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Identification, Cloning, and Sequence Analysis of the Nitrogen Regulation Gene *ntrC* of *Agrobacterium tumefaciens* C58

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We describe the cloning of an *ntrC* gene of *Agrobacterium tumefaciens* C58 by interspecific complementation of an *Escherichia coli ntrC* mutant. Restriction mapping and Southern blot analysis of the complementing clone identified a 1.7-kb *Eco*RI-*Pvu*II DNA fragment whose sequence was determined. Analysis of this sequence revealed coding regions corresponding to a complete *ntrC* gene and the C-terminal region of an *ntrB* gene. Amino acid sequence comparisons of *A. tumefaciens* NTRC protein with NTRC sequences from *Rhizobium meliloti, Bradyrhizobium* sp. (*Parasponia*), *Klebsiella pneumoniae, E. coli,* and *Salmonella typhimurium* show strong sequence conservation supporting DNA hybridization data, demonstrating strong evolutionary homology among *ntrC* genes of Rhizobiaceae. The C58 NTRC protein has been identified, by ^{35}S-labeling, in a T7 RNA polymerase (pT7-7) expression vector system.

Regulation of nitrogen metabolism in procaryotes is a complex process. In recent years several genes whose products are involved in nitrogen regulation have been identified and characterized in the enteric bacteria *Klebsiella pneumoniae* (Schroeter) Trevisan, *Salmonella typhimurium* (Loeffler) Castellani & Chalmers, and *Escherichia coli* (Migula) Castellani & Chalmers (see Kustu *et al.* 1986 and Gussin *et al.* 1986 for reviews). Such studies have recently been extended to nonenterobacterial species (Toukdarian and Kennedy 1986; Filser *et al.* 1986; Szeto *et al.* 1987; Powlowski *et al.* 1987; Rossbach *et al.* 1988). The NTRC protein (product of the *ntrC* gene) plays a central role in cellular nitrogen regulation and is responsible for the expression of *glnA* (the structural gene for glutamine synthetase), *nifA* (activator of nitrogen fixation genes in *K. pneumoniae*), and genes concerned with utilization of nonpreferred nitrogen sources such as arginine and histidine. Glutamine synthetase is responsible for the synthesis of glutamine from glutamate and ammonia (Ginsburg and Stadtman 1973), which in turn is a precursor of several important compounds, including amino acids and nucleotides. In all enterobacterial species investigated, *glnA* and *ntrC* form part of the same operon; the latter, in association with a special sigma factor encoded by an independently located gene *ntrA* (*rpoN*), regulates *glnA* (Hirschmann *et al.* 1985; Hunt and Magasanik 1985) and other genes involved in nitrogen metabolism. It has recently been shown that NTRB-NTRC proteins form one of a series of protein pairs that regulate specific metabolic functions in response to environmental stimuli (Drummond *et al.* 1986; Nixon *et al.* 1986).

The situation in Rhizobiaceae, to which the genus *Agrobacterium* belongs, is more complicated due to the presence in several species of two distinct forms of glutamine synthetase, GSI and GSII (Darrow *et al.* 1981). GSI appears similar to other procaryotic glutamine synthetases in its enzymatic structure and amino acid sequence and in modulation of activity by posttranslational modification; GSII, however, does not undergo posttranslational modification, and recently it has been shown that in *Bradyrhizobium japonicum* (Buchanan) Jordan, the gene concerned might have a eucaryotic origin (Carlson and Chelm 1986). In contrast to the operon arrangement in enterobacteria, *ntrC* in *Rhizobium meliloti* Dangeard (Szeto *et al.* 1987), *R. leguminosarum* Jordan (Filser *et al.* 1986), and *A. tumefaciens* (Smith & Townsend) Conn (Rossbach *et al.* 1987) is not linked to *glnA*.

Little is known about nitrogen regulation in *A. tumefaciens,* despite its relevance to plant genetic engineering work, its close relationship to the nitrogen-fixing *Rhizobium,* and its unique opine metabolism (see Giles and Atherly 1981). Recent evidence suggesting that several *Agrobacterium* strains fix nitrogen in free-living condition (Kanvinde *et al.* 1986; L. Kanvinde and G. R. K. Sastry, unpublished) make a detailed study of nitrogen regulation in this bacterium imperative. As a first step toward this goal, we have cloned and sequenced the *ntrC* gene from *A. tumefaciens.* Rossbach *et al.* (1987) described the cloning of *ntrC* from *A. tumefaciens.* The restriction map of their *ntrC* gene is essentially the same as ours; however, these clones show significant differences in their abilities to complement an *E. coli ntrC* mutant.

MATERIALS AND METHODS

Bacterial strains and media. Strains and plasmids used in the present work are listed in Table 1. For routine culturing of *E. coli* (at 37° C) and *A. tumefaciens* (at 30° C), Luria-Bertani (LB) medium (Miller 1972) was used. For DNA isolation, various *Agrobacterium* strains were grown in Vogel and Bonner (VB) medium (Vogel and Bonner 1956). M9 media with various supplements were prepared according to Maniatis (1982). The recipe for nitrogen-free Davis-Mingioli medium (NFDM) was taken from Cannon *et al.* (1974); the required nitrogen source such as arginine

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Nucleotide and/or amino acid sequence data is to be submitted to GenBank as accession number J03678.

was added at a final concentration of 0.2%. Thiamine (20 μg/ml) was added when necessary after autoclaving the medium. Filter-sterilized antibiotics were used at the following concentrations (μg/ml): ampicillin 50, chloramphenicol 25, kanamycin 50, and tetracycline 15 (unless otherwise specified).

