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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

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The NarX and NarQ sensor-histidine kinases control phosphorylation of the NarL and NarP response regulators in response to the respiratory oxidants nitrate and nitrite. Target operon transcription is activated by the Fnr protein in response to anaerobiosis, and it is further activated and/or repressed by the phospho-NarL and phospho-NarP proteins, which bind to heptamer DNA sequences. The location and arrangement of heptamers vary widely among different target operon control regions. We have constructed a series of mono-copy *lac* operon control region constructs in which the primary operator O1-*lac* has been replaced by 7-2-7 heptamer pairs from the *nrfA*, *nirB*, *napF*, and *fdnG* operon control regions. These constructs provide tools for dissecting various aspects of ligand interactions with sensor-kinases, sensor interactions with response regulators, and phospho-response regulator interactions with DNA targets. Expression of the *lacZ* gene from these constructs was repressed to various degrees by nitrate and nitrite. In response to nitrate, the *nrfA* and *nirB* operon 7-2-7 heptamer pairs at operator O1 each mediated greater than 100-fold repression of *lacZ* gene expression, whereas the *napF* operon 7-2-7 heptamer pair mediated approximately tenfold repression. Introduction of *narL*, *narP*, *narX*, and *narQ* null alleles in various combinations allowed the in vivo interactions between different sensor-regulator pairs to be evaluated and compared.

Enterobacteria use a variety of compounds, including nitrate (NO_3^-) and nitrite (NO_2^-), as electron acceptors for anaerobic respiration (12). Anaerobic respiratory gene expression is induced by anaerobiosis, acting through the Fnr transcriptional activator (15), and is further controlled by nitrate and nitrite, acting through the Nar regulatory system (5, 31). In *Escherichia coli*, homologous interacting two-component regulatory systems comprise the membrane-spanning sensors NarX and NarQ and the response regulators NarL and NarP. Both sensors respond to nitrate and nitrite to control the phosphorylation of both response regulators. 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. A key element for differential regulation in response to nitrate versus nitrite is ligand discrimination by the NarX sensor, which is hypothesized to act as a NarL kinase in response to nitrate but to act primarily as a phospho-NarL phosphatase in response to nitrite. By contrast, the NarQ sensor is hypothesized to act as a NarL and NarP kinase in response to both nitrate and nitrite (5, 31).

Specific DNA sites for binding phospho-NarL and phospho-NarP proteins are comprised of heptamer sequences (10, 17, 18, 33) for which a consensus sequence reads 5′-TAC YYMT-3′ (where Y = C or T and M = A or C [7]). Nar heptamers are often present as pairs of inverted repeats with 2-nucleotide (nt) spacing (7-2-7 heptamer pairs), although other arrangements are also functional (2, 4, 7). The native locations of the heptamer pairs examined in this study are shown in Fig. 1. Individual heptamers are denoted by the position of the central base pair with respect to the transcription initiation site. For example, the two heptamers in the *nirB* operon control region are centered at −74 and at −65 (Fig. 1).

The diversity of Nar heptamer organization in different control regions complicates attempts to compare phospho-NarL or phospho-NarP action at different sites. Some sites are used for transcription activation, some for repression, and some (e.g., the *napF* operon control region) for both. The NarL and NarP proteins exhibit different DNA-binding or transcription activation properties at different control regions (5, 31). Transcription of most known Nar-responsive operons is also dependent upon activation by the Fnr protein. We therefore sought to develop a versatile system that would permit in vivo analysis of the Nar regulatory circuit in isolation from these complicating factors.

Transcription of the *lacZYA* operon for lactose catabolism is activated by the cyclic AMP (cAMP) receptor protein (Crp) and is repressed by the LacI protein. The primary operator (O1-*lac*) for LacI repressor binding consists of two inverted half-sites centered at position +11 with respect to the transcription initiation site (Fig. 1 and 2A). At least one of the two auxiliary operators, centered at +411 (O2-*lac*) and at −82 (O3-*lac*), is required for maximal repression (23). The requirement for Crp activation is suppressed by the *lac* UV5 alteration in the −10 promoter element (26).

Replacement of the operator O1-*lac* with a binding site for Crp protein results in Crp-repressible *lacZYA* operon expression (14). Thus, the *lac* operators can be engineered to analyze heterologous regulatory systems. For this study, we replaced the primary operator O1-*lac* with 7-2-7 heptamer pairs from the *nrfA*, *nirB*, *napF*, and *fdnG* operon control regions (Fig. 1). These operator substitution constructs provide in vivo tools for

