The EMBO Journal Vol.1 No.4 pp.499-503, 1982

Genetic analysis of nitrogen fixation in a tropical fast-growing *Rhizobium*

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Communicated by J.Postgate
Received on 26 April 1982

The *Rhizobium* strain ORS571, which is associated with the tropical legume *Sesbania rostrata,* has the property of growing in the free-living state at the expense of ammonia or N_2 as sole nitrogen source. Five mutants, isolated as unable to form colonies on plates under conditions of nitrogen fixation, were studied. All of them, which appear as Fix$^-$ *in planta,* are *nif* mutants. With mutant 5740, nitrogenase activity of the crude extract was restored by addition of pure Mo-Fe protein of *Klebsiella pneumoniae.* A 13-kb *Bam*HI DNA fragment from the wild-type strain, which hybridized with a probe carrying the *nifHDK* genes of *K. pneumoniae,* was cloned in vector pRK290 to yield plasmid pRS1. The extent of homology between the probe and the *Bam*HI fragment was estimated at 4 kb and hybridization with *K. pneumoniae nifH, nifK,* and possibly *nifD* was detected. The pRS1 plasmid was introduced into the sesbania rhizobium *nif* mutants. Genetic complementation was observed with strain 5740(pRS1) both in the free-living state and *in planta.* It thus appears that biochemistry and genetics of nitrogen fixation in this particular *Rhizobium* strain can be performed with bacteria grown under non-symbiotic conditions.

Key words: free-living *Rhizobium* nitrogen fixer/*nif* genes cloning/nitrogenase complementation

Introduction

Strains of *Rhizobium* species, which are associated with the tropical legume *Sesbania rostrata,* display unusual features. In symbiotic association with sesbania rhizobium, the host-plant forms both root and stem nodules (Dreyfus and Dommergues, 1981b). In the free-living state, sesbania rhizobia are able to grow rapidly at the expense of NH_4^+ ions or N_2 as the sole source of nitrogen (Dreyfus *et al.*, in preparation). The latter property prompted us to study genetics of nitrogen fixation in a strain of sesbania rhizobium by using the same methodology as with *Klebsiella pneumoniae* or other free-living diazotrophs.

In *K. pneumoniae,* construction of partial diploids allowed the identification of 15 *nif* genes organized in seven transcriptional units (Merrick *et al.*, 1980). The *nifHDK* structural genes for the nitrogenase complex belong to the same operon and are carried by a 6.2-kb *Eco*RI fragment that was cloned in plasmid pSA30 (Cannon *et al.*, 1979). Using plasmid pSA30 as a probe, it was shown that the *nifHDK* genes of *K. pneumoniae* share homology with a large variety of nitrogen fixers (Mazur *et al.*, 1980; Ruvkun and Ausubel, 1980) including several rhizobia (Nuti *et al.*, 1979; Ruvkun and Ausubel, 1980; Hennecke, 1981; Hombrecher *et al.*, 1981; Prakash *et al.*, 1981). These observations were used to develop techniques for studying the symbiotic nitrogen fixation genes in *Rhizobium* species, such as *R. meliloti,* which fix nitrogen only in association with the plant (Ruvkun and Ausubel, 1981; Corbin *et al.*, 1982).

Results reported here deal with the characterization of sesbania rhizobium mutants isolated as unable to express N_2-dependent growth on plates, and with the homology between *K. pneumoniae* and sesbania rhizobium *nif* genes.

Results

Mutant growth properties and nitrogenase activity

Strain 571 and the five mutants (5701, 5702, 5721, 5731, and 5740) were grown overnight in YLS medium, washed twice with the LS base and plated on solid LSO medium supplemented with various concentrations of NH_4^+ ions (1, 2, 15 mM). The size and the number of colonies observed after 3 days of incubation were the same regardless of ammonia concentration. When plated under conditions of nitrogen fixation, the wild-type strain formed normal colonies after 5 days whereas the mutants formed small translucent colonies even after 10 days. In liquid LSO medium containing 15 mM NH_4^+ ions the growth rate of all strains was ~0.4 divisions/h. Under conditions of nitrogen fixation, a maximal growth rate of 0.16 divisions/h was obtained with strain 571 when the nicotinic acid concentration was increased to 20 mg/l or more. Under these conditions none of the mutants grew.