Isolation and manipulation of DNA. Chromosomal DNA from *A. tumefaciens* was prepared essentially following the method of Marmur (1961). Plasmid DNA was routinely isolated using the alkaline and sodium dodecyl sulfate (SDS) procedure of Birnboim and Doly (1979). Chromosomal and plasmid DNA was further purified by cesium chloride density gradient centrifugation as described in Maniatis *et al.* (1982). Restriction enzymes obtained from NBL Enzymes (Ramlington, U.K.) and Bethesda Research Laboratories (BRL) Paisley, U.K., were used according to the manufacturers' specifications. Specific DNA fragments were eluted from agarose gels by using Elutip-d minicolumns supplied by Schleicher and Schuell (Dassel, West Germany) or GeneClean from Bio-101, La Jolla, CA. Single-stranded DNA was prepared as recommended by the suppliers of the T7 sequencing kit (Pharmacia-LKB, Milton Keynes, U.K.) and further purified by using the Quiagen Tip 20 (from Diagen, Düsseldorf, West Germany). This yielded extremely pure single-stranded DNA for DNA sequencing. Vector DNA was treated with calf intestinal alkaline phosphatase as specified by the manufacturer (Boehringer Mannheim, London, U.K.). Intermolecular ligations were performed at room temperature using T4-DNA ligase and T4-DNA ligase buffer supplied by BRL at a total concentration of 80–100 μg of DNA per milliliter. The reaction mix was diluted fivefold with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) before use in transformations. The desired *E. coli* strains were made competent according to Maniatis (1982).

Table 1. Bacterial strains, plasmids, and phages

	Genotype and comments[a]	Source
Strains		
Escherichia coli		
ET8556	*ntrC* 1488	Merrick 1983
DH1	F⁻, *recA1, endA1, gyrA*96, *thi*-1, *hsdR*17 (r_k^-, m_k^+), *supE44, recA1* λ⁻	Laboratory collection
JM103	Δ(*pro lac*), *supE, thi, strA endA sbcB, hsdR*⁻, *F' traD36 proAB, lacI*^{q}, *ZΔM15* (used for M13 transformations	Laboratory collection
Agrobacterium tumefaciens		
C58	A Ti plasmidless derivative of C58 (otherwise wild type) was used.	E. W. Nester
NCPPB223	(Biotype 2)	NCPPB[b]
NCPPB2461	(Biotype 3)	NCPPB
B6	(Biotype 1)	Laboratory collection
A. radiobacter		
NCPPB1649	(Biotype 2)	NCPPB
A. rubi		
NCPPB1856	(Biotype 2)	NCPPB
A. rhizogenes		
NCPPB2655	(Biotype 1)	NCPPB
Alcaligenes eutrophus		
NCIB11842		NCIB[c]
Rhizobium leguminosarum		Laboratory collection
Plasmids		
pBR325	Ap^r, Cm^r, and Tc^r	Laboratory collection
pGP1-2	Km^r (expresses T7 RNA polymerase)	S. Tabor
pT7-7	Ap^r, T7 promoter φ 10	S. Tabor
pHW4	Ap^r, *ntrC*$^+$ in pBR325	This study
pHW5	*Ap*^{r}, *ntrC*$^+$, 2.4-kb *Eco*RI-*Cla*I of pHW4 in pBR325	This study
pHW6	Ap^r, *ntrC*$^+$, 2.4-kb *Eco*RI-*Cla*I of pHW5 in pT7-7	This study
pRmC3.8R	Tc^r, *ntrC*$^+$ of *R. meliloti* in pACYC184	F. M. Ausubel
Phages		
M13mp8		Laboratory collection
M13mp9		Laboratory collection

[a]Ap = ampicillin, Cm = chloramphenicol, Tc = tetracycline, and Km = kanamycin; and ^{r} = resistant.
[b]National Collection of Plant Pathogenic Bacteria, Harpenden, England.
[c]National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

Southern blotting. The DNA to be tested was digested with restriction enzyme(s) and transferred to Biodyne-A nylon membranes, following the procedure of Southern (1975) and using Vaccu-blot apparatus from Hybaid (Middlesex, U.K.) for the transfers. DNA fragments to be used as probes were labeled with α^{32}P-dATP, using the random priming procedure of Feinberg and Vogelstein (1983) with a specific activity of $1–6 \times 10^8$ counts per minute per microgram of DNA. Unincorporated dNTPs were removed by passing the labeled mixture through Bio-spin 30 columns supplied by Bio-Rad, Watford, Herts, U.K. Hybridizations were conducted in 50% formamide at 42° C. Membranes were normally washed under high-stringency conditions (0. 1× SSC, 0.1% SDS at 65° C) and exposed to Kodak X-ray films for 1–3 days at −70° C.

DNA sequencing. The 2.4-kilobase (kb) *Eco*RI-*Cla*I fragment of pHW5 was subcloned into *Eco*RI-*Acc*I digested M13mp8 and M13mp9. To obtain subclones of this fragment for DNA sequencing, the protocol of Dale *et al.* (1985) was used. This method offers significant advantages over several other deletion methods, because single-stranded DNA can be used for generating subclones and fewer subclones are required to obtain a complete sequence. The subclones were sized (approximately) by running the SDS-disrupted phage supernatants from various potential subclones on a 1% agarose gel overnight. Single-stranded DNA from the selected subclones was then prepared. Sequencing reactions were conducted with ^{35}S dATP and the T7 sequencing kit. The 17-base primer (−40, from New England Biolabs, Bishops' Stortford, Herts, U.K.) 5′-GTTTTCCCAGTCACGAC-3′ was used to read the *Eco*RI site of the mp9 clone containing the complete 2.4-kb *Eco*RI-*Cla*I fragment; otherwise the 17-base universal primer (included in the kit) was used. Sequencing with T7 polymerase offered a considerable advantage, because uniform band intensity was obtained and more (350–450) bases per sequencing reaction could be determined. Also, the problem of band compression in GC-rich regions was reduced.

Sequencing data were entered into a VAX/VMS computer by using Batin, then arranged into a single continuum using the Dbauto and Dbutil (Staden 1986).

Analysis of the DNA sequence, including amino acid translation and determination of codon usage, was performed with the Analyseq (Staden 1984). Protein sequence analysis with Patscan to identify sequence features was performed according to Drummond *et al.* (1986).