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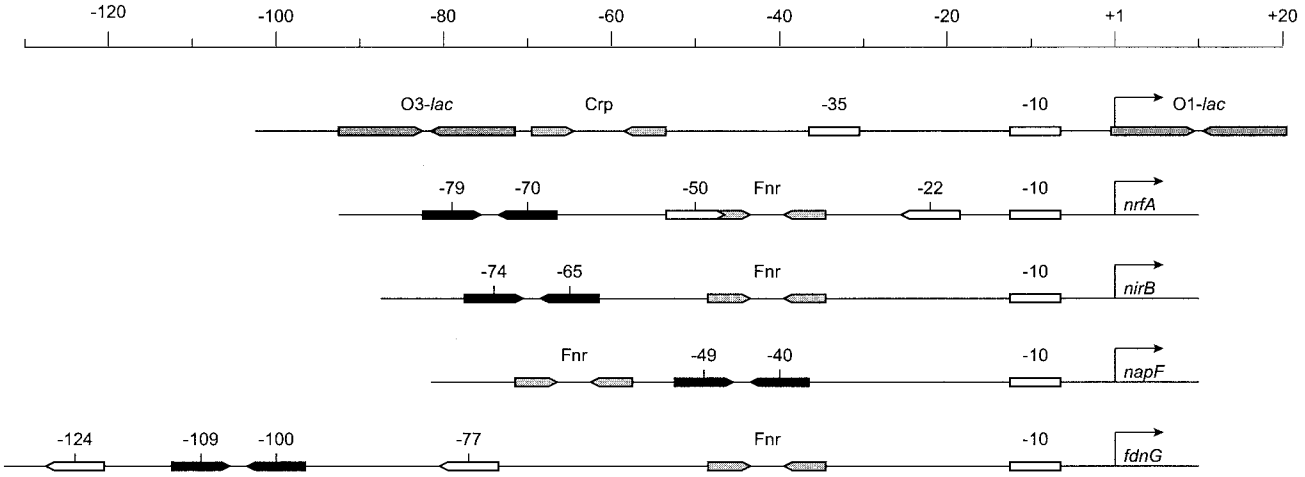

FIG. 1. The *lacZ*, *nrfA*, *nirB*, *napF*, and *fdnG* operon control regions. The scale is in nucleotides. Arrows along the nucleotide sequence represent regulatory protein binding sites: black arrows, 7-2-7 heptamer pairs studied in this work; white arrows, adjacent heptamer sequences; dark gray arrows, *lac* operators; light gray arrows, Fnr or Crp protein binding sites. White rectangles represent promoter −35 and −10 elements. Nar heptamer sequences are denoted by the positions of the central nucleotide with respect to the transcription start sites, which are shown as the thin arrows at right above each sequence schematic.

evaluating signal ligand interactions with sensor-kinases, examining sensor interactions with response regulators, and analyzing response regulator-DNA interactions.

MATERIALS AND METHODS

Strains and plasmids. Strains and plasmids are listed in Table 1. Control region sequences are depicted in Fig. 2. Genetic crosses were performed by P1*kc*-mediated generalized transduction (22). Null alleles of *nar* regulatory genes (Table 1) have been described previously (25). Standard methods were used for restriction endonuclease digestion, ligation, transformation, and PCR amplification of DNA (19).

Culture media and conditions. Defined, complex, and indicator media for genetic manipulations were used as described previously (19). Ampicillin was used at 25 mg/ml for selecting λInCh transductants (3).

Defined medium to grow cultures for enzyme assays was buffered with 3-[*N*-morpholino]propanesulfonic acid (MOPS) as previously described (30). The initial pH of this medium is set at 8.0 in order to ameliorate nitrite toxicity (32). Because the pK_a' of MOPS is 7.2, the buffering capacity of this medium continually increases as acidic fermentation products accumulate; at harvest, cultures typically had a pH value of about 7.5.

Medium for batch cultures grown to the mid-exponential phase contained glucose (80 mM) as the carbon source, and the respiratory oxidants $NaNO_3$ and $NaNO_2$ were added to 40 mM and 5 mM, respectively. Medium for overnight cultures arrested in the mid-exponential phase (13) contained glucose (6 mM), glucose plus $NaNO_3$ (4 mM and 10 mM, respectively), or glucose plus $NaNO_2$ (6 mM and 8 mM, respectively) as indicated. These concentrations were determined empirically to support growth to the mid-exponential phase (about 35 to 40 Klett units).

Cultures were grown at 37°C. Culture densities were monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.) equipped with a number 66 (red) filter. Anaerobic cultures for enzyme assays were grown in screw-cap tubes as described previously (30).

