Biochemical and genetic analysis of the nifHDKE region of *Rhizobium* ORS571

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Summary. Deletions and Tn5 insertions were obtained in a cloned 10 kb BamHI—BglII fragment carrying the *nifHDKE* region of *Rhizobium* ORS571 and were recombined into the host genome. Genetic analysis of the mutants, comparison of polypeptides synthesized under conditions of repression and depression of N₂ fixation, and biochemical complementation of crude extracts were performed. All Nif⁻ mutants were also Fix⁻. Three transcription units were identified, *nifHDK*, *nifE* and a new *nif* locus adjacent to *nifE*; no *nif* locus was found in the immediate vicinity upstream of *nifH*. Fifteen polypeptides synthesized under conditions of N₂ fixation were characterized by two-dimensional gel electrophoresis. Ten of them are likely to be *nif* products and polypeptides encoded by *nifH, nifD, nifK* and tentatively *nifE* were identified. Physiological and biochemical evidence for the functioning of the second copy of *nifH* is reported. Nitrogenase component 2 is synthesized by this copy could not be differentiated from component 2 synthesized in the wild-type strain. When the function of *nifH* copy 1 was abolished, the amount of component 2 synthesized was about 30% of that synthesized in the wild-type strain.

Key words: *Rhizobium* — Nitrogen fixation — *Nif* products — Tn5 mutagenesis

Introduction

In *Klebsiella pneumoniae*, 17 genes involved in nitrogen fixation have been identified (Dixon 1984; Elmerich 1984). The structural genes for nitrogenase are highly conserved in diazotrophs and they have been cloned from several diazotrophs using homology to *zotroph* genes (Dixon 1984; Elmerich 1984). The organization of the *nif* genes of *Rhizobium* ORS571 has been analyzed by hybridization using *K. pneumoniae nif* DNA probes. A cluster containing *nifHDKE* has been identified, and a second copy of *nifH* (referred to as *nifH* copy 2) detected (Norel et al. 1985a), and recently cloned (Norel et al. 1985b; Donald et al. 1986). Nitrogenase from the *Rhizobium* strain ORS571 was purified to homogeneity from free-living diazotrophically grown organisms (Kush et al. 1985). Component 1, a Mo—Fe protein had a *M*₂ₐ of 219,000 and consisted of two types of subunits of *M*₂ₐ 56,000 and 59,000. Component 2, a Fe-protein had a *M*₂ₐ of 74,000 with a single type of subunit of *M*₂ₐ 36,000. Nitrogenase activity was subject to "switch-off" when ammonia was added to a N₂-fixing culture (Kush et al. 1985). Switch-off resulted specifically from a decrease in activity of component 2; however, no change in the electrophoretic mobility in SDS polyacrylamide gel electrophoresis (PAGE) of component 2 subunit was observed (Kush et al. 1985). Mutants impaired in nitrogenase activity were isolated on nitrogen-free solid medium after ethyl methanesulfonate (EMS) mutagenesis, but except for mutant 5740 (Elmerich et al. 1982) and some glutamine auxotrophs (Donald and Ludwig 1984), they were not characterized.

We report here the isolation and study of Nif⁻ mutants obtained by Tn5 site-directed mutagenesis of a cloned DNA region containing the *nifHDKE* cluster and the adjacent regions. Our results provide evidence for the genetic organization of the *nifHDKE* cluster, and are essentially in agreement with the recent report by Donald et al. (1986). A new *nif* region was detected adjacent to *nifE* and no other *nif* gene was found in the immediate vicinity of *nifH*. Physiological and biochemical evidence for the functioning of *nifH* copy 2 is reported. In addition, we present data on the characterization of the *nif* products and function, using biochemical complementation and polypeptide analysis by one- and two-dimensional polyacrylamide gel electrophoresis.

