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Directed Evolution of AraC for Improved Compatibility of Arabinose- and Lactose-Inducible Promoters▽†

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Synthetic biological systems often require multiple, independently inducible promoters in order to control the expression levels of several genes; however, cross talk between the promoters limits this ability. Here, we demonstrate the directed evolution of AraC to construct an arabinose-inducible (P_{BAD}) system that is more compatible with IPTG (isopropyl-β-D-1-thiogalactopyranoside) induction of a lactose-inducible (P_{lac}) system. The constructed system is 10 times more sensitive to arabinose and tolerates IPTG significantly better than the wild type. Detailed studies indicate that the AraC dimerization domain and C terminus are important for the increased sensitivity of AraC to arabinose.

Recent advances in metabolic engineering and synthetic biology have increased the use of multiple genes (17) for various applications, such as the production of medicines (22, 24) and the construction of complex genetic circuits (21, 30). Many genetic circuits, such as inverters (32), logic gates (8), pulse generators (4), band-pass filters, and oscillators (12), have been used to develop strains of bacteria that can communicate to form two-dimensional patterns, control their population density, and attack tumor cells in response to environmental cues (1, 3, 33). It is often necessary to introduce multiple genes and control the expression levels of those genes independently in order to accomplish these tasks. To date, a number of inducible promoters have been developed for *Escherichia coli* and other bacteria (2, 11, 16) which, in theory, should make it possible to independently control the expression of more than one gene. However, some pairs of promoters suffer from cross talk (an inducer of one promoter affects the expression from another promoter), making it difficult to simultaneously and independently control the expression levels of multiple genes. In contrast to electrical systems in which system interactions are well characterized and can be isolated from one another, it is often difficult or impossible to isolate biological components from activation or inhibition by other components in the cell.

One example of cross talk involves a system containing two of the most useful promoters for gene expression in bacteria: the IPTG (isopropyl-β-D-1-thiogalactopyranoside)-inducible *lac* promoter (P_{lac}) (15) and the arabinose-inducible *araBAD* promoter (P_{BAD}) (13, 29). The *araBAD* promoter system is regulated by the transcriptional regulator AraC. In the absence of arabinose, an AraC dimer creates a 210-base-pair DNA loop by contacting two widely separated half-sites (I_1 and O_2) on the DNA and represses transcription from P_{BAD} (19, 20, 28). The binding of arabinose to AraC changes the position of the AraC dimer, causing the protein to preferentially bind different DNA-binding half-sites (I_1 and I_2). The change in configuration causes the dimer to release the DNA loop and allow transcription from P_{BAD} (14, 19, 28).

AraC is a 292-residue protein consisting of an N-terminal domain (residues 1 to 170) joined to a C-terminal DNA-binding domain (residues 178 to 292) (5, 9) by a linker of at least five residues (9). The N-terminal domain consists of the arabinose-binding and dimerization domains. Residues 7 to 18 of AraC constitute an N-terminal arm that folds over the sugar-binding pocket, and residues 2 to 6 of AraC are presumably disordered even in the presence of arabinose (28). In the crystal structure of a member of the AraC family, MarA, the C-terminal DNA-binding domain consists of two helix-turn-helix DNA-binding motifs (residues 31 to 52 and 79 to 102), which correspond to residues 198 to 219 and 246 to 269 of AraC (23).

In this study, we show that IPTG is an inhibitor of the P_{BAD} expression system. Cross talk between the P_{BAD} and P_{lac} promoters prevents them from being used simultaneously in the same cell over wide ranges of expression levels. Therefore, we constructed a mutant library of the arabinose-binding regulatory protein AraC (26) and screened mutants showing insensitivity to IPTG in order to overcome the cross talk.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* DH10B cells were grown in Luria-Bertani (LB) medium at 37°C for DNA manipulation and expression experiments. Cell growth was monitored as the optical density at a wavelength of 600 nm. Media were supplemented with ampicillin (100 μg/ml) and arabinose and/or IPTG.

