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The *Escherichia coli rhaSR-PrhaBAD* Inducible Promoter System Allows Tightly Controlled Gene Expression over a Wide Range in *Pseudomonas aeruginosa*

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ABSTRACT The *araC-ParaBAD* inducible promoter system is tightly controlled and allows gene expression to be modulated over a wide range in *Escherichia coli*, which has led to its widespread use in other bacteria. Although anecdotal evidence suggests that *araC-ParaBAD* is leaky in *Pseudomonas aeruginosa*, neither a thorough analysis of this inducible promoter system in *P. aeruginosa* nor a concerted effort to identify alternatives with improved functionality has been reported. Here, we evaluated the functionality of the *araC-ParaBAD* system in *P. aeruginosa*. Using transcriptional fusions to a *lacZ* reporter gene, we determined that the noninduced expression from *araC-ParaBAD* is high and cannot be reduced by carbon catabolite repression as it can in *E. coli*. Modulating translational initiation by altering ribosome-binding site strength reduced the noninduced activity but also decreased the maximal induced activity and narrowed the induction range. Integrating the inducible promoter system into the posttranscriptional regulatory network that controls catabolite repression in *P. aeruginosa* significantly decreased the noninduced activity and increased the induction range. In addition to these improvements in the functionality of the *araC-ParaBAD* system, we found that the *lacIq-Ptac* and *rhaSR-PrhaBAD* inducible promoter systems had significantly lower noninduced expression and were inducible over a broader range than *araC-ParaBAD*. We demonstrated that noninduced expression from the *araC-ParaBAD* system supported the function of genes involved in antibiotic resistance and tryptophan biosynthesis in *P. aeruginosa*, problems that were avoided with *rhaSR-PrhaBAD*. *rhaSR-PrhaBAD* is tightly controlled, allows gene expression over a wide range, and represents a significant improvement over *araC-ParaBAD* in *P. aeruginosa*.

IMPORTANCE We report the shortcomings of the commonly used *Escherichia coli araC-ParaBAD* inducible promoter system in *Pseudomonas aeruginosa*, successfully reengineered it to improve its functionality, and show that the *E. coli rhaSR-PrhaBAD* system is tightly controlled and allows inducible gene expression over a wide range in *P. aeruginosa*.

Pseudomonas aeruginosa is a versatile Gram-negative bacterium that inhabits a variety of different environments. It is also an opportunistic human pathogen that causes acute infections in hospitalized patients as well as chronic infections in cystic fibrosis patients. Unfortunately, *P. aeruginosa* infections are becoming difficult to treat because of the increasing prevalence of multidrug (antibiotic) resistance (1). To improve the treatment of these infections, we need to understand which gene functions are essential for the growth of *P. aeruginosa* and develop new therapeutics to inhibit them. The study of essential genes is difficult because, by definition, inactivation of an essential gene is lethal to the cell. Analysis of essential genes generally involves the construction of conditional mutants, often accomplished by controlling the expression of a gene with an inducible promoter. Inducible promoters allow the transcription of a gene to be turned on and modulated by the addition of an inducer as well as turned off when the inducer is removed. The inability to turn off gene expression can make it difficult to determine the function of a gene, particularly when low-level expression is sufficient for gene function. Inducible promoters should also allow expression over a wide range so that the induced expression can both match the native expression level and exceed it when overexpression of the gene is desired.

In the model bacterium *Escherichia coli*, the *araC-ParaBAD* inducible promoter system satisfies these criteria (2, 3). In the absence of arabinose, transcription from the *araBAD* promoter is repressed by the regulatory protein AraC. When AraC binds arabinose, it is repositioned at the *araBAD* promoter and activates transcription (4). The *araBAD* promoter is also controlled by carbon catabolite repression that prevents the transcription of genes necessary for the metabolism of less-preferred carbon sources (such as arabinose) when a preferred one (glucose) is available (5, 6). In the absence of glucose, the EIIA component of the glucose-specific phosphotransferase system (EIIA^{Glc}) is phosphorylated and stimulates adenylate cyclase to produce cyclic AMP (cAMP). The transcriptional activator protein CRP (also called the catabo-

lite activator protein, CAP) binds cAMP and activates the *araBAD* promoter. In the presence of glucose, EIIA^{Glc} transfers its phosphate to glucose as it is transported across the cell envelope and into the cell. Nonphosphorylated EIIA^{Glc} cannot stimulate adenylate cyclase, cAMP is not produced, and CRP cannot activate the *araBAD* promoter. As a result of these regulatory factors, gene expression from the *araBAD* promoter can be induced, and modulated over a wide range, by the addition of arabinose ([3]). In the absence of arabinose, the noninduced expression from the promoter is low, and it can be reduced even further by the addition of glucose. This tight control is arguably the most important feature of the inducible promoter system, particularly in the study of essential genes where high noninduced expression (leakiness) can prevent the determination of gene function. Promoter leakiness can also obscure the phenotype associated with any gene, whether essential or nonessential, when only a small amount of gene product is necessary for gene function.

The recognition of the tight control of the *araC-ParaBAD* system has prompted its use in other bacteria, including *P. aeruginosa*. While the basic regulatory features of AraC are preserved in *P. aeruginosa*, carbon catabolite repression is fundamentally different ([7]). Instead of utilizing a phosphotransferase system, *P. aeruginosa* imports glucose through an ATP-binding cassette transporter. Without EIIA^{Glc}, adenylate cyclase is not stimulated to produce cAMP in the absence of glucose. In fact, cAMP levels do not change when *P. aeruginosa* is grown with different carbon sources ([8]), and carbon source preference, the organizing principle behind catabolite repression, is different in *P. aeruginosa*. *P. aeruginosa* prefers to catabolize amino acids and organic acids rather than sugars, such as glucose ([9], [10]). The regulatory factors that enforce catabolite repression in *P. aeruginosa* are Hfq, Crc, CbrAB, and CrcZ ([7], [11]). Hfq binds mRNA necessary for the assimilation of alternative carbon sources near the ribosome-binding site (RBS) and inhibits the formation of the translation initiation complex. The small regulatory RNA CrcZ modulates Hfq availability. It contains five Hfq-binding sites and can sequester Hfq. The CbrAB two-component transcriptional regulatory system controls the amount of CrcZ according to the carbon source being used ([12]).

Despite these fundamental differences in catabolite repression, the *araC-ParaBAD* system has been used effectively to induce the expression of several genes in *P. aeruginosa* ([13]–[17]). In various cases, however, we and others have noticed that *araC-ParaBAD* may not be as tightly controlled in *P. aeruginosa* as in *E. coli*. This motivated us to examine whether *araC-ParaBAD* meets the same desired criteria in *P. aeruginosa* that provoked its widespread use in *E. coli* and other bacteria. To this end, we assessed the behavior of the *araC-ParaBAD* system in *P. aeruginosa*. We found that transcription from *araC-ParaBAD* was high in the absence of inducer and could not be decreased by catabolite repression. We then sought to improve the functionality of the system and compare it to the *lacI^q-Ptac* and *rhaSR-PrhaBAD* inducible promoter systems.

MATERIALS AND METHODS

General methods. The strains, plasmids, and oligonucleotide primers used in this study are listed in Tables 1, 2, and 3, respectively. Strains were grown in lysogeny broth (LB; 1% tryptone, 0.5% yeast extract, 1% sodium chloride) and M9 minimal medium (48 mM sodium phosphate dibasic, 22 mM potassium phosphate monobasic, 8.6 mM sodium chloride, 19

TABLE 1 Strains

Strain	Genotype
P. aeruginosa	
PA103	Serotype O11
PAO1	Serotype O5
PA14	Serotype O10
PAJM91	PA103 *attTn7::araC-ParaBAD-stRBS-lacZ frt*
PAJM423	PA103 *attTn7::araC-ParaBAD-intRBS-lacZ frt*
PAJM395	PA103 *attTn7::araC-ParaBAD-wkRBS-lacZ frt*
PAJM430	PA103 *attTn7::araC-ParaBAD-5′ UTR amiE-lacZ frt*
PAJM93	PA103 *attTn7::lacI^q-Ptac-stRBS-lacZ frt*
PAJM207	PA103 *attTn7::rhaSR-PrhaBAD-stRBS-lacZ frt*
PAJM95	PA103 *attTn7::stRBS-lacZ frt*
PAJM14	PA103 *attTn7::frt*
PAJM283	PA103 *attTn7::araC-ParaBAD-stRBS-aacC1 frt*
PAJM285	PA103 *attTn7::rhaSR-PrhaBAD-stRBS-aacC1 frt*
PAJM290	PA103 *attCTX::tet*
PAJM302	PA103 *attCTX::araC-ParaBAD-stRBS-aacC1 tet*
PAJM304	PA103 *attCTX::rhaSR-PrhaBAD-stRBS-aacC1 tet*
PAJM291	PAO1 *attCTX::tet*
PAJM308	PAO1 *attCTX::araC-ParaBAD-stRBS-aacC1 tet*
PAJM310	PAO1 *attCTX::rhaSR-PrhaBAD-stRBS-aacC1 tet*
PAJM292	PA14 *attCTX::tet*
PAJM314	PA14 *attCTX::araC-ParaBAD-stRBS-aacC1 tet*
PAJM316	PA14 *attCTX::rhaSR-PrhaBAD-stRBS-aacC1 tet*
PAJM259	PA14 *trpF::TnMar gm*
PAJM297	PA14 *trpF::TnMar gm attCTX::araC-ParaBAD-trpF tet*
PAJM326	PA14 *trpF::TnMar gm attCTX::rhaSR-PrhaBAD-trpF tet*
PAJM258	PA14 *trpC::TnMar gm*
PAJM295	PA14 *trpC::TnMar gm attCTX::araC-ParaBAD-trpC tet*
PAJM324	PA14 *trpC::TnMar gm attCTX::rhaSR-PrhaBAD-trpC tet*
PAJM235	PA14 *trpA::TnMar gm*
PAJM293	PA14 *trpA::TnMar gm attCTX::araC-ParaBAD-trpA tet*
PAJM322	PA14 *trpA::TnMar gm attCTX::rhaSR-PrhaBAD-trpA tet*
E. coli	
DH5α	F$^-$ *endA1 glnV42 thi-1 recA1 relA1 gyrA96 deoR nupG* φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *hsdR17*(r$_K$$^-$ m$_K$$^+$) λ$^-$
SM10	*thi-1 the leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r
HB101	F$^-$ *mcrB mrr hsdS20*(r$_B$$^-$ m$_B$$^-$) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*(Sm^r) *glnV44* λ$^-$

mM ammonium chloride, 2.0 mM magnesium sulfate, 0.1 mM calcium chloride) with the indicated carbon sources. When necessary for strain construction, *E. coli* strains were grown in medium supplemented with 100 μg/ml ampicillin, 30 μg/ml kanamycin, or 10 μg/ml tetracycline; *P. aeruginosa* strains were grown in medium supplemented with 30 μg/ml gentamicin, 25 μg/ml tetracycline, or 250 μg/ml carbenicillin.

