Expression of the *narX*, *narL*, *narP*, and *narQ* Genes of *Escherichia coli* K-12: Regulation of the Regulators

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The products of four *Escherichia coli* genes (*narX*, *narL*, *narQ*, and *narP*) regulate anaerobic respiratory gene expression in response to nitrate and nitrite. We used *lacZ* gene and operon fusions to monitor the expression of these *nar* regulatory genes in response to different growth conditions. Maximal expression of the *narXL* operon required molybdate, nitrate, and integration host factor. Expression of the *narP* and *narQ* genes was weakly repressed by nitrate. The NarL and NarP proteins were required for full nitrate induction of *narXL* operon expression, whereas the nitrate repression of *narP* and *narQ* expression was mediated solely by the NarL protein. *narXL* operon expression was unaffected by anaerobiosis, whereas expression of *narP* and *narQ* was induced approximately fourfold. The Fnr and ArcA proteins were not required for this anaerobic induction.

Escherichia coli is able to use alternatives to oxygen as the terminal electron acceptor of respiratory chains. The enzymes required for anaerobic respiration are synthesized only during anaerobic growth. This anaerobic induction is mediated by the activator protein Fnr (reviewed in reference 28). In addition, the synthesis of anaerobic respiratory enzymes is regulated in response to nitrate, the preferred anaerobic electron acceptor. Nitrate induces the synthesis of the formate-nitrate respiratory chain, formate dehydrogenase-N (encoded by the *fdnGHI* operon [1]) and nitrate reductase (encoded by the *narGHJI* operon [29]), and represses the synthesis of other anaerobic respiratory chain components such as fumarate reductase (encoded by the *frdABCD* operon [12]) and formate-dependent nitrite reductase (encoded by the *nrfABCDEFG* operon [4]). The product of nitrate reduction, nitrite, is also involved in regulating the expression of most anaerobic respiratory genes. For example, the *narG* and *fdnG* operons are induced weakly by nitrite (1, 23), whereas expression of the *nrfA* operon is repressed by nitrate and induced by nitrite (4).

A complex regulatory system has evolved to mediate this nitrate and nitrite control. The system consists of dual-interacting two-component regulatory systems (23) with homologous membrane-bound sensor proteins (NarX and NarQ) and homologous DNA-binding response regulators (NarL and NarP). The NarL protein binds to multiple sites in target operon control regions to regulate transcription (17, 34). For the *narG* and *fdnG* operons, these binding sites are located upstream of the transcription start point. The NarL protein binds to sites close to the transcription initiation site to repress expression of the *frdABCD* operon. In addition to NarL, the sequence-specific DNA-binding protein, integration host factor (IHF) (7), is also required for full nitrate and nitrite induction of the *narG* and *narK* operons (15, 21, 25). The *narK* gene apparently encodes a nitrite extrusion protein (24). Expression of some operons is controlled by both NarL and NarP. Most of the nitrate and nitrite induction of the *fdnG* operon is mediated by NarL, although NarP can weakly induce expression (23). In contrast, full nitrite induction of *nrfA* operon

expression can be mediated by either NarL or NarP (23, 33). The *nar* regulatory system has been recently reviewed (30).

The *narX* and *narL* genes form a complex *narXL* operon, the expression of which is subject to NarL-dependent nitrate induction (5, 32). A constitutive promoter within the *narX* coding region directs synthesis of a *narL* transcript (5, 9). The *narK* gene lies upstream of and is transcribed divergently from the *narXL* operon (32), and the *narK-narXL* intergenic region contains regulatory sequences for both promoters (2, 15, 20, 32). The *narP* and *narQ* genes are unlinked to any other *nar* genes, including each other.

Our current model to describe the Nar-mediated regulation of anaerobic enzyme synthesis implicitly assumes that the levels of the NarX, NarL, NarP, and NarQ proteins are essentially constant under different growth conditions. However, it was recently shown that expression of the *arcA* gene (which encodes a response regulator involved in the anaerobic regulation of gene expression) is directly controlled by the Fnr protein (3). This and related observations have led to a reassessment of the roles of Fnr and ArcA in the control of *cyo* operon (encoding cytochrome oxidase) expression (3, 10).

