Isolation and characterization of a *ntrC* mutant of *Bradyrhizobium* (*Parasponia*) sp. ANU289

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A mutant of *Bradyrhizobium* (*Parasponia*) sp. ANU289 affected in the regulation of nitrogen metabolism was isolated. The mutant, designated ANU293, was unable to induce ammonium transport (Amt), nitrate reductase (NR) or glutamine synthetase II (GSII) activities under conditions that induce these activities in the wild-type. However, glutamine synthetase I (GSI), which is expressed constitutively in the wild-type, was present at normal levels in the mutant. The mutant also retained the ability to fix nitrogen *in vitro* and *in planta*, although nodule development on siratro (*Macroptilium atropurpureum*) was retarded. Southern blot analysis showed that *ntrC*, the product of which is involved in regulation of nitrogen metabolism, is the site of pSUP1021 insertion in ANU293. These results indicate that the transcriptional activator NtrC is required for the expression of Amt, NR and GSII, but not GSI or nitrogenase in *Bradyrhizobium* (*Parasponia*) sp. ANU289.

Introduction

In enteric bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*, several nitrogen assimilation pathways are coordinately regulated by a central nitrogen regulation (Ntr) system (Magasanik, 1982). Three genes, *ntrA*, *ntrB* and *ntrC*, are responsible for overall nitrogen regulation in these bacteria. Genes under Ntr control include *glnA*, encoding glutamine synthetase (GS), and several others involved in the utilization of amino acids such as L-arginine, L-proline and L-histidine (Magasanik, 1982). Also under Ntr control is the gene(s) encoding ammonium (NH₄⁺) transport (*amt*; Servin-Gonzalez & Bastarrachea, 1984; Jayakumar *et al.*, 1986). In *K. pneumoniae*, the nitrogen fixation (*nif*) genes are also controlled by the Ntr system (Ausubel, 1984; Gussin *et al.*, 1986).

A Ntr system has also been shown to operate in species of *Rhizobiaceae*. Homologues of *ntrA*, *ntrB* and *ntrC* have been identified in members of the *Rhizobiaceae*, and both *ntrA* and *ntrC* have been shown to be necessary for expression of several genes involved in nitrogen assimilation

(Ausubel *et al.*, 1985; Nixon *et al.*, 1986; Pawlowski *et al.*, 1987; Ronson *et al.*, 1987; Rossbach *et al.*, 1987; Szeto *et al.*, 1987; Martin *et al.*, 1988; Shatters *et al.*, 1989; Stanley *et al.*, 1989). However, it has become apparent from these studies that the pattern of Ntr control in *Rhizobiaceae* differs from that found in the enteric bacteria. For example, whilst *glnA* is under the control of the Ntr system in enteric bacteria (Magasanik, 1982), its homologue in *Azorhizobium* and *Bradyrhizobium* species is not (Pawlowski *et al.*, 1987; Carlson *et al.*, 1987). A second, eukaryote-like glutamine synthetase (GSII, encoded by *glnII*) that is present in (brady)rhizobia is, on the other hand, regulated by the Ntr system (Carlson *et al.*, 1987; Martin *et al.*, 1988; Shatters *et al.*, 1989). Whilst transcription of the nitrogen fixation (*nif*) genes is absolutely dependent on the activator NtrC in the free-living bacterium *Klebsiella pneumoniae*, this is not the case in *Rhizobium meliloti* and *Azorhizobium caulinodans*, at least during symbiotic nitrogen fixation (Gussin *et al.*, 1986; Szeto *et al.*, 1987; Pawlowski *et al.*, 1987). Furthermore, NtrC is not necessary for the utilization of amino acids in *Rhizobiaceae* (Szeto *et al.*, 1987; Pawlowski *et al.*, 1987; Martin *et al.*, 1988).

Although differences in the pattern of Ntr control in *Rhizobiaceae* and enteric bacteria have been identified, the extent of Ntr control in the former remains unknown. In this paper we describe the isolation and characterization of a *ntrC* mutant of *Bradyrhizobium* (*Parasponia*) sp. ANU289. The mutant is affected in the regulation of not

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Abbreviations: Amt, ammonium transport; GSI, glutamine synthetase I; GSII, glutamine synthetase II; NR, nitrate reductase; Ntr, nitrogen regulation.

only glutamine synthetase II (GSII) and nitrate reductase (NR), but also NH_4^+ transport (Amt). Growth of the mutant on a variety of nitrogen sources and its nitrogen-fixing ability, both *in vitro* and *in planta*, were also studied.

