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The *ntrC* gene of *Agrobacterium tumefaciens* C58 controls glutamine synthetase (GSII) activity, growth on nitrate and chromosomal but not Ti-encoded arginine catabolism pathways

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Summary. The *ntrC* locus of *Agrobacterium tumefaciens* C58 has been cloned using the *Azorhizobium sesbaniae* ORS571 *ntrC* gene as a DNA hybridization probe. Transposon Tn*5* mutagenesis of the cloned *ntrC* locus was carried out and one Tn*5* insertion within the region of highest DNA homology with *A. sesbaniae* ORS571 *ntrC* was used for gene replacement of the wild-type C58 *ntrC* gene. The *A. tumefaciens ntrC*::Tn*5* mutant was found to be unable to grow on nitrate as sole nitrogen (N) source, to lack glutamine synthetase (GSII) activity and to be unable to use arginine (or ornithine) as sole N source, unless the Ti-encoded arginine catabolism pathway was induced with small amounts of nopaline. Thus the *A. tumefaciens ntrC* regulatory gene is essential for (transcriptional) activation of the GSII and nitrate reductase genes, as well as for the chromosomal but not the Ti-borne arginine catabolism pathways.

Key words: *Agrobacterium tumefaciens* C58 – Nitrogen regulation (*ntr*) – Opine and arginine catabolism – Glutamine synthetase – Tn*5* mutagenesis

Introduction

In enteric bacteria such as *Escherichia coli, Salmonella typhimurium* and *Klebsiella pneumoniae* a variety of nitrogen assimilation pathways are coordinately regulated by a central cellular nitrogen regulation (*ntr*) system (see Magasanik 1982). *Ntr*-controlled pathways (genes) include the glutamine synthetase (GS) gene (*glnA*) and genes involved in utilization (uptake, transport, catabolism) of a variety of amino acids such as L-histidine, L-proline, L-arginine as sole nitrogen (N) source (see Magasanik 1982; Gussin et al. 1986). In *K. pneumoniae* the nitrogen fixation (*nif*) genes are also strictly controlled by the *ntr* system (Ausubel 1984).

In bacterial species belonging to the *Rhizobiaceae* the presence/role of an *ntr* system has been analysed more recently. The cloning of *ntrC* genes and the creation of *ntrC* mutations have been described for *Rhizobium meliloti* 1021 (Ausubel et al. 1985; Szeto et al. 1987) and for *Azorhizobium sesbaniae* ORS571 (de Bruijn et al. 1987; Pawlowski et al. 1987). In *Agrobacterium* spp. *ntr* genes or *ntr* mutations have not been reported thus far. Agrobacteria are taxonomically closely related to rhizobia (White 1972; De Smedt and De Ley 1977; Holmes and Roberts 1981) and both types of these soil bacteria have a number of characteristics in common, which have prompted comparative analyses. Both the genus *Agrobacterium* and the genera *Rhizobium* and *Bradyrhizobium* are characteristic because of their highly evolved interactions with higher plants. Agrobacteria and (brady)rhizobia are also unique in that they contain two distinct glutamine synthetase (GSI, II) activities (Darrow and Knotts 1977; Fuchs and Keister 1980). GSII is quite distinct from GSI in its molecular weight, subunit composition and enzymatic properties (heat lability, lack of adenylylation; Darrow et al. 1981) and the GSII gene product of *Bradyrhizobium japonicum* has a high degree of homology with GS of higher plants (Carlson and Chelm 1986). The role of GSII in (brady)rhizobia and agrobacteria remains an open question. No clear evidence for its involvement in any aspect of plant-bacterial interactions exists to date even though both types of bacteria are subject to specialized physiological conditions with regard to N metabolism and assimilation during their interaction with plants. In nitrogen-fixing nodules (brady)rhizobia are capable of converting dinitrogen to ammonium which is primarily exported and used by the plant as a N source (see Miflin and Cullimore 1984). In return the plant provides the (brady)rhizobia with other N-containing compounds (possibly glutamate, Kahn et al. 1985) to support bacterial metabolism. In tumours agrobacteria can induce the plant to synthesize unusual compounds (opines, Petit et al. 1978) which the bacteria can utilize as sole N and carbon (C) source. In the case of *A. tumefaciens* C58 the opine specifically induced in tumours and utilized by the bacteria is nopaline and genes for both synthesis and degradation are Ti-plasmid borne (Petit et al. 1970; Bomhoff et al. 1976; Montoya et al. 1977). The primary, N-containing, breakdown product of nopaline catabolism is L-arginine. The C58 Ti plasmid encodes a separate pathway for the catabolism of the arginine thus produced, which is co-expressed with the nopaline catabolism genes (nopaline induced, Ellis et al. 1979; Holsters et al. 1980). Different pathways for the degradation of nopaline and its utilization as sole N and/or C source, have been proposed, involving both Ti and chromosomally encoded genes (Schardl and Kado 1983a, 1983b; Dessaux et al. 1986; Farrand and Dessaux 1986). Dessaux et al. (1986) have postulated that opine and arginine catabolism (genes) are likely to be under control of the same regulatory gene.

