INTERACTION BETWEEN THE NITRATE RESPIRATORY SYSTEM OF ESCHERICHIA COLI K12 AND THE NITROGEN FIXATION GENES OF KLEBSIELLA PNEUMONIAE

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SUMMARY: Hybrids were constructed between E. coli K12 chl$^-$ mutants defective in nitrate respiration and an F' plasmid carrying nitrogen fixation genes from K. pneumoniae. Examination of these hybrids showed that expression of nif$^+_{Kp}$ genes does not require a functional nitrate respiratory system, but that nitrate reductase and nitrogenase do share some Mo-processing functions. For nitrate repression of nitrogenase activity, reduction of nitrate to nitrite is not necessary, but the Mo-X cofactor encoded by chl genes is essential. Nitrate probably inhibits nitrogen fixation by affecting the membrane relationship of the nitrate and fumarate reduction systems such that the membrane cannot be energized for nitrogenase activity.

INTRODUCTION:

Evidence suggesting that the membrane-bound nitrate reductase and nitrogenase enzyme complexes may share a common molybo-protein subunit has been presented (1, 2, 3), although other studies have provided little support for this proposed commonality (4).

The nitrate respiratory complex has been well-studied in E. coli K12 (5, 6, 7). Mutants defective in their inducible formate-nitrate reductase complex have been isolated because of resistance to chlorate, which nitrate reductase can reduce to toxic chlorite (8, 9).

Therefore, the possible relationship between the nitrate respiratory system and the nitrogenase of K. pneumoniae was investigated by using hybrids constructed between various E. coli K12 mutants defective in nitrate respiration (chl$^-$) and the FN68 plasmid which carries the nitrogen fixation genes from K. pneumoniae (nif$^+_{Kp}$).

MATERIALS AND METHODS

Bacterial strains: E. coli K12 strain SB1801 his$^-$ mal$^-$ carrying the

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FN68 plasmid (F'nif^+_{Kp} his^+_{Kp} Cb^R) (10) was used as the donor in conjugation experiments with nitrate reductase (chl^-) mutants of E. coli K12, and also with pleiotropic nif^- chl^- mutants of K. pneumoniae strain M5al.

Media: Nitrogen-free medium (NFM) was that of Cannon et al. (11). Growth in liquid NFM was measured by following turbidity in a nephelometer. When necessary, NFM was supplemented with vitamin-free Casamino acids at 100 µg/ml, amino acids at 25 µg/ml and vitamins at 10^{-3} µM.

Conjugation: E. coli K12 strain SB1801 (FN68) was used as the donor in conjugation experiments with mal^+ recipient strains. Mal^+ Cb^R hybrids were selected and purified as described previously (12), and were then tested for nitrogen fixation by growth on NFM and by acetylene reduction.

Acetylene reduction assay: Rates of acetylene reduction to ethylene were measured by the method of Tubb & Postgate (13). In all cases, addition of 50 mM $(NH_4)_2$ SO_4 to the medium repressed nitrogenase activity as measured by acetylene reduction.

RESULTS AND DISCUSSION

None of the nitrate reductase mutants prevented or reduced phenotypic expression of nif^+_{Kp} genes (Table 1). Even the loss of chlC, the structural gene for a subunit of the nitrate reductase enzyme (5), did not prevent nitrogen fixation. Likewise, a functional chlA gene, coding for a Mo-X cofactor (5) is unnecessary for nitrogenase activity. However addition of 10^{-4} M MoO_4^{2-} ions was essential for full nitrogenase activity in $chlD^-$ mutants indicating an interaction between chlD and nif^+ functions.

To examine the mechanism by which nitrate inhibits nitrogen fixation, hybrids carrying the FN68 plasmid were grown in NFM with KNO_3 or KNO_2, and were tested for acetylene reduction. Only the $chlA^-$ and $chlB^-$ hybrids showed no nitrate inhibition of nitrogenase activity; these hybrids gave similar levels of acetylene reduction in NFM with or without nitrate (Table 1).