Methods

Bacterial strains. *Bradyrhizobium* (*Parasponia*) sp. ANU289 is a streptomycin-resistant, non-mucoid derivative of strain CP283 (Trinick & Galbraith, 1980). It can form effective nodules on some tropical legumes, as well as on the tropical non-legume *Parasponia*. The Tn5 mutant bank of strain ANU289 was generously provided by Dr Susan Howitt (see Howitt *et al.*, 1988, for details). The mutant isolated from this bank and described herein is designated strain ANU293.

Media and growth conditions. The basic (nitrogen-free) liquid growth medium was N-FGM (Howitt & Gresshoff, 1985), with nitrogen sources supplied at a concentration of 10 mM unless otherwise stated. Cultures (100 ml) were grown aerobically at 30 °C by shaking in 250 ml side-arm flasks at 200 r.p.m. The following solid media (containing 15 g agar l⁻¹) were also used: BMM, which is based on Bergersen's medium (Bergersen, 1969), except that 0.5 g sodium glutamate l⁻¹ and 0.5 g yeast extract l⁻¹ were present as nitrogen sources; and LB (Miller, 1972).

Enzyme assays. NH_4^+ transport activity was determined using the radioactive analogue of NH_4^+ , [¹⁴C]methylamine ([¹⁴C] MA), in a filter assay (Howitt *et al.*, 1986).

GS activity was measured using the γ -glutamyltransferase assay described by Fuchs & Keister (1980), as modified by Howitt & Gresshoff (1985). Sensitivity to heat was used to distinguish GSII activity from that of GSI: incubation of cell extracts at 50 °C for 2 h inactivates GSII, but not GSI activity in strain ANU289 (Howitt & Gresshoff, 1985).

NR activity was determined with whole cells by following NO_2^- production (Nicholas & Nason, 1957), as described previously (Howitt & Gresshoff, 1985).

In vitro nitrogenase activity was measured using the acetylene reduction assay (Dilworth, 1966) after derepression of bacterial nitrogenase in solid agar medium inside stoppered test-tubes (Bender *et al.*, 1986).

The Lowry method was used to determine protein concentration.

Southern blot analysis. Chromosomal DNA was prepared from stationary phase cultures of ANU289 and ANU293 as described by Somerville & Kahn (1983). Samples of DNA (5 µg) were digested with the restriction enzyme *EcoRI* and electrophoresed through a 0.7% agarose gel. The DNA in the gel was then nicked by depurination in 0.2 M-HCl for 10 min and denatured in 0.5 M-NaOH, 1.5 M-NaCl for 45 min, prior to transfer to a nitrocellulose filter (Sambrook *et al.*, 1989). Plasmids containing *ntrA* (3.2 kb *EcoRI* fragment), *ntrB* (0.9 kb *EcoRI* fragment), *ntrC* (1.6 kb *EcoRI* fragment), and *ntrB-ntrC* (0.7 kb *EcoRI* fragment) from *Bradyrhizobium* sp. [*Parasponiae*] strain RP501 were kindly provided by B. Tracy Nixon (Nixon *et al.*, 1986; and unpublished results from the same group). Probes for the various *ntr* genes were prepared by digesting plasmids with *EcoRI*, purifying the appropriate fragments using preparative agarose gels, and labelling 50–100 ng of the resulting DNA with [α -³²P]dCTP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹); NEN] using an oligonucleotide-labelling (random-primer) kit from Pharmacia. Hybridization was performed in 50% (v/v) formamide, 0.75 M-NaCl, 50 mM-NaH₂PO₄·2H₂O, pH 7.4, 5 mM-EDTA, 0.1% Ficoll (type 400, Pharmacia), 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin (Fraction V), 0.1% SDS, and

100 µg ml⁻¹ denatured, fragmented salmon sperm DNA at 42 °C for 20 h. The filter was then washed in 0.1 × SSC (15 mM-NaCl, 1.5 mM-sodium citrate, pH 7) plus 0.1% SDS at 65 °C for 1 h according to Sambrook *et al.* (1989). Autoradiography using Kodak X-Omat film was used to visualize hybridization of probes to the filter-bound DNA. Lambda DNA digested with *HindIII* was used for molecular size markers.