Each of the five mutants was inoculated to the stems of *S. rostrata* growing in sterile soil. All five mutants formed ineffective (Fix$^-$) nodules on the stems and no acetylene reduction was detected (data not shown). Similarly, the five mutants formed ineffective nodules on the roots of *S. rostrata* grown in test tubes (see Figure 4 for mutant 5740).

Nitrogenase activity was measured either in whole cells under conditions of derepression or in crude extracts. Results reported in Table I show that mutants 5702, 5721, and 5740 were entirely devoid of activity whereas mutant 5701 and, to a lesser extent, mutant 5731 kept some residual activity. Assays of complementation of crude extracts by *K. pneumoniae* protein 1 (Kp1) or protein 2 (Kp2) were negative except in the case of mutant 5740 with which a significant complementation by Kp1 was observed.

Cloning of sesbania rhizobium DNA homologous to the nifHDK genes of K. pneumoniae

Total DNA of strain 571 was hydrolyzed by several restriction endonucleases and hybridized with *nif* probes. Similar results were obtained either with the entire pSA30 plasmid or with the 6.2-kb purified *Eco*RI fragment containing the *nifHDK* genes. The sizes of the hybridizing restriction fragment were: 13 kb with *Bam*HI, 7 kb with *Bgl*II, 15.5 kb with *Hind*III, 16 kb with *Sal*I, and 11 kb with *Xho*I. The same fragments were detected using plasmid pCM1 as a *nif* probe.

As the sizes of the *Bam*HI and *Hind*III fragments were compatible with cloning in bacteriophage λL47.1 DNA, this vector was used to construct sesbania rhizobium gene banks. Two phages from the *Hind*III bank and three phages from

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Table I. Nitrogenase activity in sesbania rhizobium strain 571 and Fix⁻ mutants

Strain	Nitrogenase activity (units/mg protein)			
	Whole cells	Crude extracts		
		no addition	+Kp1	+Kp2
571	20	50.4	–	–
5701	3.1	1.0	1.7	0.8
5702	< 0.01	< 0.01	<0.01	<0.01
5721	< 0.01	< 0.01	<0.01	<0.01
5731	0.5	< 0.01	<0.01	<0.01
5740	< 0.01	< 0.01	3.8	<0.01

Whole cells assay: bacteria from an overnight culture in YLS medium were centrifuged, washed with LSO medium, and resuspended in the same medium at OD 0.3. Cell suspensions (10 ml) were incubated at 30°C in 50 ml Erlenmeyer flasks. The gas phase was a mixture of $Ar:O_2$ (97:3, v/v) containing 10% C_2H_2. In positive strains, C_2H_4 formation was linear from 6 h up to at least 9 h. Crude extract assay: bacteria from an overnight culture in YLS medium were centrifuged and inoculated, without washing, at OD 1 in a fermentor containing LSO medium (40 mg/l nicotinic acid). A 1 l/min flow of $N_2:O_2$ (97:3, v/v) was maintained and pH was regulated at 7.2. After 7.5 h, the bacteria were centrifuged under argon and kept in liquid nitrogen until use. In each assay 0.1 or 0.2 ml (1 – 2 mg protein) of crude extract were used. The amounts of pure Kp1 or pure Kp2 added were, respectively, 48 μg and 20 μg.

