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Nitrate reductase in *Escherichia coli* K-12: Involvement of *chlC*, *chlE*, and *chlG* loci

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We examined the properties of mutants of *E. coli* which are defective with respect to nitrate reductase activity. *chlE*::Mu *c*ts and *chlG*::Mu *c*ts mutants were all chlorate resistant, and the strains that we examined all synthesized nitrate reductase apoenzyme. We concluded that the *chlE* and *chlG* loci, like the *chlA*, *chlB*, and *chlD* loci, are involved in the synthesis or insertion of molybdenum cofactor. We identified at least four distinct phenotypic classes of *chlC*::Tn*10* mutants, all of which were fully or partially sensitive to chlorate. Two of these classes may represent lesions in the structural genes for nitrate reductase subunits A and C. Two other classes may be altered in the regulation of the expression of nitrate reductase or other anaerobic enzymes. We propose the mnemonic *nar* for naming individual genes within the *chlC* locus.

Escherichia coli will use nitrate as a terminal electron acceptor in the absence of molecular oxygen. The dispensable nature of nitrate respiration has made it amenable to genetic analysis, and most studies have focused on the terminal component, nitrate reductase. This membrane-bound enzyme, which contains nonheme iron and molybdenum cofactor, is a complex of three polypeptides. These subunits, termed A, B, and C, have molecular weights of 142,000, 60,000 and 20,000, respectively. Subunit A may encompass the active site, whereas the function of subunit B is not known (28). Subunit C is cytochrome b_{556}^{NR}, which is specifically associated with nitrate reductase (13, 24). Nitrate reductase activity is induced by nitrate only during anaerobic growth.

Most nitrate reductase-deficient mutants have been selected for by their resistance to chlorate in the absence of oxygen. Six distinct *chl* loci are known: *chlA* through *E* and *chlG* (2). Most *chl* mutants are also deficient in the activities of other molybdo-enzymes, including formate dehydrogenase. Indeed, the *chlA*, *chlB*, and *chlD* loci have been implicated in the synthesis, insertion, or processing of molybdenum cofactor (1, 27, 37). In contrast, the functions specified by *chlE* and *chlG* are unknown (15, 16, 19, 25).

DeMoss (11) has isolated *chlC* mutants whose nitrate reductase is thermolabile in vitro, and MacGregor (25) has described a single *chlC* mutant in which nitrate reductase protein is unstable in vivo. Most *chlC* mutants have only slightly reduced levels of formate dehydrogenase activity (15–17). These observations have led to the idea that the *chlC* locus includes at least one of the nitrate reductase structural genes. A gene termed *chlI*, which maps in the *chlC* region, has been described (4). *ChlI* is postulated to encode subunit C and to lie in an operon with the structural gene for at least one of the other nitrate reductase subunits.

We have identified at least four distinct phenotypic classes of mutants which have mutations in the *chlC* locus. Two of these classes have phenotypes which are consistent with those predicted for mutations in the structural genes for two of the nitrate reductase subunits. All classes are fully or partially sensitive to chlorate. Furthermore, we present evidence that mutations in *chlE* and *chlG* affect molybdenum cofactor function, rather than the biosynthesis of nitrate reductase polypeptides. We propose the designation *nar* for naming individual genes within the *chlC* locus, to distinguish these genes from the other, pleiotropic *chl* loci which are involved in the production of active molybdenum cofactor.

MATERIALS AND METHODS

Strains. *Escherichia coli* K-12 strains used in this study are listed in Table 1. RK4353 is a derivative of MC4100. Bacteriophages P1 *kc*, Mu *c*ts, and λNK370 (*b*221 *c*I857 *c*I171::Tn*10* *O* [uga] 261) were provided by R. Kadner.

Media. MacConkey nitrate agar (3) contained (per liter): 40 g of MacConkey agar base, 10 g of KNO_3, 0.5 g of $NaHCO_2$, 0.1 g of glucose, and 1 g of glycerol. MacConkey TMAO agar (8) contained (per liter): 40 g of MacConkey agar base, 4 g of glucose, and 10 g of

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

Strain	Genotype	Source
MBM7017	*araD araC*(Am) *lacZ*(Am) *trp*(Am) *relA tyrT rpsL*	M. Berman
RE103	*his pro trp lac rpsL cmlA*	CGSC[a]
W3350	*gal bio rpsL*	R. Kadner
RK7-16	*gal chlA16*	(25)
RK7-34	*gal chlD34*	(25)
RK4353	Δ*lacU169 araD139 rpsL gyrA non*	R. Kadner
RK4744	As MBM7017 but *hemA*	This work
Derivatives of RK4353		This work
RK4918	*zbh-620*::Tn*10* (Tn*10* near *chlA*)	
RK4919	*zif-621*::Tn*10* (Tn*10* near *chlB*)	
RK4920	*zcg-622*::Tn*10* (Tn*10* near *chlC*)	
RK4921	*zbh-623*::Tn*10* (Tn*10* near *chlD*)	
RK4922	*zbi-624*::Tn*10* (Tn*10* near *chlE*)	
RK4923	*zaa-625*::Tn*10* (Tn*10* near *chlG*)	
RK5200	*chlA200*::Mu *cts*	
RK5201	*chlE201*::Mu *cts*	
RK5202	*chlD202*::Mu *cts*	
RK5206	*chlG206*::Mu *cts*	
RK5208	*chlB207*::Mu *cts*	
RK5218	*chlE215*::Mu *cts*	
RK5223	*chlE220*::Mu *cts*	
RK5228	*chlE225*::Mu *cts*	
RK5230	*chlG226*::Mu *cts*	
RK5231	*chlG227*::Mu *cts*	
RK5233	*chlG229*::Mu *cts*	
RK5240	*chlE239*::Mu *cts*	
RK5255	*chlE237*::Mu *cts*	
RK5256	*chlG238*::Mu *cts*	
RK5263	*nar-200*::Tn*10* ($NarH^-$)	
RK5265	*nar-202*::Tn*10* ($NarG^-$)	
RK5266	*nar-203*::Tn*10* ($NarK^-$)	
RK5267	*nar-204*::Tn*10* ($NarI^-$)	
RK5278	*nar-215*::Tn*10* ($NarL^-$)	

[a] Courtesy of B. Bachmann, *E. coli* Genetic Stock Center.

trimethylamine *N*-oxide. MacConkey nitrate TMAO (MNT) agar was MacConkey TMAO supplemented with 2.5 g of KNO_3 per liter. Glycerol-fumarate (GF) agar was minimal medium A (29) supplemented with 0.2% glycerol and 20 mM sodium fumarate. Basal anaerobic growth medium (23) was supplemented with 1 μM $Na_2MoO_4 \cdot 2H_2O$–1 μM $Na_2Se_2O_3$–0.04% Proteose peptone. Chlorate agar was LB (29) supplemented with 0.2% glucose; $KClO_3$ was added as a separately sterilized solution to final concentrations as indicated in the text.