Symbiotic effectiveness test. Siratro (*Macroptilium atropurpureum*) seeds (12–15 mg) were soaked in concentrated sulphuric acid for 10 min, washed with 70% ethanol, and soaked in 6% sodium hypochlorite for 15 min. Seeds were subsequently rinsed with sterile distilled water and germinated on sterile water agar. After 2 d, seedlings were transplanted into sterilized Leonard jars containing Vermiculite and 1/2-strength nitrogen-free nutrient solution (Herridge, 1982) and inoculated with a mid exponential phase culture (approx. 10⁹ viable cells per jar). Plants were grown under glasshouse conditions with 16 h light and 8 h dark. Leonard jars were refilled with 1/2-strength sterile nutrient solution when necessary. Plants were harvested after 9 weeks and assayed for nitrogenase activity by measuring acetylene reduction (Dilworth, 1966). Plants were sealed inside 1 litre jars containing wet absorbent paper to prevent desiccation and 5% acetylene was injected. Reactions were carried out at room temperature (25 °C) for 60 min and the amount of ethylene produced was determined by gas chromatography. Plants were harvested and dried, after which nodules, shoots and roots were weighed. Bacteria were re-isolated from nodules using the method of Rolfe & Gresshoff (1981).

Results

Isolation and characterization of the ntrC mutant ANU293

The insertion mutant ANU293 was isolated while screening for NH_4^+ transport (*amt*) deficient mutants of strain ANU289. The screen involved plating cells onto BMM plates containing 500 mg kanamycin l⁻¹ and 100 µM-NH₄Cl as the sole nitrogen source. Preliminary experiments had established that at this concentration of ammonium, strain ANU289 expresses the Amt system and, therefore, presumably requires the transporter for good growth. Putative *amt* mutants were selected on the basis of poor growth which was manifest as less dense, more diffuse colonies. From several thousand kanamycin-resistant colonies, 13 putative *amt* mutants of strain ANU289 were identified. Of these, only ANU293 showed an Amt⁻ phenotype (determined by [¹⁴C]MA uptake) when grown to mid-exponential phase under nitrogen-limited conditions (Fig. 1). The rate of MA uptake by ANU293 was similar to that of strain ANU289 bacteroids from siratro nodules, which do not express the Amt system (Howitt *et al.*, 1986), and less than 10% of the wild-type rate of free-living cells in which the Amt system is expressed (Fig. 1).

Growth rates of the mutant and the wild-type on a variety of nitrogen sources were subsequently compared. The growth kinetics of the mutant and the wild-type

Fig. 1. Time course for [^{14}C]MA uptake by ANU293 (Δ) and the wild-type, ANU289 ($\circ$). Bacteria were grown to mid-exponential phase in N-FGM plus 10 mM-glutamate at 30 °C. Cells were washed twice and resuspended in N-FGM prior to use. Uptake was measured at 30 °C and pH 7.0 with 10 μM -[^{14}C]MA in the external medium. Results are for a typical experiment.

were almost identical when either 20 mM- NH_4Cl (doubling times of 14 and 13 h, respectively) or 20 mM-glutamine (doubling times of 9 and 8 h, respectively) were used as the sole source of nitrogen. However, the mutant grew more slowly than the wild-type on 20 mM-glutamate (25 and 16 h, respectively), and not at all on 20 mM- KNO_3 (results not shown).

The complete absence of growth of the mutant on NO_3^- suggested that it was affected in the regulation of nitrogen metabolism, rather than only NH_4^+ transport. To test this hypothesis, we investigated the ability of the mutant to induce NR and GSII activities, both of which are under Ntr control in other (brady)rhizobia (Carlson *et al.*, 1987; Martin *et al.*, 1988; Shatters *et al.*, 1989). It had been shown previously that ANU289 is able to induce the expression of NR when transferred from N-FGM containing 10 mM-glutamate to N-FGM with KNO_3 as the sole source of nitrogen (Howitt *et al.*, 1988). Fig. 2 shows the results of a similar experiment using both the mutant and wild-type. As expected, NR activity was induced in the wild-type in the presence of NO_3^- within 6 h. The mutant, however, was unable to induce NR. This explains the inability of the mutant to grow on NO_3^- .