In this paper we have examined the role of the central nitrogen regulation (*ntr*) system of *A. tumefaciens* C58 in

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Table 1. Strains and plasmids used

Strain	Relevant characteristics/genotype	Source or reference
Escherichia coli		
YMC10	*thi-1, endoA, hsdR, lacU*169	Backman et al. (1981)
YMC11	*thi-1, endoA, hsdR, lacU*169, *Δ(glnA-ntrC)*	Backman et al. (1981)
YMC17	*thi-1, endoA, hsdR, lacU*169, *ntrC*::Tn*5*	Backman et al. (1981)
GJ23	JC2926 (pGJ28) (R64*drd*11); ColE1, Mob$^+$, Tra$^+$	Van Haute et al. (1983)
Agrobacterium tumefaciens C58		
GV3101(pTiC58)	Rif^r derivative of C58; nopaline type	Holsters et al. (1980)
GV3101	Rif^r; cured of pTiC58	Holsters et al. (1980)
1-10	GV3101(pTiC58) *ntrC*::Tn*5*; Rif^r, Nm^r	This work
Azorhizobium sesbaniae ORS571		
ORS571C6, C7	*ntrC*::Tn*5*; Cb^r, Km^r	Pawlowski et al. (1987)
Rhizobium meliloti 1021		
19-1	*ntrC*::Tn*5*; Sm^r, Km^r	Szeto et al. (1987)
R. leguminosarum		
PRE	wild-type	Lie et al. (1979)
Bradyrhizobium japonicum		
USDA 110	wild-type	Adams and Chelm (1984)
Plasmids		
pACYC184	Cm^r, Tc^r	Chang and Cohen (1978)
pLAFRI	Tc^r, cos, Tra$^-$, Mob$^+$, IncP	Friedman et al. (1982)
p45-2	Gm^r derivative of pBR322; Ap^r	Koncz et al. (1984)
pSR146	*A. tumefaciens ntrC*-region in pLAFRI; Tc^r	This work
pSR1462	*A. tumefaciens ntrC*-region in pACYC184; Tc^r	This work
pRSC13	ORS571 *ntrC*-region in pACYC184	Pawlowski et al. (1987)

the regulation of Ti and chromosomal opine catabolism and arginine degradation genes. In addition we have studied the role of the *ntr* system in the regulation of the primary nitrogen (ammonium) assimilation genes of *A. tumefaciens* C58 and compared the results with analogous *ntr* regulation pathways in rhizobia.

Materials and methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Growth media. The complete medium for growing *E. coli* strains was LB (Miller 1972). For *A. tumefaciens* strains the minimal medium used was Minimal A (Miller 1972) and the complete medium TY (Beringer 1974) or LB. Nitrogen sources $(NH_4)_2\,SO_4$, glutamine and other amino acids were added to MinA to a final concentration of 0.2% unless specified in the text. Antibiotics were used in the following concentrations: Gentamycin (Gm), 100 μg/ml for *A. tumefaciens*, 10 μg/ml for *E. coli*; Kanamycin (Km), 200 μg/ml for *A. tumefaciens*, 20 μg/ml for *E. coli*; Neomycin (Nm), 400 μg/ml (in MinA medium); Rifampicin (Rif), 100 μg/ml, Tetracycline (Tc), 10 μg/ml. *A. tumefaciens* strains were grown at 28° C and *E. coli* strains at 37° C.

Plasmid DNA isolation. Large-scale plasmid DNA preparations were performed according to Ish-Horowicz and Burke (1981); small-scale plasmid DNA preparations as described by de Bruijn and Lupski (1984).

Total chromosomal DNA isolation. Agrobacterium total chromosomal DNA was isolated using a protocol modified from Meade et al. (1982). A 1.5 ml aliquot of an overnight culture was pelleted in an Eppendorf centrifuge tube, washed twice with 1 M NaCl and resuspended in 1 ml ET Buffer (0.01 M Tris, pH 8.0, 0.025 M EDTA). One hundred microlitres of a solution of lysozyme (2 mg/ml in ET Buffer) was added and cells were incubated for 20 min at 37° C. Then 0.125 ml of Sarkosyl/Pronase (10% Sarkosyl, 5 mg/ml Pronase in ET Buffer) was added and the mixture was incubated for 1 h at 37° C. The lysate was extracted twice with phenol, once with phenol/chloroform and once with chloroform. Ammonium acetate was added to a final concentration of 0.3 M and the nucleic acids were precipitated with 2.5 volumes ethanol on ice. The DNA was spooled out using a glass rod and dissolved in 0.2 ml TE Buffer (0.01 M Tris, 0.001 M EDTA).

Restriction endonuclease digestions, ligations and transformations. Restriction endonucleases were purchased from Bethesda Laboratories (BRL, Maryland, USA) and Boehringer Mannheim (Mannheim, FRG) and used according to specifications indicated by the manufacturers. Agarose electrophoresis, ligations and transformations were carried out as described in Maniatis et al. (1982).

