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RESEARCH ARTICLE

# Spot 42 Small RNA Regulates Arabinose-Inducible *araBAD* Promoter Activity by Repressing Synthesis of the High-Affinity Low-Capacity Arabinose Transporter

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**ABSTRACT** The L-arabinose-inducible *araBAD* promoter ($P_{BAD}$) enables tightly controlled and tunable expression of genes of interest in a broad range of bacterial species. It has been used successfully to study bacterial sRNA regulation, where $P_{BAD}$ drives expression of target mRNA translational fusions. Here we report that in *Escherichia coli*, Spot 42 sRNA regulates $P_{BAD}$ promoter activity by affecting arabinose uptake. We demonstrate that Spot 42 sRNA represses *araF*, a gene encoding the AraF subunit of the high-affinity low-capacity arabinose transporter AraFGH, through direct base-pairing interactions. We further show that endogenous Spot 42 sRNA is sufficient to repress *araF* expression under various growth conditions. Finally, we demonstrate this posttranscriptional repression has a biological consequence, decreasing the induction of $P_{BAD}$ at low levels of arabinose. This problem can be circumvented using strategies reported previously for avoiding all-or-none induction behavior, such as through constitutive expression of the low-affinity high-capacity arabinose transporter AraE or induction with a higher concentration of inducers. This work adds *araF* to the set of Spot 42-regulated genes, in agreement with previous studies suggesting that Spot 42, itself negatively regulated by the cyclic AMP (cAMP) receptor protein-cAMP complex, reinforces the catabolite repression network.

**IMPORTANCE** The bacterial arabinose-inducible system is widely used for titratable control of gene expression. We demonstrate here that a posttranscriptional mechanism mediated by Spot 42 sRNA contributes to the functionality of the $P_{BAD}$ system at subsaturating inducer concentrations by affecting inducer uptake. Our finding extends the inputs into the known transcriptional control for the $P_{BAD}$ system and has implications for improving its usage for tunable gene expression.

**KEYWORDS** regulatory small RNA, posttranscriptional regulation, arabinose transporter, arabinose-inducible promoter

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The *Escherichia coli* arabinose-inducible *araBAD* promoter ($P_{BAD}$) system has been widely used for controlled gene expression in a broad range of bacterial hosts ever since its first application (1) 2 decades ago, owing to the fine control of expression, wide range of induction, tight repression in the absence of an inducer, and broad host range. In this system, the master dual transcriptional regulator AraC tightly controls the arabinose transporter genes (*araE* and *araFGH*) and arabinose catabolic genes (*araBAD*) in an arabinose-inducible manner. In the absence of arabinose, dimeric apo-AraC serves as a repressor that loops DNA and blocks transcription from $P_{BAD}$. In the presence of arabinose, arabinose-bound AraC works as a transcriptional activator at the $P_{BAD}$ and transporter gene promoters ($P_E$ and $P_{FGH}$) (reviewed in reference 2). The increased catabolism of arabinose by the AraB, AraA, and AraD enzymes downregulates intracellular arabinose levels, leading to attenuated induction of $P_{BAD}$, $P_E$, and $P_{FGH}$. On the

other hand, the expression of transporters increases intracellular arabinose concentrations, further activating the transcription of the arabinose catabolic and transporter promoters. These regulatory circuits contribute to $P_{BAD}$ activity by forming negative- (for the catabolic enzymes) or positive- (for the transporters) feedback loops. Additionally, the cyclic AMP receptor protein (CRP) is critical for the activation of the *ara* genes.

The arabinose transporters are essential for arabinose uptake, and thus are critical for $P_{BAD}$ promoter activity. AraE is a low-affinity (140 to 320 $\mu$M) high-capacity arabinose/proton symporter, and AraFGH is a high-affinity ($\sim$10 $\mu$M) low-capacity ATP binding cassette (ABC) transporter, where AraF is the periplasmic arabinose-binding protein (3). The *araE* and *araFGH* genes are transcriptionally regulated by AraC and cyclic AMP (cAMP)-CRP transcription factors (2). At suboptimal arabinose concentrations, the positive feedback of these transporters leads to all-or-none induction of the $P_{BAD}$ promoter (4). Decoupling of *araE* promoter activity from the intracellular arabinose levels using a constitutive or isopropyl-$\beta$-D-thiogalactopyranoside (IPTG)-inducible $P_{lac}$ promoter overcomes the all-or-none induction pattern of the $P_{BAD}$ system, significantly improving the application of the $P_{BAD}$ system (5–7). However, no posttranscriptional control has previously been described for the $P_{BAD}$ system.

Bacterial regulatory small RNAs (sRNA) are posttranscriptional regulators that control gene expression by affecting mRNA stability and/or translation initiation through limited complementary base pairing with cognate mRNA targets (reviewed in references 8 and 9). These base-pairing interactions are catalyzed by the sRNA chaperone Hfq, which binds both sRNA and mRNA targets (reviewed in references 10 and 11). The expression of Spot 42 sRNA, encoded by *spf*, is negatively regulated by the cAMP-CRP complex, and Spot 42 sRNA is highly expressed in early exponential phase or in glucose-containing medium (12). Recent studies suggest Spot 42 is a global RNA regulator for central carbon metabolism in *E. coli* (13) and *Aliivibrio salmonicida* (14). Through base pairing with mRNAs encoding sugar transporters and catabolic enzymes for secondary carbon sources, Spot 42 reduces the leaky expression of genes for the utilization of alternative carbon sources in the presence of a preferred carbon source (glucose) and contributes to expression dynamics in changing nutrient conditions by reinforcing the role of the transcriptional activator cAMP-CRP complex for many target genes (13). Spot 42 uses three conserved unstructured regions to base-pair with cognate mRNA targets for regulation (15).

Our lab developed a genetic approach in *E. coli* for screening sRNA regulators of specific mRNA targets. The approach utilizes $P_{BAD}$-driven *lacZ* fused to an mRNA target of interest in combination with a library of plasmids that express given sRNAs (16, 17). Using this approach, we successfully identified numerous sRNA regulators for a number of mRNAs (16, 18–20). By varying the level of arabinose, the inducer for $P_{BAD}$, this approach enables tuning of fusions harboring different ribosome binding-site sequences to an intermediate level of *lacZ* reporter expression ($\sim$500 Miller units) for the screening of both positive and negative sRNA regulators.

