JOURNAL OF BACTERIOLOGY, July 1991, p. 4424–4432
0021-9193/91/144424-09$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 173, No. 14

Mutational Analysis of Nitrate Regulatory Gene *narL* in *Escherichia coli* K-12

SUSAN M. EGAN† AND VALLEY STEWART*
Section of Microbiology, Cornell University, Ithaca, New York 14853-8101

Received 25 March 1991/Accepted 10 May 1991

The *narL* gene product, NarL, is the nitrate-responsive regulator of anaerobic respiratory gene expression. We used genetic analysis of *narL* mutants to better understand the mechanism of NarL-mediated gene regulation. We selected and analyzed seven nitrate-independent *narL* mutants. Each of three independent, strongly constitutive mutants had changes of Val-88 to Ala. The other four mutants were weakly constitutive. The *narL505*(V88A) allele was largely dominant to *narL*$^+$, while *narX*$^+$ had a negative influence on its constitutive phenotype, suggesting that NarX may play a negative role in nitrate regulation. We also constructed two *narL* mutations that are analogous to previously characterized constitutive *degU* alleles. The first, *narL503*(H15L), was a recessive null allele. The second, *narL504*(D110K), functioned essentially as wild type but was dependent on *narX*$^+$ for full activity. We changed Asp-59 of NarL, which corresponds to the site of phosphorylation of other response regulators, to Asn. This change, *narL502*(D59N), was a recessive null allele, which is consistent with the hypothesis that NarL requires phosphorylation for activation. Finally, we tested the requirement for molybdate on regulation in a *narL505*(V88A) strain. Although *narL505*(V88A) conferred some nitrate-independent expression of *fdnGHI* (encoding formate dehydrogenase-N) in limiting molybdate, it required excess molybdate for full induction both in the absence and in the presence of nitrate. This finding suggests that *narL505*(V88A) did not confer molybdate-independent expression of *fdnGHI*.

Nitrate (NO_3^-) is the most energetically favorable electron acceptor available to *Escherichia coli* in the absence of oxygen. Synthesis of the enzymes involved in nitrate respiration is induced only in the absence of oxygen and the presence of nitrate. Other compounds, such as trimethylamine-*N*-oxide, dimethyl sulfoxide, and fumarate, can also be used as terminal electron acceptors, but synthesis of the enzymes involved in these respiratory chains is induced only in the absence of both oxygen and nitrate.

Formate-nitrate oxidoreductase is a major anaerobic respiratory chain which couples the oxidation of formate to the reduction of nitrate. The components of this respiratory chain, formate dehydrogenase-N and nitrate reductase, are membrane-bound enzyme complexes, each requiring molybdenum cofactor for activity. They are encoded by *fdnGHI* at 32 min and *narGHJI* at 27 min, respectively, on the *E. coli* genetic map (1; reviewed in reference 34). Fumarate respiration requires fumarate reductase, which is a membrane-bound enzyme complex encoded by *frdABCD*, at 94 min on the *E. coli* genetic map (reviewed in reference 21).

Regulation of anaerobic respiratory gene expression by anaerobiosis and nitrate is mediated by two proteins. Anaerobic regulation is mediated by the *fnr* gene product, FNR, which is a positive activator required for the transcription of a number of anaerobic genes (reviewed in references 31 and 34). Nitrate regulation is mediated by the *narL* gene product, NarL. *narL*$^+$ is required both for nitrate induction of *fdnGHI* and *narGHJI* and for nitrate repression of *frdABCD* (1, 14, 16, 33, 35, 37). The predicted amino acid sequence of NarL shares similarity with the response regulators of two-component regulatory systems (11, 23, 38; reviewed in reference 40). A proposed helix-turn-helix DNA binding domain has been identified in the LuxR family of regulatory proteins, of which NarL is a member (41).

Two-component regulatory systems consist of a histidine protein kinase and a response regulator. The histidine protein kinase, in response to an environmental signal, transfers a phosphate group to an aspartate residue in the amino-terminal domain of the response regulator. This phosphorylation activates the response regulator, which in most cases is a DNA-binding protein that regulates gene expression (reviewed in reference 40).

The *narX* gene lies just upstream of *narL* (Fig. 1). The predicted sequence of its product, NarX, shares sequence similarity with histidine protein kinases (23, 38). We previously demonstrated that in-frame deletions in *narX* confer no detectable phenotype with regard to nitrate induction of *fdnGHI* or nitrate repression of *frdABCD* expression (8). On the other hand, *narX* mutations have been isolated that lead to nitrate-independent induction of *narGHJI* and *fdnGHI* and repression of *frdABCD* (5, 17). Taken together, these results suggest a model for nitrate regulation of anaerobic respiratory genes in which NarL can be activated by either of two different nitrate-responsive histidine protein kinases: NarX, or a hypothetical second kinase which we have termed NarQ (8). This model for regulation is shown in Fig. 1.

The goal of the experiments reported here was to use a genetic analysis of *narL* mutations to better understand NarL-mediated nitrate regulation. One strong and four weak nitrate-independent *narL* alleles were isolated, sequenced, and studied by genetic complementation analysis. The strongly constitutive allele, *narL505*(V88A), conferred almost complete nitrate independence to *narGHJI* expression and was dominant to *narL*$^+$. We also constructed three alterations in *narL* by site-specific mutagenesis. Two were analogous to constitutive alleles of *degU*, a regulator of degradative exoenzyme synthesis in *Bacillus subtilis* (12). The third site-specific mutation changed Asp-59, which

* Corresponding author.
† Present address: Department of Biology, The Johns Hopkins University, Baltimore, MD 21218.

