Microbial Ecology

Endophytic Occupation of Root Nodules and Roots of *Melilotus dentatus* by *Agrobacterium tumefaciens*

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Abstract

Agrobacterium strains have been frequently isolated from the root nodules of different legumes. Various possible mechanisms have been proposed to explain the existence of these bacteria in nodules, but there is no sufficient experimental evidence to support the estimations. In this work, we proved that the *Agrobacterium* strain CCBAU 81181, which was originally isolated from the root nodules of *Onobrychis viciaefolia*, and a symbiotic strain of *Sinorhizobium meliloti* CCBAU 10062 could coinhabit the root nodules of *Melilotus dentatus*. Analyses were performed by using a fluorescence marker, reisolation of bacteria from nodules, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole cellular proteins, and polymerase chain reaction amplification of symbiotic genes. The inoculation of *A. tumefaciens* CCBAU 81181 did not affect the growth and nodulation of plants. CCBAU 81181 and 24 other *Agrobacterium* strains isolated from nodules were incapable of nodulating on their original or alternative host and 22 strains of these strains were endophytes in the roots and stems of their hosts. Also, the tumor-inducing *A. tumefaciens* strains IAM 13129^T and C58 were found capable of entering the roots of *Glycyrrhiza pallidiflora*, but did not cause pathogenic symptoms. With these results, we conclude that *A. tumefaciens* strains could be endophytic bacteria in the roots, stems, and root nodules. This finding partially explains why *Agrobacterium* strains were frequently isolated from the surface-sterilized nodules.

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Introduction

Agrobacterium and fast-growing rhizobia (*Allorhizobium*, *Rhizobium*, and *Sinorhizobium*) are closely related bacteria belonging to the family Rhizobiaceae. Although saprophytic lineages exist in both these bacterial groups, *Agrobacterium* species as phytopathogens cause the formation in various plants of crown gall or hairy roots, whereas rhizobia as symbionts form nitrogen-fixing nodules on legumes. However, *Agrobacterium* strains have been frequently isolated from root nodules of various legumes in previous studies [2, 7, 9, 11, 17, 18, 20–23, 26]. These strains have been identified as *Agrobacterium* based on numerical taxonomy and 16S rDNA-restriction fragment length polymorphism (RFLP) [9, 11, 18, 26]. Most of the *Agrobacterium* strains isolated from nodules failed to nodulate on their original hosts and they did not hybridize to *nif* and *nod* gene probes, verifying that they were not symbiotic bacteria [9, 25]. These results led us to ask: How can the agrobacteria occupy the root nodules if they have no symbiotic genes, particularly no nodulation genes? It has been hypothesized that these *Agrobacterium* strains might rapidly lose their symbiotic genes during the isolation procedure, but there is no experimental evidence to support this hypothesis. Another explanation was that they were never symbiotic, but could invade nodules as proven recently [21].

In the present study, we performed a series of experiments to check the nodulation capacity of the 25 *Agrobacterium* strains isolated from nodules in our previous studies ([9, 25], and our unpublished data) and to determine the location of an agrobacterial strain inside the plant. We also verified the effects of agrobacteria on the growth of plants.

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Materials and Methods

Bacterial Strains and Plasmids. Twenty-five *Agrobacterium* strains (Table 1) isolated from root nodules of various legumes were used in this study. These strains have been identified as *Agrobacterium* in our previous studies ([9, 25], and our unpublished data), mainly based on the numerical taxonomy and polymerase chain reaction (PCR)-based RFLP of 16S rRNA genes. The type strain of *Agrobacterium tumefaciens* IAM 13129^T and tumor-inducing strain C58 were included as references. All of the strains were grown at 28°C on TY medium (tryptone, 5 g; yeast extract, 3 g; $CaCl_2$, 0.7 g; distilled water 1 L) or YMA medium [28] (yeast extract, 3 g; mannitol, 10 g; KH_2PO_4, 0.5 g; $MgSO_4$, 0.2 g; NaCl, 0.1 g; agar, 18 g; distilled water, 1 L; pH 7.0–7.2). The *Escherichia coli* S17-1 [24] strain harboring plasmid pMP2444 [24], which was used as the donor in a conjugation test, was grown at 37°C on Luria–Bertani medium [30] supplied with 40 μg/mL gentamycin. A *gfp*-tagged derivative of *A. tumefaciens* CCBAU 81181 was grown and stored on TY or YMA medium containing 40 μg/mL gentamycin.