Plasmid construction. All DNA manipulations were performed with *E. coli* DH10B cells by using established protocols (25). To construct pBADM-*gfp*, the HindIII site in the MCS of pBAD24-*gfp* (18) was deleted by self-ligation after digestion and T4 polymerase treatment, and a new HindIII site (underlined) was

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created by the substitution of three nucleotides (AAAAGTATG → AAGCTT ATG) in front of the start codon of *araC*. Site-directed mutagenesis was performed by using a PCR method as described in the instructions for the Stratagene QuikChange site-directed mutagenesis kit.

Random mutagenesis and mutant library construction. Random mutagenesis of the *araC* gene was carried out by performing mutagenic PCR. Two oligonucleotides flanked by ClaI and HindIII restriction sites were used as forward and reverse primers, respectively. The conditions used for PCR random mutagenesis were optimized by changing the concentrations of $MnCl_2$ and $MgCl_2$ in order to obtain approximately 14% of active AraC; a 50-μl reaction mixture contained 5 μl of 10× PCR Gold buffer, 0.8 mM $MnCl_2$, 5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 25 pmol of each primer, 5 ng of template plasmid, and 2.5 U of *Taq* polymerase (Ampli*Taq* Gold DNA polymerase; Applied Biosystems). The PCR was performed with an automatic thermal cycler (MJ Research) for 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The mutagenic PCR products were then cloned into ClaI- and HindIII-digested pBADM-*gfp*.

Determination of in vivo promoter activities. Promoter activities were examined by measuring green fluorescent protein (GFP) production as an indirect, quantitative measurement of the transcriptional properties of cloned *gfp* (7, 34). The culture-averaged fluorescence was measured by using a procedure described previously (18) and expressed as relative fluorescence units (RFU). After induction for 6 h with 0.4 mM arabinose, *E. coli* cells expressing GFP in the presence of 2.5 mM IPTG were collected using fluorescence-activated cell sorting (FACS) (see Fig. S1 in the supplemental material).

RESULTS AND DISCUSSION

Reduction of P_{BAD} promoter activity in the presence of IPTG. We have found that expression from P_{BAD} is inhibited by the presence of IPTG. At relatively low concentrations of arabinose (1.25 and 0.15 mM), P_{BAD} is significantly repressed by IPTG (Fig. 1). On the other hand, the inhibition of P_{BAD} by IPTG is less dramatic in the presence of relatively high concentrations of arabinose (10 mM). Thus, IPTG's inhibition of P_{BAD} is dependent on the relative concentrations of arabinose and IPTG in the cell. The inhibition of P_{BAD} activity by IPTG can be detrimental to controlling gene expression when both IPTG- and arabinose-inducible promoters are used in the same system. Although it would be possible to overcome inhibition by saturating the system with relatively high concentrations of arabinose, this solution prevents P_{BAD} from being induced at different expression levels over a wide range of arabinose concentrations without concern for the relative concentration of IPTG in the system. There are many possibilities for how IPTG could inhibit P_{BAD} activity (e.g., inhibition of arabinose binding to AraC, arabinose transport into the cell, or AraC binding to P_{BAD}), and it is unclear which mechanism is most likely to be the cause of inhibition. One possible way to alleviate this problem is to improve the sensitivity of the system to the inducer (6). Therefore, a P_{BAD} system that is more sensitive to arabinose will be more tolerant to IPTG and more compatible with P_{lac}.

