The Influence of Repressor DNA Binding Site Architecture on Transcriptional Control

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ABSTRACT How the architecture of DNA binding sites dictates the extent of repression of promoters is not well understood. Here, we addressed the importance of the number and information content of the three direct repeats (DRs) in the binding and repression of the icdA promoter by the phosphorylated form of the global Escherichia coli repressor ArcA (ArcA-P). We show that decreasing the information content of the two sites with the highest information (DR1 and DR2) eliminated ArcA binding to all three DRs and ArcA repression of icdA. Unexpectedly, we also found that DR3 occupancy functions principally in repression, since mutation of this low-information-content site both eliminated DNA binding to DR3 and significantly weakened icdA repression, despite the fact that binding to DR1 and DR2 was intact. In addition, increasing the information content of any one of the three DRs or addition of a fourth DR increased ArcA-dependent repression but perturbed signal-dependent regulation of repression. Thus, our data show that the information content and number of DR elements are critical architectural features for maintaining a balance between high-affinity binding and signal-dependent regulation of icdA promoter function in response to changes in ArcA-P levels. Optimization of such architectural features may be a common strategy to either dampen or enhance the sensitivity of DNA binding among the members of the large OmpR/PhoB family of regulators as well as other transcription factors.

IMPORTANCE In Escherichia coli, the response regulator ArcA maintains homeostasis of redox carriers under O2-limiting conditions through a comprehensive repression of carbon oxidation pathways that require aerobic respiration to recycle redox carriers. Although a binding site architecture comprised of a variable number of sequence recognition elements has been identified within the promoter regions of ArcA-repressed operons, it is unclear how this variable architecture dictates transcriptional regulation. By dissecting the role of multiple sequence elements within the icdA promoter, we provide insight into the design principles that allow ArcA to repress transcription within diverse promoter contexts. Our data suggest that the arrangement of recognition elements is tailored to achieve sufficient repression of a given promoter while maintaining appropriate signal-dependent regulation of repression, providing insight into how diverse binding site architectures link changes in O2 with the fine-tuning of carbon oxidation pathway levels.

In Escherichia coli, the ArcAB two-component system, comprised of the membrane-bound sensor kinase ArcB and the response regulator ArcA, couples changes in the respiratory state of cells to a global transcriptional response (1). Under aerobic conditions, ArcB kinase activity is silenced, maintaining ArcA largely in the inactive, unphosphorylated state (1, 2). As O2 levels decrease, the proportion of phosphorylated ArcA increases accordingly, with maximal phosphorylation occurring under anaerobic conditions (3). Upon phosphorylation, ArcA binds extensively across the genome, regulating the expression of ~100 operons and acting predominantly as a global repressor of nonfermentative carbon oxidation pathways (4). Although the mechanism of repression has not been well studied, ArcA binding sites within the promoters of repressed operons contain a variable number of direct repeat (DR) sequence elements while almost exclusively overlapping the $\sigma^{70}$ RNA polymerase ($\sigma^{70}$-RNAP) DNA recognition elements (4). Defining how these ArcA cis-regulatory elements contribute to ArcA DNA binding and repression is critical to understanding how the ArcAB system coordinates this global reprogramming of transcription.

The DNA sequence determinants for ArcA binding have been obscured by the long, degenerate DNA elements bound by ArcA in vitro (5–10). Previous analyses of these footprinted regions have proposed a 15-bp DNA site (5′-GTTAATTAAATGTTA-3′) consisting of two adjacent direct repeats (underlined) as the minimal ArcA recognition site (11–14). However, recent analysis of the chromosomal binding regions of ArcA identified by chromatin immunoprecipitation-DNA sequencing (ChIP-seq) suggested that the minimal ArcA binding site is composed of two 10-bp direct repeat elements (5′-ATGTTAAAAA-1-ATGTTAAAAA-3′).
Furthermore, the majority of ArcA binding sites contain an additional one to three DR elements spaced by approximately one to two turns of the B-form DNA helix (11-bp or 22-bp CTC spacing) from the minimal two DR sites (4). DNase I footprinting assays suggest that these additional DR elements dictate the length of the ArcA binding site (4), providing an explanation for the long ArcA-P footprints.

The abundance of ArcA binding sites with three DR elements (4) raises the question of how ArcA dimers bind to a DNA site with an odd number of DR elements. Tandem direct repeat element recognition by an ArcA dimer is supported by the cocrystal structure of the C-terminal DNA binding domain of the closely related response regulator PhoB bound to its tandem direct repeat site as a head-to-tail dimer (15). However, the structure of the activated N-terminal regulatory domain of ArcA bound to a phosphate analog is also dimeric but with a symmetric mode of dimerization (16). These data have led to a model for ArcA and other OmpR/

(Fig. 1A) separated by a single nucleotide spacer (11 bp, center to center [CTC]). Furthermore, the majority of ArcA binding sites contain an additional one to three DR elements spaced by approximately one to two turns of the B-form DNA helix (11-bp or 22-bp CTC spacing) from the minimal two DR sites (4). DNase I footprinting assays suggest that these additional DR elements dictate the length of the ArcA binding site (4), providing an explanation for the long ArcA-P footprints.