A. tumefaciens *genomic library construction. A. tumefaciens* C58 total chromosomal DNA was partially digested with *Eco*RI, so that most of the resulting fragments were in the molecular weight range of ~20 kb, as determined by gel

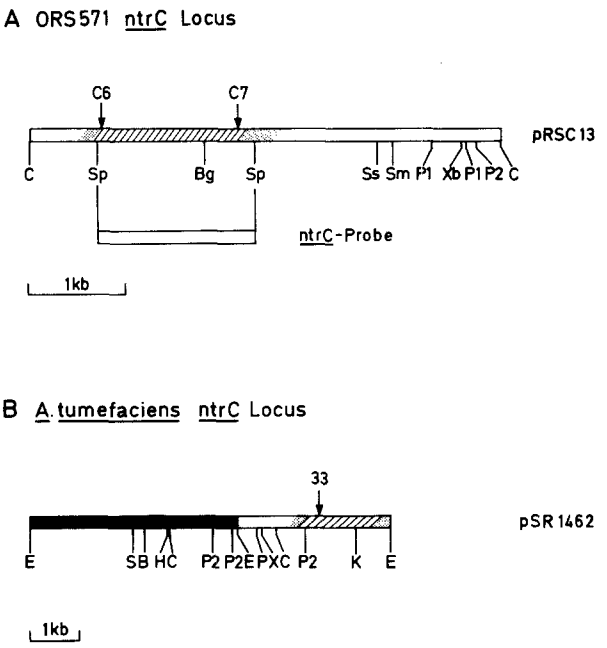

Fig. 1 A, B. Physical and genetic map of the *Azorhizobium sesbaniae* ORS571 (**A**) and *Agrobacterium tumefaciens* C58 (**B**) *ntrC* loci. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P1, *Pvu*I; P2, *Pvu*II; S, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; X, *Xho*I; Xb, *Xba*I. Data in panel **A** are based on Pawlowski et al. (1987). **A** The *boxed* region denotes *ntrC* homology to the *Escherichia coli ntrC* region; C6 and C7 indicate the position of selected *ntrC* Tn*5* insertions. **B** The *striped* region denotes homology to the ORS571 *ntrC* region; 33 indicates the position of the Tn*5* insertion used for homogenotization experiments. The *stippled* regions denote uncertainty in *ntrC* gene limits. The *black* region denotes the vector

electrophoresis. This DNA was phenolized, chloroform treated, concentrated by ethanol precipitation and ligated to the cosmid cloning vector pLAFRI (Friedman et al. 1982), which had been cut with *Eco*RI and treated with CIP (calf intestine phosphatase; Boehringer Mannheim, Mannheim, FRG). An aliquot of this ligation mixture was packaged into lambda phage particles as described by Maniatis et al. (1982) and used to infect *E. coli* strain YMCII (Backman et al. 1981).

Southern blotting and DNA hybridizations. Southern blotting was carried out as described in Maniatis et al. (1982). Nick translations were carried out using a kit purchased from Amersham (UK), as specified by the manufacturers. Colony hybridizations were carried out as described by Maas (1983).

Glutamine synthetase GS and glutamate synthase (GOGAT) assays. Agrobacterium strains were inoculated in 5 ml TY medium and grown overnight to saturation. An 0.5 ml aliquot was re-inoculated into 50 ml MinA medium (supplemented with 0.2% $(NH_4)_2 SO_4$ and 0.2% L-glutamine) and the culture incubated for 24 h. The cells were harvested and resuspended in 50 ml MinA medium (de-repressing conditions) and grown for ~10 h. Cells were harvested at 4° C, washed with 10 mM imidazole buffer (pH 7.0), containing 1% KCl, and resuspended in 10 mM imidazole buffer (pH 7.0), containing 2 mM $MgCl_2$ and 1 mM DTT. The cells were fractionated by two passages through a French Pressure Cell (Aminco, Illinois, USA) at 14000 psi. Cell debris was pelleted by centrifugation at 25000 *g* for 20 min at 4° C. The protein content of the supernatant was determined as described by Bradford (1976). GS activity was determined using the γ-glutamyl transferase assay described by Bender (1977) and activity units expressed as nmol γ-glutamylhydroxamate produced per minute per milligram protein. NADPH-dependent glutamate synthase (GOGAT) activity was determined by measuring NADPH oxidation (OD_{340} decrease) in the presence of crude extract. The reaction mixture consisted of 10 mM α-ketoglutarate, 10 mM glutamine, 0.2 mM NADPH in 100 mM Hepes, pH 7.5. GOGAT activity units were expressed as nanomol of NADPH oxidized per minute per milligram protein.

Plant tests. Young *Kalanchoe diagremontiana* plants were infected with saturated cultures of *A. tumefaciens* strains, by wounding by stem and leaves with a pasteur pipette filled with bacteria. Tumours were observed after 3–4 weeks.

Conjugation and gene-replacement experiments. Cosmid pLAFRI derivatives were mobilized from *E. coli* to rhizobial or agrobacterial strains using a triparental mating protocol (de Bruijn 1987). For the gene replacement experiment using GJ23 (Table 1) as donor strain the protocol described by de Bruijn (1987) was used. Transconjugants were selected on MinA medium supplemented with Rif, Nm and 0.2% L-glutamine.