We found that lactate-nitrate agar (39) gave unsatisfactory results for selecting Chl^+ and Nar^+ revertants or transductants. We used PN agar, which contained (per liter): 17 g of peptone (Difco Laboratories), 3 g of Proteose peptone, 5 g of NaCl, and 10 g of KNO_3. Wild-type strains gave luxuriant growth on this medium after overnight anaerobic incubation, whereas *chl* and $NarG^-$, H^-, and I^- mutants failed to grow. $NarL^-$ mutants grew slightly, but transductants were easily seen above the background lawn.

All minimal media contained 1 μg of thiamine per ml. Glucose, maltose, and sodium succinate were added as indicated at 0.2%. δ-Aminolevulinic acid was added at 10 μg/ml for *hemA* strains. Tetracycline-hydrochloride was added at 15 μg/ml, and $Na_4P_2O_7$ was added as a separately sterilized solution at 1.25 mM, as indicated in the text. Agar media contained 1.6% agar. Agar and dehydrated culture media were purchased from Difco Laboratories (Detroit, Mich.). Trimethylamine *N*-oxide was purchased from Aldrich Chemical Co. (Milwaukee, Wis.).

Culture conditions. Liquid cultures were grown anaerobically by continuously bubbling 95% N_2–5% CO_2 through the medium. Aerobic liquid cultures for nitrate reductase assays were grown in 10 ml of minimal succinate medium in 300-ml baffle-bottomed flasks which were shaken vigorously on a gyratory water bath shaker. These cultures were harvested in early-log phase by pouring them through ice into a cold container, to prevent adaptation to anaerobiosis. Plates were incubated anaerobically in Brewer jars under an atmosphere of H_2.

Isolation of mutants. (i) *chl*::Mu *cts* mutants. A saturated culture of RK4353 was swabbed onto LB agar plates, and single drops of a limiting dilution of a freshly prepared lysate of Mu *cts* were spotted on and allowed to dry. After overnight incubation at 30°C, the turbid centers from each of the resulting lysis zones were inoculated into separate culture tubes containing LB. The tubes were shaken at 30°C until the cultures reached saturation, 0.1 ml from each tube was spread on a separate 15 mM chlorate agar plate, and the plates were incubated anaerobically at 30°C overnight. Four colonies from each plate were purified on LB agar and then tested for chlorate resistance and temperature sensitivity. A single chlorate-resistant, temperature-sensitive isolate from each independent culture was retained for study.

(ii) *nar*::Tn*10* mutants. Cultures of RK4353 were grown to saturation in 100 ml of LB-maltose, sedimented, and suspended in 5 ml of 10 mM $MgSO_4$. λNK370 was added at a multiplicity of infection of 0.2 and allowed to adsorb for 30 to 45 min at room temperature. This mixture was then spread in 0.2-ml portions onto minimal glucose plates which contained tetracycline and pyrophosphate (R. Kadner, personal communication). After 2 days of incubation at 37°C, each plate contained approximately 200 tetracycline-resistant colonies. Each colony is presumed to have arisen from an independent transposition event. These plates were replica printed onto MNT-tetracycline-

pyrophosphate plates, which were then incubated anaerobically overnight at 37°C.

Tn*10* insertions near *chl* loci. Colonies arising from approximately 6,000 independent transposition events were pooled and used to prepare a generalized transducing lysate of phage P1. Suitable recipient strains were infected with this lysate, and recombinants were selected which simultaneously acquired tetracycline resistance and the donor allele for the particular gene being used. The precise location of each Tn*10* insertion was then determined by appropriate P1-mediated genetic crosses (21, 29). *zbh-620*::Tn*10*, *zbh-623*::Tn*10*, and *zbi-624*::Tn*10* were mapped with respect to *gal*, *bio*, *cmlA*, *chlD*, and *chlA* by using as recipients W3350, RE103, RK7-34, and RK7-16, respetively. In each cross, 240 tetracycline-resistant transductants were purified and tested for inheritance of the unselected donor allele(s). The map position of each of these insertions is shown in Fig. 1. *zif-621*::Tn*10* (near *chlB*), *zcg-622*::Tn*10* (near *chlC*), and *zaa-625*::Tn*10* (near *chlG*) were mapped with respect to *metE* and *chlB*; *hemA*, *tyrT*, and *chlC*; and *thr*, *leu*, and *chlG*; respectively (data not shown) (2).

Mapping of mutations. (i) *chl*::Mu *cts*. We crossed each of the *chl*-linked Tn*10* strains with each of the *chl* mutants via P1-mediated transduction and then gridded 12 tetracycline-resistant recombinants from each cross onto two sets of LB-tetracycline plates. One set of plates was incubated aerobically at 42°C, and the other set was incubated anaerobically at 30°C. After overnight incubation, the latter set of plates was overlaid with nitrite reagents as described (15). Recombinants from single Mu *cts* insertions in one of the *chl* loci simultaneously became temperature resistant and Chl$^+$ when the appropriate Tn*10* strain was the donor (18, 21). Locus assignments for the *chl*::Mu *cts* strains which are described in this paper were confirmed by retesting 50 tetracycline-resistant recombinants from each appropriate cross.

