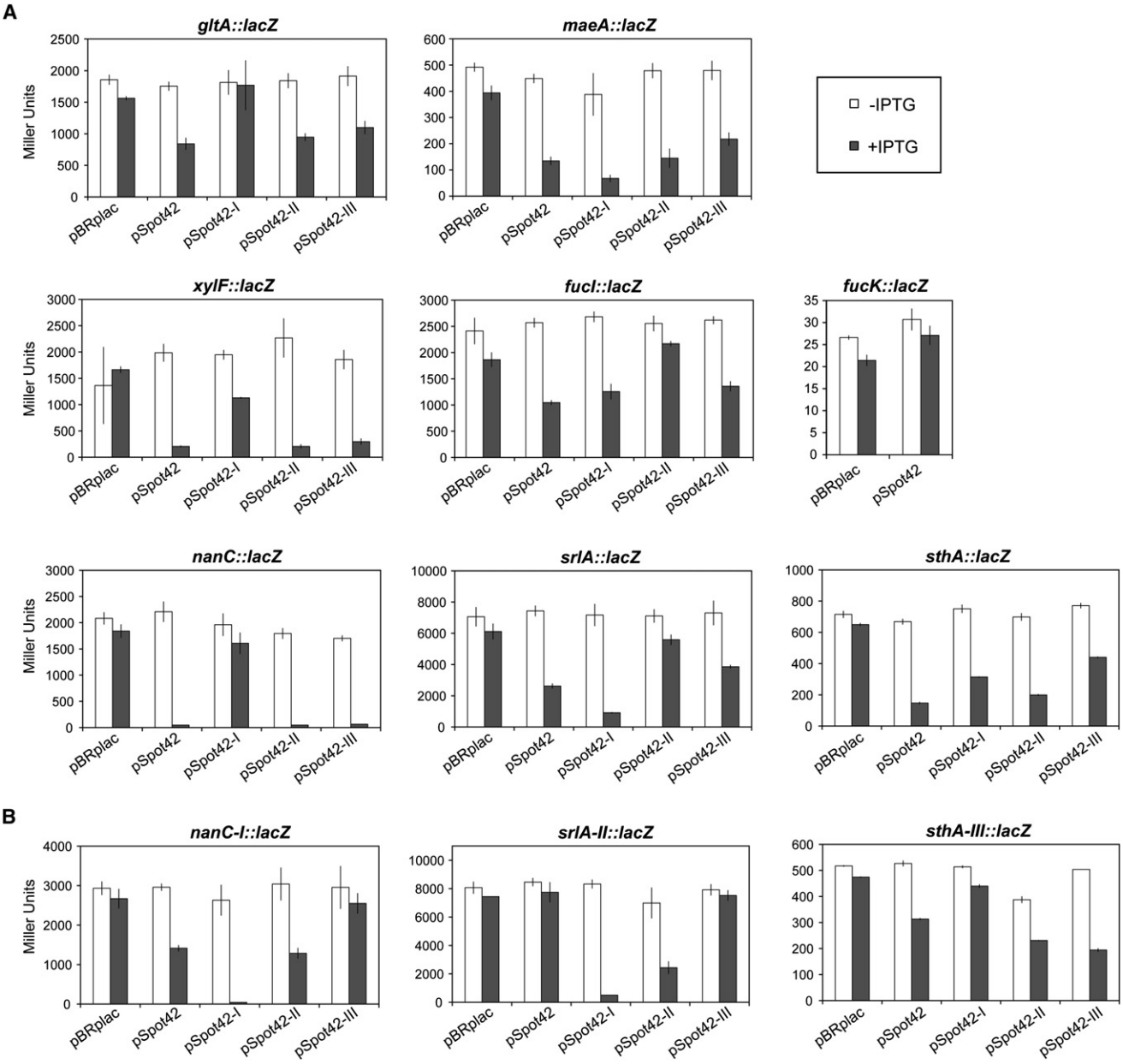

Figure 2. Mutational Analysis of Base-Pairing Interactions between Spot 42 and Target mRNAs

(A) Results for β-galactosidase assays of PM1205 Δ*spf::kanR* (GSO434) cells containing *lacZ* translational fusions.

(B) Results for β-galactosidase assays for *lacZ* translational fusions with compensatory mutations in the predicted location of base pairing with Spot 42. Derivatives of PM1205 Δ*spf::kanR* transformed with the indicated plasmid were grown in LB to an OD_{600} of ~0.1 and treated with 0.2% arabinose or 0.2% arabinose and 1 mM IPTG for 1 hr before being subjected to β-galactosidase assays. The mutations in pSpot42 variant I–III correspond to the indicated mutations in Figure 3A. The compensatory mutations in the *lacZ* translational fusions correspond to the indicated mutations in Figure 3C. The reported averages and standard deviations are from measurements of cultures from three separate colonies. See Figure S1 for predicted base-pairing interactions between Spot 42 variant I and the *srlA::lacZ* and *maeA::lacZ* fusions.

loop that regulates the expression of multiple genes—what is called a multioutput feedforward loop (Figure 5A).

To investigate the properties of the CRP-Spot 42 feedforward loop, we focused on two validated targets of Spot 42, *srlA* and *fucI*. We chose these genes because of strong evidence that both are transcriptionally activated by CRP: two CRP binding sites upstream of the *fucI* promoter have been mapped (Chen et al., 1987), and the *srlA* promoter contains a potential CRP binding site and has been shown to require CRP for transcription initiation (Yamada and Saier, 1988; Zheng et al., 2004) (Table S1). To effectively measure the protein output of this feedforward loop, we fused SPA—a composite peptide tag containing three

