

The Influence of Repressor DNA Binding Site Architecture on Transcriptional Control

Dan M. Park,* Patricia J. Kiley

Department of Biomolecular Chemistry, University of Wisconsin—Madison, Madison, Wisconsin, USA

* Present address: Dan M. Park, Lawrence Livermore National Laboratory, Livermore, California, USA.

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.

Received 22 July 2014 Accepted 25 July 2014 Published 26 August 2014

Citation Park DM, Kiley PJ. 2014. The influence of repressor DNA binding site architecture on transcriptional control. *mBio* 5(5):e01684-14. doi:10.1128/mBio.01684-14.

Editor Susan Gottesman, National Cancer Institute

Copyright © 2014 Park and Kiley. This is an open-access article distributed under the terms of the [Creative Commons Attribution-Noncommercial-ShareAlike 3.0 Unported license](#), which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

Address correspondence to Patricia J. Kiley, pjkiley@wisc.edu.

This article is a direct contribution from a Fellow of the American Academy of Microbiology.

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 σ^{70}

RNA polymerase (σ^{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')

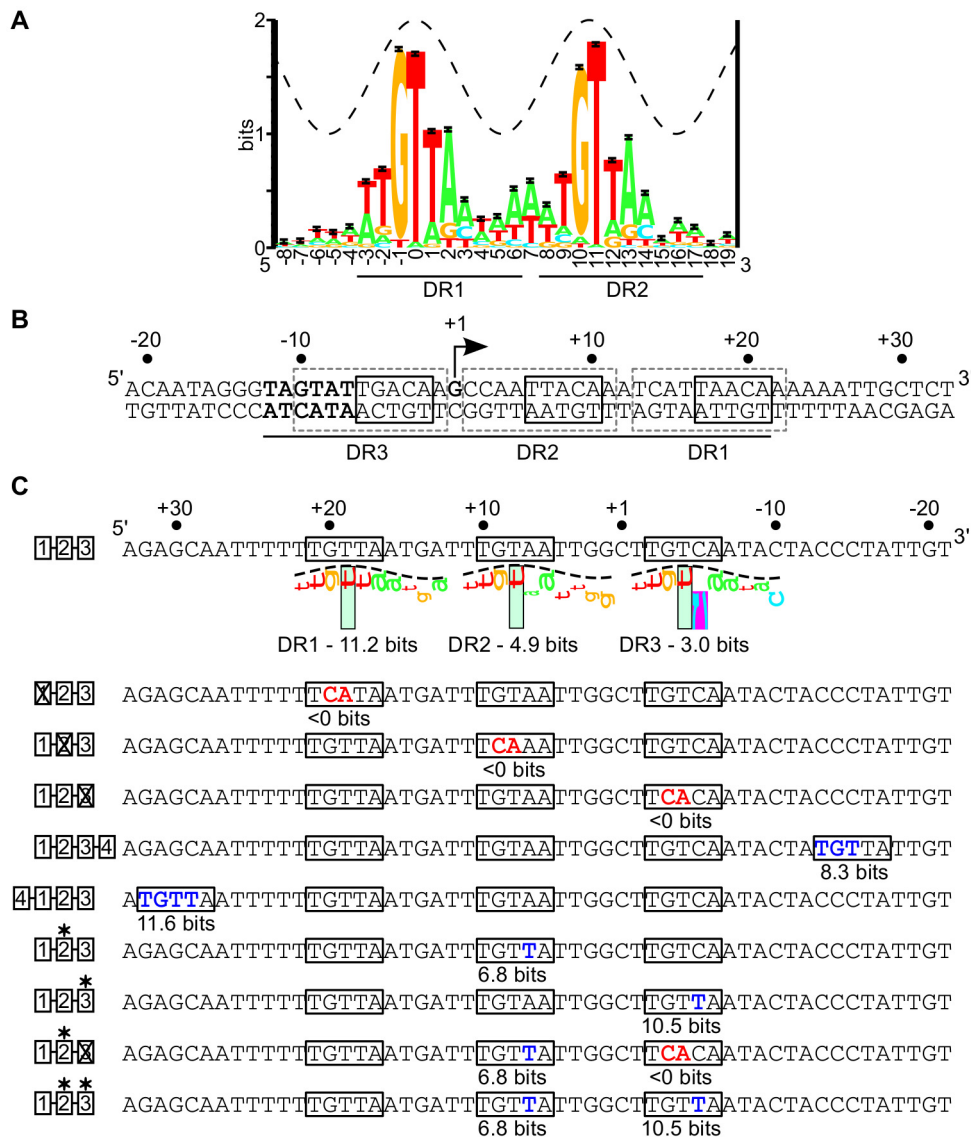


FIG 1 (A) Sequence logo for the minimal ArcA binding site consisting of two 10-bp direct repeat elements (5'-ATGTTAAAAA-1-ATGTTAAAAA-3') (4). The total sequence conservation is 15.6 ± 0.07 bits in the range from positions -3 to $+14$. The crest of the sine wave represents the major groove of B-form DNA. (B) Regulatory region of the *icdA* P_1 promoter from *E. coli*. The arrow indicates the position of the previously mapped transcription start site (5), with the σ^{70} -RNAP -10 promoter element in bold. Each of three 10-bp DR elements is indicated by dashed-line gray boxes, with the most conserved 5-bp 5'-TGTTA-3' region within each DR element indicated with a solid-line black box. The ArcA-P footprint region is indicated underneath the sequence by the black line (4). (C) Noncoding strand of the *icdA-lacZ* promoter, depicting the ArcA binding site mutations used in this study. The degree of match of each DR element to the 10-bp ArcA DR element PWM (4) is indicated in bits and visualized using sequence walkers (40). The purple box surrounding the C at position 6 indicates a contact that is more unfavorable than -4 bits and, thus, off the scale. The boxes to the left of the binding sites are the key used to indicate mutations in subsequent figures. Mutations away from the consensus in each DR element (5'-TGTTA-3' to 5'-TCATA-3') are indicated in red and labeled with an X in the cartoon, while mutations toward the consensus are indicated in blue and are labeled with an asterisk. The information content for all 10 bp of each mutated DR element is listed below the DR element.