Nodulation Tests of the Agrobacterium Strains and Nodule Isolation. The nodulation tests were performed as follows: Seeds were surface-sterilized and germinated on water–agar plates, after which the seedlings were sown in sterilized plastic cups (40 × 200 mm) half-filled with vermiculite moisturized with nitrogen-free plant nutrient solution [28].

Twenty-five strains (Table 1) were inoculated to their original hosts. There were no seeds available for *O. viciaefolia*, the original host of CCBAU 81181, so this strain was inoculated to *Melilotus dentatus* instead. *O. viciaefolia* and *M. dentatus* coexist in the same zone and share some rhizobia. Each strain was inoculated to 12 seedlings (3 seedlings/cup). The seedlings were exposed to the environment. Nodulation was observed after 1 month of growth. Reisolation from the nodules of the inoculated plants was done as described previously [28]. The nodules were separated from the roots and surface sterilized by immerging for 30 s in 95% ethanol and for 3 min in 0.1% of $HgCl_2$, followed by washing six times with sterile water. The surface-sterilized nodules were crashed and streaked in YMA plates. Colonies with different morphologies were picked up after 3 days of incubation at 28°C and further purified by repeatedly streaking in plates.

Identification of the Reisolated Strains. To identify the reisolated strains, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell proteins [26], electrophoretic plasmid

Table 1. *Agrobacterium* strains isolated from root nodules used in this study

Strain	*Origin plant*	*Root and stem colonization*[a]	*Reference*
CCBAU65207	*Crotalaria pudica*	YES	Our unpublished data
CCBAU65083	*Mimosa invisa*	NO	
CCBAU65177	*Mimosa pudica*	YES	
CCBAU65178	*Mimosa pudica*	YES	
CCBAU65185	*Mimosa pudica*	YES	
CCBAU65101	*Crotalaria pudica*	NO	
CCBAU31104	*Crotalaria pollida*	YES	
CCBAU33003	*Trifolium fragiferum*	YES	
CCBAU65175	*Mimosa pudica*	YES	
CCBAU03233	*Astragalus adsurgens*	YES	[9]
CCBAU11149	*Astragalus adsurgens*	YES	
CCBAU03165	*Astragalus adsurgens*	YES	
CCBAU01249	*Astragalus adsurgens*	YES	
CCBAU10041	*Astragalus adsurgens*	YES	
CCBAU71069	*Glycyrrhiza pallidiflora*	YES	[25]
CCBAU 71088	*Glycyrrhiza pallidiflora*	YES	
CCBAU 71098	*Caragada microphlla*	YES	
CCBAU 71097	*Caragada pruinosa*	YES	
CCBAU71270	*Sophora viciifolia*	NO	
CCBAU81022	*Vicia bungei*	YES	Our unpublished data
CCBAU81434	*Vicia angustifolia*	YES	
CCBAU85215	*Vicia sativa*	YES	
CCBAU81492	*Melilotus dentatus*	YES	
CCBAU81325	*Vicia faba*	YES	
CCBAU81181	*Onobrychis viciaefolia*[b]	YES	

[a]YES means the strain could colonize in the root and stem of original host plant as endophyte. NO means the strain could not colonize in the roots and stems of original host plant as endophyte.
[b]Seeds of *Melilotus dentatus* were used because *Onobrychis viciaefolia* seeds were unavailable.