Thus, the Mo-X cofactor coded for by chlA (5) and the association factor coded for by chlB (5) are necessary for nitrate inhibition of nitrogen fixation, indicating that molybdenum-processing functions are shared by nitrogenase and nitrate reductase. However, conversion of nitrate to nitrite is not necessary for inhibition, since nitrate still caused loss of acetylene reduction in the $chlC^-$ hybrid. Similarly in the $chlD^-$, $chlE^-$, and $chlC^-$ hybrids, nitrate still inhibited nitrogen fixation. Since the

TABLE 1. Phenotypic expression of *nif*$^{+}_{Kp}$ genes on plasmid FN68 in nitrate reductase mutants of *E. coli* K12

E. coli K12 hybrid	*chl* mutation	Acetylene Reduction		
		NFM	NFM+ KNO_3	NFM+ KNO_2
C181(FN68)	*chlA*$^{-}$	39.0	38.0	0.9
KB70(FN68)	*chlA*$^{\Delta}$	54.4	58.6	0.6
C183(FN68)	*chlB*$^{-}$	43.0	40.8	0.08
DD115(FN68)	*chlB*$^{-}$	31.2	32.2	0.08
Puig 426(FN68)	*chlC*$^{-}$	35.4	0.08	<0.01
C111(FN68)	*chlD*$^{\Delta}$	46.0	<0.01	<0.01
C113(FN68)	*chlD*$^{-}$	46.3	<0.01	<0.01
C123(FN68)	*chlD*$^{-}$	39.1	<0.01	<0.01
C197(FN68)	*chlE*$^{-}$	49.0	<0.01	<0.01
C202(FN68)	*chlE*$^{-}$	45.0	<0.01	<0.01
DD38(FN68)	*chlG*$^{-}$	56.8	0.3	0.1
SA291(FN68)	*chlA*$^{\Delta}$ *chlD*$^{\Delta}$	40.2	40.2	0.2

Acetylene reduction was measured in nmol C_2H_4/min/mg protein. KNO_2 and KNO_3 were added at final concentrations of 50 mM.

various *chl*^{R} mutations also cause loss of the formic hydrogenlyase system (6), inhibition of nitrogenase activity by nitrate is unlikely to be acting through this system.

Nitrogenase activity in all hybrids tested was inhibited by KNO_2. However, in the *chlA*$^{-}$, *chlB*$^{-}$ and *chlG*$^{-}$ hybrids, there was a reproducible low level of acetylene reduction which may indicate incomplete repression by nitrite (Table 1).

TABLE 2. Growth of *E. coli* K12 (F'nif^{+}_{Kp}) hybrids in NFM with KNO_3 or KNO_2

Hybrid	*chl* mutation	Growth		
		NFM	NFM+ KNO_3	NFM+ KNO_2
C181(FN68)	*chlA*$^{-}$	100	47	64
KB70(FN68)	*chlA*$^{\Delta}$	100	93	49
C183(FN168)	*chlB*$^{-}$	100	220	106
DD115(FN68)	*chlB*$^{-}$	100	110	75
Puig 426(FN68)	*chlC*$^{-}$	100	172	57
C111(FN68)	*chlD*$^{\Delta}$	100	155	45
C113(FN68)	*chlD*$^{-}$	100	192	75
C123(FN68)	*chlD*$^{-}$	100	148	46
C197(FN68)	*chlE*$^{-}$	100	44	61
C202(FN68)	*chlE*$^{-}$	100	46	73
DD38(FN68)	*chlG*$^{-}$	100	109	37

Growth in Pankhurst tubes was measured in a nephelometer after 24 hr incubation, and is expressed as a percentage of the NFM control. KNO_3 and KNO_2 were added to give final concentrations of 50 mM.

All four possible classes of growth response and acetylene reduction were observed when hybrids were grown in NFM with nitrate (Tables 1 and 2):

(a) inhibition of both growth and nif^{+}_{Kp} phenotypic expression (*chlE*);

(b) inhibition of growth but not of nif^{+}_{Kp} phenotypic expression (*chlA*);

(c) inhibition of nif^{+}_{Kp} phenotypic expression but not of growth (*chlC*, D, G);

(d) no inhibition of either growth or $\underline{nif}^{+}_{Kp}$ phenotypic expression ($\underline{chlB}$).

Genetic and biochemical studies indicate that only one nitrate reductase exists, and that it is a complex of two distinct components: the assimilatory nitrate reductase pathway and the nitrate respiration system (14).

All the chlorate-resistant mutants are defective in their nitrate respiration system, but because of their good growth in NFM and nitrate appear to have retained a functional assimilatory nitrate reductase pathway. However, the pleiotropic mutations in the $\underline{chlA}^{-}$ and $\underline{chlE}^{-}$ mutants appear to have perturbated both complexes.

When the same hybrids were grown in NFM + nitrite, generally the Nif^{+} phenotype was completely inhibited, and except for the $\underline{chlB}^{-}$ mutants, the extent of growth was also reduced (Table 2).

Nitrate inhibition of nitrogenase activity was examined in hybrids made with mutants of *E. coli* K12 defective in particular systems for anaerobic energization of the membrane, such as mutants defective in oxidative phosphorylation, mutants affecting the fumarate-nitrate reductase complex, and quinone mutants important in either fumarate or nitrate reduction, but in all cases nitrate completely inhibited nitrogenase activity. Thus these systems are not required for nitrate inhibition of nitrogenase activity.

