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NOTES

Cloning, Characterization, and Regulation of *nifF* from *Rhodobacter capsulatus*

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The *Rhodobacter capsulatus nifF* gene and upstream sequence were cloned by using a probe based on the N-terminal sequence of NifF. *nifF* was found to not be contained in the previously described *nif* regions I, II, and III. Comparison of the deduced amino acid sequence showed that it is highly similar to NifF from *Azotobacter vinelandii* and NifF from *Klebsiella pneumoniae*. Analysis of translational fusions demonstrated that the regulation of transcription was the same as previously reported at the protein level. Insertional mutagenesis showed that NifF contributes significantly to nitrogenase activity under normal nitrogen-fixing conditions and that it is absolutely required for nitrogen fixation under iron limitation.

Rhodobacter capsulatus is a nonsulfur photosynthetic bacterium that is able to fix atmospheric dinitrogen (N_2) under either anaerobic or microaerobic conditions when nitrogen is limiting (31). Many genes involved in the process of nitrogen fixation in *R. capsulatus* have been cloned and characterized. Unlike in *Klebsiella pneumoniae*, *nif* genes have been localized to three unlinked clusters called *nif* regions I, II, and III (5, 16, 32). Dinitrogenase reductase (Fe-protein), one of the two protein components of nitrogenase (N_2ase), is reduced by electrons which are donated by low-molecular-weight low-redox-potential electron carriers such as ferredoxins and flavodoxins. Six ferredoxin genes in *R. capsulatus*, two of which, *fdxC* (encoding ferredoxin IV [FdIV]) and *fdxN* (encoding FdI), are located in the *nif* I region and are specifically induced under nitrogen-fixing conditions, have been reported (7, 8, 21, 24, 35). Two ferredoxins, FdI and FdII, are capable of donating electrons to nitrogenase in vitro and are therefore candidates for its in vivo electron donor. In *K. pneumoniae*, it has been established that the products of two genes, *nifF* (encoding a flavodoxin) and *nifJ* (encoding a pyruvate:flavodoxin oxidoreductase), form a specific electron transport pathway to nitrogenase from pyruvate (3, 9, 19, 28). Recently, we reported that a flavodoxin (NifF) in *R. capsulatus* which is subject to *nif*-specific regulation is additionally regulated with respect to available iron and can donate electrons in vitro to N_2ase (34). At low concentrations, it was a more effective electron donor to N_2ase in vitro than were FdI and FdII. In order to further characterize this potential in vivo electron donor to N_2ase, we cloned and studied the physiological properties of the corresponding gene (*nifF*).

Identification and sequencing of the flavodoxin gene of *R. capsulatus*. To identify the gene encoding *R. capsulatus* flavodoxin, the N-terminal amino acid sequence of NifF was determined (McGill Sheldon Biotechnology Center) and a corresponding degenerate oligonucleotide probe based on residues 23 to 30 was synthesized. Southern blot DNA hybridization analysis was carried out (22) at 38°C with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–7% sodium dodecyl sulfate–10× Denhardt's reagent–100 μg of denatured salmon sperm DNA per ml and demonstrated that the *nifF* gene was not located within the previously characterized *nif* I, II, and III regions of *R. capsulatus* (results not shown). Thus, the *nifF* region represents a fourth *nif* region. Colony lift hybridization of an *R. capsulatus* cosmid library (2) gave a positive clone, pGR1, which contains a 43-kb *R. capsulatus* chromosomal DNA fragment (Fig. 1). Southern blot DNA hybridization analysis showed that *nifF* was contained within a 3.2-kb *Pst*I fragment of pGR1 (results not shown). Subcloning of this fragment in the *Pst*I site of pBluescriptII KS$^-$ yielded pGR1-1 (*nifF* oriented 3′ to 5′) (Fig. 1). Overlapping primers were used to sequence *nifF* (both strands) by the dideoxy-chain termination method of Sanger et al. (23) with a Sequenase version 2.0 DNA sequencing kit (United States Biochemical). In order to eliminate ambiguities in GC-rich regions, Sequenase polymerase was diluted 1:6, annealing (at 65°C) was for 10 min, labeling reactions were for 5 min, and a 30-min incubation at 37°C with terminal deoxynucleotidyltransferase was carried out prior to termination. According to the DNA sequence, *nifF* consists of 548 bp, starting at an ATG (nucleotide position 141) and terminating with a TGA (position 689). As deduced from the DNA sequence data, NifF is composed of 181 amino acids, with a predicted molecular mass of 19.8 kDa and an N-terminal sequence, MA KIGLFFGSDTGTTRKIAKQIKDMFDDEVM, which agrees perfectly with that determined by N-terminal sequence analysis of NifF. Sequence alignment comparisons with previously characterized flavodoxins reveal that *R. capsulatus* NifF is highly similar to NifF of *Azotobacter vinelandii* (13) as well as to NifF of *K. pneumoniae* (3, 4), with 76.7 and 67.4% similarity, respectively (Fig. 2). In fact, *R. capsulatus nifF*, when appropriately expressed, is capable of rendering a *nifF* strain of *K. pneumoniae* Nif$^+$ (6). Several potential *nif*-specific regulatory elements, including a NifA binding site (consensus, TGT-N_{10}-