Plasmid construction. Plasmid pUC18T-miniTn7T-gm ([18]) provided the backbone for the miniTn7 plasmids constructed in this study. The *araC-ParaBAD* sequence was amplified by PCR from pHERD20T ([15]) with oligonucleotide primers oJM400 and oJM401. The *lacI^q-Ptac* sequence was amplified by PCR from pMMB66HE ([19]) with oligonucleotide primers oJM402 and oJM403. The *rhaSR-PrhaBAD* sequence was amplified by PCR from *E. coli* strain W3110 genomic DNA with oligonucleotide primers oJM641 and oJM642. The pUC18T-miniTn7T-gm plasmid and PCR products were cut with SacI and PstI and then ligated to make pUC18T-miniTn7T-gm-araC-ParaBAD (pJM100), pUC18T-miniTn7T-gm-lacI^q-Ptac (pJM101), and pUC18T-miniTn7T-gm-rhaSR-PrhaBAD (pJM220), respectively. The plasmids were confirmed by PCR with oligonucleotide primers oJM414 and oJM415. DNA fragments cloned into pJM100, pJM101, or pJM220 plasmid were sequenced with oJM414 and oJM457, oJM551, or oJM730, respectively.

TABLE 2 Plasmids

Plasmid	Description or reference
pTNS3	46
pRK2013	47
pUC18T-miniTn7T-gm	48
pHERD20T	15
pMMB66HE	19
miniCTX1	22
pFLP2	24
pJM100	pUC18T-miniTn7T-gm-araC-ParaBAD (accession no. KX787911)
pJM101	pUC18T-miniTn7T-gm-lacI^q-Ptac (accession no. KX782328)
pJM220	pUC18T-miniTn7T-gm-rhaSR-PrhaBAD (accession no. KX777256)
pJM181	pUC18T-miniTn7T-gm-stRBS-lacZ
pJM179	pUC18T-miniTn7T-gm-araC-ParaBAD-stRBS-lacZ
pJM180	pUC18T-miniTn7T-gm-lacI^q-Ptac-stRBS-lacZ
pJM230	pUC18T-miniTn7T-gm-rhaSR-PrhaBAD-stRBS-lacZ
pJM299	pUC18T-miniTn7T-gm-araC-ParaBAD-intRBS-lacZ
pJM289	pUC18T-miniTn7T-gm-araC-ParaBAD-wkRBS-lacZ
pJM301	pUC18T-miniTn7T-gm-araC-ParaBAD-5′ UTR amiE
pJM302	pUC18T-miniTn7T-gm-araC-ParaBAD-5′ UTR amiE-lacZ
pJM238	pUC18T-miniTn7T-gm-araC-ParaBAD-stRBS-aacC1
pJM240	pUC18T-miniTn7T-gm-rhaSR-PrhaBAD-stRBS-aacC1
pJM251	miniCTX1-araC-ParaBAD (accession no. KX787912)
pJM252	miniCTX1-lacI^q-Ptac (accession no. KX782329)
pJM253	miniCTX1-rhaSR-PrhaBAD (accession no. KX782327)
pJM259	miniCTX1-araC-ParaBAD-stRBS-aacC1
pJM260	miniCTX1-rhaSR-PrhaBAD-stRBS-aacC1
pJM256	miniCTX1-araC-ParaBAD-PA14 trpF
pJM268	miniCTX1-rhaSR-PrhaBAD-PA14 trpF
pJM255	miniCTX1-araC-ParaBAD-PA14 trpC
pJM267	miniCTX1-rhaSR-PrhaBAD-PA14 trpC
pJM254	miniCTX1-araC-ParaBAD-PA14 trpA
pJM266	miniCTX1-rhaSR-PrhaBAD-PA14 trpA

To construct miniTn7 plasmids with *lacZ* transcriptional fusions, the *lacZ* sequence was amplified by PCR from miniCTX-lacZ (20) with oligonucleotide primers oJM524 and oJM456. oJM524 introduces a strong ribosome-binding site (stRBS; 5′-TAAGGAGG-3′) with a 7-bp spacer sequence between the RBS and start codon. The *lacZ* PCR product, as well as pUC18T-miniTn7T-gm, pJM100, pJM101, and pJM220 plasmids, were cut with PstI and HindIII and then ligated to make pUC18T-miniTn7T-gm-stRBS-lacZ (pJM181), pUC18T-miniTn7T-gm-araC-ParaBAD-stRBS-lacZ (pJM179), pUC18T-miniTn7T-gm-lacI^q-Ptac-stRBS-lacZ (pJM180), and pUC18T-miniTn7T-gm-rhaSR-PrhaBAD-stRBS-lacZ (pJM230), respectively. To build derivatives with reduced RBS strength, pUC18T-miniTn7T-gm-araC-ParaBAD-intRBS-lacZ (pJM299) and pUC18T-miniTn7T-gm-araC-ParaBAD-stRBS-lacZ (pJM289), the *lacZ* sequence was amplified by PCR with oligonucleotide primers oJM741 or oJM727, respectively, and with oJM456. oJM741 introduces an intermediate-strength RBS (intRBS) with an 8-bp spacer sequence between the RBS and start codon. oJM727 introduces a weak RBS (wkRBS) with a 7-bp spacer sequence between the RBS and start codon. Both PCR products were cut with PstI and HindIII and then ligated with pJM100 as described above.

To build a miniTn7-araC-ParaBAD plasmid with the 5′ untranslated region (UTR) of *amiE*, oligonucleotides oJM744 and oJM745 were annealed and then ligated with pJM100 that had been cut with SpeI and PstI to make pUC18T-miniTn7T-gm-araC-ParaBAD-5′ UTR amiE (pJM301). The annealed oJM744-oJM745 DNA fragment has an NcoI site that overlaps with the *amiE* start codon to simplify the construction of translational fusions. To construct a derivative with a *lacZ* translational fusion, the *lacZ* sequence was amplified by PCR from miniCTX-lacZ with oligonucleotides oJM748 and oJM749. The PCR product was then

ligated into pJM301 that had been cut with NcoI and HindIII by isothermal assembly (21) to make pUC18T-miniTn7T-gm-araC-ParaBAD-5′ UTR amiE-lacZ (pJM302).

To build miniTn7 plasmids with *aacC1* (*gm*, gentamicin acetyltransferase) transcriptional fusions, the *aacC1* sequence was amplified by PCR from pUC18T-miniTn7T-gm with oligonucleotide primers oJM669 and oJM670. oJM679 introduces a strong RBS (as described above). The *aacC1* PCR product, as well as pJM100 and pJM220 plasmids, were cut with PstI and HindIII and then ligated to make pUC18T-miniTn7T-gm-araC-ParaBAD-stRBS-aacC1 (pJM238) and pUC18T-miniTn7T-gm-rhaSR-PrhaBAD-stRBS-aacC1 (pJM240), respectively.

To make the miniCTX derivatives of each of these plasmids, miniCTX1 plasmid (22) as well as pJM100, pJM101, pJM220, pJM238, and pJM240 plasmids were cut with SacI and HindIII. The SacI-HindIII miniCTX1 and released DNA fragments were then ligated to produce miniCTX1-araC-ParaBAD (pJM251), miniCTX1-lacI^q-Ptac (pJM252), miniCTX1-rhaSR-PrhaBAD (pJM253), miniCTX1-araC-ParaBAD-stRBS-aacC1 (pJM259), and miniCTX1-rhaSR-PrhaBAD-stRBS-aacC1 (pJM260), respectively.

To make miniCTX plasmids for complementation of *trp* mutants, the *trpF*, *trpC*, and *trpA* sequences were amplified by PCR from *P. aeruginosa* strain PA14 genomic DNA with oligonucleotide primers oJM681 and oJM682, oJM679 and oJM680, and oJM676 and oJM677, respectively. Each PCR product contains the native RBS from each *trp* gene. The PCR products, as well as pJM251 and pJM253 plasmids, were cut with SpeI and HindIII and then ligated to make miniCTX1-araC-ParaBAD-PA14 trpF (pJM256), miniCTX1-rhaSR-PrhaBAD-PA14 trpF (pJM268), miniCTX1-araC-ParaBAD-PA14 trpC (pJM255), miniCTX1-rhaSR-PrhaBAD-PA14 trpC (pJM267), miniCTX1-araC-ParaBAD-PA14 trpA (pJM254), and miniCTX1-rhaSR-PrhaBAD-PA14 trpA (pJM266). DNA fragments cloned into miniCTX plasmids were confirmed with oligonucleotide primers oJM691 and oJM703. The cloned DNA fragments were sequenced with oJM703 and oJM457 (miniCTX1-araC-ParaBAD, pJM251) or oJM730 (miniCTX1-rhaSR-PrhaBAD, pJM253).