This work began as part of a project to examine the effect of sRNAs on genes identified as likely and interesting targets of sRNAs based on their enrichment by Hfq immunoprecipitation. One such gene was *recA*. The RecA protein is essential for repairing double-strand breaks (DSBs) by homologous recombination and inducing the SOS response to DNA damage in cells (reviewed in reference 21). In experiments to identify sRNA regulators of *recA* mRNA using our sRNA library approach, we initially identified Spot 42 as a strong negative regulator. However, we found that Spot 42 sRNA affects $P_{BAD}$-*recA-lacZ* expression indirectly by affecting arabinose uptake. Spot 42 sRNA achieves this by directly targeting *araF* mRNA, which encodes the periplasmic arabinose-binding protein of the high-affinity arabinose transporter. In this article, we discuss possible solutions to overcome this obstacle for using the $P_{BAD}$ system.

## RESULTS

**Spot 42 sRNA affects $P_{BAD}$-driven genes under a low L-arabinose induction condition.** We and others successfully identified sRNA regulators for a variety of mRNA

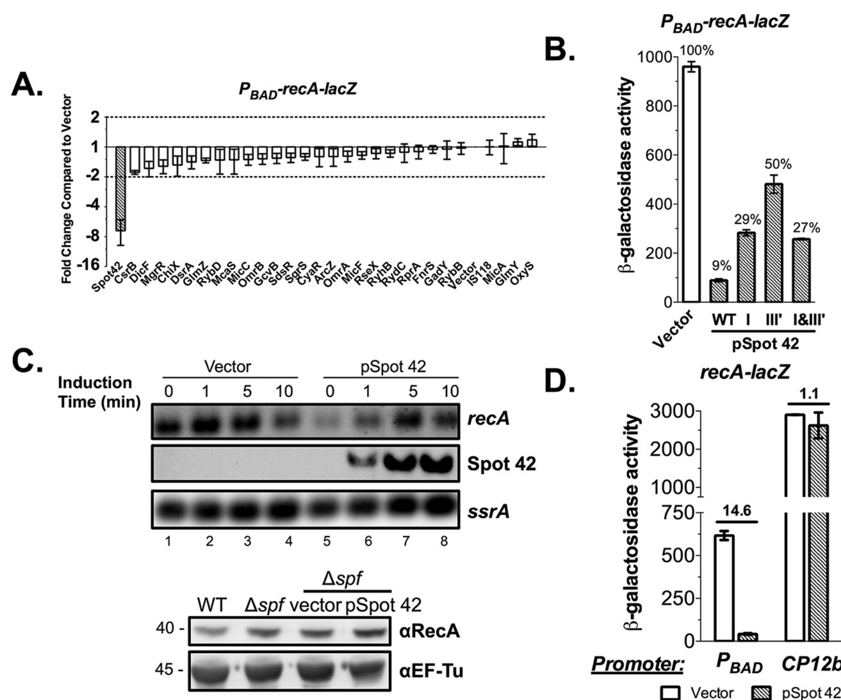


**FIG 1** Spot 42 sRNA regulates $P_{BAD}$-*recA-lacZ* expression by indirectly affecting *araBAD* promoter activity. (A) Overexpression of individual sRNAs from an *E. coli* sRNA plasmid library in the $P_{BAD}$-*recA-lacZ* reporter strain JC1005 identified Spot 42 as an sRNA repressor (>2-fold repression compared to the vector control as cutoff, shown by dotted lines). The reporter strain with sRNA plasmids was induced with 0.0002% L-arabinose and 50 μM IPTG at 37°C with shaking for 6 h before being assayed for β-galactosidase activity ($V_{max}/OD_{600}$). Expression of *recA-lacZ* was normalized to cells carrying the vector control, which gave a level of ~40 specific units. (B) β-Galactosidase activity assay of *recA-lacZ* expression in JC1005 carrying Spot 42 mutants that disrupted putative base-pairing interactions with *recA* leader sequences. Bacterial cells were grown at 37°C in LB with ampicillin (100 μg/ml), 0.0002% L-arabinose, and 50 μM IPTG for 6 h before being assayed for β-galactosidase activity. (C) Northern blot (upper panel) to measure endogenous *recA* transcripts in JC1041 (*lacI*$^{q}$ Δ*spf*) after a brief induction of Spot 42 expression (lanes 5 to 8) compared to the vector control (lanes 1 to 4). *ssrA* mRNA served as a loading control. Western blot (lower panel) to measure endogenous RecA protein in the WT (NM525,) the Δ*spf* strain (JC1041), the Δ*spf* strain carrying the vector, and the Δ*spf* strain carrying the Spot 42 plasmid treated with IPTG and grown to an $OD_{600}$ of 0.9. EF-Tu was used as a protein-loading control. (D) β-Galactosidase activity assays to measure *recA-lacZ* expression in JC1005 ($P_{BAD}$-*recA-lacZ*) and JC1058 (CP12b-*recA-lacZ*) with overproduction of Spot 42. Bacterial cells were induced with 50 μM IPTG for 6 h at 37°C in the presence (for JC1005) or absence (for JC1058) of 0.0002% L-arabinose before being assayed for β-galactosidase activity. Results of assays are expressed in Miller units. Biological triplicates were measured, and means ± SEMs are presented.

targets using an sRNA library approach combined with a chromosomally encoded, inducible translational reporter system in *E. coli* (16, 18–20, 22). In previous work (23), we found that *recA* mRNA coimmunoprecipitated with the sRNA chaperone Hfq, with a 20-fold enrichment compared to that of the total RNA sample, suggesting that Hfq-dependent sRNAs likely contribute to *recA* expression. Using the sRNA library approach to identify sRNA regulators for *recA* mRNA, we monitored *recA* expression using a $P_{BAD}$-*recA-lacZ* chromosomal translational fusion and found that overexpressing only Spot 42 strongly repressed the fusion (Fig. 1A). The overexpression of Spot 42 sRNA reduced *recA-lacZ* expression 10-fold compared with that of the vector control (Fig. 1B). In these experiments, 0.0002% (13 μM) L-arabinose was used for induction (Fig. 1A and B). Mutational analysis of Spot 42 identified single-stranded regions I and III′, described previously (13, 15), as required for *recA-lacZ* repression, but there were no additive effects when both were mutated (Fig. 1B).