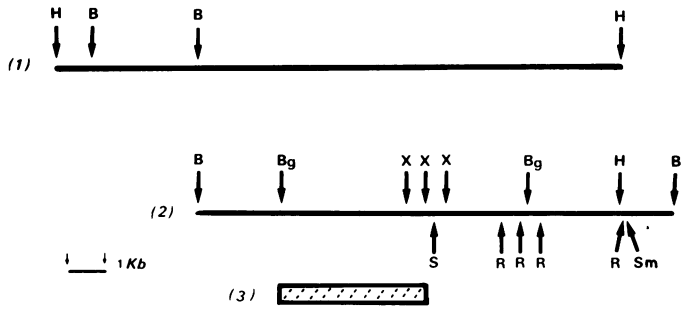

Fig. 1. Physical map of the cloned fragments of sesbania rhizobium DNA. Restriction sites: B:*Bam*HI, Bg:*Bgl*II, H:*Hind*III, R:*Eco*RI, S:*Sal*I, Sm:*Sma*I, X:*Xho*I; (1) physical map of the 15.5-kb *Hind*III fragment; (2) physical map of the 13-kb *Bam*HI fragment; (3) extent of the homology with the 6.2-kb *Eco*RI *nifHDK* fragment of *K. pneumoniae*.

the *Bam*HI bank were selected by hybridization *in situ* with plasmid pSA30 as a *nif* probe. The two *Hind*III recombinant phages were different and contained the same 15.5-kb fragment cloned in both orientations. One phage was termed λL47.Rs101, the other λL47.Rs102. The three *Bam*HI recombinant phages were similar and contained a 13-kb fragment cloned in the same orientation. One of the phages, termed λL47.Rs103, was kept for further studies.

Restriction analysis of λL47.Rs101 and λL47.Rs103 DNAs showed that they had in common an 11.5-kb *Bam*HI-*Hind*III fragment (Figure 1). To obtain more accurate data, the 13-kb *Bam*HI fragment was subcloned in plasmid pBR322 and the resulting pRS2 plasmid was used to establish the physical map of the *Bam*HI fragment (Figure 1). In addition to the restriction sites indicated, six *Pvu*II and at least eight *Ava*I sites were detected but not localized. No *Xba*I or *Hpa*I site was found.

To determine the extent of the homology between the *Bam*HI fragment of sesbania rhizobium and the *nifHDK* genes of *K. pneumoniae,* the *Bam*HI fragment was used as a probe to perform hybridization experiments with various *nif* restriction fragments of plasmid pSA30. The restriction sites used in this study are reported in Figure 2. After digestion by

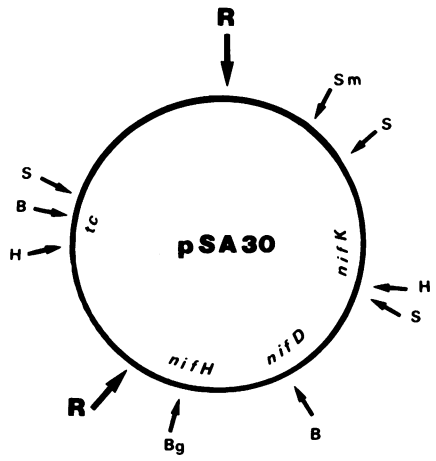

Fig.2. Physical map of plasmid pSA30. Restriction sites as in Figure 1. From Cannon *et al.*, 1979; Riedel *et al.*, 1979; Pühler and Klipp, 1981; Ruvkun and Ausubel, 1980; Ruvkun and Ausubel, 1981; Scott *et al.*, 1981; Sundaresan and Ausubel, 1981.

*Sal*I or double digestion by *Bgl*II + *Eco*RI, *Bam*HI + *Eco*RI, *Hind*III + *Eco*RI, *Sma*I + *Eco*RI, and *Hind*III + *Bgl*II, all the fragments containing *K. pneumoniae nif* DNA were found to hybridize with the probe. Conversely, using the 6.2-kb *Eco*RI fragment carried by plasmid pSA30 as a probe, hybridization experiments with various restriction fragments of plasmid pRS2 were performed. It was first established that hybridization in the *Bam*HI fragment was limited to the 7-kb *Bgl*II fragment. By double digestion with *Bgl*II + *Xho*I, it was found that hybridization occurred with the 3.6-kb *Bgl*II-*Xho*I fragment and the adjacent 0.4 *Xho*I fragment (Figure 1).