(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.

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/

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_1) (23). Thus, changes in ArcA DNA binding should change P_1 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'-TGTTA-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 O₂-dependent regulation.

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_{1icdA} , 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'-TGTTA-3' to 5'-TCATA-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_{1icdA} -*lacZ* transcriptional fusions containing the GT-to-CA (5'-TGTTA-3' to 5'-TCATA-3') mutations within each DR element under anaerobic conditions. Basal promoter activity was not altered by any binding site mutation, as all variants exhibited the same activity as the wt promoter in the absence of ArcA repression (data not shown). P_{1icdA} was repressed 14-fold by ArcA (Fig. 3A). However, disruption of either DR1 or DR2 completely abolished ArcA-dependent repression (Fig. 3A), consistent with the loss of DNA binding to all three DRs observed by DNase I footprinting (Fig. 2C and D). In contrast, disruption of DR3, 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_{1icdA} 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_{1icdA} 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'-TGTTA-3' to 5'-TGTTA-3') resulted in a 3-fold increase in anaerobic repression of P_{1icdA} (Fig. 3B). This repression still depends on DR3 function, since the additional disruption of DR3 (5'-TGTTA-3' to 5'-TCATA-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'-TGTTA-3' to 5'-TGTTA-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_{1icdA} repression by ArcA had been achieved (Fig. 3B). Assuming that these nucleotide changes simply improve DNA binding affin-

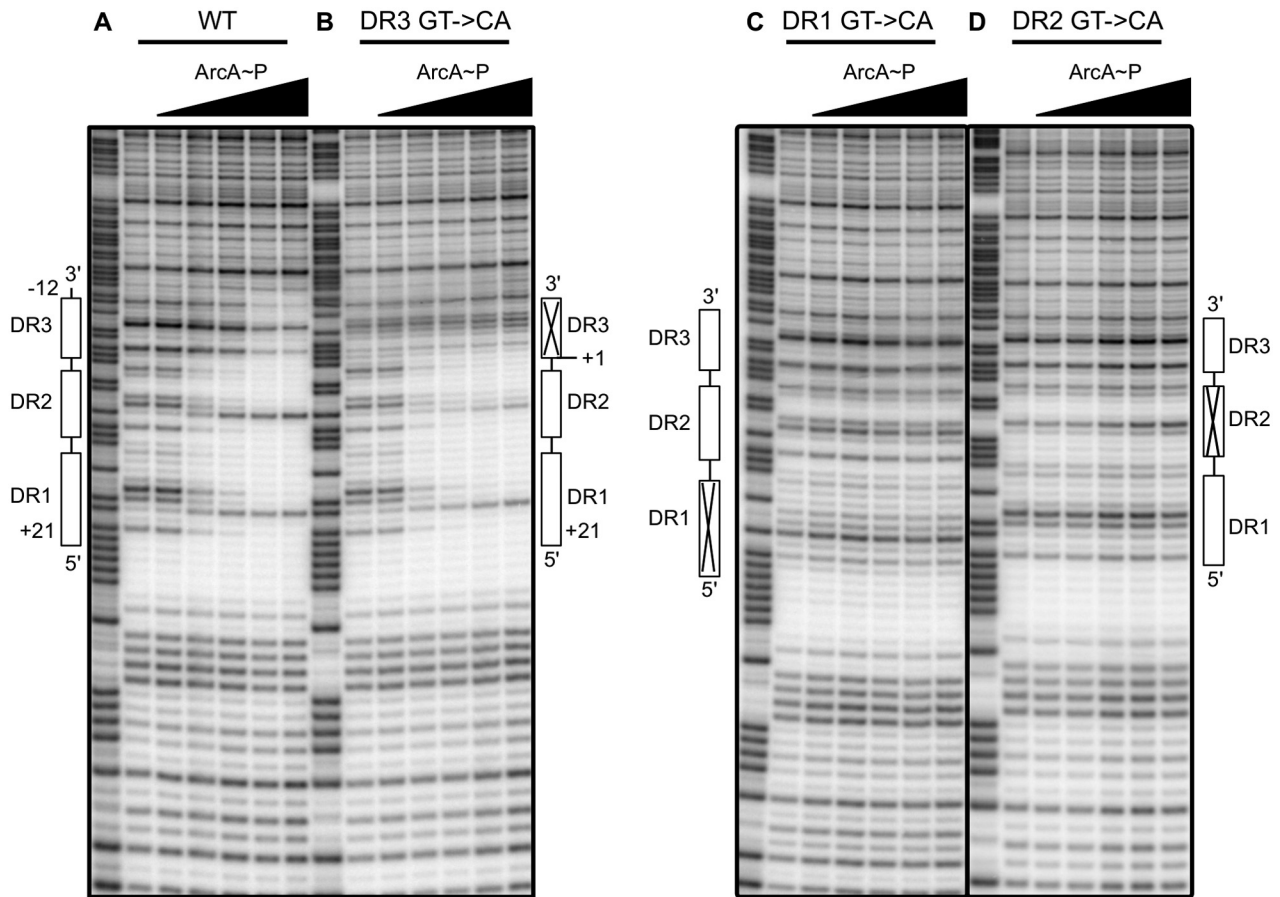


FIG 2 DNase I footprinting of ArcA binding to the wt or mutated *icdA* promoter region. (A) wt ArcA binding site containing all three DR elements; (B) elimination of DR3 by a 5'-TGTC A-3'-to-5'-TCACA-3' mutation; (C) elimination of DR1 by a 5'-TGTTA-3'-to-5'-TCATA-3' mutation; (D) elimination of DR2 by a 5'-TGTA A-3'-to-5'-TCAAA-3' mutation. The regions protected by ArcA-P are indicated with vertical lines and are numbered to indicate the position relative to the previously determined transcription start site (5). The 10-bp DR elements are indicated by open boxes, with a × representing a DR element that has been eliminated through mutation (Fig. 1C). Samples were electrophoresed with Maxam-Gilbert ladders (A + G) made using the same DNA (lane 1). ArcA-P protein concentrations are given from left to right in nM total ArcA-P protein as follows: 0, 50, 150, 300, 600, and 1,000 nM.