However, ectopic expression of Spot 42 did not substantially affect the levels of endogenous *recA* mRNA or protein (Fig. 1C), suggesting that the regulation observed might be an artifact from the $P_{BAD}$-*recA-lacZ* reporter. To test this notion, we con-

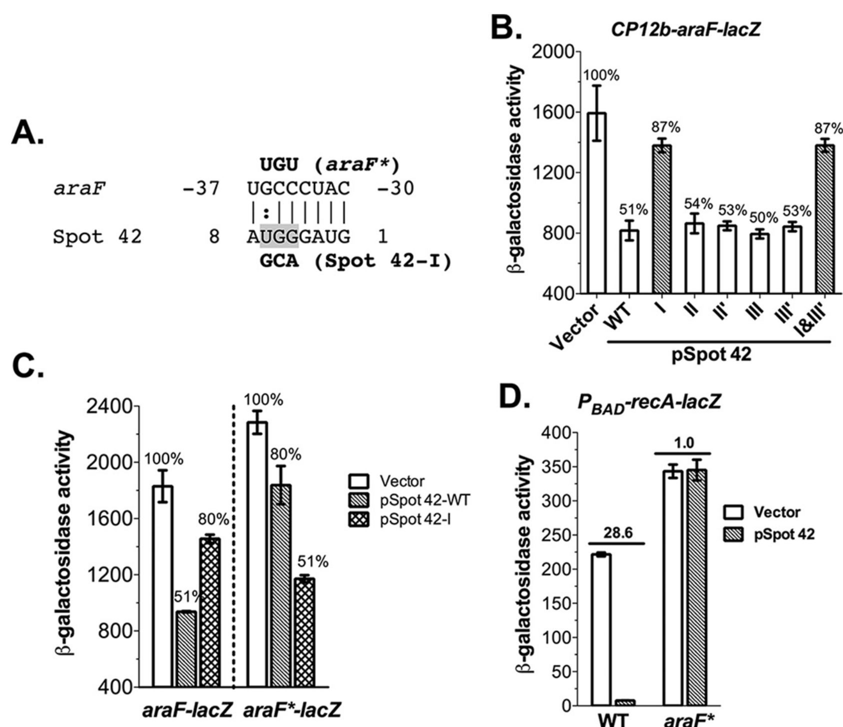


**FIG 2** Spot 42 sRNA represses *araF* expression through direct base-pairing interactions. (A) The CopraRNA-predicted RNA duplex formed between nt −37 to −30 relative to ATG of the *araF* 5′ UTR and nt 1 to 8 of Spot 42 sRNA, including wobble G-U base pairs (colon), is shown. Point mutations and compensatory mutations for Spot 42-I and *araF** are in bold. (B) β-Galactosidase activity assay of *araF-lacZ* expression in JC1100 (CP12b-*araF-lacZ*) with the vector, the WT, and six Spot 42 mutants. Assay conditions are described as for Fig. 1B except that no arabinose was added. (C) sRNA and compensatory mRNA mutagenesis analyses demonstrate direct *araF* repression by Spot 42 via base-pairing interactions. Bacterial reporter strains JC1100 (*araF-lacZ*) and JC1102 (*araF*-lacZ*) transformed with the WT, a Spot 42 mutant, or the vector were induced with 50 μM IPTG at 37°C for 6 h before being assayed for β-galactosidase activities. (D) β-Galactosidase activity assays to measure $P_{BAD}$-*recA-lacZ* expression in WT *araF* (JC1191) and specificity-mutant *araF** (JC1192) strains overproducing Spot 42. Bacterial cells were induced with 100 μM IPTG for 6 h at 37°C in the presence of 0.0002% L-arabinose before being assayed for β-galactosidase activity. Biological triplicates were assayed, and data are plotted as means ± SEMs. Results of assays are expressed in Miller units.

structed a second *recA-lacZ* reporter driven by the synthetic constitutive promoter CP12b (24) and compared it with $P_{BAD}$-*recA-lacZ*. Consistent with the results from the endogenous *recA* mRNA and protein, this CP12b-driven *recA-lacZ* reporter was completely inert to Spot 42 overexpression as compared with the vector control, whereas expression was reduced 14.6-fold with the $P_{BAD}$-*recA-lacZ* reporter (Fig. 1D), reinforcing the idea that Spot 42 sRNA repressed *recA-lacZ* expression indirectly by affecting expression from the *araBAD* promoter.

**Spot 42 sRNA represses *araF* expression through direct base-pairing interactions.** Spot 42 sRNA controls multiple genes involved in secondary carbon utilization through direct base-pairing interactions (13). To determine how Spot 42 sRNA affects the $P_{BAD}$-driven genes, we checked potential base-pairing interactions between Spot 42 sRNA and mRNAs in the $P_{BAD}$ system, including *araE*, *-F*, *-G*, *-H*, *-C*, *-B*, *-A*, and *-D*, using the CopraRNA tool (25). CopraRNA predicted a conserved base-pairing interaction between Spot 42 region I (nucleotides [nt] 1 to 8) and the *araF* 5′ untranslated region (UTR) (nt −30 to −37) (Fig. 2A). To verify the potential base-pairing interactions, we constructed an *araF-lacZ* translational fusion reporter under the control of the CP12b synthetic constitutive promoter by fusing the *araF* leader and initial translated region (nt −110 to 30 relative to the ATG) to the *lacZ* gene and then overproduced wild-type (WT) Spot 42 and six Spot 42 mutants (I, II, II′, III, III′, and I + III′) (13, 15) in the reporter strain. Overproduction of Spot 42 reduced *araF-lacZ* expression 2-fold, and only the

Spot 42 region I mutant significantly alleviated the repression (Fig. 2B). We further confirmed that Spot 42 represses *araF* through a direct base-pairing interaction by testing repression in the chromosomal compensatory mutant *araF*-lacZ* (Fig. 2C). Ectopic expression of WT Spot 42 slightly decreased *araF*-lacZ* expression, and Spot 42 mutant I, which reestablished base pairing with *araF*-lacZ*, completely restored the repression. Collectively, these results demonstrate that Spot 42 represses *araF* expression by forming a short antisense interaction between its single-stranded 5′ end and the *araF* leader.