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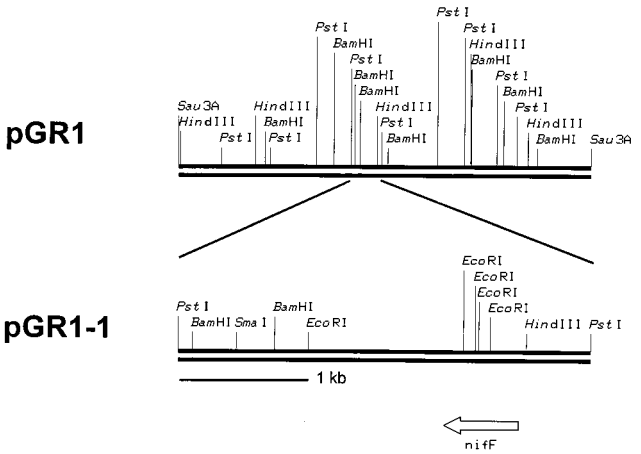

FIG. 1. Physical maps of positive *nifF* clones. pGR1 is an *R. capsulatus* DNA-pHC79 hybrid which contains approximately 43 kb of *R. capsulatus* genomic DNA, including the *nifF* gene. The 3.2-kb *Pst*I fragment of pGR1 which contains *nifF* was subcloned within the *Pst*I site of pBluescriptII KS$^-$, giving rise to pGR1-1. An arrow indicates the direction of transcription of *nifF*.

ACA [17]), a −24/−12 promoter region (consensus, −24 CT GG-N_{10}-GC-12 [17]), and a ribosome binding site, were found upstream of *nifF*. The presence and appropriate spacing of these elements suggested that *nifF* is specifically transcribed under nitrogen-fixing conditions.

Expression of *nifF-lacZ* fusions. We therefore investigated the expression of *nifF* by using strains harboring a plasmid (pUSA73) containing a *nifF-lacZ* fusion and 506 bp of the *nifF* upstream region (from the ATG) grown under different conditions and in different *R. capsulatus* mutant backgrounds. The *nifF-lacZ* fusion was constructed as follows (by standard molecular biological techniques [22]): a 0.6-kb *Bam*HI-*Msc*I fragment carrying the promoter region and the 5′ end of *nifF* was cloned into the broad-host-range *lacZ* fusion vector pPHU236 (pRK replicon) (12). This fuses the first 31 amino acids of NifF to *Escherichia coli* β-galactosidase. Conjugations (triparental mating system) were carried out as previously described (33). In wild-type B10 cells grown on RCV medium (10) with the normal complement of molybdenum and iron, the *nifF-lacZ* fusion was expressed only under nitrogen-limiting conditions (Fig. 3). Similar results were obtained with *nifH*- and *rpoN-lacZ* fusions (carried, respectively, on pPHU266 and pPHU289 [12], pRK replicons). These results confirm and amplify our previously reported immunoblot results (34). The level of expression of the *nifF-lacZ* fusion was 15 to 65% of the expression level of the *nifH-lacZ* fusion, whereas the quantity of NifF in wild-type cells is about 100-fold lower than that of NifH. We attribute this difference to the fact that the fusions were carried on multicopy plasmids; under these conditions, the quantity of transcriptional activators may become limiting. The expression of the *nifF-lacZ* fusion was examined in different *nif* gene regulatory mutants. Essentially, no expression of the *nifF*-, *nifH*-, or *rpoN-lacZ* fusion was detected in the Δ*nifA1/2* double deletion mutant RCM36 (plasmid pUSA72, which carries a 1.6-kb kanamycin cassette between the first two *Bgl*II sites of *nifA1*, was used to delete the chromosomal copy of *nifA1*), the *ntrC* mutant J61 (30), or the *rpoN* mutant AP29 (32) (results not shown). Similar results have been previously presented for *nifH* and *rpoN* fusions (11, 12). Thus, we conclude that the *nifF* gene is transcribed by a typical *nif* promoter.