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PhoB response regulators that consists of the C-terminal DNA binding domains of the dimer bound to two DRs in a head-to-tail orientation, connected via a flexible linker to the N-terminal regulatory domains that are oriented symmetrically (head to head) along a common interface (16, 17). The recent structural characterization of full-length KdpE, another OmpR/PhoB family member, bound to its direct repeat site confirmed these different domain symmetries while identifying an additional level of asymmetry resulting from intramolecular contacts between the receiver and DNA binding domains within one KdpE subunit (18). Nevertheless, full-length ArcA-P has been reported to form oligomers (19), as have both the isolated N-terminal and C-terminal domains (16), suggesting that although the minimal DNA binding unit is likely a dimer, as demonstrated for PhoB and OmpR (20–22), oligomerization beyond a dimer may explain binding to multiple direct repeats.

To gain insight into the physiological function of multiple DR element binding sites, we evaluated the role of each of the three predicted 10-bp DR elements (DR1-1, DR2-1, DR3) in ArcA-P DNA binding and repression of icdA, encoding isocitrate dehydrogenase of the tricarboxylic acid (TCA) cycle. These repeats are directionally oriented on the noncoding strand and are numbered on the basis of their order in the 5’-to-3’ direction (Fig. 1B). This particular three-DR binding site was chosen because all three DR elements were protected from DNase I cleavage when ArcA is bound (4, 5) despite both strong (DR1 [11.2-bit]) and weak (DR2 [4.9-bit] and DR3 [3.0-bit]) matches to the position weight matrix (PWM) (Fig. 1) for a single DR element (4) and because ArcA-P is the only annotated regulator of the primary icdA promoter (P_{icdA}) (23). Thus, changes in ArcA DNA binding should change P_{icdA} repression. In addition, the positions of DR3 adjacent to the −10 promoter element and of DR1 and DR2 downstream of the transcription start site (TSS) (5) (Fig. 1B) provided an opportunity to determine if there were any specific effects of DR element positioning on ArcA DNA binding and transcriptional regulation. To understand the contribution of each DR in ArcA repression of icdA, they were mutated toward or away from 5’-TGTAA-3’ (Fig. 1C), the most conserved sequence within each ArcA DR element (hereinafter referred to as the consensus) and, based on the PhoB DNA cocrystal structure (15), the region likely contacted by ArcA in the major groove. Mutant arcA alleles were used to determine the phosphorylation dependence of this regulation. Our data reveal that all three DR elements are important for full anaerobic repression of icdA and that degeneracy in these DR elements is important for preserving O_{2} dependence.

RESULTS

All three DR elements within the icdA promoter contribute to ArcA-P DNA binding in vitro. To test the role of each of the three DR elements in ArcA DNA binding to P_{icdA} DNase I footprinting assays were performed using ArcA-P and either the wild type (wt) icdA promoter fragment or those in which each DR element was individually disrupted through mutation of highly conserved GT to CA (5’-TGTAA-3’ to 5’-TGCA-3’), reducing the information content of each DR element below the theoretical lowest limit of binding (0 bits) (24) (Fig. 1C). As previously observed (4), ArcA-P protected the three DR elements of the wt promoter region from −12 to +21 relative to the TSS (Fig. 2A). As expected from previous results (4, 5), more ArcA-P (600 nM) was required to observe maximum occupancy of the lower-information-content site, DR3 (3.0 bits), than the higher-information-content sites, DR1 (11.2 bits) and DR2 (4.9 bits) (300 nM). Disruption of either DR1 or DR2 eliminated ArcA-P protection of all three DR elements, even at the highest ArcA-P levels tested (Fig. 2C and D). In contrast, when DR3 was mutated, ArcA-P binding to only DR3 was eliminated (Fig. 2B). Furthermore, the amount of ArcA-P required for maximal binding of either DR1 or DR2 was not affected by disruption of DR3, suggesting that ArcA binding to DR1 and DR2 is not enhanced by ArcA-P interactions with DR3 despite the dependence of DR3 binding on ArcA-P interactions with DR1 and DR2.

The mechanisms governing the occupancy of DR3 may be complex, since we found that an N-terminal His tag variant of ArcA-P also eliminated binding to DR3, but not DR1 and DR2 (data not shown), suggesting that protein–protein interactions may be important for stabilizing ArcA-P binding to DR3. We also found that disruption of DR3 weakened a hypersensitive band at position +8 within DR2 (Fig. 2A and B). Because DNase I is sensitive to the minor groove width (25), this change in hypersensitivity may suggest that ArcA-P bends or kinks the DNA to a greater degree when bound to all three DR elements than when bound to just DR1 and DR2. Thus, an ArcA-P dimer bound to DR1 and DR2 may also stabilize the binding of ArcA-P to DR3 by bending the DNA.