Isolation of mutants exhibiting no inhibition of P_{BAD} promoter activity by IPTG. Error-prone PCR was used to evolve AraC to increase the sensitivity of the P_{BAD} system to arabinose. Cells showing high GFP production from P_{BAD} in the presence of high concentrations of IPTG were collected using FACS and rescreened in 96-well plates. The arabinose and IPTG concentrations used to screen the mutants were determined from dose-dependent experiments that indicated what concentrations of arabinose and IPTG are required for maximal induction and repression of P_{BAD} with wild-type AraC. Twenty mutants showing high GFP expression in the presence of 0.4 mM arabinose with 2.5 mM IPTG and no background expression in the absence of arabinose were isolated. Nineteen of these mutants had the same six mutations: N6I, V65G, L133M, E165G, E169V, and a stop codon at position 280 (C280*) that leads to the deletion of 12 C-terminal amino acids (we named this mutant 1G2) (see Fig. S2 in the supplemental material). In contrast to the significantly reduced expression from the wild-type *araC*-P_{BAD} system when IPTG was added to the medium, expression from P_{BAD} with the 1G2 mutant was unaffected by IPTG (Fig. 2). All of the mutations are located outside of the arabinose-binding pocket of AraC (27, 28). Interestingly, three mutations (L133M, E165G, and E169V) are located in the coiled-coil dimerization domain (see Fig. S3 in the supplemental material), and C280* is located in the C-terminal DNA-binding domain of AraC based on the crystal structure of MarA (see Fig. S4 in the supplemental material), a homolog of the AraC DNA-binding domain (23). N6I is located on the N-terminal arm, and V65G is located on

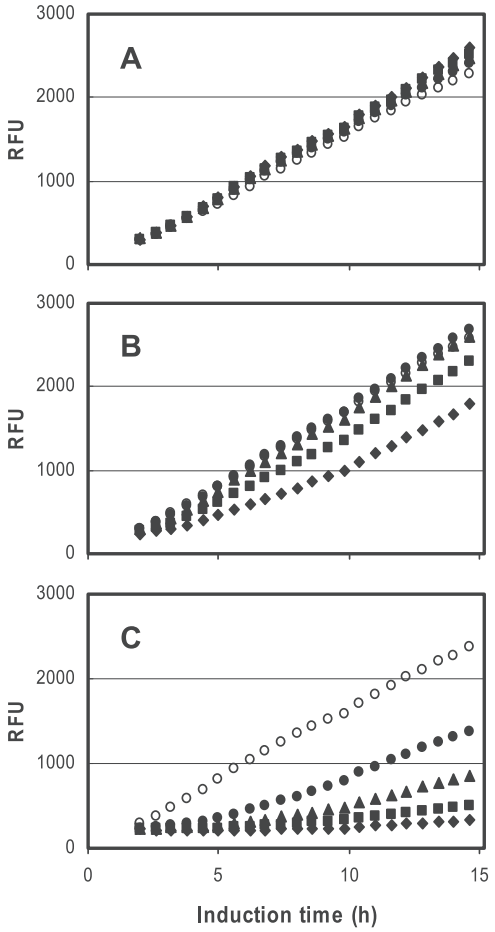

FIG. 1. Reduction of native P_{BAD} expression in the presence of IPTG. P_{BAD} expression was induced with 10 mM arabinose (A), 1.25 mM arabinose (B), and 0.15 mM arabinose (C) in the presence of different IPTG concentrations: filled diamonds, 5 mM; filled squares, 2.5 mM; filled triangles, 1.25 mM; filled circles, 0.65 mM; and open circles, 0 mM. The cells were grown in 96-well plates. The culture-averaged fluorescence was measured using a Tecan SpectraFluor Plus plate reader and expressed as RFU. The data are representative of three independent experiments.