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 hy-

persensitive 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 μ M (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 P_{1*icdA*} variant with four consecutive DR binding sites still retains O₂-dependent regulation, we measured β -galactosidase activity produced from a P_{1*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 P_{1*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-

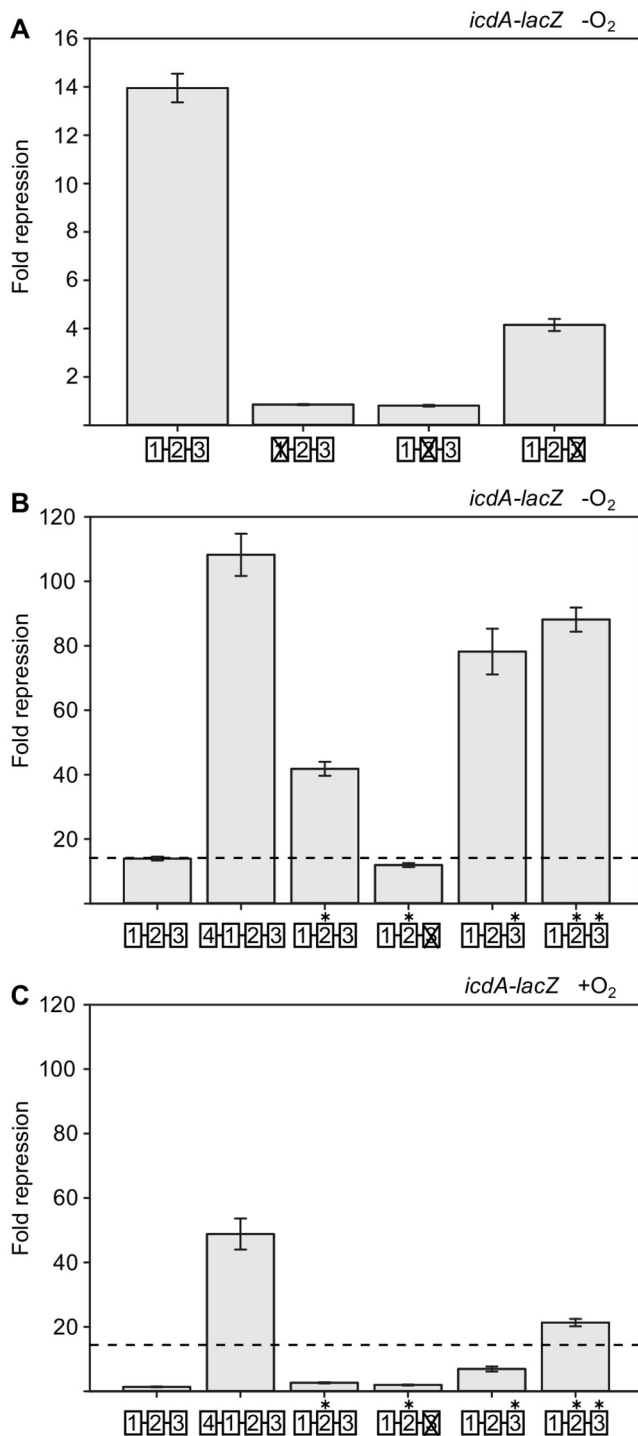


FIG 3 Effects of mutations on ArcA-dependent repression of P_{icdA} . Strains containing P_{icdA} -*lacZ* were grown in minimal medium with 0.2% glucose, and fold repression was calculated by dividing the β -galactosidase activity of a $\Delta arcA$ strain (e.g., 803 Miller units for wt P_{icdA} without O_2) by the activity of an *arcA*⁺ strain (e.g., 57 Miller units for wt P_{icdA} without O_2). The 10-bp DR elements are indicated by open boxes, with a \times representing a DR element that has been eliminated through mutation and an asterisk denoting DR elements that have been mutated toward the consensus (Fig. 1C). (A) Effects of mutations away from the consensus within each DR element assayed under anaerobic conditions ($-O_2$). (B) Effects of mutations toward the consensus within each DR element assayed under anaerobic conditions. The dotted line represents anaerobic ArcA-dependent repression of wt P_{icdA} . (C) Effects of muta-

(Continued)