analysis [15], sequencing of 16S rRNA genes amplified by PCR [14] with primers P1 (5′-TGG CTC AGA ACG AAC GCT GGC GGC-3) and P6 (5′-CCC ACT GCT GCC TCC CGT AGG AGT-3′) [26], and DNA–DNA relatedness estimated by the spectrophotometric method [7] were performed. To test the pathogenicity of *Agrobacterium* strains, the *virC* gene was amplified with primers VCF (5′-ATC ATT TGT AGC GAC T-3′) and VCR (5′-AGC TCA AAC CTG CTT C-3′) [13], by using the *A. tumefaciens* strain C58 as positive control. In PCR and DNA hybridization, DNA was extracted by the phenol–chloroform procedure [19] and hybridization was performed in triplicate.

Confirmation of Reisolated Strains' Nodulation Ability and Effect on Host Plants. This experiment was performed by using a method similar to the one mentioned above, except that the seedlings were grown in glass tubes sealed with cotton plugs, which allowed the plants to grow under aseptic conditions. The reisolated *Agrobacterium* strain CCBAU 81181 alone, the reisolated symbiotic strain CCBAU 10062 alone, and a mixture of both were inoculated to *M. dentatus*. Seedlings without bacterial inoculation were included as blank control. The height of shoots, fresh and dry weight of shoots, and the number and fresh weight of nodules were recorded, and a statistical analysis using the Tukey test (the SAS program, version 8) was performed to determine if the *Agrobacterium* strain affected the nodulation or growth of plants.

The *nodA* and *nifH* genes were also amplified using previously reported procedures [12] to confirm the existence of symbiotic genes in the reisolated strains, respectively, by using primers nifH-1 (5′-AAG TGC GTG GAG TCC GGT GG-3′)/nifH-2 (5′-GTT CGG CAA GCA TCT GCT CG-3′) [12] and nodA-1 (5′-TGC RGT GGA ARN TRN NCT GGG AAA-3′)/nodA-2 (5′-GGN CCG TCR TCR AAW GTC ARG TA-3′) [12]. The amplified *nodA* and *nifH* fragments were purified and sequenced as noted for the 16S rRNA genes.

Construction of gfp-marked A. tumefaciens Strain and its Nodulation. The plasmid pMP2444 harboring the green fluorescent protein (*gfp*) gene [24] was transformed into *E. coli* S17-1 by using a previously reported procedure [6]. The transformed *E. coli* S17-1 resistant to gentamycin was used in transconjugation with the reisolated *A. tumefaciens* strain CCBAU 81181, which has been proven to be resistant to ampicillin. The donor *E. coli* S17-1 with pMP2444 and the recipient strain CCBAU 81181 were mixed at a ratio of 1:1 (v/v) and were incubated at 28°C for 36 h on a TY plate; next, conjugants were selected on the TY medium supplied with 40 μg/mL gentamycin and 40 μg/mL ampicillin. Bacterial colonies were exposed to blue light to check the expression of *gfp* [24]. A mixture of *A. tumefaciens* CCBAU 81181 marked with *gfp* and *S. meliloti* CCBAU 10062 (1:1 v/v) was inoculated to the seedlings (about 10^7 cells/seed) of *M. dentatus*. Seedlings without inoculation, or inoculated with *A. tumefaciens* CCBAU 81181 or with *S. meliloti* CCBAU 10062 were included as controls. Plants were grown in direct sunlight in a greenhouse. The nodulation was recorded after 6 weeks, and the existence of *A. tumefaciens* CCBAU 81181 in the nodules was screened by observing the green fluorescence in the nodules under a confocal laser scanning microscope using a scanning wavelength of 488 nm.

Cohabitation of Nodules by Agrobacterium and Symbiotic Bacterium. To confirm the coexistence of symbiotic bacterium and *Agrobacterium* and to see if the *Agrobacterium* strain acquired symbiotic genes by lateral gene transfer, both the *gfp*-labeled *Agrobacterium* strain and the total DNA were isolated from the green fluorescent nodules induced by the mixture of *gfp*-tagged CCBAU 81181 and CCBAU 10062. The recovered *gfp*-labeled *Agrobacterium* colonies and the DNA were used as templates to amplify the *nodA* gene by PCR as described above.