Expression of the *ntrC* gene. The specificity of the T7 RNA polymerase for its own promoter, combined with the ability of rifampicin to selectively inhibit the host RNA polymerase, permits the exclusive expression of genes under the control of a T7 RNA polymerase promoter (Tabor and Richardson 1985). A coupled system consisting of plasmids pGP1-2 (which expresses T7 RNA polymerase) and pT7-7 containing a T7 RNA polymerase promoter, ϕ 10, and several useful cloning sites was used to express the C58 *ntrC*. The 2.4-kb *Eco*RI-*Cla*I fragment of pHW5 was cloned into *Eco*RI-*Cla*I digested pT7-7. The resulting recombinant plasmid pHW6 was used to transform *E. coli* DH1. Cells containing this recombinant plasmid were then transformed with pGP1-2. Cultures containing the two plasmids were grown in rich medium (LB) with 40 μg of ampicillin and kanamycin per milliliter until A590 equaled 0.5. At this stage, 0.2 ml of cells was centrifuged, and the cell pellet was washed with 5 ml of M9 media, centrifuged, and resuspended in 5 ml of M9 media supplemented with thiamine (20 μg/ml) and 0.01% each of 18 amino acids (minus cysteine and methionine). Cells were grown with shaking at 30° C for 2 hr, and, for induction experiments, the temperature was shifted to 42° C for 15 min; rifampicin was added (200 μg/ml), and the cells were left at 42° C for an additional 10 min. The temperature was shifted to 30° C for 20 min, and the samples were pulsed with ^{35}S methionine for 5 min at 30° C. Similar noninduced control samples involved continued growth at 30° C rather than a temperature shift to 42° C. The cells were then spun briefly, and the pellet was resuspended in 120 μl of cracking buffer (60 mM Tris-HCl, pH 6.8; 1% SDS; 1% 2-mercaptoethanol; 10% glycerol; and 0.01% bromophenol blue). The samples were incubated at 95° C for 3 min and loaded directly onto a 7.5–15% SDS-polyacrylamide gradient gel. The gel was stained with Coomassie blue, photographed, and then processed for fluorography essentially according to Hames (1986).

RESULTS

Screening of *A. tumefaciens* DNA for an *ntrC* gene. The *R. meliloti ntrC* from pRmC3.8R (Szeto *et al.* 1987) was used to probe C58 total DNA digested with *Eco*RI, *Sal*I, *Eco*RI-*Cla*I, and *Eco*RI-*Pvu*II. Hybridization and subsequent washings were carried out under high stringency. The *R. meliloti ntrC* probe hybridized strongly to a 3.1-kb *Eco*RI, 5.6-kb *Sal*I, 2.4-kb *Eco*RI-*Cla*I, and 1.7-kb *Eco*RI-*Pvu*II fragment of C58 total DNA (data not shown). The detection of such strong homology, indicating the existence of an *ntrC* gene, prompted the cloning of this gene from *A. tumefaciens*.

Isolation of the *ntrC* gene. To isolate recombinant plasmid(s) carrying the *A. tumefaciens ntrC*, the 3.1-kb region of *Eco*RI-digested C58 chromosomal DNA that hybridized to the *R. meliloti* probe was purified from a 0.7 agarose gel and ligated to pBR325 that had been *Eco*RI-digested and treated with calf intestinal phosphatase. The ligation mix was used to transform competent *E. coli* ET8556, an *ntrC* mutant (Merrick 1983). Selection for NtrC$^+$ was performed by washing the transformed cells twice with NFDM before plating on NFDM with 0.2% arginine as the sole nitrogen source and ampicillin. Plates were incubated at 37° C, and after 3 days several colonies appeared. Rapid miniscale plasmid preparations were made from several of these colonies, and all contained plasmids with a 3.1-kb insert fragment. One of these recombinants, pHW4, was selected for further analysis. To establish that pHW4 contained DNA originating from *A. tumefaciens*, the 3.1-kb *Eco*RI insert of pHW4 was labeled with ^{32}P, using the random priming method of Feinberg and Vogelstein (1983), and used to probe *Eco*RI-digested *A. tumefaciens* chromosomal DNA. This probe hybridized very strongly to a 3.1-kb *Eco*RI fragment of the C58 DNA under high-stringency conditions but did not hybridize to *E. coli* chromosomal DNA (data not shown). The restriction map of the pHW4 insert (Fig. 1) differs from that of *E. coli ntrC* (Backman *et al.* 1981), confirming the agrobacterial origin of the C58 *ntrC*. Insert pHW4 transformed *E. coli* ET8556 to a NtrC$^+$ phenotype at a high frequency, whereas ET8556 cells alone or those transformed with vector pBR325 DNA showed no growth on NFDM plus 0.2% arginine. Figure 2 summarizes the results of this experiment.

Location of *ntrC* within pHW4. A restriction map of pHW4 insert was constructed (Fig. 1) by single and double digestions with the enzymes *Cla*I, *Eco*RI, *Kpn*I, *Pvu*II, and *Xho*I. For the enzymes in common, this map is in agreement with that of Rossbach *et al.* (1987). Preliminary localization of the *ntrC* within pHW4 was achieved by hybridization of the *R. meliloti ntrC* probe (from pRmC3.8R) to different single and double digests of this plasmid. *A. tumefaciens* sequences homologous to this probe were confined to a 1.7-kb *Eco*RI-*Pvu*II fragment of pHW4 insert (Fig. 1), which corresponds to the hybridizing *Eco*RI-*Pvu*II digested C58 chromosomal DNA fragment (data not shown). The location of *ntrC* on pHW4 was further confirmed by subcloning the 2.4-kb *Eco*RI-*Cla*I fragment of pHW4 into pBR325 (the 2.4-kb *Eco*RI-*Cla*I fragment was cloned for convenience). ET8556 was