Conjugations. *P. aeruginosa* recipient strains, as well as *E. coli* donor and helper strains, were grown in 3 ml LB (with antibiotic when appropriate) at 37°C with rolling for about 8 h. One milliliter of each culture was centrifuged at 8,000 × *g* for 2 min in microcentrifuge tubes. The culture supernatants were aspirated, cell pellets were resuspended in 1 ml LB, and cell suspensions were centrifuged. Aspiration, resuspension, and centrifugation were repeated. The supernatant was aspirated and cell pellets were resuspended in 35 μl LB. Cell suspensions were spotted onto LB agar and incubated at 37°C overnight. The cells were scraped off and resuspended in LB and serially diluted 10-fold, and 100 μl of each dilution was spread on Vogel-Bonner minimal medium (VBMM; 10 mM sodium citrate tribasic, 9.5 mM citric acid, 57 mM potassium phosphate dibasic, 17 mM sodium ammonium phosphate, 1 mM magnesium sulfate, 0.1 mM calcium chloride, pH 7.0) agar with antibiotic (gentamicin or tetracycline) and incubated at 37°C overnight. Chromosomal integration of miniTn7 was confirmed by PCR with oligonucleotide primers oJM473 and oJM414, while miniCTX integration was confirmed with oDHL12 and oDHL13.

Electroporations. Recipient strains were grown in 3 ml LB in duplicate at 37°C with rolling for about 8 h. The two 3-ml cultures were pooled and then dispensed into four microcentrifuge tubes. The cultures were centrifuged at 8,000 × *g* for 2 min. Each cell pellet was resuspended in 1 ml 300 mM sucrose and centrifuged twice (23). The four cell pellets were resuspended and pooled in a total of 300 μl of 300 mM sucrose. One hundred microliters of each suspension was transferred to 1-mm-gap-width electroporation cuvettes. One hundred nanograms of pFLP2 plasmid was added to each suspension. Cells were electroporated at 1,800 V in an Eppendorf electroporator 2510. Nine hundred microliters of LB was added to each electroporation. Recovery cultures were incubated at 37°C with rolling for 1 h. Cultures were serially diluted 10-fold, spread on LB agar with antibiotic (carbenicillin), and incubated at 37°C overnight.

TABLE 3 Oligonucleotide primers

| Oligonucleotide	Sequence[a]
oJM400	cgcgagctcGAATCCCCAAATTATGACAACTTG
oJM401	cgcctgcagagcactagtCTAGCCCAAAAAAACGGGTATG
oJM402	cgcgagctcCTTGCAATTCGCGCTAACTTAC
oJM403	cgcctgcagagcactagtCTGTTTCCTGTGTGAAATTGTTATC
oJM641	cgcgagctcAAAGAGTGGAACAATGCAGG
oJM642	cgcctgcagagcactagtTGAATTTCATTACGACCAGTC
oJM414	CTAGATTTCACTTATCTGGTTGG
oJM415	ACAAAGGGAATCAGGGGATC
oJM457	ATTAGCGGATCCTACCTGAC
oJM551	CGACATCATAACGGTTCTGG
oJM730	GATACAGCGTGAATTTTCAGG
oJM524	cgcctgcagtaaggaggAACAGCTATGACCATGATTACG
oJM456	gcgaagcttTTATTTTTGACACCAGACCAACTG
oJM741	cgcctgcagtaaaggcAACAGCTATGACCATGATTACG
oJM727	cgcctgcagaggaAACAGCTATGACCATGATTACG
oJM744	ctagtTTTTTTCGTCCCGAAAAAATAACAACAAGAGGTGATATCCATGgcctgca
oJM745	tgcaggcCATGGATATCACCTCTTGTTGTTATTTTTTCGGGACGAAAAAAactag
oJM748	AACAACAAGAGGTGATATCCATGACCATGATTACGGATTCAC
oJM749	CTTCGCGAGGTACCGGGCCCATTATTTTTGACACCAGACCAACTG
oJM669	cgcctgcagtaaggaggAGCAACGATGTTACGCAGCAG
oJM670	gcgaagcttTTAGGTGGCGGTACTTGGGTC
oJM681	gcgactagtCTCACTCGTCGAGGATTCTCC
oJM682	gcgaagcttGTCGTCACATCATGTCTCGTC
oJM679	gcgactagtCCGTTTACAGAGAGGAGAACG
oJM680	gcgaagcttTCAGTCAGGATCGGCGCCCAG
oJM676	gcgactagtACCACATGCAACAGGAGTCG
oJM677	gcgaagcttTGGCGAATGCCGCTGTCAGG
oJM691	CCTTGCTGAATTAGCTTTATGC
oJM703	CTCAATGGAATTAGCTTTATGC
oJM473	GCAACCTGGCCAAGTCGGTC
oDHL12	GAGTTCAGCTGATATTGCTG
oDHL13	TCGTAGGTCTGCTGAATCAG

[a] The nucleotides shown in uppercase anneal to the PCR template, while those shown in lowercase are additional sequences that do not anneal to the template.

Excision of antibiotic resistance cassette by Flp-*FRT* recombination. Recipient strains containing chromosomal gentamicin resistance cassette flanked by *FRT* recombination sites were electroporated with pFLP2 plasmid (24). Transformants were streaked on LB with carbenicillin, as well as on LB with gentamicin, to screen for excision of the gentamicin resistance cassette by Flp recombination. Gentamicin-sensitive transformants were streaked from LB with carbenicillin to LB with 5% sucrose. Strains that have the pFLP2 plasmid are sucrose sensitive, while those that have lost the plasmid are sucrose resistant. Sucrose-resistant colonies were streaked on LB, LB with gentamicin, and LB with carbenicillin to confirm both excision of the gentamicin resistance cassette and loss of the pFLP2 plasmid.

β-Galactosidase assays. Strains were grown in 3 ml medium (LB, M9-glucose, M9-Casamino Acids, or M9-succinate) at 37°C with rolling until cell density reached an optical density at 600 nm (OD_{600}) of about 0.5. Cultures were diluted 50-fold into the 3 ml medium with or without inducer (L-arabinose, isopropyl-β-D-thiogalactopyranoside [IPTG], or L-rhamnose) and grown at 37°C with rolling until an OD_{600} of about 0.5 (mid-exponential growth phase). One milliliter of each culture was centrifuged at $10,000 \times g$ in a microcentrifuge tube for 1 min. Cell pellets were stored at −20°C. Cell pellets were thawed on ice and resuspended in 1 ml cold Z buffer (60 mM sodium phosphate dibasic, 40 mM sodium phosphate monobasic, 10 mM potassium chloride, 1 mM magnesium sulfate, pH 7.0, with 50 mM β-mercaptoethanol). One hundred microliters of each cell suspension was added to microcentrifuge tubes containing 900 μl Z buffer, 100 μl chloroform, and 50 μl 0.1% SDS. Reaction mixtures were vortexed and incubated at 30°C for 10 min. Two hundred microliters of 4 mg/ml ortho-nitrophenyl-β-galactoside (in 0.1 M phosphate buffer;

60 mM sodium phosphate dibasic, 40 mM sodium phosphate monobasic, pH 7.0) was added to each sample. Reactions were vortexed briefly and incubated at 30°C for 10 to 20 min. Four hundred microliters of 1 M sodium carbonate was added to each sample to terminate the reactions. Reaction mixtures were vortexed and centrifuged to remove cell debris. One milliliter of each reaction supernatant was transferred to disposable cuvettes. The absorbance of each reaction mixture was measured at 420 nm (A_{420}). β-Galactosidase activity (in Miller units) was calculated as $(1,000 \times A_{420})/(\text{reaction time in minutes} \times \text{cell suspension volume in } ml \times OD_{600})$.

Gentamicin resistance assays. For broth culture assays, strains were grown in 3 ml LB with or without inducer (0.2% L-arabinose or 0.03125% L-rhamnose) at 37°C with rolling until an OD_{600} of approximately 0.5. Five microliters of each culture was added to 150 μl LB or LB with gentamicin (80, 20, 5, 1.25, 0.31, or 0.078 μg/ml) in a 96-well plate. Five microliters of each culture was also added to 150 μl LB with inducer (0.2% L-arabinose or 0.03125% L-rhamnose) and gentamicin (80, 20, 5, 1.25, 0.31, or 0.078 μg/ml). Each condition was tested in triplicate. Cultures were grown at 37°C with shaking in a BioTek Synergy H1 hybrid plate reader for 8 h, and the OD_{600} was measured every 15 min. Relative growth of culture after 300 min (transition state–early stationary phase for cultures without gentamicin) was calculated (average of triplicates with gentamicin divided by average of triplicates without gentamicin). For spot dilution plate assays, strains were grown in 3 ml LB with or without inducer (0.2% L-arabinose or 0.03125% L-rhamnose) at 37°C with rolling until an OD_{600} of approximately 0.5, as described above. The cultures were then serially diluted 10-fold in LB, 5 μl of each dilution was spotted
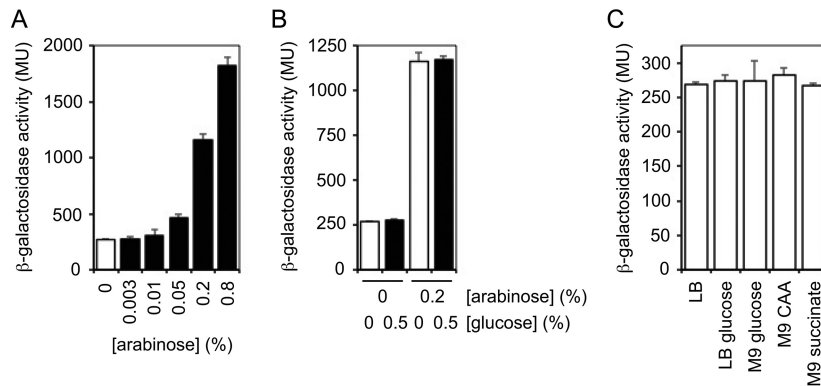
FIG 1 High noninduced expression from *araC-ParaBAD* cannot be decreased through catabolite repression in *P. aeruginosa*. The PA103 *attTn7::araC-ParaBAD-stRBS-lacZ frt* strain was grown to mid-exponential growth phase in LB or LB with increasing concentrations of arabinose (A), LB with or without 0.2% arabinose and with or without 0.5% glucose (B), or with LB, LB–0.5% glucose, M9–0.5% glucose, M9–0.5% Casamino Acids (CAA), or M9–50 mM succinate (C). β-Galactosidase activity was measured in Miller units (MU). The mean activity of three biological replicates is shown, and standard deviations are presented with error bars.

on LB agar and LB agar with gentamicin, and cells were grown at 37°C overnight.

