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The *ntrA* Gene of *Agrobacterium tumefaciens*: Identification, Cloning, and Phenotype of a Site-Directed Mutant

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A 3.6-kb *Eco*RI fragment containing the *ntrA* gene of *Agrobacterium tumefaciens* was cloned by using the homologous *ntrA* gene of *Rhizobium meliloti* as a probe. Construction of an *ntrA* mutant of *A. tumefaciens* by site-directed insertional mutagenesis demonstrated the requirement of the *ntrA* gene for nitrate utilization and C_4-dicarboxylate transport but not for *vir* gene expression or tumorigenesis.

ntrA (*glnF rpoN*) was shown to encode a sigma factor required for transcriptional activation of an NtrC-regulated promoter by binding at NtrA-specific promoter sequences in the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* (8, 9). Promoters activated by NtrA do not contain canonical −35 and −10 sequences but instead have the consensus −26 CTGGYAYR-N4-TTGCA −10 (6). The *ntrA*, *ntrB*, and *ntrC* gene products regulate the transcriptional activation of several operons involved in the assimilation of poor nitrogen sources (14). The C-terminal domain of NtrB and the N-terminal domain of NtrC protein show homology with a variety of two-component bacterial regulatory systems and therefore may share the same mode of signal transduction (17).

Genes homologous to *ntrA* of enteric bacteria were also identified in *Rhizobium meliloti* and *Rhizobium* sp. strain NGR234 (20, 29). The *Rhizobium ntrA* gene product is a coactivator of not only NtrC but also NifA and DctD. NtrC is required for the utilization of nitrate as a nitrogen source and the activation of the *nifH* gene in free-living states in response to nitrogen limitation (25). NifA activates the *nif* genes, including *nifH*, in the symbiotic state (26), whereas DctD is a positive regulator required for activation of *dctA* transcription in the free-living state. The *dctA* gene product is required for transport of the C_4-dicarboxylates succinate, fumarate, and malate. *Rhizobium ntrA* mutants are deficient in the utilization of nitrate as a sole nitrogen source and C_4-dicarboxylates as sole carbon sources (20, 24, 34). An *ntrA* mutation results in delayed root nodule formation, and nitrogen fixation does not occur (29).

It was recently reported that the plant pathogen *Pseudomonas syringae* pv. phaseolicola requires *ntrA* in the interaction with its plant host, because its *hrp* genes, which are necessary for primary symptom development and for the hypersensitive response, are under *ntrA* control. Homologs of *hrp* genes are also present in other strains of *P. syringae* and in other gram-negative plant pathogenic bacteria (3).

Considering the important role of *ntrA* in the interaction of certain bacteria with their host plants, we wished to identify the putative *ntrA* gene of *Agrobacterium tumefaciens* and determine whether it is involved in tumor formation. In this study we report the identification and cloning of *ntrA* of *A. tumefaciens*. The cloned *ntrA* gene was mutated in vitro by an insertion of the kanamycin resistance gene from Tn*5*. Then one marker exchange mutant was selected, and the phenotype of the mutant was studied.

Identification of the *ntrA* gene of *A. tumefaciens*. The strains and plasmids used in this study are described in Table 1. Two approaches were taken to show that *A. tumefaciens* has a functional *ntrA* gene. The wild-type strain A348 grows well on M9 minimal medium supplemented with succinate as the sole carbon source, suggesting that *A. tumefaciens* has a C_4-dicarboxylic acid transport system similar to that of *Rhizobium* spp. (19). Therefore, we first tested the expression of the *Rhizobium dctA* gene in *A. tumefaciens* by using an in-frame *dctA-lacZ* gene fusion in pCR63, a broad-host-range plasmid containing the entire *dct* regulon from *Rhizobium leguminosarum* (20). *A. tumefaciens* A136 and A348 containing pCR63 formed blue colonies on M9 minimal medium supplied with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and with succinate as the sole carbon source. Without pCR63, A136 and A348 formed only white colonies. Since expression of *dctA* in *Rhizobium* spp. requires a functional *ntrA* gene, this result suggests that *A. tumefaciens* also has a functional *ntrA* gene. Second, restriction fragments of strain A348 DNA were analyzed by Southern hybridization with the cloned *ntrA* gene of *Rhizobium meliloti* as a probe. The heterologous *ntrA* gene probe hybridized to a single 3.6-kb *Eco*RI fragment under conditions of high stringency, indicating that *A. tumefaciens* has a gene that is homologous to the *ntrA* gene of *R. meliloti* (data not shown). Standard procedures were used for hybridization at 42°C in a hybridization solution containing 50% formamide and then washing at 68°C (15).

