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The *narL* gene product activates the nitrate reductase operon and represses the fumarate reductase and trimethylamine *N*-oxide reductase operons in *Escherichia coli*

(*frd* gene/*tor* gene/regulation)

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ABSTRACT ***Escherichia coli*, which can utilize O_2, nitrate, fumarate, or trimethylamine *N*-oxide (Me_3NO) as terminal electron acceptor, preferentially utilizes the one with the highest redox potential. Thus O_2 prevents induction of nitrate, fumarate, and Me_3NO reductases, and nitrate curtails the induction of fumarate and Me_3NO reductases. Under anaerobic conditions the *narL* gene product, in the presence of nitrate, is known to activate transcription of the *narC* operon, which encodes nitrate reductase. This study shows that the same product plays a role in the repression by nitrate of the operons (*frd* and *tor*) that encode fumarate and Me_3NO reductases. In contrast, the anaerobic repression of ethanol dehydrogenase by nitrate does not require the *narL* product. Expression of *narL* does not require the *fnr* gene product, a pleiotropic activator that is required for full expression of *narC*, *frd*, and *tor*.**

Escherichia coli can exploit several different compounds as an exogenous acceptor for electron transport across the plasma membrane, and a network of regulation of gene expression allows the cell to take advantage of the compound with the highest redox potential (reviewed in refs. 1 and 2). Thus O_2 ($E^{\circ\prime}$ = +0.82 V) is used in preference to all other electron acceptors, and nitrate ($E^{\circ\prime}$ = +0.42 V) is used in preference to trimethylamine *N*-oxide (Me_3NO) ($E^{\circ\prime}$ = +0.13 V) and fumarate ($E^{\circ\prime}$ = +0.031 V) (3, 4). The *fnr* gene product, a pleiotropic transcriptional activator, is required for expression of the operons (*narCHI*, *frdABCD*, and *tor*) that encode nitrate, fumarate, and Me_3NO reductase complexes, respectively (5–7). O_2 decreases the concentration of the *fnr* product (8), which can account for its repression of the induction of nitrate (9–12), fumarate (13–15), and Me_3NO (16) reductases by their respective substrates.

Under anaerobic conditions nitrate becomes the most favored electron acceptor, inducing nitrate reductase (10, 17, 18) and repressing the induction of fumarate (15, 17, 19, 20) and Me_3NO (21) reductases. This study shows that the gene product responsible for the activation of the *narC* operon is involved also in the repression of *frd* and *tor*.*

MATERIALS AND METHODS

Bacterial Strains. All strains used were of *E. coli* K-12 F^-. Their sources and genotypes are given in Table 1.

Strain RK5278 was used as donor of *narL215*::Tn*10* (7) in transduction with phage P1 *vir* (24). Transductants selected and purified on tetracycline/LB agar were then screened anaerobically on MacConkey/nitrate/glycerol agar (*narL*$^+$ colonies were red; *narL* mutant colonies were red but reduced in size) and MacConkey/Me_3NO/nitrate/glucose agar (*narL*$^+$ colonies were red; *narL* mutant colonies were white) for the *nar* phenotype (7, 25). Strains ECL552 [ϕ(*frd*$^+$–*lac*) *narL215*::Tn*10*], ECL565 (*frd*$^+$ *narL215*::Tn*10*), ECL581 (*frd*$^+$ *narL215*::Tn*10*), ECL582 [*frd-4*(Oxr) *narL215*::Tn*10*], and ECL583 [*frd-1*(Con) *narL215*::Tn*10*] were thus constructed from strains ECL388, ECL392, ECL514, ECL515, and ECL511, respectively. (ϕ indicates fused genes.)

Strain RK5278 was used as a transduction donor of *narL215*::Tn*10* to strain ECL545 [ϕ(*frd*$^+$–*lac*) *chlE103*] to obtain strain ECL555 [ϕ(*frd*$^+$–*lac*) *chlE103 narL215*::Tn*10*]. Since in *chlE* background, *narL*$^+$ and *narL* mutant alleles give a similar phenotype (lacking nitrate reductase activity), the inherited *narL215* allele in strain ECL555 was verified by back-crossing into a *narL*$^+$ strain.