We examined the effects of different ammonium concentrations on *nifF-lacZ* fusion (pUSA73) expression under iron deficiency. For iron-deficient conditions, iron was omitted from the RCV base, which was prepared with highly purified deionized water (conductivity, $<10^{-7}$ S). *nifF-lacZ* fusion expression, unlike the expression of *nifH* (pPHU266) and *rpoN* (pPHU289) fusions, was not totally repressed by 15 mM ammonium (7% of 0 mM NH_4^+ expression). *R. capsulatus* cells grew more slowly and had lower final cell densities under iron-deficient conditions compared with the results under iron-sufficient conditions. This stress condition was also reflected in the decreased (two to four times) expression of a *kan-lacZ* fusion (pPHU264) (Fig. 4A). Cells starved of both nitrogen and iron expressed the *nifF-lacZ* fusion at comparable levels to expression under iron-replete conditions, whereas the expressions of *nifH*- and *rpoN-lacZ* fusions were reduced by 33 and 71%, respectively (Fig. 4A). Thus, *nifF* is highly expressed under iron limitation, again agreeing with our previously reported NifF immunoblot data (34).

Since *R. capsulatus* also possesses an alternative, iron-only nitrogenase (26, 27), we tested *nifF-lacZ* expression (pUSA73) under nitrogen limitation in the absence of molybdenum. For expression of the alternative nitrogenase, molybdenum-free AKNL medium (Mo content, <0.05 ppb) was used as previously described (25). The absence of molybdenum was confirmed by the growth of a strain carrying pKS131A, an *anfA-*

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Avine      1 .MAKIGLFFGSnTGKTRKvAKSIKKRFDDETMsDALNVNR
Rcaps      1 .MAKIGLFFGSDTGTTRKIAKQIKDMFDDEvMAKPLNVNR
Eaggl      1 .MATIGiFFGSDTGQTRKvAKLIhQKL.DGiADAPLdVRR
Kpneu      1 .MANIGiFFGtDTGKTRKIAKMIhKQL.GElADAPvNiNR
Anidu      1 .MAKIGLFyGtQTGVTQTIAESIQQEFGGESiVDLNdiAN
Avari      1 MSKKIGLFyGtQTGKTESvAEIIrDEFGnd.vVTLHdVSQ
Synec      1 .MsKIGLFFGtQTGNTEElAQAIQAAFGGSDiVELFdVAE
Ecoli      1 .MAITGiFFGSDTGNTENIAKMIQKQLGKd.vADVHdiAk

Avine     37 VSAEDFAQYQFLILGTPTLGeGeLPGLSsdCENESWEEFL
Rcaps     39 ADVaDFMAYDFLILGTPTLGDGQLPGLSAnaASESWEEFL
Eaggl     34 ATREQFlsYPVLILGTPTLGDGeLPGvEAGsQYdSWqEFT
Kpneu     33 tTlDDFMAYPVLILGTPTLGDGQLPGLEAGCESESWSEfi
Anidu     35 ADAsDLNAYDyLIiGCPTWNVGeLQS........DWEGIY
Avari     32 AeVtDLNDYQyLIiGCPTWNIGeLQS........DWEGLY
Synec     35 VDiEALRDfDQLIiGCPTWNVGeLQS........DWEALY
Ecoli     33 sSKEDLEAYDILLLGIPTWYYGeAQC........DWddFF