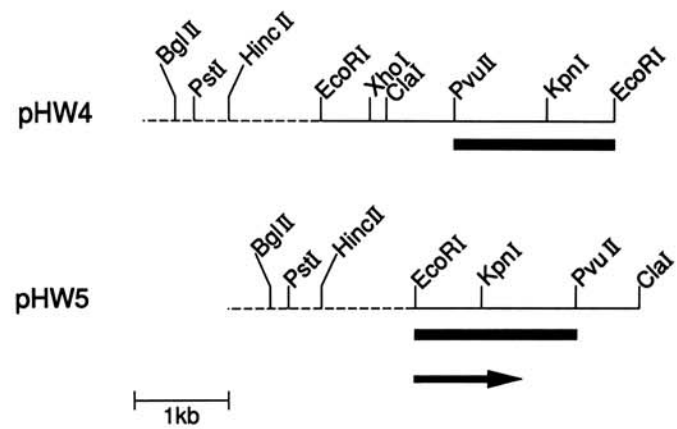

Fig. 1. Partial restriction maps of pHW4 and pHW5, showing the deduced position of *ntrC* from Southern blot data. The dashed line represents pBR325; the solid line represents *Agrobacterium tumefaciens*-derived DNA; and the bar shows the deduced position of *ntrC*. The arrow indicates the direction of transcription of *ntrC* deduced from DNA sequencing data.

transformed with the resulting plasmid, pHW5, and a random sample of 100 colonies was tested on NFDM-arginine plates with ampicillin. These ampicillin-resistant colonies, when retested, grew well on NFDM-arginine, compared with no growth for controls, showing that pHW5 must contain the functional *A. tumefaciens ntrC* gene. The ability of pHW4 and pHW5 to complement the ET8556 lesion contrasts with observations reported by Rossbach *et al.* (1987), whose clone, pSR1462, did not complement an *E. coli ntrC* mutant. Because both genes were isolated from the same *A. tumefaciens* strain and the restriction maps of the *ntrC* clones isolated are essentially similar, it is unclear why such a difference in complementation ability exists. A possible explanation is that expression may be influenced by the copy numbers of the recombinant plasmids, which would be expected to be higher for pHW4 and pHW5 (pBR325 vector) than for pSR1462 (pACYC184 vector).

DNA sequence analysis. The 2.4-kb *Eco*RI-*Cla*I fragment of pHW5 was subcloned into M13mp8 and mp9 in order to obtain DNA sequence information. Series of deletion derivatives of the M13 clones were generated and sequenced according to the single-stranded deletion method of Dale *et al.* (1985). The DNA sequence and amino acid translation of a 1.7-kb segment of this fragment is shown in Figure 3 and demonstrates the presence of an *ntrC* gene. A comparison of the predicted amino acid sequence of the *A. tumefaciens* NTRC protein with the known amino acid sequence of other NTRC proteins from *R. meliloti* (Szeto *et al.* 1987), *Bradyrhizobium* sp. (*Parasponia*) (Nixon *et al.* 1986), *K. pneumoniae* (Buikema *et al.* 1985), *E. coli* (Miranda-Ríos *et al.* 1987), and *S. typhimurium* (D. Weiss, personal communication) reveals strong conservation. The C58 NTRC sequence was also compared with the amino acid sequence of *Rhodobacter capsulatus* (Molisch) Imhoff *et al. nifR1* (an *ntrC*-equivalent in this photosynthetic bacterium, Jones and Haselkorn 1989). In particular, there is a very high degree of homology between the Rhizobiaceae NTRC proteins. Of the compared residues, 93% are identical between *A. tumefaciens* and *R. meliloti*, with 71% identical between *A. tumefaciens* and *Bradyrhizobium* sp. (*Parasponia*). Lower but highly significant homology (37%) also exists between *A. tumefaciens* and the enterobacterial *K. pneumoniae* NTRC protein. Figure 3 shows the identical amino acid residues conserved among the sequences compared.

The predicted amino acid sequence of *A. tumefaciens* NTRC was scanned with Patscan matrices as described by Drummond *et al.* (1986). Three N-terminal regions (positions 5–22, 48–69, and 95–110; Fig. 3) within domain B (Drummond *et al.* 1986) that are characteristic of NTRC-like proteins were strongly predicted. A further region at the C-terminal end of the protein corresponds to a DNA-binding domain (Fig. 3; positions 451–470; helix-turn-helix motif) identified in other NTRC proteins (Drummond *et al.* 1986). Also predicted by Patscan is a nucleotide-binding region (Fig. 3; positions 162–180, containing the conserved sequence motif -Gly-X-X-Gly-X-Gly-Lys-) at a position analogous to that in other NTRCs first identified by Drummond and Wootton (1987). Recent research has shown that NTRC, which is phosphorylated by NTRB, is capable of autodephosphorylation (Keener and Kustu 1988); however, this nucleotide-binding region does not appear to be implicated in this phenomenon, and its role remains obscure.

Codon usage in C58 *ntrC* was determined using Analyseq (Staden 1984) and was found to be very similar to that of *R. meliloti* and *Bradyrhizobium* sp. (*Parasponia*). Amino acid sequence differences between the NTRC proteins of *A. tumefaciens* and *R. meliloti* are restricted to the absence of the residue corresponding to position 3 of the *R. meliloti* sequence and two substitutions; asparagine and glycine at positions 220 and 327, respectively, replace threonine and alanine, respectively, of *R. meliloti, Bradyrhizobium* sp. (*Parasponia*), and *K. pneumoniae*. The molecular weight of the predicted NTRC polypeptide is 53,916, which is in good agreement with the estimate of 55,000 by SDS-gel electrophoresis (Fig. 4).