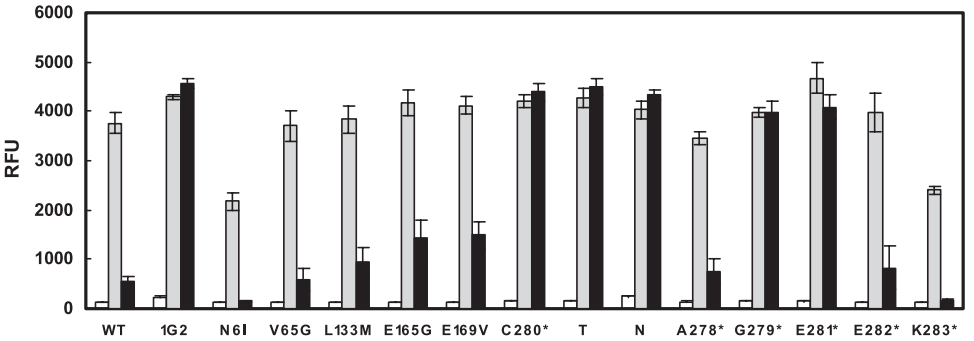

FIG. 2. Expression levels of various AraC mutants exposed to arabinose and IPTG. White bars, 0 mM arabinose and 0 mM IPTG; gray bars, 0.4 mM arabinose and 0 mM IPTG; dark-gray bars, 0.4 mM arabinose and 2.5 mM IPTG; WT, wild-type AraC; T, L133M/E165G/E169V; N, N6I/V65G/L133M/E165G/E169V. Data for single mutations N6I, V65G, L133M, E165G, and E169V and stop codons at positions 278 to 283 are also shown. The cells were grown in culture tubes. The culture-averaged fluorescence was measured using a Tecan SpectraFluor Plus plate reader and expressed as RFU. The data are representative of three independent experiments.

a β-strand of the β-barrel in the N-terminal domain (see Fig. S2 in the supplemental material).

Analysis of site-specific mutations of the 1G2 mutant. To determine which of the six mutations in AraC contributed to the change in P_{BAD} activity, we studied the effects of each individual amino acid residue and specific groups of amino acid residues (Fig. 2). Surprisingly, not all of the individual mutations increased P_{BAD}'s sensitivity to arabinose; indeed, a single mutation in the C terminus (C280*) of AraC reduced IPTG's inhibition of P_{BAD} to the same degree as that observed in the 1G2 AraC mutant. In the presence of 0.4 mM arabinose, N6I significantly reduced P_{BAD} activity; V65G, L133M, E165G, E169V, and C280* resulted in maximum P_{BAD} activity. In the presence of both 0.4 mM arabinose and 2.5 mM IPTG, the single mutation N6I also resulted in a reduction in P_{BAD} activity. V65G resulted in P_{BAD} activity comparable to that of the wild type, L133M, E165G, and E169V resulted in 1.5 to 3 times greater P_{BAD} activity than that of the wild type, and C280* resulted in 8 times greater P_{BAD} activity than that of the wild type, similar to the activity exhibited by the 1G2 mutant. Out of the six mutations, five are located in the N-terminal dimerization domain of AraC. The N-terminal dimerization domain mutations (N6I/V65G/L133M/E165G/E169V [N mutant]) jointly resulted in eight times greater P_{BAD} activity than that of the wild type. Three of the mutations are located in the coiled-coil dimerization domain (L133M/E165G/E169V [T mutant]), and they jointly resulted in eight times greater P_{BAD} activity than that of the wild type as well. Although the N mutant exhibited a phenotype similar to that of the 1G2 mutant, a double mutant with mutations N6I and E165G exhibited a phenotype similar to that of the wild type in the presence of 0.4 mM arabinose and a phenotype similar to that resulting from the N6I mutation in the presence of 0.4 mM arabinose and 2.5 mM IPTG; a double mutant with mutations N6I and C280* exhibited a phenotype similar to that of the 1G2 mutant, with a slight decrease in GFP production in the presence of 0.4 mM arabinose and 2.5 mM IPTG compared to that of the 1G2 mutant (data not shown). Therefore, even though the N6I mutation appears to have had an effect on the other five individual mutations, its effects were masked in the 1G2 mutant. From these results, we conclude that the coiled-coil dimerization domain in which three mutations (L133M, E165G, and E169V) are located and the region around amino acid 280 in the C-terminal DNA-binding domain are important for the observed improvement in P_{BAD} activity in the presence of IPTG. The three mutations in the coiled-coil domain might stabilize the interaction between the coiled-coil domains at the dimerization site in the presence of arabinose. None of the mutations in the dimerization domain showed a significant increase in background activity (Fig. 2), which suggests that the mutations affected AraC only when it was bound to arabinose.