Avine     73 PkIEGLDFSGKTvALFGLGDQVGYPENyldALGELYSFFK
Rcaps     76 PrIaDQDFSGKTIALFGLGDQVTYPLEFVnALFFLhEFFS
Eaggl     66 NTlseADLtGKTvALFGLGDQlNYSKNFVSAmRILYdLVI
Kpneu     69 SGlDDASLKGKTvALFGLGDQRGYPDNFVSgmRPLFcALS
Anidu     63 DDlDSVnFQGKKvAYFGAGDQVGYSDNFQdAmGILEEKIS
Avari     59 SElDDVDFNGKLvAYFGTGDQiGYADNFQdAiGILEEKIS
Synec     63 DDlDDVDFSGKTIAYFGAGDQVGYADNFQdAmGVLEEKIt
Ecoli     60 PTlEGIDFNGKLvALFGCGDQEDYAEYFCdALGTirdIIE

Avine    108 DRGAKiVGSWStDGYEFEsSEAVVdG.KFvGLALDLDNQs
Rcaps    112 DRGAnVVGrWPaKGYGFEDSLAVVEG.EFlGLALDQDNQa
Eaggl     98 ARGACVVGNWPREGYKFSFSAAllENNEFvGLPLDQeNQY
Kpneu    104 ARGAqmiGSWPNEGYEFSASSA.lEGDRFvGLVLDQDNQF
Anidu     98 SLGsqTVGYWPIEGYDFNeSKAV.RNNqFvGLAiDeDNQP
Avari     94 QRGgKTVGYWStDGYDFNDSKAl.RNGKFvGLALDeDNQs
Synec     99 SLGgKTVGQWPtAGYDHSeSKAE.RDGKFvGLAiDeDNQP
Ecoli     93 PRGATiVGhWPtAGYHFEASKglAdDDHFvGLAiDeDRQP

Avine    142 gKTDERvAaWLaQIAPeFGLSL*....
Rcaps    146 aLTPERLKgWLSLIAadFGLVLPA*..
Eaggl    134 DLTEERiDSWLEKIKPAVL*.......
Kpneu    138 DQTEARLASWLEEIKRTVL*.......
Anidu    130 DLTKNRiKTWvSQlKseFGL*......
Avari    127 DLTDdRiKSWvaQlKseFGL*......
Synec    132 ELTAERiQaWvaQlKPAFGL*......
Ecoli    124 ELTAERvEKWvKQlsSeGLHLDEILNA*
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FIG. 2. *R. capsulatus* NifF (Rcaps; GenBank accession no. L44290) single-letter-code amino acid protein sequence alignment with *A. vinelandii* (Avine; GenBank accession no. M20568), *Enterobacter agglomerans* (Eaggl; GenBank accession no. M38221), *K. pneumoniae* (Kpneu; GenBank accession no. X03214 and M23868), *Anacystis nidulans* (Anidu; GenBank accession no. M19116), *Anabaena variabilis* (Avari; GenBank accession no. X14577), *Synechococcus* sp. (Synec; GenBank accession no. M88253), and *E. coli* (Ecoli; accession no. M59426) flavodoxins. A black background indicates homology, while uppercase letters show identical residues and lowercase letters show similar residues. Dots indicate gaps introduced to maximize alignment; asterisks indicate stop codons. Alignment was done by the PILEUP program, University of Wisconsin Genetics Computer Group.