Results

Cloning of the A. tumefaciens *C58* ntrC *locus*

Total chromosomal DNA of *A. tumefaciens* C58 [GV3101(pTiC58); Table 1] was digested with the restriction endonucleases *Eco*RI, *Sal*I, *Bam*HI, *Hin*dIII, *Pst*I and *Cla*I, blotted and hybridized to an *Sph*I fragment of pRSC13 (Fig. 1A), carrying the *A. sesbaniae* ORS571 *ntrC* locus (Pawlowski et al. 1987). Single hybridization fragments of 3.1, 5.6, 18, 17, 6.4 and 3 kb were observed (Fig. 2B). A clone bank of *A. tumefaciens* C58 was constructed in the wide host range cosmid vector pLAFRI (Table 1), as described in Materials and methods. A total of 800 Tc^r colonies were transferred to Whatman 541 paper and hybridized to the ORS571 *ntrC* probe (Fig. 1A). One strongly hybridizing colony was observed and plasmid DNA was prepared. The DNA was digested with *Eco*RI, blotted and hybridized to the ORS571 *ntrC* probe. A single *Eco*RI fragment of 3.1 kb was found to hybridize (data not shown). This cosmid was named pSR146. The 3.1 kb *Eco*RI fragment of pSR146, carrying the region of homology with ORS571 *ntrC*, was subcloned into the *Eco*RI site of the cloning vector pACYC184 (Table 1) to form plasmid pSR1462 (Fig. 1B). Plasmid pSR1462 DNA was digested with *Eco*RI, *Sal*I, *Bam*HI, *Xho*I, *Kpn*I, *Pst*I, *Hin*dIII, *Cla*I, and *Pvu*II, to establish a restriction map of the cloned region (data not shown; Fig. 1B). Southern blots of these gels were prepared and hybridized to the ORS571 *ntrC* probe, to delimit the region of highest homology (data not shown). This analysis revealed that the *ntrC*-homologous region was confined to a 1.7 kb *Pvu*II-*Eco*RI fragment of pSR1462 (Fig. 1B).

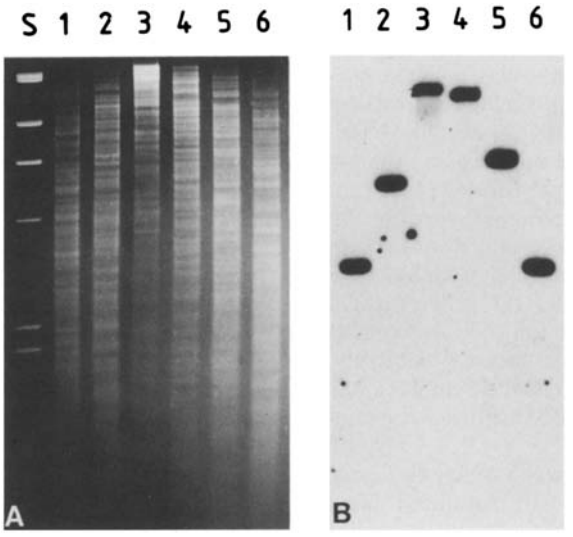

Fig. 2A, B. Hybridization of the ORS571 *ntrC* locus with *Agrobacterium tumefaciens* DNA. **A** Photograph of an ethidium bromide stained agarose gel of total chromosomal DNA of *A. tumefaciens* digested with: 1, *Eco*RI; 2, *Sal*I; 3, *Bam*HI; 4, *Hin*dIII; 5, *Pst*I; 6, *Cla*I; S, λ/*Hin*dIII size markers (23, 9.4, 6.6, 4.3, 2.3, 2.0, 0.5 kb). **B** Southern blot of the gel shown in **A**. The sizes of the restriction fragments hybridizing to the *ntrC*-probe of ORS571 are: *Eco*RI, 3.1 kb (lane 1); *Sal*I, 5.6 kb (lane 2); *Bam*HI, 18 kb (lane 3); *Hin*dIII, 17 kb (lane 4); *Pst*I, 6.4 kb (lane 5); *Cla* I, 3 kb (lane 6)

Complementation experiments with the Agrobacterium ntrC *locus and comparison with other* ntrC *loci*

To confirm that the putative *ntrC* locus carried by plasmid pSR146 corresponds to *ntrC* loci previously identified in other bacterial species, we transferred pSR146 into the *A. sesbaniae* ORS571 *ntrC*::Tn*5* strain C6 (see Fig. 1A; Pawlowski et al. 1987) and measured the ability of the transconjugant strain to fix nitrogen in the free-living state (Nif phenotype). ORS571C6 normally exhibits about 10%–15% of wild-type Nif activity (Pawlowski et al. 1987). Plasmid pSR146 reproducibly increased the level of Nif activity in ORS571C6 two- to threefold, showing (partial) complementation of the Nif$^-$ phenotype of this ORS571 *ntrC* mutant strain (data not shown). However, pSR146 was unable to complement the Ntr$^-$ phenotype of *E. coli* YMC17 (*ntrC*::Tn*5*; Table 1), as determined by testing the ability of tranconjugants to utilize arginine as N source (data not shown). We also used the putative *A. tumefaciens ntrC* locus (plasmid pSR1462) as a DNA hybridization probe to blots carrying total chromosomal DNA of *R. meliloti* 1021, a *R. meliloti ntrC*::Tn*5* strain 19-1, ORS571, *R. japonicum* USDA110, *R. leguminosarum* PRE and *E. coli* YMC10 (see Table 1). A single *Eco*RI fragment of ~3.8 kb was found to hybridize in *R. meliloti* 1021, while in *R. meliloti* 19-1 this fragment was replaced by a hybridizing fragment of 3.8+5.8 kb (Tn*5*). In the case of *A. tumefaciens* only the homologous 3.1 kb *Eco*RI fragment (see Fig. 1B) hybridized. An *Eco*RI fragment of ~15 kb of ORS571, previously shown to carry the *ntrC* locus (Pawlowski et al. 1987) and single *Eco*RI fragments of ~2.6 kb and ~8.5 kb of *B. japonicum* 110 and *R. leguminosarum* PRE respectively, also showed significant hybridization. In the case of *E. coli*, multiple *Eco*RI fragments showed weak hybridization (data not shown). These results suggest that the *A. tumefaciens ntrC* locus described here is functionally and structurally related to the corresponding loci of *A. sesbaniae* (Pawlowski et al. 1987) and *R. meliloti* (Szeto et al. 1987) and is highly conserved, as a single locus in other *Rhizobiaceae*.

