Variation of Sym Plasmid and Symbiotic Effectiveness in Isolates of *Rhizobium fredii*

CHEN WENLI LI FUDI ZHOU JUNCHU

Department of Microbiology, Huazhong Agricultural University, Wuhan, 430070

(Received January 13, 1995; Revised March 31, 1995)

ABSTRACT

The genetic diversity of symbiotic plasmids (pSyms) and symbiotic effectiveness was investigated in 11 isolates from one field population of *Rhizobium fredii* that had previously been characterized for plasmid content and serogroup polymorphisms. Two cloned DNA fragments from plasmid pSA30 and pIJ1216 were used as *nif* HDK and *nod* DABC probes respectively to identify pSym variation. Although only a single plasmid hybridized to both *nif* and *nod* probes in each strain tested, pSym size variation was great ranging from 247 to 381 MDa. In addition to pSym, all of the *R. fredii* strains studied carried 1~5 other plasmids, of which one was approximately 500MDa cryptic megaplasmid. The strains with different size of pSym significantly varied in their symbiotic effectiveness determined by their chlorophyll content, total N, dry weight of the plant and nitrogenase activity.

Key words *Rhizobium fredii*, sym plasmid, symbiotic effectiveness, variation

1 Introduction

Rhizobium strains are gram-negative soil bacteria which induce symbiotic nodules on the roots of leguminous hosts for atmospheric nitrogen fixation. Plasmids in most *Rhizobium* species carry genes controlling symbiotic functions, which are clustered on a single large plasmid termed "pSym" (Banfalvi et al., 1981; Barbour et al., 1985; Krol et al., 1982; Rosenberg et al., 1981). Two or more plasmids that carry genes controlling symbiotic functions have been found in certain strains of *R. leguminosarum* biovar *trifolii* (Hynes et al., 1990), of *R. etli* (Brom et al., 1992) and of *R. meliloti* (Hynes et al., 1986). Besides pSym(s), *Rhizobium* strains usually contain additional plasmids. The number of plasmids ranges from two to four or five, and may be as many as ten (Thurman et al., 1985). Because there is high variability, plasmid profile analysis has proven to be a useful tool for differentiating *Rhizobium* strains and determining their diversity (Glynn et al., 1985; Harrison et al., 1988; Hartmann et al., 1991; Young et al., 1988).

Differences in pSym within *R. leguminosarum* have been noted (Hombrecher et al., 1981; Young et al., 1988). *R. leguminosarum* biovar *trifolii* and *phaseoli*, and *R. fredii* contain large plasmids varying in size from 100 to 300MDa that carry genes involved in nodulation (*nod*) and symbiotic nitrogen fixation (*fix*), as well as the nitrogenase structural genes (*nif*HDK) (Beynon et al., 1980; Barbour et al., 1985; Harri-

son, et al., 1988).

In our previous study, genetic diversity have been demonstrated in *Rhizobium fredii* population from the soil of Honghu County, Hubei Province by plasmid content and serogroup polymorphisms (Chen et al., 1994). This study investigated pSym variation in a number of *R. fredii* strains from that population and assessed their symbiotic effectiveness.

2 Materials and methods

2.1 Bacterial strains and plasmids: Bacterial strains and plasmids utilized in this study are listed in Table 1. Except USDA 205, all of the *R. fredii* strains tested were isolated from the soil of Honghu County, Hubei Province (Chen et al., 1994).

Table 1 Bacterial strains and plasmids.

Strain or plasmid	Relevant Characteristics	References
<i>R. fredii</i>		
HA 1-1-4	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
HA 12-1-12	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
HA 12-1-11	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
HA 8-1-4	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
HA 9-1-9	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
HA 13-2-6	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
HA 8-1-8	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
HA 7-2-2	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
HA 13-2-1	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
HA 6-1-20	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
HA 9-1-10	Nod ⁺ Fix ⁺ , wild type	Chen W L, Li F D, Zhou J C, et al. (1994)
USDA205	Nod ⁺ Fix ⁺ , wild type	Keyser HH, Bohlool BB, Hu TS, et al. (1982)
Plasmid		
pSA30	6. 2Kbp EcoRI restriction fragment carrying the <i>nif</i> HDK of <i>K. pneumoniae</i> cloned into pACYC184	Cannon F C, Riedel G E and Ausubel F M (1979)
pIJ1216	6. 6Kbp EcoRI restriction fragment carrying the <i>nod</i> DABC of <i>R. leguminosarum</i> cloned into pKT230	Downie J A, Knight C D, Johnston A W B, et al. (1985)

2.2 Leguminous plant: Soybean (*Glycine max*) cultivar Aijiaozao was used in this

study, which was kindly provided by Institute of Oil Crops, Chinese Academy of Agricultural Sciences. It was the cultivar planted in Honghu County, where the *R. fredii* strains tested were isolated.