Genetic complementation

The 13-kb *Bam*HI fragment was subcloned at the *Bgl*II site of the pRK290 vector (Ditta *et al.*, 1980). The resulting pRS1 plasmid was used to study genetic complementation.

The transfer frequencies of pRK290 or pRS1 into our rhizobium strains were $\sim 4 \times 10^{-3}$ conjugants/recipients. Stability of the plasmids was studied in strain 571 and mutant 5740. After 30 generations in non-selective medium (YLS medium devoid of tetracycline) loss of the Tc^r character was <1% for pRK290 and ~2% for pRS1. The pRS1 plasmid was re-extracted from strain 5740 (pRS1) after growth in LSO medium containing 15 mM NH_4^+ ions and 10 μg/ml tetracycline. The restriction patterns by *Bgl*II and *Eco*RI were similar to those of the original plasmid.

Five independent clones of each mutant carrying pRS1 and one clone carrying pRK290 were patched on LSO (±Tc) plates which were incubated under conditions of nitrogen fixation. Growth was observed only for the clones of strain 5740(pRS1) (Figure 3). As shown in Table II, determination of nitrogenase activity in whole cells under conditions of nitrogenase derepression in liquid medium gave similar results. When strain 5740(pRS1) was grown in a fermentor under conditions of nitrogen fixation, the growth rate was ~0.08 divisions/h. Nitrogenase activity in the crude extract was 5.0 units/mg protein as compared with 64 for the wild-type strain grown under the same conditions.

Although introduction of pRS1 into mutant 5740 restored a significant level of nitrogenase activity, this level was relatively low compared with the wild-type. This raised the question whether the Nif^+ phenotype of strain 5740(pRS1)

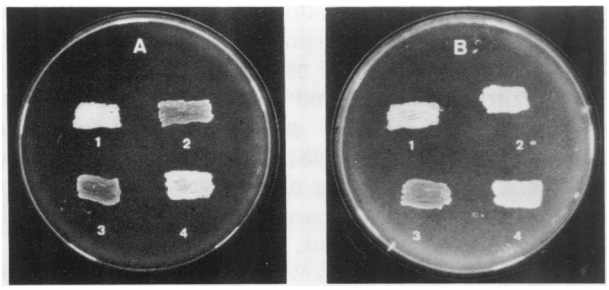

Fig.3. Complementation on solid medium of mutant 5740 by plasmid pRS1. Both plates, containing LSO medium without (**A**) or with (**B**) tetracycline, were incubated for 5 days under conditions of nitrogen fixation. Plate **A**: 1:571; 2:5740; 3:5740(pRK290); 4:5740(pRS1). Plate **B**: 1:571(pRK290); 2:571(pRS1); 3:5740(pRK290); 4:5740(pRS1).

Table II. Nitrogenase activity in whole cells of sesbania rhizobium strain 571 and mutants carrying either plasmid pRK290 or pRS1

Strain	Nitrogenase activity (units/mg protein)	
	pRK290	pRS1
571	20	10
5701	3.0	3.4
5702	< 0.01	< 0.01
5721	< 0.01	< 0.01
5731	0.5	0.4
5740	< 0.01	1.4

Experimental conditions were similar to that described in Table I. Tetracycline (10 μg/ml) was added to all media. C_2H_4 formation was measured after 8 and 9 h of incubation.

