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Substitutions at Auxiliary Operator O3 Enhance Repression by Nitrate-Responsive Regulator NarL at Synthetic *lac* Control Regions in *Escherichia coli* K-12[▽]

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We constructed monocopy *lac* operon control regions in which the operators O1-*lac* and O3-*lac* were replaced by NarL and NarP binding sites from the *nirB* or *napF* operon control regions. The results support the hypothesis that DNA-bound dimers of phospho-NarL can participate in higher-order cooperative interactions.

Anaerobic respiratory gene expression in *Escherichia coli* K-12 is controlled in part by nitrate and nitrite, acting through the Nar regulatory system. The paralogous sensors NarX and NarQ respond to nitrate and nitrite to control phosphorylation of the paralogous response regulators NarL and NarP. Phosphorylation increases the affinity of the NarL and NarP proteins for their specific DNA binding sites, from whence they activate and repress target operon expression (for a review, see reference 39).

Most phospho-NarL and -NarP binding sites consist of inverted heptamer sequences (consensus sequence, TACYYMT, where Y = C or T and M = A or C) separated by 2 nucleotides (nt) (11, 24). Binding sites with this geometry are termed 7-2-7 heptamer pairs. Specificity determinants that discriminate between binding by one regulator or the other have not yet been defined, although most sites characterized to date appear to have greater affinity for the phospho-NarL protein (11, 37).

The control regions for many nitrate-regulated operons contain heptamer sequences in addition to an essential 7-2-7 heptamer pair (for a review, see reference 39). These extra heptamers are critical for NarL-dependent regulation of *nrfA* and *fdnG* operon expression (10, 11, 19, 20, 42). Evidence suggests that a dimer of the phospho-NarL protein bound to the 7-2-7 heptamer pair has cooperative interactions with additional phospho-NarL molecules, thereby promoting their binding to the extra heptamer sequences. By contrast, phospho-NarP dimers apparently interact weakly if at all and thus are restricted to binding single 7-2-7 heptamer pairs (10, 11).

Transcription of the *lacZYA* operon for lactose catabolism is repressed by the LacI protein. The primary operator (O1-*lac*) for LacI repressor binding consists of inverted half-sites centered at position 11 with respect to the transcription initiation site (Fig. 1). At least one of the two auxiliary operators is required for maximal repression (32). LacI repressor binds cooperatively to the primary operator and to one of the auxiliary operators, thereby increasing its local concentration (for a review, see reference 29).

Previously, we described construction of synthetic *lac* operon control regions in which the primary operator O1 is replaced by 7-2-7 heptamer pairs from different Nar-regulated operons (37). Transcription from these constructs is repressed by the phospho-NarL and -NarP proteins in response to added nitrate. Here, we describe additional constructs in which the auxiliary operator O3 was also replaced by different 7-2-7 heptamer pairs. In *narL*$^+$ strains, these double-operator-substituted constructs exhibited increased repression, supporting the hypothesis that two dimers of the phospho-NarL protein bind cooperatively to specific DNA sites.

Strains. Strains are listed in Table 1. Construction of the synthetic *lac* operon control region has been described previously (37). New versions reported here, with substitutions at operator O3, were constructed by the same methods. All constructs were based on *lacZ* gene fusion plasmid pVJS3253 (37) and were crossed into bacteriophage λInCh (5).

Genetic crosses were performed by bacteriophage P1*kc*-mediated generalized transduction (26). Null alleles of *nar* regulatory genes (Table 1) have been described previously (21, 33). Standard methods were used for restriction endonuclease digestion, ligation, transformation, and PCR amplification of DNA (22).

Culture media and conditions. Defined, complex, and indicator media for genetic manipulations were used as described previously (22). The defined medium used to grow cultures for enzyme assays was buffered with 3-(*N*-morpholino)propanesulfonic acid (MOPS) as described previously (38). The medium used for overnight cultures arrested in the mid-exponential phase (13) contained glucose (6 mM) or glucose plus $NaNO_3$ (4 and 10 mM, respectively) (37). The concentrations were determined empirically to support growth to the mid-exponential phase (about 35 to 40 Klett units). Isopropyl-β-D-thiogalactoside (IPTG) was added as indicated to inactivate the LacI repressor in cultures of O1-*lac* derivatives.