Transposon Tn5 mutagenesis of plasmid pSR1462

Plasmid pSR1462 was mutagenized with Tn*5* as described by de Bruijn and Lupski (1984). A total of 42 independent pSR1462::Tn*5* plasmids were characterized by restriction analysis using *Hin*dIII and *Pvu*II to determine the position of the Tn*5* in the plasmid. Ten Tn*5* insertions were shown to be located in the cloned 3.1 kb *Eco*RI fragment, of which eight were in the 1.7 kb *Pvu*II-*Eco*RI *ntrC*-homologous region (data not shown). One Tn*5* insertion (number 33, Fig. 1B) was selected for further use. Tn*5* insertion 33 maps within the *ntrC* hybridizing region, but is flanked by stretches of DNA (>1.4 kb) on both sides long enough to allow homologous recombination (gene-replacement) in *A. tumefaciens* (see below).

Homogenotization (gene replacement) of Tn5 insertion 33

To create a chromosomal Tn*5* insertion in the putative *A. tumefaciens ntrC* locus, the Tn*5*-containing *Eco*RI fragment of pSR1462::Tn*5* 33 was recloned into the *Eco*RI site of plasmid p45-2 (Gm^r; Table 1). The resulting Gm^r, Km^r (Nm^r) plasmid (pSR1463) was tranformed into *E. coli* strain GJ23 (Table 1), to allow conjugal transfer of pSR1463 into *A. tumefaciens* GV3101 (pTiC58). Strain GJ23 carries the necessary transfer (mobilization) functions to effect the conjugal transfer of replicons carrying the pBR322 mobilization site (*bom*) such as p45-2 (and pSR1463) into *Agrobacterium* (van Haute et al. 1983). However, these replicons cannot be maintained stably in *Agrobacterium*. Therefore Nm^r GV3101(pTiC58) transconjugants represent those cases where the pSR1463 plasmid has recombined into the chromosome, a gene-replacement has occurred via homologous recombination at the putative *ntrC* locus, or where Tn*5* has transposed again from pSR1463 into the chromosome (see de Bruijn 1987). To distinguish the first two possibilities (plasmid cointegration vs gene replacement) the Nm^r GV3101(pTiC58) transconjugants were tested for Gm^r, the vector marker of pSR1463. Approximately 400 transconjugants were tested, of which approximately 25% were Gm^s, suggesting they did not contain vector sequences and therefore might represent gene replacement events. To rule out secondary Tn*5* transpositions and to confirm the location of Tn*5* in the (putative) chromosomal *ntrC* locus, total DNA was prepared from six Nm^r Gm^s GV3101(pTiC58) transconjugants, digested with *Eco*RI and *Pvu*II, blotted and probed with the pSR1462 *Eco*RI insert (Fig. 1B). One of the strains, GV3101(pTiC58)1-10, showed the expected hybridization pattern, where the wild-type *ntrC* locus bands were not observed but found to be replaced by a 3.1+5.8 (Tn*5*) kb *Eco*RI fragment and the expected *Pvu*II junction fragments (data not shown). Strain GV3101(pTiC58)1-10 was used for further analysis.

Phenotype of A. tumefaciens *GV3101(pTiC58)1-10*

Tn*5* insertion mutations in the ORS571 *ntrC* locus (C6, C7; Fig. 1A) result in a phenotype that shows poor growth on L-arginine, L-histidine and nitrate. To examine if the Tn*5* insertion mutant 33 (Fig. 1B) in GV3101(pTiC58)1-10

Table 2. Growth of *A. tumefaciens* GV3101(pTiC58), GV3101 and 1-10 on different nitrogen sources

Nitrogen Source	Strain		
	GV3101(pTiC58)	GV3101	1-10
Min A[a]	−	−	−
$+NO_3^{-}$[b]	+	+	−
$+NH_4^{+}$[b]	++	++	+
+Gln[b]	++	++	++
+Glu[b]	++	++	++
+Asn[b]	++	++	++
+Asp[b]	++	++	++
+Pro[b]	++	++	++
+His[b]	++	++	++
+Arg[b]	+	+	−/+
+Arg[b]+Nop[c]	+	+	+
+Orn[b]	−/+	−/+	−
+Orn[b]+Arg[c]	+	+	−
+Orn[b]+Nop[c]	+	−	+
+Nop[b]	++	−	++
+Nop[c]	−/+	−	−/+
+Arg[c]	−/+	−/+	−

++, good growth; +, growth; −/+, poor growth, translucent colonies; −, no growth

[a] MinA composition see Materials and methods
[b] Added to final concentration of 0.2%
[c] Added as inducer to final concentration of 0.01%