The *ntrB* gene. In *R. meliloti* (Szeto *et al.* 1987), *Bradyrhizobium* sp. (*Parasponia*) (Nixon *et al.* 1986), *K. pneumoniae* (MacFarlane and Merrick 1985), and *E. coli* (Miranda-Ríos *et al.* 1987), nitrogen-regulatory gene *ntrB*

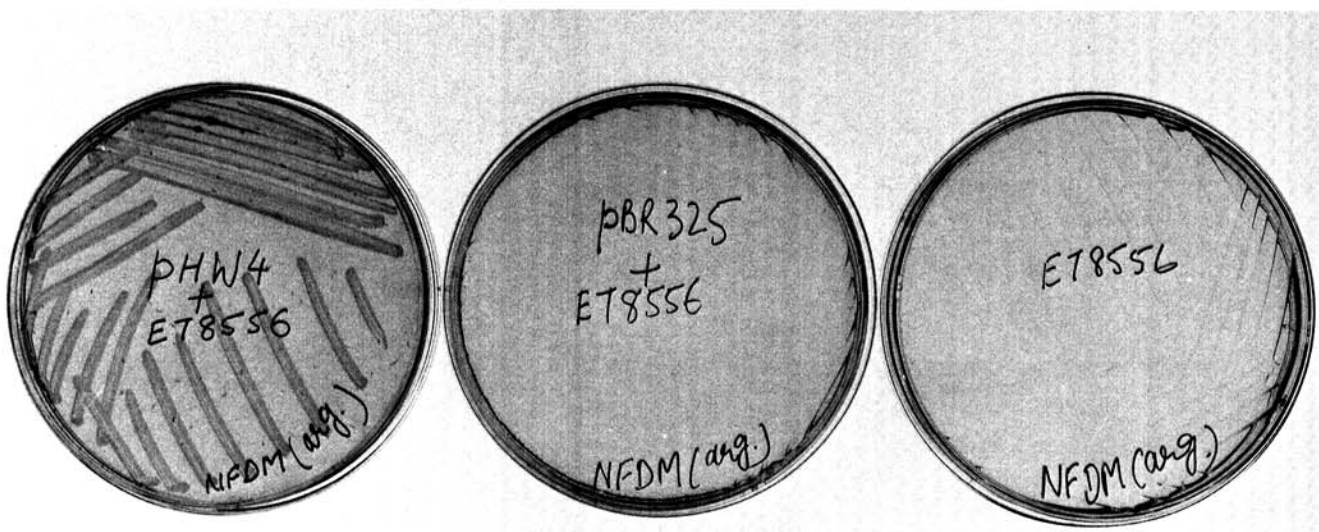

Fig. 2. Growth pattern (left to right) of ET8556 (pHW4) on nitrogen-free Davis-Mingioli medium (NFDM) with 0.2% arginine (arg) as nitrogen source, compared with ET8556 (pBR325) and with ET8556 alone on the same medium. This clearly demonstrates the ability of pHW4 to transform ET8556 to a $NtrC^+$ phenotype.

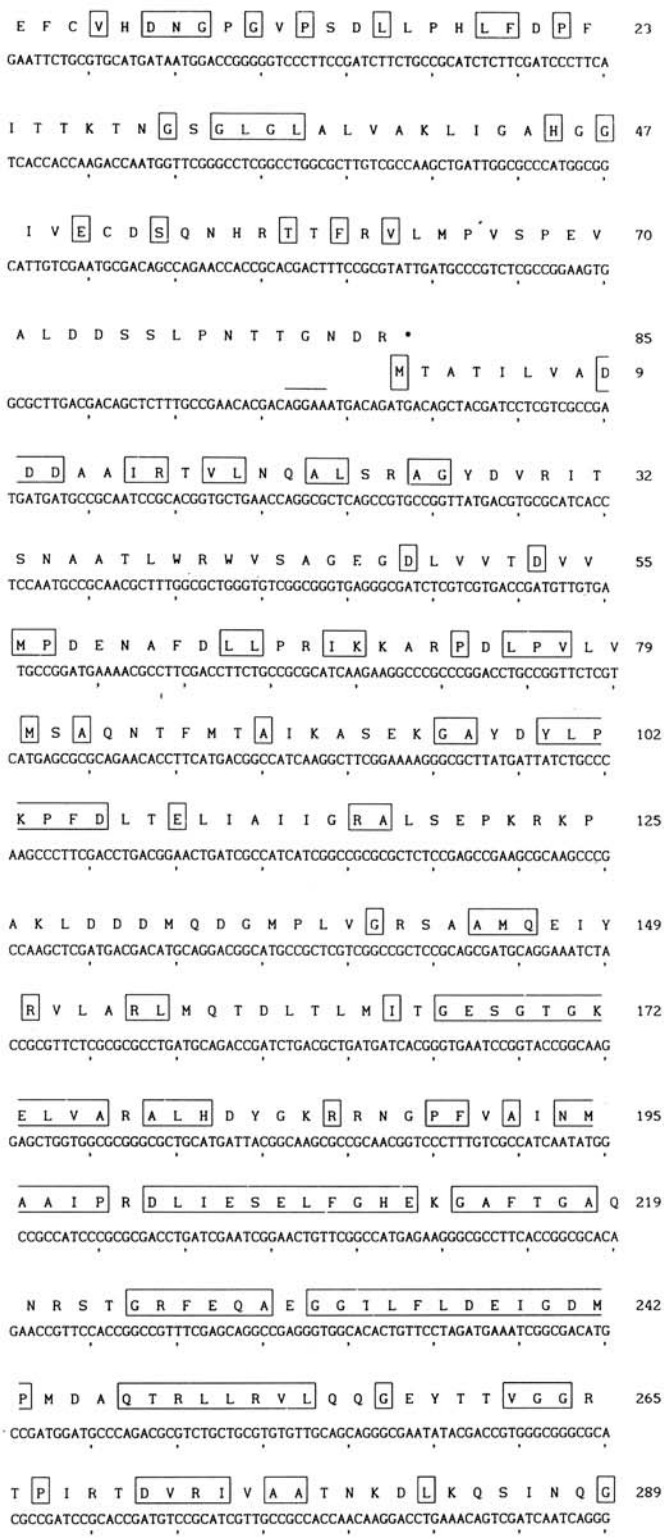

Fig. 3. Nucleotide and derived amino acid sequences of part of the 2.4-kb *Eco*RI-*Cla*I insert of pHW5, showing the complete *ntrC* gene and the 3′ end of the *ntrB* gene. The putative ribosomal binding site for *ntrC* is overscored. Boxes indicate residues conserved among the NTRB and NTRC sequences compared. The proposed C-terminal DNA-binding motif within the NTRC proteins (positions 451–470 in C58 NtrC) shows the hydrophobic residues ■ at positions 454, 458, and 460; Ala (A) at 455; Gly (G) at 459; and Ileu, Leu, or Val (O) at position 465 described as important by Drummond *et al.* (1986).

