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Enhanced N_2-fixing ability of a deletion mutant of arctic rhizobia with sainfoin (*Onobrychis viciifolia*)

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Summary. Mutagenesis provoked by exposure at elevated temperature of the cold-adapted, arctic *Rhizobium* strain N31 resulted in the generation of five deletion mutants, which exhibited loss of their smaller plasmid (200 kb), whereas the larger plasmid (>500 kb) was still present in all mutants. Deletion mutants did not show differences from the wild type in the antibiotic resistance pattern, the carbohydrates and organic acids utilization, and the growth rate at low temperature. However, deletion mutants differed from the wild type and among themselves in the ex planta nitrogenase activity, the nodulation index, and the symbiotic effectiveness. The deletion mutant *N31.6rif*^{r} showed higher nodulation index and exhibited higher nitrogenase activity and symbiotic efficiency than the other deletion mutants and the wild type. The process of deletion mutation resulted in the improvement of an arctic *Rhizobium* strain having an earlier and higher symbiotic nitrogen fixation efficiency than the wild type.

Key words: Arctic *Rhizobium* – Symbiotic efficiency – Nitrogenase – Plasmid – Deletion mutant

Introduction

Mutagenesis has been used as a process (i) for obtaining microbial strains that exhibit enhanced synthesis of economically important metabolites, and (ii) for investigating various biochemical processes. In *Rhizobium*, mutagenesis has yielded strains capable of improving symbiosis between the mutant and the host (Maier and Brill 1978; Paau 1989; Cannon et al. 1988; Shukla et al. 1989). Maier and Brill (1978) mutagenized a culture of *R. japonicum* with N-methyl-N′-nitrosoguanidine and screened the derivatives for greater symbiotic activity; they obtained two mutant derivatives nodulating the roots earlier than the wild type and expressing greater symbiotic N_2-fixing activity. Paau (1989) also mutagenized selected isolates of *R. japonicum* with N-methyl-N′-nitrosoguanidine for isolating mutants with enhanced ability to reduce acetylene. Five mutants, thus isolated, were capable of higher level occupancy of nodules in soybean fields than the wild types.

Deletion mutation in bacterial strains can be efficiently induced by growing the strains at elevated temperatures. Many fast-growing rhizobial strains lost their symbiotic properties through a process of prolonged exposure to elevated temperatures (Zurkowski and Lorkiewicz 1978). The eradication of the symbiotic capability was attributed to the loss of large endogenous plasmids or to internal deletions in these plasmids (Zurkowski and Lorkiewicz 1979). Morrison et al. (1983) reported the isolation of nonnodulating mutants of a *Rhizobium* strain by heat curing of a single large, resident plasmid. Banfalvi et al. (1981) generated several deletion mutants of *R. meliloti* by growing at 39.5 °C. Deletion in plasmid DNA was detected by gel electrophoresis. Several mutants showed increased electrophoretic mobility of a plasmid, indicating loss of a part of the plasmid.

In this paper, we (i) generated deletion mutants of an arctic rhizobial strain N31 by exposure at elevated temperature, (ii) compared the characteristics of deletion mutants with the wild-type strain N31, (iii) compared the nodulating and ex planta N_2-fixing ability of the deletion mutants, and (iv) compared the symbiotic efficiency of a deletion mutant that exhibited higher ex planta N_2-ase activity and nodulating ability than the wild type.

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Materials and methods

Mutagenesis

Arctic rhizobial strain N31 grew at 25 °C but was unable to grow at 35 °C (Prévost et al. 1987a). Strain N31 was grown in YEM (Vincent 1970) broth at 25 °C. Log-phase-grown cultures were serially diluted with K-phosphate buffer (pH 7.0; 0.05*M*) and plated on TYC solid medium (Beringer 1974). The culture plates were incubated at 35 °C for 1 week, during which no growth occurred. The plates were then incubated at 25 °C. After 2 weeks, mutant colonies appeared at a frequency of 2.5×10^{-4}. The colonies were purified and analyzed for plasmid loss or deletion in plasmid DNA.