Cultures were grown at 37°C. Culture densities were monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, NY) equipped with a number 66 (red) filter. Anaerobic cultures used for enzyme assays were grown in screw-cap tubes as described previously (38).

Enzyme assay. β-Galactosidase activities were determined at room temperature (approximately 21°C) by monitoring the

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FIG. 1. Control region sequences. The modified *lac* operon control region sequence from O3 through O1 is shown at the top. The complete sequence for the *lac* operon control region used has been described previously (37). Sequences of O1 and O3 substitutions are indicated below the corresponding *lac* operators. The deletion and insertions were employed to make the spacing variants used for the experiments whose results are shown in Fig. 2 and Tables 2 to 4. The deletion is indicated by a vertical line, whereas inserted sequences are enclosed in boxes. The transcription initiation site is labeled +1. The arrowheads indicate centers of protein-binding half-sites.

hydrolysis of *o*-nitrophenyl-β-D-galactoside in $CHCl_3$-sodium dodecyl sulfate-permeabilized cells. Specific activities are expressed in arbitrary units (26). All cultures were assayed in duplicate, and the reported values are averages from at least two independent experiments.

Nar-dependent repression of *lacZ* gene expression from O1 substitution control regions. Previously, we used site-specific mutagenesis to replace the primary operator O1-*lac* with 7-2-7 heptamer pairs from different Nar-responsive control regions (37). One of the constructs, the O1-*nirB* construct, exhibits about 100-fold repression of *lacZ* gene expression in response to nitrate, whereas the O1-*napF* construct exhibits only about 10-fold repression. However, the O1-*nirB* construct displays only about fivefold repression in a *narP*$^+$ *narL* null strain, indicating that the phospho-NarP protein binds relatively poorly at O1-*nirB*. By contrast, the O1-*napF* construct displays similar repression in both *narL*$^+$ *narP* null and *narP*$^+$ *narL* null strains, indicating that the phospho-NarL and -NarP proteins bind equally well at O1-*napF* (37).

For the experiments described below, we used constructs with the O1-*napF* substitution so that (i) enhanced repression could be more readily detected and (ii) relative levels of repression by the phospho-NarL and -NarP proteins could be compared directly.

TABLE 1. *E. coli* K-12 strains

Strain	Genotype	Reference
VJS632	F$^-$ λ$^-$ prototroph	38
VJS676	As VJS632 but Δ(*argF-lacIZYA*)*U169*	38
VJS8364	As VJS632 but Δ*lacZ*	21
Derivatives of strain VJS676		
VJS7449	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*napF lacZ*$^+$]	37
VJS7475	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*napF lacZ*$^+$]/*narL215*::Tn*10*	37
VJS7476	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*napF lacZ*$^+$]/*narP253*::Tn*10*d(Cm)	37
VJS7446	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*nirB* O1-*napF lacZ*$^+$]	This study
VJS7473	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*nirB* O1-*napF lacZ*$^+$]/*narL215*::Tn*10*	This study
VJS7474	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*nirB* O1-*napF lacZ*$^+$]/*narP253*::Tn*10*d(Cm)	This study
VJS7902	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*nirB* O1-*napF* (95 nt) *lacZ*$^+$]	This study
VJS7922	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*nirB* O1-*napF* (102 nt) *lacZ*$^+$]	This study
VJS7923	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*nirB* O1-*napF* (106 nt) *lacZ*$^+$]	This study
VJS7929	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*nirB* O1-*napF* (110 nt) *lacZ*$^+$]	This study
VJS8206	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*nirB* O1-*napF* (95 nt) *lacZ*$^+$]/*narL215*::Tn*10*	This study
VJS8207	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*nirB* O1-*napF* (95 nt) *lacZ*$^+$]/*narP253*::Tn*10*d(Cm)	This study
Derivatives of strain VJS8364		
VJS8880	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*napF lacZ*$^+$]	This study
VJS8881	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*napF lacZ*$^+$]/Δ*narL261*	This study
VJS8882	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*napF lacZ*$^+$]/Δ*narP262*	This study
VJS8883	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*napF* O1-*napF lacZ*$^+$]	This study
VJS8884	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*napF* O1-*napF lacZ*$^+$]/Δ*narL261*	This study
VJS8885	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*napF* O1-*napF lacZ*$^+$]/Δ*narP262*	This study
VJS8886	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*lac lacZ*$^+$]	This study
VJS8899	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*napF* O1-*napF* (87 nt) *lacZ*$^+$]	This study
VJS9000	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*napF* O1-*napF* (87 nt) *lacZ*$^+$]/Δ*narP262*	This study
VJS9001	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*napF* O1-*napF* (87 nt) *lacZ*$^+$]/Δ*narL261*	This study
VJS9002	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*napF* O1-*lac* (87 nt) *lacZ*$^+$]	This study
VJS9003	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*napF* O1-*lac lacZ*$^+$]	This study
VJS9004	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*napF lacZ*$^+$]/Δ*narL261* Δ*narP262*	This study
VJS9005	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*napF* O1-*napF lacZ*$^+$]/Δ*narL261* Δ*narP262*	This study
VJS9006	λ$^-$ Δ(*att*λ-*lom*)::*bla* [O3-*napF* O1-*napF* (87 nt) *lacZ*$^+$]/Δ*narL261* Δ*narP262*	This study