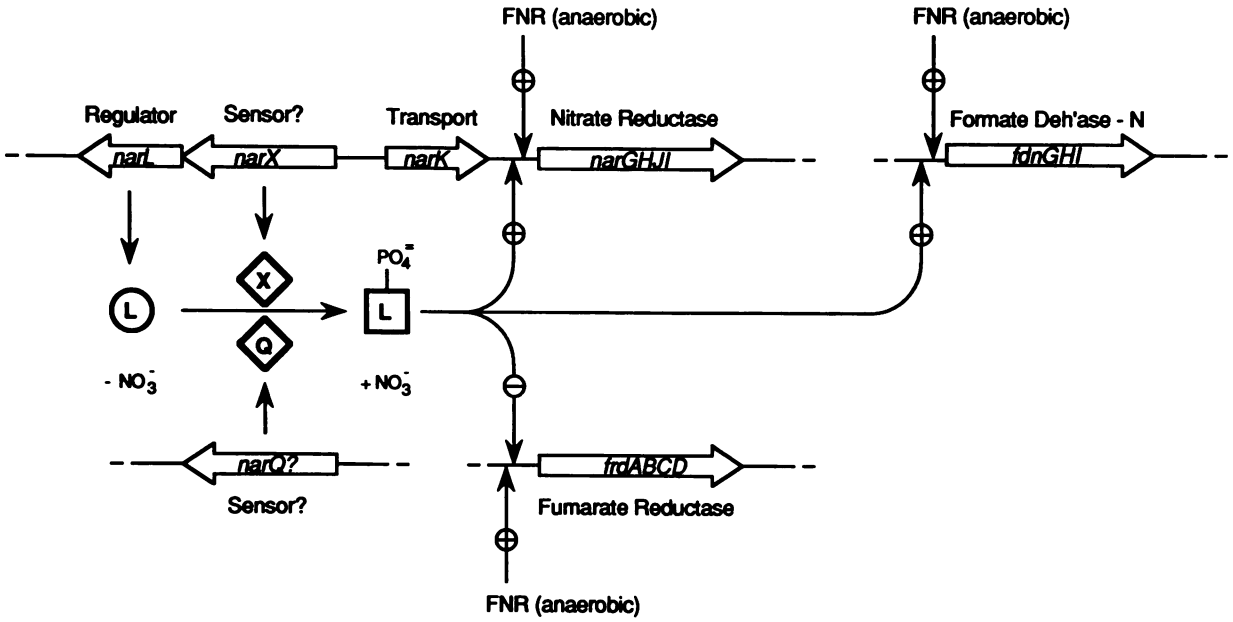

FIG. 1. Model for regulation of *narGHJI*, *fdnGHI*, and *frdABCD* expression. The *nar*, *fdn*, and *frd* regions are diagrammed schematically (not to scale). Open arrows indicate protein-coding regions and their direction of transcription. +, positive regulation (activation); −, negative regulation (repression). The *narL* gene product (NarL) is hypothesized to be a nitrate-responsive DNA-binding protein which, when activated by protein phosphorylation, activates *narGHJI* and *fdnGHI* and represses *frdABCD* transcription. The *narX* gene product and the hypothetical *narQ* gene product may be involved in modulating NarL activity. FNR is required for anaerobic induction of *narGHJI*, *fdnGHI*, and *frdABCD*. The *narK* gene product is involved in nitrate transport (24). Adapted from reference 35.

corresponds to a highly conserved aspartate which is the site of phosphorylation of other response regulators, to Asn. As expected, this was a recessive null allele. Finally, we tested the effect of limited molybdate concentration on the phenotype conferred by *narL505*(V88A).

MATERIALS AND METHODS

Strains, plasmids, and phage. *E. coli* K-12 strains, plasmids, and λ specialized transducing phage used in this study are described in Table 1. Genetic crosses were performed by using bacteriophage P1 *kc*-mediated transduction (22).

narL249::Ω was constructed by cloning the Ω-Sp element from *Bam*HI-digested pHP45Ω (26) between the *Bgl*II sites in *narL*. This deleted approximately one-third of the *narL* coding region. The resulting insertional allele was recombined into the chromosome of *E. coli* as described by Winans et al. (44).

Standard methods were used for restriction endonuclease digestion, ligation, and transformation of DNA (6). Restriction enzymes and T4 DNA ligase were from New England BioLabs, Inc. (Beverly, Mass.).

Culture media. Cultures for β-galactosidase and nitrate reductase assays were grown in 3-[*N*-morpholino]propanesulfonic acid (MOPS)-buffered minimal medium with glucose as the sole carbon source (37). The initial pH of this medium was set at 7.8. L-Tryptophan (0.2 mM) was added to all defined media, and $NaNO_3$ (40 mM) was added as indicated. For cultures of *chlD* strains, K_2SO_4 was added at 2 mM (20), and $NaMoO_4$ was added at either 0.05 or 100 μM, as indicated.

Defined, complex, and indicator media for routine genetic manipulations were used as described previously (6, 22). Ampicillin, kanamycin, and spectinomycin were used at 200, 75, and 15 μg/ml, respectively. Chloramphenicol was used at 25 μg/ml except as indicated. Agar and dehydrated media were from Difco Laboratories (Detroit, Mich.). Other components were from Sigma Chemical Co. (St. Louis, Mo.).

Culture conditions. Culture densities were monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.) equipped with a no. 66 (red) filter. Cultures were grown at 37°C. Anaerobic cultures for β-galactosidase and nitrate reductase assays were grown in screw-cap tubes as described previously (37). Plates were incubated anaerobically in Brewer jars (3).

Mutagenesis. λ specialized transducing phage were mutagenized by passage through strain ES1578, which carries the *mutD5* allele (28, 30, 45). In each case, a plate lysate was made of the phage of interest on ES1578. The mutagenized lysates, with titers of approximately 10^8 PFU/ml, were stored at 4°C.

Oligonucleotide-mediated site-specific mutagenesis followed the procedure of Vandeyar et al. (43). T4 DNA polymerase was from U.S. Biochemical Corp. (Cleveland, Ohio). The following primers were used: 5′-ATCCTGTTAA ATCTCAATA-3′ to convert Asp-59 of NarL to Asn, 5′-TTGACGATCTCCCGATGCT-3′ to convert His-15 of NarL to Leu, and 5′-CTGTTAAAAAAGATGGAACCG-3′ to convert Asp-110 of NarL to Lys. The mutagenesis was carried out on a *Bcl*I subclone of *narL* in the vector pTZ18R (U.S. Biochemical). The D59N change was identified by the loss of a *Bgl*II restriction site, while the others were identified by DNA sequence analysis. In all cases, the DNA sequence of the entire *narL* coding region was determined to ensure that no additional alterations were introduced during the mutagenesis.

