JOURNAL OF BACTERIOLOGY, Apr. 1988, p. 1999–2001
0021-9193/88/041999-03$02.00/0

Vol. 170, No. 4

Identification of a New *Bradyrhizobium japonicum* Gene (*frxA*) Encoding a Ferredoxinlike Protein

SABINE EBELING,[1] JOHN D. NOTI,[2]† AND HAUKE HENNECKE[1]*

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, CH-8092 Zurich, Switzerland,[1] and Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, New York 14853[2]

Received 26 October 1987/Accepted 13 January 1988

An open reading frame of 74 codons was identified downstream of the *nifB* gene of *Bradyrhizobium japonicum* 110. The predicted amino acid sequence shared 63% similarity with the *Rhodopseudomonas palustris* ferredoxin I sequence. We propose to name the gene *frxA*. The *frxA* gene was found to be cotranscribed with the *nifB* gene. An insertion mutation within *frxA* hardly affected nitrogen fixation activity.

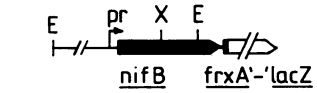
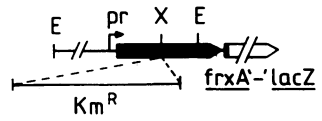
In the symbiotic gene cluster I of the soybean root nodule symbiont, *Bradyrhizobium japonicum* (strain 110), a total of nine *nif* and *fix* genes have been identified and mapped in the order *nifD, nifK, nifE, nifN, nifS, nifB, nifH, fixB,* and *fixC* (for a review, see reference 12). Although the cluster I region has been mutagenized extensively, the presence of additional genes in unmutagenized DNA or of genes without symbiotically essential function could not be excluded (9, 16). This report presents the identification of a putative ferredoxin gene located immediately downstream of *nifB*. It was named *frxA*.

The nucleotide sequence of the *B. japonicum nifB* gene was published previously (16). Upon examination of several hundred base pairs of DNA sequence on the 3′ side of *nifB* (not shown in reference 16), we detected an open reading frame of 74 codons (*frxA*). Its nucleotide sequence is shown in Fig. 1A. The presumptive start codon of *frxA* (GTG) is preceded by a well-conserved Shine-Dalgarno sequence (AAGGAG; 21) which overlaps with the *nifB* stop codon (TAA). This suggests translational coupling of *nifB* and *frxA*. From the predicted amino acid sequence (Fig. 1A), a theoretical molecular weight of 7,841 was calculated for the FrxA apoprotein. The amino acid sequence of the FrxA protein was found to exhibit significant similarity to several published ferredoxin sequences from other bacteria (Fig. 1B). For example, 40 of the 63 amino acids of ferredoxin I from *Rhodopseudomonas palustris* (15), 25 of the 61 amino acids of ferredoxin I from *Chlorobium limicola* (22), and 22 of the 66 amino acids of ferredoxin III from *Azotobacter chroococcum* (20) were found at identical positions in the *B. japonicum* FrxA protein. It is thus highly likely that the *frxA* gene encodes a ferredoxinlike protein. The functions of the other ferredoxins shown in Fig. 1B are not known; however, it is interesting that the gene for ferredoxin III in *A. chroococcum* is located closely adjacent to a second *nifH* copy which has been proposed to code for an alternative nitrogenase reductase protein of the vanadium-containing nitrogenase complex (11, 20).

Between amino acids 10 and 21 (Fig. 1B), the *B. japonicum* FrxA protein contains the cysteine arrangement CXXCXXCXXXCP which is characteristic for 4Fe:4S-type ferredoxins (10). The second cysteine cluster closer to the carboxy-terminal end is neither homologous to the clostridial-type ferredoxins nor to the *Azotobacter*-type ferredoxins but is more homologous to the ferredoxins from photosynthetic bacteria (10); all have conserved cysteines at positions 39, 51, and 55 (Fig. 1B). However, the *B. japonicum* FrxA protein is unique in lacking a cysteine at position 42 while having a cysteine at position 61 which is conserved also (and only) in *R. palustris* (Fig. 1B). The very high overall homology between the *B. japonicum* FrxA protein and the *R. palustris* ferredoxin I matches nicely with our previous finding that *B. japonicum* and *R. palustris* are phylogenetically closely related (13).