Plasmid visualization

Plasmids were vizualized by the modified Eckhardt procedure (Rosenberg et al. 1982) from early log-phase bacteria. Production of polysaccharides interfered in plasmid analysis. Therefore, bacterial cells were grown in YEM broth up to an optical density of 0.1–0.2 (at 600 nm) and washed with 0.5*M* NaCl solution. Further lysis and treatment of the cells were followed as described by Rosenberg et al. (1982). Electrophoresis was performed for 90 min at 3–4 mA and for 3 h at 40 mA. After ethidium-bromide staining of the gel, the DNA was visualized under ultraviolet light.

Cultural and physiological characteristics

All the mutant derivatives of strain N31 that showed deletion mutations were tested for antibiotic resistance, utilization of carbohydrates and organic acids, and growth at low temperatures (5 °C) as described by Prévost et al. (1987a).

Nodulation index and ex planta N_2-ase activity

Nodulation index, which is a measure of nodule number (*N*), the size of nodules (*S*), and the internal color of nodules (*C*), was calculated for each deletion mutant derivative of strain N31, as described by Gueye and Bordeleau (1988). The deletion mutants of strain N31 were assayed for ex planta nitrogenase activity in low-nitrogen liquid medium amended with 0.75% xanthan as described before (Jain et al. 1990).

Symbiotic effectiveness

A deletion mutant derivative, N31.6, exhibited greater nodulation index and ex planta nitrogenase activity than the wild type in earlier experiments. A rifampicin-resistant trait was introduced in this derivative by selecting spontaneous mutants growing on TYC solid medium agar plates (Beringer 1974), amended with 200 µg/ml rifampicin. Thus, the spontaneous, rifampicin-resistant mutant *N31.6rif*^{r} was evaluated for its symbiotic effectiveness on the temperate legume sainfoin. Dehulled, uniformly sized seeds of sainfoin cv Melrose were surface-sterilized and germinated on sterile agar (1%) for 36 h in the dark. Five uniform germinated seeds were planted in Riviera pots (Provençale de Matières Plasltiques de Marseille, France), sterilized with 0.5% Oakite solution (sanitizer no. 1, Oakite Products of Canada, Bramalea, Ontario) and containing 2.3 l of an autoclaved mixture of 50% (v/v) vermiculite in sand. The pot reservoirs were filled (1.5 l) with a nutrient solution (Bordeleau et al. 1977) supplemented with 30 mg/l N as KNO_3. Every 2 weeks, pots were flushed and refilled with fresh nutrient solution. Distilled water was added to the pots when needed. At day 14, pots were thinned to three uniform plants per pot, and they were inoculated by adding to each pot 100 ml of the nutrient solution containing approximately 10^{-7} *Rhizobium* cells per ml of the appropriate strain. Plants were grown under a 16-h light period at 400 $\mu E \cdot m^{-2} \cdot s^{-1}$ illumination at 20 °C and 8-h at 270 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 15 °C. The experimental design was a randomized complete block with eight replicates. Plants were harvested at the beginning of anthesis: 67 days (first harvest), 104 days (second harvest), and 131 days (third harvest) after sowing. Shoot were dried at 80 °C to constant weight (2 days) in a forced-air oven, weighed, ground, and analyzed for total N by a micro-Kjeldahl method (Ward and Johnston 1962).

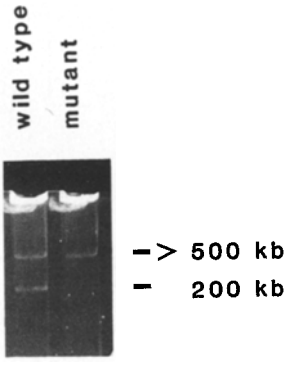

Fig. 1. Visualization of plasmids. Wild-type lane is strain N31 carrying two plasmids (> 500 and 200 kb). The mutant lane is a typical deletion mutant of heat-treated strain *N31.6* lacking the small plasmid 200 kb. Five deletion mutants had this typical plasmid profile. The method used was the Eckhardt procedure as modified by Rosenberg et al. (1982)

Results

Plasmid analysis

Plasmid analysis of arctic rhizobia strain N31 by the modified Eckhardt procedure (Rosenberg et al. 1982) demonstrated the presence of one large plasmid (> 500 kb) and one small plasmid (200 kb) (Fig. 1). The molecular weights of the plasmids of strain N-31 were calculated by comparing known molecular weights of megaplasmids of other arctic rhizobia (Caudry-Reznick et al. 1986). The strain was exposed to 35 °C for 1 week. Approximately 300 colonies, which grew after prolonged exposure to elevated temperature, were purified and tested for plasmid loss or for internal deletions in plasmids. Out of 300 mutants, 5 mutants lost their smaller plasmid (Fig. 1). In all mutants, small internal deletions of plasmids could not be detected by gel electrophoresis.

