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Requirement of Fnr and NarL Functions for Nitrate Reductase Expression in *Escherichia coli* K-12

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I used a *chlC-lac* operon fusion to study regulatory mutations which affect nitrate reductase expression in *Escherichia coli*. A NarL$^-$ mutant apparently lacks a nitrate-specific positive regulatory component. Furthermore, an *fnr* (*nirR*) mutation prevented enzyme induction under any conditions. These data are consistent with a two-step, positive control model for nitrate reductase regulation.

Escherichia coli uses nitrate as a terminal electron acceptor during anaerobic growth. The final enzyme in this respiration, nitrate reductase, is a membrane-bound complex of three polypeptides. Subunit A may contain the active site, but the function of subunit B is unknown (31). Subunit C is cytochrome b_{556}^{NR} (18). The enzyme also contains molybdenum cofactor and nonheme iron (31).

Many genetic studies of nitrate reductase have used chlorate-resistant (Chl^r) mutants, designated *chlA-E* and *chlG* (1). Most of these are pleiotropic and are apparently defective in molybdenum cofactor function (12, 15, 17, 20, 37). Pleiotropic effects (i.e., loss of molybdo-enzymes) are less pronounced in *chlC* strains. These mutants seem to be specifically defective in nitrate reductase (20, 21, 37). DeMoss (16) has isolated *chlC* mutants which have thermolabile nitrate reductase in vitro, and MacGregor (30) has described a single *chlC* mutant whose nitrate reductase is unstable in vivo. Thus, the *chlC* locus probably includes structural genes for nitrate reductase polypeptides.

We have identified four phenotypic classes of *chlC* insertion mutants, and we have proposed that individual genes in this locus be designated *nar* (37). NarG$^-$ and NarI$^-$ mutants may have lesions in the structural genes for nitrate reductase subunits A and C, respectively. These genes probably comprise a "nitrate reductase operon" with an order of promoter-*narG-narI* (5, 37; designated *chlCI* in ref. 5). A single mutant termed NarL$^-$ has a nitrate reductase activity which is not induced by nitrate. However, this strain retains normal oxygen regulation of activity. The NarK$^-$ defect is not known. Others have described mutants, termed *fnr*, *nirA*, or *nirR*, which have very low levels of several anaerobic respiratory activities (11, 27, 34).

I report here a strain which has an operon fusion of the structural gene for β-galactosidase, *lacZ*, to the promoter which controls the expression of the nitrate reductase operon. I have used this strain to study the effects that these possible regulatory mutations have on nitrate reductase expression.

MATERIALS AND METHODS

Strains. Bacterial and bacteriophage strains are shown in Table 1.

Media. MacConkey nitrate medium (2) aids in differentiating *chl*, *fnr*, and various classes of *nar* mutants (37). MNT medium (37) allows the detection of mutants defective in nitrate repression of tertiary amine *N*-oxide reductase expression. PN medium permits the selection of *chl*$^+$, *fnr*$^+$, and *nar*$^+$ transductants (37). Anaerobic liquid cultures were grown in basal anaerobic growth medium (29) supplemented with 1 μM $Na_2MoO_4 \cdot 2H_2O$–1 μM Na_2SeO_3–0.04% Proteose peptone (Difco Laboratories). LB, minimal A, and other routine media are described by Miller (33). Ampicillin was used at 25 μg/ml, and tetracycline was used at 15 μg/ml.

Culture conditions. Liquid cultures were grown anaerobically by continuously bubbling 95% N_2–5% CO_2 through the medium. Aerobic liquid cultures for enzyme assays were grown in 10 ml of minimal succinate medium in 300-ml baffle-bottom flasks which were shaken vigorously on a Gyrotory water bath shaker. These cultures were harvested in early log phase by pouring them through ice into cold containers, to prevent adaptation to anaerobiosis. Plates were incubated anaerobically in Brewer Anaerobic Jars (Becton, Dickinson & Co.) under an atmosphere of H_2 (37).