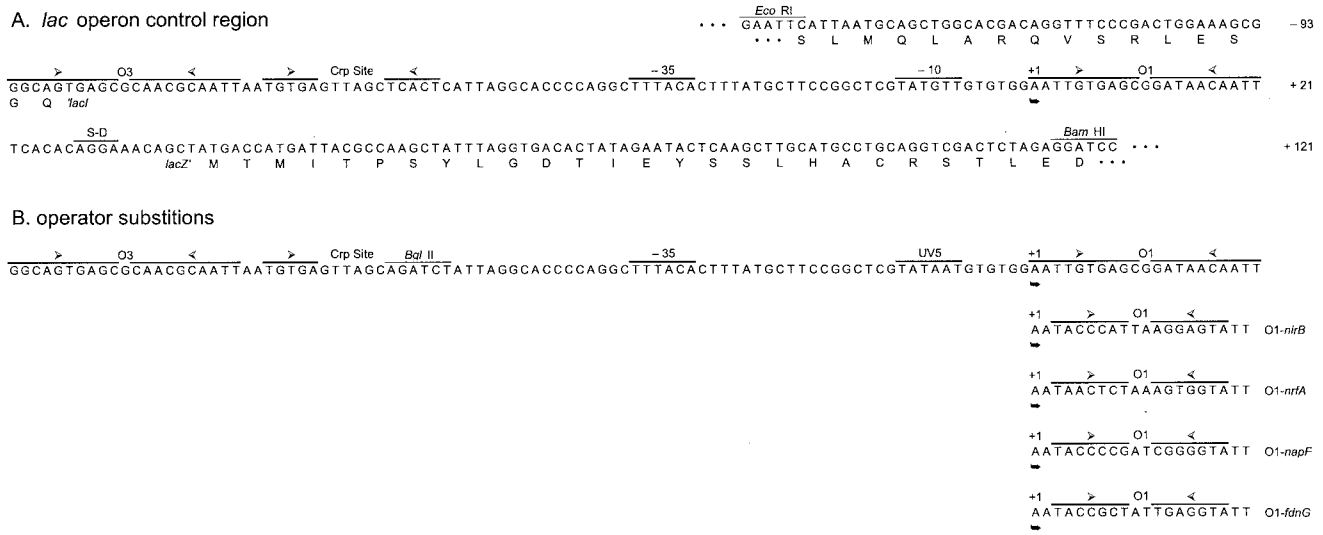

FIG. 2. Control region sequences. (A) *lac* operon control region from plasmid pALTER-1 (see also Fig. 1). (B) Modified *lac* operon control region used in this work. Substitutions at operator O1 are shown below the sequence. The transcription initiation site is labeled +1. Open arrowheads show centers of protein-binding half-sites.

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype and/or phenotype	Reference or source
E. coli		
DHB6521	Δ*lac mel gyrA supF* λInCh1 (Km^r)	3
VJS632	F^- λ^- prototroph	30
VJS676	As VJS632 but Δ(*argF-lacIZYA*)*U169*	30
Derivatives of strain VJS676 (λRS45-derived lysogens)		
VJS6959	λ [O3-*lac* O1-*lac* $lacZ^+Y^+A^+$]	This study
VJS6934	λ [O3-*lac* O1-*nrfA* $lacZ^+Y^+A^+$]	This study
VJS6311	λ [O3-*lac* O1-*nirB* $lacZ^+Y^+A^+$]	This study
VJS6313	λ [O3-*lac* O1-*nirB* $lacZ^+Y^+A^+$]/*narL215*::Tn*10*	This study
VJS6315	λ [O3-*lac* O1-*nirB* $lacZ^+Y^+A^+$]/*narP253*::Tn*10*d(Cm)	This study
VJS6317	λ [O3-*lac* O1-*nirB* $lacZ^+Y^+A^+$]/*narL215*::Tn*10 narP253*::Tn*10*d(Cm)	This study
VJS6813	λ [O3-*lac* O1-*napF* $lacZ^+Y^+A^+$]	This study
VJS6814	λ [O3-*lac* O1-*napF* $lacZ^+Y^+A^+$]/*narL215*::Tn*10*	This study
VJS6815	λ [O3-*lac* O1-*napF* $lacZ^+Y^+A^+$]/*narP253*::Tn*10*d(Cm)	This study
VJS6816	λ [O3-*lac* O1-*napF* $lacZ^+Y^+A^+$]/*narL215*::Tn*10 narP253*::Tn*10*d(Cm)	This study
VJS6945	λ [O3-*lac* O1-*fdnG* $lacZ^+Y^+A^+$]	This study
VJS6978	λ [O3-*lac* O1-*fdnG* $lacZ^+Y^+A^+$]/*narL215*::Tn*10*	This study
VJS6979	λ [O3-*lac* O1-*fdnG* $lacZ^+Y^+A^+$]/*narP253*::Tn*10*d(Cm)	This study
VJS6980	λ [O3-*lac* O1-*fdnG* $lacZ^+Y^+A^+$]/*narL215*::Tn*10 narP253*::Tn*10*d(Cm)	This study
Derivatives of strain VJS676 (λInCh-derived segregants)		
VJS7450	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*lac* $lacZ^+$]	This study
VJS7445	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*nirB* $lacZ^+$]	This study
VJS7484	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*fdnG* $lacZ^+$]	This study
VJS7489	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*nrfA* $lacZ^+$]	This study
VJS7906	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*nrfA* $lacZ^+$]/*narL249*::Ω-Sp	This study
VJS7907	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*nrfA* $lacZ^+$]/*narP253*::Tn*10*d(Cm)	This study
VJS7905	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*nrfA* $lacZ^+$]/*narQ251*::Tn*10*d(Tc)	This study
VJS7911	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*nrfA* $lacZ^+$]/*narQ251*::Tn*10*d(Tc) *narL249*::Ω-Sp	This study
VJS7912	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*nrfA* $lacZ^+$]/*narQ251*::Tn*10*d(Tc) *narP253*::Tn*10*d(Cm)	This study
VJS7904	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*nrfA* $lacZ^+$]/Δ*narX242*	This study
VJS7909	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*nrfA* $lacZ^+$]/Δ(*narXL)235*	This study
VJS7910	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*nrfA* $lacZ^+$]/Δ*narX242* *narP253*::Tn*10*d(Cm)	This study
VJS7449	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*napF* $lacZ^+$]	This study
VJS7475	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*napF* $lacZ^+$]/*narL215*::Tn*10*	This study
VJS7476	λ^- Δ(*att*λ-*lom*)::*bla* [O3-*lac* O1-*napF* $lacZ^+$]/*narP253*::Tn*10*d(Cm)	This study
Plasmids		
pRS414	Ap^r *'lacZ* gene fusion vector	28
pVJS3253	Ap^r Δ(*lacY lacA cynX tet*) derivative of pRS414	This study