(ii) *nar*::Tn*10*. We crossed each Nar$^-$ mutant strain with RK4744 via P1-mediated transduction. We then purified 100 tetracycline-resistant transductants from each cross and tested each for inheritance of the donor Nar$^-$, *tyrT*$^+$, and *hemA*$^+$ alleles. Strains which have 100% linkage between Tn*10* and Nar$^-$ are presumed to have Tn*10* integrated within a *nar* (*chlC*) gene (21). Nar was scored on MacConkey nitrate plates supplemented with δ-aminolevulinic acid. TyrT was scored on arabinose-tetrazolium plates (29) supplemented with δ-aminolevulinic acid. RK4744 is phenotypically arabinose sensitive (Ara^s); when the donor Su^0 allele replaces the Su$^+$ *tyrT* allele, the strain becomes Ara$^-$. HemA was scored by gridding transductants on two sets of minimal succinate-tryptophan plates, one set of

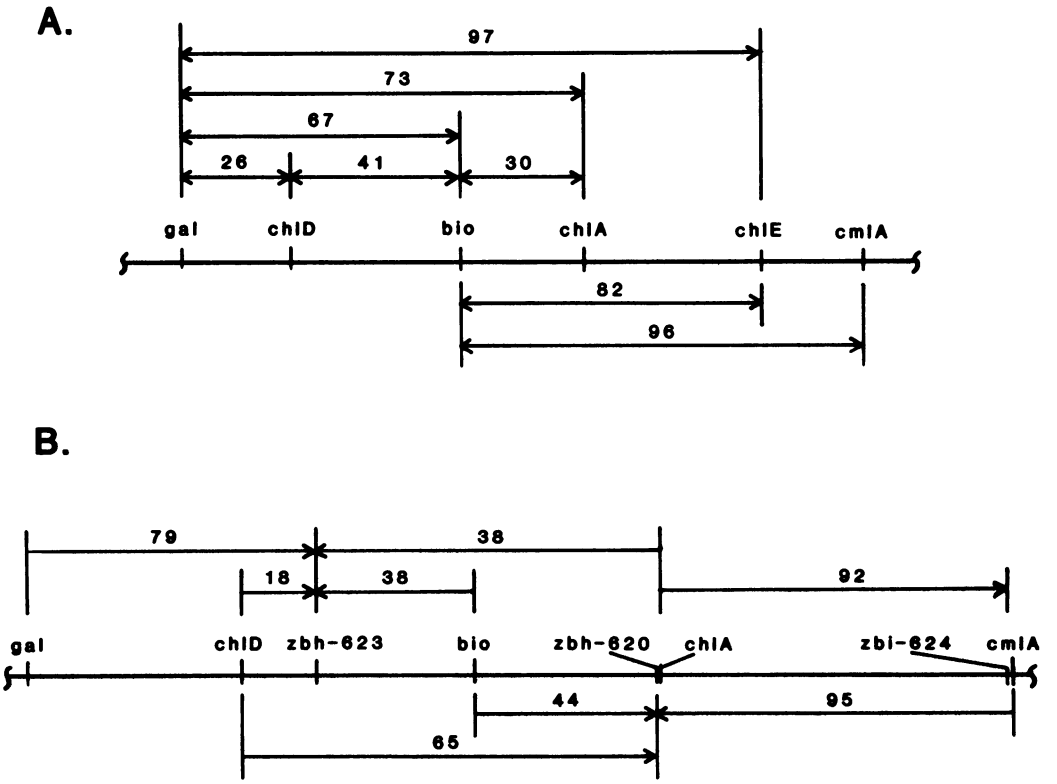

FIG. 1. Locations of Tn*10* insertions in the *chlDAE* region. (A) Genetic map of the region. Data are from references 34 and 39; figure is redrawn from reference 39. Map units are expressed as 100 minus percent cotransduction. (B) Tn*10* insertions. The locations of the Tn*10* insertions in strains RK4918 (*zbh-620*::Tn*10*), RK4921 (*zbh-623*::Tn*10*), and RK4922 (*zbi-624*::Tn*10*) were determined as described in the text. The relative positions of *zbh-620*::Tn*10* and *chlA* and of *zbi-624*::Tn*10* and *cmlA* were not determined; the orders shown are arbitrary.

which also contained δ-aminolevulinic acid. HemA$^-$ strains cannot grow oxidatively in the absence of δ-aminolevulinic acid.

Enzyme assays. We prepared crude extracts by breaking washed cells in a French pressure cell (Aminco) and subsequently removed unbroken cells by sedimentation at 3,000 × *g* for 5 min. We have described elsewhere our procedure for measuring reduced methyl viologen-linked nitrate reductase activity (28). We used the method of Pugsley and Schnaitman (33) to measure protein concentrations. We used the procedure of Bonnefoy-Orth et al. (4) to measure formate-linked nitrate reductase activity in whole cells. We used the method of Guest (17) to estimate gas production.

Immune precipitation. We have recently described our procedures for continuously labeling cultures with $H_2{}^{35}SO_4$, isolating cytoplasmic and cytoplasmic membrane fractions, immune precipitation, polyacrylamide gel electrophoresis, and radioautography (26).

Reconstitution. We have previously described our procedure for reconstituting nitrate reductase activity in vitro from mixtures of membrane and cytoplasmic fractions from different *chl* mutants (27). Briefly, we grew cultures to late-log phase in anaerobic growth medium, washed the cells, and suspended them in buffer. We broke the cells in a French pressure cell, removed the unbroken cells by sedimentation for 5 min at 3,000 × *g*, and centrifuged each preparation for 1 h at 200,000 × *g*. The supernatant fractions (S_1) were retained. We added 0.1 mM $MgCl_2$ to the S_1 fraction from RK5208 (*chlB*), incubated it for 2 h at 32°C, and then centrifuged it for 1 h at 200,000 × *g*. Samples of this supernatant (S_2) were added to equal volumes of each of the S_1 fractions in the presence of 0.1 mM $MgCl_2$. After 2 h of incubation at 32°C, we determined the viologen-linked nitrate reductase specific activity in each of the fractions.

RESULTS

Mutant isolation. We isolated 192 independent chlorate-resistant (*chl*) mutants after mutagenesis with bacteriophage Mu *c*ts. We also isolated a series of six strains, each of which has the tetracycline resistance transposon Tn*10* located adjacent to one of the *chl* loci. We used these strains as donors in P1-mediated transduction crosses and determined the map location of the affected *chl* locus in 181 of the mutant strains. Mutants whose tetracycline-resistant recombinants simultaneously acquired both Chl$^+$ and temperature resistance were presumed to carry a single Mu *c*ts prophage integrated within the *chl* locus (18, 21). Approximately 90% of the mutants were such single lysogens. The number of mutants affected in each of the *chl* loci is shown in Table 2. Mapping data for the *chlE*::Mu *c*ts mutants described in this paper are shown in Table 3. For comparison, Table 3 also includes data for a *chlA*::Mu *c*ts and a *chlD*::Mu *c*ts mutant.