To investigate whether the *narX*, *narL*, *narP*, and *narQ* genes are regulated, we constructed $\Phi(narP-lacZ)$ and $\Phi(narQ-lacZ)$ gene and operon fusions. The levels of expression of these fusions and of previously constructed $\Phi(narX-lacZ)$ and $\Phi(narL-lacZ)$ fusions were determined after growth under various conditions. The effects of *narL*, *narP*, *fnr*, and *himD* (which encodes one of the subunits of the IHF protein) null alleles on expression of the *nar* regulatory genes were also determined.

Strains, media, and assays. All strains were derived from VJS676 or its Δtrp derivative, VJS691 (32). For each gene (translational) or operon (transcriptional) fusion studied, a reference single-copy lysogen was established. The $\Phi(narX-lacZ)$ operon fusion strain, VJS2009, and the $\Phi(narL-lacZ)$ gene fusion strain, VJS2010, were described previously (5). For isogenic strain constructions, we used bacteriophage P1 *kc*-mediated transduction to introduce mutant alleles into the reference strains. The mutant alleles used were *narL215::Tn10* (29), *narP253::Tn10d(Cm)* (23), *fnr-271::Tn10* (18), *arcA1 zjj::Tn10* (13), *modB247::Tn10* (31), and $\Delta himD3::cat$ (originally from plasmid pE387 [6]). All bacterial manipulations, growth, and β -galactosidase assays were carried out as previ-

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TABLE 1. Effects of *narL*, *narP*, and *himD* null alleles on *narXL*, *narP*, *narQ*, and *narK* expression

Genotype ^a	β -Galactosidase sp act (Miller units) ^b														
	Wild-type			<i>narL narP</i> ⁺			<i>narL</i> ⁺ <i>narP</i>			<i>narL narP</i>			Δ <i>himD</i>		
	No acceptor	NO ₃ ⁻	NO ₂ ⁻	No acceptor	NO ₃ ⁻	NO ₂ ⁻	No acceptor	NO ₃ ⁻	NO ₂ ⁻	No acceptor	NO ₃ ⁻	NO ₂ ⁻	No acceptor	NO ₃ ⁻	NO ₂ ⁻
Φ (<i>narX-lacZ</i>)	140	630	320	130	200	200	130	530	220	140	150	150	140	320	240
Φ (<i>narP-lacZ</i>)	100	60	110	110	100	90	90	70	100	100	90	90	90	60	90
Φ (<i>narQ-lacZ</i>)	400	320	370	410	390	380	390	320	370	390	420	420	410	400	370
Φ (<i>narK-lacZ</i>)	15	1,740	310	15	20	15	15	1,610	190	15	15	15	15	400	110

^a All strains carry gene (*-lacZ*) or operon (*-lacZ*) fusions on single-copy λ specialized transducing phage and have *narL*, *narP*, and *himD* wild-type or null alleles as indicated.

^b Measured after anaerobic growth in defined minimal morpholinepropanesulfonic acid (MOPS) medium (32) with no acceptor, 40 mM nitrate (NO₃⁻), or 5 mM nitrite (NO₂⁻) as indicated.

ously described (32). Reported values for the β -galactosidase assay data are averaged from at least two independently grown cultures, each of which was assayed in duplicate. The standard error of the values reported was not more than 15%.