TABLE 2. Interoperator spacing influences O3-*nirB* enhancement of NarL-mediated repression at O1-*napF*

Strain	Construct	Spacing[a]	Genotype		LacZ sp act (arbitrary units)[b]		Repression (fold)
			narL	*narP*	No NO_3^-	NO_3^-	
VJS7449	O3-*lac* O1-*napF*	91	+	+	850	66	13
VJS7476	O3-*lac* O1-*napF*	91	+	−	850	71	12
VJS7475	O3-*lac* O1-*napF*	91	−	+	920	100	9.2
VJS7446	O3-*nirB* O1-*napF*	91	+	+	790	24	33
VJS7474	O3-*nirB* O1-*napF*	91	+	−	970	20	49
VJS7473	O3-*nirB* O1-*napF*	91	−	+	950	100	10
VJS7902	O3-*nirB* O1-*napF*	95	+	+	800	4	200
VJS8207	O3-*nirB* O1-*napF*	95	+	−	830	4	208
VJS8206	O3-*nirB* O1-*napF*	95	−	+	820	70	12

[a] Interoperator spacing (see Fig. 1).
[b] Strains were cultured overnight in MOPS defined medium with limiting glucose.

Enhanced repression by the O3-*nirB* substitution. The LacI repressor consists of two DNA-binding dimers assembled as a homotetramer (for reviews, see references 7, 18, 29, and 47). Full repression of *lac* operon expression requires not only the high-affinity primary operator O1-*lac* but also at least one of two lower-affinity auxiliary operators, O2-*lac* (within the *lacZ* gene) or O3-*lac* (32). Occupancy of the operator O1-*lac* by one dimer is increased through cooperative binding of the other dimer to an auxiliary operator (6, 32, 34), resulting in a more stable repressor complex (14, 17, 46). This cooperative binding accounts for the nonlinear response of *lacZYA* operon transcription to an inducer (30).

Mutant LacI repressors that form essentially normal dimers but fail to assemble into tetramers have been described (1, 2, 6, 8, 32). Mutant dimeric LacI proteins and wild-type LacI protein both repress transcription from O1-*lac* to about the same extent. However, repression by a dimeric repressor is not enhanced by the presence of an auxiliary operator (27, 32), because the repressor does not bind cooperatively to two operators (6, 8, 23, 32). Thus, specific interaction between two dimers, each bound at a separate operator, enhances the overall stability of the repression complex. Analogous observations have been made with other repressors (9, 35), including the GalR repressor (15, 23, 36).

Therefore, we reasoned that the *lac* operon control region might provide a means to study specific interactions between phospho-NarL dimers (23). Accordingly, we used site-specific mutagenesis to replace the auxiliary operator O3-*lac* (positions −92 through −72 [Fig. 1]) with 7-2-7 heptamer pairs from the *nirB* or *napF* control regions. These operators were placed in the O1-*napF*, O1-*nirB*, O1-*fdnG*, and O1-*lac* constructs (Fig. 1), forming various combinations of O3- and O1-substituted *lac* control regions.

In a *narL*$^+$ *narP*$^+$ background, the O3-*lac* O1-*napF* construct exhibited about 10-fold repression of *lacZ* gene expression in response to nitrate, whereas the O3-*nirB* construct exhibited about 30-fold repression (Table 2). Similar results were observed with the congruent O1-*nirB* and O1-*fdnG* constructs (data not shown). Thus, placing a Nar 7-2-7 heptamer pair in operator O3 resulted in a modest enhancement of nitrate repression.