Enzyme assay. β-Galactosidase activities were determined at room temperature (approximately 21°C) by following the hydrolysis of *o*-nitrophenyl-β-D-galactoside in $CHCl_3$–sodium dodecyl sulfate-permeabilized cells. Specific activities are expressed in arbitrary units (22). All cultures were assayed in duplicate, and reported values are averages from at least two independent experiments.

Construction of operator-substituted *lac* control regions. Oligonucleotide-directed site-specific mutagenesis was used to introduce substitutions into the *lac* operon control region. Mutagenesis followed either the ampicillin selection protocol (16) or the QuickChange protocol (Stratagene Cloning Systems, La Jolla, Calif.), as described previously (1). The high-fidelity thermostable DNA polymerase was Accuzyme (Bioline USA, Reno, Nev.).

Our starting point was the *lac* operon control region in plasmid pALTER-1 (16), which contains the wild-type *lac* sequence from codon Ala-331 of *lacI* through codon Thr-5 of *lacZ* (Fig. 2A). A 111-nt in-frame segment containing bacteriophage SP6 and T7 promoters and sites for several restriction endonucleases (including *Bam*HI) lies immediately downstream of codon Thr-5. We used site-specific mutagenesis to make three changes into the *lac* control region (Fig. 2). The first change introduced an upstream *Eco*RI site spanning *lacI* codons 343 to 345 (5′-GCC GAT TCA changed to 5′-GCG AAT TCA). This allows the *lac* control region to be released as a 260-nt *Eco*RI-*Bam*HI fragment. The second change introduced a *Bgl*II site spanning positions −58 through −53 (5′-TCACTC changed to 5′-AGATCT), which also destroys the promoter-proximal half-site for binding the cAMP-bound Crp protein. The third change is the UV5 substitution at the promoter −10 element (5′-TATGTT changed to 5′-TATAAT), which suppresses the Crp-binding site alteration (26). Together, these changes resulted in a *lac* control region cassette that includes the Crp-independent *lac* UV5 promoter and operators O3-*lac* and O1-*lac*. This plasmid served as template for subsequent site-specific alterations.

Following each round of mutagenesis, the DNA sequence for the 260-nt *Eco*RI-*Bam*HI fragment encompassing the *lac* control region was determined in order to eliminate isolates with spurious nucleotide substitutions. The control region cassettes were then recloned into the *lacZ* gene fusion vector pRS414 or its Δ(*lacYA*) derivative pVJS3253, which permit in-frame cloning of *Eco*RI-*Bam*HI fragments proximal to *lacZ* codon Val-10. This reconstructs a functional $lacZ^+$ gene whose transcription is governed by the upstream control region.

We experienced considerable difficulty cloning the O1-*fdnG* version into the *lacZYA* vector pRS414. As our preliminary analysis indicated that this 7-2-7 sequence effected only weak Nar-dependent repression of *lacZ* expression, we hypothesized that LacY protein overproduction in these constructs was deleterious to the host cells. We therefore deleted the region from position *Dra*I-3127

TABLE 2. Expression of the *lacZ* gene from O3-*lac* O1-*lac* constructs[a]

Strain	LacZ sp act						Repression			
	Excess Glc[b] with:			Limiting Glc[c] with:			Excess Glc with:		Limiting Glc with:	
	No addition	NO_3^-	NO_2^-	No addition	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-
VJS6959 (λRS45)	1,910	1,990	1,730	2,240	1,720	1,820	1.0	1.1	1.3	1.2
VJS7450 (λInCh)	2,070	2,120	1,800	2,040	1,390	1,380	1.0	1.2	1.5	1.5

[a] Specific activity is indicated in Miller units.
[b] Strains were cultured to the mid-exponential phase in MOPS defined medium with excess glucose, as described in Materials and Methods.
[c] Strains were cultured overnight in MOPS defined medium with limiting glucose, as described in Materials and Methods.