Molecular cloning of the *ntrA* gene of *A. tumefaciens*. General DNA manipulations were performed by standard procedures (15). Total DNA from *A. tumefaciens* A348 was partially digested with *Sau*3A and electrophoresed in an agarose gel. DNA of approximately 20 kb was electroeluted from the gel and ligated into the unique *Bam*HI site of λEMBL4 (4). Ligated molecules were packaged in vitro into lambda phage particles by using Gigapack IIXL packaging extract (Stratagene). The packaged product was used to infect the *E. coli* LE392(P2) lysogen to generate a recombinant lambda bacteriophage bank. A 3.5-kb DNA fragment of pNtr3.5BE (Table 1), which contains the *ntrA* gene of *R. meliloti*, was labeled with [α-^{32}P]dATP by nick translation (the nick translation system was from Bethesda Research Laboratories) and used to probe the recombinant phage bank by in situ plaque hybridization (15). One recombinant phage, which gave a strong hybridization signal under high-stringency conditions, was selected and designated λZL2. A

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TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Description[a]	Source or reference
R. meliloti		
Rm1021	Wild type, Sm^r	16
Rm1681	*ntrA*::Tn*5*, Sm^r, Nm^r	20
RmF250	*ntrA74*::Tn*5*-233	34
A. tumefaciens		
A136	C58 chromosome, cured of pTi	5
A348	A136 with pTiA6NC	5
A9034	A136*ntrA*::*nptII*	This study
A9121	A348*ntrA*::*nptII*	This study
E. coli		
DH5α	Transformation recipient	Bethesda Research Laboratories
LE392(P2)	P2 lysogen of LE392	Stratagene
Bacteriophages		
λEMBL4	Cloning vector	4
λZL2	*ntrA* clone	This study
Plasmids		
pGP159	*virA virG virB-lacZ*, Tc^r IncP	2
pNtr3.5BE	pUC8 with 3.5-kb insert containing *R. meliloti ntrA*	20
pHZ3.5BE	pSP329 with 3.5-kb insert containing *R. meliloti ntrA*, Tc^r IncP	H. Zhan
pCR63	pLAFR1 with *dctA-lacZ dctB dctD* insert	20
pHW21	pUC19mob with 3.6-kb *Eco*RI insert containing *ntrA*	This study
pHW219	pHW21::*nptII*	This study
pRK7813	RK2 derivative (IncP) carrying pUC9 polylinker, Tc^r	10
pTC107	pRK7813 with 3.6-kb *Eco*RI insert containing *ntrA*	This study
pUC19mob	pUC19 with *mob*	18
pIB410	*virB-lacZ*, Tc^r IncP	1

[a] Abbreviations: Sm^r, streptomycin resistance; Nm^r, neomycin resistance; Tc^r, tetracycline resistance.

TABLE 2. Growth properties of wild-type and *ntrA* mutant strains of *A. tumefaciens*[a]

Carbon or nitrogen source	Growth of:	
	A348	A9121
Arabinose (15 mM)	+	+
Glucose (15 mM)	+	+
Aspartate (15 mM)	+	+
Fumarate (7.5 mM)	+	−
Malate (7.5 mM)	+	−
Succinate (15 mM)	+	−
KNO_3 (0.5 mM)	+	−
Glutamine (0.005%)	+	+
Glutamate (15 mM)	+	+
Pyruvate (15 mM)	+	+
Octopine (0.05%)	+	+
Arginine (0.1%)	+	±

[a] Bacterial growth was tested on M9 minimal medium supplemented with different carbon or nitrogen sources as indicated and scored after 2 days of incubation at 28°C. Glucose (15 mM) was the carbon source when different nitrogen sources were provided, and NH_4Cl (18 mM) was the nitrogen source when different carbon sources were provided. Octopine was tested as a source of both carbon and nitrogen. +, With colonies; −, without colonies; ±, colonies, but smaller than those of the wild type.