We next introduced *narL* or *narP* null alleles into the strains. The O3-*nirB*-dependent enhanced repression of *lacZ* gene expression was eliminated in the *narP*$^+$ *narL* null strain but increased to nearly 50-fold in the *narL*$^+$ *narP* null strain (Table 2). Thus, the O3-*nirB*-dependent enhanced repression of *lacZ* gene expression required the *narL*$^+$ gene and appeared to be slightly inhibited by the *narP*$^+$ gene.

Interoperator spacing affects enhanced repression by the O3-*nirB* substitution. Spacing between the O3-*lac* and O1-*lac* operators is critical for effective repression, because the repressor-operator interaction depends on the helical phase of the two operators in order to allow formation of the intervening DNA loop (14, 17, 28, 29, 31). Loop formation (and therefore cooperative repressor binding) is also influenced by factors such as DNA superhelicity (12, 16, 34, 40, 44, 45) and host proteins (3, 27).

The wild-type interoperator spacing between operators O1-*lac* and O3-*lac* is nearly optimal for cooperative LacI repression (28). (Our constructs have a slightly shorter interoperator space, 91 versus 92.5 nt [Fig. 1].) We wished to determine the optimal spacing for cooperative phospho-NarL repression. We therefore varied the spacing between the O3-*nirB* and O1-*napF* operators over the range from 95 to 110 nt (Fig. 1). The variant with 95-nt spacing was constructed by filling in the 4-nt BglII overhang with Klenow polymerase, and the remaining variants were constructed by consecutive rounds of site-specific mutagenesis.

The interoperator spacing strongly affected O3-*nirB*-dependent enhanced repression of *lacZ* gene expression (Fig. 2). For the O3-*nirB* O1-*napF* constructs with 91-nt (native) and 102-nt spacing, nitrate repression of *lacZ* gene expression was about 30-fold (Table 2), whereas for the constructs with 95- and 106-nt spacing, nitrate repression of *lacZ* gene expression was about 100-fold (Fig. 2). The level of nitrate repression of *lacZ* gene expression for the construct with 110-nt spacing was intermediate, about 60-fold. These results, based on the limited number of spacing variants examined (28), are consistent with the notion that the helical phase influences the magnitude of the O3-*nirB*-dependent enhanced nitrate repression of *lacZ* gene expression (for a review, see reference 25).

The experiments described above indicated that the native O3-O1 spacing is the least optimal spacing for monitoring O3-dependent enhanced repression of *lacZ* gene expression.

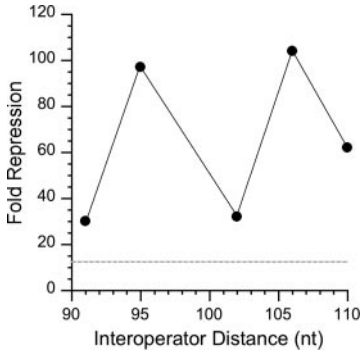

FIG. 2. Interoperator spacing influences O3-*nirB* enhancement of repression at O1-*napF*. The fold repression of *lacZ* gene expression by nitrate is plotted against the distance between the centers of operator elements O1-*napF* and O3-*nirB*. The dashed line indicates the ≤15-fold repression observed for the O3-*lacZ* O1-*napF* construct (see Table 2). Strains carry both *narL*$^+$ and *narP*$^+$ alleles.

We therefore examined the influence of *narL* and *narP* null alleles on enhanced nitrate repression of *lacZ* gene expression in the O3-*nirB* O1-*napF* construct with 95-nt spacing. In this experiment, the nitrate repression of *lacZ* expression was more than 200-fold in the *narL*$^+$ *narP* null strain, whereas the repression was only 12-fold in the *narP*$^+$ *narL* null strain (Table 2).

Enhanced repression by the O3-*napF* substitution. For the experiments described above, we used the *nirB* 7-2-7 heptamer pair substituted at operator O3. Our intention was to use a site that binds phospho-NarL strongly, in an attempt to maximize repression resulting from cooperative interactions between operators O1 and O3 (28, 31). Indeed, with nearly optimal interoperator spacing, the O3-*nirB* sequence increased NarL-dependent repression by roughly 10-fold (Fig. 2 and Table 2). As noted above, however, the O1-*nirB* operator substitution yields feeble NarP-dependent repression, suggesting that it bound phospho-NarP weakly. Thus, the O3-*nirB* substitution constructs did not permit conclusions regarding cooperative binding by the phospho-NarP protein.