Isolation of *narL* mutations. Undiluted, mutagenized lysates of the specialized transducing phage λ VJS109 and λ VJS110 (Δ*narX narL*$^+$) were mixed with cultures of strains

TABLE 1. Strains, plasmids, and phage

Strain, plasmid, or phage	Genotype	Reference or source
E. coli strains[a]		
ES1578	*mutD5 ara-14 arg*E3(Oc) *galK2 hisG4*(Oc) *kdgK51 lacY1 leuB6 mgl-51 mtl-1 rac rfbD1 rpsL31 supE44 thi-1 thr-1 tsx-33 xyl-5*	CGSC[b]
JM83	*ara* Δ(*lac-proAB*)*X111 rpsL* ϕ80dΔ(*lacZ*)*M15*	46
VJS533	As JM83 but *recA56*	37
VJS691	Δ(*argF-lac*)*U619*Δ(*trpEA*)*2*	37
VJS882	As VJS691 but *narG234*::Mu dI1734	1
VJS1250	As VJS691 but *fdnG108*::Mu dI1734	1
VJS1510	As VJS691 but *frdA401*::Mu dI1734 Δ(*narXL*)*235 recA1*	8
VJS2007	As VJS691 but *fdnG108*::Mu dI1734 Δ(*narXL*)*235 recA1*	8
VJS2534	As VJS691 but *frdA401*::Mu dI1734 *narL249*::Ω *recA1*	This work
VJS2536	As VJS691 but *frdA401*::Mu dI1734 *recA1*	This work
VJS2641	As VJS691 but *fdnG108*::Mu dI1734 Δ(*narXL*)*235 chlD247*::Tn*10*	This work
VJS2736	As VJS691 but *fdnG108*::Mu dI1734 *recA1*	This work
VJS2737	As VJS691 but *fdnG108*::Mu dI1734 *narL249*::Ω *recA1*	This work
Plasmids		
pHG165	Ap^r	32
pTZ18R	Ap^r	U.S. Biochemical
pVJS1	Ap^r, *narXL* in *Hin*dIII site of pHG165	37
pVJS211	Ap^r Km^r, pVJS1 Δ*narXL240*::Km	This work
Phage		
λp*chlC3*	*narX*$^+$ *narL*$^+$ *att*$^+$ *int*$^+$ *imm*^{21}	33
λVJS107	λp*chlC3* Δ(*narXL237*)::Km	8
λVJS109	λp*chlC3* Δ*narX238 narL*$^+$ *zch-2084*::Ω-Cm	8
λVJS110	λp*chlC3* Δ*narX242 narL*$^+$ *zch-2084*::Ω-Cm	8
λVJS119	λp*chlC3* Δ*narX238 narL503 zch-2084*::Ω-Cm	This work
λVJS121	λp*chlC3* Δ*narX238 narL504 zch-2084*::Ω-Cm	This work
λVJS124	λp*chlC3* Δ*narX238 narL502 zch-2084*::Ω-Cm	This work
λVJS125	λp*chlC3* Δ*narX238 narL505 zch-2084*::Ω-Cm	This work
λVJS126	λp*chlC3* Δ*narX238 narL506 zch-2084*::Ω-Cm	This work
λVJS128	λp*chlC3* Δ*narX242 narL508 zch-2084*::Ω-Cm	This work
λVJS130	λp*chlC3* Δ*narX242 narL507 zch-2084*::Ω-Cm	This work
λVJS134	λp*chlC3* Δ*narX238 narL509 zch-2084*::Ω-Cm	This work

[a] All strains are F^- and are λ^- except as indicated.
[b] CGSC, *E. coli* Genetic Stock Center; courtesy of B. J. Bachmann.

VJS882 [Φ(*narG-lacZ*)] and VJS1250 [Φ(*fdnG-lacZ*)]. After incubation at room temperature, the mixtures were plated on E medium (6) containing lactose (0.2 or 1%), tryptophan, and chloramphenicol and incubated anaerobically for several days. Tiny colonies above background growth were picked, and Lac^+ colonies were purified on MacConkey-lactose-chloramphenicol medium.

To determine whether the nitrate-independent Lac^+ phenotype was due to an alteration on the specialized transducing phage, genetic linkage of the Lac^+ phenotype to the specialized transducing phage was tested by using generalized P1 transduction with the marker *zbh-620*::Tn*10* (about 50% linked to λ *att* [36]). Tetracycline-resistant transductants were scored for Cm^r, carried on the specialized transducing phage, and Lac phenotype. Phage lysates were made from isolates in which the constitutive Lac^+ phenotype was linked to the λ phage. These lysates were used to transduce strains carrying Φ(*fdnG-lacZ*) and Φ(*frdA-lacZ*).

Genetic manipulation of *narL* lesions. Lesions in *narL* isolated on λ specialized transducing phage (Cm^r) were recombined onto plasmids for DNA sequence analysis. The phage were transduced into a strain carrying pVJS211 (Ap^r Km^r) and screened for single lysogens. We took advantage of the fact that this Cm^r marker, when present in single copy, conferred resistance to chloramphenicol at 25 μg/ml but when present on a multicopy plasmid conferred resistance to chloramphenicol at 200 μg/ml. The single lysogens were grown for several generations and then plated on chloramphenicol at 200 μg/ml to enrich for recombinants with the Cm^r marker on the plasmid. Pooled colonies were used to isolate plasmid DNA. Cm^r Ap^r Km^s transformants of VJS533 were isolated by selection and screening. These contained the desired recombinants, as determined by restriction analysis. Alterations in *narL* constructed by site-specific mutagenesis were recombined onto the λ specialized transducing phage λ VJS107. Our strategies and methods for transferring alleles between phage and plasmids by recombination are more fully described elsewhere (8).

All *narL* mutations were studied as single-copy lysogens in a variety of strain backgrounds. We previously found that multiple lysogens were resistant to chloramphenicol at 100 μg/ml or greater, whereas single lysogens grew poorly on this concentration of chloramphenicol (8). In this study, we verified single lysogens by testing their level of resistance to chloramphenicol.

Enzyme assays. Assays were carried out at room temperature, approximately 21°C. Cell pellets for β-galactosidase assays were suspended in 4 ml of Z buffer (22) and stored on ice. Activity was measured in $CHCl_3$-sodium dodecyl sulfate-permeabilized cells by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. Activities are expressed in terms of cell density (A_{600}), using the formula of Miller (22).

Nitrate reductase activity was measured in $CHCl_3$-sodium

TABLE 2. Nucleotide and amino acid changes of *narL* alleles

Allele	Nucleotide change			Amino acid change		
	Position[a]	From	To	Position[a]	From	To
narL502(D59N)	175	G	A	59	Asp	Asn
narL503(H15L)	44	A	T	15	His	Leu
narL504(D110K)	328	GAT	AAG	110	Asp	Lys
narL505(V88A)	263	T	C	88	Val	Ala
narL506(S80L)	239	C	T	80	Ser	Leu
narL507(S80P)	238	T	C	80	Ser	Pro
narL508(E112G)	335	A	G	112	Glu	Gly
narL509(T20I)	59	C	T	20	Thr	Ile

[a] Positions are numbered from the *narL* translation initiation site.

dodecyl sulfate-permeabilized cells by monitoring nitrite production as previously described (37). Activities are expressed in terms of cell density (A_{600}) (37).