is located upstream from the *ntrC* gene. When the sequence of the 2.4-kb *Eco*RI-*Cla*I fragment of pHW5 was examined, it also revealed the presence of the C-terminal region of an *ntrB*-like gene located immediately upstream of the *ntrC*. In *A. tumefaciens*, the *ntrB* terminator codon and the *ntrC* initiator codon overlap with no intergenic region. Presumably these genes, like those of other organisms, are cotranscribed from a promoter upstream from *ntrB*. As with other *ntrC* genes, however, the *A. tumefaciens ntrC* may have its own weak promoter. Amino acid sequence comparisons of the partial C58 NTRB sequence of 85 carboxyterminal amino acids with those of *R. meliloti, Bradyrhizobium* sp. (*Parasponia*), *K. pneumoniae, E. coli*, and *S. typhimurium* NtrBs show that 22 of these are identical in all the six NTRB C-terminal sequences compared. The C58 NTRB sequence was also compared with the *Rhodobacter capsulatus nifR2* (*ntrB*-equivalent in this bacterium, Jones and Haselkorn 1989), and significant residues were found to be conserved. The glycine-rich region of C58 NtrB (positions 30–36, Fig. 3) is reported to be conserved in all kinases described until now (Miranda-Ríos *et al.* 1987).

Protein product encoded by the pHW5 insert. Polypeptides encoded by the insert of pHW5 were identified by using the T7 RNA polymerase expression system described by Tabor and Richardson (1985). This system consists of plasmid pGP1-2 (which expresses T7 RNA polymerase in response to a temperature shift) and plasmid pT7-7, containing a T7 RNA polymerase promoter, ϕ 10, and multiple cloning sites. The 2.4-kb *Eco*RI-*Cla*I insert of pHW5 was recloned into pT7-7 (giving pHW6) to allow regulated transcription of the insert DNA in the correct

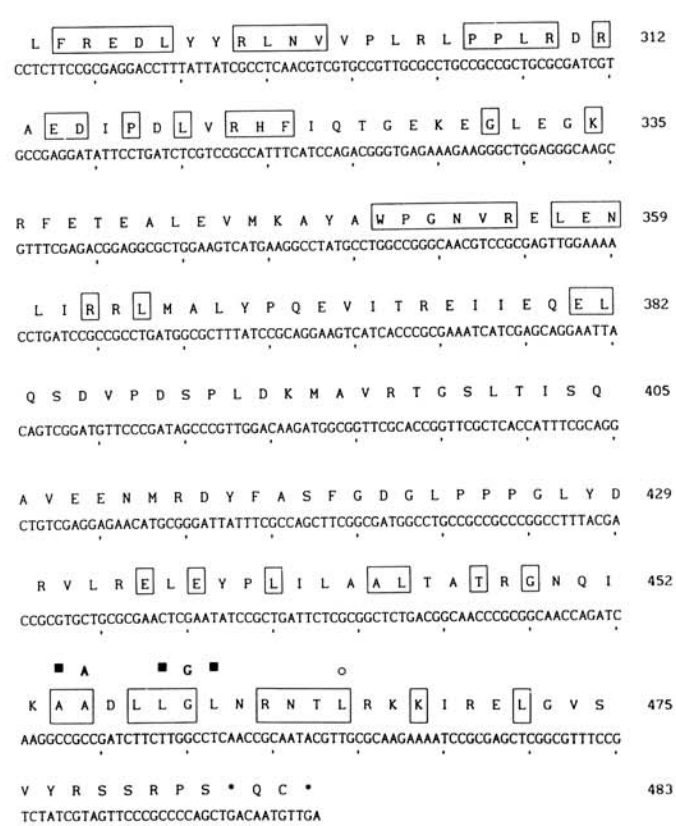

Fig. 3 *continued.*

orientation for *ntrC* expression. Figure 4A shows the polypeptides encoded by pHW6 and the pT7-7 vector. T7 RNA polymerase was induced by a temperature shift to 42° C, and host RNA polymerase was inhibited by treating with rifampicin. Uninduced cultures were treated in parallel but were maintained at 30° C, the temperature at which rifampicin uptake appears to be inhibited. Translation products of the induced and uninduced samples were labeled by including ^{35}S methionine. Cell lysates were then fractionated by SDS-PAGE. Comparison of the pHW6 uninduced and induced samples revealed increased expression of *ntrC* after induction, although low-level expression of the *ntrC* was observed in the uninduced samples and is more clearly seen on the fluorogram. The latter observation can perhaps be explained in one of two ways: the *ntrC* in most microorganisms studied possesses a weak promoter which, therefore, might be responsible for the weak expression in uninduced samples, or there may be low-level expression from the vector promoter. Another possible explanation for the appearance of the NTRC band in the uninduced samples could be the low-level expression of T7 RNA polymerase in these cells. No corresponding bands are seen in either induced or uninduced pT7-7 control samples. Figure 4B is a fluorogram of the gel lanes in Figure 4A and clearly shows significant incorporation of label at the position corresponding to the NTRC protein in the pHW6-induced sample with very little background labeling. The pT7-7 control tracks show no corresponding NTRC protein in either induced or uninduced samples. These results show the specific labeling of the NTRC protein and provide a good indication that over-expression of this protein is possible.