Expression of GSII, but not GSI, is also regulated by the nitrogen status of strain ANU289 cells (Howitt & Gresshoff, 1985). More specifically, GSII is repressed by growth on 10 mM- NH_4Cl but induced under N-limited conditions, namely 10 mM-glutamate (Howitt & Gresshoff, 1985). Table 1 shows the results of a similar

Fig. 2. Induction of NR activity. Wild-type, ANU289 ($\circ$) and the mutant, ANU293 (Δ) were grown to mid-exponential phase in N-FGM plus 10 mM-glutamate, then resuspended in N-FGM plus 10 mM- KNO_3 at $t = 0$. Induction was carried out aerobically at 30 °C. Results are for a typical experiment.

Table 1. Regulation of GS activities in ANU293, and its wild-type parent, ANU289

Cells were grown to mid-exponential phase in N-FGM plus 10 mM-glutamate as sole N-source, then transferred to N-FGM plus 10 mM- NH_4Cl for 1 d. GS activities were determined prior to and after NH_4Cl treatment. Results are for a typical experiment.

Strain	Nitrogen source	GS activity [$\mu\text{mol } \gamma\text{-glutamylhydroxamate}$ formed min^{-1} (mg protein) $^{-1}$]	
		GSI	GSII
ANU289	L-Glutamate	1.19	2.92
ANU293	L-Glutamate	0.80	0.12
ANU289	NH_4Cl	0.87	0.04
ANU293	NH_4Cl	0.91	0.05

experiment using both the mutant and the wild-type. In contrast to the wild-type, the mutant was unable to induce GSII activity during growth on glutamate. Expression of GSI in the mutant was normal.

Southern blot analysis was used to identify the site of Tn5 insertion in ANU293 (Fig. 3). *EcoRI* digests of ANU293 and ANU289 chromosomal DNA were probed with *Bradyrhizobium* sp. [*Parasponiae*] strain RP501 DNA that contains *ntrA*, *ntrB*, *ntrC* or the *ntrB-ntrC* junction. The mutation in ANU293 was not due to a simple Tn5 insertion but instead resulted from the cointegration of pSUP1021, the vector used for Tn5 mutagenesis. This was shown using as the hybridization probe ^{32}P -labelled pACYC184, the parent plasmid of pSUP1021 that has no homology to Tn5 (Simon *et al.*, 1986). pSUP1021 contains a single *EcoRI* site and its

Fig. 3. Southern blot analysis of chromosomal DNA isolated from ANU289 (odd-numbered lanes) and ANU293 (even-numbered lanes). DNA was cut with *Eco*RI, prior to gel electrophoresis, transferred to nitrocellulose filters, and hybridized to probes (see Methods for details). The DNA in each pair of lanes was hybridized to a different probe, as indicated. Plasmid pACYC184, which does not contain Tn5, is the parent of pSUP1021, the plasmid used for the mutagenesis of ANU289 (Simon *et al.*, 1986). Pointers indicate the positions of molecular size markers.

integration into the chromosome will split an *Eco*RI restriction fragment into two fragments that have a total size equal to the sum of the original fragment and the pSUP1021 cointegrate. As shown in Fig. 3, ANU293 contains DNA that hybridizes to pACYC184 (lanes 9 and 10).

There were no differences between ANU293 and ANU289 when the restriction digests were hybridized to the *ntrA* (lanes 1 and 2) or *ntrB* (lanes 3 and 4) probes. The *ntrC* probe hybridized to one restriction fragment in ANU289 but to two restriction fragments in ANU293 that are the same length as those that hybridized to pACYC184 (lanes 5 and 6). This indicates that the site of insertion of pSUP1021 is within the fragment that contains *ntrC*. The shorter of these two new bands (6.6 kb) also hybridized to the *ntrB*–*ntrC* junction fragment (lane 8) which shows that this band contains the 5'-end of the *ntrC* gene. The intensity of labelling of the two bands by the *ntrC* probe is a function of the

position of the insertion – inserts nearer to *ntrB* would have less DNA in the 6.6 kb band in common with the *ntrC* probe (see Fig. 4). In lane 6 (Fig. 3), the 6.6 kb band is labelled much less than the 9.4 kb band and this places the site of insertion to the left of the centre of the *ntrC* probe, well within the *ntrC* gene. The fact that 0.5 kb of DNA at the 3'-end of the *ntrC* probe is non-coding and, therefore, probably less conserved than the *ntrC* portion means that the deduced site of insertion would be even further toward the 5'-end of *ntrC*.