While characterizing our *chl* mutants, we observed that 15 of the 192 isolates were also *fnr* (22, 30) (data not shown). *fnr* mutations were cryptic because Chl$^-$ was phenotypically dominant to Fnr$^-$ in many respects (unpublished data). We initially detected *fnr* lesions while mapping *chl*::Mu *c*ts mutations. In the nitrite reagent overlay method that we were using to score transductants (15), we observed that *chl*$^+$ recombinants from *fnr*$^+$ strains produced a dark, distinct purple color, whereas *chl*$^+$ recombinants from *fnr* strains produced a light, "diffuse" pink color. Subsequently, we tested our strains for their ability to form fumarate reductase by examining their growth on GF medium. Fumarate reductase activity is required for anaerobic growth on this medium. Fumarate reductase is not a molybdo-enzyme, and it is unaffected in *chl* mutants. However, this activity is absent in *fnr* mutants (22). We routinely tested all of our strains for the Fnr character, and we only show data for Fnr$^+$ strains in this paper. We did not detect *fnr* mutations in any of our *nar* (*chlC*) mutants.

Subsequently, we developed a differential medium for detecting mutants that are specifically altered in nitrate reductase expression. This medium is adapted from the method that Casse et al. applied to the study of *Salmonella typhimurium* (5). We considered three observations

TABLE 2. Distribution of mutants recovered at each *chl* locus

Medium	No. of mutants at (locus):					
	chlA	*chlB*	*chlC*	*chlD*	*chlE*	*chlG*
Chlorate	78	9	1[a]	51	35	8
MNT	0	0	16	0	0	0

[a] Selected on low-level (5 mM) chlorate.

TABLE 3. Number of *chl*$^+$ recombinants in crosses between Tn*10* insertion strains and *chl*::Mu *c*ts mutants[a]

Recipient	Locus	No. of recombinants with (donor)[b]:		
		RK4921	RK4918	RK4922
RK5202	*chlD*	42	12	1
RK5200	*chlA*	41	50	10
RK5201	*chlE*	12	21	39
RK5218	*chlE*	4	14	41
RK5223	*chlE*	4	13	44
RK5228	*chlE*	6	8	44
RK5240	*chlE*	8	14	35
RK5255	*chlE*	6	11	38

[a] Fifty tetracycline-resistant recombinants from each cross were tested for Chl phenotype and temperature sensitivity.

[b] The positions of Tn*10* insertions are shown in Fig. 1. RK4921 contains *zbh-623*::Tn*10* (near *chlD*), RK4918 contains *zbh-620*::Tn*10* (near *chlA*), and RK4922 contains *zbi-624*::Tn*10* (near *chlE*).

in designing this medium. First, the presence of functioning nitrate reductase inhibits the formation of many other enzymes which are involved in anaerobic metabolism. However, if nitrate reductase activity is inhibited, then these other anaerobic enzymes are expressed (9, 30, 40). Second, a differential medium for detecting tertiary amine oxide reductase (TOR) activity has been described (8). *E. coli* colonies growing anaerobically on glucose accumulate acid. Trimethylamine, the reduction product of trimethylamine *N*-oxide, is a strong base. Thus, the pH difference between TOR-proficient and TOR-deficient strains is distinguished by the dyes present in MacConkey TMAO medium. Third, TOR is a molybdo-enzyme (35), and pleiotropic *chl* mutants lack this activity (8). Therefore, we reasoned that MacConkey TMAO medium supplemented with nitrate (i.e., MNT medium) would differentiate mutants which are specifically altered in nitrate reductase formation. Colonies of wild-type and *chl* strains were red on this medium, whereas colonies of strains that are derepressed for TOR expression were white or pink.

We used MNT medium to screen approximately 50,000 colonies which arose from independent insertions of Tn*10* and identified 16 light-colored isolates (Table 2). We designated these strains as Nar$^-$. Preliminary experiments indicated that all of these mutants were affected in the *chlC* locus, and we confirmed this by examining the linkages between the *nar* lesions and *hemA* and *tyrT* (*supF*), two genes which flank *chlC* (17; Table 4). In 15 of these mutants, tetracycline resistance was 100% linked to Nar$^-$, which indicates that Tn*10* is integrated within the affected *nar* gene (21).

Finally, we performed one experiment to screen tetracycline-resistant colonies for resistance to a low concentration (i.e., 5 mM) of chlorate. Only 1 isolate of 73 was sensitive to 25 mM chlorate. The mutation in this strain (RK5278) was designated *nar-215*::Tn*10* (NarL$^-$), and it also mapped at the *chlC* locus (Table 4). RK5278 and the wild-type parent were indistinguishable after growth on MNT medium.

TABLE 4. Linkage of *nar*::Tn*10* mutations to *hemA* and *tyrT*[a]

Donor	Class	No. of recombinants			
		Tyr$^-$ Hem$^-$	Tyr$^+$ Hem$^+$	Tyr$^-$ Hem$^+$	Tyr$^+$ Hem$^-$
RK5265	NarH$^-$	5	35	3	57
RK5263	NarG$^-$	1	24	0	75
RK5267	NarI$^-$	1	26	0	73
RK5266	NarK$^-$	8	39	9	44
RK5278	NarL$^-$	17	33	17	33

[a] Each *nar*::Tn*10* strain was crossed with RK4744, and 100 tetracycline-resistant recombinants were tested for their Tyr and Hem phenotypes.

Characterization of Nar$^-$ mutants. Our preliminary experiments distinguished five apparently distinct classes of Nar$^-$ mutants, NarG$^-$ through NarL$^-$. We found seven NarG$^-$, one NarH$^-$, two NarI$^-$, and six NarK$^-$ mutants, in addition to the NarL$^-$ mutant mentioned above. We selected a single representative from each class for detailed analysis.

We found that MacConkey nitrate medium (3) was invaluable for differentiating various Nar$^-$ mutants. Upon anaerobic incubation, wild-type strains formed large, salmon-colored colonies on this medium, whereas Chl$^-$ and Fnr$^-$ mutants formed minute, red colonies. Colonies of NarG$^-$, NarH$^-$, and NarI$^-$ strains were small and pale pink, and those of NarK$^-$ mutants were large and yellow-white. Our NarL$^-$ strain formed medium-sized, dark-red colonies, which were easily distinguished from Chl$^-$ or Fnr$^-$ colonies. These various phenotypes were distinct even when many colonies were gridded on a plate in a patch pattern, and we now routinely use MacConkey nitrate medium to score the Nar or Chl phenotypes of recombinants from genetic crosses.

Mutants of the NarG$^-$ through NarK$^-$ classes were all completely sensitive to 5 mM chlorate, which is the lowest concentration that we tested. (Fnr$^-$ mutants were also sensitive to 5 mM chlorate.) The NarL$^-$ mutant formed very small colonies in the presence of 5 mM chlorate; subsequent aerobic incubation allowed these colonies to grow to full size. We termed this phenotype "chlorate-inhibited" (Chl^i), as it was clearly distinct from both chlorate resistance (Chl^r) and chlorate sensitivity (Chl^s). The NarL$^-$ mutant was fully sensitive to 10 mM chlorate. In contrast, all of the *chl*::Mu *cts* mutants we tested were resistant to 25 mM chlorate, which is the highest concentration that we used.