(at codon 4 of *lacY*) to *Ngo*MIV-6815 in plasmid pRS414. This deletion removed the *'lacY*, *lacA*, *cynX'* and *'tet* sequences, thereby reducing the 10.6-kb plasmid pRS414 to the 7.0-kb plasmid pVJS3253. This vector proved suitable for cloning all constructs, including one that retains the O1-*lac* operator sequence, and therefore was employed for all subsequent experiments.

Plasmid pRS414-based constructs were crossed into bacteriophage λRS45 (27), and monocopy lysogens were identified by a whole-colony PCR test (24). The smaller size of plasmid pVJS3253 makes it compatible with the packaging limit of bacteriophage λInCh (3). These constructs were placed in monocopy in the host chromosome as described previously (3).

RESULTS

Comparison of bacteriophage λ vectors and culture conditions. We modified the *lac* control region as described in Materials and Methods to make transcription initiation independent of the cAMP-responsive Crp protein (26). Four different Nar 7-2-7 sequences, from the *nrfA*, *nirB*, *napF*, and *fdnG* control regions, were then substituted in place of the O1-*lac* primary operator (Fig. 1 and 2B). We made monocopy derivatives of most *lac* O1 substitution control regions as both λRS45 lysogens and as λInCh segregants as described in Materials and Methods. The λInCh procedure provides antibiotic selection for specialized transducing phage, and it also provides a more direct route to isolating monocopy constructs. The final step of the λInCh procedure results in segregation of the λ prophage (3).

We used two methods for growing cultures in defined MOPS-buffered medium. The first was to culture strains to the mid-exponential phase in medium containing excess glucose, following past practice (30). The second method was to culture strains overnight in medium containing limiting glucose, such that growth arrested in the mid-exponential phase (13). We empirically adjusted the glucose, nitrate, and nitrite concentrations so that growth arrested at approximately the same density irrespective of added electron acceptor (see Materials and Methods). Because we used different culture methods and different bacteriophage λ derivatives at different times during the course of this work, we compared the expression of O1-*lac* constructs (in *lacI* null strains) in all four combinations of phage systems and growth conditions used. The levels of *lacZ* gene expression were very similar in the λRS45 and λInCh versions and under the two different culture conditions (Table 2). Overall, we judge the two monocopy methods and two culture conditions to yield essentially identical results.

The glucose-limited overnight cultures exhibited a slight (1.5-fold or less) decrease in *lacZ* gene expression during growth with the electron acceptors nitrate or nitrite; this decrease was less pronounced in the glucose-excess cultures (Table 2). Previous studies have demonstrated that catabolite repression of *lac* operon expression is relieved during anaerobic growth and restored upon addition of nitrate as an alternative electron acceptor (9). We therefore attribute the slight decrease in *lacZ* gene expression in nitrate- or nitrite-supplemented cultures to residual catabolite repression elicited by the electron acceptor during anaerobic growth.

Effects of nitrate and nitrite on *lacZ* gene expression from O1 substitution control regions. We used site-specific mutagenesis to replace the primary operator O1-*lac* with 7-2-7 heptamer pairs from four different Nar-responsive control regions (Fig. 2B). Each construct in this series retains the O3-*lac* operator. Two of the constructs, O1-*nrfA* and O1-*nirB*, exhibited about 100-fold repression of *lacZ* gene expression in response to nitrate and five- to tenfold repression in response to nitrite (Table 3). By contrast, the O1-*napF* construct exhibited only about tenfold repression of *lacZ* gene expression in response to nitrate (Table 3). Finally, the O1-*fdnG* construct exhibited less than twofold repression of *lacZ* gene expression in response to nitrate (Table 3). Similar results were obtained for all four constructs with both excess glucose and glucose-limited overnight cultures (data not shown; see Tables 4 and 5).

The nonrepressed level of *lacZ* gene expression from the O1-*napF* construct was about threefold lower than that from the other constructs (Table 3). Conversely, the nonrepressed level of *lacZ* gene expression from the O1-*nrfA* construct was about 1.5-fold higher than that from the other constructs (Table 3). Mutations at positions +6 and +10 within O1-*lac* affect *lac* promoter function (20). Thus, it is possible that certain of the operator substitutions we studied had mild effects on *lacUV5* promoter strength.