Screening of various members of Rhizobiaceae for an *ntrC*-like gene. Because the gene *ntrC* is of central importance in nitrogen regulation, the *A. tumefaciens ntrC* gene was used as a hybridization probe to screen other members of Rhizobiaceae. The *A. tumefaciens* strains NCPPB223, NCPPB2461, and B6; *A. radiobacter* (Beijerinck & van Delden) Conn NCPPB1649; *A. rubi* (Hildebrand) Starr & Weiss NCPPB1856; *A. rhizogenes* (Riker *et al.*) Conn NCPPB2655; and *Rhizobium leguminosarum* were used for this study. In addition, *Alcaligenes eutrophus* Davis NCIB11842 was also included. Total DNA was digested with *Eco*RI, separated by electrophoresis, transferred to a Biodyne-A nylon

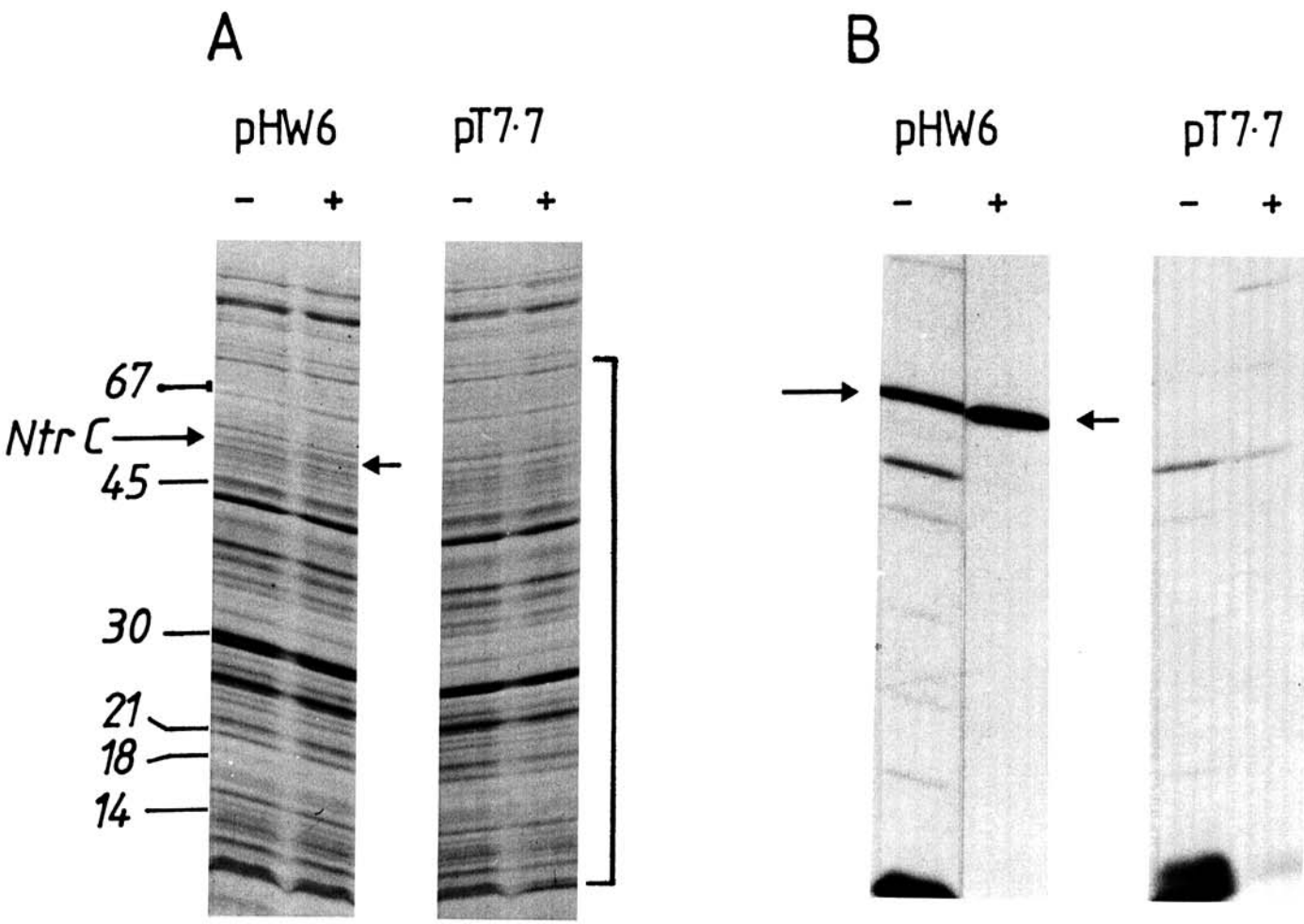

Fig. 4. Expression of the *Agrobacterium tumefaciens ntrC* by T7 RNA polymerase in induced (+) and uninduced (−) samples. **A**, SDS-polyacrylamide gel stained with Coomassie blue. Size markers (kDa) are indicated. The arrows show the position of the NTRC protein, which is clearly visible after induction at 42° C. **B**, Fluorogram of part of the gel (bracketed) shown in **A**. The most prominently labeled band (indicated by arrows) following induction corresponds to the NTRC protein. Poor rifampicin uptake in the uninduced sample resulted in the labeling of a wide range of proteins including NTRC. The control lanes with pT7-7 show no band corresponding to NTRC.

membrane, and probed with the ^{32}P-labeled 1.7-kb *Eco*RI-*Pvu*II fragment of pHW4 to look for homologous sequences. Hybridization was conducted under high-stringency conditions and filters were washed at high stringency (0.1× SSC, 0.1% SDS at 65° C for 3 × 30 min) before autoradiography for 24 hr. As can be seen from Figure 5, despite the strain-specific restriction fragment-length polymorphisms, all the strains tested show a strong signal indicating that an *ntrC*-like gene exists in all these members of Rhizobiaceae and in *A. eutrophus*.

DISCUSSION

Our results clearly show that *A. tumefaciens* C58 possesses a regulatory gene structurally and functionally related to the *ntrC* genes from *R. meliloti, Bradyrhizobium* sp. (*Parasponia*), *K. pneumoniae, E. coli,* and *S. typhimurium* and located downstream from an *ntrB* gene, suggesting the presence of a "two component regulatory system" (Nixon *et al.* 1986) in *A. tumefaciens*.

The region of *ntrB* of C58 sequenced during this work consists of the C-terminal, which is common to several other regulatory proteins (Nixon *et al.* 1986). Nixon *et al.* (1986) also reported that this region is crucial for disseminating information about environmental conditions to the regulatory proteins sharing N-terminal homology to the *ntrC* product.