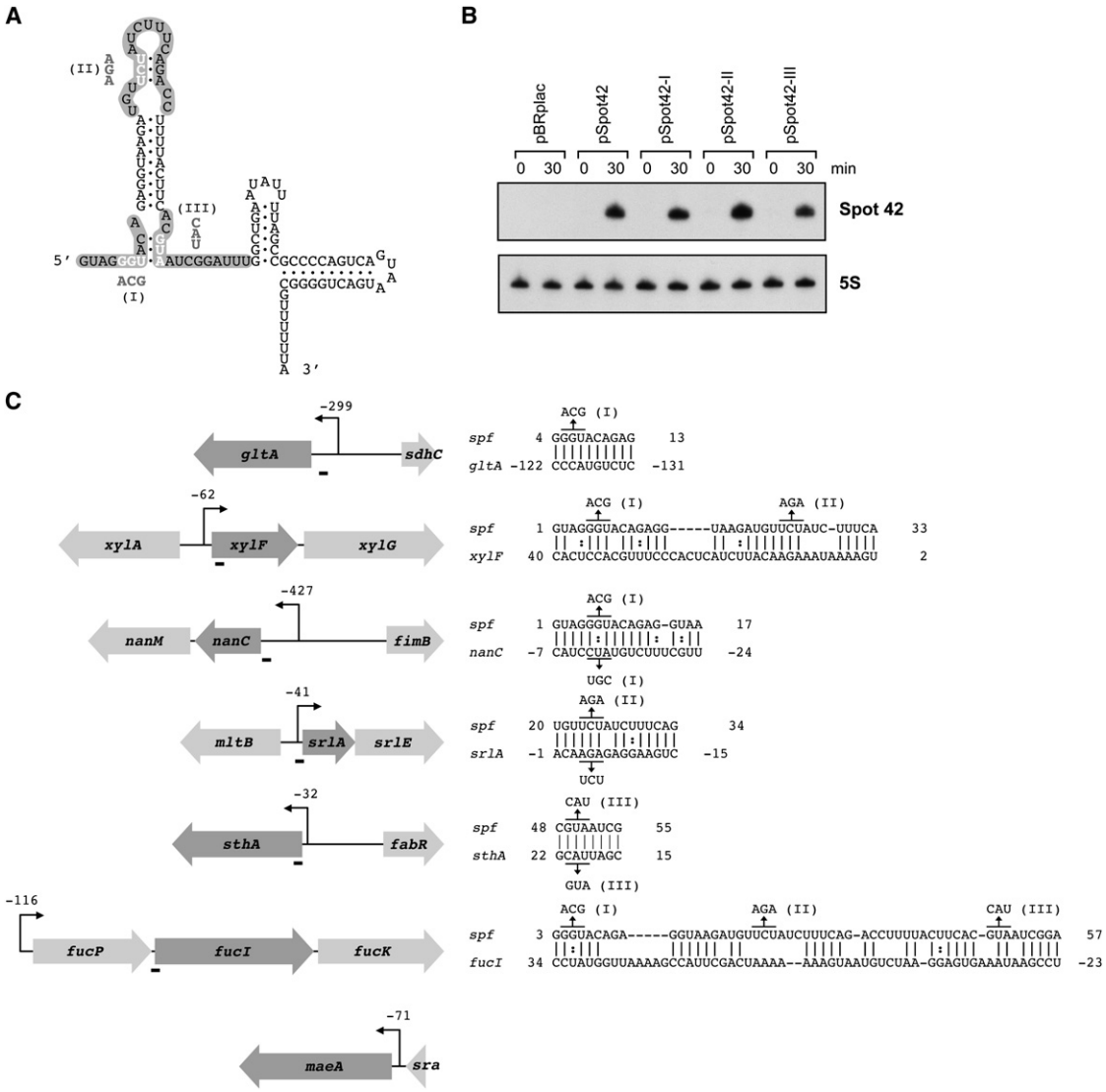

Figure 3. Predicted Base-Pairing Interactions between the Single-Stranded Regions of Spot 42 and Target mRNAs

(A) Secondary structure of Spot 42, reported previously (Møller et al., 2002b). The three single-stranded regions are highlighted in gray. Three consecutive nucleotides (white) in each single-stranded region were mutated to disrupt predicted base-pairing interactions with target mRNAs (I–III).

(B) Northern blot analysis of NM525 Δ*spf::kanR* (GSO433) cells transformed with pBRplac, pSpot42, or pSpot42 variants I–III. Cells were grown in LB to an OD_{600} of ~0.3, treated with 1 mM IPTG, and incubated for 0 min or 30 min. An equimolar concentration of probes spf.north1 and spf.north2 were used to detect all Spot 42 variants on the same membrane. 5S RNA served as a loading control.

(C) Genes identified by microarray analysis and base-pairing interactions with Spot 42 predicted by the folding algorithm NUPACK. NUPACK did not predict any significant base-pairing interactions between Spot 42 and *maeA*. Mutations introduced into pSpot42 and the *lacZ* translational fusions are designated. The bar below each target gene designates the predicted location of base pairing with Spot 42. The number above each promoter, indicated as a dark arrow, specifies the number of nucleotides between a transcriptional start site and the start codon of the first gene in the operon.

FLAG epitopes—to the 3′ end of the genomic copy of *srlA* or *fucI* (Zeghouf et al., 2004).

Spot 42 Can Reduce Leaky Expression under Catabolite Repression

We first evaluated how the CRP-Spot 42 feedforward loop regulates the expression of target genes under steady-state conditions. In addition to each wild-type strain containing the full feedforward loop (WT), two mutant strains were generated with reduced loops: one reduced to direct regulation by CRP through deletion of *spf* (Δ*spf*) and another with the entire loop disrupted through deletion of both *spf* and *crp* (Δ*spf* Δ*crp*) (Figure 5A). Strains were grown in LB to activate CRP or in LB and glucose to deactivate CRP since the Δ*crp* strains showed no growth in casamino acid-enriched M9 in the absence of glucose. Quantitative western blot analysis then was performed to measure the