had a similar phenotype, we tested the ability of strain 1-10 to utilize a variety of nitrogen sources for growth (Table 2; Fig. 3). Strain 1-10 grew as well as wild-type GV3101(pTiC58) on ammonia, L-glutamine, L-glutamate, L-asparagine, L-aspartate, L-proline and L-histidine (Table 2; Fig. 3, Panel H). However, no growth of strain 1-10 on nitrate (Fig. 3B) and poor growth on arginine (Fig. 3C) was observed (Table 2). We also examined the ability of strain 1-10 to utilize nopaline as sole N source, in comparison with GV3101(pTiC58) and its derivative that lacks the Ti plasmid GV3101 (Table 1). Strain 1-10 grew as well as GV3101(pTiC58) on 0.2% nopaline, while GV3101 showed no growth (Table 2). The nopaline catabolism pathway is Ti-borne and inducible by nopaline. Arginine and ornithine have been proposed to be intermediates in the nopaline catabolism pathway. Further catabolism of these compounds in mediated by both a Ti-encoded (nopaline-induced) and chromosomal pathway(s). To determine which steps of the pathways might be under control of the *ntrC*-like gene, we examined the ability of GV3101(pTiC58), GV3101 and strain 1-10 to grow on L-arginine and L-ornithine in the presence and absence of 'inducers' (0.01% nopaline, 0.01% L-arginine). The addition of 0.01% nopaline restored normal growth of strain 1-10 on L-arginine (Table 2; Fig. 3D) suggesting that in a *ntrC* strain the chromosomal arginine utilization genes are poorly expressed and that the Ti-encoded, *ntrC*-independent, pathway can only function when induced by nopaline. L-ornithine could not be used as N source by any of the strains tested (Table 2; Fig. 3E). Addition of 0.01% L-arginine restored growth on ornithine to GV3101 with or without the Ti plasmid, but not to strain 1-10 (Table 2; Fig. 3F). However, addition of 0.01% nopaline restored growth to GV3101(pTiC58) and strain 1-10, but not to GV3101 (Table 2; Fig. 3G).

Glutamine synthetase (GS) and glutamate synthase (GOGAT) enzyme activities in strain 1-10

In enteric bacteria the *ntr* system not only regulates amino acid catabolism (utilization) genes but also the *glnA* gene, the product of which (GS), in combination with glutamate

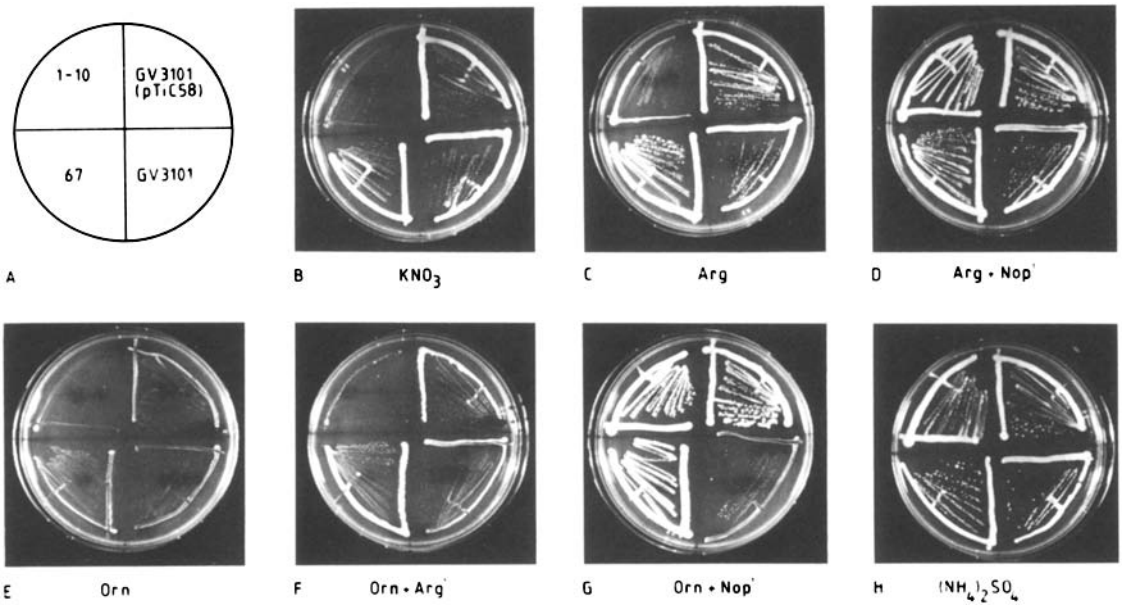

Fig. 3A–H. Growth of agrobacterial strains on Minimal A medium supplemented with various nitrogen sources. **A** Plan of strains; 1-10, *ntrC*::Tn*5* in GV3101 (pTiC58); GV3101(pTiC58), C58 Rif^r (Table 1); 67, uncharacterized Tn*5* insertion in GV3101(pTiC58); GV3101, pTi cured derivative of C58 Rif^r (Table 1). Minimal A plates were supplemented with the following nitrogen sources at 0.2%: **B** potassium nitrate; **C** L-arginine; **D** L-arginine and nopaline (0.01%) as inducer; **E** L-ornithine; **F** L-ornithine and L-arginine (0.01%) as inducer; **G** L-ornithine and nopaline (0.01%) as inducer; **H** ammonium sulphate

