Expression of *Klebsiella pneumoniae* Nitrogen Fixation Genes in Nitrate Reductase Mutants of *Escherichia coli*

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SUMMARY

Nitrate reductase (*nar*) *A*, *B* and *E* mutants of *Escherichia coli* with plasmids carrying *Klebsiella pneumoniae* nitrogen fixation (*nif*) genes reduced acetylene independently of added molybdate, but *narD* mutants showed pleiotropic dependence on the concentration of added molybdate for expression of both *nar* and *nif*. No complementation of *nar* mutations by *nif* occurred; nitrite but not nitrate repressed *nif* in *nar* hosts. Derepression of *nif* occurred in molybdenum-deficient *narD* (*nif*) strains since nitrogenase peptides were present. *nifB* mutants, thought to have a lesion in the pathway of molybdenum to nitrogenase, as well as *nif* deletion mutants, had normal nitrate reductase activity.

INTRODUCTION

Nitrogenase and nitrate reductase are both multimeric molybdoproteins and correlations in structure, function and incidence have been suggested. In pot studies, a correlation between the nitrate reductase content of *Rhizobium japonicum* and symbiotic efficiency was noted (see Cheniae & Evans, 1960; Evans & Russell, 1971), which gained support at the genetic level from the observation by Kondorosi *et al.* (1973) that nitrate reductase mutants of *R. meliloti* had lost effectiveness in plant nodulation but regained it on reversion (Sik *et al.*, 1976). At the molecular level, a molybdenum-containing fragment generated by acid treatment of nitrogenase, or other molybdoenzymes, complements nitrate reductase activity in the *nit-1* mutant of *Neurospora crassa* (see Pan *et al.*, 1976). Acid-treated nitrogenase restored acetylene-reducing activity to extracts of the *nifB* class of nitrogen-fixation mutants of *Klebsiella pneumoniae* (St John *et al.*, 1975). *Klebsiella* Nif⁻ mutants which pleiotropically showed chlorate resistance were mentioned by Streicher, Gurney & Valentine (1971), and Tubb (1974) described a regulatory Nif⁻ mutant which was pleiotropically negative in assimilatory nitrate reduction.

Among the characterized nitrate reductase mutants in *Escherichia coli* (Venables, 1972) is a class, *narD*, in which nitrate reductase activity is restored by high levels of added molybdate (Glaser & De Moss, 1971; Sperl & De Moss, 1975). This organism contains Pichinoty's (1973) class A nitrate reductase for which chlorate is a substrate and is converted to the toxic chlorite; chlorate resistance thus correlates with absence of dissimilatory nitrate reductase activity and the *chl* and *nar* genotypes are identical. We shall use the *nar* nomenclature in this paper, which reports studies on the expression of *K. pneumoniae* *nif* genes introduced into various *nar* mutants of *E. coli*, and on the nitrate reductase activity of certain *nif* mutants of *K. pneumoniae*.

METHODS

Organisms and culture. Table 1 lists the bacteria used, their genotypes and sources. They were maintained on nutrient agar slopes, with appropriate selection for drug-resistance markers in strains carrying *nif* plasmids.

For most tests we used a low-nitrogen medium (NFDM; Cannon *et al.*, 1974) containing Casamino acids ($100\ \mu\text{g ml}^{-1}$) but with no added molybdate. Concentrated phosphate buffer was extracted with 8-hydroxyquinoline and CHCl_3 before being autoclaved and added to the medium, which was prepared and used in acid-washed glassware. Sterile sodium molybdate, sodium nitrate or sodium nitrite was added as necessary and the following supplements required by the *nar* strains were included if needed ($\mu\text{g ml}^{-1}$): thiamin, 1; L-leucine, L-threonine and L-histidine, 20; biotin, 1. Complete media were Oxoid no. 2 broth solidified with 1.5% (w/v) agar with, if necessary, the following additions ($\mu\text{g ml}^{-1}$): streptomycin, 200; carbenicillin, 100; kanamycin, 20. For chlorate resistance tests, KClO_3 was added to $50\ \mu\text{mol ml}^{-1}$.

Plasmid transfer. *Escherichia coli* 1C5466 carrying the F prime plasmid FN68 was mated with *E. coli nar* recipients in broth at 37°C for 1 to 1.5 h and exconjugants were selected for Sm^r , Cb^r . The same donor carrying the P prime plasmid RP41 was mated with *nar* recipients on nutrient agar for 6 h at 37°C and exconjugants were selected for Sm^r , Cb^r , Km^r .

Transduction. Bacteriophage P1c1r100 Km was used as described by Goldberg, Bender & Streicher (1974) to transduce various *his*-linked *nif* mutations into *K. pneumoniae hisD2 nif*⁺ strain kp5022. Phage lysates were prepared from P1c1r100 Km lysogens of the *his*⁺ *nif* mutants. The recipient strain kp5022 was infected with phage and subsequently plated on minimal glucose medium to select for His⁺ transductants. Colonies were patched on NFDM agar plates and incubated anaerobically to screen for cotransduction of *nif*.