Cultural and physiological characteristics

Five mutants that exhibited loss in their plasmid DNA were checked for resistance against eight antibiotics (Table 1). The deletion mutants did not show different antibiotic resistance pattern than the wild type.

All the deletion mutants and the wild type were able to utilize glucose, mannitol, citrate, succinate, and fumarate; α-ketoglutarate was not utilized (Table 1). In a previous study (Prévost et al. 1987a), the wild-type strain

Table 1. Cultural and physiological characteristics of wild-type N31 strain of arctic *Rhizobium* and its deletion mutants

	Strains						
	N31	*N31.6*	*N31.6rif*^{r}	*N31.78*	*N31.155*	*N31.217*	*N31.300*
Antibiotic resistance[a]							
Ap 10	s	s	s	s	s	s	s
Cm 10	s	s	s	s	s	s	s
Er 10	r	r	r	r	r	r	r
Km 10	r	r	r	r	r	r	r
Nal 20	r	r	r	r	r	r	r
Rif 60	s	s	r	s	s	s	s
Sm 2	r	r	r	r	r	r	r
Tc 2	s	s	s	s	s	s	s
Utilization of carbon sources[b]							
Glucose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+
Succinate	+	+	+	+	+	+	+
Fumarate	+	+	+	+	+	+	+
α-keto-glutarate	−	−	−	−	−	−	−
Mean generation time on YEM[c]							
At 5 °C	82	81	81	82	83	82	81
At 25 °C	4.5	4.3	4.3	4.4	4.8	4.6	4.4

[a] Plates of YEM-containing antibiotics were inoculated and incubated at 25 °C for 7 days. The antibiotics used were in μg/ml: ampicillin (Ap 10), chloramphenicol (Cm 10), erythromycin (Er 10), kanamycin (Km 10), nalidixic acid (Nal 20), rifampicin (Rif 60), streptomycin (Sm 2), tetracyclin (Tc 2). Strains were recorded resistant (r) when growth occurred, and sensitive (s) when no growth or very poor growth occurred

[b] Strains were tested for their ability to grow with different carbohydrates (glucose or mannitol at 1%) and organic acids (citrate, succinate, fumarate, α-ketoglutarate, each at 2, 10, and 20 m*M*) as sole source of carbon in a defined medium enriched with thiamine (100 μg/l) and panthotenic acid (100 μg/l), according to a method previously described (Antoun et al. 1984)

[c] Strains were cultured in 50-ml Erlenmeyer flasks containing 10 ml of liquid YEM medium (Vincent 1970) for 4 days at 25 °C or 40 days at 5 °C on a rotary shaker (125 rpm). Cells from 1 ml of culture were sampled periodically (every 4 h at 25 °C, 5 days at 5 °C), washed twice in saline, resuspended in 1 ml 1 *N* NaOH, then heated at 90 °C for 10 min prior to protein determination by the modified Lowry procedure (Hanson and Phillips 1981), using the Folin reaction and bovine albumin as standard. All determinations were in triplicate. The mean generation time was calculated during the exponential phase

N31 was shown to utilized these organic acids and carbohydrates.

In liquid YEM medium, growth of all the deletion mutants was compared with the wild type. At 5 and 25 °C, there were no significant differences in the growth rates of the deletion mutants and the wild type (Table 1). Mean generation time of the wild type was 82 and 4.5 h at 5 and 25 °C, respectively.

Nodulation index and ex planta N_2-ase activity

Nodulation index of all the deletion mutants and the wild-type strain N31 was compared (Table 2). Nodulation index was higher (6) for deletion mutants *N31.6rif*^{r} and *N31.6* than for the wild-type strain and other deletion mutants (4 or less).

There were significant differences in ex planta N_2-ase activity for the deletion mutants and the wild-type strain N31 (Table 3). Four of the mutants showed significantly lower N_2-ase activity than the wild type. One mutant, *N31.6*, exhibited 19.8% higher N_2-ase activity than the wild type. The rifampicin-resistant trait was introduced into the deletion mutant *N31.6* for further inoculation studies and genetic characterization experiments. There were no significant differences ($P>0.05$) in ex planta N_2-ase activity among wild-type strain N31, mutant *N31.6*, and mutant *N31.6rif*^{r}, although both the mutants, *N31.6* and *N31.6rif*^{r}, showed 19.8 and 15.8% greater activity, respectively, than the wild-type strain N31 (Table 3).

