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ORIGINAL ARTICLE

# Characterization of root-nodule bacteria isolated from the medicinal legume *Indigofera tinctoria*

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**Abstract** Fourteen root-nodule bacteria isolated from the medicinal legume *Indigofera tinctoria* were characterized for their phenotypic features including growth curves, utilization of carbon and nitrogen sources, antibiotic resistance, vitamin requirement and growth under different conditions. The partial sequences of the 16S rDNA of these strains were obtained and BLASTN analysis revealed that the microsymbionts of *I. tinctoria* were related to members of five distinct genera: *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Cupriavidus* and *Pseudoalteromonas*. The partial *nif*H gene of *Pseudoalteromonas*-like strain DASA 57075 had 96% similarity with *nif*H genes of members of *Bradyrhizobium*. The partial *nod*C gene of *Pseudoalteromonas*-like strain DASA 57075 showed 88% similarity with the *nod*C gene of several rhizobia including *Sinorhizobium*, *Bradyrhizobium* and *Mesorhizobium*. We propose a bacterium that is related to *Pseudoalteromonas* from the gamma-class of Proteobacteria as a new legume symbiont. This is also the first report that the same species of legume can be nodulated by bacteria from up to five different genera in three distinct classes.

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**Keywords** *nif*H gene · *nod*C gene · *Indigofera tinctoria* · *Pseudoalteromonas*-like strain · Root-nodule bacteria · 16S rDNA

## Introduction

*Indigofera tinctoria* (true indigo) is a leguminous plant that is widely popular in many applications of traditional medicine, especially in Thailand, China, India and West Africa. In Thailand this medicinal legume is applied to relieve fever, cold, headache, diuretic fatigue, constipation, wounds and symptoms of venomous snake bite. Recently, the biological activities and medicinal properties of *I. tinctoria* have been studied scientifically. Interestingly, a significant number of compounds from *I. tinctoria* demonstrate various biological activities and pharmacological actions. Phytochemical compounds from *I. tinctoria* have been found to possess antioxidant activity (Sreepriya et al. 2001; Bakasso et al. 2008); antidyslipidemic activity (Narender et al. 2006; Puri et al. 2007); hepatoprotective effects (Anand et al. 1981; Singh et al. 2001, 2006); protective effects against endotoxin (Sreepriya et al. 2001) and liver injury (Anand et al. 1981); anticancer effects against chronic myelocytic leukemia (Han 1994); and inhibitory effects against cyclops, the carriers of dracunculiasis (Kamal and Mangla 1987). In addition to its medicinal properties, *I. tinctoria* has been used since ancient times as one of the original sources of indigo dye for textiles. The plant is also used widely as a green manure to improve soil fertility.

Like other legumes, *I. tinctoria* can be nodulated by a specific group of bacteria. Generally, root- and stem-nodule bacteria symbiotically nodulate and fix nitrogen in association with certain leguminous plants, providing the nitrogen

source for the plant and reducing the need for artificial fertilizers, which can be expensive and cause environmental problems (Pongsilp and Boonkerd 2007). Although the active substances of *I. tinctoria* have been studied widely and its medicinal products are hugely popular, so far the microsymbionts of this plant have not been studied. Hence, in this study, we examined the phenotypic characteristics of 14 strains isolated from root nodules of *I. tinctoria* grown in Thailand, and obtained partial nucleotide sequences of 16S rDNA, and *nif*H and *nod*C genes.

## Materials and methods

### Bacteria and culture conditions

The 14 bacterial strains used in this study are listed in Table 1. Bacteria were isolated from root nodules of *I. tinctoria* grown in Thailand. Nodulation tests with their original hosts were carried out, and the strains were profiled using random amplified polymorphic DNA (RAPD) analysis. Strains belonging to different clusters of the dendrograms constructed by RAPD profiles were selected for further analysis (Pongsilp and Nuntagij 2009). Yeast-Mannitol (YM) medium (Keele et al. 1969) was used for growth and maintenance.

### Sequence analysis of partial 16S rDNA and phylogenetic analysis

Partial 16S rDNA of each strain was amplified using universal primers UN16S 926f (5′-AAACTYAAAKGAATTGACG G-3′) and UN16S 1392r (5′-ACGGGCGGTGTGTRC-3′; Lane 1991). PCR reactions were carried out and the products purified as described by Pongsilp et al. (2002) except that the purified PCR products were sequenced by Bio Basic (Markham, ON, Canada). The nucleotide sequences were aligned using BLASTN program (http://www.ncbi.nlm.nih.gov/). Reference 16S rDNA sequences were selected and downloaded from GenBank (http://www.ncbi.nlm.nih.gov/). Phylogenetic trees for the DNA sequences were constructed using the Neighbor-Joining method (Saitou and Nei 1987). Sequences were taken together in the calculations of levels of sequence similarity using CLUSTALW 1.74 (Higgins et al. 1992). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. Phylogenetic trees were conducted in the MEGA4 suite of program (Tamura et al. 2007).