Inhibition of *araF* by Spot 42 should reduce arabinose import and thus block induction of the promoter of the $P_{BAD}$-*recA-lacZ* reporter at low arabinose concentrations. To confirm this, the *araF** mutation that abolishes Spot 42 repression in the *araF*-lacZ* fusion (Fig. 2C) was introduced into the native *araF* gene in the $P_{BAD}$-*recA-lacZ* reporter strain. In this strain (*araF**), Spot 42 no longer repressed $P_{BAD}$-*recA-lacZ* (Fig. 2D), demonstrating that repression of $P_{BAD}$ by Spot 42 fully depends on the pairing with *araF*. The induction level of $P_{BAD}$-*recA-lacZ* in this strain increased (1.5-fold compared with that in WT *araF*), presumably reflecting the failure of endogenous Spot 42 to repress arabinose import (see below, Fig. 3B). In addition, we tested cells without the *araFGH* operon in the $P_{BAD}$-*recA-lacZ* background to determine how overproducing Spot 42 affects *recA-lacZ* expression (see Fig. S1 in the supplemental material). Consistent with the notion that AraFGH is the major transporter at low concentrations of arabinose, the deletion of *araFGH* drastically decreased (14-fold) *recA-lacZ* induction when 0.0002% arabinose was used (compare open bars in Fig. S1A). Overexpressing Spot 42 in the Δ*araFGH* strain reduced *recA-lacZ* expression 2.9-fold, much less than the 33.6-fold reduction in the *araFGH*$^+$ host, consistent with AraFGH as the major target for Spot 42-mediated inhibition of $P_{BAD}$ activity at low arabinose concentrations. The remaining repression in the Δ*araFGH* strain also suggests that, in the absence of AraFGH, Spot 42 likely has other targets in this regulon. Interestingly, region III′ of Spot 42, important for $P_{BAD}$-*recA-lacZ* repression (Fig. 1B), was completely dispensable for *araF-lacZ* repression (Fig. 2B), further indicating that Spot 42 might affect $P_{BAD}$ activity through base pairing with other unknown targets involved in arabinose transport or utilization. At higher concentrations of arabinose, the effect of deleting *araFGH* was much less dramatic (see Fig. S1B), which is consistent with participation from other transporters in arabinose entry.

**Endogenous Spot 42 sRNA is sufficient to affect *araF* expression during early exponential cell growth or growth in glucose medium.** In *E. coli*, the cellular levels of Spot 42 sRNA are high in the early exponential phase and low during the remaining phases of cell growth (12). To test if endogenous levels of Spot 42 are sufficient to affect *araF* expression, arabinose uptake, and thus *araBAD* promoter activity, we compared *araF-lacZ* expression in WT and Spot 42 deletion (Δ*spf*) strains grown in LB medium at exponential and stationary phases (blue bars in Fig. 3A and B). In early exponential phase, deletion of Spot 42 increased *araF-lacZ* expression by 2.2-fold compared with that of the WT (Fig. 3A, compare bars 1 and 2), whereas only a slight increase (1.3-fold) was observed in stationary phase (Fig. 3B, bars 1 and 2), consistent with high levels of Spot 42 in early exponential phase and low in stationary phase (Fig. 3C). Importantly, in the *araF*-lacZ* reporter strains, where WT Spot 42 cannot form complementary base pairing with *araF**, reporter expression in the WT Spot 42 background was 2.3-fold higher than that of *araF-lacZ* in exponential phase (Fig. 3A, bar 5 versus bar 1), and deletion of Spot 42 did not further increase *araF*-lacZ* expression (Fig. 3A, compare bar 6 with bar 5). A similar pattern was seen in stationary phase, and the modest increase seen for the *araF-lacZ* fusion (Fig. 3B, compare bar 2 with bar 1) was eliminated in the *araF*-lacZ* fusion (Fig. 3B, compare bar 6 with bar 5). In summary, deleting Spot 42 (Δ*spf*) and removing the pairing site (*araF**) in both growth stages similarly increased the expression of the fusion. Taken together, these results support a base-pairing interaction between Spot 42 and *araF* that is required for repression and is seen most dramatically in exponential phase, where levels of Spot 42 are high.