Complementation of tryptophan auxotrophy. Strains were grown in 3 ml M9 minimal medium supplemented with 50 mM succinate, as well as with 1 mM L-tryptophan or 0.003125% L-rhamnose when indicated. Cultures were grown at 37°C with rolling for about 15 h and the OD_{600} was measured. Strains were also grown in 200 μl M9 with 50 mM succinate, as well as with L-rhamnose (0.006, 0.003, 0.0015, 0.0008, 0.0004, and 0.0002%) or L-tryptophan (1.0, 0.25, 0.0625, and 0.0156 mM), as indicated. Each condition was tested in triplicate. These cultures were grown at 37°C with shaking in a BioTek Synergy H1 hybrid plate reader for 8 h, and the OD_{600} was measured every 15 min.

Accession number(s). The annotated nucleotide sequences of plasmids pJM100, pJM101, pJM220, pJM251, pJM252, and pJM253 were submitted to GenBank under accession numbers KX787911, KX782328, KX777256, KX787912, KX782329, and KX782327, respectively.

RESULTS

Gene expression from *araC-ParaBAD* is leaky in the absence of arabinose in *P. aeruginosa*. Our initial analysis of the *araC-ParaBAD* inducible promoter system was motivated by two important questions. First, how much gene expression occurs from the *araBAD* promoter in the absence of arabinose? In other words, how tightly controlled is the inducible promoter system? Second, is this noninduced gene expression decreased by catabolite repression in the presence of glucose? To address these questions, we constructed a miniTn7 delivery plasmid with the *araC-ParaBAD* inducible promoter system (pUC18T-miniTn7T-gm-araC-Para-BAD). This new plasmid differs from the previous version built in our laboratory (17) in that the new plasmid contains the *araBAD* transcriptional initiation sequences but lacks any translational initiation sequences. This new design allowed maximum flexibility in the manipulation and use of the inducible promoter system. We then built a transcriptional fusion of the *araBAD* promoter with the *E. coli lacZ* gene that encodes the enzyme β-galactosidase. We engineered the transcriptional fusion to contain a strong ribosome-binding site to allow us to accurately measure low-level *lacZ* gene expression. The miniTn7-araC-ParaBAD-lacZ reporter was integrated in single copy into the chromosome of *P. aeruginosa* strain PA103. The strain bearing the reporter was grown to mid-exponential growth phase in rich medium (lysogeny broth [LB]) and β-galactosidase activity was measured. As shown in

Fig. 1A, we observed an increase in β-galactosidase activity in the presence of increasing concentrations of arabinose between 0.003 and 0.8%. These results met the expectation that the *araC-Para-BAD* system would be inducible over a range of inducer concentrations. In the absence of arabinose, however, we still observed significant β-galactosidase activity. While the maximal inducible activity measured was about 1,800 Miller units (MU), the noninduced activity was about 270 MU. This noninduced activity is similar to the amount of activity we have observed with some constitutive promoters (data not shown). Therefore, it appears that there is leaky expression from the *araC-ParaBAD* system in the absence of inducer in *P. aeruginosa*.

To determine if gene expression from the *araC-ParaBAD* inducible promoter system is subjected to catabolite repression in *P. aeruginosa*, we began by testing if the addition of glucose can decrease *lacZ* expression. The strain containing the *lacZ* reporter was grown in rich medium with and without 0.5% glucose. If the *araBAD* promoter was controlled by catabolite repression as it is in *E. coli*, then β-galactosidase activity should be decreased in the presence of glucose. Instead, we observed no difference in reporter activity in the presence of glucose compared to that in its absence (Fig. 1B). To test if the addition of glucose could decrease the induced expression, we also grew the reporter strain with and without 0.5% glucose in rich medium with 0.2% arabinose. Again, we observed the same reporter activity in the presence and absence of glucose (Fig. 1B). To rule out the possibility that catabolite repression by glucose was suppressed in rich medium, we also assessed noninduced expression in M9 minimal medium supplemented with 0.5% glucose. As observed in rich medium, glucose did not change the noninduced expression (Fig. 1C). *P. aeruginosa* preferentially utilizes amino acids and organic acids as carbon sources over sugars. Accordingly, amino acids and organic acids (such as succinate and citrate) elicit strong catabolite repression. Glucose, on the other hand, stimulates weak catabolite repression. We considered the possibility that growth with amino acids and organic acids could impose catabolite repression on the *araBAD* promoter. Growth in M9 minimal medium supplemented with 0.5% Casamino Acids or 50 mM succinate had no effect on the noninduced expression from the *araBAD* promoter (Fig. 1C). These data show that there is leaky expression from the *araC-*

ParaBAD inducible promoter system in the absence of arabinose in *P. aeruginosa*, and that this noninduced activity cannot be decreased through catabolite repression.

Modulating translational initiation affects both noninduced and maximal induced expression from *araC-ParaBAD*. Two important features of an inducible promoter system are low noninduced expression and high maximum induced expression, resulting in a wide range within which the expression of a gene can be modulated. Once we determined that the noninduced expression from the *araC-ParaBAD* system cannot be decreased by the addition of glucose or preferred carbon sources, such as Casamino Acids or succinate, we explored ways to mitigate the noninduced activity. One way to minimize the manifestation of the noninduced expression is to decrease translational initiation by reducing ribosome-binding site (RBS) strength. We reasoned that we might be able to offset high transcription of *lacZ* with low translation of the mRNA and limit the amount of the protein gene product synthesized in the absence of inducer. A caveat of this approach, however, is that reducing translational initiation could decrease both the noninduced and the maximal induced activities. If the noninduced activity decreased more than maximal induced activity, then there might be an RBS that restricts the noninduced activity and preserves the maximal induced activity. To attempt to find such an RBS, we constructed *lacZ* transcriptional fusions with three different RBS: strong (CTGCAG**TAAGGAGG**AACAGC T**ATG**), intermediate (CTGCA**GAGG**AAACAGCT**ATG**), and weak (CTGCAGTA**AGG**CAACAGCT**ATG**) (where boldface indicates the mRNA ribosome-binding site nucleotides that base pair with the 16S rRNA, as well as the start codon nucleotides). The strong RBS, the same as the one used in the transcriptional fusion described above, is complementary to the 3′ end of the *P. aeruginosa* 16S rRNA (UGGAUC**ACCUCCUUA**-3′ [where boldface indicates the nucleotides that base pair with the ribosome-binding site]), the region of the 30S ribosomal subunit that recognizes mRNA and drives the formation of the translation initiation complex. These sequences have a Gibbs free energy of binding (ΔG) of −13.82 kcal/mol (calculated using OligoAnalyzer 3.1 from Integrated DNA Technologies). The intermediate and weak RBS have ΔG of −6.24 and −4.67 kcal/mol, respectively. The intermediate RBS slightly diminished the noninduced β-galactosidase activity compared to that of the strong RBS, while the weak RBS decreased the noninduced activity more than 5-fold (Fig. 2A). As anticipated, however, both the intermediate and weak RBS also caused a reduction in the maximal induced activity (Fig. 2B). In fact, the maximal induced activity decreased more than the noninduced activity, leading to decreased induction ratios (the ratio of the maximal induced activity to the noninduced activity) (Fig. 2C). Therefore, reducing RBS strength incompletely achieved our goals of decreasing the noninduced expression and widening the range within which gene expression can be induced. Although the effects of the noninduced activity were reduced, it came at the cost of a lower maximal induced level and contracted the range within which gene expression could be modulated.

The 5′ UTR of *P. aeruginosa amiE* improved the functionality of *araC-ParaBAD*. Another approach we envisioned to minimize the appearance of the high-level noninduced expression of the *araC-ParaBAD* system was to integrate it into the regulatory network that governs catabolite repression in *P. aeruginosa*. During growth with a preferred carbon source, *P. aeruginosa* blocks translation of mRNA gene products needed to catabolize nonpre-
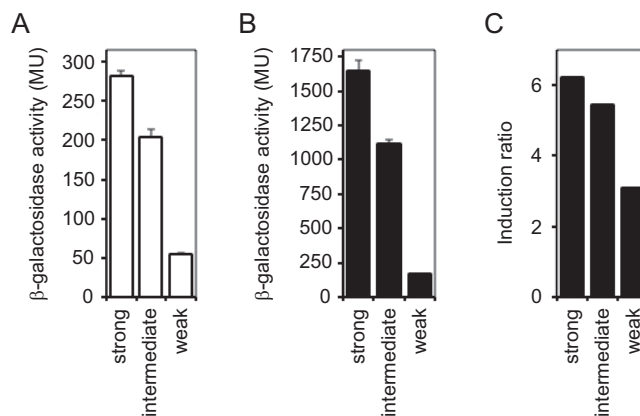
FIG 2 Decreased translational initiation decreased both noninduced and maximally induced expression from *araC-ParaBAD*. (A) PA103 *attTn7::araC-ParaBAD-stRBS-lacZ frt* (strong), PA103 *attTn7::araC-ParaBAD-intRBS-lacZ frt* (intermediate), and PA103 *attTn7::araC-ParaBAD-wkRBS-lacZ frt* (weak) were grown to mid-exponential growth phase in LB without arabinose. β-Galactosidase activity was measured. (B) Cells were grown to mid-exponential growth phase in LB with 0.8% arabinose. (C) Comparison of induction ratios with strong RBS, intermediate RBS, and weak RBS. Induction ratio is maximum induced activity to noninduced activity.