The FN68 plasmid did not restore any nitrate reductase activity to $\underline{chl}^{-}$ *E. coli* K12 hybrids, and thus does not carry functions to complement defects in the nitrate respiratory system.

Two $\underline{nif}^{+}$ mutants of *K. pneumoniae* strain M5a1, *nif*88 and *nif*105, which are probably defective in nitrogenase component II, and which are also defective in nitrate reduction (15), were used as recipients for the FN68 plasmid and were then tested for nitrogen fixation (Table 3). In the *nif*88(FN68) hybrid no acetylene reduction was detected, which indicates that phenotypic expression of the plasmid-borne $\underline{nif}^{+}_{Kp}$ genes is abolished by the chromosomal $\underline{nif}^{-}$ mutation. As expected, no acetylene reduction was obtained by this

TABLE 3. Phenotypic expression of *nif*$^{+}_{Kp}$ genes on plasmid FN68 in mutants of *K. pneumoniae* defective in both nitrogen fixation and nitrate reduction

Hybrid	Medium	Acetylene reduction (nmol C_2H_4/min/mg protein)	Growth
*nif*88(FN68)	NFM	<0.01	100
	NFM + NO_3^-	<0.01	77
	NFM + NH_4^+	<0.01	208
*nif*105(FN68)	NFM	8.0	100
	NFM + NO_3^-	30.0	98
	NFM + NH_4^+	<0.01	141
M5a1 *nif*$^{+}$	NFM	51.3	100
	NFM + NO_3^-	<0.01	84
	NFM + NH_4^+	<0.01	201

NO_3^- and NH_4^+ were added to NFM in Pankhurst tubes to give final concentrations of 50 mM. Growth was assessed after 24 hr incubation, by measuring turbidity in a nephelometer, and is expressed as a percentage of the NFM control.

hybrid in the presence of either nitrate or ammonia (Table 3). In marked contrast, hybrid *nif*105(FN68) reduced acetylene at a low level in NFM, but also showed a nitrate-dependent, four-fold stimulation of acetylene reduction (Table 3). This result could not be explained simply on the basis of better growth with nitrate, since growth was similar in NFM with or without nitrate (Table 3). A similar stimulation of nitrogenase activity by nitrate has been reported for soybean bacteroids (16) and also for *Spirillum lipoferum* (17). The F'*nif*$^{+}_{Kp}$ plasmid was expressed normally in other *nif*$^{-}$ *chl*^{S} mutants of *K. pneumoniae*.

These results, using *nif*$^{+}_{Kp}$ hybrids defective in nitrate respiration,

show that regulation of growth and nitrogenase activity is complex. Nitrate inhibition of nitrogen fixation may be manifested by affecting the proposed functional or organizational relationship between the fumarate and nitrate reductase systems (18) such that competition by electron carriers in the membrane prevents efficient electron transport to fumarate. Without a functional fumarate reduction system, a Nif^- phenotype occurs (19). This interpretation would also explain the results obtained with a chl^R mutant of A. vinelandii (20), where nitrate inhibited the activity but not the formation of nitrogenase. Since the $chlA^-$ and $chlB^-$ hybrids reduced acetylene even in the presence of nitrate, these functions must directly affect the proposed competition by electron carriers. However, the situation is more complicated, since nitrate actually stimulated nitrogenase activity in Klebsiella pneumoniae nif105(FN68) hybrid. The simplest explanation of this result is that the hybrid nitrogenase enzyme complex formed is less able to be activated by the normal electron transport system of Klebsiella, but addition of nitrate presumably affects the proposed competition by electron carriers such that the modified nitrogenase complex can now be fully activated.

The results obtained with $chlA^-$, $chlB^-$ and $chlD^-$ hybrids suggest that nitrate reductase and nitrogenase share Mo-processing functions. This commonality could explain the pleiotropy observed in Rhizobium meliloti (1), where some nitrate reductase mutants also lacked nitrogenase activity. Since these mutants were also altered in their nodulation capacity, it appears that defects in the nitrate respiration pathway may also affect the Rhizobium-plant interactions.

Pagan et al. (4), using nitrate reductase mutants of Rhizobium sp. 32H1, concluded that nitrate probably inhibited nitrogenase activity by its reduction to nitrite, although they did not exclude the possibility that nitrate or nitrite competed with nitrogenase for electrons, thereby lowering nitrogenase activity. These two possibilities could not be distinguished in Rhizobium due to the lack of well-defined mutants available in E. coli

K12, which have allowed a clearer analysis of the problem.

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