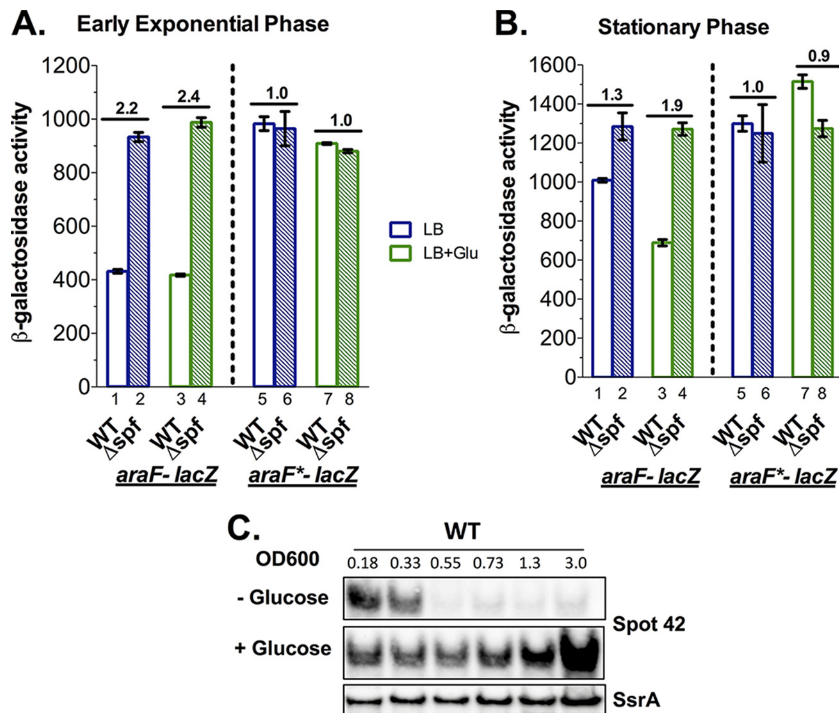


**FIG 3** Endogenous levels of Spot 42 sRNA are sufficient to repress *araF* expression. β-Galactosidase activity assays of *araF-lacZ* expression in WT (JC1100) and Δ*spf* (JC1109) strains and *araF*-lacZ* expression in WT (JC1102) and Δ*spf* (JC1110) strains in early exponential (A) and stationary (B) phases. Bacterial cells were grown at 37°C in LB or LB plus 0.2% glucose (LB+Glu) and assayed at an $OD_{600}$ of 0.3 (exponential phase) or 3.0 (stationary phase) for β-galactosidase activity. Results of assays are expressed in Miller units. Fold changes in β-galactosidase activity in Δ*spf* cells compared with that in the WT are shown above the bars. (C) Northern blot to measure Spot 42 sRNA levels during growth of JC1100 cells in the presence or absence of 0.2% glucose. *ssrA* mRNA served as a loading control.

The transcription of *spf*, encoding Spot 42, is negatively regulated by the cAMP-CRP complex and is highly induced in glucose medium (12) (Fig. 3C). We also tested how the endogenous Spot 42 affects *araF* expression in the same strains discussed above, when cells were grown in LB with 0.2% glucose (green bars in Fig. 3A and B). There was no significant effect of glucose on either Spot 42 levels (Fig. 3C) or Spot 42-dependent repression of *araF-lacZ* in early exponential phase (Fig. 3A). In the WT strain, *araF-lacZ* was expressed at the same level as in LB and was increased 2.4-fold with deletion of Spot 42 (Δ*spf*) in LB with glucose (Fig. 3A, compare bar 4 with bar 3), similar to the 2.2-fold increase in expression in LB (Fig. 3A, compare bar 2 with bar 1). In contrast, in stationary phase, Spot 42, which was barely detectable without glucose in the WT strain, was highly abundant in cells grown with glucose (Fig. 3C). Consistent with the higher level of Spot 42, the *araF-lacZ* fusion was expressed at a lower level than in LB (Fig. 3B, compare bar 3 with bar 1). Therefore, the deletion of *spf* had a larger effect, increasing the expression by 1.9-fold (Fig. 3B, compare bar 4 with bar 3). The effects of glucose on the *araF-lacZ* fusion were entirely abolished in the *spf* null mutant (Fig. 3B, compare bar 4 with bar 2). Notably, *araF*-lacZ* expression was not affected by the presence of glucose or Spot 42, and expression levels were similar to those of *araF-lacZ* cells deleted for *spf* (Fig. 3A and B, bars 7 and 8 compared with bars 2 and 4). Altogether, these results demonstrate that when levels of endogenous Spot 42 are high, either in early exponential phase or when cells are grown with glucose, Spot 42 is sufficient to repress *araF* expression in a sequence-specific manner.

Since either multicopy or endogenous Spot 42 was sufficient to repress *araF* expression, we further tested the effect of Spot 42 on *E. coli* cell adaptation and growth in low arabinose medium. We first compared the growth of *E. coli* transformed with vector or Spot 42 plasmids in rich (LB) medium and in minimal medium with low

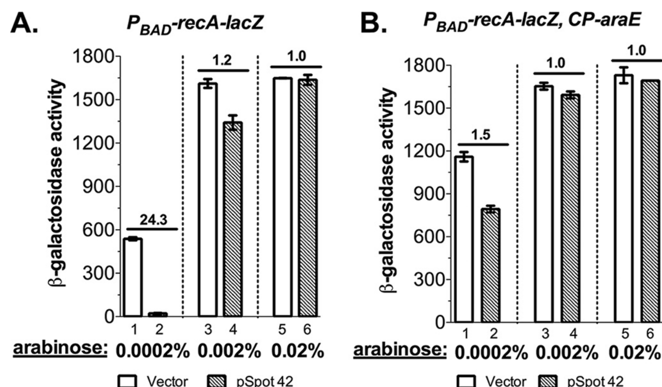


**FIG 4** High arabinose induction concentrations or constitutive expression of the AraE transporter is sufficient to prevent Spot 42 repression of the *araBAD* promoter. (A) β-Galactosidase activity assays of $P_{BAD}$-*recA-lacZ* expression in JC1005 harboring a plasmid encoding Spot 42 in the presence of 100 μM IPTG and increasing doses of arabinose (0.0002%, 0.002%, and 0.02%). (B) β-Galactosidase activity assays of *recA-lacZ* expression in a strain constitutively expressing the low-affinity high-capacity arabinose transporter AraE (JC1095) with the Spot 42 plasmid in the presence of 100 μM IPTG and increasing doses of arabinose (0.0002%, 0.002%, and 0.02%). Biological triplicates were assayed, and data are plotted as means ± SEMs. Results of assays are expressed in Miller units.

(0.0002%) or high (0.2%) arabinose. The growth of cells was unaffected by Spot 42 overexpression in LB (see Fig. S2A in the supplemental material), was slightly slower in minimal 0.2% arabinose medium (Fig. S2B), and was dramatically slower in minimal 0.0002% arabinose medium (Fig. S2C) compared with the growth of the vector controls and cells in which Spot 42 was not induced. This growth defect is fully consistent with the role of Spot 42 in controlling AraFGH-mediated arabinose transport at low levels of arabinose. In a second experiment, we tested the adaptations of WT and Δ*spf* cells when switched from minimal glucose to minimal arabinose medium (see Fig. S3). The Spot 42 deletion strain and the WT strain grew in identical manners after shifts from glucose minimal medium to either LB or minimal glucose medium. The Δ*spf* strain had a slight but insignificant growth advantage in 0.2% (high) arabinose medium. At the 0.0002% (low) arabinose concentration, there was a modest but statistically significant growth advantage of the cells devoid of Spot 42 (see Fig. S3A and B). This growth advantage of Δ*spf* cells is consistent with Spot 42 repressing the AraFGH transporter, causing a somewhat slower shift from glucose to arabinose.