ferred carbon sources (7). In this form of catabolite repression, the RNA-binding protein Hfq binds short sequences, called CA (for catabolite activity) motifs, in the 5′ UTR of target mRNA and blocks translation by occluding nearby RBS (11). During growth with a nonpreferred carbon source, such as glucose, the small RNA CrcZ sequesters Hfq and allows translation of target mRNA (12). We thought that if we appended the 5′ UTR of such a target gene to the *lacZ* reporter gene, then perhaps we could subject *lacZ* to catabolite repression and decrease noninduced *araC-ParaBAD* activity without affecting the maximal induced activity. To this end, we inserted a portion of the 5′ UTR of the well-studied catabolite-repressed gene *amiE* into our miniTn7-araC-ParaBAD-lacZ reporter between the *ParaBAD* transcriptional start site and the *lacZ* start codon (Fig. 3A). It is worth noting that the RBS in the 5′ UTR *amiE* should be considered approximately intermediate in strength (the sequence is AAC**AAGAGGT**GATATCC**ATG** [where boldface indicates the mRNA ribosome-binding site nucleotides that base pair with the 16S rRNA, as well as the start codon nucleotides]; ΔG of −7.58 kcal/mol, with an 8-nucleotide spacing between RBS and start codon). The *P. aeruginosa amiEBCRS* operon encodes enzymes necessary to metabolize aliphatic amides such as acetamide (25). The *amiE* gene is preceded by the 134-bp untranslated region. The *amiE*-distal portion of the 5′ UTR contains a transcriptional terminator (*amiL*) that prevents read-through transcription of the *ami* operon in the absence of aliphatic amides. To eliminate that regulation, we used the *amiE*-proximal 40 bp of the 5′ UTR. This removes the *amiL* transcriptional terminator (ΔG of −29.10 kcal/mol) but preserves a putative 7-bp stem-loop (TTTTTCGTCCCGAAAAA; ΔG of −3.80 kcal/mol) and the CA motif. It is not known if this putative stem-loop is important for Hfq binding and catabolite repression. If this portion of the 5′ UTR *amiE* is sufficient for catabolite repression, then growth with amino acids (preferred carbon source, strong catabolite repression) should restrict *lacZ* translation and result in low noninduced β-galactosidase activity with *araC-ParaBAD* (Fig. 3B). Growth with glucose (nonpreferred carbon source, weak catabolite repres-
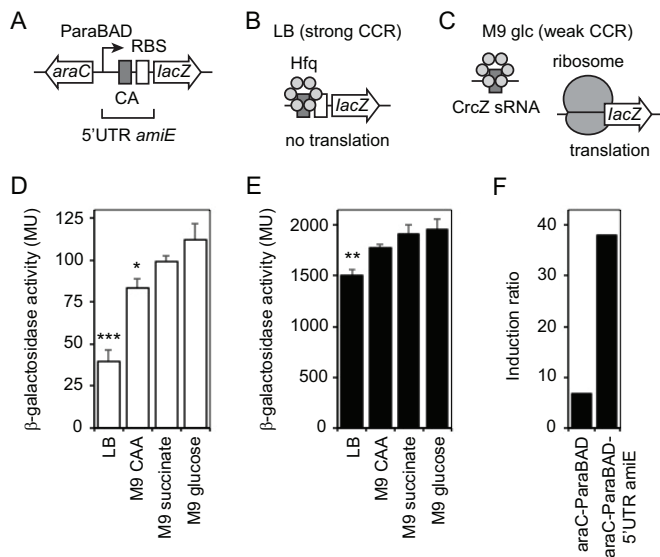
FIG 3 5′ UTR of *P. aeruginosa amiE* decreased noninduced expression from *araC-ParaBAD* through carbon catabolite repression. (A) Design of *araC-ParaBAD* inducible promoter system with 5′ UTR of *P. aeruginosa amiE* and *lacZ* reporter. (B) Inhibition of *lacZ* mRNA translation by strong carbon catabolite repression (CCR) in LB medium. Hfq binds CA motif in 5′ UTR *amiE* and blocks formation of translation initiation complex. (C) Translation of *lacZ* mRNA under weak carbon catabolite repression (CCR) in M9 glucose. CrcZ sRNA sequesters Hfq and allows formation of translation initiation complex. (D) PA103 *attTn7::araC-ParaBAD-5′ UTR amiE-lacZ frt* strain was grown to mid-exponential growth phase in LB, M9–0.5% Casamino Acids (CAA), M9–50 mM succinate, and M9–0.5% glucose without arabinose. β-Galactosidase activity was measured. Statistical significance was determined using one-way analysis of variance followed by Dunnett's test comparing each to M9 glucose (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). (E) Cells were grown to mid-exponential growth phase with 0.8% arabinose in LB, M9–0.5% Casamino Acids (CAA), M9–50 mM succinate, and M9–0.5% glucose. Statistical significance was determined as described above. (F) Comparison of induction ratios without 5′ UTR *amiE* and with 5′ UTR *amiE* in LB. Induction ratio is maximum induced activity to noninduced activity.

sion), on the other hand, should allow *lacZ* translation and lead to higher noninduced activity as well as high maximal induced activity (Fig. 3C).

To determine if the 5′ UTR *amiE* could decrease the noninduced expression of the *araC-ParaBAD* system through catabolite repression, the araC-ParaBAD-5′ UTR amiE-lacZ reporter strain was grown to mid-exponential growth phase in LB and M9 minimal medium with either Casamino Acids, succinate, or glucose, and then β-galactosidase activity was measured. As shown in Fig. 3D, the lowest noninduced activity was observed when the strain was grown in LB, a medium rich in amino acids. The noninduced activity progressively increased in M9 minimal medium supplemented with Casamino Acids, succinate, and glucose. In contrast, the noninduced expression from the *araBAD* promoter without the 5′ UTR *amiE* was not reduced in the presence of amino acids or succinate (as shown in Fig. 1C). Therefore, the 40-bp *amiE*-proximal portion of the 5′ UTR *amiE* appeared to allow catabolite repression of the *lacZ* reporter. Although the 5′ UTR *amiE*-*lacZ* reporter activity was affected in a manner consistent with catabolite repression, the magnitude of the effect was lower than that expected based on previous studies with *amiE* (12).

Irrespective of the mild catabolite repression, inclusion of the

5′ UTR *amiE* did diminish the noninduced *araC-ParaBAD* activity while only modestly reducing the maximal induced activity. When the araC-ParaBAD-5′UTR amiE-lacZ reporter strain was grown with 0.8% arabinose (maximal induction) in LB and M9 Casamino Acids, succinate, or glucose, the maximal induced activity was affected in a way reflective of mild catabolite repression. As observed for the noninduced activity, the maximal induced activity was the lowest in LB and progressively increased in M9 Casamino Acids, M9 succinate, and M9 glucose (Fig. 3E). When grown in LB, the maximal induced activity was reduced only about 17% with the 5′ UTR of *amiE* compared to without it (1,500 and 1,800 MU, respectively; compare Fig. 1A and 3E). The net effect of decreased noninduced activity and largely unaffected maximal induced activity was an increased induction ratio with the 5′ UTR *amiE* compared to that without it (Fig. 3F). The induction ratio was 5.6-fold higher with the 5′ UTR *amiE* than without it when grown in LB.