Construction of *narP*, *narQ*, and *narK* gene and operon fusions. Gene and operon fusions of *lacZ* to *narP*, *narQ*, and *narK* were first constructed on plasmids and then transferred to λ phage by in vivo recombination, using the vectors of Simons et al. (27). The in vivo transcription start sites of the *narX*, *narP*, and *narQ* genes have not yet been reported. Therefore, each of the fusion constructs included a large region of upstream DNA to ensure that all regulatory elements were present. The *narP* fusions were constructed by cloning the 527-bp *EcoRI*-*BclI* fragment of plasmid pVJS334 (23) between the *EcoRI* and *BamHI* sites of plasmids pRS414 and pRS415 (27) to make *lacZ* gene and operon fusions, respectively. The fusion junction is at codon 14 of the *narP* gene, and the restriction fragment includes 486 bp of DNA upstream of the *narP* coding region. This 486-bp region encompasses all of the DNA between *narP* and an upstream IS5 insertion element (23). For the *narQ* fusions, the ~2.9-kb *PvuII* fragment of the *narQ*⁺ plasmid (described in reference 22) was cloned into the *SmaI* sites of plasmids pVJS414 and pVJS415. The correct orientation of the cloned fragment was confirmed by DNA sequencing. The fusion junction is at codon 182 of the *narQ* gene, and the fusion includes over 2 kb of upstream DNA. The *narK-lacZ* gene fusion was constructed by cloning the ~0.4-kb *BamHI*-*BstYI* fragment of plasmid pVJS1116 (17) into the *BamHI* site of plasmid pRS414. The correct orientation of the insert was confirmed by restriction enzyme digest analysis. The fusion junction is at codon 22 of the *narK* gene, and the fusion includes 315 bp of upstream DNA which extends to position -23 bp with respect to the translation start site of the divergently transcribed *narX* gene. The transcription initiation site of *narK* is located 25 bp upstream of its start codon (15). All of these fusions were recombined onto λ RS45 (27), and single-copy lysogens of VJS676 were identified by the Ter test as described previously (27). The construction of the *narX* and *narL* fusions was described previously (5). The gene and operon fusions of *lacZ* to *narX* were constructed from DNA fragments which include all of the region between the divergently transcribed *narX* and *narK* genes.

Strains with the Φ (*narQ-lacZ*) or Φ (*narX-lacZ*) gene fusion expressed low levels of β -galactosidase (data not shown and reference 5). Therefore, in the following experiments we used *narX* and *narQ* operon fusions to *lacZ*. The β -galactosidase activity (expressed in Miller units [19]) of the Φ (*narP-lacZ*) and Φ (*narL-lacZ*) gene fusion strains, although lower than that of the operon fusions (data not shown and reference

5), was sufficient for use in these experiments. For each pair of fusions, the overall patterns of nitrate and nitrite regulation were indistinguishable (data not shown and reference 5).

Regulation by nitrate and nitrite. We first examined the effects of nitrate and nitrite on the expression of the *narXL*, *narP*, and *narQ* fusions (Table 1). Note that both the *narX* and *narL* genes are expressed from the promoter upstream of *narX*. Therefore, the regulation of the Φ (*narX-lacZ*) operon fusion indicates changes in the expression of both *narX* and *narL*. Expression of the *narXL* operon was induced four- to five-fold by nitrate and approximately twofold by nitrite (Table 1). Expression of Φ (*narP-lacZ*) and Φ (*narQ-lacZ*) was repressed weakly, but reproducibly, by nitrate and unaffected by nitrite. The NarX and NarL proteins alone are sufficient for normal nitrate regulation of the *narG*, *fdnG*, and *frdA* operons (23). Therefore, the nitrate induction of *narXL* operon expression and the nitrate repression of *narP* and *narQ* expression have a physiological rationale.

Next we investigated the roles of the response regulators, NarL and NarP, in the observed regulation (Table 1). Introduction of a *narL* null allele abolished most, but not all, of the nitrate and nitrite induction of Φ (*narX-lacZ*) expression, as observed previously (5). In a *narP* null strain, the nitrate and nitrite induction of *narXL* operon expression was only slightly reduced. Finally, in a *narL narP* double-null strain, the induction of Φ (*narX-lacZ*) expression by nitrate or nitrite was abolished. These results indicate that both the NarL and NarP proteins are involved in induction of the *narXL* operon by nitrate or nitrite, a pattern similar to that observed for the *narG* and *fdnG* operons (23). The effects of *narL* and *narP* null alleles on the expression of Φ (*narP-lacZ*) and Φ (*narQ-lacZ*) were similar: the *narL* allele abolished the weak repression by nitrate, and the *narP* allele had no effect.