Isolation and Counting of Endophytic Agrobacterium in Roots and Stems. The roots of 1-month-old *M. dentatus* seedlings inoculated with CCBAU 81181 were cut off, weighed, and surface-sterilized for nodule isolation according to previously reported procedures [28]. Next, 1 g fresh roots was ground in 9 mL sterile water. The root extracts were diluted and spread on the YMA plates. After incubation at 28°C for 72 h, the colonies were counted and the colony forming units per gram of fresh roots was calculated. To clarify whether endophytic occupation is a universal phenomenon for *A. tumfaciens* strains, the other 24 strains listed in Table 1 were inoculated to their original hosts. Two tumor-inducing *A. tumefaciens* strains, IAM 13129^T and C58, were inoculated on *Astragalus adsurgens*, *Glycyrrhiza pallidiflora*, *M. dentatus*, and *Trifolium fragiferum*. The strain CCBAU 81181 was also inoculated to *M. dentatus*, *A. adsurgens*, and *Mimosa pudica*. The inoculated bacteria were reisolated from the roots and stems of seedlings after 1 month of growth.

Results

Nodulation of Agrobacterium Strains. Most legume plants inoculated with *Agrobacterium* strains (Table 1) did not form nodules, except for the *M. dentatus* seedlings inoculated with CCBAU 81181, a strain originally isolated from *Onobrychis viciaefolia*. In the case of *M. dentatus* seedlings inoculated with CCBAU 81181, nodules were observed and reisolated. Two

different colony types were obtained from some nodules of *M. dentatus* inoculated with CCBAU 81181. One was similar to CCBAU 81181, and was named CCBAU 81181R. It had colonies about 3 mm in diameter after a 72-h incubation period. The other colony was about 1.5 mm in diameter and was designated as CCBAU 10062.

Identification of the Reisolated Strains. The results of SDS–PAGE of whole-cell proteins (Fig. 1A) and electrophoretic analysis of plasmid (Fig. 1B) showed that the strain CCBAU 81181R was identical to the original inoculum CCBAU 81181, but different from CCBAU 10062. CCBAU 10062 was identified as *S. meliloti* because it had 81.5% of DNA–DNA relatedness and the 16S rRNA gene sequence (Fig. 2) was almost identical to *Sinorhizobium meliloti* USDA 1002^T. The strain CCBAU 81181 was recognized as *A. tumefaciens* because it had 73.2% of DNA–DNA relatedness and 16S rRNA gene sequences were identical to *A. tumefaciens* IAM 13129^T. In the PCR amplification of the *virC* gene, no product was obtained from *Agrobacterium* strains CCBAU 81181 and CCBAU 81181R (data not shown), whereas the positive control *A. tumefaciens* C58 had a corresponding band (700 bp).

Nodulation Ability of the Reisolated Strains. In the nodulation tests under aseptic conditions, no nodule was formed in the *M. dentatus* seedlings inoculated with *A. tumefaciens* CCBAU 81181. Nodules were found in the seedlings inoculated with *S. meliloti* CCBAU 10062, or with a mixture of CCBAU 10062 and CCBAU 81181 (Table 2). To confirm the existence of *A. tumefaciens* CCBAU 81181 in the nodules, 19 nodules randomly selected from four seedlings of *M. dentatus* inoculated with a mixture of CCBAU 10062 and CCBAU 81181 were isolated, and the isolates were identified by SDS–PAGE of whole-cell proteins. The results indicated that six out of 19 nodules contained both CCBAU 10062 and CCBAU 81181, whereas the rest only contained CCBAU 10062.