The *lacI*q-*Ptac* and *rhaSR-PrhaBAD* inducible promoter systems are tightly controlled in *P. aeruginosa*. While we explored modifications to improve the *araC-ParaBAD* system, an alternative was to simply use different inducible promoter systems. Perhaps the most commonly used inducible promoter system is the IPTG-inducible *E. coli lacI-Plac*. A less commonly used but tightly controlled inducible promoter system is the rhamnose-inducible *E. coli rhaSR-PrhaBAD* (26, 27). Although Valvano and coworkers showed that the *E. coli rhaSR-PrhaBAD* system is tightly controlled in *Burkholderia cenocepacia* (28) and used it to identify and study essential genes (29–32), the functionality of this system in *P. aeruginosa* has not been reported. We constructed and tested two alternative inducible promoter systems for use in *P. aeruginosa*, miniTn7-lacIq-Ptac and miniTn7-rhaSR-PrhaBAD. The *tac* promoter is a hybrid promoter derived from the *E. coli trp* and *lacUV5* promoters (33, 34). This hybrid promoter has low noninduced expression and higher maximal induced expression than the wild-type *lac* promoter in *E. coli*. With the same *lacZ* transcriptional reporter gene (with a strong RBS) used to evaluate *araC-ParaBAD* (Fig. 1), gene expression from both *lacI*q-*Ptac* and *rhaSR-PrhaBAD* was inducible over a range of inducer concentrations (Fig. 4A and B). The *rhaSR-PrhaBAD* system, however, is much more sensitive to inducer than *araC-ParaBAD*. Maximal induced expression from *araC-ParaBAD* occurred with 0.8% arabinose, while maximal induced expression from *rhaSR-PrhaBAD* occurred with 0.01% rhamnose (80 times more sensitive than *araC-ParaBAD*). The maximal induced expression was maintained up to 0.8% rhamnose (data not shown). Most importantly, the noninduced expression from both *lacI*q-*Ptac* and *rhaSR-PrhaBAD* was significantly lower than that with *araC-ParaBAD* (about 4- and 11-fold lower, respectively) (Fig. 4C). As observed with *araC-ParaBAD*, the noninduced expression with both *lacI*q-*Ptac* and *rhaSR-PrhaBAD* was unaffected by the addition of glucose. Additionally, both of these inducible promoter systems maintained high maximal induced expression. The maximal induced expression from *lacI*q-*Ptac* was about the same as that of *araC-ParaBAD*, while it was slightly lower from *rhaSR-PrhaBAD* (about 30% lower). The induction ratio was 3.5-fold higher with *lacI*q-*Ptac* than with *araC-ParaBAD*, while it was 7.6-fold higher with *rhaSR-PrhaBAD* (Fig. 4D). Therefore, both *lacI*q-*Ptac* and *rhaSR-PrhaBAD* satisfy the two most important criteria of inducible promoter systems. They both drive low-level gene expression in the absence of inducer and high max-
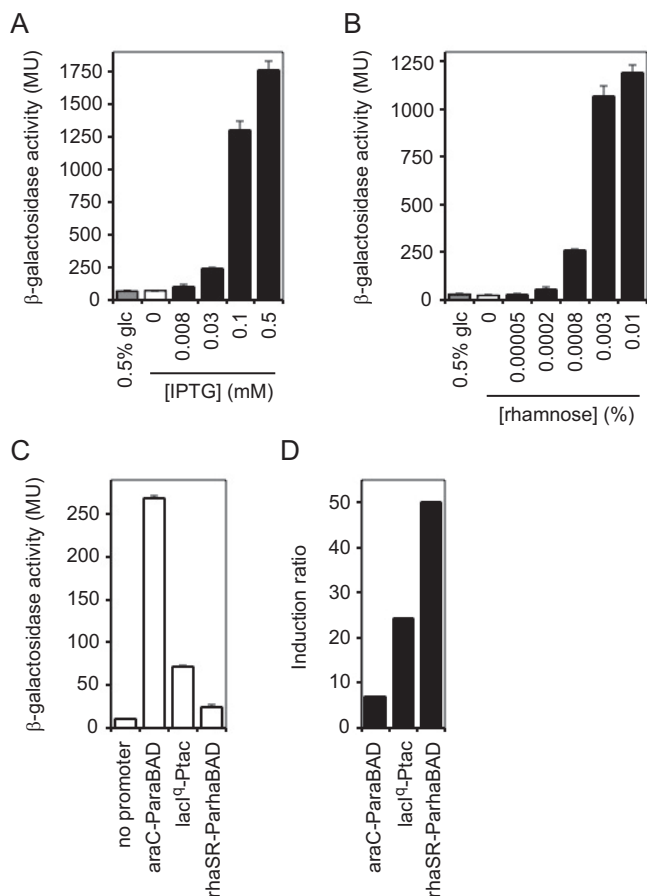
FIG 4 Lower noninduced expression from *lacI*q-*Ptac* and *rhaSR-PrhaBAD*. (A) PA103 *attTn7::lacI*q-*Ptac-stRBS-lacZ frt* strain was grown to mid-exponential growth phase in LB with or without glucose (glc) and with or without increasing concentrations of IPTG. β-Galactosidase activity was measured. (B) PA103 *attTn7::rhaSR-PrhaBAD-stRBS-lacZ frt* strain was grown to mid-exponential growth phase in LB with or without glucose and with or without increasing concentrations of rhamnose. β-Galactosidase activity was measured. (C) Comparison of noninduced activities of *araC-ParaBAD*, *lacI*q-*Ptac*, and *rhaSR-PrhaBAD*. (D) Comparison of induction ratios of *araC-ParaBAD*, *lacI*q-*Ptac*, and *rhaSR-PrhaBAD*. Induction ratio is maximum induced activity to noninduced activity.

imal induced expression in the presence of inducer, leading to a wide range within which a gene can be expressed. Of the three inducible promoter systems tested, the *rhaSR-PrhaBAD* system performed the best. It was the most tightly controlled, allowed expression over the widest range, and was the most sensitive to inducer.

Physiological consequences of noninduced gene expression from *araC-ParaBAD* but not *rhaSR-PrhaBAD*. To investigate the consequences of the different levels of noninduced expression from *araC-ParaBAD* and *rhaSR-PrhaBAD* on gene function, we examined a conditionally essential gene whose function resulted in a simple phenotype. We chose to use the *aacC1* gene (from the miniTn7-gm delivery plasmid) (18) encoding a gentamicin acetyltransferase that inactivates gentamicin and leads to resistance to the antibiotic. In the absence of *aacC1* expression, *P. aeruginosa* is sensitive to gentamicin. If the noninduced expression from *araC-ParaBAD* or *rhaSR-PrhaBAD* was sufficient for gentamicin acetyltransferase gene function, then the cells contain-

ing the *aacC1* gene under the control of these promoters would become resistant to gentamicin in the absence of inducer. We cloned the *aacC1* gene into our miniTn7-araC-ParaBAD and miniTn7-rhaSR-PrhaBAD plasmids, integrated them into the *P. aeruginosa* chromosome (and excised the *FRT*-flanked *aacC1* from the miniTn7 backbone), and measured gentamicin resistance. *P. aeruginosa* with either empty miniTn7, miniTn7-araC-ParaBAD-aacC1, or miniTn7-rhaSR-PrhaBAD-aacC1 was grown to mid-exponential growth phase in LB and then diluted and grown in LB with or without increasing concentrations of gentamicin (from 0.08 to 20 μg/ml). The isogenic negative-control strain, the wild type with an empty miniTn7, grew in the presence of gentamicin until a MIC of 5 μg/ml (Fig. 5A, white triangles). The strain with miniTn7-araC-ParaBAD-aacC1 grew until a MIC of 20 μg/ml (Fig. 5A, white squares). This increased gentamicin resistance showed that the noninduced expression from *araC-ParaBAD* was sufficient for *aacC1* gene function. The strain with miniTn7-rhaSR-PrhaBAD-aacC1, on the other hand, exhibited the same gentamicin sensitivity as the control strain, indicating that the noninduced expression from *rhaSR-PrhaBAD* was not sufficient for *aacC1* gene function (Fig. 5A, white circles). This strain grew in the presence of 50 μg/ml gentamicin with 0.003125% rhamnose (Fig. 5A, black circles), indicating that the miniTn7-rhaSR-PrhaBAD-aacC1 construct was functional. To confirm these results in a different experimental context, each strain was grown to mid-exponential phase in LB, serially diluted 10-fold, and then spotted and grown on LB and LB with 10 μg/ml gentamicin. The strains bearing either an empty miniTn7 or miniTn7-rhaSR-PrhaBAD-aacC1 grew on LB but were unable to grow on LB with gentamicin, while the strain with miniTn7-araC-ParaBAD-aacC1 grew equally well on LB and LB with gentamicin (Fig. 5B). These results clearly show that the leakiness of the *araC-ParaBAD* system has physiological consequences, a problem not observed with the *rhaSR-PrhaBAD* system.

In these experiments, the inducible promoter systems and gentamicin acetyltransferase gene were integrated at the Tn*7* transposon insertion site (*attTn7*) near the chromosomal origin of replication (18, 35). To eliminate the possibility that this phenotypic consequence was unique to this particular chromosomal locus, we built strains with the inducible promoter systems and gentamicin acetyltransferase gene at the CTX bacteriophage integration site (*attCTX*) on the opposite side of the circular chromosome near the terminus of replication (22). We constructed miniCTX-araC-ParaBAD-aacC1 and miniCTX-rhaSR-PrhaBAD-aacC1 plasmids and integrated them into the *P. aeruginosa* chromosome. The strains were then grown to mid-exponential phase in LB, serially diluted, spotted, and grown on LB and LB with gentamicin. As observed at the Tn*7* insertion site, the *attCTX::araC-ParaBAD-aacC1* strain was gentamicin resistant, while the *attCTX::rhaSR-PrhaBAD-aacC1* strain was gentamicin sensitive (Fig. 5C). We also wanted to rule out the possibility that this phenotype is exclusive to a certain *P. aeruginosa* strain. Each of the experiments described to this point were performed with the *P. aeruginosa* strain PA103. Thus, we also integrated miniCTX-araC-ParaBAD-aacC1 and miniCTX-rhaSR-PrhaBAD-aacC1 in the common laboratory strains PAO1 and PA14. Both the PAO1 and PA14 strains with *attCTX::araC-ParaBAD-aacC1* were gentamicin resistant, while both strains with *attCTX::rhaSR-PrhaBAD-aacC1* were gentamicin sensitive (Fig. 5D and E). Therefore, the physiological consequences of the noninduced gene expression from *araC-ParaBAD* were observed at
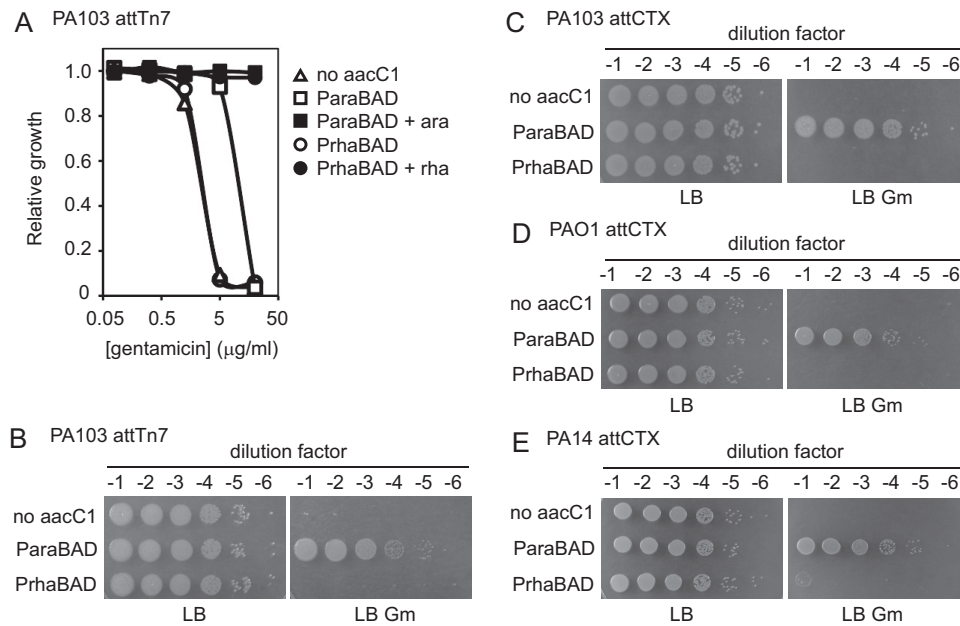
FIG 5 Noninduced expression of *aacC1* from *araC-ParaBAD* but not *rhaSR-PrhaBAD* is sufficient for gentamicin resistance at different chromosomal loci and in different strains. (A) PA103 *attTn7::frt* (no *aacC1*; triangles), PA103 *attTn7::araC-ParaBAD-stRBS-aacC1 frt* (*ParaBAD*; squares), and PA103 *attTn7::rhaSR-PrhaBAD-stRBS-aacC1 frt* (*PrhaBAD*; circles) strains were grown to mid-exponential growth phase in LB without inducer (empty shapes) or with inducer (arabinose or rhamnose; filled squares or circles, respectively). Cultures were diluted into LB with or without gentamicin and with or without inducer. Cultures were grown in 96-well plates at 37°C with shaking. Relative growth (OD_{600} of culture with gentamicin/without gentamicin) was calculated after 300 min (5 h). (B to E) Strains were grown to mid-exponential growth phase in LB. Cultures were serially diluted 10-fold and spotted onto LB and LB with 10 µg/ml gentamicin. Plates were grown at 37°C overnight. (B) PA103 *attTn7::frt* (no *aacC1*), PA103 *attTn7::araC-ParaBAD-stRBS-aacC1 frt* (*ParaBAD*), and PA103 *attTn7::rhaSR-PrhaBAD-stRBS-aacC1 frt* (*PrhaBAD*) strains. (C) PA103 *attCTX::tet* (no *aacC1*), PA103 *attCTX::araC-ParaBAD-stRBS-aacC1 tet* (*ParaBAD*), and PA103 *attCTX::rhaSR-PrhaBAD-stRBS-aacC1 tet* (*PrhaBAD*) strains. (D) PAO1 *attCTX::tet* (no *aacC1*), PAO1 *attCTX::araC-ParaBAD-stRBS-aacC1 tet* (*ParaBAD*), and PAO1 *attCTX::rhaSR-PrhaBAD-stRBS-aacC1 tet* (*PrhaBAD*) strains. (E) PA14 *attCTX::tet* (no *aacC1*), PA14 *attCTX::araC-ParaBAD-stRBS-aacC1 tet* (*ParaBAD*), and PA14 *attCTX::rhaSR-PrhaBAD-stRBS-aacC1 tet* (*PrhaBAD*) strains.