Effects of *narL* and *narP* null alleles on *lacZ* gene expression

TABLE 3. Expression of the *lacZ* gene from O3-*lac* O1 substitution constructs[a]

Construct	LacZ sp act[b] with:			Repression with:	
	No addition	NO_3^-	NO_2^-	NO_3^-	NO_2^-
O3-*lac* O1-*lac*	2,700	2,660	2,360	1.0	1.1
O3-*lac* O1-*nrfA*	3,920	33	420	120	9.3
O3-*lac* O1-*nirB*	2,700	23	430	120	6.3
O3-*lac* O1-*napF*	810	74	360	11	2.3
O3-*lac* O1-*fdnG*	2,140	1,100	1,860	1.9	1.2

[a] Specific activity is indicated in Miller units.
[b] Strains were cultured to the mid-exponential phase in MOPS defined medium with excess glucose, as described in Materials and Methods. All carry λRS45-derived prophages.

TABLE 4. Effects of *narL* and *narP* null alleles on expression of the *lacZ* gene from O3-*lac* O1 substitution constructs[a]

Construct	Genotype		LacZ sp act[b] with:			Repression with:	
	narL	*narP*	No addition	NO_3^-	NO_2^-	NO_3^-	NO_2^-
O3-*lac* O1-*nirB*	+	+	3,390	30	360	110	9.4
	+	−	3,350	39	520	86	6.4
	−	+	3,470	680	1,010	5.1	3.4
	−	−	3,270	2,330	2,860	1.4	1.1
O3-*lac* O1-*napF*	+	+	1,150	85	290	14	4.0
	+	−	1,160	100	650	12	1.8
	−	+	1,210	140	170	8.6	7.1
	−	−	1,170	760	1,050	1.5	1.1

[a] Specific activity is indicated in Miller units.
[b] Strains were cultured overnight in MOPS defined medium with limiting glucose. All carry λRS45-derived prophages.

from O1-substitution control regions. We next examined repression of *lacZ* gene expression from the O1-*nirB*, O1-*napF*, and O1-*nrfA* constructs in strains carrying null alleles of the *narL* and *narP* genes encoding the two Nar response regulators. Expression of the *lacZ* gene from the O1-*nirB* construct was strongly repressed by nitrate in the $narL^+$ *narP* null strain (86-fold) (Table 4) but only weakly repressed in the $narP^+$ *narL* null strain (5.1-fold). By contrast, *lacZ* gene expression from the O1-*napF* construct was repressed to a similar degree by nitrate in both the $narL^+$ *narP* null and the $narP^+$ *narL* null strains (12- versus 8.6-fold) (Table 4). Expression of the *lacZ* gene from the O1-*nrfA* construct was strongly repressed by nitrate in both the $narL^+$ *narP* null and the $narP^+$ *narL* null strains (100- versus 36-fold) (Table 5, lines 1 to 3).

Maximal nitrite repression of *lacZ* gene expression from the O1-*nirB* construct required both $narL^+$ and $narP^+$ (Table 4). By contrast, nitrite repression of *lacZ* gene expression from the O1-*napF* construct was due almost exclusively to $narP^+$ (7.1- versus 1.8-fold) (Table 4). Indeed, the $narL^+$ allele antagonized the $narP^+$-dependent nitrite repression of *lacZ* gene expression from the O1-*napF* construct (4.0- versus 7.1-fold) (Table 4). Nitrite repression of *lacZ* gene expression from the O1-*nrfA* construct was likewise dependent largely upon $narP^+$ (19- versus 5.7-fold) (Table 5, lines 1 to 3).

Effects of *narX* and *narQ* null alleles on *lacZ* gene expression from the O1-*nrfA* control region. We next wished to examine the effects of null alleles of the *narX* and *narQ* genes encoding the two Nar sensors. We chose the O1-*nrfA* construct because it exhibited robust nitrate repression of *lacZ* gene expression in both *narL* null and *narP* null strains, as described in the preceding section.

In the $narX^+$ *narQ* null strain, effective nitrate repression of *lacZ* gene expression from the O1-*nrfA* construct required $narL^+$, and nitrite repression of *lacZ* gene expression was weak irrespective of $narL^+$ or $narP^+$ (Table 5). These observations are consistent with the idea that the NarX protein responds preferentially to nitrate. Conversely, the $narQ^+$ *narX* null strain exhibited very strong repression of *lacZ* gene expression by nitrate or nitrite irrespective of $narL^+$ or $narP^+$ (Table 5). This indicates that the NarX protein acts to inhibit the influence of the NarQ protein with respect to nitrite signaling.

DISCUSSION

Replacing the O1-*lac* operator with a binding site for Crp protein results in Crp-dependent repression of *lacUV5* promoter transcription (14). Other examples in which the *lac* promoter (or artificial promoters based on *lac*) have been used to examine repression by heterologous regulators include the anaerobic activator protein Fnr (38) and the quorum-sensing regulatory protein LuxR (11).