Genetic methods. (i) Isolation of *nar*::Mu d1 mutants. Samples of a saturated culture of RK4353 were mixed with a Mu d1 lysate for a multiplicity of infection of roughly 0.2. After 20 min of incubation at 30°C, dilutions of these mixtures were plated on MNT-ampicillin medium (9). These plates were incubated anaerobically overnight at 30°C, and nine independent

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

Strain	Genotype	Source
E. coli strains		
MC4100	*ΔlacU169 araD139 rpsL*	(8)
RK4353	as MC4100 but *gyrA non*	R. Kadner
Derivatives of RK4353		
RK4746	*trp*	This study
RK4748	*pyrF*	This study
RK4922	*zcg-622*::Tn*10* (between *hemA* and *chlC*)	(37)
RK4942	*zcj-630*::Tn*10* (near *fnr*)	This study
RK4943	*zcj-637*::Tn*10* (between *pyrF* and *fnr*)	This study
RK5212	*chlE209*::Mu *cts fnr-250*	(37)
RK5264	*nar-201*::Tn*10* (NarG$^-$)	(37)
RK5265	*nar-202*::Tn*10* (NarG$^-$)	(37)
RK5266	*nar-203*::Tn*10* (NarK$^-$)	(37)
RK5268	*nar-205*::Tn*10* (NarG$^-$)	(37)
RK5269	*nar-206*::Tn*10* (NarG$^-$)	(37)
RK5272	*nar-209*::Tn*10* (NarG$^-$)	(37)
RK5273	*nar-210*::Tn*10* (NarG$^-$)	(37)
RK5277	*nar-214*::Tn*10* (NarG$^-$)	(37)
RK5278	*nar-215*::Tn*10* (NarL$^-$)	(37)
RK5280	*nar-215*::Tn*10 recA* λ NEM540	This study
RK5281	*nar-215*::Tn*10 recA* λ p*chlC3*	This study
RK5282	*nar-216*::Mu d1 (NarG$^-$)	This study
RK5284	as RK5282 but λp1(209) Δ(Mu) Φ(*nar-lac*)*218*	This study
Derivatives of RK5284		This study
RK5285	*zcg-622*::Tn*10*	
RK5286	*nar-203*::Tn*10* (NarK$^-$)	
RK5287	*nar-215*::Tn*10* (NarL$^-$)	
RK5288	*fnr-250 zcj-637*::Tn*10*	
RK5289	*nar-215*::Tn*10 recA* λ NEM540	
RK5290	*nar-215*::Tn*10 recA* λ p*chlC3*	
Phage strains		
P1 *kc*		R. Kadner
Mu d1	Mu *cts* d1(Ap^r *lac*)	(9)[a]
λp1(209)	*lacYZO'-ΔW209-trp'AB'*::(+Mu)	(8)[b]
λ pΦ(*nar-lac*)*1*	as λp1(209) but Φ(*nar-lac*)*1*	This study
λ pΦ(*nar-lac*)*9*	as λp1(209) but Φ(*nar-lac*)*9*	This study
λ NEM540	*imm*^{21}	(6)[c]
λ p*chlC3*	as λ NEM540 but *chlC3*	This study
λgt7-*ara6*	*cI b522 nin-5 ara6*	(13)[d]

[a] Courtesy of M. Casadaban.
[b] R. Kadner collection.
[c] Courtesy of M. Smith.
[d] Courtesy of R. Davis.

light-colored isolates were purified by single-colony isolation on MacConkey nitrate-ampicillin medium.

(ii) Isolation of λ pΦ(*nar-lac*). Strain RK5282 was swabbed onto LB agar, and drops of a lysate of λp1(209) were spotted on and allowed to dry. Samples from the centers of the resulting lysis zones were streaked directly onto LB plates that had been spread with 10^9 PFU of λgt7-*ara6*. λ-Resistant colonies were purified on LB and tested for λ immunity, arabinose phenotype (Ara), and Mu d1 lysogeny. λ-Immune, Ara$^-$, Mu-immune clones were streaked onto LB plates and incubated at 42°C overnight. Many temperature-resistant survivors retained all of the parental phenotypes except for Mu immunity and ampicillin resistance (25, 26). Several such segregants were treated with UV light to prepare phage stocks (33). Nine independent Lac$^+$ transducing phage lines were retained for further study (4, 8, 25).

(iii) Selection for *nar*$^+$ transduction by λ derivatives. Saturated cultures (0.1 ml each) of *nar* strains were mixed with 0.1 ml of a λ stock dilution. The mixtures were incubated for 15 min, and each was spread on a separate PN plate. I also observed that anaerobiosis promotes lytic growth of λ (32). I took two steps to minimize lysis: I incubated the plates aerobically for 3 to 6 h before their overnight anaerobic incubation, and

I incorporated 1.25 mM $Na_4P_2O_7$ into the medium to reduce phage adsorption.