Strain RK5266 was used as a transduction donor of *narK203*::Tn*10* to strain ECL388 to obtain strain ECL551 [ϕ(*frd*$^+$–*lac*) *narK203*::Tn*10*]. Transductants selected and purified on tetracycline/LB agar were screened anaerobically on the two MacConkey agars. On MacConkey/nitrate/glycerol agar, *narK*$^+$ colonies were red; *narK* mutant colonies were yellow to white (7). On MacConkey/Me_3NO/nitrate/glucose agar, *narK*$^+$ colonies were red; *narK* mutant colonies were white.

Strain ECL545 (*chlE103*) was isolated from strain ECL388 (*chl*$^+$) after 2 days of anaerobic incubation on chlorate/xylose agar. The mutation was shown to be at the *chlE* locus by transducing the *zbi-624*::Tn*10* from strain RK4922 (80% linkage to *chlE*$^+$) into strain ECL545. Among the tetracycline-resistant transductants, 85% grew anaerobically on DL-lactate/nitrate agar (inheritance of *chlE*$^+$).

Strain ECL323 was used as a transduction donor of *fnr-1 zci*::Tn*10* to strain ECL388 to obtain strain ECL557 [ϕ(*frd*$^+$–*lac*) *fnr-1 zci*::Tn*10*]. Transductants selected and purified on tetracycline/LB agar were screened for the inheritance of *fnr-1*, as manifested by the loss of ability to grow anaerobically on DL-lactate/nitrate agar (10% cotransduction).

Growth of Cells. For routine cultures, LB medium (1.0% tryptone/0.5% yeast extract/0.5% NaCl) was used. For enzyme assays, cells were grown in a mineral medium buffered at pH 7.6 by 0.1 M 3-(*N*-morpholino)propanesulfonate (Mops) and supplemented with 0.03% casein acid hydrolysate (to stimulate growth) and other appropriate compounds (26). Aerobic cultures were grown in 40 ml of Mops medium vigorously agitated in 300-ml flasks and harvested in midexponential phase (approximately 100 Klett units, no. 42 filter). Anaerobic cultures were grown in 50-ml screw-capped test tubes filled to the top with Mops medium and left undisturbed for 16 hr. In all experiments involving the expression of the *frd* operon, glycerol was added to promote the induction by fumarate (27). Agar plates were incubated anaerobically in sealed jars containing an atmosphere of H_2

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Abbreviation: Me_3NO, trimethylamine *N*-oxide.

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Table 1. *Escherichia coli* K-12 F⁻ strains

Strain	Derived from	Genotype and phenotype	Source or ref.
RK4922		*zbi-624*::Tn*10 araD139 gyrA ΔlacU169 non rpsL thi*	7
RK5263		*narH200*::Tn*10 araD139 gyrA ΔlacU169 non rpsL thi*	7
RK5265		*narC202*::Tn*10 araD139 gyrA ΔlacU169 non rpsL thi*	7
RK5267		*narI204*::Tn*10 araD139 gyrA ΔlacU169 non rpsL thi*	7
RK5266		*narK203*::Tn*10 araD139 gyrA ΔlacU169 non rpsL thi*	7
RK5278		*narL215*::Tn*10 araD139 gyrA ΔlacU169 non rpsL thi*	7
ECL323		*fnr-1 zci*::Tn*10 araD139 fldB ΔlacU169 ptsF25 relA rpsL thi*	22
ECL388		*frd-101*::λpl(209) *araD139 fldB ΔglpD103 ΔlacU169 ptsF25 relA rpsL sdh-9 thi* Gal⁻	20
ECL392		*frd*⁺ *araD139 fldB glpA101*::λpl(209) *ΔglpD103 ΔlacU169 ptsF25 relA rpsL sdh-9 thi* Gal⁻	23
ECL511		*frd-1*(Con)	23
ECL514		*frd*⁺	20
ECL515		*frd-4*(Oxr)	20
ECL545	ECL388	*frd-101*::λpl(209) *chlE103*	This work
ECL551	ECL388	*frd-101*::λpl(209) *narK203*::Tn*10*	This work
ECL552	ECL388	*frd-101*::λpl(209) *narL215*::Tn*10*	This work
ECL555	ECL545	*frd-101*::λpl(209) *chlE103 narL215*::Tn*10*	This work
ECL557	ECL388	*frd-101*::λpl(209) *fnr-1 zci*::Tn*10*	This work
ECL565	ECL392	*frd*⁺ *narL215*::Tn*10 glpA101*::λpl(209)	This work
ECL581	ECL514	*frd*⁺ *narL215*::Tn*10*	This work
ECL582	ECL515	*frd-4*(Oxr) *narL215*::Tn*10*	This work
ECL583	ECL511	*frd-1*(Con) *narL215*::Tn*10*	This work

Gal⁻, galactose-negative; *frd*(Con), cis-dominant promoter mutation rendering operon expression constitutive; *frd*(Oxr), cis-dominant promoter mutation rendering operon expression resistant to O_2.

and CO_2 (GasPak Anaerobic System, Baltimore Biological Laboratory Microbiology System).