Both nitrate reductase and β-galactosidase assays were performed on strains carrying Φ(*frdA-lacZ*). These cells were resuspended in 0.32 M potassium phosphate, pH 7.1 (37), and the β-galactosidase activity was measured in 0.5 volume of Z buffer and 0.5 volume of potassium phosphate buffer plus cell suspension. Control experiments established that the specific activity of β-galactosidase was unaltered by this difference in procedure.

All cultures were assayed in duplicate. Reported values are averaged from two independent experiments.

DNA sequence analysis. DNA sequencing was carried out by using the dideoxynucleotide chain termination method with modified T7 DNA polymerase (42) and [α-^{35}S]dATP labeling (2). Double-stranded plasmid DNA was used for sequencing (19). The complete *narL* sequence for each mutant was determined on at least one strand. Modified T7 DNA polymerase (Sequenase 2.0) and other sequencing reagents were from U.S. Biochemical, and [α-^{35}S]dATP was from Amersham Corp. (Arlington Heights, Ill.). Oligonucleotide primers were synthesized at the Oligonucleotide Synthesis Facility of the Cornell University Biotechnology Program.

RESULTS

Isolation of nitrate-independent *narL* mutations. We mutagenized λ VJS109 and λ VJS110, which carry Δ*narX238* *narL*$^+$ and Δ*narX242* *narL*$^+$, respectively, by passage through a *mutD5* strain, ES1578 (28, 30, 45). The mutagenized lysate was mixed with cultures of either VJS882 or VJS1250, which are both *narX*$^+$ *narL*$^+$ and carry Φ(*narG-lacZ*) and Φ(*fdnG-lacZ*) operon fusions, respectively. The mixtures were plated on minimal lactose-chloramphenicol medium lacking nitrate, and the plates were incubated anaerobically for several days. Tiny colonies above background growth were picked and purified on MacConkey-lactose medium. Lac$^+$ isolates were found only among lysogens of VJS1250. The efficiency of lysogeny was roughly 10^{-1}, and the frequency of Lac$^+$ colonies was approximately 10^{-5}/PFU.

Generalized transduction analysis using phage P1 was used to determine whether the mutations were linked to the λ specialized transducing phage. In most of the isolates tested, we found that the Lac$^+$ phenotype, conferred by the *narL* alterations, was linked to the λ specialized transducing phage. All of these mutants conferred a strong Lac$^+$ phenotype on VJS1250 on MacConkey-lactose medium both in the presence and in the absence of nitrate. Twenty-one isolates were generated by this screening procedure. Seven independent isolates were chosen for detailed analysis.

Sequence analysis of nitrate-independent *narL* mutations. Each of the alleles isolated by the foregoing procedure was recombined onto a plasmid as described in Materials and Methods. The resulting plasmids were subjected to DNA sequence analysis using oligonucleotide primers complementary to sequences in *narL*. The nucleotide sequence of the entire *narL* coding region was determined, and a single base substitution was found in each case. Of the seven mutations sequenced, five unique base changes were found, with the Val-88-to-Ala change isolated three times (Table 2 and Fig. 2).

Genetic analysis of *narL* mutations. Single lysogens of the λ specialized transducing phage carrying the *narL* mutations were constructed in a variety of strain backgrounds. We wished to determine the effect of each of the mutations on nitrate induction of *narGHJI* and Φ(*fdnG-lacZ*) expression and on nitrate repression of Φ(*frdA-lacZ*) expression. Each mutation was assayed in *narX*$^+$ *narL*::Ω, *narX*$^+$ *narL*$^+$ and Δ(*narXL*) strain backgrounds to test whether it was dominant or recessive to *narL*$^+$ and to examine the effect of *narX*$^+$.

Effect of *narL505*(V88A) on nitrate regulation. Of the five different constitutive mutations assayed, *narL505*(V88A) was the only one with a strong constitutive phenotype. Nitrate reductase activity after growth in the absence of nitrate was sharply elevated in all strains carrying *narL505*(V88A), with about 120, 80, and 190% of the wild-type-induced activity in *narX*$^+$ *narL*::Ω, *narX*$^+$ *narL*$^+$, and Δ(*narXL*) strains, respectively (Table 3). Overall, the phenotype of *narL505*(V88A) was not as strong for nitrate-independent expression of Φ(*fdnG-lacZ*). Expression of this fusion was 16, 11, and 100% of the wild-type-induced level in *narX*$^+$ *narL*::Ω, *narX*$^+$ *narL*$^+$, and Δ(*narXL*) strains, respectively (Table 3). Finally, *narL505*(V88A) had only a slight effect on expression of Φ(*frdA-lacZ*). Expression in *narX*$^+$ *narL*::Ω and *narX*$^+$ *narL*$^+$ strains was essentially wild type, while expression in a Δ(*narXL*) strain in the absence of nitrate was repressed to about 20% of the derepressed level (Table 3).

Additional *narL* mutations. Four weakly nitrate-independent mutations of *narL* were found, *narL506* to *-509*. The amino acid changes in these mutants are shown in Table 2 and Fig. 2. They conferred a maximum of two- to threefold nitrate independence on *narGHJI* expression, twofold independence on Φ(*fdnG-lacZ*) expression, and no significant nitrate-independent repression of Φ(*frdA-lacZ*) expression. *narL508*(E112G) conferred nitrate independence only in

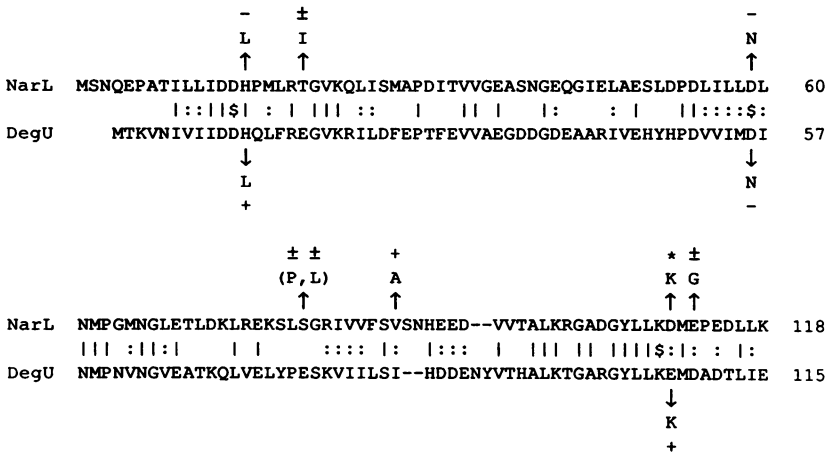

FIG. 2. Sequence alignment of the amino-terminal regions of NarL and DegU in the standard single-letter code. Arrows indicate residues changed in mutants and their relative phenotypes. Dashes in the sequence represent gaps in the alignment. Symbols: |, identical residues; :, analogous residues; $, invariant residues of response regulators; +, constitutive phenotype; ±, low-level constitutive phenotype; −, null phenotype; *, partially *narX*$^+$-dependent phenotype. Analogous residues are Arg-His-Lys, Asn-Gln, Asp-Glu, Ile-Val-Leu-Met, and Ser-Thr (modified from reference 7). The alignment is adapted from reference 38.

narX$^+$ *narL*::Ω strains, while each of the others conferred nitrate independence only in Δ(*narXL*) strains (data not shown).