Comparison of *narXL* and *narK* regulation. The approximately 300-bp *narK-narXL* intergenic region contains the control regions for both operons. Since the two control regions may overlap, we investigated the similarities in the regulation of the *narXL* and *narK* genes. We determined the effects of nitrate and nitrite on the expression of Φ (*narK-lacZ*) in various *nar* regulatory mutants (Table 1). As observed previously (2, 15, 32), Φ (*narK-lacZ*) expression was induced by nitrate during anaerobic growth, albeit to a much greater level than the four- to fivefold induction of Φ (*narX-lacZ*) expression (Table 1). We also found that, like *narXL* expression, *narK* expression was induced by nitrite. Once again, the induction of *narK* was much greater than that of *narXL*. A *narL* null allele abolished the nitrate and nitrite induction of Φ (*narK-lacZ*) expression. In a *narP* null strain, the nitrite induction of Φ (*narK-lacZ*) expression was reduced but the nitrate induction was

TABLE 2. Effects of an *fnr* null mutation on expression of *narX*, *narP*, *narQ*, and *narL*

Genotype ^a	β -Galactosidase sp act (Miller units) ^b											
	Wild type						<i>fnr</i> -271::Tn10					
	Aerobic			Anaerobic			Aerobic			Anaerobic		
	No acceptor	NO ₃ ⁻	NO ₂ ⁻	No acceptor	NO ₃ ⁻	NO ₂ ⁻	No acceptor	NO ₃ ⁻	NO ₂ ⁻	No acceptor	NO ₃ ⁻	NO ₂ ⁻
$\Phi(narX-lacZ)$	170	240	160	130	610	240	130	—	—	170	620	550
$\Phi(narP-lacZ)$	30	20	30	110	60	100	30	—	—	100	40	80
$\Phi(narQ-lacZ)$	100	80	80	440	280	320	90	—	—	440	560	480
$\Phi(narL-lacZ)$	30	—	—	50	—	—	40	—	—	50	—	—

^a All strains carry gene (*-lacZ*) or operon (*-lacZ*) fusions on single-copy λ specialized transducing phage and have an *fnr* wild-type or null allele as indicated.

^b Measured after aerobic or anaerobic growth in defined minimal MOPS medium (32) with no acceptor, 40 mM nitrate (NO₃⁻), or 5 mM nitrite (NO₂⁻) as indicated. —, not determined.

unaffected. The regulatory patterns of the *narX* and *narK* promoters were therefore similar. Both were induced by nitrate or nitrite. Both the NarL and NarP proteins were required for full nitrate and nitrite induction of $\Phi(narX-lacZ)$ expression and for full nitrite induction of $\Phi(narK-lacZ)$ expression. Several binding sites for the NarL protein have been identified at positions -220 to -150 and -110 to -60 of the *narK* control region (2, 17). It seems likely that the control regions of the *narK* and *narX* promoters overlap and that some or all of these NarL binding sites are involved in activation of both the *narX* and *narK* promoters.

Effects of IHF. Nitrate induction of $\Phi(narG-lacZ)$ expression is strongly dependent on IHF, which binds to the *narG* operon control region (21, 25). A deletion of the *himD* gene, which encodes one of the two subunits of IHF, results in a smaller but significant reduction in nitrate induction of $\Phi(narX-lacZ)$ and $\Phi(narK-lacZ)$ expression (15, 21) (Table 1), and IHF specifically binds to the *narK-narX* intergenic control region (17). We wished to determine whether the IHF protein is also involved in regulating $\Phi(narP-lacZ)$ and $\Phi(narQ-lacZ)$ expression. A *himD* null allele had no effect on $\Phi(narP-lacZ)$ expression, whereas it eliminated the weak nitrate repression of $\Phi(narQ-lacZ)$ expression (Table 1). This effect on $\Phi(narQ-lacZ)$ expression is likely an indirect consequence of decreased *narXL* operon expression in the *himD* mutant (Table 1).

