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Perturbation of *nifT* Expression in *Klebsiella pneumoniae* Has Limited Effect on Nitrogen Fixation

HOLLY M. SIMON, MARY J. HOMER,† AND GARY P. ROBERTS*

Department of Bacteriology and the Center for the Study of Nitrogen Fixation, University of Wisconsin—Madison, Madison, Wisconsin 53706

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In the nitrogenase system of *Klebsiella pneumoniae*, *nifT* is located between *nifDK*, the structural genes for dinitrogenase, and *nifY*, whose product is involved in nitrogenase maturation. It is, therefore, a reasonable hypothesis that the NifT protein might also have a role in the maturation of nitrogenase. However, the phenotypic characterization of *nifT* and *nifT*-overexpressing strains for effects on the regulation, maturation, and activity of nitrogenase identified no properties that were distinct from those of the wild type. We conclude that the *K. pneumoniae* NifT protein is not essential for nitrogen fixation under the conditions examined.

Extensive biochemical and genetic analysis has rendered the nitrogenase system of *Klebsiella pneumoniae* one of the best studied among both free-living and symbiotic nitrogen-fixing organisms. Nitrogenase is the enzyme responsible for reducing atmospheric N_2 to ammonia and is made up of two enzymes, dinitrogenase (also referred to as component I or the MoFe protein) and dinitrogenase reductase (also referred to as component II or the Fe protein) (7). Dinitrogenase is a 240-kDa $\alpha_2\beta_2$ tetramer encoded by *nifD* and *nifK* and is responsible for the reduction of N_2. It contains an iron-molybdenum cofactor (FeMo-co) at the site of substrate reduction. Dinitrogenase reductase is an α_2 dimer of about 60 kDa and is encoded by *nifH*. During catalysis, it specifically donates electrons to dinitrogenase. Dinitrogenase reductase has also been shown to have additional roles in the maturation of dinitrogenase (1, 16).

An open reading frame of 216 bp positioned downstream of *nifK* was first designated *nifT* in *K. pneumoniae* by Arnold et al. (2). An undesignated open reading frame, similarly positioned downstream of *nifK*, had previously been identified in *Azotobacter vinelandii* (5) and was subsequently also designated *nifT* (11). The predicted *K. pneumoniae* and *A. vinelandii* NifT proteins are 40% identical to one another. Additionally, NifT is homologous to the amino-terminal end of the *mosB* gene product from *Rhizobium meliloti* (35 to 37% identity) (15). MosB appears to be required for rhizopine biosynthesis; however, the specific functions of its multiple domains have not been elucidated (15).

The conservation of *nifT* in both *K. pneumoniae* and *A. vinelandii*, together with its cotranscription with *nifHDK*, suggested a possible role in nitrogenase function for the gene product. Neither an insertion mutation nor a deletion of the entire *nifT* gene in *A. vinelandii* had an obvious effect on diazotrophic growth (11). Similar mutations made in *nifY* of *K. pneumoniae* also resulted in strains still capable of diazotrophic growth, but further investigations have shown that NifY is an integral player in nitrogenase maturation (10). This finding demonstrates that proteins functioning in important roles in nitrogenase maturation may not be absolutely required for diazotrophic growth, most likely because other proteins are able to substitute for their functions. The existence of such strains with mutations in genes coding for important but not essential gene products led us to examine strains with aberrant *nifT* expression for an indication of the function of NifT in nitrogenase maturation. We constructed both an in-frame, chromosomal deletion within *nifT* of *K. pneumoniae* and a *nifT* expression plasmid. Strains harboring the *nifT* deletion or the *nifT* expression vector were examined for effects on regulation, activity, and maturation of the nitrogenase proteins.