To examine this point, we made constructs with O3-*napF* operator substitutions (Table 3). In *narL*$^+$ *narP* null strains, the O3-*napF* substitution resulted in repression that was threefold greater than that observed in O3-*lac* strains. The enhancement was even more pronounced (more than fivefold) with a construct in which the interoperator spacing was decreased to 87 nt (constructed by deleting the GATC overhang resulting from BglII digestion). By contrast, repression in the *narP*$^+$ *narL* null strains was increased less than twofold in the presence of the O3-*napF* operator substitution (Table 3). This indicates that the phospho-NarP protein has only weak cooperative interactions that result in enhanced O3-dependent repression.

The level of repression with the O3-*napF* operator substitution was lower in *narL*$^+$ *narP*$^+$ strains than in *narL*$^+$ *narP* null strains but was similar in the O3-*lac* strain (Table 3). This indicates that the phospho-NarP protein, which competes with the phospho-NarL protein for binding to the *napF* site, inhibits the enhanced repression resulting from phospho-NarL cooperative interactions. This further suggests that the phospho-NarP protein has weak, if any, cooperative interactions.

Control experiments. In the *lacZYA* operon control region, the auxiliary operators cannot mediate repression in the absence of the primary operator, O1-*lac* (27, 32). To document this for the synthetic *lac* control regions described here, we made O3-*napF* O1-*lac* constructs with 91-nt (native) and 87-nt interoperator spacing. Neither construct exhibited Nar-dependent repression (Table 4). This established that the repression observed with O3 substitution constructs (Tables 2 and 3; Fig. 2) was wholly dependent upon substitution also at operator O1.

Analysis of NarL-NarP chimeras. The NarL protein dimerizes through its carboxyl-terminal DNA-binding domain (24). Since the phospho-NarL and -NarP proteins differ in the ability to have cooperative interactions, we hypothesized that this is a property of the corresponding amino-terminal receiver domains. As one attempt to examine this, we constructed chimeric NarL-NarP proteins by fusing the receiver domain from one protein to the DNA-binding effector domain of the other

TABLE 3. Interoperator spacing influences O3-*napF* enhancement of NarL-mediated repression at O1-*napF*

Strain	Construct	Spacing[a]	Genotype		LacZ sp act (arbitrary units)[b]		Repression (fold)
			narL	*narP*	No NO_3^-	NO_3^-	
VJS8880	O3-*lac* O1-*napF*	91	+	+	1,190	100	12
VJS8882	O3-*lac* O1-*napF*	91	+	−	1,170	110	11
VJS8881	O3-*lac* O1-*napF*	91	−	+	1,020	140	7.3
VJS9004	O3-*lac* O1-*napF*	91	−	−	1,010	650	1.6
VJS8883	O3-*napF* O1-*napF*	91	+	+	1,150	55	21
VJS8885	O3-*napF* O1-*napF*	91	+	−	1,200	35	34
VJS8884	O3-*napF* O1-*napF*	91	−	+	1,100	130	8.5
VJS9005	O3-*napF* O1-*napF*	91	−	−	1,100	720	1.5
VJS8899	O3-*napF* O1-*napF*	87	+	+	870	23	38
VJS9001	O3-*napF* O1-*napF*	87	+	−	980	17	58
VJS9000	O3-*napF* O1-*napF*	87	−	+	1,100	100	11
VJS9006	O3-*napF* O1-*napF*	87	−	−	970	620	1.6

[a] Interoperator spacing (see Fig. 1).
[b] Strains were cultured overnight in MOPS defined medium with limiting glucose.