**Bypass of Spot 42 sRNA effect on the $P_{BAD}$ system by high arabinose concentrations or by constitutive expression of the AraE arabinose transporter.** Our lab has successfully used the $P_{BAD}$ system to identify and test sRNA regulators (16, 18–20, 26), and we found that Spot 42 was not always repressive in these other cases, suggesting that something about the conditions used for $P_{BAD}$-*recA-lacZ* caused the problem. We noticed that in several cases, Spot 42 strongly affected $P_{BAD}$-driven genes at low concentrations of L-arabinose but direct base-pairing interactions were hard to verify (19; unpublished data). The translation of *recA* was apparently robust, and thus, low levels of arabinose (0.0002%) were used for induction (Fig. 1). AraFGH is a high-affinity low-capacity ABC family arabinose transporter and is very efficient at transporting low concentrations of arabinose. Once extracellular arabinose surpasses a threshold concentration, the low-affinity high-capacity transporter AraE takes over (3) (see Fig. S1B). We measured the expression of $P_{BAD}$-*recA-lacZ* under various concentrations of L-arabinose in the presence of multicopy Spot 42 (Fig. 4A). The 24-fold repression of *recA-lacZ* with Spot 42 overexpression in LB containing 0.0002% (13 μM) L-arabinose was largely eliminated (1.2-fold repression) with 0.002% (130 μM) L-arabinose and completely ablated with 0.02% (1.3 mM) L-arabinose (Fig. 4A). This arabinose dose-dependent control of *recA-lacZ* expression by Spot 42 is consistent with our finding that it is an indirect effect, caused by Spot 42 repression of AraF, inhibiting arabinose uptake (and thus induction of the

$P_{BAD}$-*recA-lacZ* fusion) at low levels of arabinose. This striking result also demonstrates that higher concentrations of arabinose might eliminate the effect on the expression of $P_{BAD}$-driven genes from Spot 42. Such higher concentrations were used in many of our previous studies (16, 18, 19, 26), explaining why this problem was not previously appreciated.

The arabinose-inducible promoter has an all-or-none expression pattern (1, 4), and constitutive expression of the low-affinity high-capacity AraE transporter overcomes this problem (6, 7). We tested if constitutive expression of the AraE symporter is sufficient to bypass the requirement for the AraFGH transporter, thus overcoming the inhibition by Spot 42 at low levels of arabinose. We introduced the *araE* gene controlled by the constitutive promoter CP18 (6) into the $P_{BAD}$-*recA-lacZ* reporter strain and measured the effect of Spot 42 on *recA-lacZ* repression under various concentrations of arabinose (Fig. 4B). Constitutive expression of *araE* largely eliminated the inhibition on $P_{BAD}$-*recA-lacZ* expression by Spot 42 even at the very low concentration (0.0002%) of L-arabinose (compare 1.5-fold repression in Fig. 4B [bar 2 versus bar 1] with 24-fold repression in Fig. 4A [bar 2 versus bar 1]). When induced with higher concentrations of arabinose (0.002% and 0.02%), *recA-lacZ* expression was completely independent of Spot 42 expression (Fig. 4B). Induction of the $P_{BAD}$ promoter was also better at the very low arabinose concentration in the *araE* constitutive strain, which is reflected by the higher level of $P_{BAD}$-*recA-lacZ* expression (compare bar 1 in Fig. 4B with bar 1 in Fig. 4A). In both strains, 0.002% (130 $\mu$M) arabinose was sufficient for full induction (Fig. 4A and B, bars 3). Altogether, these data illustrate that we can manage the side effect of Spot 42 sRNA on $P_{BAD}$-driven genes either by using higher concentrations of arabinose for induction or by working in a strain that constitutively expresses the AraE symporter.

## DISCUSSION

Here we described the unexpected finding that Spot 42 translational repression of *araFGH*, encoding the high-affinity arabinose transporter, perturbs induction of the $P_{BAD}$ system in *E. coli* at subsaturating concentrations of the inducer. Induction of the $P_{BAD}$ system with either saturating concentrations of inducers or constitutive expression of the low-affinity high-capacity transporter AraE is sufficient to overcome the effect of Spot 42.

The *araF* gene encodes the periplasmic arabinose-binding protein of the high-affinity low-capacity arabinose transporter AraFGH and is the first gene in the operon encoding all components of this system. Our results are in good agreement with the consensus role of Spot 42 sRNA in controlling the utilization of alternative carbon sources. In the presence of glucose, the preferred carbon source, Moller et al. (27) demonstrated that highly induced Spot 42 downregulates GalK, the galactokinase in the *galETKM* operon essential for galactose catabolism, through base pairing with the *galK* mRNA Shine-Dalgarno sequence, thereby blocking *galK* translation. More recently, Beisel and Storz expanded the role of Spot 42 in carbon catabolite repression by identifying Spot 42-repressed genes from multiple operons involved in the uptake and utilization of nonfavored carbon sources, including xylose, fucose, galactose, sorbitol, and lactate (13). As the cAMP-CRP complex activates many of these sugar utilization genes and also represses *spf* (Spot 42 encoding gene), these regulatory pathways form a feedforward loop where Spot 42 reduces the leaky expression of target genes in glucose medium and contributes to expression dynamics of target genes under changing nutrient conditions. By identifying the high-affinity arabinose transporter as a new target of Spot 42, our finding further expands the repertoire of Spot 42-controlled carbon utilization genes, reinforcing it as a global RNA regulator for central carbon metabolism. It seems quite possible that the use of other catabolite-repressed promoters with coregulated permeases is also subject to Spot 42 regulation in *E. coli* and related bacteria. For instance, it was shown that the xylose ABC transporter *xylF* is repressed by Spot 42 (13). Because many of these genes are not well expressed in the

absence of the inducing sugar, they may not be detected in most experiments, as was the case for the *araFGH* operon in previous studies (13, 23).