The close proximity of the *B. japonicum frxA* gene to the 3′ end of *nifB* suggested that both genes might be cotranscribed. This assumption was tested by measuring the expression of the *frxA* gene with help of a *frxA′-′lacZ* fusion. By using plasmid pMC1403 (4), a translational fusion of *lacZ* to codon 13 of *frxA* (at an *Esp*I site within *frxA*) was constructed, resulting in plasmid pRJ4237. pRJ4237 also carries the complete *nifB* gene together with the *nifB* promoter (Table 1) (5, 16). When the positive regulatory gene of *Klebsiella pneumoniae, nifA*, on pMC71A (1) was provided in *trans*, the *Escherichia coli* MC1061 host cells (4) expressed eight times more β-galactosidase activity than without *nifA* (Table 1). A derivative of pRJ4237 was constructed which carried an insertion of the Tn*5*-derived kanamycin resistance cassette (2.35-kilobase-pair *Xho*I fragment) within

TABLE 1. Expression of a *frxA′-lacZ* fusion in *E. coli* MC1061 and its dependence on *nifA* and on the *nifB* promoter[a]

Plasmid carrying frxA′-′lacZ fusion	Relevant structure (⟵ 500 bp)	β-Galactosidase activity in E.coli (U)	
		− nifA	+ nifA (pMC71A)
pRJ4237		10.4	84.2
pRJ4238		3.1	4.0

[a] For further details, see text. Abbreviations: bp, base pairs; pr, *nifB* promoter; E, *Eco*RI; X, *Xho*I.

* Corresponding author.
† Present address: Triton Biosciences, Inc., Alameda, CA 94501.

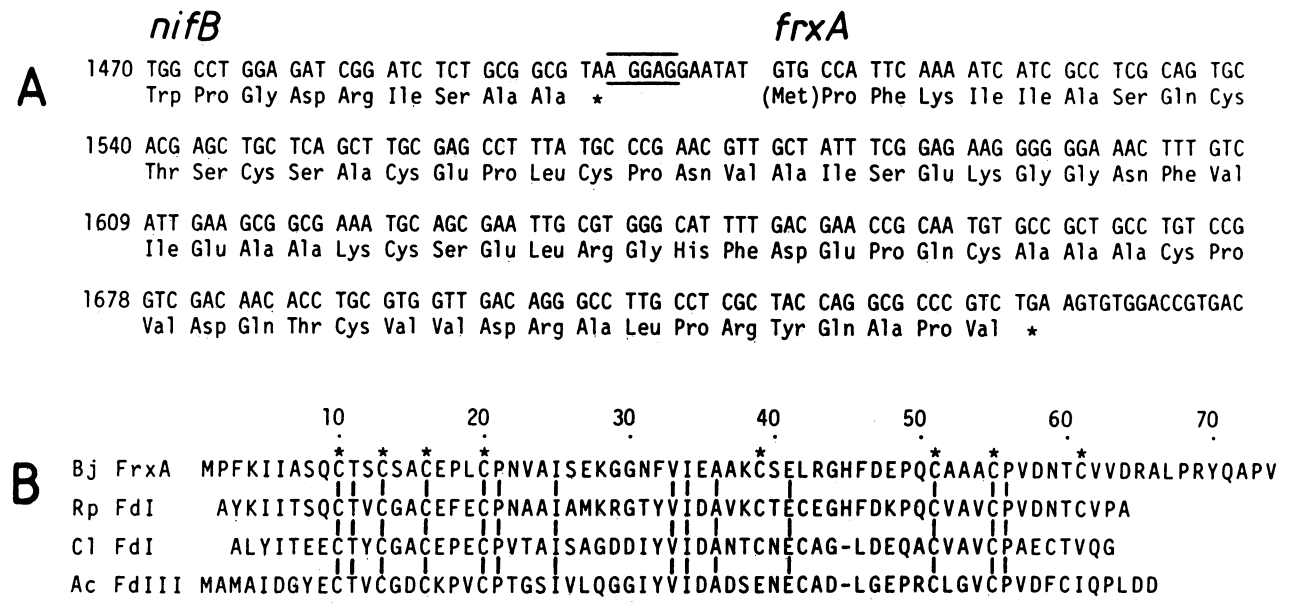
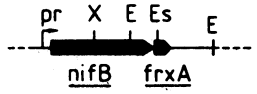
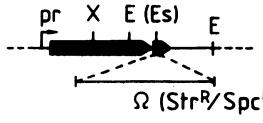