was due to complementation or to recombination. To answer this question, ~100 cells of strain 5740(pRS1) were plated on LSO medium with or without ammonia. Colonies grown on the LSO medium were homogeneous in size and their number was similar to that obtained on the LSO + NH_4^+ plates. This excluded the possibility that recombination occurred at a high level. It can also be seen in Table II that nitrogenase activity of strain 571(pRS1) was lower than that of strain 571(pRK290). Actually, introduction of both plasmids into strain 571 modified the kinetics of nitrogenase derepression. In the absence of plasmids, the lag phase preceding the appearance of nitrogenase activity was ~3 h and a linear production of C_2H_4 was observed after 6–7 h. When the cells contained pRK290, the activity was delayed by 1 h. When the cells contained pRS1, the lag phase was 5 h and the linear rate of C_2H_4 production was only a half of that observed in the other cases.

Complementation of mutant 5740 by plasmid pRS1 was also checked *in planta*. As shown in Figure 4, plants inoculated with mutant 5740 were similar to the uninoculated controls, whereas plants inoculated with strain 5740(pRS1) were as vigorous as those inoculated with the wild-type strain. Four weeks after inoculation, the bacteria contained in the root nodules of plants inoculated with the wild-type strain or strain 5740(pRS1) were examined. By plating on YLS medium, ~2 x10^{10} cells were found in both cases from ~150 mg (fresh weight) nodules per plant. In the case of the plant inoculated with strain 5740(pRS1), 90% of the clones were Tc^r and 10% were Tc^s. Ten clones of each category were patched on LSO medium and checked for growth under conditions of nitrogen fixation. All the Tc^r clones appeared as

Fig. 4. Complementation *in planta* of mutant 5740 by plasmid pRS1. **1:** uninoculated control; **2, 3, 4:** inoculated respectively with 571, 5740, and 5740(pRS1). The picture was taken 3 weeks after root inoculation.

Nif^+ and the Tc^s as Nif^-. This excluded a contamination of the plant by the wild-type strain. The stability *in planta* of plasmid pRS1 in *Rhizobium* appeared to be similar to that observed under free-living conditions.

Discussion

In general, these results show that study of the *nif* genes of sesbania rhizobium strain 571 can be performed *ex planta,* using the methodology previously developed for free-living nitrogen fixers (e.g., *K. pneumoniae*). As expected from the physiological properties of sesbania rhizobium, it was possible to isolate mutants on plates, to characterize them biochemically and to perform genetic analysis by complementation with the aid of plasmid pRK290 as a vector of sesbania rhizobium DNA.

Mutants 5701, 5702, 5721, 5731, and 5740 isolated as unable to form colonies on nitrogen-free solid medium displayed very little or no residual acetylene reducing activity. Such a Nif^- phenotype can be due to a mutation in a *nif* gene or, as it was shown in other nitrogen fixers, to a mutation impairing an enzyme involved in the general nitrogen metabolism of the cells, such as the *ntr* system or glutamate synthase (Darrow, 1980). The five Nif^- mutants studied here are unlikely to be *ntr* or *asm* mutants since they grow as well as the wild-type strain in minimal medium containing ammonia as the only source of nitrogen even at a concentration as low as 1 mM.

Acetylene-reducing activity measured in a crude extract of

the wild-type strain was of the same order of magnitude as that of regular free-living nitrogen fixers and at least 10 times higher than the maximum value reported for *Rhizobium* sp. 32HI in pure culture (Bergersen *et al.*, 1976). Assays of complementation of mutant crude extracts by Kp1 or Kp2 were positive only in the case of mutant 5740, which was complemented by Kp1 but not by Kp2. This strongly suggests that mutation *nif-5740* impairs a structural gene for nitrogenase. However, in the absence of information on the structure of sesbania rhizobium nitrogenase and on the nature of the *nif-5740* mutation, it is too early to decide which gene involved in the formation of a functional nitrogenase is impaired. This is the second example of interspecific biochemical complementation of nitrogenase activity in *Rhizobium,* since Maier and Brill (1976) reported complementation by nitrogenase reductase (Component II) from *Azotobacter vinelandii* of a crude extract from bacteroids of a *R. japonicum* Fix^- mutant.