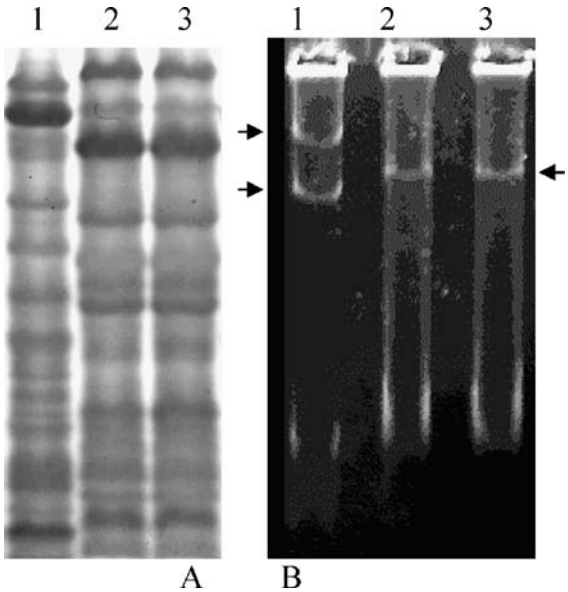

Figure 1. Identification of the nodule isolates from an *Agrobacterium*-inoculated seedling of *Melilotus dentatus*. (A) SDS-PAGE of whole-cell proteins: *lane 1*, reisolated strain CCBAU 10062; *lane 2*, reisolated strain CCBAU 81181R; *lane 3*, *Aagrobacterium tumefaciens* CCBAU 81181. (B) Electrophoretic plasmid patterns: *lane 1*, reisolated strain CCBAU 10062; *lane 2*, reisolated strain CCBAU 81181R; *lane 3*, *A. tumefaciens* CCBAU 81181.

In the PCR amplification of *nodA* and *nifH* genes, no products were obtained from *A. tumefaciens* CCBAU 81181 and CCBAU 81181R (data not shown). Because these strains also failed to nodulate on the host, we concluded that CCBAU 81181 was a nonsymbiotic bacterium. The expected *nodA* (650 bp) and *nifH* (550 bp) bands were observed from *S. meliloti* CCBAU 10062. The *nodA* sequence similarity was 99% between CCBAU 10062 (GenBank accession number DQ023314) and *S. meliloti*. The *nifH* sequence similarity was 98% between CCBAU 10062 (DQ019317) and *S. meliloti*. These results further confirmed that CCBAU 10062 was a symbiotic strain related to *S. meliloti*.

Effect of A. tumefaciens CCBAU 81181 on Plants. As shown in Table 2, there were no significant differences between CCBAU 81181-inoculated and noninoculated seedlings of *M. dentatus* on the growth of plants, indicating that the *A. tumefaciens* CCBAU 81181 did not cause apparent damage to the plants. Significant differences were detected in shoot height and shoot fresh and dry weight between the CCBAU 10062-inoculated seedlings and the blank controls or between the seedlings inoculated with a mixture of CCBAU 10062 and CCBAU 81181 and blank controls. The data in Table 2 showed that *A. tumefaciens* CCBAU 81181 increased the fresh weight of the nodules, but no significant difference was detected between the plants inoculated with CCBAU 10062 alone and with the mixture of CCBAU 10062 and CCBAU 81181 in shoot length, shoot fresh weight, and nodule numbers. These results indicated that *A. tumefaciens* CCBAU 81181 did not affect the nodulation and growth of plants.

Direct Evidence of A. tumefaciens in Nodules. From the nodules of *M. dentatus* plants inoculated with a mixture of *gfp*-labeled CCBAU 81181 and *S. meliloti* CCBAU 10062, we observed fluorescence corresponding to CCBAU 81181 in the center of some nodules (Fig. 3)—a direct evidence that the nonsymbiotic *Agrobacterium* strain could occupy the nodule tissues.