We selected for constitutive alleles of *narL* in *narL*$^+$ strains, expecting that all of the constitutive alleles would be dominant to *narL*$^+$. However, these weakly constitutive alleles were fully recessive to *narL*$^+$ when assayed after growth in liquid cultures (data not shown). We do not understand the basis for these differences.

Construction of mutational changes in *narL*. Site-specific mutagenesis was used to construct three additional alterations in *narL*. Two changes were designed to mimic constitutive mutants of DegU, a response regulator in the same subclass as NarL (12, 38). The alterations in *narL* changed His-15 to Leu and Asp-110 to Lys.

The other alteration changed Asp-59 of NarL to Asn. In sequence alignment comparisons, this Asp residue is conserved in all response regulators (Fig. 2), and it has been shown to be the site of phosphorylation, and thereby activation, of CheY, OmpR, and VirG (15, 18, 27; reviewed in reference 40).

Analysis of site-specific *narL* mutations. All three site-specific *narL* mutations were studied as single-copy lysogens after recombination onto λ specialized transducing phage. Neither of the *narL* alterations that were analogous to constitutive alleles of *degU* conferred constitutive phenotypes. *narL503*(H15L) abolished nitrate induction of Φ(*fdnG-lacZ*) and nitrate repression of Φ(*frdA-lacZ*) in *narX*$^+$ *narL*::Ω strains (Table 4). (The residual threefold induction of Φ(*fdnG-lacZ*) in the presence of nitrate is also observed in *narL* null mutants carrying this fusion [1, 8].) *narL503*(H15L) was recessive to *narL*$^+$, as shown by the essentially normal nitrate regulation in *narX*$^+$ *narL*$^+$ strains (Table 4). This allele also abolished nitrate induction of

TABLE 3. Complementation analysis of *narL504*(D110K) and *narL505*(V88A)

narL allele[a]	Enzyme sp act[b]					
	narX$^+$ *narL*::Ω[c]		*narX*$^+$ *narL*$^+$[c]		Δ(*narXL*)[c]	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
narGHJI[d]						
narL$^+$	31	645	12	670	16	580
narL504(D110K)	58	780	10	565	6	160
narL505(V88A)	790	700	540	845	1,075	500
Φ(*fdnG-lacZ*)[e]						
narL$^+$	6	840	5	970	12	1,065
narL504(D110K)	6	640	5	980	6	220
narL505(V88A)	135	815	110	955	1,050	1,170
Φ(*frdA-lacZ*)[f]						
narL$^+$	56	4	52	4	47	4
narL504(D110K)	59	11	49	4	35	34
narL505(V88A)	44	4	50	4	10	4

[a] *narL* allele on the λΔ*narX* specialized transducing phage at the λ attachment site.
[b] Determined as described in Materials and Methods and expressed in arbitrary units. Cultures were grown anaerobically with or without nitrate as indicated.
[c] *narX* and *narL* alleles at the *nar* locus.
[d] Nitrate reductase enzyme activity from *narGHJI*.
[e] β-Galactosidase activity from a Φ(*fdnG-lacZ*) operon fusion.
[f] β-Galactosidase activity from a Φ(*frdA-lacZ*) operon fusion.

TABLE 4. Complementation analysis of *narL502*(D59N) and *narL503*(H15L)

narL allele[a]	β-Galactosidase sp act[b]			
	narX$^+$ *narL*::Ω[c]		*narX*$^+$ *narL*$^+$[c]	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
Φ(*fdn-lacZ*)[d]				
narL$^+$	5	1,010	5	925
narL502(D59N)	7	23	6	800
narL503(H15L)	6	25	5	870
Φ(*frd-lacZ*)[e]				
narL$^+$	53	2	50	2
narL502(D59N)	54	75	62	5
narL503(H15L)	44	72	51	2

[a] *narL* allele on the λΔ*narX* specialized transducing phage at the λ attachment site.
[b] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown anaerobically with or without nitrate as indicated.
[c] *narX* and *narL* alleles at the *nar* locus.
[d] β-Galactosidase activity from a Φ(*fdnG-lacZ*) operon fusion.
[e] β-Galactosidase activity from a Φ(*frdA-lacZ*) operon fusion.

Φ(*fdnG-lacZ*) in a Δ(*narXL*) strain background (data not shown).

narL504(D110K) functioned essentially as wild type for nitrate regulation in both *narX*$^+$ *narL*::Ω and *narX*$^+$ *narL*$^+$ backgrounds. Interestingly, *narL504*(D110K) appeared to be somewhat dependent on *narX*$^+$, as shown by the reduced nitrate regulation in Δ(*narXL*) strain backgrounds (Table 3).

narL502(D59N) abolished NarL-mediated nitrate regulation of Φ(*fdnG-lacZ*) and Φ(*frdA-lacZ*) expression in *narX*$^+$ *narL*::Ω strain backgrounds. This allele was recessive to wild-type *narL*, as shown by the essentially normal nitrate regulation in *narX*$^+$ *narL*$^+$ strain backgrounds (Table 4). This allele also abolished nitrate induction of Φ(*fdnG-lacZ*) in a Δ(*narXL*) strain background (data not shown).

Effect of molybdate on regulation by *narL505*(V88A). Limiting *E. coli* for molybdate prevents full induction of nitrate reductase and formate dehydrogenase-N synthesis and also prevents full repression of fumarate reductase synthesis (9, 13). We accomplished molybdate limitation by using a *chlD* strain, defective in molybdate uptake (29). In the presence of nitrate, with low or high molybdate, *narL505*(V88A) was indistinguishable from *narL*$^+$. Each induced expression of Φ(*fdnG-lacZ*) in limiting molybdate to a level of about 25 to 30% of the level in excess molybdate (Table 5). In the absence of nitrate, the *narL505*(V88A) strain expressed Φ(*fdnG-lacZ*) in limiting molybdate, but to a level slightly lower than that in the presence of nitrate. Excess molybdate allowed full induction in the *narL505*(V88A) strain in the absence of nitrate (Table 5).