The $P_{BAD}$ system is a popular tool for inducible gene expression (28), and understanding the regulatory details will help improve the use of this system. It was previously recognized that one of the issues for the $P_{BAD}$ system was the all-or-none induction pattern at low concentrations of inducers (4). This problem can be solved by decoupling the transport of the inducer from the induction of the transporter genes (*araE* and *araFGH*). This has been achieved via constitutive expression of the transporter genes (6, 7) and by expressing a promiscuous LacY mutant (LacYA177C) transporter with relaxed specificity in an *araE*, *araFGH*, and *araBAD*-null strain (29). We demonstrated here that Spot 42 sRNA downregulates the high-affinity transporter AraFGH, thus affecting $P_{BAD}$ activity at low concentrations of inducers. To produce recombinant proteins with the $P_{BAD}$ expression system, high concentrations of an inducer will likely be used, and thus the effect of endogenous Spot 42 on AraF will be subtle (Fig. 3). However, for experiments in which levels of Spot 42 are high (early exponential phase or growth in glucose medium), endogenous levels of Spot 42 will reinforce the all-or-none behavior of the *ara* regulon by repressing basal levels of *araFGH* expression and thus may confound the interpretation of effects expected from the $P_{BAD}$-controlled protein. Many other sugar utilization pathways are regulated by Spot 42 (13), and some of them (e.g., D-xylose and L-rhamnose) also show all-or-none responses (30). Thus, it is very likely that Spot 42 also contributes to complex single-cell behaviors in these pathways. Our results also suggest that Spot 42-mediated inhibition of $P_{BAD}$ at low inducer concentrations may involve other components of the arabinose utilization system. This is suggested by the observation that the III′ mutant of Spot 42 interfered with repression (Fig. 1B), although it did not affect *araF* repression (Fig. 2B) and moderately repressed $P_{BAD}$-*recA-lacZ* in cells lacking *araFGH* (see Fig. S1A). However, these other effects were not seen when the AraFGH system was resistant to Spot 42 (Fig. 2D), suggesting that Spot 42 likely affects the expression of lower-affinity transporters. Nevertheless, given the effectiveness of constitutive *araE* expression for homogeneous induction of the $P_{BAD}$ genes (6, 7) as well as bypassing Spot 42 effects, incorporating constitutive expression of arabinose transporters is likely to be the best general solution.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The bacterial strains used in this study are derivatives of the *E. coli* K-12 strain MG1655. Bacterial strains and plasmids are listed in Table S1 in the supplemental material. Primers, probes, and synthetic gene fragments are listed in Table S2. Spot 42 deletion strains and *araE* constitutive expression strains were constructed using phage P1*vir* transductions as previously described by Miller (31). The chromosomal inducible translational fusion $P_{BAD}$-*recA-lacZ* reporter strain (JC1005) was generated by λ Red recombineering in strain PM1805 with PCR products of the *recA* 5′ UTR and the first 10 codons (nt −50 to 30 relative to the ATG) amplified using oligonucleotides JC33/JC28 and selected on sucrose minimal agar plates (32). The constitutive CP12b-*recA-lacZ* reporter strain (JC1058) was generated by replacing the *araBAD* promoter in JC1005 with a *kan*-CP12b gene fragment amplified from JC1051 using primers JC63/JC71 and selected on LB agar plates with kanamycin (Kan). The CP12b-*araF-lacZ* (JC1100) and CP12b-*araF*-lacZ* (JC1102) reporter strains were generated using synthetic DNA gene fragments, gblocks (IDT, Coralville, IA) named $uP_{BAD}$-*zeo*-CP12b-*araF-lacZ* and $uP_{BAD}$-*zeo*-CP12b-*araF*-lacZ* (DNA sequences are listed in Table S2), which contain the *araF* 5′ UTR and the first 10 codons (nt −110 to 30 relative to the ATG). These were introduced into PM1805 for recombineering (32, 33) and selected on LB agar plates with zeocin (Zeo). These gblocks contain a sequence of 40 bp homologous to the region upstream of $P_{BAD}$ ($uP_{BAD}$) in PM1805, enabling replacement of $P_{BAD}$ with the *zeo*-CP12b cassette. The Δ*araFGH* derivative of JC1005 (JC1180) was generated via λ Red recombineering in JC1005 with a PCR product amplified from JCS1001, using oligonucleotides JC149/JC150, and selected on LB agar plates with Kan. Derivatives of JC1005 harboring the chromosomal WT *araF* (JC1191) or specificity mutant *araF** (JC1192) were constructed by homologous recombination of PCR products amplified from JC1100 or JC1102, respectively, using oligonucleotides JC164/JC165 into JC1186. JC1186 is a derivative of JC1005 in which a *kan-sacB* cassette, amplified from NM543 using oligonucleotides JC167/JC163, was inserted in the native *araF* leader. This enabled the counterselection of final recombinants containing *araF* or *araF** on minimal sucrose agar. All chromosomal constructs in the reporter strains were verified by PCR and Sanger sequencing.

sRNA genes in the library were cloned in the pBRplac vector plasmid under the control of an isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible promoter (16). Various Spot 42 sRNA mu-

tants were either obtained from G. Storz (NIH) or generated by QuikChange mutagenesis as described by Beisel et al. (13, 15). Specifically, the pSpot 42 I + III′ mutant was constructed by QuikChange site-directed mutagenesis (Agilent Technologies) using pSpot 42 I as the DNA template and Spot 42-III′.fwd/Spot 42-III′.rev as primers (15). The construct was verified by Sanger sequencing.

All bacterial strains were grown in Lennox broth medium or M63 salt (KD Medical, Columbia, MD). The M63 medium was supplemented with 0.001% vitamin $B_1$, 0.0001% biotin, 0.1% Casamino Acids, and either glucose or arabinose as the carbon source, as indicated. When needed, glucose was supplemented at a final concentration of 0.2%. A 0.0002% (13 $\mu$M) concentration of arabinose was used for $P_{BAD}$ induction except where otherwise indicated, and 50 $\mu$M or 100 $\mu$M IPTG was used for sRNA induction. The following concentrations of antibiotics were used: ampicillin, 100 $\mu$g/ml; chloramphenicol, 10 $\mu$g/ml; kanamycin, 25 $\mu$g/ml; and zeocin, 25 $\mu$g/ml.