FIG. 1. Sequence analysis of *frxA*. (A) Nucleotide sequence of the 3′ end of *nifB* and the complete *frxA* gene. The numbering system continues that given in reference 16. The putative Shine-Dalgarno sequence for *frxA* is underlined and overscored. (B) Comparison of the predicted amino acid sequence of the FrxA protein with ferredoxins (Fd) from *R. palustris* (Rp), *C. limicola* (Cl), and *A. chroococcum* (Ac); for references, see text. Identical amino acids in all four sequences are connected by vertical lines. The eight cysteines in the *B. japonicum* (Bj) FrxA protein (*) are indicated.

the *nifB*-internal *Xho*I site (9). This plasmid, pRJ4238, resulted in only background β-galactosidase activity, even in the presence of *nifA* (Table 1). These data strongly suggest (i) that the *frxA* gene is translated in *E. coli*, (ii) that the expression of *frxA* is *nifA* dependent, and (iii) that *frxA* is transcribed from the *nifB* promoter.

To test whether or not *frxA* has any function in nitrogen fixation, a mutation was created by in vitro insertion of an Ω interposon (17) into the *Esp*I site at codon 14 of *frxA* (Table 2). The mutation was marker exchanged into the genome of *B. japonicum* 110 *spc*4 (18), giving rise to strain X16. The N_2 fixation phenotype of mutant X16 was measured in soybean root-nodule symbiosis (Fix) and in free-living, microaerobic cultures (Nif) (Table 2). The experiments showed that the Fix activity of mutant X16 was similar to wild-type activity, whereas there was an approximately 50% reduction in Nif activity ex planta. In conclusion, under the assay conditions used in our laboratory, the *frxA* gene is not absolutely

TABLE 2. Phenotypic analysis of a *frxA* mutant (strain X16)[a]

B. japonicum strain	Relevant genomic structure	Acetylene reduction activity	
	⊢ 500 bp	in symbiosis	in free-living culture
		μMol C_2H_2 × $hr^{-1} \times g^{-1}$	nMol C_2H_2 × ml^{-1}
110 spc 4 (wild-type)		144.2	8.1
X16		129.8	4.3

[a] For further details on the analysis and construction of the mutation, see text. Abbreviations: bp, base pairs; pr, *nifB* promoter; E, *Eco*RI; Es, *Esp*I; X, *Xho*I.

TABLE 3. Amino acid composition of three different ferredoxins (Fd) of *B. japonicum*

Amino acid	FdI[a] (FrxA)	FdII[b]	FdIII[c] (FixX)
Ala	10	7	5
Arg	3	0	7
Asp/Asn	3/3	5	5/4
Cys	8	8	5
Glu/Gln	5/3	5	7/2
Gly	3	2	9
His	1	1	4
Ile	4	4	3
Leu	3	1	8
Lys	3	3	4
Met	1	0	1
Phe	3	1	2
Pro	7	5	7
Ser	5	4	3
Thr	2	2	5
Trp	0	0	1
Tyr	1	1	5
Val	6	5	11

[a] Predicted from the nucleotide sequence of the *frxA* gene, with putative N-terminal methionine included (Fig. 1).
[b] Determined experimentally by amino acid analysis of the purified protein; data are taken from Carter et al. (3).
[c] Predicted from the nucleotide sequence of the *fixX* gene, with N-terminal methionine included (T. Zürcher, personal communication).