Hybridizations performed with the restricted pRS2 plasmid revealed that the region of homology with the 6.2-kb *nif* *Eco*RI probe was limited to a 4-kb fragment formed by a 3.6-kb *Bgl*II-*Xho*I fragment and a contiguous 0.4-kb *Xho*I fragment (Figure 1). However, when the 13-kb *Bam*HI fragment was in turn used as a probe to hybridize with the restricted pSA30 plasmid, homology was found throughout the entire 6.2-kb *K. pneumoniae nif Eco*RI fragment. In particular the *Eco*RI-*Bgl*II and *Sma*I-*Eco*I fragments located at each extremity of the *Eco*RI fragment (Figure 2) showed a strong hybridization with the probe. It is not possible, since no heteroduplex analysis has been done yet, to determine which parts of the 6.2-kb *K. pneumoniae nif Eco*RI fragment do not hybridize with the homologous 4-kb sesbania rhizobium *nif* *Bgl*II-*Xho*I fragment. However, some conclusions may be drawn from the hybridization experiments. Hybridization of the *Eco*RI-*Bgl*II fragment of plasmid pSA30 (Figure 2) with the sesbania rhizobium *Bam*HI fragment used as a probe, strongly suggests that the *Bam*HI fragment carries a *nif* gene corresponding to *K. pneumoniae nifH* (Sundaresan and Ausubel, 1981; Scott *et al.*, 1981). In addition, hybridization of the central *Sal*I fragment suggests the existence of a *nif* gene corresponding to *K. pneumoniae nifK* (Riedel *et al.*, 1979; Ruvkun and Ausubel, 1981) but the presence of a gene corresponding to *K. pneumoniae nifD* is not excluded. The same reasoning with the distal *Sma*I-*Eco*RI fragment suggests the existence of *nif* genes corresponding to *K. pneumoniae nifY* (Pühler and Klipp, 1981) or *K. pneumoniae, nifE* (Riedel *et al.*, 1979) or both. This differs slightly from previous observations made in other *Rhizobium* species where no hybridization with plasmid pCM1 was found (Prakash *et al.*, 1981) and where no homology with *K. pneumoniae nifK* was detected (Bånfalvi *et al.*, 1981; Hennecke, 1981; Ruvkun and Ausubel, 1981). In *R. meliloti* and *R. japonicum,* homology was limited to *nifH* and *nifD* and the size of the homologous fragment was ~2.6 kb.

Genetic complementation of the *nif-5740* mutation by plasmid pRS1 does not re-establish the level of nitrogenase activity of the wild-type strain (7% in whole cells under conditions of derepression, 9% in crude extracts). However, on the 13-kb *Bam*HI DNA fragment carried by plasmid pRS1, the region of homology corresponds to a 4-kb sequence located in the middle of the fragment, and it is very likely that the complementing gene is transcribed from its own promotor. The relatively low level of complementation could be due to at least two reasons. First, introduction of plasmid pRS1 into the wild-type strain delays derepression of nitrogenase activity which levels at ~50% of the maximal value. This could be due to the titration by plasmid pRS1 of a positive effector (the equivalent of the *K. pneumoniae nifA* product: Buchanan-Wollaston *et al.*, 1981). Second, complementation of oligomeric proteins such as nitrogenase occurs frequently at a relatively low level as observed in *K. pneumoniae* with *nifK* and *nifD* point mutations.