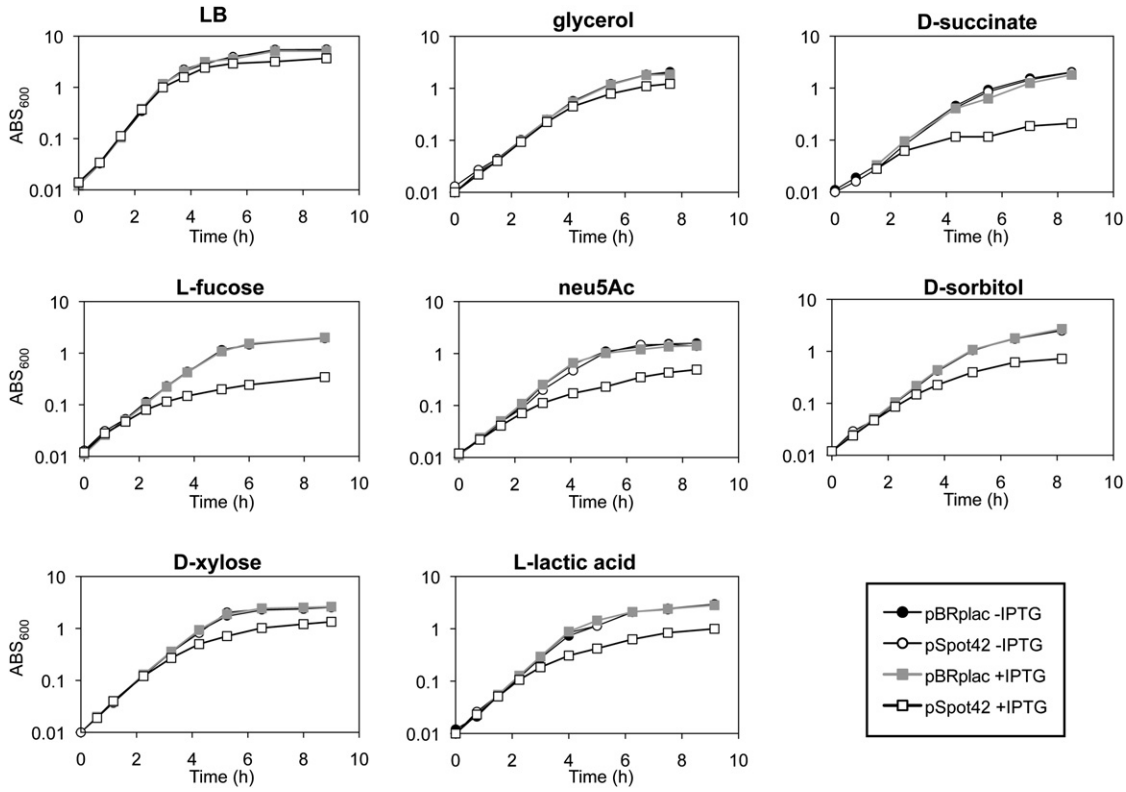

Figure 4. Limited Growth on Nonpreferred Carbon Sources following Spot 42 Overexpression

NM525 Δ*spf::kanR* (GSO433) cells transformed with pBRplac or pSpot42 were grown overnight in LB or in casamino acid-enriched M9 containing the indicated carbon source and diluted to an OD_{600} of 0.01 into the same type of media with or without 1 mM IPTG. OD_{600} was measured at different times during cell growth. Applied concentration of specific carbon sources: 0.4% glycerol, 0.8% sodium succinate hexahydrate, 0.2% L-fucose, 2 mM N-acetylneuraminic acid (neu5Ac), 0.2% D-sorbitol, 0.2% D-xylose, and 60 mM L-lactic acid. Results are representative of two independent experiments. See Figure S2 for growth data for extended time periods, for media lacking casamino acids, and for strains containing a deletion of Spot 42 target genes.

relative levels of SrlA-SPA and FucI-SPA under each experimental condition (Figures 5B, 5C, S3A, and S3B).

This analysis showed that glucose reduced SrlA-SPA protein levels ~60-fold in the WT strain (Figure 5B). In the Δ*spf* strain, glucose only reduced protein levels ~10-fold, consistent with a loss of Spot 42-mediated repression. Further deletion of *crp* eliminated the detectable expression of SrlA-SPA, showing that CRP is required for *srlA* transcription, as observed previously (Yamada and Saier, 1988). We obtained similar results when the WT and Δ*spf* strains were grown in casamino acid-enriched M9 containing glycerol (Figure S3C). We also obtained comparable results using an *srlA-lacZ* fusion, where glucose reduced fusion levels 61-fold in the WT strain and 37-fold in the Δ*spf* strain (Figure S3E). These results suggest that Spot 42 acts to reduce leaky expression of *srlA* in the presence of glucose.

Glucose reduced FucI-SPA protein levels ~3-fold in the WT strain. In the Δ*spf* strain, glucose also reduced FucI-SPA levels ~3-fold. Further deletion of *crp* reduced protein levels in the presence and absence of glucose. In this strain, FucI-SPA levels were slightly lower in the presence of glucose likely due to faster growth. We obtained similar results when the WT and Δ*spf* strains were grown in casamino acid-enriched M9 containing glycerol (Figure S3D). We also tested a *fucI-lacZ* fusion, where glucose reduced fusion levels 6.2-fold in the WT strain and 4.7-fold in the Δ*spf* strain (Figure S3F). This small difference in repression by glucose would be difficult to detect by quantitative western blot analysis. Overall, our steady-state results indicate that, as part of the CRP-Spot 42 feedforward loop, Spot 42 can reduce leaky expression under repressing conditions.

Spot 42 Influences the Dynamics of Catabolite Repression

Coherent feedforward loops comprised of transcription regulators can confer altered regulatory dynamics in comparison to direct regulation, as predicted by computational modeling and observed in multiple experimental systems (Kalir et al., 2005; Mangan and Alon, 2003; Mangan et al., 2003). To examine the regulatory dynamics conferred by the CRP-Spot 42 feedforward loop, we measured how quickly SrlA-SPA and FucI-SPA levels change after CRP is activated or deactivated. The deactivation of CRP was achieved by growing cells in casamino acid-enriched M9 containing glycerol and adding glucose, while the activation of CRP was achieved by growing cells in casamino acid-enriched M9 containing glycerol and glucose and adding cAMP.

Under conditions of CRP deactivation, the deletion of *spf* delayed gene repression (Figures 6 and S4). For both SrlA-SPA