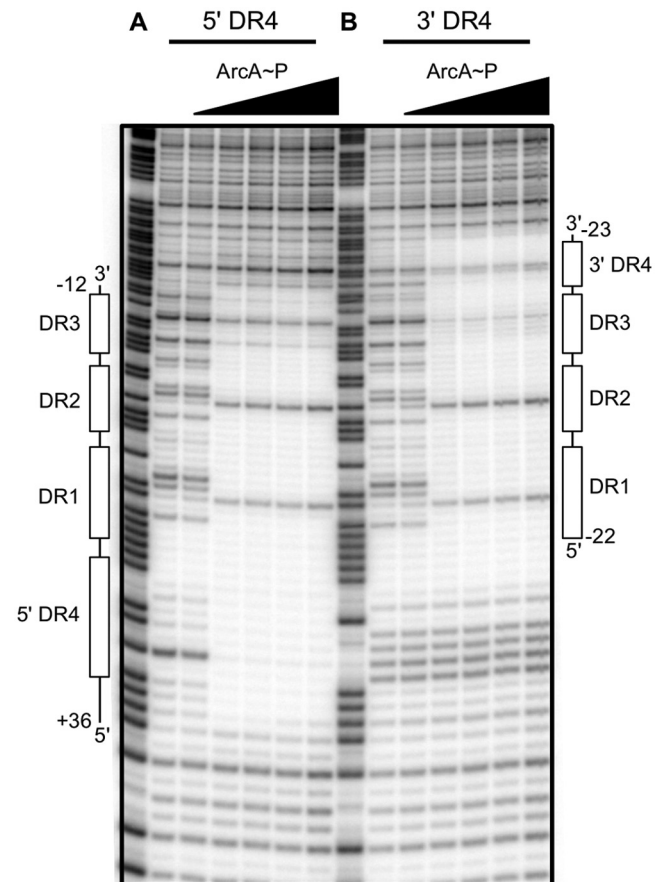


FIG 4 DNase I footprinting of ArcA binding to the *icdA* promoter region containing four DR elements. (A) Fourth DR element (5'-TGTTA-3') located 5' of DR1; (B) fourth DR element (5'-TGTTA-3') located 3' of DR3. The regions protected by ArcA-P are indicated with vertical lines, with DR elements indicated by open boxes. The numbers indicate positions relative to the previously determined transcription start site. Samples were electrophoresed with Maxam-Gilbert ladders (A + G) made using the same DNA (lane 1). ArcA-P protein concentrations are given from left to right in nM total ArcA-P protein as follows: 0, 50, 150, 300, 600, and 1,000 nM.

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 O_2 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 O_2 -dependent regulation of ArcA DNA binding.

We also tested whether the degeneracy of DR2 and DR3 (Fig. 1C) is important for maintaining O_2 -dependent regulation of P_{icdA} by assaying the variants where the sites were mutated toward the consensus. Improving DR2 or DR3 toward the con-

Figure Legend Continued

tions toward the consensus within each DR element assayed under aerobic conditions. Error bars represent the standard errors of results from at least three independent replicates. We note that P_{icdA} expression in the construct with a fourth DR element was about 18% higher in a $\Delta arcA$ background than in the other strains tested (data not shown).

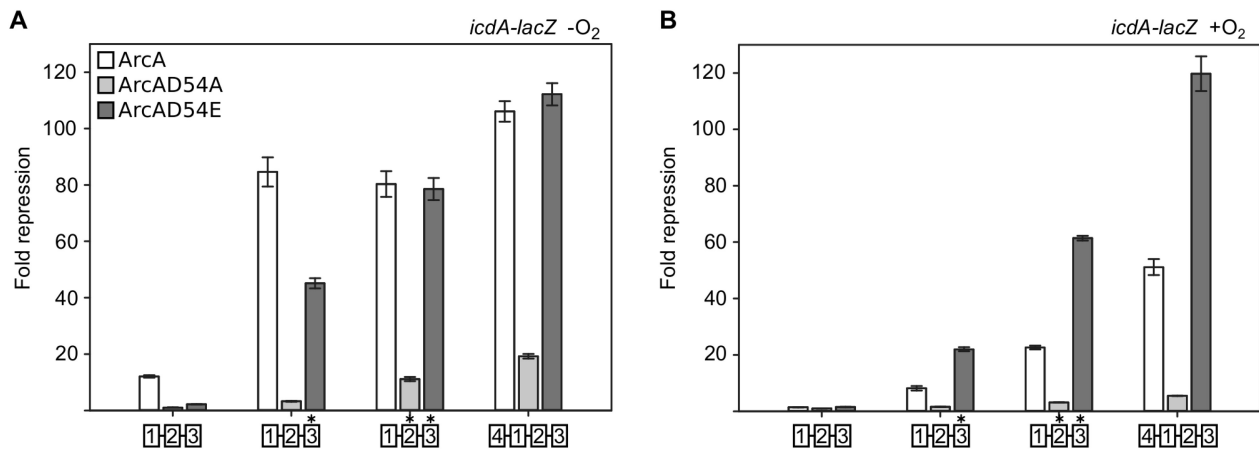


FIG 5 Phosphorylation dependence of ArcA repression of P_{1cdA} in strains with strengthened ArcA binding sites. The fold repression of P_{1cdA} -lacZ in strains containing *arcA*-FRT-*cat*-FRT (white bars), *arcA*(D54A)-FRT-*cat*-FRT (light-gray bars), or *arcA*(D54E)-FRT-*cat*-FRT (dark-gray bars) was determined from cells grown under anaerobic (A) or aerobic (B) conditions and calculated by dividing the β -galactosidase activity of a Δ *arcA* 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.

sensus also increased aerobic P_{1cdA} 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_{1cdA} 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 O₂-dependent relief of repression.

Enhanced ArcA repression is still dependent on phosphorylation. To test whether the enhanced repression of P_{1cdA} 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_{1cdA} -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_{1cdA} , 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_{1cdA} constructs with strengthened binding sites even though repression of wt P_{1cdA} 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_{1cdA} 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 O₂-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 cocystal 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