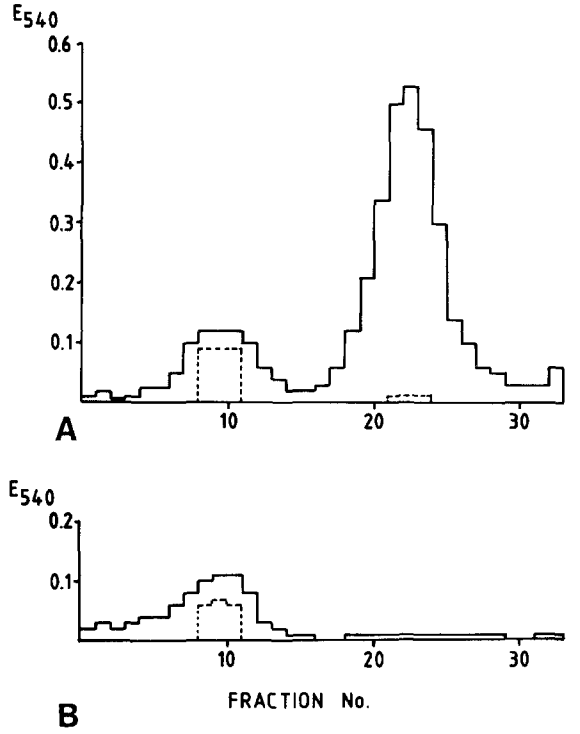

Fig. 4A, B. Glutamine synthetase activity profiles of crude extracts of: **A** GV3101(pTiC58); **B** 1-10 (*ntrC*::Tn*5*) fractionated on sucrose gradients. One millilitre of freshly prepared crude cell extracts obtained by French press treatment (see Materials and methods) was loaded on a 5%–20% sucrose gradient in 10 mM imidazole buffer containing 1 mM $MnCl_2$ (pH 7.0). Samples were centrifuged at 150000 *g* for 16 h at 4° C, gradients were fractionated and GS transferase activity of each fraction was determined according to Bender et al. (1977). The optical density of the reaction mixture at 540 nm (E_{540}) is indicated on the y-axis, the fraction number on the x-axis. The *broken lines* indicate the E_{540} of the peak fractions after heat treatment of the samples at 50° C. The *left hand* peak represents GSI and *right hand* peak GSII activity

synthase (GOGAT), is responsible for assimilation of ammonium at concentrations of <10 mM (Magasanik 1982). In *Rhizobiaceae* the GS-GOGAT pathway has been proposed to be the primary pathway for ammonium assimilation (see Miflin and Cullimore 1984). It was therefore of interest to determine whether the *A. tumefaciens ntrC*::Tn*5* mutant strain 1-10 was affected in GS or GOGAT activity. The study of GS expression in *Agrobacterium* is complicated by the presence of two distinct GS (I, II) enzymes. They can be distinguished by molecular weight and by the fact that GSII activity is extremely heat labile. Thus whole cell extracts were made from N-starved cultures of GV3101(pTiC58) and strain 1-10 as described in Materials and methods. Using the γ-glutamyl transferase assay of Bender et al. (1977), GS activity of extracts were determined, before and after heat treatment at 50° C (see Materials and methods). Strain 1-10 was found to have considerably lower total GS activity than GV3101(pTiC58) (295 units vs 1579 units), which was essentially heat stable (290 units for 1-10 and 522 units for GV3101(pTiC58) after heat inactivation). The extracts were applied to sucrose gradients and the fractions assayed for GS activity. While GV3101(pTiC58) extract clearly exhibited two peaks of GS activity (GSI, II), strain 1-10 only showed a GSI peak (Fig. 4). GOGAT assays were also performed (see Materials and methods), but no effect of the *ntrC*::Tn*5* mutation on NADPH-GOGAT activity was observed; 4630 units for 1-10 versus 5630 units for GV3101(pTiC58).

Virulence test of strain 1-10

In order to determine if the *ntrC*::Tn*5* mutation of strain 1-10 had an effect on virulence, we infected young *Kalanchoe diagremontiana* plants with cultures of 1-10, GV3101(pTiC58) and GV3101. Strains 1-10 and GV3101(pTiC58) induced normal tumours on stems and leaves after 3–4 weeks, while GV3101 induced no tumour formation (data not shown).

Discussion

We have described the cloning and characterization of the *ntrC* gene of *A. tumefaciens* C58. The *A. sesbaniae* ORS571 *ntrC* locus described by Pawlowski et al. (1987) was used as heterologous gene probe in order to clone the *A. tumefaciens ntrC* locus, since it proved to have a high degree of homology with its *A. tumefaciens* counterpart (Fig. 2). Only a single *ntrC*-homologous fragment was observed in *A. tumefaciens* DNA, in contrast to the multiple *ntrC/nifA* homologous bands observed in *A. sesbaniae* (Pawlowski et al. 1987) and *B. japonicum* (Chelm et al. 1985). A *ntrC*::Tn*5* mutant of *A. tumefaciens* was constructed, using the protocol described by van Haute et al. (1983), which was found to represent a useful alternative to the homogenotization protocol described by Ruvkun and Ausubel (1981); for a comparison see de Bruijn (1987).

The phenotype of the *A. tumefaciens ntrC*::Tn*5* strain 1-10 has a number of features in common with that of analogous *ntrC* strain of other bacterial species. The inability of strain 1-10 to grow on nitrate as sole N source represents a commonly observed $NtrC^-$ phenotype. *NtrC* mutants unable to utilize nitrate have been described for *R. meliloti* (Szeto et al. 1987), *Azotobacter vinelandii* (Toukdarian and Kennedy 1986), *A. sesbaniae* ORS571 (Pawlowski et al. 1987; P. Ratet and F. de Bruijn, unpublished observations) and *Azospirillum brasilense* (Pedrosa and Yates 1984). All other observed phenotypes are variable. For example the ability to derepress the nitrogen fixation (*nif*) genes in *ntrC* mutants varies with the bacterial species examined. While *K. pneumoniae* (de Bruijn and Ausubel 1981; Espin et al. 1981), *A. brasilense* (Pedrosa and Yates 1984), *A. sesbaniae* (Pawlowski et al. 1987; de Bruijn et al. 1987) and *Rhodopseudomonas capsulata ntrC* mutants are essentially Nif^-, analogous *ntrC* mutants of *A. vinelandii* (Toukdarian and Kennedy 1986) and *R. meliloti* (Szeto et al. 1987) are unaffected in nitrogen fixation.