Regulation by anaerobiosis. The genes required for anaerobic respiration are expressed only during anaerobic growth, and this control is mediated by the activator protein Fnr. To determine if anaerobiosis and Fnr play a role in expression of the *nar* regulatory genes, we measured the expression of *narX*, *narP*, *narQ*, and *narL* during aerobic and anaerobic growth in *fnr*⁺ and *fnr* null strains (Table 2). Basal expression levels of $\Phi(narX-lacZ)$ were similar during aerobic or anaerobic growth. However, induction by nitrate was significantly reduced during aerobic growth. The reasons for this are unclear, but part of the explanation could be the lower levels of the NarP and NarQ proteins during aerobic growth (see below). Expression of $\Phi(narX-lacZ)$ was unaffected by a *fnr* null allele with the exception that induction by nitrite during anaerobic growth was increased. Expression of *narK* is induced during anaerobic growth and is almost completely dependent on the Fnr protein (15, 32). Therefore, Fnr must bind to the region of DNA between *narK* and *narX*, but it apparently activates only the *narK* promoter. An apparent Fnr binding site required for normal *narK* expression has been identified at position -41.5 bp with respect to the *narK* transcription initiation site (2).

Expression of both *narP* and *narQ* was induced approximately fourfold by anaerobiosis under all growth conditions (Table 2). However, the Fnr protein was not required for this

anaerobic induction since levels of expression of $\Phi(narP-lacZ)$ and $\Phi(narQ-lacZ)$ were similar in wild-type and *fnr* null strains. The ArcA-ArcB two-component regulatory system is also involved in the anaerobic regulation of many genes in *E. coli* (14). However, we found that expression of $\Phi(narP-lacZ)$ and $\Phi(narQ-lacZ)$ was also unaffected by an *arcA* null allele (data not shown).

The cell must be ready to respond quickly to nitrate availability when the oxygen supply is depleted. The observation that the basal expression of the *narXL* operon is not dependent on anaerobiosis is consistent with this requirement. Conversely, the anaerobic induction of *narP* and *narQ* suggests that these gene products are not required for a fast adaptation to nitrate respiration. Once again, this is consistent with the observation that NarX and NarL are sufficient for normal nitrate regulation of some operons (see above).

Levels of expression of the $\Phi(narL-lacZ)$ fusion were similar during aerobic and anaerobic growth and were unaffected by an *fnr* null allele (Table 2). Note that the β -galactosidase activity from the $\Phi(narL-lacZ)$ gene fusion (in strain VJS2010) is due only to expression from the *narL* promoter within the *narX* coding region (5). Therefore, this *narL* promoter is constitutive with respect to anaerobiosis as well as to nitrate and nitrite (5).

Molybdate regulation. Molybdate is required for nitrate-dependent regulation of several NarL-regulated operons, including *narG* and *fdnG* operon induction and *frdA* operon repression (8, 11). Molybdate limitation is achieved by using a *mod* (formerly *chlD*) strain defective in molybdate uptake (26) and adding 2 mM K₂SO₄ to the growth medium (16). The limitation is relieved by growth in the presence of 100 μ M molybdate. We measured the expression of each of the $\Phi(nar-lacZ)$ fusions during anaerobic growth under molybdate-limiting conditions (Table 3). In all cases, the basal anaerobic expression was unaffected by molybdate limitation. However, molybdate was required for normal induction of $\Phi(narX-lacZ)$ expression by nitrate and for the nitrate-dependent repression of $\Phi(narP-lacZ)$ and $\Phi(narQ-lacZ)$ expression (Table 3). The mechanism by which molybdate regulates expression of the *narG*, *fdnG*, and *frdA* operons is unknown. However, the NarL protein mediates the nitrate regulation of these operons. Perhaps the lower level of NarL (and NarX) during molybdate limitation (Table 3) at least partly accounts for some of the aberrant nitrate regulation.