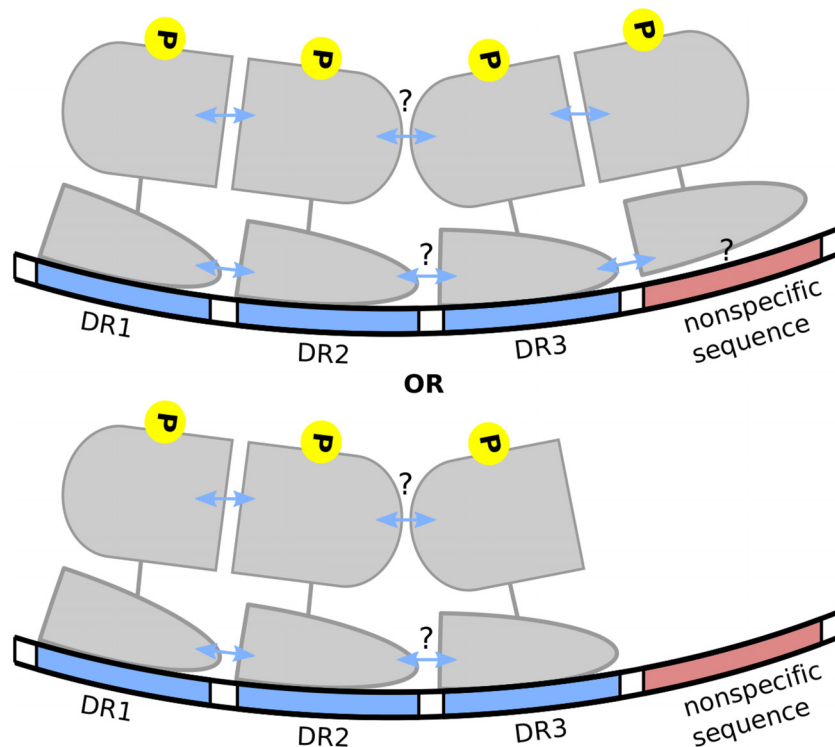


FIG 6 Model for ArcA-P binding to a three-DR binding site. The orientation and protein-protein contacts between the N- and C-terminal domains within an ArcA-P dimer are based on crystallographic data from ArcA and PhoB, respectively (15, 16). Energetically favorable contacts are indicated in blue, while contacts likely to be less favorable are indicated in red. We propose that two ArcA-P dimers bind to a three-DR site in a cooperative manner; the first dimer binds to DR1 and DR2, and a second dimer binds to DR3 and adjacent nonspecific sequences. A favorable energetic contribution from the interaction between ArcA-P dimers is likely required to overcome the poor binding affinity of an ArcA-P dimer to DR3 and adjacent nonspecific sequence. Alternatively, it is possible that dimerization is not required for binding to DR3; ArcA-P may bind to DR3 as a monomer. Potential regions of interaction between ArcA-P molecules in both scenarios are marked with question marks

(20, 21). 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 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 -2 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 *icdA* was reduced by replacing the nonspecific sequence adjacent to DR3 with a fourth DR element, the increase in binding affinity resulted in the promoter bound by ArcA-P in a highly cooperative manner. A similar switch-like occupancy of ArcA-P was also observed for the four DR elements at the *astC* promoter (4). Data obtained using an *icdA-lacZ* reporter fusion indicate that strengthening DR3 toward the consensus likely also enhances binding affinity. Indeed, all three DR elements at the *acs* promoter are bound over a narrow range of ArcA-P levels, likely due to a greater energetic contribution to ArcA-P binding provided by a stronger DR3 element (4). Thus, the combinatorial effect of strong or weak DR elements may be used to either dampen or enhance the concentration-sensitive occupancy by ArcA-P compared to that of a site with only two DRs.

Physiological significance of multiple DR binding sites. The configuration of the ArcA DR elements may also provide a mechanism for achieving a stepwise response to changes in O₂, as suggested for OmpR-P dimer binding to the *ompF* and *ompC* promoters in response to changes in osmolarity (32). For example, under aerobic conditions, ArcA-P levels are likely insufficient for appreciable binding to the *icdA* promoter; thus, *icdA* expression is high, consistent with the need for isocitrate dehydrogenase for carbon oxidation in the TCA cycle. However, as O₂ becomes limiting, ArcA-P levels likely increase (3), perhaps allowing an ArcA-P dimer to bind DR1 and DR2, reducing *icdA* expression to an intermediate level. As O₂ is further depleted, ArcA-P levels likely increase more, and we expect binding to all three DRs, reducing *icdA* expression to levels optimal for anaerobic metabolism. Experimental support for this model came from showing that O₂-dependent regulation was disrupted at *icdA* either by adding a fourth DR element or by improving DR2 and DR3. Thus, these results suggest that the degeneracy in DR2 and DR3 and the absence of a recognizable fourth DR element is important for maintaining the balance between strong, but not complete, anaerobic repression and O₂-dependent relief of repression.

Given the function of the majority of ArcA-repressed operons in aerobic respiratory metabolism, this balance between high-affinity ArcA-P binding and maintenance of O₂-dependent regulation is likely widely applicable to genomic ArcA binding sites.

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 aerobic 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 FruR (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