Enzyme assays. Crude extracts were prepared by breaking washed cells in a French pressure cell (Aminco), with subsequent removal of unbroken cells by sedimentation at 3,000 × *g* for 5 min. MacGregor et al. (31) describe the method for measuring reduced methyl viologen-linked nitrate reductase activity. Miller (33) describes the β-galactosidase assay, and Pugsley and Schnaitman (35) describe the procedure to measure protein concentrations.

RESULTS

***fnr* mutations.** We fortuitously isolated several *fnr chl* double mutants during the course of isolating *chl* mutants (37). I crossed eight of these strains to *chl*$^+$ to compare their Fnr phenotypes. I chose *fnr-250* as a typical mutation for later studies.

Characteristically, *fnr* strains have very reduced activities of anaerobic respiratory enzymes. I used differential or selective media to examine the presence of formate hydrogenlyase (21) and nitrite (34), nitrate, fumarate, and tertiary amine *N*-oxide (37) reductases. All of the *fnr* strains I tested appeared to lack all of these activities. In addition, all of these mutants were chlorate-sensitive (27).

Generalized transduction studies show that the *fnr* locus is roughly 10% linked to *pyrF* and less than 5% linked to *trp* (27). I crossed RK5212 (*fnr-250*) with RK4746 (*trp*) and RK4748 (*pyrF*) via P1-mediated transduction (33) and scored prototrophic recombinants for inheritance of *fnr*. Of 100 *pyrF*$^+$ recombinants, 12 were Fnr$^-$, whereas none of the 100 *trp*$^+$ recombinants was Fnr$^-$. I also isolated several Tn*10* insertions adjacent to *fnr-250* and used one of these (*zcj-630*::Tn*10*) to quickly map other independent *fnr* lesions. All eight mutations I examined had over 90% linkage to this insertion (15, 24, 37).

***nar*::Mu d1 mutants.** We have recently described our use of MNT medium to specifically isolate *chlC* (*nar*) mutants (37). I recovered three NarG$^-$, two NarI$^-$, and four NarK$^-$ independent mutants after mutagenesis with Mu d1. Two of these strains were phenotypically Lac$^+$ only when grown anaerobically in the presence of nitrate. I designated the mutations in these two strains as *nar-216*::Mu d1 (NarG$^-$), and *nar-217*::Mu d1 (NarI$^-$).

I isolated a strain termed RK4920 which carries a Tn*10* insertion between *chlC* and *hemA* (21, 24, 37). I used this strain as a donor in P1-mediated transduction crosses and found that both of the *nar*::Mu d1 mutations showed approximately 50% linkage to this insertion. This linkage value is characteristic for all *nar* mutations that I have tested.

Isolation of λ pΦ(*nar-lac*). I used the method of Komeda and Iino (26) to replace Mu sequences with a λ prophage at the site of the *nar-216*::Mu d1 insertion. I used two such segregants to isolate independent λ transducing phage lines, λ pΦ(*nar-lac*)*1* (isolated from strain RK5284) and λ pΦ(*nar-lac*)*9*. Both of these lines gave rise to lysogens which retained the parental Φ(*nar-lac*) regulation of β-galactosidase.

I also observed anomolous behavior of the λ pΦ transducing phage (25). Most of the RK4353 lysogens derived from these phage were either Lac$^-$ or had a constitutive Lac phenotype. The presence of a λ *imm*^{21} helper prophage had little effect on lysogen formation. However, some lysogens were Lac$^+$ only when grown anaerobically in the presence of nitrate. This mode of regulation was stably inherited in these lysogens.

Berman and Beckwith (4) have described a general method to test candidate operon fusion transducing phage. They reasoned that phage which carry a given fusion should recombine with certain polar mutations in the gene of interest. The resulting wild-type recombinants are Lac$^-$, since the polar mutation now blocks transcription from the operon fusion promoter. Both of the λ pΦ(*nar-lac*) transducing phage recombined with *nar*::Tn*10* mutations to yield *nar*$^+$ transductants (Table 2). Roughly 25% of the transductants remained Lac$^+$. This phenomenon has been observed by others and is presumed to result from dilysogen formation (14).

Regulation of Φ(*nar-lac*). Showe and DeMoss (36) found that induction of nitrate reductase is prevented in the presence of oxygen, occurs to a low level in the absence of oxygen, and reaches maximum levels in the presence of nitrate. Other groups (10, 19) have shown that β-galactosidase activity is regulated in an analogous manner in Φ(*chlC-lac*) strains. β-galactosidase activity in strain RK5285, Φ(*nar-lac*), showed the same two-step induction pattern (Table 3).