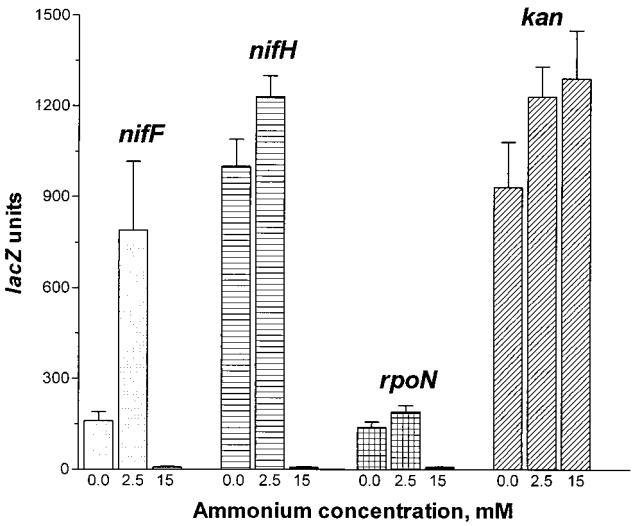

FIG. 3. The expression of β-galactosidase from different fusion plasmids carried by strain B10 (wild type) under various ammonium concentrations. Plasmid pUSA73 contains *nifF-lacZ*, pPHU266 contains *nifH-lacZ*, pPHU289 contains *rpoN-lacZ*, and pPHU264 contains *kan-lacZ*. Nitrogen-sufficient conditions correspond to 15 mM NH_4^+, while nitrogen-limiting conditions were either 2.5 or 0 mM NH_4^+. β-Galactosidase activity assays (in *lacZ* units) were carried out by the method of Miller (18) and Hübner et al. (12). All assays were carried out in duplicate. Data are the averages of 4 to 10 independent experiments. Bars indicate standard deviations.

lacZ fusion-containing vector, which has 17-fold-increased expression in the absence of Mo. Under molybdenum-deficient conditions, all tested *nif* genes were still repressed by ammonium. Interestingly, in the absence of ammonium, *nifF* was highly expressed, whereas *nifH-lacZ* expression (pPHU266) was reduced by 50% and *rpoN-lacZ* expression (pPHU289) was unaltered compared with expression under molybdenum-sufficient conditions (Fig. 4B). Clearly, the *nifF* gene is expressed under conditions in which the alternative nitrogenase is expressed; therefore, NifF may be able to donate electrons in vivo to the alternative nitrogenase system.

***nifF* mutant strains.** Previously, a strain with FdI deleted was shown to grow under nitrogen-fixing conditions, to have in vivo nitrogenase activity lower than that of the wild type, and to be affected in the amounts and stabilities of nitrogenase proteins synthesized (14, 15). These results suggest that an electron carrier in addition to FdI is capable of driving in vivo nitrogenase activity. In an attempt to determine the physiological role of NifF, we created a mutant strain in which the chromosomal copy of *nifF* was inactivated. A 360-bp *Hin*dIII-*Eco*RI partial digest fragment carrying the 5′ end of *nifF* was cloned in the suicide vector pPHU281 (11). Subsequently, a 1.3-kb *Sma*I fragment from pUC4-KIXX was cloned in the *Msc*I site within *nifF*. The plasmid carrying the *kan* gene read in the same direction as *nifF* was named pUSA64; the opposite orientation yielded pUSA65. Plasmids pUSA64 and pUSA65 were used to inactivate the *nifF* gene of *R. capsulatus*. Kanamycin-resistant and tetracycline-sensitive double recombinants were observed with pUSA64 at a frequency of 5%. No double crossover events were found among more than 600 exconjugants with plasmid pUSA65. (This suggests that a gene upstream of *nifF* is lethal when expressed from the *kan* promoter.) The resulting strain, RCM39, was shown by Southern hybridization to contain an interrupted *nifF*. In addition, this analysis, as well as immunoblotting results (not shown), demonstrated that *R. capsulatus* contains only a single copy of this gene. The *nifF* strain RCM39 was found to grow in both the presence and absence of molybdenum, with N_2 as the sole nitrogen source. Thus, NifF seems to be dispensable for both the conventional Mo nitrogenase and the Fe-only alternative nitrogenase. However, the in vivo nitrogenase activity of this strain grown under nitrogen-limited (Mo-replete) conditions was only 55% of that of the wild-type strain (120 nmol of $C_2H_4 \cdot min^{-1} \cdot mg$ of $protein^{-1}$), whereas the amounts of N_2ase proteins were normal, as determined by immunoblotting (results not shown). Thus, NifF plays an important role in supporting maximal nitrogenase activity under these conditions. Similar results were obtained with a *nifF* mutant of *A. vinelandii*, which gave 70% of wild-type activity (1). In this regard, it is interesting that *nifF* strains of *K. pneumoniae*, although reportedly incapable of dinitrogen-dependent growth, have low but significant levels of in vivo N_2ase activity (2 to 5% of wild-type activity) (19, 20, 29).