TABLE 5. Effect of molybdate on Φ(*fdnG-lacZ*) regulation by *narL505*(V88A)

narL allele[a]	β-Galactosidase sp act[b]			
	0.05 μM MoO_4^{2-}		100 μM MoO_4^{2-}	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
narL$^+$	9	180	9	645
narL505(V88A)	92	195	720	785

[a] *narL* allele on the λΔ*narX* specialized transducing phage at the λ attachment site. The strains are Δ(*narXL*) at the *nar* locus.
[b] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown anaerobically with or without nitrate, and with low or high molybdate, as indicated.

DISCUSSION

Isolation of *narL* mutations. Our experiments were designed to explore the function of the *narL* gene product by using genetic analysis of mutants. We used a *mutD5* strain to mutagenize λ specialized transducing phage carrying *narL*$^+$ (28, 30, 45). We selected for *narL* mutations that conferred nitrate-independent expression of *narGHJI* or *fdnGHI*. We lysogenized *narX*$^+$ *narL*$^+$ strains carrying the Φ(*narG-lacZ*) or Φ(*fdnG-lacZ*) operon fusion with phage from the mutagenized lysates and plated the mixtures on minimal lactose medium lacking nitrate. We found Lac$^+$ isolates only in the strain carrying Φ(*fdnG-lacZ*). Each of the seven mutants characterized allowed sufficient expression from Φ(*fdnG-lacZ*) or Φ(*narG-lacZ*) to be dark red (Lac$^+$) on MacConkey-lactose medium in the absence of nitrate, while wild-type strains were white (Lac$^-$) on this medium. Three independent isolates had the same amino acid change, Val-88 to Ala, and a strong constitutive phenotype (Tables 2 and 3; Fig. 2). The other four mutants had a variety of amino acid changes, but each conferred a maximum of only two- to threefold-higher levels than wild type in the absence of nitrate (data not shown).

Analysis of *narL505*(V88A). The mutation isolated three times was a Val-88-to-Ala change in NarL (Fig. 2). The analogous residue of CheY, Ala-88, lies very near the acidic pocket which forms the site of protein phosphorylation (39; reviewed in reference 40). Interestingly, an Ala-to-Val change at the analogous position in Spo0A allows sporulation of *B. subtilis* in the absence of nutrient deprivation (25). This *spo0A*(A87V) allele is dependent on *spo0B*$^+$ and *spo0F*$^+$ for activity, both of which are proposed to encode parts of a phosphorelay leading to phosphorylation of Spo0A (4, 25). These comparisons imply that NarL505(V88A) may require phosphorylation for activity.

One of our Val-88-to-Ala isolates, NarL505(V88A), was subjected to thorough complementation analysis on a single-copy λ specialized transducing phage. The mutant was analyzed for nitrate regulation by measuring nitrate reductase expression from *narGHJI* and by measuring β-galactosidase expression from Φ(*fdnG-lacZ*) and Φ(*frdA-lacZ*) in *narX*$^+$ *narL*::Ω, *narX*$^+$ *narL*$^+$, and Δ(*narXL*) strains. The overall level of constitutivity with *narL505*(V88A) was highest for expression of *narGHJI*, intermediate for expression of Φ(*fdnG-lacZ*), and lowest for expression of Φ(*frdA-lacZ*) (Table 3). The differences in magnitude of the effects of *narL505*(V88A) suggests that these operons may have different sensitivities to NarL-mediated regulation, perhaps reflecting different affinities for NarL binding. We recently found that the phenotype of polar insertions in *narX* is due to decreased expression of *narL* (8). The sensitivity of each of these operons for NarL-mediated regulation is consistent with the previous finding that polar insertions in *narX* have the smallest effect on *narGHJI* induction, an intermediate effect on *fdnGHI* induction, and the greatest effect on *frdABCD* repression (1, 35, 37). We previously proposed that different conformations of NarL might be involved in activation and repression (38). A more economical hypothesis, suggested by our current results, is that different sensitivities (binding affinities) may account for differences in activation and repression.

The constitutive phenotype of *narL505*(V88A) was largely dominant to wild-type *narL*, as illustrated by its phenotypes

in *narX*$^+$ *narL*::Ω and *narX*$^+$ *narL*$^+$ strains (Table 3). The levels of constitutivity conferred by *narL505*(V88A) were always highest in *narX* strains (Table 3). This finding implies that *narX*$^+$ can have a negative effect on NarL.

If NarL505(V88A) is phosphorylation dependent, two models could explain the nitrate-independence of the encoding allele. The first is that this protein is a more efficient phosphoacceptor than is wild-type NarL. The phosphotransfer to NarL505(V88A) could either be from low-level kinase activity of NarX and NarQ in the absence of nitrate or from one or more of the other histidine protein kinases in *E. coli*. The second model is that phosphorylated NarL505(V88A) is less susceptible to dephosphorylation than is wild-type NarL. In this case, low-level kinase activity in the absence of nitrate would lead to an accumulation of phosphorylated NarL505(V88A). Further studies are necessary to explore these ideas.

Additional nitrate-independent *narL* mutations. The four low-level constitutive *narL* mutations isolated showed the same pattern as *narL505*(V88A) for nitrate-independent effects on the three operons examined, with the strongest effects on *narGHJI* expression, intermediate effects on Φ(*fdnG-lacZ*) expression, and virtually no effect on Φ(*frdA-lacZ*) expression (data not shown). This result further supports the hypothesis that these operons differ in their sensitivities to NarL-mediated regulation.

Analysis of *narL503*(H15L). We used site-specific mutagenesis to construct two alterations analogous to constitutive mutants of the DegU protein of *B. subtilis* (12). DegU is a response regulator in the same sequence subclass as NarL (38). Henner et al. analyzed several *degU* mutants isolated for overexpression of degradative exoenzymes. Two of the mutants have changes of His-12 to Leu and Glu-107 to Lys (12).

DegU(H12L) changes the residue following one of the three invariant residues of response regulators, corresponding to Asp-13 of CheY (Fig. 2). Asp-13 is proposed, on the basis of the three-dimensional structure of CheY, to participate in the formation of an acidic pocket involved in the phosphoacceptor chemistry of the protein (reviewed in reference 40). This region of DegU shares a high degree of sequence similarity with NarL (Fig. 2). However, the corresponding change, *narL503*(H15L), did not lead to constitutive activation and repression by NarL, but rather abolished its ability to regulate gene expression in response to nitrate. This inactive form of NarL was recessive to wild-type NarL, demonstrating that it is a null allele (Table 4).