Characteristically, Chl^r mutants are pleiotropic in that they lack (or have reduced) enzyme activities of all known molybdo-enzymes, including nitrate reductase, formate dehydrogenase (15, 16), and TOR (8). We examined the presence of these latter two activities in Nar$^-$ mutants by using physiological tests. We estimated formate hydrogenlyase (i.e., formate dehydrogenase plus hydrogenase) proficiency by examining the mutants' abilities to produce gas in glucose-supplemented medium, and we detected TOR expression by examining the strains' phenotypes after anaerobic growth on MacConkey TMAO medium. None of the Nar$^-$ mutants was distinguishable from the wild type by either of these tests.

Nitrate reductase activities of Nar$^-$ mutants. We assayed nitrate reductase activity by using

reduced methyl viologen, which donates electrons directly to nitrate reductase itself independent of the cytochrome (20). Table 5 shows the nitrate reductase specific activities in crude extracts of each of the Nar$^-$ mutants. Each mutant class had a unique, reproducible level of activity relative to that of the wild type.

We consistently found that RK5265 (NarG$^-$) had approximately 1% of the wild-type nitrate reductase activity (Table 5). Since nitrate reductase activity is undetectable in many classes of Chl^r mutants, we investigated the nature of the residual activity in RK5265. We grew cultures of RK5265 and the wild-type parent RK4353 in liquid media supplemented with nitrate, fumarate, or trimethylamine *N*-oxide. Nitrate reductase activity in RK4353 was induced approximately 10-fold by growth on the nitrate-supplemented medium, but the activity in RK5265 remained at the same low level, irrespective of the growth medium (data not shown). We also tested the in vitro effect of sodium azide, which is a strong inhibitor of nitrate reductase activity (14). Azide inhibited nitrate reductase activity to roughly the same extent in both RK4353 and RK5265 (data not shown).

Bonnefoy-Orth et al. (4) have described mutants which they term *chlI*, whose mutations map at the *chlC* locus. These mutants are characterized as having nearly wild-type levels of viologen-linked nitrate reductase activity, but only about 10% of wild-type levels of formate-linked nitrate reductase activity (assayed in whole cells). Table 5 shows that the formate-linked activities in the NarG$^-$, NarH$^-$, and NarI$^-$ mutants were all roughly 10% or less than the wild-type activity. The formate-linked activity in the NarI$^-$ mutant was significantly less than its viologen-linked activity. The NarK$^-$ mutant had high levels of activity assayed by either method, whereas the NarL$^-$ activities were relatively low.

We investigated the regulation of nitrate reductase formation in NarK$^-$ and NarL$^-$ mutants. We grew cultures of RK4353, RK5266 (NarK$^-$) and RK5278 (NarL$^-$) under three sets of conditions: aerobic with no nitrate, anaerobic with no nitrate, and anaerobic with nitrate. Table 6 shows the viologen-linked nitrate reductase activities for these strains under each of these conditions. For all three strains, enzyme activity was nearly absent when cultures were grown aerobically, and it was present at comparably low levels when cultures were grown anaerobically in the absence of nitrate. Enzyme activity was induced by nitrate in the wild-type and the NarK$^-$ mutant, and it was not induced by nitrate in the NarL$^-$ mutant.

TABLE 6. Methyl viologen-nitrate reductase activities of Nar$^-$ mutants grown aerobically, anaerobically, and anaerobically with nitrate

Strain	Class	Growth	μmol of NO_2^- per min per mg of protein	
			Expt 1	Expt 2
RK4353	Wild type	$+O_2$, $-NO_3^-$	0.0061	0.0024
RK5266	NarK$^-$		0.013	0.0030
RK5278	NarL$^-$		0.0035	0.0021
RK4353	Wild type	$-O_2$, $-NO_3^-$	0.064	0.038
RK5266	NarK$^-$		0.046	0.035
RK5278	NarL$^-$		0.060	0.039
RK4353	Wild type	$-O_2$, $+NO_3^-$	0.81	0.43
RK5266	NarK$^-$		1.1	0.70
RK5278	NarL$^-$		0.036	0.021

Nitrate reductase protein in Nar$^-$ mutants. We used antiserum directed against subunits A and B of nitrate reductase to precipitate this enzyme from cytoplasmic and Triton X-100-soluble (i.e., cytoplasmic membrane) fractions from the wild-type strain and each of the *nar*::Tn*10* mutants (24). The precipitates were resolved by gel electrophoresis, and the resultant band patterns are shown in Fig. 2. Both the wild-type and NarK$^-$ strains clearly produced all three subunits in substantial amounts, whereas the NarL$^-$ strain produced at least small amounts of intact subunits A and B. We could not detect

TABLE 5. Reduced methyl viologen-nitrate reductase and formate-nitrate reductase activities of Nar$^-$ mutants

Strain	Class	MVH[a] activity (μmol of NO_2^- per min per mg of protein)		Formate activity (μmol of NO_2^- per min per 10^9 cells)	
		Expt 1	Expt 2	Expt 1	Expt 2
RK4353	Wild type	0.83	0.76	0.067	0.097
RK5265	NarG$^-$	0.0092	0.0074	0.0032	0.0015
RK5263	NarH$^-$	0.089	0.088	0.0030	0.0087
RK5267	NarI$^-$	0.49	0.37	0.0092	0.0098
RK5266	NarK$^-$	1.2	0.98	0.055	0.083
RK5278	NarL$^-$	0.044	0.019	0.018	0.045

[a] MVH, Methyl viologen.