All three DR elements are required for repression of icdA in vivo. How ArcA binding to each DR element contributes to icdA repression was determined by measuring β-galactosidase activity produced from P_{icdA}-lacZ transcriptional fusions containing the GT-to-CA (5’-TGTAA-3’ to 5’-TGCA-3’), which did not perturb ArcA binding to DR1 and DR2 (Fig. 2B), showed an ~3.5-fold loss in repression (Fig. 3A). This result suggests that in vivo occupancy of DR1 and DR2 is sufficient to direct a moderate amount of P_{icdA} repression but that additional occupancy of DR3 is required for maximal repression, perhaps because it overlaps the −10 promoter element.

The three DRs of P_{icdA} are suboptimal for maximal repression. Since DR2 and DR3 contain a lower information content than DR1, we tested whether mutations that improve the information content affect repression under anaerobic conditions. Mutation of DR2 toward the consensus (5’-TGTAA-3’ to 5’-TGTA-3’) resulted in a 3-fold increase in anaerobic repression of P_{icdA} (Fig. 3B). This repression still depends on DR3 function, since the additional disruption of DR3 (5’-TGTCA-3’ to 5’-TACA-3’) caused the same 3-fold reduction in repression as observed when DR3 was disrupted in an otherwise wt icdA sequence (Fig. 3A and B). When just DR3 was mutated toward the consensus (5’-TGTCA-3’ to 5’-TGTA-3’), repression was increased 6-fold (Fig. 3B). Improving both DR2 and DR3 toward the consensus resulted in a level of repression similar to that observed with a consensus DR3 element alone, suggesting that maximal P_{icdA} repression by ArcA had been achieved (Fig. 3B). Assuming that these nucleotide changes simply improve DNA binding affin-
ity, the enhanced anaerobic repression suggests that the three DRs of wt icdA are not completely occupied by ArcA under our standard anaerobic growth conditions.

**A fourth DR element enhances ArcA binding affinity.** Although our DNase I footprinting analysis suggests that an ArcA-P dimer bound to DR1 and DR2 stabilizes the binding of ArcA-P to DR3, whether ArcA-P binds as a dimer or as a monomer to DR3 is an open question. The lack of DNase I protection of the DNA sequence adjacent to DR3 suggests that if a dimer is bound, then this sequence either contributes only weakly or not at all to stabilizing the binding of the second dimer. To determine whether adding a fourth DR element facilitates ArcA-P binding and increases the footprint length, a consensus DR element (5′ = -TGTTA-3′) was added at the same spacing (11 bp CTC) to either the 3′ or the 5′ end of the three-DR ArcA binding site within the icdA promoter region and DNase I footprinting experiments were performed. For both variants, the ArcA-P footprint encompassed all four DR elements, and the apparent ArcA-P DNA binding affinity was noticeably increased compared to that with the wt binding site (Fig. 4A and B). In addition, protection of the entire four-DR site occurred over a very narrow increase in ArcA-P levels (≤4-fold), suggesting that cooperativity was also enhanced. Notably, the hypersensitive sites at positions +8 and +19 were unaffected by binding to a fourth repeat, suggesting bending or kinking similar to that with the wt binding site. Finally, as with the wt icdA fragment, binding depended on phosphorylation, since no binding was observed with unphosphorylated ArcA at protein concentrations up to 1 μM (data not shown).

**Adding a fourth DR element or improving DR2 or DR3 disrupts O2-dependent regulation of ArcA DNA binding.** Despite the potential for enhancement of DNA binding, multiple consecutive high-information-content DR sites are relatively rare in the E. coli genome (4), raising the question of whether there is a tradeoff between DNA binding and the ability to respond to the regulatory signal. To test whether the P1 icdA variant with four consecutive DR binding sites still retains O2-dependent regulation, we measured β-galactosidase activity produced from a P1 icdA- lacZ transcriptional fusion containing either the 3′ or the 5′ DR4 element and compared it to that produced with the wt promoter under anaerobic or aerobic conditions. As expected, ArcA-dependent repression of wt P1 icdA was largely relieved in the presence of O2 (Fig. 3C), consistent with the known reduction in ArcA-P levels under aerobic conditions (3). However, addition of DR4 to the 5′ end not only resulted in an 8-fold increase in repres-
sion compared to the repression with the wt binding site under anaerobic conditions (Fig. 3B) but also increased repression by ArcA under aerobic conditions to nearly the same magnitude observed under anaerobic conditions, indicating that ArcA repression of this variant site was no longer O2 sensitive (Fig. 3C). The addition of DR4 to the 3'-end disrupted promoter function, preventing assessment of ArcA repression (data not shown). The simplest interpretation of these results is that strengthening binding affinity disrupts O2-dependent regulation of ArcA DNA binding.

