INTERACTION BETWEEN THE OXIDATIVE PHOSPHORYLATION GENES OF *ESCHERICHIA COLI* K12 AND THE NITROGEN FIXATION GENE CLUSTER OF *KLEBSIELLA PNEUMONIAE*

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Summary

Hybrids were constructed between *E.coli* K12 *unc*$^-$ mutants uncoupled in oxidative phosphorylation, and thus defective in ATP biosynthesis, and an F' plasmid carrying nitrogen fixation genes from *Klebsiella pneumoniae*. Examination of these hybrids showed that expression of *nif*$^+_{Kp}$ genes in *E.coli* K12 does not require coupling of oxidative phosphorylation but needs the contribution of an anaerobic electron transport system involving fumarate reduction. The *nif*$_{Kp}$ cluster of genes does not contain functions able to complement a defective Mg^{2+}-ATPase aggregate but does contain a function(s) which appears to interact with the *uncB*$^-$ mutant over the formation of a redox system.

Introduction

Nitrogen fixation is a biological process by which atmospheric nitrogen is converted to ammonia by the complex enzyme nitrogenase (1). This enzyme, specified by *nif* genes (2), requires ATP as well as a suitable reductant for activity (1).

The bacterium *Klebsiella pneumoniae*, which is closely related to *Escherichia coli* (a non-nitrogen fixer) has been extensively studied and shown to fix nitrogen under anaerobic conditions (3). However, little is known of the electron transport pathway that couples the oxidation of substrate to

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reduction of the nitrogenase enzyme, but a similarity was suggested between Klebsiella and E. coli in the generation and transport of low-potential reducing power from pyruvate (3). In fact, hybrids can be made between K. pneumoniae and E. coli C and K12 strains and shown to fix nitrogen (4,5,6).

Nitrogen fixation thus depends on appropriate electron transport reactions, adequate energy supplies, and probably on the incorporation of the nitrogenase enzyme into an organisational unit associated with membranes. Because of the availability of genetically and biochemically well characterized mutants in E. coli, we have constructed hybrid strains carrying an F-prime nif^{+}_{Kp} plasmid (nif^{+} genes from K. pneumoniae) to study which energy pathways might be coupled to nitrogenase activity.

Materials and Methods

Bacterial strains: E. coli K12 strain SB1801 his^{-} mal^{-} carrying the FN68 plasmid (F' nif^{+}_{Kp} his^{+}_{Kp} Cb^{R}) which carries about 3 minutes of the E. coli K12 ^{Kp}genome from shiA to metG (5) was used as the donor in conjugation experiments because the nif^{+}_{Kp} genes are stably linked to the carbenicillin resistance marker (Cb^{R}).

Recipients used were the isogenic E. coli K12 strains AN259 $argH^{-}$ $entA^{-}$, AN249 $uncA^{-}$ $argH^{-}$ $entA^{-}$; AN283 $uncB^{-}$ $argH^{-}$ $entA^{-}$; AN285 unc405 $argH^{-}$ $entA^{-}$ (7,8), and AN771 $uncC^{-}$ $argH^{-}$ $entA^{-}$ (Cox, unpublished).

For retransfer of the plasmid, SB1801 was used as the recipient strain.

Media: EMB and LB glucose media have been described elsewhere (6). Minimal medium (MM) was that of Davis and Mingioli (9). Glucose peptone water with bromocresol purple was used for hydrogenlyase assays (10).

Nitrogen-free medium was that of Cannon et al. (11); NFM plates were incubated at 30°C under 99% N_2/1% CO_2 by the method of Hill (12). Growth in liquid NFM was measured by following turbidity in Pankhurst tubes. When necessary, NFM and MM were supplemented with appropriate amino acids at 25 µg/ml and vitamins at 10^{-3} µM.

Anaerobic growth of unc^{-} mutants was tested on MM under 99% N_2/1% CO_2 by the method of Hill, or under H_2 in a gas jar.