When used, fumarate and pyruvate were added to 20 mM; glycerol was added to 0.2 mM [a concentration that gives an inductive effect without causing excessive accumulation of L-glycerol 3-phosphate by mutants blocked in its dehydrogenation (28)]; and nitrate, Me_3NO, and D-xylose were added to 10 mM. Potassium chlorate was added at 2 mg/ml, and tetracycline was added at 20 μg/ml.

Enzyme Assays. For assay of fumarate reductase, nitrate reductase, Me_3NO reductase, or ethanol dehydrogenase, cells were washed once with 1 mM DL-dithiothreitol and 10 mM potassium phosphate (pH 7.0) and resuspended in the same solution for sonic disruption. Cellular debris was removed by centrifugation at 10,000 × *g* for 30 min. Fumarate reductase activity was assayed anaerobically by monitoring the reoxidation of reduced benzyl viologen at 500 nm (22). Similar procedures were used for the assay of nitrate and Me_3NO reductase activities. For ethanol dehydrogenase assay, the cell extract was further clarified by centrifugation at 100,000 × *g* for 60 min to remove most of the NADH oxidase activity. The assay was carried out at pH 10 by monitoring the reduction of NAD at 340 nm (29). Protein concentrations were estimated with bovine serum albumin as the standard (30). Specific activity of the enzymes was expressed in nanomoles per minute per milligram of protein at 30°C.

β-Galactosidase activity was assayed in unbroken cells (rendered permeable by sodium dodecyl sulfate and chloroform) at 30°C by monitoring the hydrolysis of *o*-nitrophenyl β-D-galactoside at 420 nm, and the specific activity was expressed in units according to Miller (31).

RESULTS

Effects of *nar* and *chl* Mutations on the Expression of the *frd* Operon. Throughout our studies, the effects on *frd* expression were tested on cells grown in media containing both fumarate and glycerol, since such a medium gave strong anaerobic induction of fumarate reductase (27). We first examined several classes of mutants affected in nitrate reduction for clues to the mechanism of repression of fumarate reductase by nitrate. The nitrate reductase complex consists of three different subunits encoded by *narC*, *narH*, and *narI*, which constitute an operon (7, 32, 33). When we examined strains RK5265 (*narC*::Tn*10*), RK5263 (*narH*::Tn*10*), and RK5267 (*narI*::Tn*10*), in which nitrate reductase activity was abolished by the insertion of Tn*10* into one of the structural genes, the ability of nitrate to counteract the anaerobic induction of fumarate reductase was found to remain intact (data not shown). The proteins encoded by these genes therefore did not have a direct role in nitrate repression.

Since *E. coli* is known to have a minor nitrate reductase (7, 34), the results from the *nar* mutants described above could not exclude nitrite or a further reduction product as the true corepressor. Both the major and the minor nitrate reductases require the molybdenum cofactor as the prosthetic group, and the synthesis of this cofactor in turn depends on the *chlE* gene product (35). A *chlE* mutation should therefore totally block the reduction of nitrate, thus also allowing it to be maintained at an increased intracellular concentration. In such a mutant we found that nitrate repression was intensified (see below), suggesting that nitrate acts directly as a corepressor.