Symbiotic effectiveness of deletion mutant N31.6rif^r

Symbiotic performance of deletion mutant *N31.6rif*^{r} with sainfoin was compared by inoculating plants with the mutant and the wild-type strains. At the first harvest, the shoot dry weight (SDW) yield of sainfoin inoculated with mutant *N31.6rif*^{r} was significantly higher ($P<0.05$) than that of the N31 treatment (Table 4). At the second and third harvests, the SDW yield of sainfoin plants

Table 2. Nodulation index of wild-type N31 strain of arctic *Rhizobium* and its deletion mutants

	Host plant							
	Sainfoin				*A. alpinus*			
	Nodulation[a]							
Strains	N	S	C	I	N	S	C	I
N31	2	2	1	4	2	2	1	4
N31.6	3	2	1	6	3	2	1	6
N31.6rif^r	3	2	1	6	3	2	1	6
N31.78	2	2	1	4	2	1	1	2
N31.155	2	2	1	4	2	2	1	4
N31.217	3	1	1	3	2	2	1	4
N31.300	3	1	1	3	3	1	1	3

[a] Each strain was tested for ability to nodulate *Astragalus alpinus* (Sainfoin) and *Onobrychis viciifolia* under sterile conditions. Surface-sterilized seeds were sown in 200 × 25 mm sterile, cotton-plugged glass tubes containing 20 ml of vermiculite and 15 ml of a nitrogen-free nutrient solution (Bordeleau et al. 1977), and placed in a controlled-environment growth cabinet programmed for 350 μE m^{-2} s^{-1} illumination at 20 °C for 16 h, alternating with 270 μE m^{-2} s^{-1} at 15 °C for 8 h. After 1 week, the seedlings in the stoppered tubes were inoculated with 10^6–10^7 cells of the various mutants. Six weeks after inoculation, root systems were examined for nodulation. Nodule number [*N*] was rated on a scale from 0 (no nodule) to 3 (many nodules); nodule size [*S*] was from 1 (small nodule) to 2 (big nodule), and nodule internal color [*C*] was from 0 (white) to 1 (red). Nodulation index [*I*] was calculated as ($N \times S \times C$). Tests were performed twice in four replicates

Table 3. Ex planta nitrogenase activity (acetylene reduction activity) of wild-type N31 strain of arctic *Rhizobium* and its deletion mutants

Strains	Nitrogenase activity (nmol $C_2H_4 \times$ mg protein^{-1})
Wild-type N31	149.56 a*
Deletion mutants	
N31.6	179.10 a
N31.6rif^r	173.18 a
N31.78	71.12 b
N31.155	20.32 c
N31.217	58.31 cb
N31.300	21.40 c

* Means followed by the same letter are not significantly different ($P \leq 0.05$) according to the Duncan's multiple range test. Means are from ten replicates

inoculated with *N31.6rif*^{r} was significantly ($P < 0.05$) higher than the yield obtained with both uninoculated controls. Total SDW of all three harvests was highest with the mutant *N31.6rif*^{r}, 6.6% higher than the wild strain N31, 9.5% higher than the uninoculated plants receiving combined N, and 73.3% higher than the uninoculated plants not receiving any combined nitrogen.

Table 4. Effects of the wild-type strain N31 and the deletion mutant strain *N31.6rif*^{r} of arctic *Rhizobium* on the shoot dry matter yield of sainfoin

Treatments	Yield shoot dry weight (gram/plant)			
	1st cut	2nd cut	3rd cut	Total cuts
Inoculated				
Wild-type N31	1.3146c*	2.5117a	2.0838a	5.9100 a
Mutant *N31.6rif*^{r}	1.9300b	2.4671a	1.9333ab	6.3304a
Uninoculated				
Low nitrogen (30 mg/l NO_3-N)	0.9313c	0.3442c	0.4175c	1.6929b
High nitrogen (140 mg/l NO_3-N)	3.1017a	1.0725b	1.5575b	5.7317a