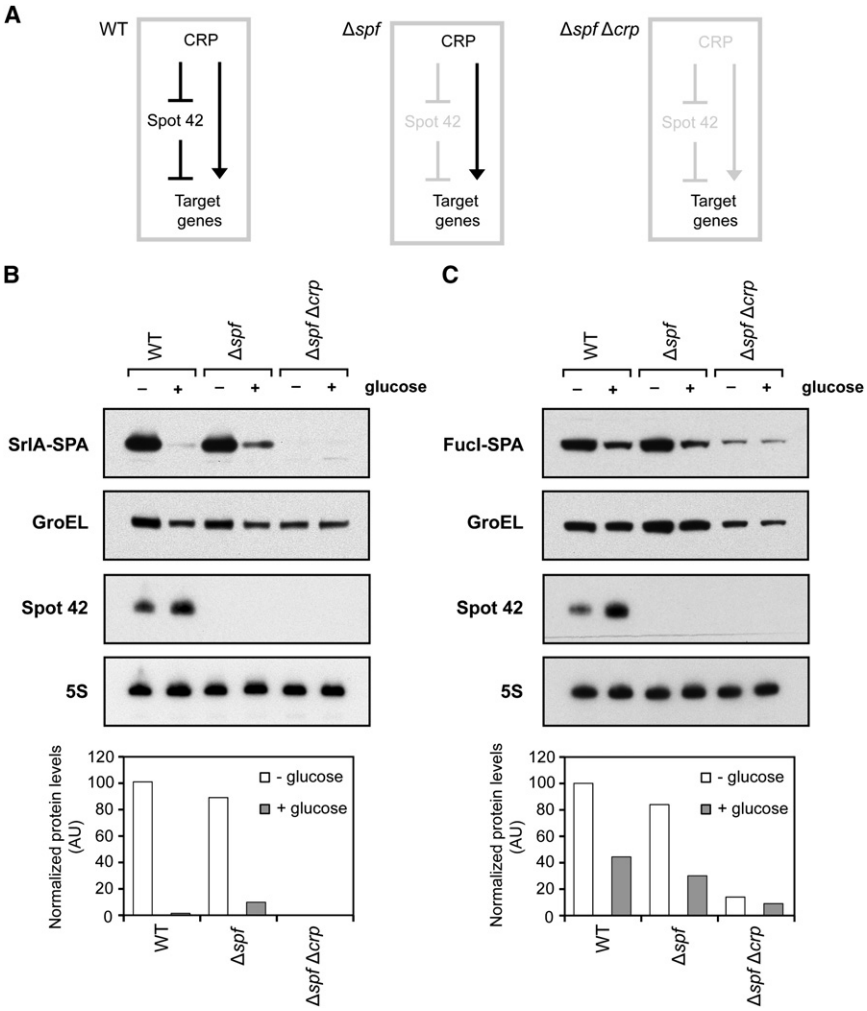

Figure 5. Steady-State Behavior of the CRP-Spot 42 Feedforward Loop

(A) CRP and Spot 42 participate in a multioutput coherent feedforward loop. CRP positively regulates target expression, directly at the transcriptional level and indirectly by repressing Spot 42. Mutations were introduced into the wild-type SPA fusion strain (WT) that reduces the loop to direct regulation by CRP (Δ*spf*) or eliminates regulation by both CRP and Spot 42 (Δ*spf* Δ*crp*).

(B) Results from quantitative western blot analysis for *srlA-SPA* strains.

(C) Results from quantitative western blot analysis for *fucI-SPA* strains. For both (B) and (C), strains were grown in LB or LB containing 0.2% glucose. Northern blot analysis was used to detect the levels of Spot 42. GroEL and 5S RNA served as loading controls. Results are representative of two independent experiments. See Figure S3 for dilution series, for data for cells grown in casamino acid-enriched M9 containing glycerol or casamino acid-enriched M9 containing glycerol and glucose, for β-galactosidase assay results for *srlA-lacZ* and *fucI-lacZ* fusion strains, and steady-state data for strains containing a destabilized version of SrlA-SPA.

and FucI-SPA, the addition of glucose had a negligible effect on protein levels over the first ~30 min (Figures 6A, 6C, S4E, and S4G). After this time interval, protein levels in the WT strain began to decline, while protein levels in the Δ*spf* strain required an additional ~60 min before declining. The difference in the dynamics of gene repression between the WT and Δ*spf* strains cannot be attributed to a difference in the growth rate (Figures S4I and S4J). In support of Spot 42 contributing to gene repression after ~30 min, Spot 42 reached maximal expression after ~20 min following glucose addition (Figures S4K and S4M). Western blotting results were confirmed through two independent experiments, although the results for FucI-SPA were more prone to variability due to the smaller change in protein levels following glucose addition. These results show that, in the context of the CRP-Spot 42 feedforward loop, Spot 42 acts to accelerate the repression of target genes.

Under conditions of CRP activation, the deletion of *spf* accelerated gene activation (Figures 6 and S4). For SrlA-SPA, the addition of cAMP resulted in a rapid increase in protein levels that peaked at ~45 min for the WT strain and ~20 min for the Δ*spf* strain (Figures 6B and S4F). For FucI-SPA, the addition of cAMP also resulted in a rapid increase in protein levels that peaked at ~30 min for the WT strain and ~10 min for the Δ*spf* strain (Figures 6D and S4H). In support of Spot 42 contributing to the delay in gene activation over an ~30 min time interval, Spot 42 levels declined over ~20 min following the addition of cAMP (Figures S4L and S4M). These results suggest that, in the context of the CRP-Spot 42 feedforward loop, Spot 42 acts to delay the activation of target genes.

We also measured the steady-state behavior and dynamics of the CRP-Spot 42 feedforward loop using a destabilized version of SrlA-SPA. A frameshift mutation was introduced into the SrlA binding partner SrlE, which reduced the half-life of SrlA-SPA to ~2 min (data not shown). Quantitative western blot analysis showed that the stabilized and destabilized versions of SrlA-SPA behaved similarly under steady-state conditions (Figures 5B and S3G) and following the addition of cAMP (Figures 6B and S4O). Compared to the stabilized version of SrlA-SPA, the addition of glucose resulted in a much more rapid decrease in destabilized SrlA-SPA levels for the WT and Δ*spf* strains. However, destabilized SrlA-SPA levels rebounded ~30 min after glucose addition for the Δ*spf* strain but only partially rebounded for the WT strain, consistent with the repressive role of Spot 42 (Figure S4N).

Altogether, our results suggest that inclusion of Spot 42 in the CRP-Spot 42 feedforward loop can affect the dynamics of target gene regulation in two ways: (1) accelerate gene repression when the preferred carbon source appears, and (2) delay gene activation when the preferred carbon source disappears.