In spite of the relatively low level of complementation, strain 5740(pRS1) appeared to be as efficient *in planta* as the wild-type strain (Figure 4) at least for the first 4 weeks after inoculation of the roots. This raises a more general question that has not yet been considered in *Rhizobium,* which is that of the relationship between nitrogenase activity determined in whole cells or crude extracts of a given strain and the ability of that strain to support plant growth. In particular, this introduces the possibility of isolating Nif^- mutants that appear leaky on plates which could not be selected directly *in planta.*

Questions still entirely open are that of the localization of sesbania rhizobium *nif* genes: are they plasmidic as in the fast-growing temperate rhizobia (Bånfalvi *et al.*, 1981; Hombrecher *et al.*, 1981; Prakash *et al.*, 1981; Rosenberg *et al.*, 1981) or chromosomal? What is their kinship with the *nif* genes of the other *Rhizobium* species? Finally, in sesbania rhizobium, one may wonder whether all the *nif* genes required for growth in the free-living state are also required for nitrogen fixation in the nodules and *vice versa.*

Materials and methods

Bacterial strains, plasmids, and bacteriophage

Sesbania rhizobium wild-type strain was ORS571 (Dreyfus and Dommergues, 1981a), termed here 571. *Escherichia coli* strains were HB101 (r_k^- m_k^- Str^r λ^r), LE392 (r_k^- m_k^- *supE supF*), C600(P2) (r_k^- m_k^- P2 lysogen), BHB2688 and BHB2690 (Hohn and Collins, 1980). Plasmids were pRK290 (Tc^r IncP Tra^-) (Ditta *et al.*, 1980), pBR322 (Bolivar *et al.*, 1977), pSA30 and pCM1 (Cannon *et al.*, 1979) which carry, respectively, the *nifHDK* and *nifB-K* genes of *K. pneumoniae.* Bacteriophage was λL47.1 (Loenen and Brammar, 1980).

Media and growth conditions for Rhizobium

The LS base contained per liter: D-L sodium lactate, 5 g; disodium succinate $6H_2O$, 5 g; K_2HPO_4, 1.67 g; KH_2PO_4, 0.87 g; $MgSO_4.7H_2O$, 0.1 g; NaCl, 0.05 g; $CaCl_2.2H_2O$, 40 mg; $FeCl_3.6H_2O$, 10 mg; $Na_2MoO_4.2H_2O$, 5 mg; $MnSO_4.H_2O$, 2.5 mg; $ZnSO_4.7H_2O$, 0.7 mg; $CoSO_4.7H_2O$, 0.14 mg; $CuSO_4.5H_2O$, 0.12 mg; H_3BO_3, 0.03 mg; pH 6.8. The LSO medium was the LS base supplemented with 0.02 mg/l biotin, 4 mg/l nicotinic acid and 4 mg/l calcium pantothenate. The YLS medium was the LSO medium supplemented with 1 g/l $(NH_4)_2SO_4$ and 1 g/l Difco Yeast Extract. All cultures were carried out at 30°C in Erlenmeyer flasks or in a 1.5 l Biolafitte fermentor containing 1 l of medium. With NH_4^+-containing media, the gas phase was air and under conditions of nitrogen fixation or nitrogenase derepression, the gas phase was a mixture of N_2:O_2 or Ar:O_2 (97:3, v/v). When the bacteria were grown in a fermentor, the pH was regulated at 7.2 with 1 N HCl. The same gas phase conditons were used for cultures on solid media.

Isolation of mutants

Ethyl-methane-sulfonate mutagenesis was performed as described by Miller (1972). Mutagenized bacteria were grown overnight in YLS medium, washed once with LSO medium, and plated on LSO solid medium. After 3 days of incubation under N_2:O_2 (97:3, v/v) tiny colonies were toothpicked on LSO, LSO supplemented with 15 mM NH_4^+, and YLS plates. Clones growing on ammonia-supplemented LSO and not on LSO plates were checked for nitrogenase activity. About 40 000 colonies from two mutagenized cultures were examined. Of 50 small colonies tested, five mutants, designated 5701, 5702, 5721, 5731, and 5740, were kept for further studies.