2.3 Isolation of plasmid DNA and plasmid visualization: *R. fredii* strains were grown in PA media (Peptone $4\text{g} \cdot \text{L}^{-1}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ $0.5\text{g} \cdot \text{L}^{-1}$, pH 6.8~7.0) overnight (to a concentration of approximately 10^7 cells ml^{-1}) following inoculation with actively growing culture. Cell culture (1ml) was centrifuged and to the pellet was added 50 μl of lysis mix (1mg lysozyme ml^{-1} , 1 unit RNAase ml^{-1} in 25% W/V sucrose and $0.025\text{mol} \cdot \text{L}^{-1}$ Tris/HCl). This mixture was immediately transferred to the sample wells of a 0.65% (W/V) agarose gel, a single wide well containing 0.4% (W/V) agarose and 1% (W/V) SDS was incorporated behind these wells. A horizontal gel kit was used to run gels at 100V for 5h in Tris/borate buffer (pH8.3). Gels were stained in a solution of $1\mu\text{g}$ ethidium bromide ml^{-1} for 40 min.

Plasmid size was determined by calibration against the relative mobilities of four plasmids in USDA205 whose sizes were known as 7, 120, 230, 450MDa respectively (Broughton W J, Heycke N, Meyer H Z A, et al., 1984).

2.4 Southern blotting and hybridization: Plasmid DNA were Southern blotted from the gel to a Gene Screen (Du pont, product of NEN) following the manufacturer's instruction. Two DNA probes were used in this study, 6.2Kbp EcoRI restriction fragment from pSA30 and 6.6Kbp EcoRI restriction fragment from pIJ1216. Labelling of probes, hybridization to the membrane, and detection of hybrid DNA were done with the Nonradioactive DNA Labelling and Detection Kit from Boehringer Mannheim, according to the instructions provided with the kit.

2.5 Plant inoculation tests: Plants were grown in the assembly pot similar to Leonard jar designed by this laboratory. Sand was filled in a plastic cup with a small hole at the bottom through which a wick was anchored. The cup was loaded onto a glass bottle containing Fahraeus nitrogen-free solution (Fahraeus, 1957). The whole device was sterilized at 121°C for 120 min. Seeds of soybean (*Glycine max*) cultivar Aijiaoao were pre-immersed in 95% ethanol for 5 min, surface-sterilized with 0.1% HgCl_2 for 5 min and rinsed with sterile water for 10 times. Sterilized seeds were germinated on water agar plate for 2 days in darkness at 28°C . The resulting seedlings were planted singly in chamber sand pot and inoculated with 1 ml of late log-phase culture (10^8 cells ml^{-1}) of each strain grown in yeast extract mannitol (YEM) broth culture when the cotyledons opened. There were 8 replicates per treatment and the experiment was arranged in a completely randomized design. Uninoculated plants were served as controls. Throughout the duration of the plant test, no nodules were found in the uninoculated control plants, showing the absence of rhizobial contamination. Plants were grown in a green house under natural light and harvested at 40 days after inoculation.

Effectiveness of rhizobial strains was determined by chlorophyll content, nitrogenase activity, total N and dry weight of the soybean shoots.

2.6 Chlorophyll measurement: Chlorophyll content determination was done by some modification of the method described by Mirza N A, Bohlool B B and Somasegaran P (1990). At harvest, using a standard single-hole punch 12 leaf discs (6.35mm dia) were cut from leaflets of each plant taken at random but excluding the youngest and the oldest trifoliate leaves. After weighed, leaf discs were transferred immediately to

screw-capped glass vials containing 5ml of 95% ethanol. Chlorophyll was extracted by keeping the vials at 85°C for 30 min. Then 95% ethanol was added to the 10 ml mark of vials. Chlorophyll (a + b) concentration was determined spectrophotometrically at 645nm and 663 nm.