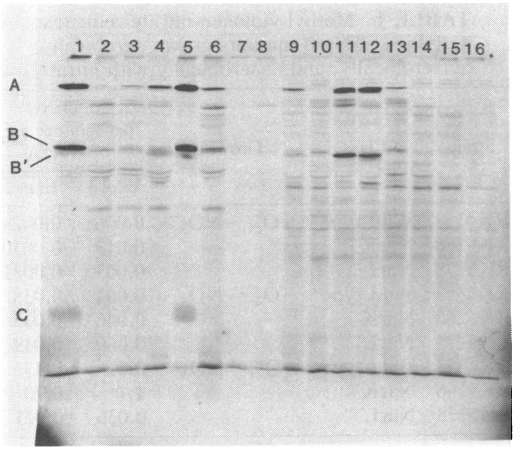

FIG. 2. Nitrate reductase protein in *nar*::Tn*10* mutants. Anaerobic cultures were continuously labeled with ^{35}S, and both the membrane and cytoplasmic fractions were subjected to immune precipitation. The precipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the labeled proteins were visualized by radioautography. In all samples except controls, equal numbers of counts per minute were loaded on the gel. For the preimmune controls, volumes equal to those added for the immune precipitates were loaded. A, B, B', and C mark the positions of these subunits. Lanes 1 through 8 contain membrane fractions, and lanes 9 through 16 contain cytoplasmic fractions. Lanes 7 and 15 and 8 and 16 contain preimmune serum precipitates of RK4353 (wild type), and RK5263 ($NarH^-$), respectively. Lanes 1 and 9, RK4353 (wild type); lanes 2 and 10, RK5265 ($NarG^-$); lanes 3 and 11, RK5263 ($NarH^-$); lanes 4 and 12, RK5267 ($NarI^-$); lanes 5 and 13, RK5266 ($NarK^-$); lanes 6 and 14, RK5278 ($NarL^-$).

subunit A in either the membrane or the cytoplasmic fractions of the $NarG^-$ mutant; however, this strain may have retained subunit B in the membrane fraction. The $NarH^-$ and $NarI^-$ strains accumulated subunits A and B in both fractions. Subunit B from the membrane fraction of the $NarH^-$ strain appeared to be predominately in the modified, or B form, whereas that from the $NarI^-$ strain seemed to be mostly in the unmodified B' form (26).

***chlE* mutants.** We chose six of our *chlE*::Mu *c*ts mutants for further analysis. All of these strains were Chl^r, all failed to produce gas, and all appeared to be deficient in TOR. We could not detect nitrate reductase activity in these strains.

We examined the nitrate reductase polypeptides in the membrane fractions of these mutants, and the results are shown in Fig. 3. Nitrate reductase subunits A, B, and C had electrophoretic mobilities similar to those of the wild-type parent. Subunit B from the mutants migrated as a more pronounced doublet due to incomplete post-translational modification (27).

We previously found that *chlB* mutants accumulate molybdenum cofactor in the cytoplasm and that *chlA* mutants synthesize nitrate reductase apoenzyme. In vitro incubation of a *chlB* soluble fraction with the membrane fraction from a *chlA* mutant leads to reconstitution of nitrate reductase activity (27). We performed similar experiments with three of our *chlE*::Mu *c*ts mutants, and we included a *chlA*::Mu *c*ts mutant as a control. Table 7 shows that all three *chlE* mutants provided reconstitution of enzyme activity to levels similar to those given by the *chlA* mutant.

We previously reported *chl* mutants RK7-3, RK7-13, and RK7-44 to be defective in the *chlE* locus (25). Our recent data, using the Tn*10* strains described above, clearly showed that, in fact, these strains are all mutated at the *chlD* locus (data not shown). In addition, our current isolates of RK7-3 and RK7-44 were also *fnr*. We do not know whether the original 1970 isolates of these strains were *fnr*.

***chlG* mutants.** We unexpectedly (15, 19) recovered eight *chlG*::Mu *c*ts mutants from the chlorate resistance selections. These strains

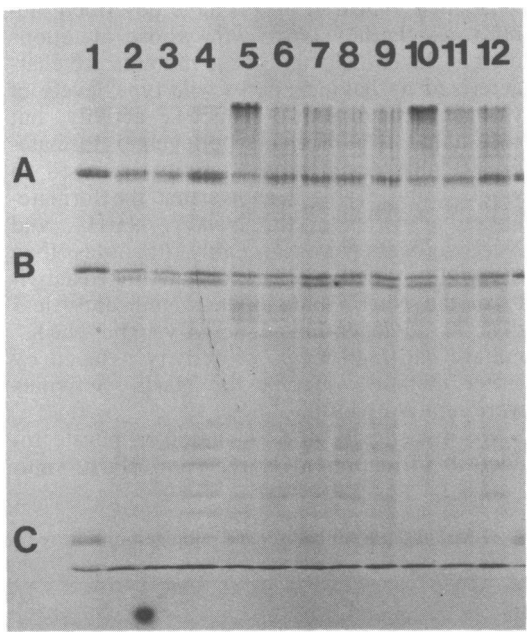

FIG. 3. Nitrate reductase protein in membrane fractions from *chlE*::Mu *c*ts and *chlG*::Mu *c*ts mutants. Procedure is as described in the legend to Fig. 2. Equal numbers of counts per minute were loaded on the gel. Only the membrane fraction precipitates are shown. Lane 1, wild type (RK4353). Lanes 2 through 7, *chlE*::Mu *c*ts mutants (RK5201, RK5218, RK5223, RK5228, RK5240, RK5255). Lanes 8 through 12, *chlG*::Mu *c*ts mutants (RK5206, RK5230, RK5231, RK5233, RK5256).

TABLE 7. Methyl viologen-nitrate reductase activities in reconstituted extracts of *chl*::Mu *cts* mutants[a]

Strain	Locus	μmol of NO_2^- per min per mg of protein	
		Extract alone[b]	Extract plus *chlB* fraction[c]
RK5200	*chlA*	<0.00001	0.069
RK5201	*chlE*	<0.00001	0.033
RK5228	*chlE*	<0.00001	0.034
RK5255	*chlE*	<0.00001	0.029
RK5206	*chlG*	0.0025	0.028
RK5231	*chlG*	0.0029	0.030
RK5256	*chlG*	0.0044	0.021

[a] Equal volumes of mutant extract and *chlB* strain cytoplasm were mixed and incubated for 2 h before determining activity.

[b] No increase in specific activity was observed by incubating extract plus buffer.

[c] Specific activity of the *chlB* S_2 fraction (RK5208) was 0.0044.

were fully Chl^r, made reduced amounts of gas, and retained about 1.5% of the wild-type level of nitrate reductase activity. These strains appeared to be deficient in the expression of TOR, as estimated by their phenotypes on MacConkey TMAO medium.