* Means followed by the same letter in the same column are not significantly different ($P \leq 0.05$) according to the Duncan's multiple range test. Mean are from eight pot replicates containing three plants each

Table 5. Effects of the wild-type strain N31 and the deletion mutant strain *N31.6rif*^{r} of arctic *Rhizobium* on the shoot nitrogen content of sainfoin

Treatments	Shoot nitrogen content (gram/plant)			
	1st cut	2nd cut	3rd cut	Total cuts
Inoculated				
Wild-type N31	20b*	49a	34b	103b
Mutant *N31.6rif*^{r}	35a	45a	42a	122a
Uninoculated				
Low nitrogen (30 mg/l NO_3-N)	9c	4c	6d	19d
High nitrogen (140 mg/l NO_3-N)	39a	18b	26c	82c

* Means followed by the same letter in the same column are not significantly different ($P \leq 0.05$) according to the Duncan's multiple range test. Mean are from eight pot replicates containing three plants each

There were pronounced differences in the total N-content (TNC) of sainfoin plants inoculated with strain N31 and the mutant *N31.6rif*^{r} (Table 5). At the first harvest, the TNC of sainfoin plants inoculated with *N31.6rif*^{r} was 74.8% higher (significant at $P < 0.05$) than the plants inoculated with N31, and more than four times higher than the uninoculated low-nitrogen plants. At the second harvest, the TNC of sainfoin plants that received combined N as NH_4NO_3 decreased significantly, while the TNC of plants inoculated with N31 and *N31.6rif*^{r} was significantly higher ($P < 0.05$) than that of the uninoculated control plants. The results of the third harvest were similar to those obtained at second harvest. TNC of all three harvests of plants inoculated with *N31.6rif*^{r} was

significantly higher ($P<0.05$) than that of the plants inoculated with strain N31 and the uninoculated plants.

Percentage of nitrogen derived from the atmosphere (NDF) through symbiotic nitrogen fixation was calculated for plants inoculated with strain N31 and *N31.6rif*^{r} (Prévost et al. 1987c). Higher percentage values of NDF were obtained with strain *N31.6rif*^{r} (84.5%) than with strain N31 (81.5%).

Discussion

The arctic rhizobial strain N31, used in this study, was isolated from arctic legume plants (Prévost et al. 1987a). At low temperatures, nitrogenase activity as well as growth of arctic rhizobia was better than that of temperate homologous rhizobia with sainfoin (Prévost et al. 1987b). This apparent adaptation of arctic rhizobia to low temperatures can be advantageous over other rhizobia, especially when temperature is limiting for both bacterial growth and nitrogenase activity during cold phases of the growing season.

Researchers working with *Rhizobium* spp. have long been developing inoculants that can promote higher levels of N_2-fixation in legumes. In several cases, mutagenesis was used as a method for obtaining strains of bacteria with higher symbiotic efficiency (Maier and Brill 1975; Cho et al. 1985; Shukla et al. 1989; Paau 1989). Chemical mutagens were used to mutagenize rhizobial strains, followed by screening for efficient strains. We used deletion mutation by heat treatment as a method for isolating superior, nitrogen-fixing strains of arctic rhizobia.

Five deletion mutants of arctic rhizobial strain N31 were isolated in which smaller plasmid (200 kb) was missing. Deletion in larger plasmid, if it occurred, could not be detected by gel electrophoresis. Banfalvi et al. (1981) generated several deletion mutants of *R. meliloti* by heat treatment, but only in four out of eight mutants could deletions in plasmid(s) be detected by gel electrophoresis, although all eight derivatives had lost *nod* and *nif* genes. Loss of plasmid(s) or internal deletions in plasmids by heating has been demonstrated in *Rhizobium* (Zurkoski and Lorkiewicz 1979) and in other bacterial species (May et al. 1964; Terawaki et al. 1967). The loss of plasmids or genes at elevated temperatures may be due to temperature-sensitive replication of plasmids or genes (Terawaki et al. 1967).

Ex planta nitrogenase activity of the mutant strain (*N31.6rif*^{r}) was higher than for the wild type. Since ex planta N_2-ase activity is measured without the host and under artificial conditions of growth (e.g., nutrients and O_2), differences in symbiotic nitrogen fixation could be much more significant. Maier and Brill (1978) were unable to demonstrate significant differences in ex planta N_2-ase activity of the mutants and the wild-type strains of *R. japonicum*, but symbiotic N_2-fixing activity of the mutants was significantly greater than that of the wild-type strain. Ex planta nitrogen fixation efficiency is probably not a reliable predictor of symbiotic nitrogen fixation efficiency.