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
MG1655	F ⁻ λ ⁻ <i>rph-1</i>	This laboratory
PK9416	MG1655 Δ <i>arcA</i>	4
PK9483	MG1655 P _{<i>icdA</i>} (-58GGTGA-54)- <i>lacZ</i>	4
PK9484	PK9416 P _{<i>icdA</i>} (-58GGTGA-54)- <i>lacZ</i>	4
PK9494	MG1655 P _{<i>icdA</i>} (-58GGTGA-54, 19TG20)- <i>lacZ</i>	This study
PK9495	PK9416 P _{<i>icdA</i>} (-58GGTGA-54, 19TG20)- <i>lacZ</i>	This study
PK9486	MG1655 P _{<i>icdA</i>} (-58GGTGA-54, 8TG9)- <i>lacZ</i>	This study
PK9487	PK9416 P _{<i>icdA</i>} (-58GGTGA-54, 8TG9)- <i>lacZ</i>	This study
PK9496	MG1655 P _{<i>icdA</i>} (-58GGTGA-54, -4TG-3)- <i>lacZ</i>	This study
PK9497	PK9416 P _{<i>icdA</i>} (-58GGTGA-54, -4TG-3)- <i>lacZ</i>	This study
PK9915	MG1655 P _{<i>icdA</i>} (-58GGTGA-54, 29AACA32)- <i>lacZ</i>	This study
PK9916	PK9416 P _{<i>icdA</i>} (-58GGTGA-54, 29AACA32)- <i>lacZ</i>	This study
PK9917	MG1655 P _{<i>icdA</i>} (-58GGTGA-54, -15ACA-13)- <i>lacZ</i>	This study
PK9918	PK9416 P _{<i>icdA</i>} (-58GGTGA-54, -15ACA-13)- <i>lacZ</i>	This study
PK9924	MG1655 P _{<i>icdA</i>} (-58GGTGA-54, -5A)- <i>lacZ</i>	This study
PK9925	PK9416 P _{<i>icdA</i>} (-58GGTGA-54, -5A)- <i>lacZ</i>	This study
PK9941	MG1655 P _{<i>icdA</i>} (-58GGTGA-54, 7A)- <i>lacZ</i>	This study
PK9942	PK9416 P _{<i>icdA</i>} (-58GGTGA-54, 7A)- <i>lacZ</i>	This study
PK9943	MG1655 P _{<i>icdA</i>} (-58GGTGA-54, 7A, -5A)- <i>lacZ</i>	This study
PK9944	PK9416 P _{<i>icdA</i>} (-58GGTGA-54, 7A, -5A)- <i>lacZ</i>	This study
PK10967	MG1655 P _{<i>icdA</i>} (-58GGTGA-54, 7A, -4TG-3)- <i>lacZ</i>	This study
PK10968	PK9416 P _{<i>icdA</i>} (-58GGTGA-54, 7A, -4TG-3)- <i>lacZ</i>	This study
BW25993	<i>lacI</i> ^q Δ <i>lacZ</i> _{NJ16} <i>hsdR514</i> Δ <i>araBAD</i> _{AH33} Δ <i>rhaBAD</i> _{LD78}	45
PK9970	PK9483 <i>arcA::cat</i>	This study
PK9973	PK9915 <i>arcA::cat</i>	This study
PK9971	PK9924 <i>arcA::cat</i>	This study
PK9972	PK9943 <i>arcA::cat</i>	This study
PK9980	PK9483 <i>arcA-D54A::cat</i>	This study
PK9983	PK9915 <i>arcA-D54A::cat</i>	This study
PK9981	PK9924 <i>arcA-D54A::cat</i>	This study
PK9982	PK9943 <i>arcA-D54A::cat</i>	This study
PK9975	PK9483 <i>arcA-D54E::cat</i>	This study
PK9978	PK9915 <i>arcA-D54E::cat</i>	This study
PK9976	PK9924 <i>arcA-D54E::cat</i>	This study
PK9977	PK9943 <i>arcA-D54E::cat</i>	This study
Plasmids		
pKD46	Phage λ <i>gam-bet-exo</i> genes under P _{<i>araB</i>} control	B. L. Wanner
pKD13	FRT- <i>kan</i> -FRT	K. A. Datsenko and B. L. Wanner
pKD32	FRT- <i>cat</i> -FRT	B. L. Wanner
pPK7035	<i>kan</i> gene from pHP45Ω and BamHI-NdeI fragment from pRS1553 into pBR322	37
pPK9476	pPK7035 P _{<i>icdA</i>} (-58GGTGA-54)- <i>lacZ</i>	4
pPK9477	pPK7035 P _{<i>icdA</i>} (-58GGTGA-54 19TG20)- <i>lacZ</i>	This study
pPK9908	pPK7035 P _{<i>icdA</i>} (-58GGTGA-54 8TG9)- <i>lacZ</i>	This study
pPK9909	pPK7035 P _{<i>icdA</i>} (-58GGTGA-54 -4TG-3)- <i>lacZ</i>	This study
pPK9913	pPK7035 P _{<i>icdA</i>} (-58GGTGA-54 29AACA32)- <i>lacZ</i>	This study
pPK9914	pPK7035 P _{<i>icdA</i>} (-58GGTGA-54 -15ACA-13)- <i>lacZ</i>	This study
pPK15001	pPK7035 P _{<i>icdA</i>} (-58GGTGA-54, 7A, -4TG-3)- <i>lacZ</i>	This study
pPK9965	<i>arcA</i> in pBR322	This study
pPK9966	BamHI FRT- <i>cat</i> -FRT in pPK9965	This study
pPK9431	Ap ^r ; His ₆ - <i>arcA</i> cloned into the NheI and XhoI sites of pET-21d	4

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 pPK9965. 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'-GGTAGCAAACATGCAGACCCCGCACATTCTTATCG-3') and a primer with homology to the region downstream of *arcA* (5'-GCGC CGTTTTTTTTGACGGTGGTAAAGCCGATTAGTGTAGGCTGGAG CTGCTTC-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

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 DR1 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 upwards 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 μ g/ml) or tetracycline (final concentration, 10 μ g/ml) was added, and cells were placed 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 Δ *arcA* 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 His₆ 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, pPK9909, 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 subsequently purified with Elutip-d columns (Schleicher and Schuell). ArcA was phosphorylated by incubating it with 50 mM disodium carbamyl phosphate (Sigma-Aldrich) in 50 mM Tris, pH 7.9, 150 mM NaCl, and 10 mM MgCl₂ for 1 h at 30°C (6) and immediately used in the binding assays. Footprinting assays were performed by incubating phosphorylated ArcA with labeled DNA (~5 nM) for 10 min at 30°C in 40 mM Tris (pH 7.9), 30 mM KCl, 100 μ g/ml bovine serum albumin (BSA), and 1 mM DTT followed by the addition of 2 μ g/ml DNase I (Worthington) for 30 s. The DNase I reaction was terminated by the addition of sodium acetate and EDTA to final concentrations of 300 mM and 20 mM, respectively. The reaction mix was ethanol precipitated, resuspended in urea loading dye, heated for 60 s at 90°C, and loaded onto a 7 M urea–8% polyacrylamide gel in 0.5 \times Tris-borate-EDTA (TBE) buffer. An A+G ladder was made by formic acid modification of the radiolabeled DNA, followed by piperidine cleavage (44). The reaction products were visualized by phosphorimaging.

ACKNOWLEDGMENTS

We thank Wilma Ross for assistance with DNase I footprinting experiments and Erin Mettert for critically reading the manuscript.

This work was supported by NIH grant GM45844 to P.J.K. D.M.P. was supported in part by the DOE BACTER Program (grant DE-FG02-04ER25627).