We also tested whether the degeneracy of DR2 and DR3 (Fig. 1C) is important for maintaining O2-dependent regulation of P1icdA by assaying the variants where the sites were mutated toward the consensus. Improving DR2 or DR3 toward the consensus compared to the repression with the wt binding site under anaerobic conditions (Fig. 3B) but also increased repression by ArcA under aerobic conditions to nearly the same magnitude observed under anaerobic conditions, indicating that ArcA repression of this variant site was no longer O2 sensitive (Fig. 3C). The addition of DR4 to the 3'-end disrupted promoter function, preventing assessment of ArcA repression (data not shown). The simplest interpretation of these results is that strengthening binding affinity disrupts O2-dependent regulation of ArcA DNA binding.

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sensus also increased aerobic $P_{icdA}$ repression compared to that with the wild-type binding site, but the effect was more pronounced with a consensus DR3 element (~2.5-fold versus 7-fold repression) (Fig. 3C). $P_{icdA}$ with both consensus DR2 and DR3 elements was even more repressed by ArcA under aerobic conditions (21-fold) than with either consensus site alone, suggesting that there was an additive effect (Fig. 3C). Together, these results suggest that improving binding affinity through the use of consensus DR elements disrupts the signal-dependent regulation of ArcA DNA binding, suggesting that the degeneracy of DR2 and DR3 is important for balancing anaerobic repression with $O_2$-dependent relief of repression.

**Enhanced ArcA repression is still dependent on phosphorylation.** To test whether the enhanced repression of $P_{icdA}$ with mutant ArcA binding sites is still dependent on phosphorylation, the aspartate residue at position 54 (site of phosphorylation [19]) in the chromosomal copy of *arcA* was mutated to yield either alanine or glutamate, preventing phosphorylation from ArcB (19). The D54A variant reduced the repression of all $P_{icdA}$-*lacZ* constructs compared to that with the wt protein under both aerobic and anaerobic growth conditions (Fig. 5A). This suggests that, independent of the strength of the binding site, repression is largely dependent on the phosphorylated form of ArcA. This result is consistent with the failure of unphosphorylated ArcA to bind to the four DR sites in vitro (data not shown). Thus, the elevated aerobic repression with the strengthened ArcA binding sites appears to result from increased occupancy of the small amount of ArcA-P likely present during aerobic conditions.

We expected ArcA(D54E) to similarly reduce the repression of $P_{icdA}$ since this substitution has previously been shown to prevent both phosphorylation from ArcB and binding to the *pfl* promoter (19). Surprisingly, ArcA(D54E) still strongly repressed $P_{icdA}$ constructs with strengthened binding sites even though repression of wt $P_{icdA}$ was largely eliminated; repression of the construct with a consensus DR3 element was reduced by only 2-fold, while repression of constructs with consensus DR2 and DR3 elements or a fourth DR element was indistinguishable from that observed with the wt protein under anaerobic conditions (Fig. 5B). Furthermore, under aerobic conditions, ArcA(D54E) repression of $P_{icdA}$ was increased compared to that of wt ArcA for all binding sites tested (Fig. 5B). Thus, D54E ArcA appears to partially mimic phosphorylated ArcA. An aspartate-to-glutamate substitution has previously been shown to elicit constitutive activity in some response regulators (26).

**DISCUSSION**

The results presented here provide new insight into the plasticity of the DNA elements that can control transcriptional repression. Our data suggest that for *icdA*, the arrangement of multiple DNA binding elements appears to be tailored to achieve both sufficient DNA binding affinity and repression by ArcA while maintaining $O_2$-dependent regulation. We propose that the distribution of DNA binding information across several DR elements may be a design principle to achieve the appropriate level of repression and to tune the signal-dependent regulation of target genes for both ArcA and other repressors.

**Interaction of ArcA with three DR elements of *icdA*.** Our analysis of the three DR elements of the *icdA* promoter indicate that ArcA-P binding to the lowest-information-content site, DR3, is stabilized by ArcA-P bound to DR1 and DR2, suggestive of a cooperative DNA binding mechanism. The lack of an observable defect in binding to DR1 or DR2 when DR3 was eliminated suggests that the cooperative energy is predominantly partitioned toward binding of DR3, as expected for sites with large differences in intrinsic levels of binding energy (27). Since ~67 genomic sites have an odd number of DR elements (4), cooperativity is likely an important determinant for ArcA binding genome-wide.