TABLE 4. Neither O3-*napF* nor interoperator spacing influences expression from O1-*lac*

Strain	Construct	Spacing[a]	Genotype		LacZ sp act (arbitrary units)[b]		Repression (fold)
			narL	*narP*	No NO_3^-	NO_3^-	
VJS8886	O3-*lac* O1-*lac*	91	+	+	3,290	2,050	1.6
VJS9003	O3-*napF* O1-*lac*	91	+	+	2,680	2,210	1.2
VJS9002	O3-*napF* O1-*lac*	87	+	+	2,770	2,200	1.3

[a] Interoperator spacing (see Fig. 1).
[b] Strains were cultured overnight in MOPS defined medium with limiting glucose and 0.1 M IPTG.

protein. We made four chimeras with all possible combinations of receiver-linker-effector arrangements, based on a study of OmpR-PhoB chimeras (43). As observed in other studies of chimeric response regulators (4, 41, 43), transcription control by these NarL-NarP chimeras responded poorly or not at all to inducing signal, compromising analysis of enhanced repression at O3 substitution constructs. Although the results (data not shown) were consistent with the notion that the NarL receiver domain mediates cooperative interaction, the overall magnitude of the response was weak.

Concluding remarks. This study exploited well-documented observations that cooperative interactions between LacI dimers, mediated by formation of a DNA loop, can enhance repression of *lacZYA* operon transcription by about 50-fold (32). We were curious to determine whether the *lac* control region could provide an artificial means to examine cooperative interactions between DNA-binding proteins unrelated to the LacI repressor. Previous studies revealed cooperative binding by phospho-NarL dimers to immediately adjacent sites in the *fdnG* and *nrfA* operon control regions (10, 11, 19, 20, 42). Accordingly, binding to the O1 and O3 operator substitutions, spaced approximately 90 nt apart, represents a nonnative context for the Nar regulators. On the other hand, the Nar system allows internal comparison of otherwise similar proteins that differ in the ability to form cooperative interactions.

The operator substitution O3-*napF* enhanced NarL-dependent repression from an O1-*napF* synthetic *lac* control region up to fivefold, whereas NarP-dependent repression was virtually unaltered (Table 3). This result provides independent support for the hypothesis that phospho-NarL dimers have cooperative interactions in binding to specific DNA sites, whereas the phospho-NarP protein has poor or no cooperative interactions. Additional analysis with the operator substitution O3-*nirB* revealed NarL-dependent repression that was enhanced up to 15-fold (Table 2) and was dependent on the spacing between the two operators (Fig. 2). Thus, the results mimic those obtained for the native *lac* system and suggest that the *lac* control region may provide a general assay for analyzing cooperative protein-DNA interactions.