To study the effects of possible regulatory mutations, I constructed *nar* and *fnr* derivatives of the operon fusion strain RK5284. Regulation of β-galactosidase activity in these strains paralleled that of nitrate reductase activity in the parental mutant strains (37; Table 3). The NarK$^-$ lesion (in strain RK5286) had little obvious effect on regulation. The NarL$^-$ derivative (RK5287) showed normal oxygen regulation, but enzyme activity failed to be further induced by nitrate. The presence of the *fnr* mutation (RK5288) prevented induction under any conditions.

NarL$^-$. A phage λ-hybrid pool formed from RK4353 DNA was generously provided by Barbara Mann (13). I used *recA* derivatives of representative *nar* strains to screen this pool for specialized transducing phage carrying parts of

TABLE 2. Transduction of independent *nar*::Tn*10* mutants to *nar*$^+$, using λ pΦ(*nar-lac*) transducing phage[a]

Strain	No. of transductants	
	λ pΦ(*nar-lac*)*1*	λ pΦ(*nar-lac*)*9*
RK5264	5	1
RK5265	>50[b]	>50[b]
RK5268	0	0
RK5269	4	3
RK5272	9	0
RK5273	2	0
RK5277	0	0

[a] Transduction was performed as described in the text. Each transductant was purified by single colony isolation on MacConkey nitrate medium before being tested for its Lac and λ immunity phenotypes.

[b] A total of 24 transductants were purified and tested.

the *chlC* locus. An isolate designated λ p*chlC3* complemented the NarL$^-$ mutation *nar-215*::Tn*10*. I used this phage to prepare lysogens of various *recA nar* strains. None of the other *nar* mutations I tested was complemented by λ p*chlC3*. In addition, λ p*chlC3* failed to recombine with any NarG$^-$ or NarI$^-$ mutations, as assayed by spot testing on PN medium. In all of these experiments, λ p*chlC3* showed strong complementation of the NarL$^-$ mutation.

I prepared lysogens of a *recA* NarL$^-$ strain, using both λ p*chlC3* (RK5281) and the parental phage λNEM540 (RK5280). Table 4 shows the reduced methyl viologen-linked nitrate reductase activities in both of these strains after anaerobic growth in the presence or absence of nitrate. λ p*chlC3* complemented the NarL$^-$ mutation and thus allowed wild-type levels of enzyme induction by nitrate. The same observation held true for regulation of Φ(*nar-lac*). β-Galactosidase activity was induced by nitrate only in the λ p*chlC3* lysogen (RK5290) of a *recA* Φ(*nar-lac*) NarL$^-$ strain (Table 4). β-Galactosidase activity was not induced by nitrate in the λ NEM540 lysogen (RK5289).

We have described our use of MacConkey nitrate medium (2, 37) to differentiate classes of *nar* mutants. I have found this differentiation to be reliable and nearly unambiguous. On this medium, NarL$^-$ strains all formed characteristic medium-sized, dark red colonies. Likewise, wild-type colonies were large and salmon colored, whereas NarG$^-$ colonies were small and pale pink. One expects that NarG$^-$ (lack of nitrate reductase) would be phenotypically dominant to NarL$^-$ (low levels of nitrate reductase activity). In fact, all of the Φ(*nar-lac*) (i.e., NarG$^-$) NarL$^-$ double mutants I constructed during this work had the characteristic NarL$^-$ phenotype on MacConkey nitrate medium. This phenotype is not due to nitrate reductase activity; strains RK5289 and RK5290 had only NarG$^-$ levels of nitrate reductase activity (Table 4). When lysogenized with λ p*chlC3*, the NarG$^-$ NarL$^-$ strain (RK5290) was phenotypically NarG$^-$ on MacConkey nitrate medium.

DISCUSSION

Showe and DeMoss (36) examined the regulation of nitrate reductase formation in *E. coli*. They showed that aerobic cultures have extremely low levels of activity, whereas anaerobic cultures have a significant activity. Addition of nitrate to an anaerobic culture further induces nitrate reductase activity some 20-fold. Nitrate has no effect on the activity in an aerobic culture. These workers suggested that this two-step induction is a consequence of two separate regulatory signals.