An unanswered question is what the stoichiometry of ArcA-P binding to DR1, -2, and -3 is. It is possible that ArcA-P binds to the *icdA* promoter as a dimer of dimers; one dimer binds DR1 and DR2, as depicted in the PhoB and KdpE DNA cocrystal structures (15, 18), and the second dimer binds DR3 but only weakly to adjacent DNA sequence, such that no footprint is observed (Fig. 6). This model is supported by the requirement for phosphorylation of ArcA to bind to DR3, which is also known to promote dimer formation among OmpR/PhoB response regulators.

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**FIG 5** Phosphorylation dependence of ArcA repression of $P_{icdA}$ in strains with strengthened ArcA binding sites. The fold repression of $P_{icdA}$-*lacZ* in strains containing *arcA*-FRT-cat-FRT (white bars), *arcA*(D54A)-FRT-cat-FRT (light-gray bars), or *arcA*(D54E)-FRT-cat-FRT (dark-gray bars) was determined from cells grown under anaerobic (A) or aerobic (B) conditions and calculated by dividing the β-galactosidase activity of a ΔarcA strain by the activity with each of the *arcA* alleles. Asterisks denote DR elements that have been mutated toward the consensus. Error bars represent the standard errors of results from at least three independent replicates.
However, phosphorylation may simply eliminate an interaction between the regulatory and DNA-binding domains, allowing ArcA-P to bind as a monomer to DR3 (Fig. 6). Thus, additional studies are necessary to determine the stoichiometry of ArcA binding to the icdA promoter and whether this stoichiometry is shared among other ArcA sites with three DR elements. In either scenario, the predominance of three DR sites with 11-bp CTC spacing between each DR in the E. coli genome, together with our previous finding that ArcA-P did not bind to a predicted DR3 element in which the CTC spacing was separated by an additional bp (4), suggests that protein-protein interactions between correctly spaced subunits is important for cooperative ArcA binding to the icdA promoter and whether this stoichiometry is shared among other ArcA sites with three DR elements.

In either scenario, the predominance of three DR sites with 11-bp CTC spacing between each DR in the E. coli genome, together with our previous finding that ArcA-P did not bind to a predicted DR3 element in which the CTC spacing was separated by an additional bp (4), suggests that protein-protein interactions between correctly spaced subunits is important for cooperative ArcA binding to multiple DRs. Because the C-terminal domain of ArcA binds as a dimer to two adjacent DRs, one can envision that binding of an ArcA-P dimer to DR1 and DR2 stabilizes a second dimer or a monomer via protein interactions with DR3. Additionally, the hypersensitive site observed when all three (or four) DR elements of icdA were occupied may indicate a requirement for DNA bending to facilitate these protein-protein interactions. Our finding that an N-terminally His-tagged variant of ArcA failed to stabilize binding to DR3 suggests that the His tag specifically disrupts the mechanism needed to enhance the energetics of DR3 binding site occupancy. Additional work is needed to define the molecular interactions that stabilize ArcA-P binding to DR3 elements, but tagged protein variants may not recapitulate this important property of response regulators.

Maximizing repression by binding DR3. The analysis of the effects of mutations eliminating individual DR elements suggests that DR1 and DR2 determine the overall strength of ArcA binding and that all three DR elements contribute to repression. However, the fact that DR3 overlaps the −10 hexamer as opposed to DR1 and DR2, which are located between positions +2 and +22 (Fig. 1B), suggests that ArcA binding to DR3 may interfere with the initial binding of RNA polymerase to form the closed complex, as has been shown for the Lac repressor bound to the Lac operator that overlaps the TSS (28). Furthermore, more-effective repression was observed when the Lac and Tet operators overlapped the −10 and −35 promoter elements than when they overlapped those placed downstream of the TSS (29–31). We do not expect this particular role of DR3 in icdA to be broadly applicable to all multiple-DR-element ArcA binding sites because of differences in both the strengths and the locations of DR3 elements relative to the TSS (4). Furthermore, because ArcA DNA elements are direct repeats, they can be found either in the same or in the opposite orientation from the promoter elements, providing additional flexibility for coding repressor information within a constrained sequence space. There are several instances where all three ArcA DR elements overlap the promoter elements or where DR3 is found downstream of the TSS and may thus play a role more akin to those of DR1 and DR2 of icdA. This flexible property of response regulators may also be confined to repressors, since acti-
vators are likely to be located in specific positions because of the typical requirement to interact with RNA polymerase.

The combinatorial effect of weak versus strong DR elements can create a range of responses to ArcA levels. At icdA, the differences in binding affinity of an ArcA-P dimer for DR1/DR2 versus DR3 increases the amount of ArcA-P required for full occupancy in vitro. Assuming that ArcA-P binds the same way in vivo, this binding site architecture would extend the sensitivity to ArcA-P levels by increasing the amount of ArcA-P required for maximal repression. This property may be a feature shared with other response regulators, since in the case of OmpR, the binding of an OmpR-P dimer to box 1 (two DR elements) at the ompF and ompC promoters occurs at a lower concentration of OmpR-P than does binding of OmpR-P to adjacent OmpR boxes (32, 33). Similarly, binding of PhoB-P to the upstream PhoB box at the pstS promoter occurs at a lower concentration of PhoB-P than when it binds to the adjacent, downstream box (34).