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REFERENCES

1. **Alberti, S., S. Oehler, B. von Wilcken-Bergmann, H. Krämer, and B. Müller-Hill.** 1991. Dimer-to-tetramer assembly of Lac repressor involves a leucine heptad repeat. New Biol. **3:**57–62.
2. **Alberti, S., S. Oehler, B. von Wilcken-Bergmann, and B. Müller-Hill.** 1993. Genetic analysis of the leucine heptad repeats of Lac repressor: evidence for a 4-helical bundle. EMBO J. **12:**3227–3236.
3. **Becker, N. A., J. D. Kahn, and L. J. Maher III.** 2005. Bacterial repression loops require enhanced DNA flexibility. J. Mol. Biol. **349:**716–730.
4. **Bock, A., M. Bantscheff, A. L. Perraud, K. Rippe, V. Weiss, M. O. Glocker, and R. Gross.** 2001. Rational design and molecular characterization of a chimaeric response regulator protein. J. Mol. Biol. **310:**283–290.
5. **Boyd, D., D. S. Weiss, J. C. Chen, and J. Beckwith.** 2000. Towards single-copy gene expression systems making gene cloning physiologically relevant: lambda InCh, a simple *Escherichia coli* plasmid-chromosome shuttle system. J. Bacteriol. **182:**842–847.
6. **Brenowitz, M., N. Mandal, A. Pickar, E. Jamison, and S. Adhya.** 1991. DNA-binding properties of a *lac* repressor mutant incapable of forming tetramers. J. Biol. Chem. **266:**1281–1288.
7. **Chakerian, A. E., and K. S. Matthews.** 1992. Effect of *lac* repressor oligomerization on regulatory outcome. Mol. Microbiol. **6:**963–968.
8. **Chakerian, A. E., V. M. Tesmer, S. P. Manly, J. K. Brackett, M. J. Lynch, J. T. Hoh, and K. S. Matthews.** 1991. Evidence for leucine zipper motif in lactose repressor protein. J. Biol. Chem. **266:**1371–1374.
9. **Court, D. L., A. B. Oppenheim, and S. L. Adhya.** 2007. A new look at bacteriophage lambda genetic networks. J. Bacteriol. **189:**298–304.
10. **Darwin, A. J., J. Li, and V. Stewart.** 1996. Analysis of nitrate regulatory protein NarL-binding sites in the *fdnG* and *narG* operon control regions of *Escherichia coli* K-12. Mol. Microbiol. **20:**621–632.
11. **Darwin, A. J., K. L. Tyson, S. J. Busby, and V. Stewart.** 1997. Differential regulation by the homologous response regulators NarL and NarP of *Escherichia coli* K-12 depends on DNA binding site arrangement. Mol. Microbiol. **25:**583–595.
12. **Eismann, E. R., and B. Müller-Hill.** 1990. *lac* repressor forms stable loops in vitro with supercoiled wild-type *lac* DNA containing all three natural *lac* operators. J. Mol. Biol. **213:**763–775.
13. **Haldimann, A., L. L. Daniels, and B. L. Wanner.** 1998. Use of new methods for construction of tightly regulated arabinose and rhamnose promoter fusions in studies of the *Escherichia coli* phosphate regulon. J. Bacteriol. **180:**1277–1286.
14. **Hsieh, W. T., P. A. Whitson, K. S. Matthews, and R. D. Wells.** 1987. Influence of sequence and distance between two operators on interaction with the *lac* repressor. J. Biol. Chem. **262:**14583–14591.
15. **Irani, M. H., L. Orosz, and S. Adhya.** 1983. A control element within a structural gene: the *gal* operon of *Escherichia coli*. Cell **32:**783–788.
16. **Krämer, H., M. Amouyal, A. Nordheim, and B. Müller-Hill.** 1988. DNA supercoiling changes the spacing requirement of two *lac* operators for DNA loop formation with *lac* repressor. EMBO J. **7:**547–556.
17. **Krämer, H., M. Niemoller, M. Amouyal, B. Revet, B. von Wilcken-Bergmann, and B. Müller-Hill.** 1987. *lac* repressor forms loops with linear DNA carrying two suitably spaced *lac* operators. EMBO J. **6:**1481–1491.
18. **Lewis, M.** 2005. The *lac* repressor. C. R. Biol. **328:**521–548.
19. **Li, J., S. Kustu, and V. Stewart.** 1994. In vitro interaction of nitrate-responsive regulatory protein NarL with DNA target sequences in the *fdnG*, *narG*, *narK* and *frdA* operon control regions of *Escherichia coli* K-12. J. Mol. Biol. **241:**150–165.
20. **Li, J., and V. Stewart.** 1992. Localization of upstream sequence elements required for nitrate and anaerobic induction of *fdn* (formate dehydrogenase-N) operon expression in *Escherichia coli* K-12. J. Bacteriol. **174:**4935–4942.
21. **Lin, H.-Y., P. J. Bledsoe, and V. Stewart.** 2007. Activation of *yeaR-yoaG* operon transcription by nitrate-responsive regulator NarL is independent of oxygen-responsive regulator Fnr in *Escherichia coli* K-12. J. Bacteriol. **189:**7539–7548.
22. **Maloy, S. R., V. J. Stewart, and R. K. Taylor.** 1996. Genetic analysis of pathogeneic bacteria. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. **Mandal, N., W. Su, R. Haber, S. Adhya, and H. Echols.** 1990. DNA looping in cellular repression of transcription of the galactose operon. Genes Dev. **4:**410–418.
24. **Maris, A. E., M. R. Sawaya, M. Kaczor-Grzeskowiak, M. R. Jarvis, S. M. Bearson, M. L. Kopka, I. Schröder, R. P. Gunsalus, and R. E. Dickerson.**