Cohabitation of A. tumefaciens and S. meliloti in the Same Nodules. In this study, the *nodA* gene was amplified from all four green fluorescent nodules (data

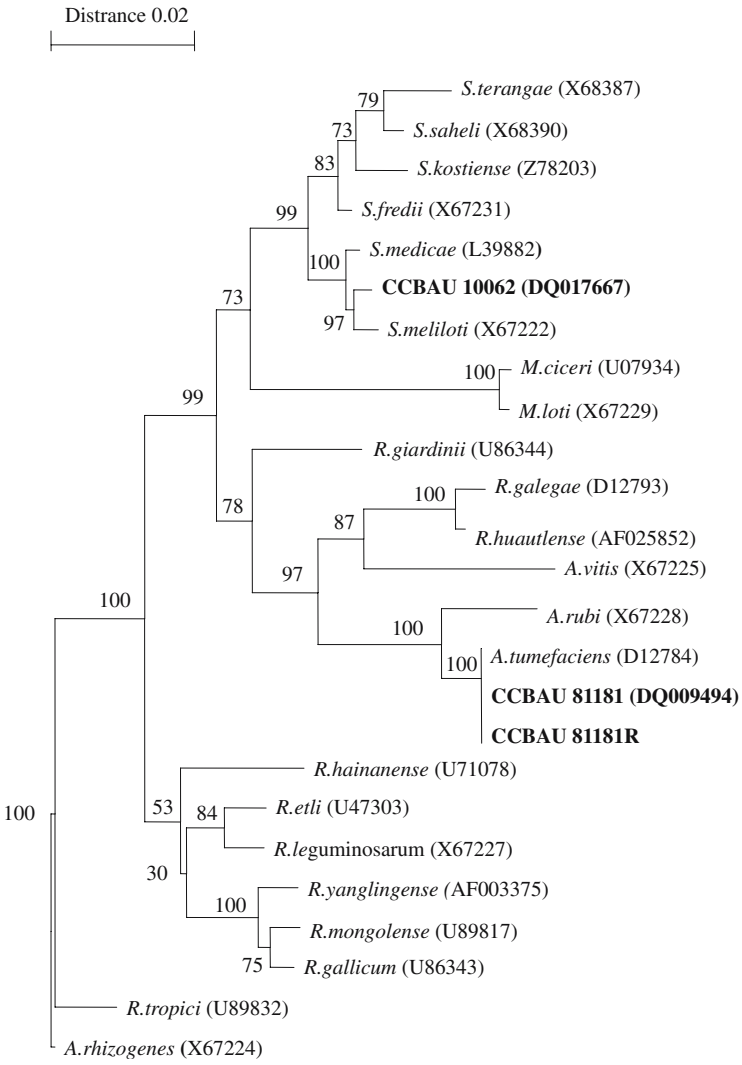

Figure 2. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships among CCBAU 81181, CCBAU 81181R, CCBAU 10062, and other species within the genera *Agrobacterium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*. Sequences were aligned by using the program in the package of Clustal X (Thompson *et al.*, 1994). The neighbor-joining tree was reconstructed and bootstrapped with 1000 replications of each sequence. The scale bar represents 0.02% substitutions of nucleotide.

not shown), but not from the reisolated *gfp*-labeled *Agrobacterium* colonies. These results supplied indisputable evidence that the *Agrobacterium* and *Sinorhizobium* strains coexisted in the nodules, consistent with the fact that both the symbiotic and nonsymbiotic strains were isolated from a single nodule. Results of electrophoretic analysis of plasmid (Fig. 1B) proved the *Agrobacterium* strain CCBAU 81181 did not acquire the symbiotic genes inside the nodules. This observation supported the theory of Mhamdi *et al.* [21] that nodule colonization by *A. tumefaciens* did not result from symbiotic gene transfer.