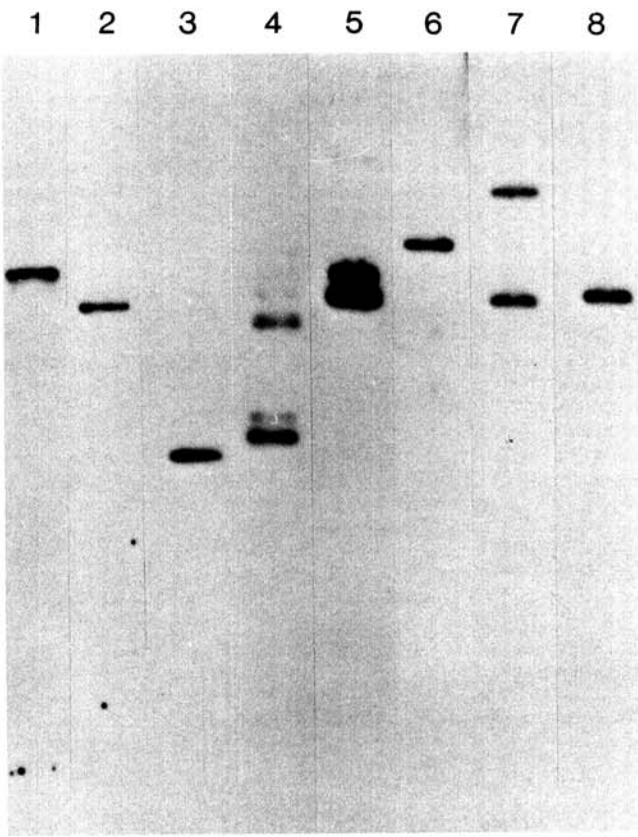

Fig. 5. High-stringency hybridization of the *Agrobacterium tumefaciens* C58 *ntrC* to *Eco*RI-digested total DNA from different members of Rhizobiaceae and to *Alcaligenes eutrophus*. Strains used: **1**, NCPPB1856; **2**, NCPPB223; **3**, NCPPB2461; **4**, B6; **5**, *Rhizobium leguminosarum*; **6**, NCIB11842; **7**, NCPPB1649; and **8**, NCPPB2655 (see Table 1). The strong hybridization signals demonstrate the high degree of evolutionary conservation of the *ntrC* gene.

The *A. tumefaciens, R. meliloti, Bradyrhizobium* sp. (*Parasponia*), and *K. pneumoniae ntrC* gene products share three major regions of homology, supporting the proposal that the *ntrC* product consists of three functional domains (Drummond *et al.* 1986; Nixon *et al.* 1986). The N-terminal region of the C58 *ntrC* product also shows homology to the N-terminal regions of several other regulatory proteins and perhaps functions in receiving the information regarding the nitrogen status of the cell by interacting with *ntrB* product (Drummond *et al.* 1986; MacNeil *et al.* 1982; Nixon *et al.* 1986). The central conserved portion of the C58 *ntrC* product reveals a high degree of homology to *nifA* products from several species (Buikema *et al.* 1985; Nixon *et al.* 1986). As all of these proteins act as transcriptional activators in conjuction with the *ntrA* sigma factor (Szeto *et al.* 1987), it is quite probable that this region is needed for interaction with *ntrA* product. The conserved C-terminal region of the C58 *ntrC* product shows the typical helix-turn-helix motif of the DNA binding proteins and probably represents a DNA binding domain (Drummond *et al.* 1986).

The high degree of homology observed between *ntrC* of *A. tumefaciens* and *R. meliloti* and also several other members of Rhizobiaceae is probably not surprising in view of the conserved nature of the *ntrC*-type genes among species so far examined (Drummond *et al.* 1986). Interestingly, probing the C58 chromosomal DNA with *K. pneumoniae ntrA* and *ntrB* did not reveal any detectable hybridization even under conditions of low stringency (H. Wardhan and G. R. K. Sastry, unpublished). However, we have found the C-terminal region of an *ntrB*-like gene situated directly upstream from the *ntrC* in C58. Amino acid sequence comparison of this C-terminal region with that of *K. pneumoniae* NTRB shows conservation of significant residues. Our demonstration of the ability of C58 *ntrC* to complement the *E. coli ntrC* mutant implies that C58 NtrC must interact with the host NTRA and NTRB proteins and suggests significant conservation between these classes of proteins from C58 and *E. coli*. The work reported here paves the way for further work on the *ntrB* and *ntrC* genes of *A. tumefaciens*. Because of the successful expression of *ntrC* using the T7 RNA polymerase expression system, it should be feasible to overproduce NtrC for protein analysis and mutagenesis studies.

The presence of closely located C58 *ntrB* and *ntrC* (data presented in this paper) and the lack of any linkage between C58 *ntrC* and *glnA* (Rossbach *et al.* 1987) is in contrast to the situation in enterobacteria, where *glnA* and *ntrBC* constitute a single operon. *A. tumefaciens* contains two glutamine synthetases, and recently a locus glnT (which is expressed only under special physiological conditions) has been described by Rossbach *et al.* (1988). C58 *ntrC* has been shown to regulate GSII (Rossbach *et al.* 1987) but not *glnA*, which explains the lack of linkage between *glnA* and *ntrBC* in this bacterium. We have recently isolated a clone from a C58 library that also complements ET8556, but its restriction map differs from that of pHW4. Whether this clone carries an *ntrC*-like gene and whether it regulates *glnA* remains to be seen. It will also be interesting to study the effect of *ntrC* mutations on the nitrogen-fixing ability of *A. tumefaciens*, because several *Agrobacterium* strains including C58 grow well on nitrogen-free medium, reduce

acetylene, and also incorporate ^{15}N when grown under an atmosphere enriched with the isotope (Kanvinde *et al.* 1986; L. Kanvinde and G. R. K. Sastry, unpublished).

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