In contrast, in a *narL* mutant that failed to produce the activator protein for the *narC* operon (36), the induction of fumarate reductase became highly resistant to nitrate (data not shown). Since fumarate reductase is a membrane-protein complex and its activity therefore might not accurately reflect

Table 2. Effect of nitrate on anaerobically induced levels of φ(*frd*⁺-*lac*) in wild-type and mutant background

Strain	Genotype	Expression of *frd*, β-galactosidase units		
		− NO_3	+ NO_3	− NO_3/+ NO_3
ECL388	φ(*frd*⁺-*lac*)	730	140	5.2
ECL545	φ(*frd*⁺-*lac*) *chlE*	650	16	40
ECL552	φ(*frd*⁺-*lac*) *narL*	700	770	0.9
ECL555	φ(*frd*⁺-*lac*) *narL chlE*	860	720	1.2
ECL551	φ(*frd*⁺-*lac*) *narK*	780	170	4.6

Cells were grown anaerobically in fumarate/glycerol/xylose medium without nitrate (− NO_3) or with nitrate (+ NO_3).

Table 3. Effects of *narL* and *fnr* mutations on aerobic and anaerobic expressions of $\phi(frd^+\text{-}lac)$ and *narC* operons

Strain	Genotype	Expression of *frd*, β-galactosidase units						Expression of *narC*,* nitrate reductase units			
		Aerobic			Anaerobic			Aerobic		Anaerobic	
		− NO_3	+ NO_3	− NO_3/+ NO_3	− NO_3	+ NO_3	− NO_3/+ NO_3	− NO_3	+ NO_3	− NO_3	+ NO_3
ECL388	$\phi(frd^+\text{-}lac)$	120	22	5.5	770	150	5.1	10	42	140	1800
ECL552	$\phi(frd^+\text{-}lac)$ *narL*	130	130	1.0	700	720	0.97	17	4	170	64
ECL557	$\phi(frd^+\text{-}lac)$ *fnr*	110	21	5.2	120	29	4.1	44	46	21	19

The growth conditions were as given for Table 2.
*The growth medium was supplemented with 1 μM sodium molybdate, a cofactor of the enzyme.

the level of enzyme induction, we also examined the expression of a hybrid operon with the frd^+ promoter fused to the *lac* structural genes, $\phi(frd^+\text{-}lac)$. Table 2 shows that the β-galactosidase activity in strain ECL388 [$\phi(frd^+\text{-}lac)$] was decreased by a factor of 5 by nitrate when the cells were grown anaerobically in a fumarate/glycerol/xylose medium. The *chlE* mutation in strain ECL545 intensified the nitrate repression by an order of magnitude. The *narL* mutation in strain ECL552, in contrast, abolished the nitrate effect. Nitrate was also without effect in strain ECL555 bearing both the *narL* and the *chlE* mutations. The gene *narK*, closely linked to *narL*, is thought to have a role in nitrate regulation of other electron acceptor systems (7). However, a Tn*10*-insertion mutation in this gene did not significantly change the nitrate repression of the $\phi(frd^+\text{-}lac)$ in strain ECL551. The results therefore show that the repression of *frd* by nitrate was accentuated when it could not be reduced to nitrite and this repression depended on the *narL* product but not on the *narK* product.

Effects of Aerobiosis and the *fnr* Mutation on Nitrate Repression. As Table 3 shows, in strain ECL388 [$\phi(frd^+\text{-}lac)$] the induced level of expression of the *frd* operon (as indicated by the synthesis of β-galactosidase) was lowered by a factor of about 6 and the induced level of expression of the *narC* operon (as indicated by nitrate reductase activity) was lowered by a factor of about 40 by O_2. Both aerobically and anaerobically, nitrate decreased *frd* expression by a factor of 5. In strain ECL552 [$\phi(frd^+\text{-}lac)$ *narL*], the *narL* mutation prevented nitrate from repressing the aerobic or anaerobic expression of *frd* and from inducing *narC* under either of the respiratory conditions. These results indicate that aerobic repression of *frd* and aerobic induction of *narC* by nitrate also depended on the *narL* product. This in turn implies that the expression of *narL* was not subject to aerobic repression. In strain ECL557 [$\phi(frd^+\text{-}lac)$ *fnr*], the *fnr* mutation prevented significant anaerobic induction of *frd* and *narC*, but did not prevent nitrate from lowering the aerobic or anaerobic expression of *frd*. Thus the *fnr* product was not required for the *narL*-mediated repression of *frd*.