TABLE 1. Acetylene reduction by *unc*$^-$ (F'*nif*$^+_{Kp}$) hybrids

Nif class	Hybrid strain	*unc* gene	Acetylene reduction - fumarate	Acetylene reduction + fumarate
I	AN249	*uncA*$^-$	0.10	0.10
I	AN285	*unc405*$^-$	0.10	10.50
I	AN771	*uncC*$^-$	0.05	0.05
II	AN283	*uncB*$^-$	45.10	50.00
II	AN259	*unc*$^+$	42.20	43.50

Acetylene reduction, (nmol/min/mg protein). Fumarate was added at final concentrations of 5 and 10 mM. Both concentrations gave similar rates of acetylene reduction.

Matings: *E. coli* K12 strain SB1801 containing the FN68 plasmid was used as the donor. Hybrids were made with *E. coli* K12 recipient strains mutant in the oxidative phosphorylation (*unc*) system. All recipient strains were *mal*$^+$. Mating procedure is described by Bernstein *et al* (13), and mating mixtures were incubated at 30°C for two hours. *Mal*$^+$ *Cb*^{R} hybrids were selected on EMB-maltose-carbenicillin (300 µg/ml) plates at 30°C. Hybrid isolates were purified by restreaking three times on the selection medium, and were then tested for nitrogen fixation by growth on NFM and by acetylene reduction.

Acetylene reduction assay: Rates of formation of ethylene from acetylene were measured by the method of Tubb & Postgate (14). Acetylene reduction was measured by cultures grown in NFM with and without 100 µg/ml vitamin-free casamino acids to ensure optimal growth without repression of nitrogenase activity. In all cases, addition of 5mM $(NH_4)_2SO_4$ to the culture medium repressed nitrogenase activity as measured by acetylene reduction.

Hydrogenlyase assay: This enzyme system which produces H_2 and CO_2 from formate anaerobically was measured as described elsewhere (15).

Results and Discussion

The FN68 plasmid (F' *nif*$^+_{Kp}$ *his*$^+$ *Cb*^{R}) was transferred into well-defined mutants of *E. coli* K12 defective in oxidative

phosphorylation (*unc*$^-$ mutants). These hybrids fall into 2 classes (Table 1). Class I hybrids have a defective *Nif* phenotype; they grow slowly on nitrogen free medium, reduce acetylene at a very slow rate and occur in the background of either AN249 (*uncA*$^-$, defective Mg^{2+}-ATPase) or AN285 (*unc*405, lacks the Mg^{2+}-ATPase aggregate). Another class I hybrid was in the background of AN771, an *uncC*$^-$ mutant defective in coupling between the Mg^{2+}-ATPase aggregate and the electron transport chain like the *uncB*$^-$ mutant AN283, but of a different complementation group (Cox, personal communication). These hybrids show a rate of acetylene reduction of about 0.1% to 0.2% of that found with the isogenic parental hybrid AN259 (FN68), indicating that a functional Mg^{2+}-ATPase aggregate is necessary for nitrogen fixation.

Moreover, no complementation occurs between these two *unc*$^-$ mutants and the F'*nif*$^+_{Kp}$ plasmid, indicating that the *nif*$_{Kp}$ region lacks functions which will restore a normal Mg^{2+}-ATPase aggregate.

All the *unc*$^-$ mutants diminish the formation of fumarate from glucose under anaerobic conditions, the effect being most marked in those strains having a defective Mg^{2+}-ATPase aggregate (8). Thus mutants AN249(*uncA*$^-$) and AN285(*unc*405$^-$), which are of this latter type, require added fumarate for anaerobic growth in a minimal medium with glucose as sole carbon source, as well as for the anaerobic uptake of inorganic phosphate and serine (8). When fumarate was added to the AN285(FN68) hybrid a hundredfold stimulation in its level of nitrogen fixation (acetylene reduction)was observed. In contrast, the AN249 (FN68) hybrid showed no response to added fumarate (Table 1). These results indicate that