Enzyme activity. Nitrate reductase activity was assessed qualitatively as nitrite formed either after growth for 16 to 20 h at 37°C in stationary tubes of Oxoid dextrose/peptone broth containing 0.1% (w/v) KNO_3 or after anaerobic growth under argon in the Mo-free low-nitrogen glucose medium containing NaNO_3 ($10\ \text{mg ml}^{-1}$). A more precise assessment was obtained by anaerobic culture for 16 to 20 h in NFDM plus Casamino acids; NaNO_3 was added to $0.25\ \text{mg ml}^{-1}$ and, after 30 to 60 min at 37°C , nitrite formation in 1 ml of culture was assessed colorimetrically and graded visually. Diazotization of sulphanilic acid by a portion of culture followed by coupling with 'Cleve's acid' (Trollope & Evans, 1975) or β -naphthylamine was used to obtain a pink coloration.

Nitrogenase activity was assessed by the acetylene-reduction method. Cultures in nutrient broth were diluted 1:50 in 3.5 ml portions of NFDM plus Casamino acids medium in 5 ml sterile bijou bottles capped with Suba-Seal rubber closures. After shaking at 30°C for 16 to 36 h to allow derepression of nitrogenase synthesis, 1 ml C_2H_2 was injected and 30 to 60 min later the amount of C_2H_4 formed was measured on a Pye gas chromatograph with a 20 cm column of Porapak N and a flame ionization detector working in a stream of N_2 .

Electrophoresis. Derepressed cells grown at 30°C under N_2 in NFDM plus Casamino acids for 36 to 48 h, and repressed control cells grown similarly but with NH_4Cl ($1\ \text{mg ml}^{-1}$) were centrifuged, and the pellet was resuspended in 0.2 ml buffer containing sodium dodecyl sulphate ($20\ \text{mg ml}^{-1}$) and heated in a boiling water bath for 2 min. Extract ($75\ \mu\text{l}$ containing about $250\ \mu\text{g}$ bacterial protein) was loaded into each sample well of an SDS-polyacrylamide slab gel prepared according to the Ortec buffer recipes (Kennedy *et al.*, 1976) in order to ensure that the two subunit peptides of nitrogenase Mo-Fe protein resolved into two bands

Table 1. *Bacterial strains and plasmids*

Strain	Genotype*	Source or reference
<i>E. coli</i> K12		
R594	<i>thr leu thi bio strA gal</i>	Venables (1972)
C198	R594 <i>narA</i>	Venables (1972)
C218	R594 <i>narB</i>	Venables (1972)
C202	R594 <i>narE</i>	Venables (1972)
W602	<i>thr leu thi bio strA</i>	Venables (1972)
C111	W602 <i>narD</i>	Venables (1972)
JC5466	<i>trp recA56 his spc</i>	N. Willetts
<i>K. pneumoniae</i> M5a1		
KP5022	<i>hisD2 nif⁺ str</i>	Streicher <i>et al.</i> (1974)
Δ52	Δ(<i>gnd-his-nif</i>)	S. Streicher
Δ107	Δ(<i>gnd-his-nif</i>)	R. A. Dixon
UN142	<i>nifH hisD</i>	St John <i>et al.</i> (1975)
UN150	<i>nifB hisD</i>	St John <i>et al.</i> (1975)
UN316	<i>nifD hisD</i>	St John <i>et al.</i> (1975)
UN318	<i>nifE hisD</i>	St John <i>et al.</i> (1975)
UN328	<i>nifB hisD</i>	St John <i>et al.</i> (1975)
UN364	<i>nifH hisD</i>	St John <i>et al.</i> (1975)
UN587	<i>nifF his</i>	St John <i>et al.</i> (1975)
Plasmids		
FN68	F' <i>his nif</i> Cb ^r	Cannon, Dixon & Postgate (1976a)
RP41	P' <i>his nif</i> Cb ^r Km ^r Tc ^r	Dixon, Cannon & Kondorosi (1976)

* Symbols for chromosome markers after Bachmann, Low & Taylor (1976). Symbols for plasmid-determined drug resistance are: Cb, carbenicillin; Km, kanamycin; Tc, tetracycline.

during electrophoresis. Gels were stained with Coomassie blue. In these conditions, de-repressed cells yield about 28 bands on the gels of which three prominent ones correspond in position to the two subunits of nitrogenase Mo-Fe protein and the single subunit of nitrogenase Fe protein. These three are absent from gels from NH₄⁺-repressed cells. Nitrogenase represents 10 to 15% of total cell protein in fully-derepressed cells. Nitrogenase levels lowered more than 5- to 10-fold (10 to 20% of fully-derepressed levels) would not be detected by this method.