We examined the nitrate reductase protein composition of five *chlG*::Mu *cts* mutants by immune precipitation (Fig. 3). These strains, like the *chlE*::Mu *cts* mutants, produced intact subunits A, B, and C with some unmodified subunit B. We also tested these strains for reconstitution of nitrate reductase activity in vitro (Table 7). The behavior of all three strains was similar to that of the *chlA* and *chlE* mutants.

del Campillo-Campbell and Campbell have found that biotin sulfoxide reductase is a molybdo-enzyme and that pleiotropic *chl* mutants lack this activity (10). The locus defined by one of the mutations they studied, *bisD*, appears to be identical to *chlG*. They found that biotin sulfoxide reductase activity is increased when the *bisD* strain is cultured in the presence of relatively high levels of MoO_4^{-2}. We extended this observation to nitrate reductase activity with three of our *chlG*::Mu *cts* mutants, strains RK5206, RK5231, and RK5266. We found that the nitrate reductase specific activity in these mutants was increased approximately threefold with respect to the wild-type increase when the cells were cultured in basal anaerobic medium supplemented with 10 mM Na_2MoO_4 (data not shown). Higher MoO_4^{-2} concentrations significantly inhibited cell growth; we are therefore hesitant to draw any conclusions concerning phenotypic restoration of enzyme activity at these concentrations.

DISCUSSION

The nitrate reductase activity from a variety of procaryotic and eucaryotic organisms has been extensively studied from both a genetic and a physiological viewpoint. Most genetic studies have used mutants which were selected as being chlorate resistant. In all cases of which we are aware, a number of unlinked *chl* loci have been identified. *E. coli* is typical, as *chl* mutations have been found at six distinct loci.

Most studies of nitrate reductase have employed either spontaneous or chemical mutagen-induced mutants. We utilized insertion elements to generate a set of isogenic, independent *chl* mutants of *E. coli*. The advantages of insertion mutations are that they are easily defined: the affected gene suffers complete loss of function, and the mutations are not usually allelic, so that mutants with different phenotypes are probably affected in different genes. Since "mutagenesis" is performed at low "dosage," most mutants differ from the wild type by only a single lesion. Finally, insertion mutations are completely polar on the expression of distal genes in an operon (18, 21).

We initially isolated and mapped a number of *chl*::Mu *cts* mutations which were selected as conferring chlorate resistance. Since the functions of the *chlA*, *chlB* (27), and *chlD* (37) loci are reasonably well defined, we have not characterized these mutants extensively. In contrast, very little information exists for the *chlE* (16, 25) and *chlG* (15, 19) loci, so we focused our attention on these mutants.

The *chlE*::Mu *cts* mutants that we examined were phenotypically identical to *chlA* mutants in all of the criteria that we tested: apoenzyme composition (including cytochrome b_{566}^{NR}), pleiotropy, and behavior in the in vitro reconstitution system. Using spectral analyses, Hackett and Bragg (personal communication) also detected wild-type cytochrome patterns in the three *chlE*::Mu *cts* strains that they studied. Thus, in contrast to our earlier report suggesting that *chlE* encodes a nitrate reductase polypeptide (25), we propose that the *chlE* function, like the *chlA* function, is required for the synthesis or insertion of molybdenum cofactor.

Our *chlG*::Mu *cts* mutants also retained intact apo-nitrate reductase and were complemented by a *chlB* soluble extract in our in vitro reconstitution assay. In accordance with the results of del Campillo-Campbell and Campbell (10), we observed that molybdate promoted a small but highly reproducible phenotypic restoration of nitrate reductase activity. Thus, our *chlG* mutants resemble *chlD* mutants, both in this respect and in the fact that both classes of mutants are phenotypically leaky with respect to

molybdo-enzyme activities (10, 37). Sperl and DeMoss suggested that the *chlD* function facilitates the activation of nitrate reductase at normal, low concentrations of molybdate. One possible explanation for the role of *chlG* is that it provides another component required for this process.

The cumulative evidence from both our own work and that of other laboratories (1, 8, 10, 15) strongly supports the molybdenum cofactor hypothesis stated by Dubourdieu et al. (12). This hypothesis is that the pleiotropic *chl* loci (*chlA*, *B*, *D*, *E*, and *G*) are all involved in the synthesis, processing, or insertion of molybdenum cofactor. Pleiotropic *chl* mutants of *E. coli* and other enteric bacteria lack the activities of all known molybdo-enzymes: nitrate reductase, formate dehydrogenase (16), TOR (8, 35), tetrathionate reductase (6, 31), and biotin sulfoxide reductase (10). Anaerobic enzymes which do not require molybdenum, such as fumarate reductase and nitrite reductase, are unaffected in *chl* mutants. All of the pleiotropic *chl* mutants are resistant to relatively high levels of chlorate, and purified nitrate reductase and TOR have both been shown to have a significant chlorate reductase activity (14, 35). We speculate that the "chlorate reductase" activity of *E. coli* is the sum of the activities of some or all of the molybdo-enzymes listed above and that single-step resistance to high levels of chlorate occurs only by the loss of a component which is common to all of these enzymes. We suggest that molybdenum cofactor is one such component.

The synthesis of large amounts of nonfunctional apomolybdo-enzymes in *chl* mutants may provide pressure for the selection of secondary mutations in genes which regulate anaerobic respiratory pathways. We found that a number of the *chl* mutants which we isolated for this study had each acquired an additional mutation in the *fnr* locus. Mutations at *fnr*, which is unlinked to any of the *chl* loci, result in the loss of expression of several anaerobic enzymes, including nitrate reductase, nitrite reductase, fumarate reductase (22, 30), and TOR (unpublished data). The *fnr* gene product has been proposed to act as a positive regulator for the expression of anaerobic enzymes in *E. coli* (30). We speculate that some of the conflicting data that have been reported concerning the biochemical and physiological phenotypes of *chl* mutants have been due to the presence of cryptic *fnr* mutations in some of the strains studied.

Our insertion mutations in the *chlC* locus are phenotypically very different from insertion mutations in the other *chl* loci described above. We did not recover any *chlC* mutants among the nearly 200 mutants selected for high-level chlorate resistance. We have used the mnemonic Nar to designate the phenotypes of these mutants, and we propose that individual genes identified at this locus be termed *nar* to distinguish them from the other, pleiotropic *chl* loci. We caution the reader not to confuse the *nar* genes of the older literature (17, 38) with the *nar* mutations we report herein. Using MNT medium, we recovered four phenotypic classes of Tn*10* insertion mutations ($NarG^-$, H^-, I^-, and K^-). We also isolated a single Tn*10* insertion mutant, $NarL^-$, by selecting for resistance to a low level of chlorate.