Endophytic Agrobacterium in Roots and Stems. Although inoculation of CCBAU 81181 did not induce the formation of nodules on *M. dentatus*, it was reisolated as

Table 2. Effects of *Agrobacterium tumefaciens* CCABU 81181 on growth and nodulation of plants

Strains	*Shoot height (cm)*	*Shoot fresh weight (g/plant)*	*Shoot dry weight (g/plant)*	*Nodule number (per plant)*	*Nodule fresh weight (g/plant)*
Blank control	15.8 ± 1.3a*	0.123 ± 0.016a	0.031 ± 0.005a	0	0
CCBAU81181	18.1 ± 1.9a	0.202 ± 0.073a	0.051 ± 0.022a	0	0
CCBAU10062	33.7 ± 3.1b*	0.881 ± 0.200b	0.183 ± 0.030b	35.5 ± 14.059a	0.017 ± 0.006a
CCBAU81181 + CCBAU10062	33.7 ± 1.8b	0.974 ± 0.205b	0.201 ± 0.036b	22.5 ± 5.196a	0.028 ± 0.007b
$Pr > F$ ($\alpha = 0.05$)	<0.0001	<0.0001	<0.0001	0.1335	0.0474

The letters a and b indicate different Tukey grouping.

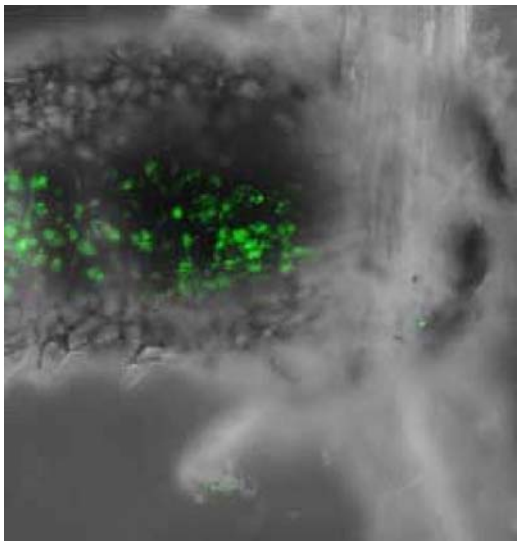

Figure 3. Pictures showing the existence of *Agrobacterium* strain (green fluorescence) inside the nodules. The nodules from seedling of *M. dentatus* inoculated with a mixture of GFP-labeled *A. tumefaciens* CCBAU 81181 and *S. meliloti* CCBAU 10062 were observed under confocus laser scanning microscope.

endophytes from the surface-sterilized roots and stems of the inoculated seedlings at 3.0×10^6 CFU/g fresh roots and 2.0×10^5 CFU/g fresh shoots. This result indicated that *A. tumefaciens* CCBAU 81181 occupied not only the nodules, but also the roots and stems. The endophytic occupation was also confirmed in 21 other *Agrobacterium* strains isolated from the nodules (Table 1), indicating that agrobacterial association with root nodules was commonplace in legumes. The tumor-inducing strains IAM 13129^T and C58 were also reisolated from the roots and stems of *G. pallidiflora*, although they did not invade the other three legumes, *A. adsurgens*, *M. dentatus*, and *T. fragiferum*. The invasion of these two tumor-inducing strains did not cause crown gall or other symptoms on the seedlings of *G. pallidiflora*. CCBAU 81181 was reisolated from the roots and stems of inoculated seedlings of *M. dentatus*, *A. adsurgens*, and *M. pudica*, demonstrating that the endophytic colonization of *A. tumefaciens* CCBAU 81181 was unspecific.

Discussion

Recently, Bala and Giller [3] and Chen *et al.* [5] reported that some *Agrobacterium*-like strains could form effective nodules on some legume plants, including *Leucaena leucocephala* and soybean plants. An earlier work [16] also demonstrated that *A. tumefaciens* strains could induce ineffective nodules in lucerne plants. In contrast, many *Agrobacterium* strains isolated from root nodules failed to nodulate on their original hosts [2, 7, 9, 11, 17, 18, 20–23, 25, 29]. The mechanism by which these nonsymbiotic strains enter the nodules remains unclear [20], although the coexistence of *Agrobacterium* strains and symbiotic bacteria in nodules has been proven [21].