Effect of the *narL* Mutation on Nitrate Repression of *frd* Operons with Altered Promoters. Two classes of presumptive *frd* promoter mutations (cis-dominant) have been described. The *frd*(Oxr) mutation rendered the synthesis of fumarate reductase resistant to the effect of O_2 without significantly affecting either nitrate repression or the requirement of fumarate as an inducer. The *frd*(Con) mutation, on the other hand, rendered the synthesis of fumarate reductase constitutive—i.e., resistant to both O_2 and nitrate and independent of fumarate (20). Table 4 shows that the *narL* mutation strongly reduced the sensitivity of frd^+ and *frd*(Oxr) to anaerobic nitrate repression but did not significantly alter the expression pattern of *frd*(Con). The residual nitrate effect on the level of fumarate reductase activity in strains ECL581 (frd^+ *narL*), ECL582 [*frd*(Oxr) *narL*], and ECL583 [*frd*(Con) *narL*] was probably post-transcriptional.

Regulation of Me_3NO Reductase and Ethanol Dehydrogenase. In strain ECL392 (tor^+) grown anaerobically on xylose, Me_3NO induced the level of its reductase about 8-fold (data not shown). As previously reported (21), the induced level was lowered by a factor of 2 by nitrate (Table 5). However, the *narL* mutation inexplicably allowed the Me_3NO reductase to be induced to a higher level in the presence of nitrate. Possible effects of *chlE* mutation on the induction of Me_3NO reductase could not be studied because the resulting absence of the molybdenum cofactor deprives the enzyme of its prosthetic group.

The presence of fumarate did not lower the induced level of Me_3NO reductase significantly. Likewise, the presence of Me_3NO had little effect on the induced level of fumarate reductase (data not shown).

Nitrate also repressed the level of ethanol dehydrogenase, which plays a role in fermentative but not in respiratory growth. This nitrate effect, however, is not significantly influenced by the *narL* mutation (Table 5).

DISCUSSION

A classical model for the specific control of gene expression involves three elements: an effector, a regulatory protein, and a site within the target operator. Nitrate repression of fumarate reductase was shown to involve all three elements. (*i*) The enhancement of repression by *chlE* mutation, which totally blocks the enzymic reduction of nitrate, demonstrated that nitrate itself was an effector. A more recent study showed molybdate to be a coeffector both in *narC* activation and in *frd* repression (unpublished results). (*ii*) Relief of the repression by the *narL* mutation indicated that the gene product, which activated the *narC* operon (36), was also required for repression of the *frd* operon. This interpretation is consistent with the finding that, in the absence of the *narL* protein, even raising the intracellular concentration of nitrate with the help of a *chlE* mutation had no repressive effect on *frd* expression. (*iii*) In the cis-dominant *frd*(Oxr) mutants, synthesis of fumarate reductase was resistant to O_2 but not to nitrate repression (20). This would suggest that in the promoter region of the *frd* operon there is a specific site for nitrate-mediated regulation that is distinct from the site for O_2-mediated regulation, and, as would be expected, in these mutants nitrate control was eliminated by the *narL* mutation.

Table 4. Effect of the *narL* mutation on anaerobic expression of frd^+, *frd*(Oxr), and *frd*(Con) alleles

Strain	Genotype	Fumarate reductase, units		
		− NO_3	+ NO_3	− NO_3+/+ NO_3
ECL514	frd^+	1100	180	6.1
ECL581	frd^+ *narL*	1100	620	1.8
ECL515	*frd*(Oxr)	3100	480	6.5
ECL582	*frd*(Oxr) *narL*	3300	2000	1.7
ECL511	*frd*(Con)	1100	850	1.3
ECL583	*frd*(Con) *narL*	1200	780	1.6

The growth conditions were as given for Table 2.

Table 5. Effects of *narL* mutation on the ability of nitrate to repress anaerobic expression of *tor*$^+$ and *adhE*$^+$ operons

Strain	Genotype	Me_3NO reductase,* units			Ethanol dehydrogenase,† units		
		$-NO_3$	$+NO_3$	$-NO_3/+NO_3$	$-NO_3$	$+NO_3$	$-NO_3/+NO_3$
ECL392	*tor*$^+$ *adhE*$^+$	2000	1000	2.0	93	26	3.6
ECL565	*tor*$^+$ *adhE*$^+$ *narL*	1600	2600	0.6	110	20	5.5

All cultures were grown anaerobically in a xylose medium without nitrate ($-NO_3$) or with nitrate ($+NO_3$).
*The growth medium was supplemented with Me_3NO and 1 μM molybdate.
†The growth medium was supplemented with pyruvate.