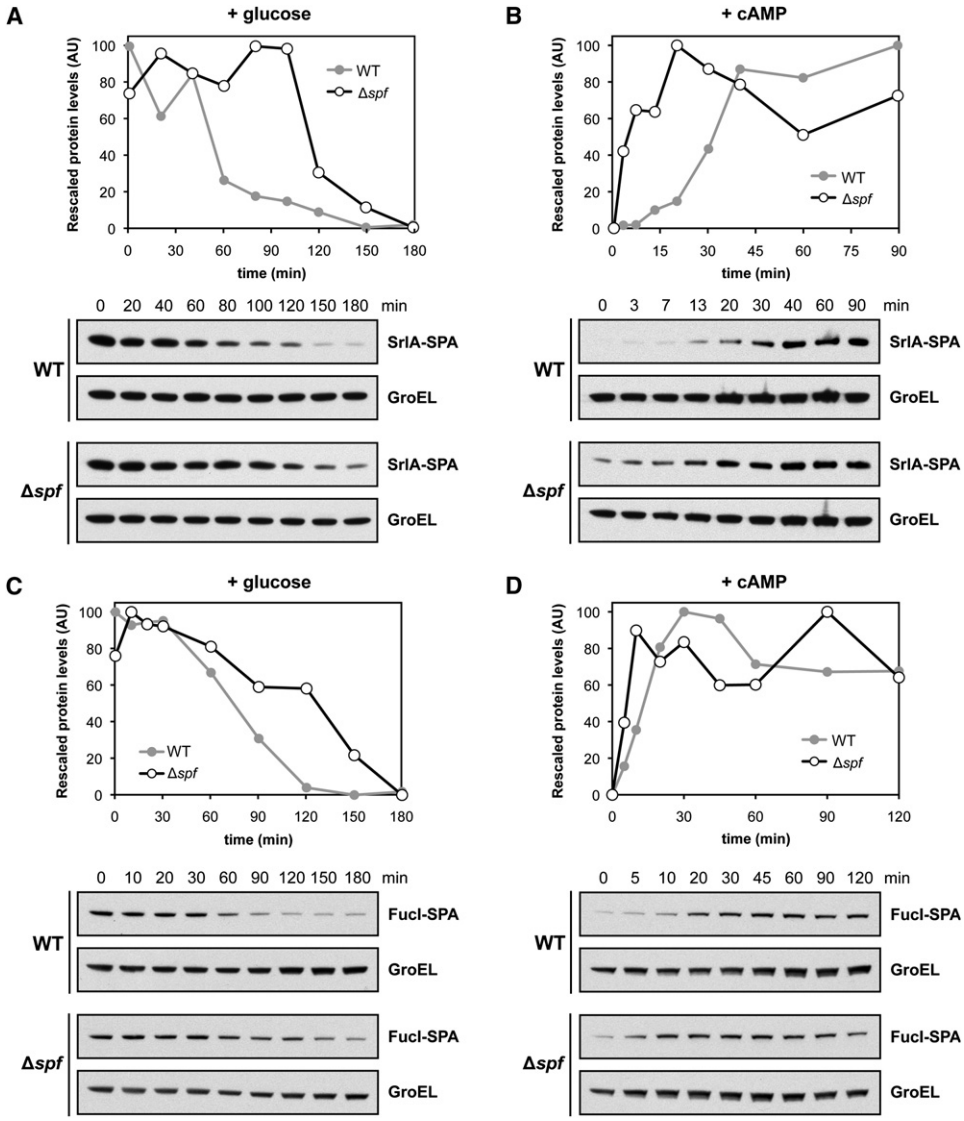

Figure 6. Regulatory Dynamics of the CRP-Spot 42 Feedforward Loop

(A) Time course for SrlA-SPA levels following deactivation of CRP. *srlA-SPA* strains containing the full loop (WT) or the loop reduced to direct regulation by CRP (Δ*spf*) were grown in casamino acid-enriched M9 containing 0.4% glycerol in multiple tubes, and 0.2% glucose was added to each tube at the indicated time prior to harvesting the cultures. Normalized protein levels calculated from quantitative western blot analysis were rescaled to span 0–100.

(B) Time course for SrlA-SPA levels following activation of CRP. WT and Δ*spf* strains were grown in casamino acid-enriched M9 containing 0.4% glycerol and 0.2% glucose in multiple tubes, and 10 mM cAMP was added to each tube at the indicated time prior to harvesting the cultures.

(C) Time course for FucI-SPA levels following deactivation of CRP. *fucI-SPA* strains containing the full loop (WT) or the loop reduced to direct regulation by CRP (Δ*spf*) were grown as described in (A).

(D) Time course for FucI-SPA levels following activation of CRP. WT and Δ*spf* strains were grown as described in (B). Results are representative of three independent experiments. See Figure S4 for dilution series, for an independent set of time course data, for growth rates of the *srlA-SPA* and *fucI-SPA* strains, and for time course data for strains expressing a destabilized version of SrlA-SPA.

DISCUSSION

Physiological Role of Spot 42

In this study we identified and validated numerous targets of the Hfq-binding sRNA Spot 42 in addition to the previously known target *galK*. The identified targets are involved in many aspects of cellular metabolism, including central and secondary metabolism, oxidation of NADPH, and the uptake and catabolism of diverse nonpreferred carbon sources. We also showed that Spot 42 overexpression reduced growth on several nonpreferred carbon sources transported or metabolized by the identified gene targets. These growth phenotypes build on the previous observation that overexpression of Spot 42 reduces growth on succinate (Rice and Dahlberg, 1982). The growth phenotype on succinate may be associated with some of the target genes identified by microarray analysis or genes regulated by Spot 42 at the

level of translation not detected by the microarrays. Taken together, these results suggest that Spot 42 makes multiple contributions to catabolite repression.

A previous study revealed that different sugar transport and catabolism genes show complex transcriptional responses to glucose and their respective carbon sources under steady-state conditions, despite sharing similar regulatory schemes (Kaplan et al., 2008). We found that Spot 42 regulates numerous transport and catabolism genes that also respond to glucose and other specific carbon sources. Among these genes are a few (*fucI*, *galK*) that were investigated by Kaplan et al. Thus, posttranscriptional regulation of metabolic genes introduces yet another layer of complexity in the cellular responses to changing nutrient conditions.

Feedforward Loops that Incorporate sRNAs

We found that Spot 42 participates with the global regulator CRP in a coherent feedforward loop that regulates multiple genes. This loop is comprised of three regulatory interactions: CRP represses Spot 42, CRP activates target genes, and Spot 42 represses target genes. One possible contribution of this feedforward loop, as shown for the regulation of *srlA* and *fucI*, is reducing leaky gene expression under steady-state, repressing conditions. By reducing the leaky expression of multiple genes unnecessary for glucose catabolism, Spot 42 could help divert metabolic resources toward cell growth and the consumption of glucose.