2002. Dimerization allows DNA target site recognition by the NarL response regulator. Nat. Struct. Biol. **9:**771–778.
25. **Matthews, K. S.** 1992. DNA looping. Microbiol. Rev. **56:**123–136.
26. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
27. **Müller, J., A. Barker, S. Oehler, and B. Müller-Hill.** 1998. Dimeric *lac* repressors exhibit phase-dependent co-operativity. J. Mol. Biol. **284:**851–857.
28. **Müller, J., S. Oehler, and B. Müller-Hill.** 1996. Repression of *lac* promoter as a function of distance, phase and quality of an auxiliary *lac* operator. J. Mol. Biol. **257:**21–29.
29. **Müller-Hill, B.** 1998. The function of auxiliary operators. Mol. Microbiol. **29:**13–18.
30. **Oehler, S., S. Alberti, and B. Müller-Hill.** 2006. Induction of the *lac* promoter in the absence of DNA loops and the stoichiometry of induction. Nucleic Acids Res. **34:**606–612.
31. **Oehler, S., M. Amouyal, P. Kolkhof, B. von Wilcken-Bergmann, and B. Müller-Hill.** 1994. Quality and position of the three *lac* operators of *E. coli* define efficiency of repression. EMBO J. **13:**3348–3355.
32. **Oehler, S., E. R. Eismann, H. Krämer, and B. Müller-Hill.** 1990. The three operators of the *lac* operon cooperate in repression. EMBO J. **9:**973–979.
33. **Rabin, R. S., and V. Stewart.** 1993. Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in *Escherichia coli* K-12. J. Bacteriol. **175:** 3259–3268.
34. **Sasse-Dwight, S., and J. D. Gralla.** 1988. Probing co-operative DNA-binding in vivo. The *lac* O1:O3 interaction. J. Mol. Biol. **202:**107–119.
35. **Schleif, R.** 2000. Regulation of the L-arabinose operon of *Escherichia coli*. Trends Genet. **16:**559–565.
36. **Semsey, S., M. Geanacopoulos, D. E. Lewis, and S. Adhya.** 2002. Operator-bound GalR dimers close DNA loops by direct interaction: tetramerization and inducer binding. EMBO J. **21:**4349–4356.
37. **Stewart, V., and P. J. Bledsoe.** 2003. Synthetic *lac* operator substitutions for studying the nitrate- and nitrite-responsive NarX-NarL and NarQ-NarP two-component regulatory systems of *Escherichia coli* K-12. J. Bacteriol. **185:** 2104–2111.
38. **Stewart, V., and J. Parales, Jr.** 1988. Identification and expression of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. J. Bacteriol. **170:**1589–1597.
39. **Stewart, V., and R. S. Rabin.** 1995. Dual sensors and dual response regulators interact to control nitrate- and nitrite-responsive gene expression in *Escherichia coli*, p. 233–252. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. ASM Press, Washington, DC.
40. **Swigon, D., B. D. Coleman, and W. K. Olson.** 2006. Modeling the Lac repressor-operator assembly: the influence of DNA looping on Lac repressor conformation. Proc. Natl. Acad. Sci. USA **103:**9879–9884.
41. **Tapparel, C., A. Monod, and W. L. Kelley.** 2006. The DNA-binding domain of the *Escherichia coli* CpxR two-component response regulator is constitutively active and cannot be fully attenuated by fused adjacent heterologous regulatory domains. Microbiology **152:**431–441.
42. **Tyson, K. L., J. A. Cole, and S. J. W. Busby.** 1994. Nitrite and nitrate regulation at the promoters of two *Escherichia coli* operons encoding nitrite reductase: identification of common target heptamers for both NarP- and NarL-dependent regulation. Mol. Microbiol. **13:**1045–1055.
43. **Walthers, D., V. K. Tran, and L. J. Kenney.** 2003. Interdomain linkers of homologous response regulators determine their mechanism of action. J. Bacteriol. **185:**317–324.
44. **Whitson, P. A., W. T. Hsieh, R. D. Wells, and K. S. Matthews.** 1987. Supercoiling facilitates *lac* operator-repressor-pseudooperator interactions. J. Biol. Chem. **262:**4943–4946.
45. **Whitson, P. A., W. T. Hsieh, R. D. Wells, and K. S. Matthews.** 1987. Influence of supercoiling and sequence context on operator DNA binding with *lac* repressor. J. Biol. Chem. **262:**14592–14599.
46. **Whitson, P. A., and K. S. Matthews.** 1986. Dissociation of the lactose repressor-operator DNA complex: effects of size and sequence context of operator-containing DNA. Biochemistry **25:**3845–3852.
47. **Wilson, C. J., H. Zhan, L. Swint-Kruse, and K. S. Matthews.** 2007. The lactose repressor system: paradigms for regulation, allosteric behavior and protein folding. Cell. Mol. Life Sci. **64:**3–16.