2.7 Acetylene reduction assay: Nitrogenase activity was measured by acetylene reduction performed on the intact root system including nodules. Roots were separated from shoots at the cotyledon scars and placed in a 20 ml sealed vial. The reaction was started with an air gas phase containing 2% (V/V) acetylene. After incubating for 1h samples were injected into a Hitach 163 gas chromatography with a Porapak N 80~100 column and nitrogen carrier gas at 90°C. Nitrogenase activity was expressed as micromoles of ethylene produced per milligram of nodule fresh weight per hour.

2.8 Kjeldahl determination of plant total N: The plant tops were oven dried at 65°C for 48h and dry weight recorded. 0.2g of the finely ground plant material was subjected to Kjeldahl digestion for 1h at 440°C. Total N was then determined by a semi-micro-Kjeldahl procedure.

2.9 Statistical analysis: Each experiment was analysed separately by analysis of variance for a completely-randomized design. The Duncan's Multiple Range Test was used to compare the means of strain treatments.

3 Results

3.1 Plasmid profiles and plasmid characterization

The isolates of *R. fredii* examined exhibited much variation in plasmid profile (In Cover Page 3 Fig. 1a, 2a). Two to six plasmids were reproducibly detected in each strain. By calibration against the relative mobilities of four plasmids in USDA205 with known sizes of 70 120 230 450 MDa respectively (Broughton W J, Heycke N, Meyer H Z A, et al., 1984), the plasmid sizes of the tested strains ranged from 12 to 530 MDa (Table 2). A characteristic large plasmid (about 500MDa) was present in all the strains. This diversity of plasmid profiles demonstrated the existence of a great heterogeneity of plasmid pools within *R. fredii* strains examined.

3.2 Location of symbiotic genes on plasmids

Eckhardt gels used for visualization of plasmids were transferred to a Gene Screen, then hybridized to *nif* HDK and *nod* DABC probes respectively (In Cover Page 3 Fig. 1b, 2b). In each strain, only one plasmid band hybridized to the *nif* probe. This plasmid band also hybridized to the *nod* probe. The sizes of the hybridized plasmids (pSym) ranged from 247 to 381MDa (Table 2). The other megaplasmids harbored by the *R. fredii* strains studied did not hybridize to the *nif* or *nod* probe.

3.3 Effectiveness of rhizobial strains

All of the *R. fredii* strains examined effectively nodulated soybean cultivar Aijiaozao with efficient nitrogen fixation (Table 3). HA12-1-12 was apparently the most effective strain as judged by chlorophyll content, total N, nitrogenase activity and plant dry weight. Significant difference between *R. fredii* strains tested was found in chlorophyll content and other parameters. The effectiveness of strains with different plasmid profile varied.

Table 2 The number and approximate sizes of the plasmids of each strain tested.

Strain	No. of plasmids	Sizes of plasmids (MDa) *
HA1-1-4	2	490, <u>318</u>
HA12-1-12	3	490, <u>275</u> , 201
HA12-1-11	3	497, <u>259</u> , 156
HA 8-1-4	3	478, <u>326</u> , 112
HA 9-1-9	4	472, <u>247</u> , 186, 70
HA13-2-6	4	490, <u>326</u> , 113, 59
HA 8-1-8	5	523, <u>354</u> , 275, 203, 56
HA 7-2-2	5	530, <u>381</u> , 148, 94, 55
HA13-2-1	5	510, <u>282</u> , 201, 124, 40
HA 6-1-20	6	510, <u>296</u> , 203, 70, 32, 12
HA 9-1-10	6	454, <u>314</u> , 250, 164, 64, 40

* The numbers underlined correspond to sizes of plasmids showing hybridization to *nif*HDK and *nod*DABC probes in experiments such as that shown on Fig. 1, 2

Table 3 Effectiveness of *R. fredii* strains on soybean.

Strain	Chlorophyll (a + b) content (mg · g ⁻¹)	Plant dry weight (g)	Toal N (mg. plant ⁻¹)	Amount of acetylene reduced (μ molC ₂ H ₄ · g ⁻¹ hr ⁻¹)
CK	0.307f *	0.265d	2.833h	0
USDA205	1.142e	0.461c	9.128g	5.136g
HA12-1-11	1.627d	0.536bc	8.409g	6.565f
HA13-2-1	1.631d	0.553bc	11.778f	6.878f
HA 1-1-4	1.713cd	0.560b	12.905f	7.035f
HA 8-1-8	1.822c	0.578b	13.320f	9.671e
HA13-2-6	2.173b	0.643ab	17.756e	12.265d
HA 7-2-2	2.178b	0.661ab	16.208de	13.202cd
HA 6-1-20	2.228ab	0.675ab	18.894cd	13.465c
HA 8-1-4	2.238ab	0.688a	20.739bc	13.975c
HA 9-1-10	2.324ab	0.706a	21.501b	15.435b
HA 9-1-9	2.340ab	0.720a	23.179b	17.146a
HA12-1-12	2.372a	0.809a	28.705a	17.220a