Reduced leakiness could be a considerable benefit of other coherent feedforward loops, yet this property has been rarely discussed. One explanation is that coherent feedforward loops have been studied primarily in the context of transcriptional regulation. Here, two transcription regulators bind a single promoter in order to regulate expression of the downstream gene. Regulation often is described as requiring both regulators to bind the promoter (A *and* B to regulate C) or at least one regulator to bind the promoter (A *or* B to regulate C) (Alon, 2007). For either mode of coregulation, gene expression is either *on* or *off* depending on the combination of regulators present. When the feedforward loop is composed of a transcription regulator and an sRNA, regulation occurs under two different mechanisms—transcription and posttranscription/translation. By separating the mechanisms of regulation, both regulators can influence gene expression independently and subsequently reduce leaky expression.

We found that the CRP-Spot 42 coherent feedforward loop also showed altered regulatory dynamics in comparison to direct regulation by CRP. Transcriptional coherent feedforward loops with the same configuration as the CRP-Spot 42 loop have been predicted to show a delay when the loop is activated but similar dynamics when the loop is deactivated (Mangan and Alon, 2003). Equivalent dynamics were predicted for coherent feedforward loops composed of an sRNA and a transcription regulator (Shimoni et al., 2007). While our results revealed a delay when CRP was activated, our results also revealed a faster decrease in protein levels when CRP was deactivated (Figure 6). The faster decrease in protein levels may be attributed to Spot 42 inducing the degradation or translational repression of the *srlA* and *fucI* mRNAs. The ability to accelerate gene repression may allow cells to adapt more rapidly to new environmental conditions, such as the presence of glucose. This ability is not associated with feedforward loops incorporating transcription factors, highlighting one potential advantage of sRNAs over proteins.

There is compounding evidence that other bacterial sRNAs besides Spot 42 participate in feedforward loops. The sRNAs MicC and MicF both appear to form coherent feedforward loops with the sensory regulator OmpR to control outer membrane protein biosynthesis (Chen et al., 2004; Mizuno et al., 1984; Shimoni et al., 2007). Furthermore, the ongoing identification of gene targets of Hfq-binding sRNAs suggests that additional sRNAs participate in feedforward loops with their transcription regulator. For example, the genes *mqo*, *sodA*, *yebZ*, and *yobA* were found, by microarray analysis, to be repressed by the anaerobically induced sRNA FnrS, and the promoters of these genes contain potential binding sites for the FnrS regulator FNR (Boysen et al., 2010; Durand and Storz, 2010). As a second example, the *sdh* operon is directly regulated by the iron-responsive sRNA RyhB, and the promoter of this operon contains a potential binding site for the RyhB regulator Fur (Massé and Gottesman, 2002; Zhang et al., 2005). Finally, the genes *gabD*, *modC*, and *fepA* were identified by microarray analysis following overexpression of the CRP-activated sRNA CyaR (De Lay and Gottesman, 2009). A CRP binding site was mapped in the promoter of *gabD* and is required for transcription (Germer et al., 2001; Marschall et al., 1998), while the promoters of *modC* and *fepA* each contain a potential CRP binding site (Zhang et al., 2005; Zheng et al., 2004). Thus, the feedforward loop may represent a prevalent regulatory circuit that includes base-pairing sRNAs. It would be interesting to test if these sRNAs participate in the proposed feedforward loops and, if so, to determine how these loops contribute to the response to changes in environmental conditions.

The inclusion of base-pairing sRNAs in feedforward loops is similar to that observed for microRNAs (miRNAs), the regulatory counterparts to base-pairing sRNAs, in most eukaryotes (reviewed in Carthew and Sontheimer, 2009). Many miRNAs appear to participate in coherent feedforward loops within developmental circuits (Hornstein and Shomron, 2006; Tsang et al., 2007). The miRNA-containing feedforward loops are hypothesized to reduce leaky expression of developmental genes and thus maintain the developmental state of the cell (Hornstein and Shomron, 2006). A similar reduction in leaky expression also was achieved with an engineered coherent feedforward loop comprised of a transcription repressor and a synthetic miRNA (Deans et al., 2007). Although recent studies have focused on the regulatory contributions of miRNAs in feedforward loops under steady-state conditions, our work demonstrates that the dynamics of these feedforward loops also may be influenced by the inclusion of regulatory RNAs. Thus, sRNAs in bacteria and eukaryotes may play key roles in shaping the steady-state behavior and dynamics of signaling and developmental pathways.

EXPERIMENTAL PROCEDURES

Plasmid and Strain Construction

The plasmids and oligonucleotides used in this study are listed in Table S3. The bacterial strains used in this study are all derivatives of *E. coli* K-12 MG1655

and are listed in Table S3. Plasmid and strain construction is described in Supplemental Experimental Procedures.

Growth Conditions

All strains were grown by shaking at 250 rpm at 37°C, unless otherwise noted. Strains were grown in LB media or in 1X M9 salts supplemented with 10 μg/ml thiamine, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.2% casamino acids (casamino acid-enriched M9). Cell density was obtained by measuring the OD_{600} using an Ultrospec 3000 UV/Visible spectrophotometer (GE Healthcare, Piscataway, NJ).

RNA Isolation

Total RNA was isolated using the hot phenol extraction procedure (Aiba et al., 1981). The concentration of RNA following ethanol precipitation was determined using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Microarray Analysis

NM525 cells harboring pBRplac or pSpot42 were grown in LB to an OD_{600} of ~0.4 and treated with 1 mM IPTG. After 7 min, total RNA was isolated as described above. cDNA preparation and hybridization to the GeneChip *E. coli* Genome 2.0 array (Affymetrix, Santa Clara, CA) was conducted as described previously (Durand and Storz, 2010).

Northern Blot Analysis

Northern blot analysis was conducted as described previously (Durand and Storz, 2010). The RNA Millennium Marker (Ambion, Austin, TX) was used as a size marker on the agarose gels, and the Perfect RNA Marker (EMD Chemicals, Gibbstown, NJ) was used as a size marker on the polyacrylamide gels.

5′ RACE Analysis

5′ RACE was performed on *srlA* and *sthA*, generally as described previously (Mandin and Gottesman, 2009). See the Supplemental Experimental Procedures for further details.