REFERENCES

- Malpica R, Sandoval GR, Rodriguez C, Franco B, Georgellis D. 2006. Signaling by the arc two-component system provides a link between the redox state of the quinone pool and gene expression. *Antioxid. Redox Signal.* 8:781–795. <http://dx.doi.org/10.1089/ars.2006.8.781>.
- Malpica R, Franco B, Rodriguez C, Kwon O, Georgellis D. 2004. Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. *Proc. Natl. Acad. Sci. U. S. A.* 101:13318–13323. <http://dx.doi.org/10.1073/pnas.0403064101>.
- Rolfe MD, Ter Beek A, Graham AI, Trotter EW, Asif HM, Sanguinetti G, de Mattos JT, Poole RK, Green J. 2011. Transcript profiling and inference of *Escherichia coli* K-12 ArcA activity across the range of physiologically relevant oxygen concentrations. *J. Biol. Chem.* 286:10147–10154. <http://dx.doi.org/10.1074/jbc.M110.211144>.
- Park DM, Akhtar MS, Ansari AZ, Landick R, Kiley PJ. 2013. The bacterial response regulator ArcA uses a diverse binding site architecture to regulate carbon oxidation globally. *PLoS Genet.* 9:e1003839. <http://dx.doi.org/10.1371/journal.pgen.1003839>.
- Chao G, Shen J, Tseng CP, Park SJ, Gunsalus RP. 1997. Aerobic regulation of isocitrate dehydrogenase gene (*icd*) expression in *Escherichia coli* by the *arcA* and *fmr* gene products. *J. Bacteriol.* 179:4299–4304.
- Lynch AS, Lin EC. 1996. Transcriptional control mediated by the ArcA two-component response regulator protein of *Escherichia coli*: characterization of DNA binding at target promoters. *J. Bacteriol.* 178:6238–6249.
- Cunningham L, Georgellis D, Green J, Guest JR. 1998. Co-regulation of lipoamide dehydrogenase and 2-oxoglutarate dehydrogenase synthesis in *Escherichia coli*: characterisation of an ArcA binding site in the *lpd* promoter. *FEMS Microbiol. Lett.* 169:403–408. <http://dx.doi.org/10.1111/j.1574-6968.1998.tb13347.x>.
- Pellicer MT, Lynch AS, De Wulf P, Boyd D, Aguilar J, Lin EC. 1999. A mutational study of the ArcA-P binding sequences in the *aldA* promoter of *Escherichia coli*. *Mol. Gen. Genet.* 261:170–176. <http://dx.doi.org/10.1007/s004380050954>.
- Pellicer MT, Fernandez C, Badia J, Aguilar J, Lin EC, Baldom L. 1999. Cross-induction of *glc* and *ace* operons of *Escherichia coli* attributable to pathway intersection. Characterization of the *glc* promoter. *J. Biol. Chem.* 274:1745–1752. <http://dx.doi.org/10.1074/jbc.274.3.1745>.
- Drupal N, Sawers G. 1995. Purification of ArcA and analysis of its specific interaction with the *pfl* promoter-regulatory region. *Mol. Microbiol.* 16:597–607. <http://dx.doi.org/10.1111/j.1365-2958.1995.tb02422.x>.
- Gerasimova AV, Gelfand MS, Makeev VY, Mironov AA, Favorov AV. 2003. ArcA regulator of gamma-proteobacteria: identification of the binding signal and description of the regulon. *Biophysics* 48:S21–S25.
- McGuire AM, De Wulf P, Church GM, Lin EC. 1999. A weight matrix for binding recognition by the redox-response regulator ArcA-P of *Escherichia coli*. *Mol. Microbiol.* 32:219–221. <http://dx.doi.org/10.1046/j.1365-2958.1999.01347.x>.
- Liu X, De Wulf P. 2004. Probing the ArcA-P modulon of *Escherichia coli* by whole genome transcriptional analysis and sequence recognition profiling. *J. Biol. Chem.* 279:12588–12597. <http://dx.doi.org/10.1074/jbc.M313454200>.
- Wang X, Gao H, Shen Y, Weinstock GM, Zhou J, Palzkill T. 2008. A high-throughput percentage-of-binding strategy to measure binding energies in DNA-protein interactions: application to genome-scale site discovery. *Nucleic Acids Res.* 36:4863–4871. <http://dx.doi.org/10.1093/nar/gkn477>.
- Blanco AG, Sola M, Gomis-Rüth FX, Coll M. 2002. Tandem DNA recognition by PhoB, a two-component signal transduction transcriptional activator. *Structure* 10:701–713. [http://dx.doi.org/10.1016/S0969-2126\(02\)00761-X](http://dx.doi.org/10.1016/S0969-2126(02)00761-X).
- Toro-Roman A, Mack TR, Stock AM. 2005. Structural analysis and solution studies of the activated regulatory domain of the response regulator ArcA: a symmetric dimer mediated by the alpha4-beta5-alpha5 face. *J. Mol. Biol.* 349:11–26. <http://dx.doi.org/10.1016/j.jmb.2005.03.059>.
- Gao R, Mack TR, Stock AM. 2007. Bacterial response regulators: versatile