Nitrogen fixation by ANU293

Unlike *Rhizobium* species, *Bradyrhizobium* species can be induced to fix nitrogen *in vitro*. Using the method of Bender *et al.* (1986) it was found that the mutant was able to reduce acetylene at rates identical to those of wild-type cells (results not shown), indicating that the mutant retained the ability to derepress nitrogenase *in vitro*.

Table 2. Symbiotic performance of the *ntrC* mutant

Siratro seedlings were inoculated with either ANU293 or ANU289 and grown under sterile conditions in a glasshouse. Uninoculated plants were also included as a control. Plants were harvested after 9 weeks of growth. Values are means $\pm$ standard errors at the 95% confidence interval.

Inoculum	No. of plants	Nodule fresh weight (mg per plant)	Dry weight of plant part (mg per plant)			Nitrogenase activity mmol C ₂ H ₄ formed h ⁻¹	
			Nodules	Shoot	Root	(g nodule fresh weight) ⁻¹	(g dry weight) ⁻¹
ANU289	23	121 $\pm$ 34	16 $\pm$ 2	121 $\pm$ 13	48 $\pm$ 6	12 $\pm$ 3	74 $\pm$ 10
ANU293	26	46 $\pm$ 20	7 $\pm$ 1	48 $\pm$ 2	24 $\pm$ 2	10 $\pm$ 3	62 $\pm$ 20
None	34	0	0	17 $\pm$ 1	25 $\pm$ 1	0	0

Symbiotic phenotype of ANU293

To test the symbiotic effectiveness of ANU293, siratro seedlings were inoculated with either mutant or wild-type strains and grown under sterile conditions. Uninoculated plants were included as a control. After 9 weeks, both sets of inoculated plants appeared healthy and had dark green leaves. In contrast, plants without inoculum were stunted and chlorotic with pale, yellow leaves. Bacteria re-isolated from nodules induced by the mutant retained kanamycin resistance and showed the same growth characteristics as ANU293. Plants were harvested and nitrogenase activity of intact plants measured using acetylene reduction. Nodule fresh weights and nodule, shoot and root dry weights were also determined (Table 2). Specific nitrogenase activity of nodules induced by the mutant ANU293 was not significantly different from that of nodules induced by the wild-type. However, nodule, root and shoot dry weights of plants inoculated with ANU293 were significantly lower than control plants inoculated with ANU289, although they were higher than those of uninoculated plants (Table 2). The ability of mutant ANU293 to induce nodules on siratro roots was subsequently tested using the method of Mathews *et al.* (1989). There was no significant difference between the mutant and the wild-type in their ability to initiate nodules on siratro (results not shown). This suggests that the reduced growth of plants inoculated with the mutant was a result of retarded nodule development rather than decreased or delayed nodule initiation.

Discussion

The results presented above indicate that ANU293 is an *ntrC* mutant of ANU289. The pleiotropic effects of this mutation on the expression of several genes involved in nitrogen metabolism, namely those encoding Amt, NR and GSII, are consistent with this conclusion.

Fig. 4. Physical map of the *ntrB*-*ntrC* region of *Bradyrhizobium* (*Parasponia*) sp. ANU289 based on the results of Fig. 3 and a comparison with published results of Nixon *et al.* (1986). *EcoRI* restriction sites are marked with the letter R. Also shown are the *Bradyrhizobium* sp. [*Parasponiae*] strain RP501 *EcoRI* fragments that were used as probes for the Southern blot analysis shown in Fig. 3. The *EcoRI* site shown in parentheses is present in strain RP501 but apparently not in strain ANU289.

The results of the Southern blot analysis shown in Fig. 3 suggest that *ntrB* and *ntrC* are adjacent in ANU289. Furthermore, the position of *EcoRI* sites in and around *ntrB*-*ntrC* in ANU289 are similar to those in *Bradyrhizobium* sp. [*Parasponiae*] strain RP501 (Nixon *et al.*, 1986), except for the absence of the one *EcoRI* site within *ntrC* (see Fig. 4). It seems likely that *ntrB* and *ntrC* are part of a single operon, as they are in *Bradyrhizobium* sp. [*Parasponiae*] strain RP501 (Nixon *et al.*, 1986). In contrast, *ntrA* appears to be unlinked to *ntrB* and *ntrC*.