Bonnefoy-Orth et al. (4) have recently proposed that the structural genes for nitrate reductase subunits A and C are arranged in an operon at the *chlC* locus, with transcription occurring clockwise with respect to the *E. coli* genetic map. Our results are consistent with, and provide further evidence for, this hypothesis. Our $NarG^-$ mutant appeared to lack nitrate reductase subunits A and C, and the $NarH^-$ and $NarI^-$ mutants appeared to lack only subunit C. Given the polar nature of Tn*10* insertions, this result is consistent with an operon arrangement of promoter-*narG-narI*. Our results from three-point transduction crosses (Table 4 and unpublished data) also suggest this proposed gene order.

Our antiserum was raised against purified subunits A and B, so that we could detect subunit C only if it was associated with subunits A or B or both and thus coprecipitated (24, 26). N. Hackett and P. Bragg (personal communication) have used low-temperature difference spectroscopy and redox titration to examine the cytochrome content of several of our strains. They found that the cytochrome complement of our $NarK^-$ strains was identical to that of the wild type. The $NarG^-$ and $NarI^-$ strains that they examined appeared to lack only a cytochrome, which is probably cytochrome b_{556}^{NR} (i.e., subunit C).

Our preimmune serum recognized a polypeptide with an apparent molecular weight of 60,000; therefore, we could not unequivocally determine whether the $NarG^-$ strain produced subunit B. However, all of the strains (including the wild type), except the $NarG^-$ mutant, appeared to accumulate subunit B′ in the cytoplasm (Fig. 2) (26). We take this as presumptive evidence that the $NarG^-$ strain did not stably produce subunit B. Clearly, further analyses are required to identify the structural gene for subunit B.

We found that our $NarG^-$ mutants retained approximately 1% of the wild-type nitrate reductase activity (Table 5). If the *nar-202*::Tn*10* mutation in strain RK5265 defines the structural gene for the catalytic (A) subunit, then this insertion mutant would be expected to com-

pletely lack nitrate reductase activity. We found that this residual activity was not induced by nitrate; we therefore suggest that it is merely a gratuitous activity contributed by some other molybdo-enzyme(s).

ChlI mutants (4) have a high level of enzyme activity when assayed with reduced viologen as the electron donor, but a low activity when assayed with physiological donors such as formate. It was hypothesized that these mutants are affected in the structural gene for apocytochrome b_{556}^{NR}. Our NarI$^-$ mutations resulted in a very similar phenotype, mapped in the same position as *chlI*, and appeared to result in the loss of cytochrome b_{556}^{NR}. We suggest that *nar-204*::Tn*10* (NarI$^-$) defines the same gene as that defined by the *chlI* mutations.

We only isolated a single NarH$^-$ insertion mutant. This strain had a "weak NarI$^-$" phenotype; that is, the viologen-linked nitrate reductase activity, although still significantly higher than that of the NarG$^-$ mutant, was approximately fivefold lower than that of the NarI$^-$ mutant (Table 5). In addition, subunit B from the NarH$^-$ membrane fraction appeared to be predominantly in the modified form, whereas that from the NarI$^-$ strain appeared to be mostly unmodified (Fig. 2) (26). Thus, the phenotypes of strains RK5263 (NarH$^-$) and RK5267 (NarI$^-$) are somewhat distinct. We are currently analyzing these mutations to ascertain whether they define separate genes.

Our data imply that the NarL$^-$ phenotype reflects the loss of a nitrate-sensitive positive regulator for nitrate reductase expression. Nitrate reductase activity in the NarL$^-$ mutant was indifferent to induction by nitrate. The data in Table 6 are consistent with the findings of Showe and DeMoss (36), who determined that induction of nitrate reductase is prevented in the presence of oxygen, occurs at a low level in the absence of oxygen, and reaches maximum levels in the presence of nitrate. This observation implies that the regulation of nitrate reductase expression occurs on two independent levels (36).

We speculate that the level of nitrate reductase activity in this mutant is sufficient to mediate at least partial nitrate repression of other anaerobic enzymes, which include most of the known molybdo-enzymes in *E. coli*. However, the remaining low level of nitrate reductase activity is not sufficient to make the cells completely chlorate sensitive. We only selected a single NarL$^-$ insertion mutant, but recently we isolated several NarL$^-$ mutants while isolating pseudorevertants of NarI$^-$ strains. Our preliminary characterization indicates that these NarL$^-$ mutations are very similar to the one we have described here.

We isolated several NarK$^-$ mutants as being derepressed for TOR in the presence of nitrate (9, 30, 40). Since nitrate reductase seemed to be functional in these mutants, the gene defined by *nar-203*::Tn*10* (NarK$^-$) might encode a component which mediates this nitrate repression. We stress that we have not assayed the activities of any of these repressed enzymes in this mutant. Clark and Cronan (7) isolated mutations termed *adh*, which result in the overproduction of alcohol dehydrogenase, an enzyme which is normally subject to nitrate repression. These *adh* mutations, like the NarK$^-$ lesions, map in the *chlC* locus. Meanwhile, Pascal et al. (32) have identified a mutation termed *ana*, which prevents the expression of some nitrate-repressed enzymes. *ana* also maps very close to the *chlC* locus.

These observations have led us to devise a model to guide our future experimental approach. Others (30) have suggested that *fnr* encodes a positive regulator that activates the transcription of genes which encode anaerobic respiratory enzymes. Nitrate respiration is the preferred anaerobic pathway, so that a second regulatory system may be required to repress transcription of other anaerobic respiratory pathways when nitrate respiration is functioning. This putative regulatory system would have to counter the effects of *fnr* regulation on these alternate pathways. We postulate that the NarK$^-$ phenotype reflects the loss of a gene product(s) which responds to nitrate respiration and acts to repress the transcription of the other respiratory enzymes. The *ana* mutation might be an alternate allele of *narK*, or perhaps it defines a separate gene involved in this regulatory mechanism.

Previous work (11, 16, 17, 25) has indicated that the *chlC* locus contains at least one of the nitrate reductase structural genes. Our data lend support to this hypothesis and suggest that the structural genes for nitrate reductase subunits A and C (at least) are arranged in an operon at this locus (4). Our work is also consistent with the study by Glaser and DeMoss (15), who showed that most *chlC* mutants remain chlorate sensitive. In addition, our data support the conclusion drawn from recent studies (7, 32) which suggest that the *chlC* locus also contains regulatory genes. We do not suppose that we have identified all the genes involved in nitrate respiration and its regulation, because our mutant isolation scheme has had two constraints: we demanded null mutations by virtue of insertion element mutagenesis, and we largely relied on the TOR-derepressed phenotype to identify mutants. Further genetic analysis, using various mutagens and differentiation protocols, will be necessary to fully define this system.

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