We describe here a series of monocopy *lacUV5*-based promoter constructs in which the native O1-*lac* and O3-*lac* operators are replaced by 7-2-7 heptamer pairs from the Nar-regulated *nrfA*, *nirB*, *napF*, and *fdnG* operons. Repression of *lacZ* gene expression from these O1-substitution constructs reflects at least four parameters: (i) signal ligand (nitrate or nitrite), (ii) ligand interactions with sensor-kinases (NarX and

TABLE 5. Effects of *narX*, *narQ*, *narL*, and *narP* null alleles on expression of the *lacZ* gene from the O3-*lac* O1-*nrfA* construct[a]

Genotype				LacZ sp act[b] with:			Repression with:	
narL	*narP*	*narX*	*narQ*	No addition	NO_3^-	NO_2^-	NO_3^-	NO_2^-
+	+	+	+	6,660	60	470	110	14
+	−	+	+	6,460	65	1,140	100	5.7
−	+	+	+	6,580	180	340	36	19
+	+	+	−	6,180	90	1,590	69	3.9
+	−	+	−	6,500	90	1,520	72	4.3
−	+	+	−	3,420	520	660	6.6	5.2
+	+	−	+	6,840	40	60	170	110
+	−	−	+	6,700	60	60	110	110
−	+	−	+	6,580	50	60	130	110

[a] Specific activity is indicated in Miller units.
[b] Strains were cultured overnight in MOPS defined medium with limiting glucose. All are λInCh-derived segregants.

NarQ), (iii) sensor interactions with response regulators (NarL and NarP), and (iv) phospho-response regulator interactions with DNA targets.

Relative affinities for binding phospho-NarL and phospho-NarP proteins. The inverted sequence symmetry of 7-2-7 heptamers pairs, coupled with analysis of single- and double-nucleotide substitutions, strongly suggests that they are bound by dimers of phospho-NarL or phospho-NarP protein (33). This conclusion is supported by recent X-ray analysis of the NarL protein carboxyl-terminal DNA-binding domain cocrystallized with a 7-2-7 heptamer pair oligonucleotide (21). Although phospho-NarL can also bind to heptamers that are deployed in other configurations (2, 4, 7, 17), we focus here on the 7-2-7 heptamer pairs that comprise the sole or principal NarL and NarP protein binding sites in their respective control regions (Fig. 1).

The tetrameric LacI repressor effects about 20-fold repression of *lacZYA* operon expression in constructs lacking both auxiliary operators O2-*lac* and O3-*lac* (23). The greater-than-100-fold repression of *lacZ* gene expression by nitrate from the O1-*nrfA* and O1-*nirB* constructs (Table 3) therefore constituted a robust response that suggests high-affinity protein-DNA interactions. The O1-*napF* construct exhibited about tenfold repression of *lacZ* gene expression by nitrate, whereas the O1-*fdnG* construct exhibited less than twofold repression of *lacZ* gene expression by nitrate (Table 3). These results imply relative in vivo binding affinities, from high to low, of *nrfA*, *nirB* > *napF* > *fdnG*. This is consistent with the conclusions from previous studies, as described below.

The O1-*nrfA* construct. During growth in batch cultures, expression of the *nrfABCDEFG* operon encoding periplasmic nitrite reductase is induced by nitrite and repressed by nitrate (25, 29, 34, 35). Full-level nitrite induction is observed in either *narL*$^+$ *narP* null or *narP*$^+$ *narL* null strains and requires the 7-2-7 heptamer pair at −79 and −70 (25, 34). Qualitative DNase I protection studies revealed maximal protection of the *nrfA* 7-2-7 heptamer pair at relatively low concentrations of either maltose binding protein (MBP)-NarL or MBP-NarP protein phosphorylated with acetyl phosphate (7). Thus, previous in vivo and in vitro studies indicate that phospho-NarL and phospho-NarP proteins both bind the *nrfA* operon 7-2-7 heptamer pair with relatively high affinities. During growth with nitrate, phospho-NarL protein (but not phospho-NarP protein) binds also to lower-affinity heptamers at positions −50 and −22 (Fig. 1) to repress *nrfA* operon expression (7, 34).

Comparison of *lacZ* gene expression from the O1-*nrfA* construct in *narL*$^+$ *narP* null and *narP*$^+$ *narL* null strains suggests that the *nrfA* operon 7-2-7 heptamer pair was bound with similar affinities by either phospho-NarL or phospho-NarP protein during growth with nitrate (Table 5), fully consistent with these previous studies.

The O1-*nirB* construct. During growth in batch cultures, expression of the *nirBDC* operon encoding NADH-nitrite reductase is induced by both nitrate and nitrite (33–35). Nitrite induction and full-level nitrate induction require the 7-2-7 heptamer pair at −74 and −65 and the *narL*$^+$ gene, whereas the *narP*$^+$ gene is required only for full-level nitrate induction (33, 34). The phospho-NarL and phospho-NarP proteins are not direct activators of *nirB* operon expression, but rather counter the effects of negative regulatory proteins (39). In qualitative DNase I protection studies, maximal protection of the *nirB* 7-2-7 heptamer sequence was observed at relatively low concentrations of MBP-NarL protein but at considerably higher concentrations of MBP-NarP protein (7). Thus, previous in vivo and in vitro studies indicate that phospho-NarL protein binds the *nirB* operon 7-2-7 heptamer pair with higher affinity than phospho-NarP protein.