* Values are the means of eight replications

Means having the same letters within a column were not significantly different by Duncan's Multiple Range Test at 1% probability

4 Discussion

This investigation reveals genetic diversity among 11 *R. fredii* strains with different plasmid profile from Honghu County, Hubei Province. The results of location of symbiotic genes indicated that a great variation in size of pSym existed in *R. fredii* strains examined. These results corroborate the report of pSym size differences by Hombrecher et al. (1981), Krol et al. (1982) in *R. leguminosarum* bv. *viceae*, and by Harrison et al. (1988) in *R. leguminosarum* bv. *trifolii*. A number of explanations can be put forward to account for this diversity. The instability and tendency of pSym to be deleted under particular conditions have been recognized by Zurkowski (1982); presumably conditions within natural environments may occasionally favor such deletions, thereby generating size differences in indigenous pSyms. Ronson et al. (1983) showed the readiness of pSym to recombine with cryptic plasmids which could result in a pSym carrying a large proportion of non-symbiotic DNA. Moreover, recombination between different pSyms has been reported (Christensen et al., 1983).

In all the strains examined, a single plasmid ranging from 247 to 381 MDa was shown to contain the symbiotic determinants. A characteristic plasmid, about 500MDa, was present in each strain. Unlike *R. meliloti* (Banfalvi et al., 1981; Rosenberg et al., 1981), this megaplasmid does not seem to contain *nif*HDK or *nod* DABC genes. However, on the basis of its presence in all strains, this megaplasmid may specifically encode some unknown genetic information relevant to either the symbiosis or the survival of the bacteria in the soil, and it merits further study.

The significant difference between *R. fredii* strains studied was observed in their effectiveness. Contrary to the work of Thurman et al. (1985), we did not find a negative correlation between the total number of plasmids or pSyms and dry weight of plants. It showed that the effectiveness of strains with different size of pSym varied.

ACKNOWLEDGEMENT

This work is part of the joint EC-project no. TS3-CT92-0127 and was supported in part by the National Natural Science Foundation of China (NSFC).

REFERENCES

- Banfalvi Z, Sakanyan V, Konec C, et al., 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *Rhizobium meliloti*. *Mol. Gen. Genet.*, **184**: 318~325
- Barbour W M, J N Mathis, G H Elkan, 1985. Evidence for plasmid-and chromosome-borne multiple *nif* genes in *Rhizobium fredii*. *Appl. Environ. Microbiol.*, **50**: 41~44
- Beynon J L, J E Beringer, A W B Johnston, 1980. Plasmids and host range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. *J. Gen. Microbiol.*, **120**: 421~429
- Brom S, Garcia de los Santos A, T Stepkowsky, et al., 1992. Different plasmids of *Rhizobium leguminosarum* bv. *phaseoli* are required for optimal symbiotic performance. *J. Bacteriol.*, **174**: 5183~5189
- Broughton W J, N Heycke, H Z A Meyer et al., 1984. Plasmid-linked *nif* and *nod* genes in fast growing rhizobia that nodulate *Glycine max*, *Psophocarpus tetragonolobus*, and *Vigna unguiculata*. *Proc.*