Expression of the *tor* operon also appears to be influenced by the *narL* product, yet the regulatory pattern differs from that of the *frd* operon. First, the nitrate effect on *tor* expression is relatively weak. Second, in the *narL* mutant, nitrate enhanced the induced level of Me_3NO reductase. This peculiarity remains to be explained.

A single regulatory protein with activator and repressor functions is not unprecedented. It has been found, for example, in the *ara* system for the catabolism of D-arabinose (37) and in the *gln* system for nitrogen utilization (38, 39) in *E. coli*. In these cases the regulatory protein acts oppositely on the same set of genes under different biochemical conditions, whereas in *narL* regulation it appears that the same protein acts oppositely on different sets of genes under the same biochemical condition. We cannot exclude, however, two alternative possibilities. One is that the *narL* locus includes two genes: one encoding the activator protein for the *narC* operon, and the other encoding the repressor protein for the *frd* and *tor* operons; and a Tn*10* insertion in the upstream gene could simultaneously abolish two gene functions by a polar effect. However, an analysis of the degree of *narC* induction and that of *frd* repression as a function of nitrate or molybdate concentration gave no evidence for two distinct affinity constants (unpublished data). Alternatively, it is conceivable that the *narL* product serves exclusively as a gene activator and that the negative effect on *frd* expression resulted from stimulation of the synthesis of a separate repressing element. A fascinating example of regulation through an intermediary is the negative autogenous control of the *crp* gene, which plays a central role in catabolite repression. The CRP protein activates the divergent transcription in the *crp* promoter region of an antisense RNA capable of blocking further transcription of the mRNA by duplex formation (40, 41). Thus, proof of a direct role of the *narL* product as both an activator and repressor awaits *in vitro* evidence.

The ability of nitrate to lower further the aerobic level of *frd* expression in the wild-type strain but not in the *narL* mutant would indicate that the synthesis of the *narL* protein, unlike that of nitrate reductase, is not subject to strong O_2 repression. On similar grounds, *narL* expression, unlike that of the *narC*, is not strongly dependent on the presence of the pleiotropic activator encoded by *fnr*.

Although the hierarchical order imposed by the regulatory system for electron acceptance gives O_2 precedence over nitrate, and nitrate over fumarate, the temporal order of evolutionary appearance of the pathways was likely to be the reverse. Fumarate is postulated to have been exploited initially simply as a hydrogen sink in primitive anaerobes, since the compound could be generated endogenously from pyruvate via oxaloacetate and malate. Fumarate reduction would be catalyzed by a soluble enzyme with an activity not coupled to proton extrusion (still true for the present day *Veillonella alcalescens*). With the emergence of the *b* cytochromes and their association with fumarate reductase in the plasma membrane, it became possible to generate protonmotive force by the reduction of fumarate. The appearance of the *b* cytochromes in turn made possible the evolution of an electron transport chain with nitrate as the terminal acceptor (for assays on the evolution of membrane bioenergetics, see refs. 42 and 43). Nitrate is believed to have been available as an electron acceptor before aerobic respiration was made possible by the O_2 released from photosynthesis (44). As each redox system with a greater thermodynamic advantage was acquired, it was also important to prevent the electrons from being siphoned through less rewarding channels. For instance, the diversion of electrons from the respiratory pathway utilizing exogenous fumarate to the ethanol fermentation pathway not only would deprive the cell of the energy generated by proton extrusion (45, 46) but also would require consumption of the acetyl-CoA that can be profitably used as a carbon source or to synthesize ATP from ADP. In *Klebsiella pneumoniae* the presence of fumarate during anaerobic growth lowered the level of ethanol dehydrogenase by a factor of 4 (29). The mechanism by which the operation of an electron transport system reduces the synthesis of hydrogen disposal enzymes in fermentative pathways is obscure, even though we have shown that the repression of ethanol dehydrogenase by nitrate in *E. coli* did not involve the *narL* product. After the emergence of nitrate respiration, the *narL* product probably first evolved as a specific activator protein for the *narC* operon and subsequently broadened its role to include repression. This would require also the coevolution of a control site for the *narL* protein by each target operator. Finally, with the emergence of aerobic metabolism, all the operators involved in anaerobic respiration would eventually have to have been endowed with a site for the FNR regulatory protein. The base sequence would suggest that *fnr* arose from a *crp* gene likely to have been already in existence during the era of glycolytic fermentation (47).

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