two distinct chromosomal loci and in three different *P. aeruginosa* strains. Furthermore, the tightly controlled *rhaSR-PrhaBAD* system prevented the antibiotic resistance phenotype at both chromosomal loci and in each of the three strains tested.

Modulation of growth rate through inducible gene expression from *rhaSR-PrhaBAD*. With the knowledge that the noninduced expression from *araC-ParaBAD* was sufficient for the function of a conditionally essential nonchromosomal gene, we examined whether this noninduced expression could also be problematic with conditionally essential chromosomal genes. When provided with a suitable carbon and nitrogen source, wild-type *P. aeruginosa* can synthesize all of the compounds it needs to grow, such as nucleic acids, amino acids, lipids, and sugars. The genes responsible for the synthesis of amino acids, for example, are conditionally essential. When an amino acid is available in the growth medium, the genes necessary for its synthesis are dispensable for growth. When the amino acid is absent, the bacterium must make it and the biosynthesis genes become essential for growth. We took advantage of the conditional essentiality of the tryptophan biosynthesis genes to examine the effects of noninduced expression from *araC-ParaBAD* and *rhaSR-PrhaBAD* inducible promoter systems on chromosomal *P. aeruginosa* genes.

To determine if the noninduced expression from *araC-ParaBAD* was sufficient for the tryptophan biosynthesis gene function, we cloned *trpF*, *trpC*, and *trpA* from *P. aeruginosa* strain PA14 into our miniCTX-araC-ParaBAD and miniCTX-rhaSR-PrhaBAD plasmids. We choose to work with these *trp* genes because each

gene is either alone (not part of an operon) or is located at the 3′ end of an operon (see Fig. S1 in the supplemental material) (36–38), meaning that transposon insertions would only disrupt the target genes and not affect the expression of downstream genes. Importantly, each of the *trp* genes have different translation initiation sequences (RBS and start codons). To ensure that we were testing the outcome of gene expression with a variety of different native translation initiation sequences, we preserved these sequences (*trpF*, CAAC**AGGAG**TCGAAAGC**ATG**, 8-nucleotide spacing between RBS and start codon; *trpC*, AGAG**AGGAG**AAC GCACA**GTG**, 8-nucleotide spacing between RBS and start codon; and *trpA*, TC**GAGG**ATTCTCCGTCG**TTG**, 11-nucleotide spacing between RBS and start codon) in each miniCTX1 construct. After the miniCTX constructs were integrated into the chromosome of *P. aeruginosa* strain PA14 *trpF*, *trpC*, and *trpA* transposon insertion mutants (39), we grew the auxotrophic mutants and their complemented derivatives in M9 minimal medium supplemented with succinate or M9 succinate with tryptophan. We began these experiments by comparing the wild-type PA14 and the PA14 *trpF* mutant. The wild-type strain grew equally well in M9 succinate with and without tryptophan, while the *trpF* mutant only grew in M9 succinate with tryptophan (Fig. 6A). The *trpF* mutant complemented with *attCTX::araC-ParaBAD-trpF* grew as well in M9 succinate as the wild-type strain. This showed that the noninduced expression from *araC-ParaBAD* was sufficient for *trpF* function. The *trpF* mutant complemented with *attCTX::rhaSR-PrhaBAD-trpF* did not grow in M9 succinate, indicating
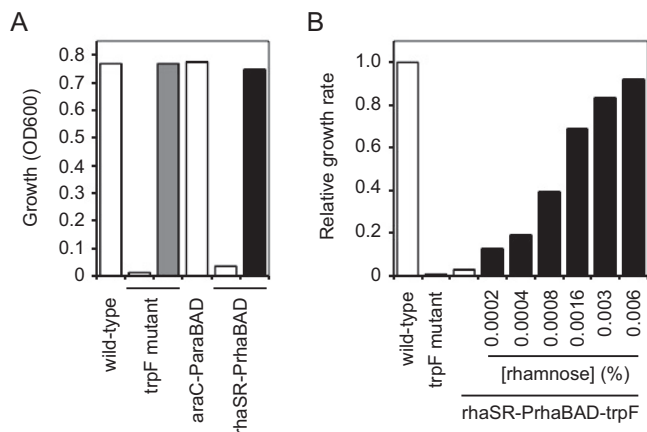
FIG 6 Complementation of tryptophan auxotrophy and modulation of growth rate with inducible expression of tryptophan biosynthesis gene from *rhaSR-PrhaBAD*. (A) PA14 (wild-type), PA14 *trpF*::Tn*Mar* (*trpF* mutant), PA14 *trpF*::Tn*Mar attCTX*::*araC-ParaBAD-trpF tet* (*araC-ParaBAD*), and PA14 *trpF*::Tn*Mar attCTX*::*rhaSR-PrhaBAD-trpF tet* (*rhaSR-PrhaBAD*) strains were grown overnight (about 15 h) in M9 succinate (white bars), M9 succinate with tryptophan (gray bar), or M9 succinate with rhamnose (black bar). The measured OD_{600} of each overnight culture is shown. (B) PA14 (wild-type), PA14 *trpF*::Tn*Mar* (*trpF* mutant), and PA14 *trpF*::Tn*Mar attCTX*:: *rhaSR-PrhaBAD-trpF tet* (rhaSR-PrhaBAD-*trpF*) cultures were grown in M9 succinate (white bars) or M9 succinate with increasing concentrations of rhamnose (black bars) in 96-well plates at 37°C with shaking for 8 h. Relative growth rates were calculated with respect to the wild-type PA14 culture.

that the noninduced expression from *rhaSR-PrhaBAD* was insufficient for *trpF* function. The strain grew well in M9 succinate with 0.003% rhamnose, confirming that the *attCTX*::*rhaSR-PrhaBAD-trpF* construct was functional. We observed the same results with the complemented *trpC* and *trpA* mutants (see Fig. S2), despite the fact that the genes have different translational initiation sequences and encode different enzymes. Together these results show that the noninduced expression from *araC-ParaBAD* can be problematic with conditionally essential chromosomal genes with different translation initiation sequences. Importantly, this problem can be resolved by utilizing the more tightly controlled *rhaSR-PrhaBAD* system.

Using our miniTn7-*lacZ* reporter in *P. aeruginosa* strain PA103, we showed that the *rhaSR-PrhaBAD* inducible promoter system allowed gene expression over a wide range (Fig. 4B). With the assumption that gene expression from *rhaSR-PrhaBAD* can be similarly tuned in *P. aeruginosa* strain PA14, we expected to be able to modulate tryptophan biosynthesis by inducing different levels of *trpF* gene expression. To test this assumption, we grew wild-type PA14, the *trpF* mutant, and the *trpF* mutant complemented with miniCTX-rhaSR-PrhaBAD in M9 succinate or M9 succinate with increasing concentrations of rhamnose (Fig. 6B; also see Fig. S3 in the supplemental material). In minimal medium, the rate of cell growth should be proportional to cellular tryptophan synthesis. If *trpF* gene expression from rhaSR-PrhaBAD is tunable, then the growth rate of the *attCTX-rhaSR-PrhaBAD-trpF* strain should increase with increasing rhamnose concentration. As shown in Fig. 6B, we observed an increase in growth rate of the *trpF* mutant complemented with miniCTX-rhaSR-PrhaBAD in the presence of increasing concentrations of rhamnose from 0.002 to 0.006%. When grown in M9 succinate with 0.0002% rhamnose, the growth rate of the complemented

mutant was nearly 10 times slower than that of the wild type. As the concentration of rhamnose was increased, we observed a progressive increase in growth rate until the wild-type growth rate was reached with 0.006% rhamnose. These results not only demonstrate the utility of the rhaSR-PrhaBAD inducible promoter system but also provide an experimental platform to precisely tune *P. aeruginosa* metabolism and growth.