Transcription of genes under Ntr control in bacteria requires NtrA, a unique sigma factor which combines with the RNA polymerase core enzyme, and the regulatory protein NtrC (or NRI; Hirschman *et al.*, 1985; Hunt & Magasanik, 1985; Merrick & Gibbons, 1985). NtrC has sequence-specific DNA-binding activity (Ames & Nikaido, 1985) which has been implicated in transcriptional activation (Reitzer & Magasanik, 1986). Expression of the *ntrC* gene occurs

when cells are grown in N-limited media (Magasanik, 1982). The phenotype of ANU293 is similar to those of other (brady)rhizobial *ntrC* mutants. For example, *Bradyrhizobium japonicum ntrC* mutants are unable to utilize KNO_3 as the sole nitrogen source, nor are they able to transcribe the *glnII* gene (encoding GSII) in aerobic, nitrogen-starved cultures. Expression of *glnA* (encoding GSI), on the other hand, is not affected by the lesion in *ntrC* (Martin *et al.*, 1988). *Rhizobium meliloti* and *Azorhizobium caulinodans ntrC* mutants, like ANU293, are able to form effective nodules on their host plants, although *A. caulinodans ntrC* mutants exhibit delayed nodulation and reduced nitrogen fixation activity (Szeto *et al.*, 1987; Pawlowski *et al.* 1987).

In enteric bacteria, the Ntr system controls the utilization of urea, histidine, proline and arginine (Magasanik, 1982). In contrast, results with *Bradyrhizobium* and *Rhizobium* species suggest that utilization of histidine, proline and arginine is not dependent on the Ntr system in these genera (Ronson *et al.*, 1987; Martin *et al.*, 1988). Nonetheless, Szeto *et al.* (1987) found that while *ntrC* is not absolutely required for growth of *R. meliloti* on a variety of amino acids, *ntrC* mutants do suffer a growth disadvantage when arginine or proline is the sole nitrogen source. *A. caulinodans ntrC* mutants, on the other hand, grow more slowly than the wild-type when arginine or histidine, but not proline, is provided as the primary N-source (Pawlowski *et al.*, 1987). ANU293 grew more slowly than the wild-type on arginine and glutamate but showed normal growth rates on urea, proline and histidine (results not shown). The reduced growth rates of ANU293 on glutamate and arginine may be a consequence of the Amt^- phenotype, as was found for an Amt^- mutant of *Klebsiella pneumoniae* (Castorph & Kleiner, 1984).

The ability of the *ntrC* mutant ANU293 to fix nitrogen at normal rates, both *in vitro* and *in planta*, suggests that transcription of *nif* genes is not dependent on NtrC in strain ANU289. A similar conclusion was drawn for *R. meliloti*, at least for *nif* regulation *in planta* (Szeto *et al.*, 1987). However, the *ex planta* transcription of several *R. meliloti nif* genes does require NtrC. The situation appears to be different again in *A. caulinodans* where NtrC exerts a profound effect on the induction of *nif* gene transcription both *ex planta* and *in planta* (Pawlowski *et al.*, 1987).

Although the mutant was able to infect siratro efficiently and form effective nodules, plant growth was slower, suggesting that nodule development was delayed. Prior to the onset of nitrogen fixation, rhizobia require a net input of reduced nitrogen for growth and replication. Nitrogen compounds supplied by the plant may include ammonia and amino acids derived from seed reserves. Since the mutant grows poorly on some amino acids and

is unable to scavenge NH_4^+ because of its Amt^- phenotype, under the conditions likely to prevail in the developing nodule the mutant may exhibit slower growth rates than the wild-type. This could delay bacteroid development and the onset of nitrogen fixation, which in turn would manifest itself in a reduced rate of plant growth relative to wild-type inoculated plants.

Conclusion

We have isolated a *ntrC* mutant of *Bradyrhizobium (Parasponia)* sp. ANU289, and designated it ANU293. The phenotype of ANU293 indicates that expression of Amt , NR and GSII, but not nitrogenase or GSI, are dependent on the transcriptional activator NtrC in ANU289.

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