The O1-*nirB* construct exhibited strong repression of *lacZ* gene expression by nitrate in the *narL*$^+$ *narP* null strain but only weak repression in the *narP*$^+$ *narL* null strain (Table 4). This is fully consistent with conclusions drawn from the studies summarized above. The DNA sequence determinants of this differential binding by phospho-NarL and phospho-NarP protein remain to be determined.

The O1-*napF* construct. During growth in batch cultures, expression of the *napFDAGHBC* operon encoding periplasmic nitrate reductase is induced by nitrite and to a lesser degree by nitrate (6, 25, 36). Nitrite and nitrate induction require the *narP*$^+$ gene and the 7-2-7 heptamer pair at −49 and −40. The phospho-NarL protein antagonizes phospho-NarP-dependent transcription activation by competing for binding to the 7-2-7 heptamer pair (6, 8). In qualitative DNase I protection studies, maximal protection of the *napF* 7-2-7 heptamer sequence was observed at relatively low concentrations of MBP-NarL protein but at considerably higher concentrations of MBP-NarP protein (6).

In response to nitrate, *lacZ* gene expression from the O1-*napF* construct was repressed to a similar degree in both *narL*$^+$ *narP* null and *narP*$^+$ *narL* null strains (Table 4). In response to nitrite, however, effective repression required the *narP*$^+$ allele (Table 4). These results are consistent with conclusions drawn from the in vivo studies summarized above. However, the relative affinities for phospho-NarL and phospho-NarP inferred from the in vivo data reported here do not correlate with the previous qualitative DNase I protection results (6). Certainly, phospho-NarP protein is an effective activator of *napF* operon transcription (6, 8, 25), suggesting that it probably has a relatively high affinity for the *napF* operon 7-2-7 heptamer pair (Table 4).

The O1-*fdnG* construct. During growth in batch cultures, expression of the *fdnGHI* operon encoding respiratory formate dehydrogenase is induced by nitrate and to a much lesser degree by nitrite (18, 25). Nitrite induction requires the *narL*$^+$ gene, the 7-2-7 heptamer pair at −109 and −100, and an additional heptamer at −77 (4, 17, 18). In qualitative DNase I protection studies, maximal protection of the *fdnG* operon 7-2-7 heptamer sequence required relatively high concentrations of MBP-NarL protein compared to that of the *nirB* and *nrfA* operon 7-2-7 heptamer sequences (7).

Expression of the *lacZ* gene from the O1-*fdnG* construct was repressed by only twofold during growth in nitrate (Table 3). Although congruent with the qualitative DNase I protection results summarized above, this result surprised us because *fdnG* operon expression is induced about 100-fold by nitrate (4, 18, 25). Further analysis is required to understand the determinants for the affinities, both in vivo and in vitro, of different 7-2-7 heptamer pairs for binding the phospho-NarL and phospho-NarP proteins.

Sensor-regulator interactions. The O1-*nrfA* construct exhibited strong repression of *lacZ* gene expression by either phos-

pho-NarL or phospho-NarP protein (Table 5). Therefore, we chose this construct to further examine the effects of null alleles of the *narX* and *narQ* genes encoding the sensor-kinases. Previous studies have suggested that the NarX sensor responds preferentially to nitrate, whereas the NarQ sensor responds equally well to both nitrate and nitrite (5, 31).

In the *narX*$^+$ *narQ* null strain, strong nitrate repression of *lacZ* gene expression from the O1-*nrfA* construct required the *narL*$^+$ allele. Furthermore, nitrite repression of *lacZ* gene expression was significantly decreased by the *narQ* null allele irrespective of *narL*$^+$ or *narP*$^+$ (Table 5). These results support the idea that NarX-NarL forms a cognate two-component pair that responds preferentially to nitrate (5, 31).

By striking contrast, the *narQ*$^+$ *narX* null strain exhibited strong repression of *lacZ* gene expression by either nitrate or nitrite irrespective of *narL*$^+$ and *narP*$^+$ (Table 5). This suggests that the NarQ sensor can partner effectively with either the NarP or the NarL response regulator in response to either nitrate or nitrite. Thus, the NarQ sensor appears to be less selective than the NarX sensor with respect to both signal ligand and response regulator.

Previous studies led to the conclusion that negative regulation (i.e., phospho-NarL phosphatase activity) by the NarX protein is a critical aspect of differential target operon expression in response to nitrate versus nitrite (25, 29, 37). The results summarized in Table 5 support this idea. In *narX*$^+$ *narQ*$^+$ strains, nitrate, but not nitrite, elicited strong repression of *lacZ* gene expression from the O1-*nrfA* construct. By contrast, in *narQ*$^+$ *narX* null strains, both nitrate and nitrite were equally strong signals for repression. Therefore, the NarX protein serves to antagonize NarQ function, especially in response to nitrite (5, 31).

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