β-Galactosidase Assays

Three colonies from each transformed strain were inoculated into separate tubes containing LB and grown overnight. Overnight cultures were diluted into 3 ml of LB to an OD_{600} of 0.01 and grown to an OD_{600} of ~0.1, and either 0.2% arabinose or 0.2% arabinose with 1 mM IPTG was added to each culture. After 1 hr, cultures were subjected to β-galactosidase assays as described previously (Miller, 1972).

Quantitative Western Blot Analysis

For the steady-state measurements, overnight cultures were diluted to an OD_{600} of 0.01 in the same type of media and grown to an OD_{600} of 0.4–0.6. For the dynamic measurements, overnight cultures were diluted to an OD_{600} of 0.01 into nine or ten tubes containing the same type of media and grown to an OD_{600} of 0.4–0.6. At the indicated time prior to harvesting the cells, 0.2% glucose (CRP-deactivated) or 10 mM cAMP (CRP-activated) was added to each tube. Supplementing the media with cAMP rapidly activates CRP even in the presence of glucose, as shown previously (Kaplan et al., 2008; Mangan et al., 2003).

To harvest the cells, 1 ml of each culture was mixed with 0.4 ml of ice-cold 1× PBS, and cells were pelleted and frozen on dry ice. Total protein from an equal number of cells was resolved on a Novex 15-well 16% tricine gel (Invitrogen, Carlsbad, CA) and transferred to a 0.45 μm pore nitrocellulose membrane (Invitrogen, Carlsbad, CA). The SPA tag was detected using the mouse monoclonal anti-FLAG M2-alkaline phosphatase antibody (Sigma-Aldrich, St. Louis) with the Lumi-Phos WB reagent (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. The loading control GroEL was detected with the mouse anti-GroEL monoclonal antibody (Abcam, Cambridge, MA), and the Immunopure goat anti-mouse IgG-horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA) with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions.

Protein levels were calculated using the ImageJ software package (http://rsb.info.nih.gov/ij/). Background intensity measured directly below each band was subtracted, and a dilution series in the same gel was used to calculate relative protein levels. For each time point, relative protein levels of the SPA fusion were divided by relative protein levels of the loading control GroEL to yield normalized protein levels. For the dynamic measurements, normalized protein levels were rescaled to span 0–100 in order to directly compare the dynamics between strains.

ACCESSION NUMBERS

The complete microarray data set from the three independent experiments was deposited in NCBI GEO (GSE24875).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, four figures, and three tables and can be found with this article online at doi:10.1016/j.molcel.2010.12.027.

ACKNOWLEDGMENTS

We thank E. Fozo, R. Fuchs, S. Gottesman, M. Goulian, E. Hobbs, B. Janson, M. Thomason, and L. Waters for their comments on this manuscript and S. Durand for technical assistance. P. Mandin kindly provided the *E. coli* strain PM1205 and the plasmid pSpot42, and N. Majdalani kindly provided the *E. coli* strain NM525. Work carried out in the laboratory of G.S. is supported by the Intramural Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. C.L.B. is a Gordon and Betty Moore Foundation Fellow of the Life Sciences Research Foundation.

Received: August 13, 2010
Revised: October 29, 2010
Accepted: December 1, 2010
Published: February 3, 2011

REFERENCES

Aiba, H., Adhya, S., and de Crombrugghe, B. (1981). Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. J. Biol. Chem. *256*, 11905–11910.

Alon, U. (2007). Network motifs: theory and experimental approaches. Nat. Rev. Genet. *8*, 450–461.

Ansong, C., Yoon, H., Porwollik, S., Mottaz-Brewer, H., Petritis, B.O., Jaitly, N., Adkins, J.N., McClelland, M., Heffron, F., and Smith, R.D. (2009). Global systems-level analysis of Hfq and SmpB deletion mutants in *Salmonella*: implications for virulence and global protein translation. PLoS ONE *4*, e4809.

Beisel, C.L., and Storz, G. (2010). Base pairing small RNAs and their roles in global regulatory networks. FEMS Microbiol. Rev. *34*, 866–882.

Boysen, A., Møller-Jensen, J., Kallipolitis, B., Valentin-Hansen, P., and Overgaard, M. (2010). Translational regulation of gene expression by an anaerobically induced small non-coding RNA in *Escherichia coli*. J. Biol. Chem. *285*, 10690–10702.

Carthew, R.W., and Sontheimer, E.J. (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell *136*, 642–655.

Chen, Y.M., Zhu, Y., and Lin, E.C. (1987). The organization of the *fuc* regulon specifying L-fucose dissimilation in *Escherichia coli* K12 as determined by gene cloning. Mol. Gen. Genet. *210*, 331–337.

Chen, S., Zhang, A., Blyn, L.B., and Storz, G. (2004). MicC, a second small-RNA regulator of Omp protein expression in *Escherichia coli*. J. Bacteriol. *186*, 6689–6697.

Chiang, B.Y., Chen, T.C., Pai, C.H., Chou, C.C., Chen, H.H., Ko, T.P., Hsu, W.H., Chang, C.Y., Wu, W.F., Wang, A.H., and Lin, C.H. (2010). Protein S-thiolation by Glutathionylspermidine (Gsp): the role of *Escherichia coli* Gsp synthetASE/amidase in redox regulation. J. Biol. Chem. *285*, 25345–25353.

De Lay, N., and Gottesman, S. (2009). The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior. J. Bacteriol. *191*, 461–476.

Deans, T.L., Cantor, C.R., and Collins, J.J. (2007). A tunable genetic switch based on RNAi and repressor proteins for regulating gene expression in mammalian cells. Cell *130*, 363–372.

Durand, S., and Storz, G. (2010). Reprogramming of anaerobic metabolism by the FnrS small RNA. Mol. Microbiol. *75*, 1215–1231.

Germer, J., Becker, G., Metzner, M., and Hengge-Aronis, R. (2001). Role of activator site position and a distal UP-element half-site for sigma factor selectivity at a CRP/H-NS-activated σ^S-dependent promoter in *Escherichia coli*. Mol. Microbiol. *41*, 705–716.

Görke, B., and Stülke, J. (2008). Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat. Rev. Microbiol. *6*, 613–624.

Hornstein, E., and Shomron, N. (2006). Canalization of development by microRNAs. Nat. Genet. Suppl. *38*, S20–S24.