The general method of operon fusion has proven to be invaluable for studying bacterial gene expression. The regulation of *lac* expression is thought to provide a specific measure for transcriptional regulation of a given operon. Thus, operon fusions allow one to detect and analyze regulatory mutations (4, 8, 9).

Several groups have reported *chlC-lac* operon fusions (10, 19; this work). In at least two cases, β-galactosidase activity was subject to two-step regulation by oxygen and nitrate (10; this work). Thus, the principal controls of nitrate reductase expression act at the level of transcription.

Kaprálek et al. (23) concluded that oxygen inhibits nitrate reductase expression at two levels. Oxygen prevents transcription of nitrate reductase genes and also interferes with a post-transcriptional step (perhaps assembly). β-Galactosidase activity was higher than expected in the aerobic operon fusion culture, considering the very low aerobic level of nitrate reductase activity (36, 37). Perhaps oxygen inactivation of nitrate reductase activity accounts for this discrepancy. I observed comparable magnitudes of

TABLE 3. β-Galactosidase activities of Φ(*nar-lac*) derivatives grown aerobically, anaerobically, and anaerobically with nitrate

Strain	Class	μmol of ONP[a] per min/mg of protein		
		$+O_2$	$-O_2$	$-O_2$, $+NO_3^-$
RK5285	WT[b]	0.0052	0.012	0.14
RK5286	NarK$^-$	0.0046	0.0092	0.12
RK5287	NarL$^-$	0.0050	0.0084	0.0075
RK5288	Fnr$^-$	0.0053	0.0033	0.0033

[a] ONP, *o*-Nitrophenol.

[b] WT, Wild type.

TABLE 4. Complementation of *nar-215*::Tn*10* (NarL$^-$) by λ p*chlC3* during anaerobic growth with or without nitrate

Strain	Class[a]	MVH-nitrate reductase (μmol of NO_2^- per min/mg of protein)		β-Galactosidase (μmol of ONP per min/mg of protein)	
		$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
RK5280	λ NEM540	0.077	0.075	ND[b]	ND
RK5281	λ p*chlC3*	0.093	1.4	ND	ND
RK5289	Φ(*nar-lac*) λ NEM540	<0.001	<0.001	0.0015	0.0016
RK5290	Φ(*nar-lac*) λ p*chlC3*	<0.001	<0.001	0.0020	0.083

[a] Each strain is *nar-215*::Tn*10 recA*.
[b] ND, Not determined.

nitrate induction in anaerobic cultures for both nitrate reductase and β-galactosidase activities.

I present genetic evidence to support the two-step model for induction of nitrate reductase activity. The *fnr* mutation resulted in a complete lack of induction, whereas the NarL$^-$ mutant lacked only the second, nitrate-specific step of induction. These two mutations together can account for the full range of nitrate reductase induction found in wild-type *E. coli*. In addition, a specialized transducing phage (λ p*chlC3*) fully complemented the NarL$^-$ defect in *trans* with respect to both nitrate reductase and β-galactosidase activities.

The NarL$^-$ mutation resulted from the integration of a highly polar insertion element (24). Thus, the putative activator protein could be encoded by a gene promoter distal to the *nar-215*::Tn*10* mutation. Indeed, the NarL$^-$ phenotype appeared to be complex. I have not identified other components that are influenced by the NarL$^-$ mutation.

Several groups (11, 27, 34, 37) have isolated mutations termed *fnr*, *nirA*, or *nirR*, which result in very low levels of anaerobic respiratory enzymes. One proposal (11, 34) is that this locus encodes a positive regulatory protein which activates the transcription of anaerobic respiratory enzyme structural genes. The data in Table 3 are consistent with and provide further support for this hypothesis. These data do not obviate other possible models (10).

These results may be taken as a first indication that nitrate reductase expression is subject to positive regulation (3, 7, 22, 28). Both the Fnr and NarL functions are required for maximum induction. This is formally analogous to arabinose (*ara*) gene regulation, which is subject to both pathway-specific (AraC) and "global" (catabolite activation) forms of positive control (28).

We previously reported that NarK$^-$ strains appeared to be wild type with respect to physiological nitrate reductase activity. These strains were isolated as being derepressed for tertiary amine *N*-oxide reductase activity in the presence of nitrate (37). The NarK$^-$ mutation had little effect on the regulation of nitrate reductase expression. In addition, on MacConkey nitrate medium, NarK$^-$ was phenotypically recessive to NarG$^-$. The possible involvement of NarK function(s) in the regulation of other anaerobic enzymes remains unproven.

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