- regulatory strategies from common domains. *Trends Biochem. Sci.* 32: 225–234. <http://dx.doi.org/10.1016/j.tibs.2007.03.002>.
18. Narayanan A, Kumar S, Evrard AN, Paul LN, Yernool DA. 2014. An asymmetric heterodomain interface stabilizes a response regulator-DNA complex. *Nat. Commun* 5:3282. <http://dx.doi.org/10.1038/ncomms4282>.
 19. Jeon Y, Lee YS, Han JS, Kim JB, Hwang DS. 2001. Multimerization of phosphorylated and non-phosphorylated ArcA is necessary for the response regulator function of the Arc two-component signal transduction system. *J. Biol. Chem.* 276:40873–40879. <http://dx.doi.org/10.1074/jbc.M104855200>.
 20. Mack TR, Gao R, Stock AM. 2009. Probing the roles of the two different dimers mediated by the receiver domain of the response regulator PhoB. *J. Mol. Biol.* 389:349–364. <http://dx.doi.org/10.1016/j.jmb.2009.04.014>.
 21. Barbieri CM, Wu T, Stock AM. 2013. Comprehensive analysis of OmpR phosphorylation, dimerization, and DNA binding supports a canonical model for activation. *J. Mol. Biol.* 425:1612–1626. <http://dx.doi.org/10.1016/j.jmb.2013.02.003>.
 22. Ritzefeld M, Walhorn V, Kleineberg C, Bieker A, Kock K, Herrmann C, Anselmetti D, Sewald N. 2013. Cooperative binding of PhoB(DBD) to its cognate DNA sequence—a combined application of single-molecule and ensemble methods. *Biochemistry* 52:8177–8186. <http://dx.doi.org/10.1021/bi400718r>.
 23. Keseler IM, Collado-Vides J, Santos-Zavaleta A, Peralta-Gil M, Gama-Castro S, Muñiz-Rascado L, Bonavides-Martinez C, Paley S, Krummenacker M, Altman T, Kaipa P, Spaulding A, Pacheco J, Latendresse M, Fulcher C, Sarker M, Shearer AG, Mackie A, Paulsen I, Gunsalus RP, Karp PD. 2011. EcoCyc: a comprehensive database of *Escherichia coli* biology. *Nucleic Acids Res.* 39:D583–D590. <http://dx.doi.org/10.1093/nar/gkq1143>.
 24. Schneider TD. 1997. Information content of individual genetic sequences. *J. Theor. Biol.* 189:427–441. <http://dx.doi.org/10.1006/jtbi.1997.0540>.
 25. Drew HR, Travers AA. 1984. DNA structural variations in the *E. coli tyrT* promoter. *Cell* 37:491–502. [http://dx.doi.org/10.1016/0092-8674\(84\)90379-9](http://dx.doi.org/10.1016/0092-8674(84)90379-9).
 26. Smith JG, Latiolais JA, Guanga GP, Pennington JD, Silversmith RE, Bourret RB. 2004. A search for amino acid substitutions that universally activate response regulators. *Mol. Microbiol.* 51:887–901. <http://dx.doi.org/10.1046/j.1365-2958.2003.03882.x>.
 27. Ackers GK, Shea MA, Smith FR. 1983. Free energy coupling within macromolecules. The chemical work of ligand binding at the individual sites in co-operative systems. *J. Mol. Biol.* 170:223–242. [http://dx.doi.org/10.1016/S0022-2836\(83\)80234-4](http://dx.doi.org/10.1016/S0022-2836(83)80234-4).
 28. Schlax PJ, Capp MW, Record MT, Jr.. 1995. Inhibition of transcription initiation by *lac* repressor. *J. Mol. Biol.* 245:331–350. <http://dx.doi.org/10.1006/jmbi.1994.0028>.
 29. Elledge SJ, Davis RW. 1989. Position and density effects on repression by stationary and mobile DNA-binding proteins. *Genes Dev.* 3:185–197. <http://dx.doi.org/10.1101/gad.3.2.185>.
 30. Lanzer M, Bujard H. 1988. Promoters largely determine the efficiency of repressor action. *Proc. Natl. Acad. Sci. U. S. A.* 85:8973–8977. <http://dx.doi.org/10.1073/pnas.85.23.8973>.
 31. Cox RS, III, Surette MG, Elowitz MB. 2007. Programming gene expression with combinatorial promoters. *Mol. Syst. Biol.* 3:145. <http://dx.doi.org/10.1038/msb4100187>.
 32. Yoshida T, Qin L, Egger LA, Inouye M. 2006. Transcription regulation of *ompF* and *ompC* by a single transcription factor, OmpR. *J. Biol. Chem.* 281:17114–17123. <http://dx.doi.org/10.1074/jbc.M602112200>.
 33. Rampersaud A, Harlocker SL, Inouye M. 1994. The OmpR protein of *Escherichia coli* binds to sites in the *ompF* promoter region in a hierarchical manner determined by its degree of phosphorylation. *J. Biol. Chem.* 269: 12559–12566.
 34. Makino K, Amemura M, Kawamoto T, Kimura S, Shinagawa H, Nakata A, Suzuki M. 1996. DNA binding of PhoB and its interaction with RNA polymerase. *J. Mol. Biol.* 259:15–26. <http://dx.doi.org/10.1006/jmbi.1996.0298>.
 35. Chen Z, Lewis KA, Shultzaberger RK, Lyakhov IG, Zheng M, Doan B, Storz G, Schneider TD. 2007. Discovery of *fur* binding site clusters in *Escherichia coli* by information theory models. *Nucleic Acids Res.* 35: 6762–6777. <http://dx.doi.org/10.1093/nar/gkm631>.
 36. Butala M, Zgur-Bertok D, Busby SJ. 2009. The bacterial LexA transcriptional repressor. *Cell. Mol. Life Sci.* 66:82–93. <http://dx.doi.org/10.1007/s00018-008-8378-6>.
 37. Kang Y, Weber KD, Qiu Y, Kiley PJ, Blattner FR. 2005. Genome-wide expression analysis indicates that FNR of *Escherichia coli* K-12 regulates a large number of genes of unknown function. *J. Bacteriol.* 187:1135–1160. <http://dx.doi.org/10.1128/JB.187.3.1135-1160.2005>.
 38. Prost JF, Nègre D, Oudot C, Murakami K, Ishihama A, Cozzone AJ, Cortay JC. 1999. Cra-dependent transcriptional activation of the *icd* gene of *Escherichia coli*. *J. Bacteriol.* 181:893–898.
 39. Nesbit AD, Giel JL, Rose JC, Kiley PJ. 2009. Sequence-specific binding to a subset of IscR-regulated promoters does not require IscR Fe-S cluster ligation. *J. Mol. Biol.* 387:28–41. <http://dx.doi.org/10.1016/j.jmb.2009.01.055>.
 40. Schneider TD. 1997. Sequence walkers: a graphical method to display how binding proteins interact with DNA or RNA sequences. *Nucleic Acids Res.* 25:4408–4415. <http://dx.doi.org/10.1093/nar/25.21.4408>.
 41. Neidhardt FC, Bloch PL, Smith DF. 1974. Culture medium for enterobacteria. *J. Bacteriol.* 119:736–747.
 42. Miller JH. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 43. Ku HH. 1966. Notes on the use of propagation of error formulas. *J. Res. Natl. Bureau Stand.* 70C:263–273.
 44. Maxam AM, Gilbert W. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499–560. [http://dx.doi.org/10.1016/S0076-6879\(80\)65059-9](http://dx.doi.org/10.1016/S0076-6879(80)65059-9).
 45. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97:6640–6645. <http://dx.doi.org/10.1073/pnas.120163297>.