RESULTS

The *nar*⁺ strains *E. coli* R594 and W602 and the four nitrate reductase mutants (*narA*, *narB*, *narD*, *narE*) into which the plasmids FN68 or RP41 were introduced showed normal nitrogenase activities in media with molybdate at 10⁻⁴ M. All parent strains and *nar* mutants without plasmid were inactive. The *narD* mutants, but none of the other strains carrying RP41, showed a requirement for added molybdenum comparable to that reported by Glaser & De Moss (1971) for nitrate reduction (Table 2). The possession of RP41 did not restore the Nar⁺ phenotype to any of the mutants, whether in growth tests, in nitrite formation or in restoration of chlorate sensitivity. Comparable checks for chlorate sensitivity or nitrite formation were negative in *nar* mutant strains carrying FN68.

The dependence of nitrogenase activity on molybdate concentration in a *narD* background was investigated further. When *E. coli* C111(RP41) was grown without added molybdate, addition of molybdate followed by acetylene 2 h later restored the Nif⁺ phenotype (Table 3). When chloramphenicol (100 µg ml⁻¹) was added shortly before the molybdate, it prevented subsequent expression of acetylene-reducing activity. SDS-polyacrylamide gel electrophoresis of extracts from C111(RP41) grown without added molybdate showed the presence of nitrogenase proteins in normal derepressed amounts. Inactive nitrogenase was

Table 2. *Nitrogenase and nitrate reductase activities of nar⁺ and nar strains of E. coli*

Nitrogenase activity is expressed as nmol C₂H₄ formed min⁻¹ (mg bacterial protein)⁻¹: average of two to four tests. Nitrite formation was determined colorimetrically as described in Methods: ++, dark pink, strongly positive; +, light pink; -, colourless, negative. Growth on nitrate was indicated by colony formation or cell turbidity in liquid medium: +, growth; -, no growth. s, sensitive; R, resistant; NT, not tested.

Strain	Nitrogenase activity		Nitrate reductase activity detected by :		
	No Na ₂ MoO ₄ added	100 µM-Na ₂ MoO ₄ added	Nitrite formation	Growth on nitrate	Chlorate resistance
R594 <i>nar</i> ⁺	NT	0	++	+	S
R594(RP41)	75	85	++	+	S
W602 <i>nar</i> ⁺	NT	0	+	+	NT
W602(RP41)	35	42	+	+	NT
C198 <i>narA</i>	NT	0	-	-	R
C198(RP41)	65	53	-	-	R
C218 <i>narB</i>	NT	0	-	-	R
C218(RP41)	25	27	-	-	R
C202 <i>narE</i>	NT	0	-	-	R
C202(RP41)	15	25	-	-	R
CI11 <i>narD</i>	0	0	(- MoO ₄ ²⁻) -	-	NT
			(+ MoO ₄ ²⁻) +	+	NT
CI11(RP41)	< 0.3	30	(- MoO ₄ ²⁻) -	-	NT
			(+ MoO ₄ ²⁻) +	+	NT

Table 3. *Molybdate dependence of nitrogenase activity in E. coli CI11(RP41) narD*

Bacteria were grown without added molybdate as described in Methods. After 20 h molybdate was added and the cultures were shaken for 2 h. Acetylene was then added to each bottle and ethylene formation was measured after 30 to 60 min. Nitrogenase activity is expressed as nmol C₂H₄ formed min⁻¹ (mg protein)⁻¹.

Na ₂ MoO ₄ added (M)	Nitrogenase activity
0	< 0.5
10 ⁻⁸	< 0.5
10 ⁻⁷	< 0.5
5 × 10 ⁻⁷	1.4
10 ⁻⁶	2
5 × 10 ⁻⁶	19
10 ⁻⁵	18
10 ⁻⁴	20
10 ⁻³	16

thus present in the Mo-deficient *narD* (*nif*) mutant and protein synthesis was necessary for its activation after addition of molybdate.

Repression of nitrogenase by nitrate. Nitrate, like ammonia, prevents expression of nitrogenase activity but several mechanisms other than direct co-repression at the genetical level by nitrate might operate. For example, Sorger (1969) showed that a *chl^r* mutant of the aerobe *Azotobacter vinelandii* escaped nitrate repression of nitrogenase activity. Nitrogenase activities of various *E. coli* strains carrying *nif* genes on RP41 were tested after growth in NFDM plus Casamino acids with nitrite or nitrate. Nitrite repressed nitrogenase activity in all strains tested, but the *narA*(RP41) and *narB*(RP41) strains grown in nitrate showed nearly normal levels of acetylene reduction (Table 4) (*narD* and *narE* were not tested).