Concluding remarks. A complicated regulatory system has evolved to regulate gene expression in response to nitrate and nitrite in *E. coli*. Dual homologous sensor proteins respond to nitrate and nitrite and interact with dual homologous response regulators in similar but distinct ways. The DNA binding sites

TABLE 3. Effects of molybdate on expression of *narX*, *narP*, *narQ*, and *narL*

Fusion ^a	β -Galactosidase sp act (Miller units) ^b			
	-NO ₃ ⁻		+NO ₃ ⁻	
	-MoO ₄ ²⁻	+MoO ₄ ²⁻	-MoO ₄ ²⁻	+MoO ₄ ²⁻
$\Phi(narX-lacZ)$	120	100	220	610
$\Phi(narP-lacZ)$	90	90	80	50
$\Phi(narQ-lacZ)$	350	350	430	290
$\Phi(narL-lacZ)$	50	40	50	40

^a All strains carry gene (-'lacZ) or operon (-lacZ) fusions on single-copy λ specialized transducing phage and have the *modB247::Tn10* allele.

^b Cultures were grown anaerobically in defined minimal MOPS medium (32) with or without 40 mM nitrate (NO₃⁻) and 100 μ M molybdate (MoO₄²⁻) as indicated.

of the response regulators are widely diverse in number, organization, and spacing. The complexity of this system allows for the precise control of gene expression in response to constant changes in the extracellular concentrations of nitrate and nitrite. The work presented here has revealed another level of complexity in the *nar* regulatory system. The genes encoding the four components of the system are all regulated. The precise concentrations of the sensors and response regulators may be important for efficient adaptation to varying nitrate and nitrite concentrations.

Our results indicate that the expression of the *narXL* operon is increased when nitrate becomes available during anaerobic growth (Table 1). This may allow for fine tuning of target operon expression. It has been proposed that *narG*, *fdnG*, and *frdA* operon expression is differentially sensitive to NarL-dependent regulation. For example, insertions in *narX*, which reduce expression of *narL* due to polarity, cause a slight decrease in nitrate induction of *narG* but almost eliminate nitrate repression of *frdA* (5, 32). When nitrate becomes available, nitrate reductase and formate dehydrogenase-N are synthesized and assembled into an active respiratory chain. As this is accomplished, and with continued nitrate availability, the next priority would be to cease synthesis of alternative anaerobic respiratory enzymes such as fumarate reductase. Thus, the basal levels of NarL and NarX might be sufficient for the first phase (formate-nitrate oxidoreductase synthesis), whereas elevated levels of NarL and NarX, resulting from nitrate induction of *narXL* operon expression, might be required for full repression of *frdA* operon expression. The nitrate induction of *narXL* operon expression, coupled with the varying sensitivities of the *narG*, *fdnG*, and *frdA* control regions to the availability of phospho-NarL, might aid in establishing these priorities in the correct order.

Our model for Nar-mediated regulation has implicitly assumed that regulatory protein synthesis is not greatly affected by null alleles of other regulators (23, 30). The results presented in this report largely support this assumption; for example, the expression of the *narP* and *narQ* genes appears to be only modestly affected in *narL* null mutants (Table 1). However, *narX* gene expression is more noticeably reduced in a *narL* mutant (Table 1), although we are uncertain whether the resulting lower level of NarX protein would significantly alter NarP-mediated regulation, given that the NarQ protein provides similar functions (23). By contrast, the recently observed Fnr-dependent control of *arcA* gene expression has subjected models for Fnr and ArcA control of gene expression to reevaluation (3, 10).

In the *nar* regulatory system, it is ultimately the concentra-

tion of active (phosphorylated) NarL and NarP which determines the level of expression of target genes. We can now conclude that the concentrations of the phosphorylated response regulators are the final result of at least three variable factors: first, expression of the *narL* and *narP* genes themselves; second, the kinase/phosphatase activity of the two sensor proteins NarX and NarQ, which is controlled by nitrate and nitrite concentrations (23, 30); and third, the amounts of the two sensor proteins which are available to phosphorylate the response regulators, which depend on the level of expression of the *narX* and *narQ* genes.

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