Materials and methods

*Bacterial strains and plasmids.* The wild-type *Rhizobium* strain was ORS571 (Dreyfus and Dommergues 1981). Mutants 5740, 5751 and 5795 were Nif⁻ derivatives of ORS571 obtained after EMS mutagenesis. Mutant 5740 has been previously described and is impaired in nitrogenase protein 1 activity (Elmerich et al. 1982); mutant 5751 is a prototroph whereas 5795 requires glutamine for growth. Other mutants carried insertions or deletions shown in Fig. 1. *Escherichia coli* strains were S17.1 (*pro thi recA RP4-2, Te::Mu–Km::Tn7 'Tra' IncP-1*) (Simon et al. 1984), C600 (*trp his recA rif*) and HB101 (*pro leu recA*) (our labo–
jugation experiments. Plasmids pUC4K (Messing and
deletions in the
Fig. 1. Localisation of Tn5 insertions and of in vitro generated
0.6 kb
pRS6 was pRK290. The vector for pRS25, pRS46, pRS47, pRS51,
and pRS52 was pSUP202. The R1 fragment corresponds to a
boxed arrows, the molecular weight of the nif products
are indicated in kDa. The vector for pRS8, pRS81, pRS82, and
pRS6 was pRK290. The vector for pRS25, pRS46, pRS47, pRS51,
and pRS52 was pSUP202. The R1 fragment corresponds to a
0.6 kb XhoI fragment, purified from pRS25 which was used as
an intragenic probe for nifH. Plasmid pRS8 has been previously
described (Norel et al. 1985a). The Km r cartridge in pRS51
was a 2.2 kb XhoI fragment from Tn5. The Km' cartridge in pRS51
and pRS52 was a 1.5 kb BamHI fragment from pUC4K.

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Conjugation and site-directed mutagenesis. Plasmids in
E. coli S17.1 were transferred to Rhizobium according to
Simon et al. (1984). In other cases, tri-parental matings were
performed, using pRK2013 as a mobilizing agent. For Tn5
mutagenesis in E. coli, plasmids derived either from
pSUP202 or pRK290 were introduced by transformation into
E. coli S17.1—Tn5, (a S17.1 derivative containing Tn5 in
the chromosome, provided by Dr. Mazodier). Independent
transformants were mated with E. coli C600. After
conjugation on solid LB medium for 2 h and resolation
on selective LB Rif Km medium, Tn5 was localized by
restriction mapping. The mutations were subsequently
transferred into the strain ORS571 genome by homologous
recombination according to Ruvkun et al. (1982) and Si-
mon et al. (1984). Plasmid pPH111 was used to cure strain
ORS571 of pRK290 derivatives.

DNA techniques. Plasmid purification and construction
were performed using conventional techniques previously
described (Elmerich et al. 1982). Details on plasmid con-
struction are reported in the legend of Fig. 1.

Physical localisation of Tn5 or of the kanamycin cartridge
in the ORS571 genome. Correct homogenotization was
checked in deletion mutants 57151, 57152, 57182 (Table 1)
and in all Tn5 mutants shown in Fig. 1, except mutant
carrying insertion 44 which was only used as a Nif +
control. Total DNA of the mutants was extracted according to
El-
merich et al. (1982) from a 10 ml culture in LSN medium.
Restriction, Southern blotting and hybridization were per-
formed as described (Elmerich et al. 1982). In each case
the restricted sample was loaded on two different gels to
perform hybridization with a nif probe and with an inser-
tion probe. DNA from the Tn5 mutant carrying insertion
44 (Fig. 2) and from the deletion strain 57182 was digested with
BamHI, BglII, HindIII and Smal. Restricted DNA was
probed with the R1 fragment (see Fig. 1) and the 2.2 kb
XhoI fragment from Tn5 carrying the Km' gene. DNA from
the deletion strains 57151 and 57152 was digested with
Hin-
dIII, since the Km' cartridge from pUC4K which was substi-
tuted into the deleted fragment contained a single HindIII
site. Restricted DNA was probed with the HindIII fragment
from pRS46 and with pUC4K. DNA from the other inser-
tion mutants was digested with BglII and probed with the
BamHI—Sall fragment purified from pRS25 and with
DNA of pBR322::Tn5.

Polypeptide analysis. Cells grown in complete medium were
centrified, washed and transferred, at OD600 0.3, into
25 ml flasks containing 5 ml LSO medium, under an atmos-
phere of Ar/O2 (97/3, v/v). After three hours incubation at
37° C, with shaking, 10 µCi [35S]-methionine (SMM31b
CEA, 1,075 Ci/mmole) were added. After 30 min, 5 mg me-
thionine was added. Cells were centrifuged anaerobically,
with washed LSO medium, with methionine, and re-
suspended in 100 µl Tris-HCl 100 mM, pH 7.5 buffer. The
suspension was sonicated twice for 30 s with a Branson
B-12 sonifier, treated with 1 µl of 5 mg/ml DNPase. At this
step, samples could be kept frozen at ~80° C or subjected
directly to electrophoresis. The isoelectric focusing was per-