Table 4. Nitrate and nitrite repression of nitrogenase in nar mutants of *E. coli*

Cultures of *nar* mutants and of the parent type carrying *nif* plasmids were grown anaerobically in NFDM plus Casamino acids at 37 °C with or without the supplements listed. Nitrogenase activity is expressed as nmol C₂H₄ formed min⁻¹ (mg protein)⁻¹.

Strain	Supplement (20 mM)	Nitrogenase activity
R594(RP41) <i>nar</i> ⁺	None	75
	NaNO ₃	1.7
	NaNO ₂	< 0.2
C198(RP41) <i>narA</i>	None	51
	NaNO ₃	39
	NaNO ₂	< 0.2
C218(RP41) <i>narB</i>	None	68
	NaNO ₃	34
	NaNO ₂	0.5

SDS-polyacrylamide gel electrophoresis of extracts prepared from these strains showed no nitrogenase proteins in nitrite-grown cells nor in *nar*⁺ nitrate-grown cells. It follows that nitrate is not itself a co-repressor but must be metabolized to nitrite in order to repress synthesis of nitrogenase.

Nitrogenase and the nifB locus. The *nif* cluster includes the gene *nifB* which is probably concerned with the incorporation of Mo into nitrogenase. In view of the pleiotropy between *nif* and *narD*, the question arises whether *nifB* shows pleiotropy with nitrate reduction. All the UN strains in Table 1 (*nifF*, *nifH*, *nifD*, *nifE*, as well as *nifB*) were chlorate-resistant and lacked nitrate reductase activity; hence they were unsuitable for a direct test of this question. His⁺ revertants of the UN strains were therefore selected and their mutant *nif* genes were separately transduced into *K. pneumoniae* strain Kp5022. Two His⁺ Nif⁻ strains were picked from each transduction; all showed full nitrate reductase activity, including the *nifB* transductants. Strains Δ52 and Δ107 of *K. pneumoniae* which have chromosomal deletions extending through *nifB* (Dixon *et al.*, 1977) grew normally with nitrate as nitrogen source and formed nitrite.

DISCUSSION

The expression of *K. pneumoniae nif* genes in *nar* mutants of *E. coli* shows clear pleiotropy between *nif* expression and mutation in the *narD* gene, which is involved in assimilation of the molybdenum necessary for nitrate reductase activity. An earlier report to the contrary (Cannon *et al.*, 1976b) was mistaken and arose from a confusion of medium recipes in our laboratory. The probability is that the *narD* locus specifies a molybdenum insertase rather than an uptake protein, the insertion taking place at high molybdate concentration (Sperl & De Moss, 1975). Evidently *nar* and *nif* share some Mo-processing functions. The *nifB* locus is clearly quite distinct from *narD*. It shows no pleiotropy with *nar* expression; the *narD* locus maps at 17 min in *E. coli* compared with 39 min for *hisD*; *nifB* is part of the *nif* cluster, close to *hisD* in *K. pneumoniae*, and is presumably absent from *E. coli*. It follows that *narD* mutants of *K. pneumoniae* might be pleiotropically Nif⁻ and the converse event, Nif⁻ mutants which show a Nar⁻ phenotype, could be of the *narD* class as well as of the regulatory class already described. They should be readily detectable by their chlorate resistance; some of those mentioned by Streicher *et al.* (1971) may have been of this class, but the frequencies of co-transduction with *hisD* suggest that some may have been fortuitous double mutants: the rate of forward mutation to Chl^r in *K. pneumoniae* can be very high (unpublished observations).

The *narD* mutation is always somewhat leaky: faint nitrite colorations occur and, in our hands, marginally low levels of *nif* expression are detected if a *nif* plasmid is present. This leakiness can be attributed to contaminating Mo in the media used, since there was obviously sufficient, without added Mo, to satisfy the requirements of the various Nif⁺ *E. coli* and *K. pneumoniae* strains used in our experiments.

Our finding that Mo-deficient *E. coli narD*(RP41) contains normal amounts of the three nitrogenase peptides is in contrast to the results of Brill, Steiner & Shah (1974), who found no antigenically cross-reacting material to either the Mo-Fe or Fe nitrogenase proteins in *K. pneumoniae* derepressed in the absence of Mo. The absence of nitrogenase proteins could be explained by their use of non-growing N₂-dependent cells in which net protein synthesis was severely reduced and derepression thereby possibly impaired. In our experiment, nitrogenase activity was not necessary for growth since cells were provided with a non-repressive source of nitrogen and we could detect formation of inactive nitrogenase. An unlikely possibility is that *nif* derepression is Mo-dependent in *K. pneumoniae* but not in *E. coli* carrying *nif* genes. In any case our experiments provide no evidence for Mo being an inducer of *nif* gene expression.

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