required for nitrogen fixation, but it may contribute to maximal nitrogen fixation activity under certain conditions of bacterial or plant cultivation. A leaky nitrogen fixation phenotype has recently also been shown for an insertion mutation in the *B. japonicum nifS* gene (9). In this context, it is of interest to recall that the *frxA* gene is in the same operon as *nifB* (Fig. 1; Table 1) and that the *nifB* gene product has been suggested to function in the processing of the nitrogenase iron-molybdenum cofactor (19). It is possible, therefore, that the *frxA* product is involved in an oxidation-reduction step of cofactor synthesis under physiological conditions other than those used by us to test the phenotype of mutant X16. One such condition could be molybdenum starvation; this idea is attractive in view of the fact that the FrxA protein shares considerable homology with the *A. chroococcum* ferredoxin III (Fig. 1B) whose function may be to support the activity of an alternative nitrogenase that is induced under nitrogen-fixing conditions in the absence of molybdenum (20). In the complete *K. pneumoniae nif* cluster, which is known to carry all genes sufficient to encode a functional molybdenum-containing nitrogenase (6), a *frxA*-like gene has not been detected thus far.

Another explanation for the lack of a clear phenotype associated with the *frxA* mutation in strain X16 could be that *B. japonicum* possesses additional, functionally analogous ferredoxins substituting for the missing FrxA protein. This assumption is corroborated by the following: (i) the recent identification of a *B. japonicum fixX* gene (T. Zürcher, personal communication) which codes for a ferredoxinlike protein (7, 8, 14), and (ii) the earlier purification of a ferredoxin from soybean root-nodule bacteroids (3). In Table 3, we present a comparison of the amino acid composition of the latter ferredoxin (herein called FdII) with the amino acid composition of the FrxA and FixX proteins. The data clearly show that all three ferredoxins are different, but the FdII and FrxA proteins appear to be more similar to each other than to the FixX protein (Table 3). This observation is further substantiated by the comparison of the FrxA amino acid sequence with the amino acid sequences of the published FixX proteins from *Rhizobium meliloti* and *Rhizobium trifolii* (7, 8, 14); they show little, if any, similarity (results not shown). Thus, the *B. japonicum* FdII protein would be the more likely candidate in substituting the function of FrxA. Answers to these open questions may be obtained after cloning and mutagenizing the FdII-encoding, third *B. japonicum* ferredoxin gene. With regard to *frxA*, it will be of interest to see whether a homologous gene also exists in fast-growing *Rhizobium* species or other diazotrophic bacteria. That this may indeed be the case was indicated by information given in the Addendum in Proof in reference 2.

We thank our colleague T. Zürcher for allowing us to use his data on *fixX* in Table 3. H. Paul is thanked for typing the manuscript.

S.E. was supported in part by a Graduate Fellowship from the Fonds der Basler Chemischen Industrie.