The *nifT* deletion mutation was first constructed on the plasmid pNF111, which is a pUC19 derivative (19) carrying a 1.4-kb *Eco*RI-*Sal*I fragment containing *nifT*. pNF111 was partially digested with *Ava*II and incubated with the large fragment of DNA polymerase I (Klenow fragment) to make the ends flush. The plasmid was then digested with *Bsa*AI and religated, resulting in an in-frame deletion of 114 bp of *nifT*, referred to as Δ*nifT6294*. Restriction enzymes were purchased from New England Biolabs (Beverly, Mass.) or Promega Corp. (Madison, Wis.). The construction was confirmed by DNA sequencing, using the Sequenase kit from Bio-Rad (Hercules, Calif.) (data not shown). The deletion was cloned into pJR4, a derivative of the R6K vector pGP704 (14), which requires the product of the *pir* gene in order to replicate. Wild-type *K. pneumoniae* UN, which has been previously described (13), lacks *pir*, so selection of the drug resistances encoded by pJR4 resulted in a strain with the plasmid integrated into its chromosome by homologous recombination. Isolates that had undergone recombination and lost the plasmid and the wild-type copy of the *nifT* region but that retained the copy with the deletion were obtained by screening for loss of the plasmid drug resistances after nonselective growth. Replacement of the wild-type copy of *nifT* with the deletion mutant copy was confirmed by Southern analysis (data not shown), and the *nifT* deletion strain was designated UN5472.

We created a *nifT* expression vector by cloning a *Hin*dIII-*Sal*I fragment containing *nifT* downstream of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter in expression vector pKK223-3 (6), generating the plasmid pNF118. pNF118 was introduced into strain UN5350, creating the strain UN5407. UN5350 is wild-type UN harboring an F′ that encodes the *lacI*^{q} repressor, allowing for regulation of expression from the P_{tac} promoter. A band migrating at approximately 8.4 kDa was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15 to 20% polyacrylamide) only in extracts of UN5407 (*nif*$^+$/pNF118) that had been grown in the presence of IPTG (data not shown).

* Corresponding author. Phone: (608) 262-3567. Fax: (608) 262-9865. Electronic mail address: groberts@bact.wisc.edu.

Present address: Department of Pharmacology, Mayo Foundation, Rochester, MN 55905.

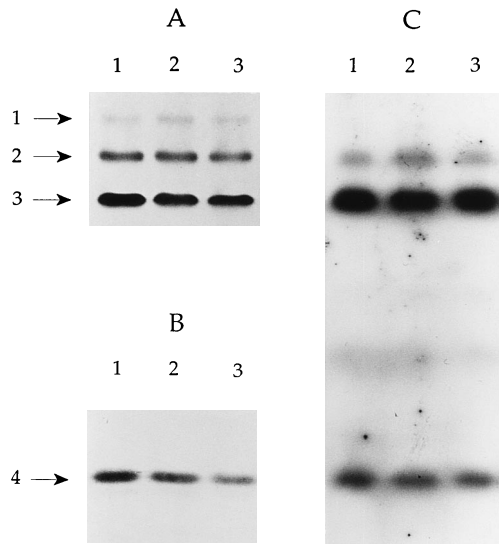

FIG. 1. Nondenaturing gel electrophoretic analysis of dinitrogenase and dinitrogenase reductase antigens and iron-containing proteins in *nifT* strains. Extracts of wild-type, *nifT*, and *nifT*-overexpressing strains which had been derepressed for *nif* expression were resolved on anoxic, nondenaturing polyacrylamide gels and were developed with antibodies to dinitrogenase (A) and dinitrogenase reductase (B) on immunoblots. (C) Similar extracts were likewise electrophoresed, and the gel was stained for iron. The three panels are positioned such that bands in panels A and B are in the same vertical orientation as those in panel C. Lanes 1, extract from the wild type, UN (*nif*$^+$); lanes 2, extract from UN5472 (*nifT*); lanes 3, extract from UN5407 (*nif*$^+$/pNF118). Arrows 1, 2, and 3 indicate the positions of the upper, middle, and lower species of dinitrogenase (see text), respectively, in panels A and C. Arrow 4 indicates the position of dinitrogenase reductase in panels B and C.