On the other hand, promoters with three or more DR elements of high information content appear to result in ArcA-P occupancy over a very narrow range of protein concentrations. For example, when the disparity in ArcA-P binding affinities at over a very narrow range of protein concentrations. For example, of high information content appear to result in ArcA-P occupancy of the promoter occurs at a lower concentration of PhoB-P than when it responds to ArcA-P with a switch-like behavior as cells become limited for O2. For the engineered icdA promoter containing a four-DR site, it seems likely that the affinity of ArcA-P for this site is so strong that the concentration of ArcA-P present under anaerobic conditions is sufficient to occupy this site so that an O2-dependent change in repression cannot be observed. Nevertheless, our data provide a model for how the ArcA binding site architecture may be optimized to achieve regulatory logic schemes not possible with a canonical two-DR binding site. This plasticity in the promoter architecture likely plays an important role in linking the redox-sensing properties of the ArcAB two-component system with the fine-tuning of expression of carbon oxidation pathway levels.

The incorporation of plasticity in the binding site architectures that we observed for ArcA may be a common regulatory strategy for other global transcriptional repressors (e.g., Fur, LexA). Like ArcA, Fur binding sites are variable in length (30 to 103 bp) and contain multiple Fur recognition elements of differing predicted strengths and locations with respect to the promoter elements (35). Although the physiological basis for this plasticity is unknown, it may similarly impose a differential sensitivity of regulatory target expression to changes in Fe-Fur concentrations. Furthermore, although LexA-regulated genes typically have only one LexA binding site, differences in the strengths and locations of these sites alter the absolute level and sensitivity of expression (36). In a few cases, adjacent LexA sites are bound in a cooperative manner, further enhancing the sensitivity to changes in signal (36), as hypothesized for the ArcA binding sites located upstream of acs and astC. Given the conserved dimerization mode and binding of direct repeat DNA sites among response regulators within the OmpR/PhoB family (16), this architectural plasticity may be a common regulatory strategy, particularly for regulators that act as repressors at many targets.

MATERIALS AND METHODS

Strain construction. An icdA promoter-lacZ fusion was constructed as described previously (37) by amplifying the region from −50 to −330 with respect to the start of translation using primers flanked by XhoI or BamHI restriction sites. The icdA fragment contains two promoters: one whose expression is dependent on ArcA (P1) and a second whose expression is dependent on FurR (P2) (5, 38). To examine icdA expression from only P1, transcription from P2 was eliminated using QuikChange site-directed mutagenesis (Stratagene) as described previously (39) to mutate the −10 site from 5′-CATTAT-3′ to 5′-CGGTGA-3′, generating pPK9476. Mutations within the ArcA binding site of the icdA promoter were similarly generated using pPK9476 as a template (mutations are numbered with respect to P1 in Table 1). These lacZ promoter constructs

Furthermore, it may explain why there are many three-DR sites without identifiable fourth DR elements in the E. coli genome and, additionally, why the average strength of DR elements decreases as the number of DR elements in the binding site increases (4). Nevertheless, both the strength of the promoter and the incorporation of other regulator binding sites should at least partially dictate the specific ArcA binding site architecture required to achieve optimal regulation, with four DR sites apparently necessary at some promoters.

It will also be informative to determine how expression of other ArcA-dependent promoters (e.g., acs and astC) with a strong DR3 and/or DR4 respond to changes in O2. The saturation of ArcA-P binding to these sites over a narrow range of ArcA-P concentrations in vitro (4) suggests that these promoters may respond to ArcA-P with a switch-like behavior as cells become limited for O2. For the engineered icdA promoter containing a four-DR site, it seems likely that the affinity of ArcA-P for this site is so strong that the concentration of ArcA-P present under anaerobic conditions is sufficient to occupy this site so that an O2-dependent change in repression cannot be observed. Nevertheless, our data provide a model for how the ArcA binding site architecture may be optimized to achieve regulatory logic schemes not possible with a canonical two-DR binding site. This plasticity in the promoter architecture likely plays an important role in linking the redox-sensing properties of the ArcAB two-component system with the fine-tuning of expression of carbon oxidation pathway levels.

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were then recombined into the chromosomal lac operon as previously described (37) and then transduced using P1 vir into MG1655 and PK9416 to form the strain derivatives listed in Table 1.