Nitrogenase assays

Nitrogenase activity was measured, either in whole cells or in crude extracts, by the acetylene reduction test (Elmerich *et al.*, 1978). One unit of activity was defined as 1 nmol of ethylene produced/min. Crude extracts were obtained by

the method described by Eady *et al.* (1972) using a 0.1 M Tris-HCl buffer at pH 8.1. Pure nitrogenase (Kp1) and nitrogenase reductase (Kp2) of *K. pneumoniae* were prepared by J.Houmard according to Eady *et al.* (1972).

Isolation of DNA

Total DNA from sesbania rhizobium strain 571 was prepared according to the method of Marmur (1961) modified by a supplementary treatment with proteinase K. Plasmid DNA from *E. coli* strains was purified according to Humphreys *et al.* (1975). For rapid extraction from 1 ml culture, the method of Birboim and Doly (1979) was used. Plasmid purification from strain 5740(pRS1) was performed by the technique of Hirsch *et al.* (1980). Bacteriophage DNA was extracted from CsCl gradient purified particles using SDS deproteinisation (Loenen and Brammar, 1980).

Construction of bacteriophage and plasmid clones

Restriction endonucleases were from Biolabs. T_4 DNA ligase was from Boehringer. Digestions and ligations were performed according to the manufacturer's recommendations. Horizontal gel electrophoresis of DNA samples used conditions previously described (Elmerich *et al.*, 1978). Restriction fragments were recovered from the gels by electroelution. DNA probes were labelled with [α^{32}P]dATP (Amersham) according to Rigby *et al.* (1977). Filter blot hybridizations were performed by the methods of Southern (1975) or Benton and Davis (1977). Two gene banks of sesbania rhizobium strain 571 were constructed using bacteriophage λL47.1 DNA as a vector. For each bank, 5 μg bacterial DNA and 5 μg vector DNA were hydrolyzed, either with *Hind*III or *Bam*HI, mixed and ligated. Aliquots of the ligation mixture were packaged *in vitro* in λ heads (Hohn and Collins, 1980) and phage samples were plated on *E. coli* strain C600(P2). In both cases ~300 plaques were toothpicked onto overlays of *E. coli* strain LE392. Plates were used for filter hybridization with plasmid pSA30 DNA as a probe. Hybridizing phages were purified and their DNA was extracted. A 13-kb *Bam*HI fragment from one of the recombinant phages was subsequently cloned at the *Bgl*II site of plasmid pRK290 and at the *Bam*HI site of plasmid pBR322. After transformation of *E. coli* strain HB101 (Cohen *et al.*, 1972), recombinant plasmids were isolated. The plasmid derived from pRK290 was termed pRS1, whereas that obtained from pBR322 was termed pRS2. Plasmids pRK290 and pRS1 were introduced into the various sesbania rhizobium strains according to the procedure of Ditta *et al.* (1980).

Plant tests

S. rostrata seeds were surface sterilized and germinated as previously described (Dreyfus and Dommergues, 1981b). Germinated seeds were transferred to sterile 30 ml test tubes capped with aluminium foil and filled with Jensen medium (Vincent, 1970). The plants were grown at 25°C under artificial light (14 h/day). Two days after transfer, the Jensen medium was inoculated with 10^8 bacterial cells from an overnight culture in YLS medium. All experiments were incubated for 4 weeks and performed in triplicate. To recover the sesbania rhizobium strains, the root nodules were excised, surface sterilized in 0.01% $HgCl_2$ for 10 min, washed, and extracted by mechanical blending in LSO medium.

Acknowledgements

The authors wish to thank Dr. R.Haselkorn for discussions and suggestions while this work was in progress and for the gift of plasmids pRK290 and pRK2013, Drs. G.Zeikus and Y.Dommergues for critical reading of the manuscript, Dr. P.Tiollais for the gift of bacteriophage λL47.1, Drs. C.Franche and L.Sibold for help in some experiments, and G.Lépée for skilful technical assistance. This work was supported by research funds from the University Paris VII and from Elf-Aquitaine and Entreprise Minière et Chimique.

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