Both the *nifT* mutant and the *nifT* expressor were examined under a variety of conditions to seek effects on nitrogenase activity. Expression of *nifT* from pNF118 was induced by the addition of 0.5 mM IPTG. In vivo nitrogenase activity was examined over time in the course of serine derepressions in which the normal amounts of iron (180 mM) and molybdenum (10 mM) were added. Representative activities in strains which were grown, derepressed, and assayed for nitrogenase as described previously (8) were as follows: 29 nmol of ethylene per min per ml for UN (*nif*$^+$), 29 nmol of ethylene per min per ml for UN5472 (*nifT*), and 27 nmol of ethylene per min per ml for UN5407 (*nif*$^+$/pNF118). The optical density at 600 nm at the time of harvest was 1.2 to 1.3. Additional studies, in which the levels of iron and molybdenum were lowered in various combinations or iron and molybdenum were left out of the medium altogether, were done (data not shown). No significant difference in in vivo nitrogenase activity for UN5472 or UN5407 compared with that for UN was observed under any condition tested.

In order to test for a role of NifT in the maturation of nitrogenase, Western blot (immunoblot) analysis of native gels was performed to monitor the formation of nitrogenase components in extracts from UN5472 (*nifT*) and UN5407 (*nif*$^+$/pNF118). Strains were derepressed as described above, and cell extracts were prepared as described previously (10). Equivalent amounts of protein, as determined by a bicinchoninic acid assay (18), were applied to an anoxic, nondenaturing polyacrylamide gel as described previously (10). The protocols used for Western blotting and developing (3) were modified according to the method of Brandner et al. (4). Figure 1 shows Western blots of anoxic native gels; the first panel (Fig. 1A) was developed with antibodies to dinitrogenase, and the second panel (Fig. 1B) was developed with antibodies to dinitrogenase reductase. Arrows 1 and 2 indicate bands representing partially processed forms of dinitrogenase, while arrow 3 indicates holodinitrogenase and arrow 4 indicates dinitrogenase reductase. Both the electrophoretic positions and the levels of accumulation of dinitrogenase and dinitrogenase reductase antigens for both UN5472 and UN5407 were similar to those for the wild type. The slight decrease in dinitrogenase reductase (Fig. 1B, lane 3) was not consistently seen. There were also no detectable differences observed on Western blots from anoxic native gels that were developed with antibodies to NifY (data not shown).

To determine if the iron content of any of the nitrogenase components in UN5472 (*nifT*) or UN5407 (*nif*$^+$/pNF118) differed from that observed for those components in UN (*nif*$^+$), extracts were electrophoresed on anoxic native gels and stained for iron (12). Bands corresponding to both nitrogenase subunits appeared to have similar amounts of iron in all three strains (Fig. 1C).

To determine the activities of the nitrogenase components, in vitro activity assays were performed as described previously (17), and representative activities in crude extracts were as follows: 35 nmol of ethylene per min per mg for UN (*nif*$^+$), 37 nmol of ethylene per min per mg for UN5472 (*nifT*), and 31 nmol of ethylene per min per mg for UN5407 (*nif*$^+$/pNF118). When purified nitrogenase components were added to these crude extracts (17), the levels of active dinitrogenase and dinitrogenase reductase were comparable in all three. These results corroborate the observations made in the analyses of native gels and the in vivo studies.

Summary. Although we have been unable to detect a function for NifT, it is nonetheless conceivable that it has an important role in nitrogenase maturation. By analogy, a *nifY* strain has 60 to 80% of the nitrogenase activity of the wild-type strain yet NifY has been shown to be an integral factor in dinitrogenase maturation (10). NifY performs at least some of the functions of the gamma protein of *A. vinelandii* (9), which is a chaperone-insertase responsible for converting apodinitrogenase to holodinitrogenase by the insertion of the FeMo-co. In the case of NifY, it is our working hypothesis that *K. pneumoniae* contains other proteins that are capable of performing its function to a significant degree. Similarly, we consider it likely either that our laboratory conditions do not demand NifT function for nitrogen fixation or that another protein is able to substitute for NifT in its absence. While we have no supportive data, potential roles for NifT include involvement in a step in the insertion of the FeMo-co into apodinitrogenase; involvement in formation or insertion of the P clusters of dinitrogenase, which are unique [8Fe-8S] double cubanes probably involved in the reduction of the FeMo-co (7); or involvement in dinitrogenase reductase maturation.

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