Chromosomally encoded arcA mutants in which aspartate at position 54 was replaced with glutamate or alanine were constructed in several steps. First, the arcA open reading frame (codons 1 to 238) was amplified using primers flanked by HindIII and BamHI and cloned into pBR322, generating pPK9465. The cat cassette from pKD32, which has flanking FRT (FLP recognition target) sites, was then cloned into the BamHI site, 6 bp after the arcA termination codon. The arcA gene on the resulting plasmid, pPK9966, was then mutated using QuikChange (Stratagene) site-directed mutagenesis to create the D54A and D54E mutants. The arcA-cat fragments were PCR amplified using a primer with homology to the region upstream of arcA (5′-GGTAGGAAACACGACTACCCCGGCGGATCTTTTCTAG-3′) and a primer with homology to the region downstream of arcA (5′-GCCG CTTGTTTTTTTTGCGCCGAGGTAGGAGCGGCTGGAG CTTGCTTC-3′), and the DNA was electroporated into BW25993/pKD46. The correct recombinants were selected for chloramphenicol (Cm) resistance, confirmed with DNA sequencing, and then transduced with P1 vir into the desired icdA promoter-lacZ fusion strains (Table 1). Placement of the cat cassette downstream of arcA did not

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| MG1655            | F- ρ- rph-1 | This laboratory     |
| PK9416            | MG1655 ΔarcA | 4                   |
| PK9483            | MG1655 P\text{\textsc{sub}} (-58GTTG-54)-lacZ | 4                   |
| PK9484            | PK9416 P\text{\textsc{sub}} (-58GTTG-54)-lacZ | 4                   |
| PK9494            | MG1655 P\text{\textsc{sub}} (-58GTTG-54, 19TG20)-lacZ | This study         |
| PK9495            | PK9416 P\text{\textsc{sub}} (-58GTTG-54, 19TG20)-lacZ | This study         |
| PK9486            | MG1655 P\text{\textsc{sub}} (-58GTTG-54, 8TG9)-lacZ | This study         |
| PK9487            | PK9416 P\text{\textsc{sub}} (-58GTTG-54, 8TG9)-lacZ | This study         |
| PK9496            | MG1655 P\text{\textsc{sub}} (-58GTTG-54, -4TG-3)-lacZ | This study         |
|PK9497 | PK9416 P\text{\textsc{sub}} (-58GTTG-54, -4TG-3)-lacZ | This study |
| PK9915            | MG1655 P\text{\textsc{sub}} (-58GTTG-54, 29ACA32)-lacZ | This study         |
| PK9916            | PK9416 P\text{\textsc{sub}} (-58GTTG-54, 29ACA32)-lacZ | This study |
| PK9917            | MG1655 P\text{\textsc{sub}} (-58GTTG-54, -15ACA-13)-lacZ | This study         |
| PK9918            | PK9416 P\text{\textsc{sub}} (-58GTTG-54, -15ACA-13)-lacZ | This study         |
| PK9924            | MG1655 P\text{\textsc{sub}} (-58GTTG-54, -5A)-lacZ | This study         |
| PK9925            | PK9416 P\text{\textsc{sub}} (-58GTTG-54, -5A)-lacZ | This study         |
| PK9941            | MG1655 P\text{\textsc{sub}} (-58GTTG-54, 7A)-lacZ | This study         |
| PK9942            | PK9416 P\text{\textsc{sub}} (-58GTTG-54, 7A)-lacZ | This study         |
| PK9943            | MG1655 P\text{\textsc{sub}} (-58GTTG-54, 7A, -5A)-lacZ | This study         |
| PK9944            | PK9416 P\text{\textsc{sub}} (-58GTTG-54, 7A, -5A)-lacZ | This study         |
| PK10967           | MG1655 P\text{\textsc{sub}} (-58GTTG-54, 7A, -4TG-3)-lacZ | This study         |
| PK10968           | PK9416 P\text{\textsc{sub}} (-58GTTG-54, 7A, -4TG-3)-lacZ | This study         |
| BW25993           | lacZ ΔaraBAD ΔrhaBAD ΔA1132 ΔA1178 | 45                 |
| PK9970            | PK9485 arcA-cat | This study         |
| PK9973            | PK9915 arcA:cat | This study         |
| PK9971            | PK9924 arcA:cat | This study         |
| PK9972            | PK9943 arcA:cat | This study         |
| PK9980            | PK9483 arcA-D54A:cat | This study |
| PK9983            | PK9915 arcA-D54A:cat | This study |
| PK9981            | PK9924 arcA-D54A:cat | This study |
| PK9982            | PK9943 arcA-D54A:cat | This study         |
| PK9975            | PK9483 arcA-D54E:cat | This study         |
| PK9978            | PK9915 arcA-D54E:cat | This study |
| PK9976            | PK9924 arcA-D54E:cat | This study         |
| PK9977            | PK9943 arcA-D54E:cat | This study         |
| Plasmids          |             |                     |
| pKD46             | Phage λ gam-bet-exo genes under P\text{\textsc{aro}} control | B. L. Wanner |
| pKD13             | FRT-kan-FRT | K. A. Datsenko and B. L. Wanner |
| pKD32             | FRT-cat-FRT | B. L. Wanner |
| pPK7035           | kan gene from pHP450 and BamHI-Ndel fragment from pRS1553 into pBR322 | 37                 |
| pPK9476           | pPK7035 P\text{\textsc{sub}} (-58GTTG-54)-lacZ | 4                   |
| pPK9477           | pPK7035 P\text{\textsc{sub}} (-58GTTG-54, 19TG20)-lacZ | This study         |
| pPK9908           | pPK7035 P\text{\textsc{sub}} (-58GTTG-54, 8TG9)-lacZ | This study         |
| pPK9909           | pPK7035 P\text{\textsc{sub}} (-58GTTG-54, 8TG9)-lacZ | This study         |
| pPK9913           | pPK7035 P\text{\textsc{sub}} (-58GTTG-54, -4TG-3)-lacZ | This study         |
| pPK9914           | pPK7035 P\text{\textsc{sub}} (-58GTTG-54, -15ACA-13)-lacZ | This study         |
| pPK15001          | pPK7035 P\text{\textsc{sub}} (-58GTTG-54, 7A, -4TG-3)-lacZ | This study         |
| pPK9965           | arcA in pBR322 | This study         |
| pPK9966           | BamHI FRT-cat-FRT in pPK9965 | This study |
| pPK9431           | Ap\textsuperscript{r}; His\textsubscript{r}; arcA cloned into the Nhel and Xhol sites of pET-21d | 4                   |
alter ArcA activity, as icdA promoter-lacZ activity was comparable to that of the wt arcA+ strain for all binding sites tested (Fig. 3B and C and 5A and B).