LITERATURE CITED

1. **Buchanan-Wollaston, V., M. C. Cannon, J. L. Beynon, and F. C. Cannon.** 1981. Role of the *nifA* gene product in the regulation of *nif* expression in *Klebsiella pneumoniae*. Nature (London) **294:** 776–778.
2. **Buikema, W. J., J. A. Klingensmith, S. L. Gibbons, and F. M. Ausubel.** 1987. Conservation of structure and location of *Rhizobium meliloti* and *Klebsiella pneumoniae nifB* genes. J. Bacteriol. **169:**1120–1126.
3. **Carter, K. R., J. Rawlings, W. H. Orme-Johnson, R. R. Becker, and H. J. Evans.** 1980. Purification and characterization of a ferredoxin from *Rhizobium japonicum* bacteroids. J. Biol. Chem. **255:**4213–4233.
4. **Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou.** 1983. β-Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. **100:**293–308.
5. **Chelm, B. K., T. A. Carlson, and T. H. Adams.** 1985. Isolation, characterization and expression of *Bradyrhizobium japonicum nif* and glutamine synthetase genes, p. 217. *In* H. J. Evans, P. J. Bottomley, and W. E. Newton (ed.), Nitrogen fixation research progress. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
6. **Dixon, R. A.** 1984. Genetic complexity of nitrogen fixation. J. Gen. Microbiol. **130:**2745–2755.
7. **Dusha, I., S. Kovalenko, Z. Banfalvi, and A. Kondorosi.** 1987. *Rhizobium meliloti* insertion element ISRm2 and its use for identification of the *fixX* gene. J. Bacteriol. **169:**1403–1409.
8. **Earl, C. D., C. W. Ronson, and F. M. Ausubel.** 1987. Genetic and structural analysis of the *Rhizobium meliloti fixA, fixB, fixC,* and *fixX* genes. J. Bacteriol. **169:**1127–1136.
9. **Ebeling, S., M. Hahn, H. M. Fischer, and H. Hennecke.** 1987. Identification of *nifE*-, *nifN*- and *nifS*-like genes in *Bradyrhizobium japonicum*. Mol. Gen. Genet. **207:**503–508.
10. **George, D. G., L. T. Hunt, L. S. L. Yeh, and W. C. Barker.** 1985. New perspectives on bacterial ferredoxin evolution. J. Mol. Evol. **22:**20–31.
11. **Hales, B. J., E. E. Case, J. E. Morningstar, M. F. Dzeda, and L. A. Mauterer.** 1986. Isolation of a new vanadium-containing nitrogenase from *Azotobacter vinelandii*. Biochemistry **25:** 7251–7255.
12. **Hennecke, H., H. M. Fischer, S. Ebeling, M. Gubler, B. Thöny, M. Göttfert, J. Lamb, M. Hahn, T. Ramseier, B. Regensburger, A. Alvarez-Morales, and D. Studer.** 1987. *Nif, fix* and *nod* gene clusters in *Bradyrhizobium japonicum*, and *nifA*-mediated control of symbiotic nitrogen fixation, p. 191–196. *In* D. P. S. Verma and N. Brisson (ed.), Molecular genetics of plant-microbe interaction. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
13. **Hennecke, H., K. Kaluza, B. Thöny, M. Fuhrmann, W. Ludwig, and E. Stackebrandt.** 1985. Concurrent evolution of nitrogenase genes and 16S rRNA in *Rhizobium* species and other nitrogen fixing bacteria. Arch. Microbiol. **142:**342–348.
14. **Iismaa, S. E., and J. M. Watson.** 1987. A gene upstream of the *Rhizobium trifolii nifA* gene encodes a ferredoxin-like protein. Nucleic Acids Res. **15:**3180.
15. **Minami, Y., S. Wakabayashi, F. Yamada, K. Wada, W. G. Zumft, and H. Matsubara.** 1984. Ferredoxins from the photosynthetic purple non-sulfur bacterium *Rhodopseudomonas palustris*. Isolation and amino acid sequence of ferredoxin I. J. Biochem. **96:**585–591.
16. **Noti, J. D., O. Folkerts, A. N. Turken, and A. A. Szalay.** 1986. Organization and characterization of genes essential for symbiotic nitrogen fixation from *Bradyrhizobium japonicum* I110. J. Bacteriol. **167:**774–783.
17. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
18. **Regensburger, B., and H. Hennecke.** 1983. RNA polymerase from *Rhizobium japonicum*. Arch. Microbiol. **135:**103–109.
19. **Roberts, G. P., and W. J. Brill.** 1981. Genetics and regulation of nitrogen fixation. Annu. Rev. Microbiol. **35:**207–235.
20. **Robson, R., P. Woodley, and R. Jones.** 1986. Second gene (*nifH**) coding for a nitrogenase iron protein in *Azotobacter chroococcum* is adjacent to a gene coding for a ferredoxin-like protein. EMBO J. **5:**1159–1163.
21. **Shine, J., and L. Dalgarno.** 1975. Determinant of cistron specificity in bacterial ribosomes. Nature (London) **254:**34–38.
22. **Tanaka, M., M. Haniu, K. T. Yasunobu, M. C. W. Evans, and K. K. Rao.** 1974. Amino acid sequence of ferredoxin from a photosynthetic green bacterium, *Chlorobium limicola*. Biochemistry **13:**2953–2959.