Importantly, the *nifF* strain RCM39 was incapable of dini-

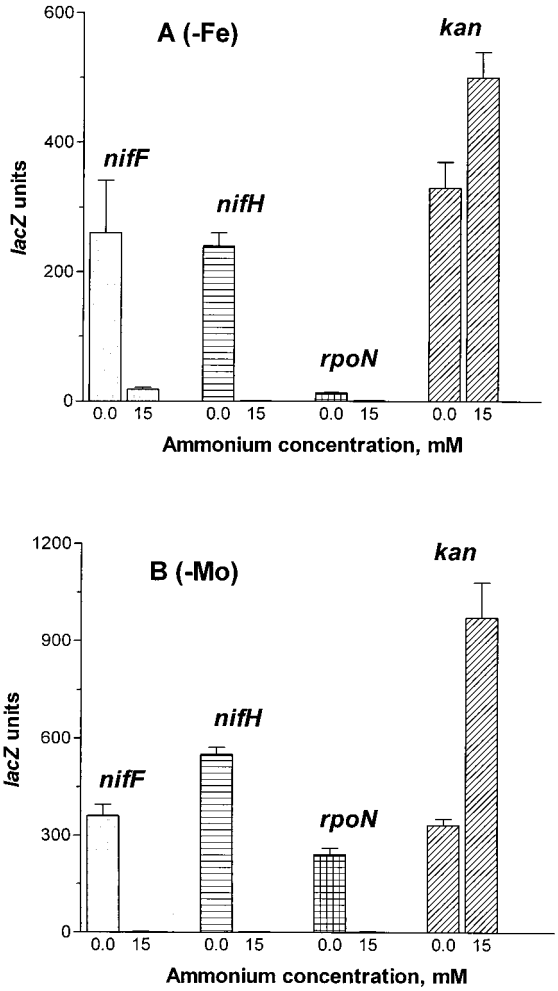

FIG. 4. The effects of iron or molybdenum availability on the expression of β-galactosidase from different fusion plasmids carried by strain B10 (wild type) under different ammonium concentrations. Plasmid pUSA73 contains *nifF-lacZ*, pPHU266 contains *nifH-lacZ*, pPHU289 contains *rpoN-lacZ*, and pPHU264 contains *kan-lacZ*. Cells were grown in the absence of iron (−Fe; A) or molybdenum (−Mo; B). Nitrogen sufficiency corresponds to 15 mM NH_4^+, while the nitrogen-limiting condition is 0 mM NH_4^+. β-Galactosidase activity assays (in *lacZ* units) were carried out by the method of Miller (18) and Hübner et al. (12). All assays were carried out in duplicate. Data are the averages of 4 to 10 independent experiments. Bars indicate standard deviations.

trogen-dependent growth when iron was removed from the medium, indicating that it is ordinarily capable of donating electrons to N_2ase in vivo and is essential for nitrogen fixation when iron is limited. Under these conditions, the levels of active auxiliary electron carriers, such as FdI, may be too low to effectively drive N_2ase. Additional control of NifF expression vis-à-vis a deficiency in available iron could serve two purposes, to ensure sufficient levels of NifF under nitrogen-fixing conditions and to provide an alternate low-potential electron carrier in the presence of excess ammonium, when FdII is thought to play an essential role (21).

Nucleotide sequence accession number. The nucleotide sequence data reported here have been entered into the GenBank database under accession no. L44290.

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