Kalir, S., Mangan, S., and Alon, U. (2005). A coherent feed-forward loop with a SUM input function prolongs flagella expression in *Escherichia coli*. Mol. Syst. Biol. *1*, 1–6. Published online March 29, 2005. 10.1038/msb4100010.

Kaplan, S., Bren, A., Zaslaver, A., Dekel, E., and Alon, U. (2008). Diverse two-dimensional input functions control bacterial sugar genes. Mol. Cell *29*, 786–792.

Mandin, P., and Gottesman, S. (2009). A genetic approach for finding small RNAs regulators of genes of interest identifies RybC as regulating the DpiA/DpiB two-component system. Mol. Microbiol. *72*, 551–565.

Mangan, S., and Alon, U. (2003). Structure and function of the feed-forward loop network motif. Proc. Natl. Acad. Sci. USA *100*, 11980–11985.

Mangan, S., Zaslaver, A., and Alon, U. (2003). The coherent feedforward loop serves as a sign-sensitive delay element in transcription networks. J. Mol. Biol. *334*, 197–204.

Marschall, C., Labrousse, V., Kreimer, M., Weichart, D., Kolb, A., and Hengge-Aronis, R. (1998). Molecular analysis of the regulation of *csiD*, a carbon starvation-inducible gene in *Escherichia coli* that is exclusively dependent on σ^s and requires activation by cAMP-CRP. J. Mol. Biol. *276*, 339–353.

Massé, E., and Gottesman, S. (2002). A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc. Natl. Acad. Sci. USA *99*, 4620–4625.

Miller, J.H. (1972). Experiments in Molecular Genetics (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D., and Alon, U. (2002). Network motifs: simple building blocks of complex networks. Science *298*, 824–827.

Mizuno, T., Chou, M.Y., and Inouye, M. (1984). A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proc. Natl. Acad. Sci. USA *81*, 1966–1970.

Møller, T., Franch, T., Højrup, P., Keene, D.R., Bächinger, H.P., Brennan, R.G., and Valentin-Hansen, P. (2002a). Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. Mol. Cell *9*, 23–30.

Møller, T., Franch, T., Udesen, C., Gerdes, K., and Valentin-Hansen, P. (2002b). Spot 42 RNA mediates discoordinate expression of the *E. coli* galactose operon. Genes Dev. *16*, 1696–1706.

Polayes, D.A., Rice, P.W., Garner, M.M., and Dahlberg, J.E. (1988). Cyclic AMP-cyclic AMP receptor protein as a repressor of transcription of the *spf* gene of *Escherichia coli*. J. Bacteriol. *170*, 3110–3114.

Rice, P.W., and Dahlberg, J.E. (1982). A gene between *polA* and *glnA* retards growth of *Escherichia coli* when present in multiple copies: physiological effects of the gene for spot 42 RNA. J. Bacteriol. *152*, 1196–1210.

Sahagan, B.G., and Dahlberg, J.E. (1979a). A small, unstable RNA molecule of *Escherichia coli*: spot 42 RNA. I. Nucleotide sequence analysis. J. Mol. Biol. *131*, 573–592.

Sahagan, B.G., and Dahlberg, J.E. (1979b). A small, unstable RNA molecule of *Escherichia coli*: spot 42 RNA. II. Accumulation and distribution. J. Mol. Biol. *131*, 593–605.

Shen-Orr, S.S., Milo, R., Mangan, S., and Alon, U. (2002). Network motifs in the transcriptional regulation network of *Escherichia coli*. Nat. Genet. *31*, 64–68.

Shimoni, Y., Friedlander, G., Hetzroni, G., Niv, G., Altuvia, S., Biham, O., and Margalit, H. (2007). Regulation of gene expression by small non-coding RNAs: a quantitative view. Mol. Syst. Biol. *3*, 138.

Sittka, A., Lucchini, S., Papenfort, K., Sharma, C.M., Rolle, K., Binnewies, T.T., Hinton, J.C., and Vogel, J. (2008). Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. PLoS Genet. *4*, e1000163.

Tsang, J., Zhu, J., and van Oudenaarden, A. (2007). MicroRNA-mediated feedback and feedforward loops are recurrent network motifs in mammals. Mol. Cell *26*, 753–767.

Urbanowski, M.L., Stauffer, L.T., and Stauffer, G.V. (2000). The *gcvB* gene encodes a small untranslated RNA involved in expression of the dipeptide and oligopeptide transport systems in *Escherichia coli*. Mol. Microbiol. *37*, 856–868.

Vogel, J. (2009). A rough guide to the non-coding RNA world of *Salmonella*. Mol. Microbiol. *71*, 1–11.

Waters, L.S., and Storz, G. (2009). Regulatory RNAs in bacteria. Cell *136*, 615–628.

Yamada, M., and Saier, M.H., Jr. (1988). Positive and negative regulators for glucitol (*gut*) operon expression in *Escherichia coli*. J. Mol. Biol. *203*, 569–583.

Zadeh, J.N., Steenberg, C.D., Bois, J.S., Wolfe, B.R., Pierce, M.B., Khan, A.R., Dirks, R.M., and Pierce, N.A. (2011). NUPACK: Analysis and design of nucleic acid systems. J. Comput. Chem. *32*, 170–173.

Zeghouf, M., Li, J., Butland, G., Borkowska, A., Canadien, V., Richards, D., Beattie, B., Emili, A., and Greenblatt, J.F. (2004). Sequential Peptide Affinity (SPA) system for the identification of mammalian and bacterial protein complexes. J. Proteome Res. *3*, 463–468.

Zhang, A., Wassarman, K.M., Rosenow, C., Tjaden, B.C., Storz, G., and Gottesman, S. (2003). Global analysis of small RNA and mRNA targets of Hfq. Mol. Microbiol. *50*, 1111–1124.

Zhang, Z., Gosset, G., Barabote, R., Gonzalez, C.S., Cuevas, W.A., and Saier, M.H., Jr. (2005). Functional interactions between the carbon and iron utilization regulators, Crp and Fur, in *Escherichia coli*. J. Bacteriol. *187*, 980–990.

Zheng, D., Constantinidou, C., Hobman, J.L., and Minchin, S.D. (2004). Identification of the CRP regulon using in vitro and in vivo transcriptional profiling. Nucleic Acids Res. *32*, 5874–5893.