After this report was submitted for publication, Dahl et al. published a paper demonstrating that the DegU(H12L) protein is not phosphorylated in vitro (5a). Surprisingly, DegU(H12L) depends on *degS*$^+$ for activity, implying that DegU may be activated by two mechanisms, one phosphorylation dependent and the other phosphorylation independent (5a). Thus, the observation that the analogous change in *narL*(H15L) conferred a null phenotype is explicable, if this mutant protein is not a substrate for kinase activity. This result also suggests that NarL may not share the phosphorylation-independent mechanism of activation suggested for DegU (5a).

Analysis of *narL504*(D110K). DegU(E107K) also changes the residue following one of the three invariant amino acids of response regulators, corresponding to Lys-109 of CheY (Fig. 2). This lysine residue is located near the acidic pocket in the three-dimensional structure of CheY, but its role in activation of response regulators is not known (39; reviewed in reference 40). The corresponding alteration, *narL504* (D110K), did not have a constitutive phenotype. Interestingly, it functioned essentially as wild type except in Δ(*narXL*) strains, suggesting that it is somewhat dependent on NarX for function (Table 4). This finding further suggests that NarX is involved in NarL-mediated nitrate regulation. Based on the current model for NarL-mediated nitrate regulation, the *narL504*(D110K) allele may be defective in a positive interaction with NarQ (Fig. 1).

Analysis of *narL502*(D59N). The residue corresponding to Asp-57 of CheY is invariant in response regulators and has been shown to be the site of protein phosphorylation in CheY, OmpR, and VirG (15, 18, 27; reviewed in reference 40). Since NarL-mediated nitrate regulation does not require NarX, the most obvious candidate histidine protein kinase (8), it was unclear whether NarL functions like other response regulators. We changed Asp-59 of NarL to Asn (*narL502*) to determine whether protein phosphorylation is involved in activation of NarL.

narL502(D59N) was incapable of activating expression of Φ(*fdnG-lacZ*) or repressing expression of Φ(*frdA-lacZ*) in response to nitrate, and it was recessive to wild-type *narL*, demonstrating that it is a null allele (Table 4). These results are consistent with the hypothesis that activation of NarL in the presence of nitrate requires phosphorylation of Asp-59.

Does gene regulation in *narL505*(V88A) strains require molybdate? *narGHJI* and *fdnGHI* induction and *frdABCD* repression require molybdate in addition to *narL*$^+$, anaerobiosis, and nitrate (reviewed in reference 34). One question is whether this molybdate regulation acts through NarL, as has been suggested (17), or whether molybdate regulation is mediated by a separate regulatory system.

We wished to determine whether the *narL505*(V88A) allele, which conferred a nitrate-independent constitutive phenotype, also conferred a molybdate-independent constitutive phenotype. In practice, molybdate limitation is achieved by using strains with lesions in *chlD*, a gene required for molybdate uptake (29). *chlD* strains are limited for molybdate, as measured by molybdoenzyme activities and gene regulation. Provision of relatively high levels of molybdate in the culture medium phenotypically suppresses this defect (9, 10, 13; reviewed in reference 34).

We examined the regulation of Φ(*fdnG-lacZ*) expression in *narL*$^+$ or *narL505*(V88A) Δ*narX chlD*::Tn*10* strains grown with limiting or with excess molybdate. The *narL*$^+$ strain exhibited only about 30% of normal induction when grown in limiting molybdate. This residual induction probably reflects incomplete molybdate limitation under these growth conditions.

Expression of Φ(*fdnG-lacZ*) in the *narL505*(V88A) strain was completely independent of nitrate in the molybdenum-excess culture, as expected (Tables 4 and 5). However, nitrate-induced Φ(*fdnG-lacZ*) expression in the molybdate-limited culture was not enhanced by *narL505*(V88A) (Table 5). Thus, we conclude that this allele, while conferring full nitrate independence in this strain background, remained fully dependent on molybdate for the nitrate-induced level of Φ(*fdnG-lacZ*) expression. If nitrate and molybdate both act through NarL by the identical mechanism, one would expect nitrate-independent alleles such as *narL505*(V88A) to confer molybdate independence as well.

Conclusions are complicated, however, by examining molybdate-limited Φ(*fdnG-lacZ*) expression in the absence of nitrate (Table 5). While the *narL*$^+$ strain exhibited the expected low level of Φ(*fdnG-lacZ*) expression, the *narL505*(V88A) strain exhibited approximately 50% of the molybdate-limited, nitrate-induced level. Thus, at least some

measure of the constitutivity conferred by *narL505*(V88A) was retained in the molybdate-limited cultures.

We believe these data suggest that molybdate-mediated and nitrate-mediated regulation occur by separate mechanisms. However, they do not exclude the possibility that molybdate and nitrate both act through NarL. The actual mechanism of molybdate-mediated gene regulation remains a challenge for future studies.

ACKNOWLEDGMENTS

We thank B. J. Bachmann for the culture of strain ES1578, and we thank L. A. Collins, S. Kustu, J. B. Stock, and D. S. Weiss for helpful discussions. We are grateful to L. A. Collins for constructing *narL249*::Ω. T. Thannhauser synthesized oligonucleotides.

This study was supported by Public Health Service grant GM36877 from the National Institute of General Medical Sciences.

REFERENCES

1. **Berg, B. L., and V. Stewart.** 1990. Structural genes for nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. Genetics **125**:691–702.
2. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
3. **Brewer, J. H., and D. L. Allgeier.** 1966. Safe self-contained carbon dioxide-hydrogen anaerobic system. Appl. Microbiol. **14**:985–988.
4. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. Initiation of sporulation in *Bacillus subtilis* is controlled by a multicomponent phosphorelay. Cell **64**:545–552.
5. **Collins, L. A., S. M. Egan, and V. Stewart.** Unpublished data.

5a. **Dahl, M. K., T. Msadek, F. Kunst, and G. Rapoport.** 1991. Mutational analysis of the *Bacillus subtilis* DegU regulator and its phosphorylation by the DegS protein kinase. J. Bacteriol. **173**:2539–2547.

6. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. A manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Doolittle, R. F.** 1987. Of urfs and orfs. A primer on how to analyze derived amino acid sequences. University Science Books, Mill Valley, Calif.
8. **Egan, S. M., and V. Stewart.** 1990. Nitrate regulation of anaerobic respiratory gene expression in *narX* deletion mutants of *Escherichia coli* K-12. J. Bacteriol. **172**:5020–5029.
9. **Giordano, G., B. A. Haddock, and D. H. Boxer.** 1980. Molybdenum limited growth achieved either phenotypically or genotypically and its effect on the synthesis of formate dehydrogenase and nitrate reductase by *Escherichia coli* K12. FEMS Microbiol. Lett. **8**:229–235.
10. **Glaser, J. H., and J. A. DeMoss.** 1971. Phenotypic restoration by molybdate of nitrate reductase activity in *chlD* mutants of *Escherichia coli*. J. Bacteriol. **108**:854–860.
11. **Gunsalus, R. P., L. V. Kalman, and R. R. Stewart.** 1989. Nucleotide sequence of the *narL* gene that is involved in global regulation of nitrate controlled respiratory genes of *Escherichia coli*. Nucleic Acids Res. **17**:1965–1975.
12. **Henner, D. J., M. Yang, and E. Ferrari.** 1988. Localization of *Bacillus subtilis sacU*(Hy) mutations to two linked genes with similarities to the conserved procaryotic family of two-component signalling systems. J. Bacteriol. **170**:5102–5109.
13. **Iuchi, S., and E. C. C. Lin.** 1987. A molybdenum effector of fumarate reductase repression and nitrate reductase induction in *Escherichia coli*. J. Bacteriol. **169**:3720–3725.
14. **Iuchi, S., and E. C. C. Lin.** 1987. The *narL* gene product activates the nitrate reductase operon and represses the fumarate reductase and trimethylamine *N*-oxide reductase operons in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84**:3901–3905.
15. **Jin, S. G., R. K. Prusti, T. Roitsch, R. G. Ankenbauer, and E. W. Nester.** 1990. Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role in biological activity of VirG. J. Bacteriol. **172**:4945–4950.
16. **Kalman, L. V., and R. P. Gunsalus.** 1989. Identification of a second gene involved in global regulation of fumarate reductase and other nitrate-controlled genes for anaerobic respiration in *Escherichia coli*. J. Bacteriol. **171**:3810–3816.
17. **Kalman, L. V., and R. P. Gunsalus.** 1990. Nitrate-independent and molybdenum-independent signal transduction mutations in *narX* that alter regulation of anaerobic respiratory genes in *Escherichia coli*. J. Bacteriol. **172**:7049–7056.
18. **Kanamaru, K., H. Aiba, and T. Mizuno.** 1990. Transmembrane signal transduction and osmoregulation in *Escherichia coli*. 1. Analysis by site-directed mutagenesis of the amino acid residues involved in phosphotransfer between the two regulatory components, EnvZ and OmpR. J. Biochem. **108**:483–487.
19. **Kraft, R., J. Tardiff, K. S. Krauter, and L. A. Leinwand.** 1988. Using mini-prep plasmid DNA for sequencing double stranded templates with Sequenase™. BioTechniques **6**:544–547.
20. **Lee, J. H., J. C. Wendt, and K. T. Shanmugam.** 1990. Identification of a new gene, *molR*, essential for utilization of molybdate by *Escherichia coli*. J. Bacteriol. **172**:2079–2087.
21. **Lin, E. C. C., and D. R. Kuritzkes.** 1987. Pathways for anaerobic electron transport, p. 201–221. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
22. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Nohno, T., S. Noji, S. Taniguchi, and T. Saito.** 1989. The *narX* and *narL* genes encoding the nitrate-sensing regulators of *Escherichia coli* are homologous to a family of prokaryotic two-component regulatory genes. Nucleic Acids Res. **17**:2947–2957.
24. **Noji, S., T. Nohno, T. Saito, and S. Taniguchi.** 1989. The *narK* gene product participates in nitrate transport induced in *Escherichia coli* nitrate-respiring cells. FEBS Lett. **252**:139–143.
25. **Olmedo, G., E. G. Ninfa, J. Stock, and P. Youngman.** 1990. Novel mutations that alter the regulation of sporulation in *Bacillus subtilis*. Evidence that phosphorylation of regulatory protein Spo0A controls the initiation of sporulation. J. Mol. Biol. **215**:359–372.
26. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29**:303–313.
27. **Sanders, D. A., B. L. Gillececastro, A. M. Stock, A. L. Burlingame, and D. E. Koshland.** 1989. Identification of the site of phosphorylation of the chemotaxis response regulator protein, CheY. J. Biol. Chem. **264**:21770–21778.
28. **Schaaper, R. M.** 1988. Mechanisms of mutagenesis in the *Escherichia coli* mutator *mutD5*: role of DNA mismatch repair. Proc. Natl. Acad. Sci. USA **85**:8126–8130.
29. **Scott, D., and N. K. Amy.** 1989. Molybdenum accumulation in *chlD* mutants of *Escherichia coli*. J. Bacteriol. **171**:1284–1287.
30. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Spiro, S., and J. R. Guest.** 1990. FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. FEMS Microbiol. Rev. **75**:399–428.
32. **Stewart, G. S. A. B., S. Lubinsky-Mink, C. G. Jackson, A. Kassel, and J. Kuhn.** 1986. pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression. Plasmid **15**:172–181.
33. **Stewart, V.** 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. J. Bacteriol. **151**:1325–1330.
34. **Stewart, V.** 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. Microbiol. Rev. **52**:190–232.
35. **Stewart, V., and B. L. Berg.** 1988. Influence of *nar* (nitrate reductase) genes on nitrate inhibition of formate-hydrogen lyase and fumarate reductase gene expression in *Escherichia coli* K-12. J. Bacteriol. **170**:4437–4444.
36. **Stewart, V., and C. H. MacGregor.** 1982. Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. J. Bacteriol. **151**:788–799.

37. **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.
38. **Stewart, V., J. Parales, Jr., and S. M. Merkel.** 1989. Structure of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. J. Bacteriol. **171**:2229–2234.
39. **Stock, A. M., J. M. Mottonen, J. B. Stock, and C. E. Schutt.** 1989. Three-dimensional structure of CheY, the response regulator of bacterial chemotaxis. Nature (London) **337**:745–749.
40. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53**:450–490.
41. **Stout, V., A. Torres-Cabassa, M. R. Maurizi, D. Gutnick, and S. Gottesman.** 1991. RcsA, an unstable positive regulator of capsular polysaccharide synthesis. J. Bacteriol. **173**:1738–1747.
42. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA **84**:4767–4771.
43. **Vandeyar, M. A., M. P. Weiner, C. J. Hutton, and C. A. Batt.** 1988. A simple and rapid method for the selection of oligodeoxynucleotide-directed mutants. Gene **65**:129–133.
44. **Winans, S. C., S. J. Elledge, J. H. Krueger, and G. C. Walker.** 1985. Site-directed insertion and deletion mutagenesis with cloned fragments in *Escherichia coli*. J. Bacteriol. **161**:1219–1221.
45. **Wu, T. H., C. H. Clarke, and M. G. Marinus.** 1990. Specificity of *Escherichia coli mutD* and *mutL* mutator strains. Gene **87**:1–5.
46. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.