**sRNA library screen and $\beta$-galactosidase activity assay.** The sRNA overexpression library screen was carried out as described by Mandin and Gottesman (16). Briefly, 29 *E. coli* sRNAs were individually transformed into the $P_{BAD}$-*recA*-*lacZ* reporter strain JC1005 using the transformation and storage solution (TSS) method (34). Transformants were inoculated into a 96-well microtiter plate containing 100 $\mu$l LB plus 100 $\mu$g/ml ampicillin, 0.0002% arabinose, and 50 $\mu$M IPTG to induce expression of the sRNAs and reporter gene and were grown at 37°C for 6 h with shaking at 225 rpm. The optical densities of the cells at 600 nm ($OD_{600}$) were measured at the endpoint, and bacterial cells were permeabilized by adding 50 $\mu$l permeabilization buffer (100 mM Tris-HCl [H 7.8], 32 mM $Na_2HPO_4$, 8 mM dithiothreitol [DTT], 8 mM EDTA, 4% Triton X-100, 200 $\mu$g/ml polymyxin B). The $\beta$-galactosidase activities were measured as a function ($V_{max}$) of $OD_{420}$ versus time after the addition of 50 $\mu$l of an *o*-nitrophenyl-$\beta$-D-galactopyranoside (ONPG; 4 mg/ml) solution by using the SpectraMax 250 microtiter plate reader (Molecular Devices) as described by Majdalani et al. (35) and Zhou et al. (36). The final $\beta$-galactosidase activities for this assay were determined as specific units calculated as the $V_{max}$ normalized to $OD_{600}$. These units, used only in Fig. 1A, are about 25 times smaller than Miller units.

*lacZ* expression in all other experiments, in various reporter strains, was determined by $\beta$-galactosidase activity assay as described by Miller (31). Unless otherwise noted, bacterial cultures were assayed at an $OD_{600}$ of ~3.0. Biological triplicates were assayed, and data were plotted as means ± standard errors (SEMs).

**RNA extraction and Northern blot analysis.** To measure the accumulation of endogenous Spot 42 sRNA during cell growth, overnight cultures were diluted 500-fold into fresh LB medium in the presence or absence of 0.2% glucose and incubated at 37°C with shaking at 225 rpm. A 700-$\mu$l sample was collected from each culture when the $OD_{600}$ reached ~0.2, and then additional samples were taken every 15 min until cells reached an $OD_{600}$ of ~3.0. To determine the effect of ectopic expression of Spot 42 on endogenous *recA* mRNA levels, cells carrying vector plasmid control (pBRplac) or pSpot 42, in which Spot 42 is expressed from the *lac* promoter, were induced with IPTG (100 $\mu$M) at an $OD_{600}$ of ~0.4, and a 700-$\mu$l sample was collected at time zero and at 1, 5, and 10 min postinduction. Cellular total RNA was extracted from each sample using the hot phenol method as described by Masse et al. (37) and probed with the biotinylated *recA* probe (see Table S2).

Northern blot analyses of Spot 42 sRNA were conducted as previously described (38). Briefly, 3 $\mu$g of total RNA from each sample were resolved on a Bio-Rad Criterion 10% Tris-borate-EDTA (TBE)–urea polyacrylamide gel in 1× TBE buffer at 100 V for 1 h. The resolved RNA samples were then transferred to a Zeta-probe GT membrane (Bio-Rad) by wet electroblotting at 200 mA for 2 h in 0.5× TBE and cross-linked to the membrane by UV irradiation. The resulting membrane was hybridized with the biotinylated Spot 42 and *ssrA* probes (see Table S2) in ULTRAhyb solution (Ambion) at 42°C overnight and further incubated with a streptavidin-conjugated alkaline phosphatase. The blot was then developed using the BrightStar BioDetect kit (Ambion) according to the manufacturer's instructions. Northern blot to detect *recA* mRNA was performed as previously described (38). Northern blots were imaged by capturing the chemifluorescence using the image analyzer LAS-3000 (Fujifilm Life Science).

**Western blotting.** Western blotting to determine the endogenous RecA protein levels was performed using standard procedures. Bacterial cells were diluted 500-fold from overnight cultures into fresh LB plus 0.2% glucose medium. For overexpressing sRNAs from plasmids, ampicillin and IPTG (100 $\mu$M) were added to the medium.

Bacterial cultures were collected at an $OD_{600}$ of ~0.9, and a volume equivalent to 1 ml of cells at an $OD_{600}$ of 1 was pelleted, washed once with phosphate-buffered saline (PBS) (KD Medical), and lysed by adding 100 $\mu$l of 1× SDS sample buffer (New England BioLabs) and boiling for 10 min. A 10-$\mu$l sample of the supernatant was resolved on a 12% bis-Tris polyacrylamide NuPAGE gel (Invitrogen) in 1× morpholinepropanesulfonic acid (MOPS) buffer (Invitrogen). Protein samples were then transferred onto a 0.2-$\mu$m nitrocellulose membrane using an iBlot gel transfer device (Invitrogen). The membrane was blocked for 30 min in PBS with 0.1% Tween 20 (PBST) and 5% nonfat dry milk and probed with rabbit polyclonal anti-RecA antibodies (Abcam; 1:1,000) in PBST with 5% nonfat dry milk at room temperature for 1 h. After three PBST washes, the membrane was probed with alkaline phosphatase-conjugated anti-rabbit IgG antibodies (Cell Signaling Technology; 1:2,500) for another hour. The membrane was washed three additional times with PBST and then developed using the Novex AP chemiluminescent substrate (Thermo Fisher Scientific). Western images were acquired by capturing the chemifluorescence using the image analyzer LAS-3000 (Fujifilm Life Science).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00691-16.

**TEXT S1,** PDF file, 0.4 MB.

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