In the present study, nodules were found on the seedlings of *M. dentatus* inoculated with *Agrobacterium* strain CCBAU 81181. However, the nodule isolation results showed that the nodules were infected not only by the inoculated CCBAU 81181, but also by an additional bacterium, CCBAU 10062. The SDS–PAGE of proteins (Fig. 1A), analysis of plasmid patterns (Fig. 1B), sequencing of 16S rRNA (Fig. 2), and DNA–DNA hybridization demonstrated that CCBAU 81181R was identical to the original inoculum, and CCBAU 81181 was related to *A. tumefaciens* strain and had no tumor-inducing genes or symbiotic genes before and after the reisolation from nodules. The other strain, CCBAU 10062, was identified as a symbiotic bacterium belonging to *S. meliloti* based on the analyses noted above, in addition to sequencing of *nodA* and *nifH* genes. *S. meliloti* CCBAU 10062 might be an airborne contaminant because the seedlings were exposed to the environment in this nodulation experiment and alfalfa nodulating bacteria were also employed in the same laboratory.

In agreement with several previous reports [8, 20, 21, 23], the 25 *Agrobacterium* strains used in this study could not nodulate on their original hosts, although they were isolated from root nodules [9, 11]. However, both reisolation and *gfp* gene labeling showed that the non-symbiotic *A. tumefaciens* CCBAU 81181 could occupy the nodules formed by symbiotic bacteria, as reported previously [21]. We concluded that the *Agrobacterium* cells and the symbiotic *S. meliloti* strain CCBAU 10062 coexisted inside the nodules, because both strains were isolated from a single nodule and the symbiotic genes of the *S. meliloti* strain (*nodA* and *nifH*) were amplified from the nodules occupied by the *Agrobacterium* strain.

Statistical analysis of the nodule numbers and plant growth (Fig. 2) demonstrated that the occupation of *Agrobacterium* in the nodules did not affect the growth of plants, indicating that the *Agrobacterium* strains live inside the nodules as endophytic bacteria according to the definition of Hallmann *et al.* [10]. The amount of CFU, at 3×10^6/g roots, also fits the range of endophytic bacteria. The finding of CCBAU 81181 as endophytic bacterium in the roots of *M. dentatus* indicated that the existence of symbiotic bacteria was not necessary for the invasion of plants by *A. tumefaciens* CCBAU 81181, and that the *Agrobacterium* strain might invade the plant roots or nodules by cracks, just like the *Bradyrhizobium* strain BTA-1 [27] or other endophytic bacteria [1, 4]. Although the quantity of this strain in roots reached as high as 3×10^6 CFU/g fresh tissue, CCBAU 81181 only occupied six of 19 nodules, indicating that the nodule

infection and the root infection might be independent cases. It is also note worthy that the nodule occupation frequency (6/19) was much higher in the laboratory than in the field. This might be attributable to competition with the indigenous bacteria in the field.

In this study, we demonstrated that endophytic habitation was common for *A. tumefaciens* strains isolated from nodules and for phytopathogenic strains such as IAM 13129^T and C58. The endophytic occupation of phytopathogenic *Agrobacterium* strains meant that these bacteria may have some secondary host in their life cycle. The cross-infection of strain CCBAU 81181 with *M. dentatus*, *A. adsurgens*, and *M. pudica* showed that the endophytic occupation of *Agrobacterium* strains was not specific, and this may explain why nonsymbiotic *Agrobacterium* strains have been isolated from nodules of many different legume plants.

In conclusion, our results proved that the root nodule isolate *A. tumefaciens* CCBAU 81181 was not a symbiont or phytopathogen, but was an endophytic bacterium in the nodules, stems, and roots. The endophytic occupation of roots and stems was a common feature for the *Agrobacterium* strains, including some tumor-inducing strains. Also, the nodule occupation by *A. tumefaciens* strains may have no specificity. Our findings could partially explain why some *Agrobacterium* strains have been isolated from nodules. The coexistence of *Agrobacterium* and symbiotic rhizobia is an important ecological event because the coexisting bacteria may have more opportunities to exchange their genomic information, as revealed in other reports [3, 5]. Our results also demonstrated that the pathogenic strains may have secondary hosts in their life cycle.

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