3.6-kb *Eco*RI fragment from λZL2, which contained all of the hybridized sequences, was subcloned into the unique *Eco*RI sites of pUC19mob and pRK7813 to make the plasmids pHW21 and pTC107, respectively (Table 1). These plasmids were used in subsequent analyses.

To prove that the 3.6-kb *Eco*RI fragment contains a functional *ntrA* gene, plasmid pTC107 was mobilized into the *R. meliloti ntrA* mutant RmF250. Tetracycline-resistant transconjugants were selected and tested for growth on M9 medium with succinate as a sole carbon source. Plasmid pTC107 complemented RmF250 fully for growth on M9 succinate medium.

Construction of an *ntrA* mutant of *A. tumefaciens*. Restriction fragment analysis of pHW21 showed that the 3.6-kb fragment contains a unique *Xho*I site that splits the 3.6-kb *Eco*RI fragment into 2.0- and 1.6-kb fragments. Both of the fragments hybridized to the *ntrA* gene probe of *R. meliloti* in Southern hybridization (data not shown). We conclude that the *Xho*I site is located in the coding region of the *ntrA* gene of *A. tumefaciens*.

A 3.27-kb *Hin*dIII fragment from Tn*5* (11) that contains the kanamycin resistance gene was electroeluted from an agarose gel and made blunt ended with Klenow enzyme and deoxynucleoside triphosphate by standard procedures (15). The fragment was ligated into the *Xho*I site of pHW21, which was made blunt ended as above. The resulting plasmid, pHW219, was introduced into *A. tumefaciens* A136 and A348 by electroporation. Because pHW219 cannot replicate in *A. tumefaciens*, the selected kanamycin-resistant and carbenicillin-sensitive colonies should come from gene replacement events and were presumed to be *ntrA* mutants of *A. tumefaciens*. To confirm this, the mutants were analyzed by Southern hybridization. All selected mutants had a 7.5-kb *Eco*RI fragment that hybridized to the *ntrA* gene probe of *R. meliloti* instead of the 3.6-kb fragment of A136 and A348 (data not shown). Two mutants, A9034 derived from A136 and A9121 derived from A348, were studied further.

Phenotype of the *ntrA* mutant of *A. tumefaciens*. A9121, like the *R. meliloti ntrA* mutants, could not grow on M9 minimal medium plates supplemented with succinate, fumarate, or malate as the sole carbon source or with nitrate as the sole nitrogen source; A9121 exhibited a reduced-growth phenotype with arginine as the sole nitrogen source (Table 2). When pTC107 or pHZ3.5BE was introduced, strain A9121 regained the ability to utilize nitrate and succinate. This result strongly suggests that the *A. tumefaciens* C_4-dicarboxylic acid transport system and the nitrate reductase gene are under *ntrA* control, as in *R. meliloti* (20). One difference is that A9121 still can use aspartate as a sole carbon source, unlike the *R. meliloti ntrA* and *dct* mutants (31, 32).

The nitrogen regulatory gene *ntrC* of *A. tumefaciens* was identified and cloned previously (30). The *ntrC* gene controls glutamine synthetase activity, growth on nitrate, and chromosomal arginine catabolism pathways (21). Our finding that the *ntrA* mutant of *A. tumefaciens* cannot utilize nitrate as a sole nitrogen source shows that expression of the *nas* (nitrate reductase) gene of *A. tumefaciens* is *ntrA* dependent. The intermediate arginine growth phenotype is similar to the previously reported *ntrC* mutant phenotype (21).