The evidence of increased N_2-fixing ability of the deletion mutant *N31.6rif*^{r} was apparent in test-tube and pot inoculation studies. In test-tube studies, nodulation index was in the order of 6 for the mutant *N31.6* and *N31.6rif*^{r}, 4 for N31 wild-type and less than 4 for other deletion mutants. In pot inoculation experiments, the mutant *N31.6rif*^{r} was symbiotically superior to the wild-type strain N31 as evaluated by the total SDW of all three harvests. Furthermore, the *N31.6rif*^{r} mutant expressed an early symbiotic, nitrogen fixation efficiency, as seen by the highest SDW and total N-content produced at the first harvest. This is an advantageous agronomic trait desirable to establish a beneficial symbiotic system. An enhanced nitrogen-fixing ability at the early period of symbiosis might be due to an earlier colonization of the root system by the mutant, since it exhibited a slightly shorter generation time (4.5 versus 4.3 h, Table 1) and a higher nodulation index (6 versus 4, Table 2) than the wild type. In a later period, this advantage diminished as maximum colonization and nodulation of the root system by the applied rhizobia reached an equilibrium with the plant development; such phenomenon was previously reported for perennial legumes (Bordeleau et al. 1977). Characteristics of growth at 5 °C, the most important traits of arctic rhizobia, were retained by the deletion mutant *N31.6rif*^{r}, although the strain was exposed to elevated temperature.

All the deletion mutants were similar to the wild type in (i) antibiotic resistance, (ii) growth at low temperature, and (iii) carbohydrate and organic acid utilization. This proves that all the deletion mutants were variants of the wild-type strain N31 and not contaminants.

The reasons for increased N_2-fixing ability of mutant strains have been suggested but not proven. Maier and Brill (1978) suggested that increased symbiotic efficiency of *R. japonicum* mutants may be due to mutation in genes involved in H_2-metabolism or nodulation. Paau (1989) isolated *R. japonicum* mutants that outcompeted indigenous populations in nodulating soybean plants under field conditions. Cho et al. (1985) isolated *R. japonicum* mutants capable of more rapid nodulation of soybean plants. Both studies indicate that mutation in nodulation genes was probably involved in increased N_2-fixing ability of the mutants. Cannon et al. (1988) attempted to make precise modifications to the regulation of *nif* (nitrogen-fixing) genes and *dct* (C_4-dicarboxylate transport) genes of *Rhizobia* spp., but the yield response conclusions were conflicting. Modification in the *nif A* gene, which activates *nif* expression, significantly affected plant

growth both positively and negatively (Cannon et al. 1988). Similarly, the *dct A* gene, one of the four *dct* genes, also influenced nitrogen fixation and plant growth significantly (Cannon et al. 1988). Introduction of nonrhizobial genes in rhizobia can also affect plant growth: the presence of the PSUP 104 plasmid (containing *E. coli* genes; Simon et al. 1983) in *R. meliloti* had a significant detrimental effect on the growth of alfalfa (Cannon et al. 1988).

The process of deletion mutation has never been used for the improvement of *Rhizobium* strains. One would expect a negative effect of a deletion mutant in which certain essential genes are lost. On the other hand, deletion mutation may delete certain N_2-fixation limiting genes and may, thus, enhance N_2-fixation. We observed a negative effect of deletion mutation in four out of five deletion mutants and a positive effect with the deletion mutant *N31.6rif*^{r} on ex planta N_2-ase activity. It remains to be seen whether or not the deletion mutant *N31.6rif*^{r} is competitive for nodule occupancy in the presence of indigenous *Rhizobium* populations, and whether is is able to fix atmospheric N_2 in the presence of combined nitrogen, as has been demonstrated in *R. japonicum* mutants (Maier and Brill 1978).

Since one group of deletion mutants exhibited a positive effect and another group exhibited a negative effect on ex planta N_2-ase activity, it is likely that deletion mutations occurred in the 500-kb megaplasmid and/or in the genome. Further genetic analysis of this mutant strain will enable us to identify genes controlling symbiosis between *Rhizobium* and legumes.

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