**Determination of the information content of DR elements.** A 10-bp ArcA DR element, PWM, derived from the conservation of bases within aligned DRI and DR2 elements from 128 sequences bound by ArcA in vivo (4) was used to guide the design of binding site mutations. The information content of each mutant DR element was determined by the scan program (24) and is indicated in bits (Fig. 1C). Greater information content should reflect stronger ArcA binding (24). Sequence walkers (40) were used to visualize how DR elements were evaluated by the PWM. Nucleotides extending upward represent favorable DNA contacts, while letters extending downward represent unfavorable contacts.

**β-Galactosidase assays.** All strains were grown in MOPS minimal medium (41) with 0.2% glucose at 37°C and sparged with a gas mix of 95% N2 and 5% CO2 (anaerobic) or 70% N2, 5% CO2, and 25% O2 (aerobic). Cells were harvested during mid-log growth (optical density at 600 nm [OD600] of ~0.3 on a PerkinElmer Lambda 25 UV/visible-light spectrophotometer). To terminate cell growth and any further protein synthesis, chloramphenicol (final concentration, 20 μg/ml) or tetracycline (final concentration, 10 μg/ml) was added, and cells were plated on ice until assayed for β-galactosidase activity (42). β-Galactosidase assays were repeated at least three times, and fold repression was calculated by dividing the β-galactosidase activity of a ΔarcA strain by the activity of an arcA+ strain. Standard errors for data plotted as “fold repression” were calculated using a formula for propagation of standard error (43).

**Overexpression and purification of His6-ArcA.** E. coli BL21 (DE3) plysS, containing the PK9431 gene, was grown at 37°C until an OD600 of ~0.4 was reached. A final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, and cells were incubated at 30°C. Cells were harvested, suspended in 5 mM imidazole buffer containing 20 mM Tris-Cl (pH 7.9) and 0.5 M NaCl, and lysed by sonication. His6-ArcA was isolated from cell lysates by passing them over a Ni-nitrilotriacetic acid (NTA) column preequilibrated with 5 mM imidazole, washing the column extensively with the same buffer and then with 20 and 50 mM imidazole, and then eluting with 100 mM imidazole. Fractions containing the overexpressed His6-ArcA, determined by electrophoresis, were dialyzed against 50 mM Tris-Cl, pH 7.5, 0.1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.2 M NaCl. The His tag was removed from ArcA by overnight incubation with tobacco etch virus (TEV) protease at 4°C and passage over a Ni-NTA-agarose column (Qiagen). The protein concentration of ArcA (reported here as monomers) was determined as previously described (4).

**DNase I footprinting.** icdA promoter fragments were isolated from pPK9476, pPK9477, pPK9908, pPK9913, pPK9914, and pPK15001 (Table 1) after digestion with XhoI and BamHI. Sequenase (version 2.0 (USB Scientific) was used to 3′-end radiolabel the BamHI end of the fragment with [α-32P]dGTP (PerkinElmer). Labeled DNA fragments were isolated from a nondenaturing 5% acrylamide gel and were visualized by phos- phorimaging.

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