To determine whether *ntrA* plays any role in *vir* gene expression, we measured the *vir* gene induction in nitrogen-limiting and nitrogen-rich media by using a *virB-lacZ* fusion. The *vir* gene induction in strains A9121 and A348 did not differ significantly under the conditions tested (Table 3). We also tested *vir* gene induction in the Ti plasmid-cured strains A136 and A9034. In this case we used pGP159, which

TABLE 3. Assay of *vir* gene induction[a]

Nitrogen source	β-Galactosidase activity (U)			
	A136	A9034	A348	A9121
NH_4Cl (18 mM)	3,900	1,340	4,000	4,100
Glutamine (0.005%)	4,100	1,500	3,800	4,200
Glutamine (0.2%)	4,000	1,420	4,000	4,100

[a] A136 and A9034 contained pGP159, and A348 and A9121 contained pIB410. β-Galactosidase activity was assayed as described by Stachel et al. (23). The induction medium used was that of Winans et al. (33) plus the indicated nitrogen source and glutamine concentrations. All cultures were incubated at 28°C for 18 h before induction was measured.

contains native *virA*, *virG*, and the *virB-lacZ* fusion gene. *vir* gene expression in the *ntrA* mutant A9034 was about one-third of that in A136 (Table 3).

Whether this reduction is related to the observation that the *Rhizobium ntrA* mutant expresses the *nodABC-lacZ* fusion gene to only about one-third of the level of the wild-type strain under nitrogen-limited conditions (24) is not clear. One possible explanation is that a factor required for full *vir* gene induction can be provided by both the chromosome and the Ti plasmid. The Ti plasmid-encoded gene is not *ntrA* regulated, whereas the chromosomal-encoded gene is. The loss of the factor caused by *ntrA* mutation can be complemented by the Ti plasmid-determined function. Thus, *vir* gene induction in the Ti plasmid-containing *ntrA* mutant is not reduced.

The virulence of strain A9121 was tested on several plants. Tumor formation was assayed by wounding young leaves (*Bryophyllum diagremontiana*) or stems (*Nicotiana tabacum*, *N. glauca*, *Zinnia elegans*, and sunflower) with a sterile scalpel and then inoculating the appropriate strains with a toothpick. Tumors were scored after 3 to 4 weeks. In all cases, the tumorigenicity of A9121 was not significantly different from that of A348. We also used a series of 10-fold dilutions of A9121 cells (10^9 to 10^3 cells per ml) to infect *N. glauca* leaf disks to examine whether A9121 had decreased virulence. No significant difference between A9121 and A348 was observed. Thus the *ntrA* gene of *A. tumefaciens* is not involved in tumor formation.

The *ntrA* gene product, σ^{54}, has physiologically diverse roles in different bacteria (13). In *Pseudomonas aeruginosa*, a gene encoding flagellin is under σ^{54} control (27). When *A. tumefaciens* infects plants in nature, chemotaxis to the plant may play an important role in the early stages of infection (7, 22). We tested the chemotactic activity of A9121 toward glucose and arabinose by a previously described method (1). We did not see any difference between A9121 and A348. Electron microscopy revealed that both A9121 and A348 have normal flagella, indicating that the *ntrA* mutation does not affect flagellum formation.

Since the cloned *ntrA* gene can complement the C_4-dicarboxylate utilization deficiency of *ntrA* mutants of *R. meliloti*, the functional *ntrA* gene of *A. tumefaciens* must be the same as that of *R. meliloti*. The fact that the *A. tumefaciens ntrA* mutant is unable to utilize succinate, fumarate, and malate but can use other carbon sources is evidence that the *A. tumefaciens ntrA* mutant, like *R. meliloti*, *Azotobacter vinelandii*, and *Pseudomonas putida ntrA* mutants, is deficient in C_4-dicarboxylate transport (12, 20, 28). Thus, the *dctA* gene of *A. tumefaciens* must require σ^{54} for its transcription. It appears that *ntrA* control of C_4-dicarboxylate transport in nonenteric bacteria is a general phenomenon. Although the *A. tumefaciens ntrA* mutant cannot utilize succinate, it grows well in AB defined, MG/L complex, and induction media. It seems that the C_4-dicarboxylate transport system of *A. tumefaciens* is not involved in its pathogenicity, since the *ntrA* mutant has normal virulence on a variety of plants.

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