Unlike the mutations in the N-terminal dimerization domain, only one mutation at amino acid 280 was sufficient to produce the same phenotype as that of the 1G2 mutant. The C280* mutation by itself resulted in a phenotype similar to that of the 1G2 mutant; therefore, we truncated the C terminus of AraC to different lengths in order to further study the effects of the C-terminal mutation. Interestingly, G279* and E281* resulted in phenotypes similar to that resulting from C280*, and not one of the three mutations produced a significant increase in background activity from P_{BAD} (Fig. 2). The absence of a significant increase in background activity from the C-terminal deletion mutations indicates that the three amino acid residues affect P_{BAD} activity only when arabinose is bound to the N-terminal dimerization domain. The three amino acid residues are not any of the ones previously identified to be part of the arm–DNA-binding domain interaction in AraC (31). It has also been suggested that charged amino acid residues at the C terminus of AraC might help to stabilize the protein (10); however, it is not clear whether the charge of the C-terminal amino acid residue contributes to the mutant systems' increased sensitivity to arabinose. Both amino acids at positions 281 and 282 in wild-type AraC are glutamic acids, but E282* resulted in increased sensitivity for arabinose while K283* did not.

Comparison of promoter activities between wild-type AraC and 1G2 AraC. To further characterize the 1G2 mutant, we performed dose-dependent experiments with different concentrations of arabinose and IPTG, using a P_{BAD} expression system containing wild-type, 1G2, C280*, or T AraC (Fig. 3). GFP production was under the control of AraC. The system with wild-type AraC showed almost no induction with 0.005 mM arabinose, while that with 1G2 AraC showed significant levels of GFP production with the same concentration of arabinose.

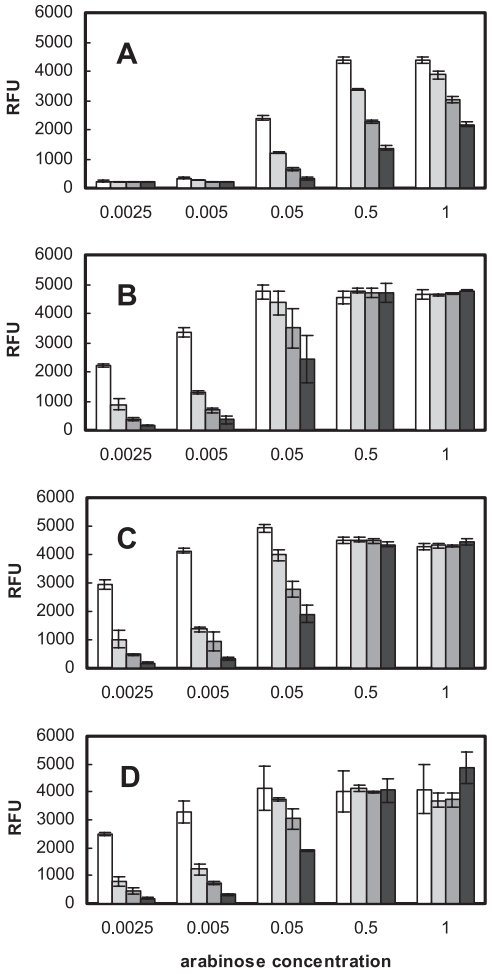

FIG. 3. Comparison of P_{BAD} activities between wild-type AraC (A), the 1G2 mutant (B), the C280* mutant (C), and the T mutant (D). The cells were exposed to various arabinose and IPTG concentrations. White bars, 0 mM IPTG; light-gray bars, 0.2 mM IPTG; gray bars, 0.5 mM IPTG; dark-gray bars, 1 mM IPTG. The cells were grown in culture tubes. The culture-averaged fluorescence was measured using a Tecan SpectraFluor Plus plate reader and expressed as RFU. The data are representative of three independent experiments.