TABLE 2. Expression of F'*nif*$^{+}_{Kp}$ plasmid transferred from Nif defective *unc*$^{-}$ (F'*nif*$^{+}_{Kp}$) hybrids back to strain SB1801

Strains	Phenotype		
	Carbenicillin resistance	His^{+}	Nif^{+} (C_2H_2 reduction)
SB1801	-	-	< 0.01
SB1801(FN68)	+	+	50.10
SB1801-249(FN68)	+	+	45.20
SB1801-285(FN68)	+	+	43.70
SB1801-771 (FN68)	+	+	34.10

C_2H_2 reduction, in nmol/min/mg protein.

fumarate reduction plays an important role in the fixation of nitrogen in *E. coli* K12. Thus a functional Mg^{2+}-ATPase aggregate is necessary not for coupling of oxidative phosphorylation but for the normal synthesis of fumarate from glucose which is then used in the supply of energy for active nitrogen fixation.

Class II hybrids are phenotypically *Nif*$^{+}$ and reduce acetylene at rates comparable with *K. pneumoniae* strain M5a1, the bacterium from which the *nif*$^{+}_{Kp}$ genes were derived. Examples of these hybrids are the parent strain AN259 and its isogenic *uncB*$^{-}$ mutant, AN283 (Table 1). Although strain AN283 is defective in coupling between the Mg^{2+}-ATPase aggregate and the electron transport chain (7,8) its hybrid, AN283(FN68) shows a high rate of nitrogen fixation. AN283(*uncB*$^{-}$) does not require fumarate for anaerobic growth

or for expression of the *nif*$^{+}_{Kp}$ genes carried on the FN68 plasmid, but does need addition of fumarate for anaerobic uptake of inorganic phosphate and serine (8). These results further confirm that coupling is unnecessary in *E. coli* K12 for expression of *nif*$^{+}_{Kp}$ genes.

To check that the *nif*$^{+}_{Kp}$ genes were still present on the FN68 plasmid in class I hybrids, the Nif-defective hybrids were used as donor strains in conjugation experiments with recipient strain SB1801 (Table 2). When the plasmid was transferred into SB1801 from any of the Nif-defective hybrids it was possible to recover *Nif*$^{+}$, *His*$^{+}$ and *Cb*^{R} colonies which showed that the *nif*$^{+}_{Kp}$ genes had not been lost by segregation of the plasmid in any of the class I hybrids.

The presence of the FN68 plasmid made no difference to the Suc^{-} phenotype exhibited by the various *unc*$^{-}$ mutants (7), so that no function is present on the FN68 plasmid to complement this defect.

Both AN283(*uncB*$^{-}$) and its hybrid AN283(FN68) produce acid under H_2, N_2 or argon as indicated by a change in the colour of the dye bromocresol purple to yellow. In addition, AN283, but not its hybrid, bleaches the dye under these anaerobic conditions. Since this dye can be also bleached by sodium dithionite or by a crude preparation of ferridoxin from *E. coli* but not by ascorbic acid, AN283 must overproduce a redox system which is poised at a level between ascorbic acid and dithionite. Thus the presence of the F'*nif*$^{+}_{Kp}$ plasmid in AN283(*uncB*$^{-}$) appears to cause a loss of overproduction of the redox system, presumably by complementation between the mutant and some function(s) on the plasmid. The presence of the plasmid did not affect the response of the other *unc*$^{-}$

strains in these hydrogenlyase assays; all produced acid and gas and did not bleach the dye.

These results show that expression of *nif*$^{+}_{Kp}$ genes in *E. coli* K12 does not require the coupled Mg^{2+}-stimulated ATPase activity but does need the contribution of an anaerobic electron transport system involving fumarate reduction. This was further confirmed with a fumarate reductase mutant AN472(*frd*-1)(8), whose hybrid gave a class I Nif-defective response (Skotnicki and Rolfe, in preparation).

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