The effect of *ntrC* mutations on the utilization of amino acids as sole N sources is even more diverse. *NtrC* mutants of *E. coli*, *S. typhimurium* and *K. pneumoniae* are disturbed in the utilization (catabolism, transport) of histidine, proline and arginine as N sources (Pahel and Tyler 1979; Kustu et al. 1979; de Bruijn and Ausubel 1981). *A. sesbaniae ntrC* mutants are disturbed in utilization of arginine, histidine and to some degree proline (Pawlowski et al. 1987). *R. meliloti ntrC* mutants, however, grow as well as the wild-type strain on arginine, proline or histidine (Szeto et al. 1987). In the case of the *A. tumefaciens ntrC* mutant, only growth

on arginine appeared to be affected (Table 2). Here the situation is further complicated by the presence of a Ti plasmid encoded, as well as a chromosomal arginine catabolism pathway (Dessaux et al. 1986). Our evidence indicates that it is the chromosomal arginine utilization pathway which is arginine-induced and *ntrC*-controlled. The Ti-encoded pathway appears to be *ntrC*-independent but nopaline-inducible. Both arginine catabolism pathways have been proposed to proceed via ornithine (Schardl and Kado 1983b; Dessaux et al. 1986). The catabolism of ornithine via the chromosomal pathway again appears to be arginine-inducible and *ntrC*-controlled, while the Ti-encoded pathway is nopaline-inducible and *ntrC*-independent. If the Ti-encoded nopaline – arginine – ornithine conversion proceeds via proline (Dessaux et al. 1986; Farrand and Dessaux 1986), then the further catabolism of proline via Δ pyrrolidone-5-carboxylate (P-5-C) to glutamate via a chromosomal pathway is probably *ntrC*-independent, since growth on proline is undisturbed in the *ntrC* mutant. Thus *ntrC* appears to control only the genes encoding arginase and ornithine-transaminase, which catalyse the first and second step of the chromosomal arginine catabolism pathway (Dessaux et al. 1986). Ntr control and arginine induction of the ornithine-transaminase gene, suggested by our results, contrasts with the finding that the activity of this enzyme appears to be low level constitutive (Dessaux et al. 1986). Further experiments quantitating enzyme levels in the *ntrC* strain are necessary to clarify this point. It is clear, however, that the nopaline (arginine/ornithine) utilization pathway encoded by the Ti plasmid has given *Agrobacterium* the capacity to grow on this unusual N (and C) source independent of the central nitrogen control system.

The effect of *ntrC* mutations on glutamine synthetase (GS) activity also appears to be bacterial species dependent. *NtrC* mutant of *E. coli*, *S. typhimurium* and *K. pneumoniae* are essentially GS^- ($GlnA^-$; Pahel and Tyler 1979; Kustu et al. 1979; de Bruijn and Ausubel 1981). *NtrC* mutants of *A. brasilense* have 30% of wild-type GS activity (Pedrosa and Yates 1984). *NtrC* mutants of *A. sesbaniae* ORS571 have wild-type GS activity (de Bruijn et al. 1987; Pawlowski et al. 1987). In other members of the *Rhizobiaceae*, which have two GS enzymes (GSI, II) the *ntr* effect on GS activity has to be subdivided according to GSI or II activity. Our results with *A. tumefaciens ntrC* strain 1-10 clearly establishes for the first time that GSII activity is strictly controlled by *ntrC* while GSI is not. Using the *R. meliloti* 1021 *ntrC*::Tn*5* strain 19-1 (Szeto et al. 1987), we have shown that GSII activity is completely absent in this rhizobial mutant as well (S. Rossbach and F. de Bruijn, unpublished observations). That the $NtrC^-$ phenotype of *A. tumefaciens* strain 1-10 is not an indirect effect of the absence of GSII activity has been shown by analysing growth of a strain carrying an insertion mutation in the GSII structural gene; this mutant strain grows normally on nitrate and arginine (S. Rossbach, J. Schell and F.J. de Bruijn, unpublished results). The *A. tumefaciens ntrC* gene appears to be dispensible for virulence, since normal tumour formation on *Kalanchoe* and *Sesbania rostrata* was observed with strain 1-10. This supports the notion that neither the *ntr* system, nor GSII plays a particular role in the interaction of agrobacteria with their host plant.

Thus, our studies show that *A. tumefaciens* has a central, nitrogen regulation (*ntrC*) gene, which controls expression of the glutamine synthetase II, nitrate reductase and chromosomal but not Ti-encoded arginine catabolism genes. The apparently divergent roles the conserved *ntrC* gene plays in the various bacterial backgrounds which have been examined may be a true reflection of the often very different physiological growth conditions (availability of N sources) the different bacterial species are exposed to. Identification and characterization of additional *ntr*-related loci such as *ntrA*, *glnB* and *glnD* in *Agrobacterium* is in progess to elucidate further the role of the *ntr* system in controlling nitrogen assimilation processes in this organism, in comparison with other members of the *Rhizobiaceae*.

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