- Natl. Acad. Sci. USA.*, **81**: 3093~3097
- Cannon F C, G E Riedel, F M Ausubel, 1979. Overlapping sequences of *Klebsiella pneumoniae* nif DNA cloned and characterized. *Mol. Gen. Genet.*, **174**: 59~66
- Chen W L, F D Li, J C Zhou, et al., 1994. Diversity of *Rhizobium fredii* strains in the soil of Honghu County, Hubei Province. *J. Huazhong Agricultural University*, **13**: 581~587 (in Chinese)
- Christensen A H, K R Schubert, 1983. Identification of a *Rhizobium trifolii* plasmid coding for nitrogen fixation and nodulation genes and its interaction with pJB5JL, a *Rhizobium leguminosarum* plasmid. *J. Bacteriol.*, **156**: 592~599
- Downie J A, C D Knight, A W B Johnston, et al., 1985. Identification of genes and gene products involved in the nodulation of peas by *Rhizobium leguminosarum*. *Mol. Gen. Genet.*, **198**: 255~262
- Fahraeus G, 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J. Gen. Microbiol.*, **16**: 374~381
- Glynn P, P Higgins, A Squartini, et al., 1985. Stain identification in *Rhizobium trifolii* using DNA restriction analysis, plasmid DNA profiles and intrinsic antibiotic resistance. *FEMS Microbiol. Lett.*, **30**: 177~182
- Harrison S P, D G Jones, P H D Schunmann, et al., 1988. Variation in *Rhizobium leguminosarum* biovar *trifolii* Sym plasmids and the association with effectiveness of nitrogen fixation. *J. Gen. Microbiol.*, **134**: 2721~2730
- Hartmann A, N Amarger, 1991. Genotypic diversity of an indigenous *Rhizobium meliloti* field population assessed by plasmid profiles, DNA fingerprinting, and insertion sequence typing. *Can. J. Microbiol.*, **37**: 600~608
- Hombrecher G, N J Brewin, A W B Johnston, 1981. Linkage of genes for nitrogenase and nodulation ability on plasmids in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. *Mol. Gen. Genet.*, **182**: 133~136
- Hynes M F, N F McGregor, 1990. Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium Leguminosarum*. *Mol. Microbiol.*, **4**: 567~574
- Hynes M F, R Simon, P Muller, et al., 1986. The two megaplasmids of *Rhizobium meliloti* are involved in the effective nodulation of alfalfa. *Mol. Gen. Genet.*, **202**: 356~362
- Keyser H H, B B Bohlool, T S Hu, et al., 1982. Fast-growing rhizobia isolated from root nodules of soybean. *Science*, **215**: 1631~1632
- Krol A J M, J G J Hontelez, A van Kammen, 1982. Only one of the large plasmids in *Rhizobium leguminosarum* strain PRE is strongly expressed in the endosymbiotic state. *J. Gen. Microbiol.*, **128**: 1839~1847
- Mirza N A, B B Bohlool, P Somasegaran, 1990. Non-destructive chlorophyll assay for screening of strains of *Bradyrhizobium iaponicum*. *Soil Biol. Biochem.*, **22**: 203~207
- Ronson C W, D B Scott, 1983. Identification, broad host range mobilization and mutagenesis of a *Rhizobium trifolii* Sym: R68.45 cointegrate plasmid. In: Puhler A ed., *Molecular Genetics of Bacteria-plant Interaction*, Berlin and Heidelberg: Springer, 177~187
- Rosenberg C, P Boistard, J Denarie, et al., 1981. Genes controlling early and late functions in symbioses are located on a megaplasmid in *Rhizobium meliloti*. *Mol. Gen. Genet.*, **184**: 326~333
- Thurman N P, D M Lewis, D G Jones, 1985. The relationship of plasmid number to growth, acid tolerance and symbiotic efficiency in isolates of *Rhizobium trifolii*. *J. Appl. Bacteriol.*, **58**: 1~6
- Young J P W, W Wexler, 1988. Sym plasmid and chromosomal genotypes are correlated in field populations of *Rhizobium leguminosarum*. *J. Gen., Microbiol.*, **134**: 2731~2739
- Zurkowski W, 1982. Molecular mechanism for loss of nodulation properties of *Rhizobium trifolii*. *J. Bacteriol.*, **150**: 999~1007

Fig. 1 Plasmid profiles and location of *nif* genes in *R. fredii* strains

a. Plasmid electrophoretic pattern on a 0.65% agarose gel

b. Hybridization with *nif* HDK probe

A. pSA30 DNA; B. N. reference strain *R. fredii* USDA205; C. HA9-1-10; D. HA6-1-20; E. HA13-2-1; F. HA7-2-2; G. HA8-1-8; H. HA13-2-6; I. HA9-1-9; J. HA8-1-4; K. HA12-1-11; L. HA12-1-12; M. HA1-1-4

Fig. 2 Plasmid profiles and location of *nod* genes in *R. fredii* strains

a. Plasmid electrophoretic pattern on a 0.65% agarose gel

b. Hybridization with *nod*DABC probe

A. pIJ1216; B. N. reference strain *R. fredii* USDA205; C. HA9-1-10; D. HA6-1-20; E. HA13-2-1; F. HA7-2-2; G. HA8-1-8; H. HA13-2-6; I. HA9-1-9; J. HA8-1-4; K. HA12-1-11; L. HA12-1-12; M. HA1-1-4