DISCUSSION

The aims of the work described here were to describe the shortcomings of the *araC-ParaBAD* inducible promoter system and reengineer it to improve its functionality in *P. aeruginosa*, as well as to identify a system that is tightly controlled in the absence of inducer and inducible over a wide range of expression levels. These criteria are important because a failure to achieve tight control of gene expression can obscure the study of gene function, while a narrow range of inducible gene expression can make it difficult to match an induced expression level with the native expression level. Our data show that the *araC-ParaBAD* inducible promoter system is not tightly controlled in the absence of arabinose in *P. aeruginosa*, as it is in *E. coli*, and the leakiness cannot be reduced through carbon catabolite repression. Although *araC-ParaBAD* exhibits high maximally induced expression, the high noninduced expression narrows the range within which expression can be modulated. Both the *lacI^q-Ptac* and *rhaSR-PrhaBAD* inducible promoter systems display significantly lower noninduced expression than *araC-ParaBAD*, and they both maintain high maximal induced expression. Consequently, expression from *lacI^q-Ptac* and *rhaSR-PrhaBAD* is inducible over a broader range than that of *araC-ParaBAD*. Of these three inducible promoter systems, *rhaSR-PrhaBAD* is the most tightly controlled and allows expression over the widest range.

Although our data describe the different behaviors of these inducible promoter systems in *P. aeruginosa*, they do not necessarily explain why the differences exist. In *E. coli*, the promoters of many catabolic genes (including the *ara*, *lac*, and *rha* operons) tend to be weak and need to be activated by transcription factors to allow the formation of the RNA polymerase-promoter complex and transcriptional initiation (40). Alterations that strengthen these promoters by making the sequence closer to the consensus sequence recognized by RNA polymerase (**TTGACA**-17-**TA TAAT** in *E. coli*) can eliminate the need for activation. (DNA sequences shown in boldface indicate the consensus promoter sequence recognized by σ^70-RNA polymerase in *E. coli*.) The *lacUV5* promoter (**TT**T**ACA**-18-**TATAAT**), for example, is a derivative of the *lac* promoter (**TT**T**ACA**-18-**TAT**GTT) with mutations that strengthen the promoter and suppress the need for cAMP-CRP activation (41). The *tac* promoter (**TTGACA**-16-**TATAAT**) used in this study is a *trp-lacUV5* hybrid that strengthens the promoter even further (33). If promoter strength was the only factor that contributes to noninduced expression, then the *tac* promoter may be expected to be the least tightly controlled. Presumably, the architecture of the regulatory system and the cellular levels of the regulatory proteins are also important factors. The regulatory system that controls the *tac* promoter is comprised of a single regulatory protein, the LacI transcriptional repressor. LacI binds to an operator site that overlaps the promoter and prevents the formation of the RNA polymerase-promoter complex. The *lacI^q* gene has a mutation that strengthens the promoter that controls expression of *lacI*, leading to an increase in

the amount of LacI produced, thereby decreasing noninduced expression from the *tac* promoter (42, 43). The *rhaBAD* promoter (AG**G**TCG-17-**TA**GACT) is weak in *E. coli*. Activation of the *rhaBAD* promoter occurs through a regulatory cascade involving RhaR and RhaS (26). RhaR is a transcription factor that binds rhamnose and activates the transcription of the *rhaS* gene, as well as its own gene. When RhaS, also a transcription factor, accumulates to sufficient amounts, it activates transcription from the *rhaBAD* promoter. Perhaps this architecture creates a regulatory buffer that maintains low noninduced expression (27). The *araBAD* promoter (C**TGAC**G-18-**TA**CTG**T**) is also quite weak in *E. coli*. It is repressed by AraC (in the absence of arabinose) and then activated by the arabinose-AraC complex. One possible explanation for its leakiness is that the cellular levels of AraC are lower and the promoter is stronger in *P. aeruginosa* than *E. coli*. In this scenario, AraC repression may be less complete and the need for activation by arabinose-AraC could be diminished in *P. aeruginosa* compared to that in *E. coli*.

Despite its leakiness in *P. aeruginosa*, the *araC-ParaBAD* system has been used to induce the expression of several genes (13–17). For some applications of inducible promoter systems, tightly controlled gene expression may not be particularly important. In cases where the amount of gene product necessary for gene function is large, the leaky gene expression from *araC-ParaBAD* may be insufficient for gene function. On the other hand, there are presumably many other cases where only a small amount of gene product is needed for gene function. In this study, we presented two distinct examples that demonstrate the problems associated with the *araC-ParaBAD* system in *P. aeruginosa*. Noninduced expression of a gentamicin acetyltransferase gene (*aacC1*) from *araC-ParaBAD* was sufficient to confer gentamicin resistance. Similarly, noninduced expression of three different tryptophan biosynthesis genes (*trpF*, *trpC*, and *trpA*) from *araC-ParaBAD* was sufficient to support tryptophan biosynthesis and cell growth in minimal medium. The leakiness of the *araC-ParaBAD* system was already encountered in a study of essential cell envelope biosynthesis genes (14). To overcome this problem, the authors constructed a suicide plasmid (pBEM10) for integration of *araC-ParaBAD* upstream of target genes at their native chromosomal loci. The distinguishing feature of this integration plasmid was the inclusion of a weak RBS (TTG**GG**CTAACCTTCTGAAAAGCTT**ATG**; ΔG of -3.09 kcal/mol; 19-nucleotide RBS start codon spacing). This weak RBS would offset the effects of *araC-ParaBAD* leakiness by decreasing translational initiation and accumulation of the resulting protein gene product. Subsequently, miniCTX1 derivatives that preserve this weak RBS were constructed and used to study other essential envelope biosynthesis genes (16, 44). As we demonstrated in our study, decreasing RBS strength can reduce the problem of promoter leakiness, but it also reduces the maximal induced amount of gene product and narrows the range within which the gene product can be modulated. Therefore, the existing method for overcoming the problems with the *araC-ParaBAD* system incompletely achieves the goals of tightly controlled noninduced expression and high maximum induced expression, allowing a broad range of inducible gene expression. Furthermore, this approach does not resolve the problem when the gene product in not a protein, such as noncoding RNAs that are not translated into proteins.

In addition to modulating RBS strength, we also explored an alternative approach to mitigate the effects of *araC-ParaBAD*

leakiness. We introduced a portion of the 5′ UTR from the *amiE* gene downstream of *araC-ParaBAD* in an attempt to subject target genes to carbon catabolite repression (translational inhibition by Hfq) in *P. aeruginosa*. The expectation was that the addition of the 5′ UTR would allow us to decrease translation of target mRNA when the cells were grown with a preferred carbon source (such as amino acids, which elicit strong catabolite repression). Indeed, the 5′ UTR of *amiE* improved the functionality of the *araC-ParaBAD* systems by decreasing the noninduced activity of the *lacZ* gene about 6-fold and increasing the induction ratio about 5-fold in rich medium (LB). The reduction was more modest than we expected, however, when cells were grown in minimal medium supplemented with Casamino Acids compared to minimal medium with glucose. The 5′ UTR of the *amiE* gene is 134 bp in length and contains a transcriptional terminator (*amiL*) that prevents readthrough transcription of the *ami* operon in the absence of aliphatic amides, followed by a 40-bp region with the CA motif recognized by Hfq (11, 12). Using a translational *lacZ* fusion, the 134-bp 5′ UTR resulted in an approximately 2-fold decrease in activity in minimal medium supplemented with succinate compared to glucose and about a 10-fold decreased compared to activity in minimal medium with mannitol (12). We observed only about a 10% decrease in activity with our *amiE-lacZ* translational fusion in minimal medium with succinate compared to glucose. These results suggest that the CA motif is not sufficient for catabolite repression of *amiE* and indicate that additional upstream sequences also are involved. A more complete understanding of catabolite repression in *P. aeruginosa* should allow the design and implementation of a posttranscriptional regulatory module that could augment the usefulness of *araC-ParaBAD* as well as other inducible and constitutive promoter systems.

Although our primary motivation to identify an inducible promoter system that is tightly controlled in *P. aeruginosa* was the analysis of essential gene function with the aim of developing new antibiotics to treat *P. aeruginosa* infections, the utility of tightly controlled inducible promoters is not restricted to the study of essential genes. They can also be applied to understanding essential cellular functions maintained through parallel gene pathways. Such parallel pathways are employed by cells to promote phenotypic stability despite cellular and environmental variability. Genes in these pathways are often synthetically lethal, meaning that mutants in individual gene pathways are viable and mutant combinations are lethal. The study of synthetic lethal genes often involves conditional expression of a gene with an inducible promoter that allows inactivation of its synthetic lethal gene pairs. Tightly controlled inducible promoters can also be used to modify genetic circuits to understand the design principles of natural circuits, as well as to replace natural genetic circuits with controllable synthetic ones that have altered functionality. Such genetic engineering can also be applied to precisely alter cell metabolism and improve a targeted cellular function (45). In this context, tightly controlled inducible promoters are used to shift metabolic flow toward a desired product by amplifying the desired pathway or limiting an alternative pathway. The improved and expanded repertoire of inducible promoter systems provided in this work, in particular *rhaSR-PrhaBAD*, should help progress toward an understanding of gene function and the engineering of metabolic capabilities in *P. aeruginosa*.

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