At 0.05 mM arabinose, 1G2 AraC showed maximal GFP production without significant inhibition by IPTG (20% decrease in GFP expression with 0.5 mM [10×] IPTG), while the wild-type AraC showed moderate GFP production (50% compared to GFP production by wild-type AraC with 0.5 mM arabinose) with significant inhibition by IPTG (70% decrease in GFP expression with 0.5 mM IPTG). Therefore, the P_{BAD} system containing 1G2 AraC is significantly more sensitive to arabinose (10-fold) and tolerant to IPTG than that containing wild-type AraC.

Dose-dependent experiments were also performed with the C280* and T mutants in order to determine whether the mutations in different domains of AraC would produce different expression profiles in the presence of different concentrations of arabinose and IPTG. Any changes in the expression profiles of the T and C280* mutants compared to that of the 1G2 mutant would provide clues to the mechanisms by which the mutations independently increase the sensitivity of the system to the inducer. The expression patterns of P_{BAD} under the control of C280* AraC and T AraC are identical to that under the control of 1G2 AraC: 10-fold-increased sensitivity to arabinose, saturation at 0.5 mM arabinose, and significantly improved tolerance for IPTG compared to wild-type AraC. Therefore, more experiments are required in order to determine how the mutations in different domains of AraC independently produce the same phenotype.

Although cross talk itself was not completely removed, the P_{BAD} system we constructed will be important for the regulatable production of proteins that require high expression levels in the presence of IPTG, especially when the systems are constructed with low-copy-number plasmids. A system using P_{BAD} on a low-copy-number plasmid would not produce as much AraC and thus would be more difficult to induce at low arabinose concentrations. Furthermore, the inhibition of the P_{BAD} system by IPTG would be more noticeable when there is less AraC in the cell. However, a mutant P_{BAD} system that is more sensitive to arabinose would alleviate these problems without increasing background expression, because the protein-ligand complex is more likely to form and activate transcription from P_{BAD} even in the presence of IPTG.

In conclusion, although the mechanism by which IPTG inhibits the activity of P_{BAD} remains unclear, we have successfully engineered a P_{BAD} expression system that is more compatible with IPTG-induced P_{lac} systems. The engineered P_{BAD} system is more sensitive to arabinose and tolerant to IPTG, allowing for the simultaneous and independent control of the expression levels of multiple genes from both P_{BAD} and P_{lac}. We have shown that arabinose transport into the cell is probably not inhibited by IPTG, because all of the mutant phenotypes resulted from mutations in AraC. We were unable to determine the exact mechanism by which the different mutations in AraC reduced IPTG inhibition of the P_{BAD} system and increased its sensitivity to arabinose. It is possible that the mutations changed AraC's affinity for arabinose or its affinity for the promoter in the presence of arabinose or IPTG. However, studies of mutants carrying individual mutations derived from the 1G2 mutant indicate that both the coiled-coil dimerization domain and the C-terminal DNA-binding domain of AraC are important for the activity of the protein and a reduction in P_{BAD}'s inhibition by IPTG. A single mutation at C280* reduced P_{BAD}'s sensitivity to IPTG to the same degree as that observed in the 1G2 mutant. Our results also provide insight into the mechanism by which AraC regulates P_{BAD} and the role of specific amino acid residues in that regulation. Improvements to our engineered P_{BAD} system could be made by applying more rounds of random mutagenesis to our mutant AraC or P_{BAD} and by screening for new mutants that are even less sensitive to IPTG in order to further reduce cross talk between P_{BAD} and P_{lac}. A better understanding of how IPTG interacts with the P_{BAD} system would allow directed mutagenesis techniques to be applied in order to alleviate inhibition by IPTG.

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