### Phenotypic features

The bacterial strains were characterized by examining the following features: (1) growth curve; (2) acid and alkaline production; (3) colony morphology; (4) utilization of 52 compounds (each at a concentration of 0.1% w/v) as sole carbon source; (5) utilization of 30 compounds (each at a concentration of 0.1% w/v) as sole nitrogen source; (6) tolerance to ten antibiotics; (7) requirement for ten vitamins; (8) growth at pH 5.0, 6.5 and 8.0; (9) growth in YM broth supplemented with NaCl at concentrations of 0 M, 0.2 M and 1.0 M; and (10) growth at 20, 30 and 40°C.

Growth curves as well as growth at different pH, concentrations of NaCl and temperature were determined. Each strain was grown at 30°C at 200 rpm for 3–5 days and

**Table 1** Accession numbers and related genera of root-nodule symbionts of *Indigofera tinctoria*

| Strain | Accession number | 16S rDNA gene sequence identity | Related genus |
|---|---|---|---|
| DASA 57010 | GQ241303 | 87% | *Rhizobium* |
| DASA 57027 | GQ241304 | 99% | *Rhizobium* |
| DASA 57053 | GQ241305 | 97% | *Rhizobium* |
| DASA 57065 | GQ241306 | 88% | *Rhizobium* |
| DASA 57076 | GQ241307 | 98% | *Rhizobium* |
| DASA 57015 | GQ241308 | 96% | *Sinorhizobium* |
| DASA 57019 | GQ241309 | 96% | *Bradyrhizobium* |
| DASA 57009 | GQ241300 | 84% | *Cupriavidus* |
| DASA 57020 | GQ241301 | 83% | *Cupriavidus* |
| DASA 57038 | GQ241302 | 84% | *Cupriavidus* |
| DASA 57003 | GQ241297 | 84% | *Pseudoalteromonas* |
| DASA 57004 | GQ241296 | 91% | *Pseudoalteromonas* |
| DASA 57066 | GQ241298 | 84% | *Pseudoalteromonas* |
| DASA 57075 | GQ241299 | 95% | *Pseudoalteromonas* |

used as inoculum. The total cell counts of inoculum were examined by the standard plate count method and inoculated into YM broth adjusted to the desired pH or concentration of NaCl. The initial cell density of each strain was $1.00\times10^7$ colony forming units (CFU)/ml. To examine growth at different pH, cells were cultured at 28°C in YM broth containing NaCl 0.1 g/l (0 M) and the pH of medium was controlled by adding HEPES buffer, the pH of which was adjusted to achieve a final concentration of 0.1 M in the medium at the desired pH. To examine growth at different concentrations of NaCl, cells were cultured at 28°C and the pH of medium was controlled at 7.0 using HEPES buffer. To examine growth at different temperatures, cells were cultured in YM broth containing NaCl 0.1 g/l (0 M) and the pH of medium was controlled at 7.0 using HEPES buffer. The cultures were shaken at 200 rpm for 8 days. The cell numbers were measured by the standard plate count method. Acid and alkaline production was examined on YM agar plates containing 25 µg/ml bromthymol blue as a pH indicator. Utilization of compounds as sole carbon and nitrogen sources was performed on basal medium (Sneath 1986) without yeast extract, and agar was replaced with agarose by the method described by Ji and Wilson (2002). To test antibiotic resistance, bacterial cultures were spread on YM agar plates and antibiotic discs (Oxoid, Basingstoke, UK) were placed on the agar surface. Antibiotic susceptibility was observed as clear zones around antibiotic discs. Requirement for vitamins was assessed as described by Watson et al. (2001) on Bergersen's synthetic medium (BSM; Bergersen 1961) in which thiamine and biotin were replaced with various vitamins, and agar was replaced with agarose.

### Sequence analysis of partial *nif*H and *nod*C

The *nifH* and *nodC* genes of selected strains were analyzed. Approximately 360 bp of the *nif*H gene was amplified using the primer pair Zehr-nifHf (5′-TG YGAYC CNAARGCNGA-3′) and Zehr-nifHr (5′-ADNGCCATC ATYTCNCC-3′; Zehr and McReynolds 1989). PCR reactions were carried out as described by Chowdhury et al. (2009). Approximately 930 bp of the *nod*C gene was amplified using the primer pair *nodC*F (5′-AYGTHGT YGAYGACGGTTC-3′) and *nodC*I (5′-CGYGACAG CCANTCKCTATTG-3′; Laguerre et al. 2001). PCR reactions were carried out as described by Laguerre et al. (2001). Negative controls (no DNA added) were included in all sets of reactions. PCR products were separated using 1% agarose gel in TBE buffer and purified using a QIA Quick Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR products were sequenced by Bio Basic. The nucleotide sequences were aligned using BLASTN (http://www.ncbi.nlm.nih.gov/).

## Results and discussion

### Sequence analysis of partial 16S rDNA and phylogenetic analysis

All strains could nodulate *I. tinctoria* and belonged to different clusters of the dendrograms constructed from RAPD profiles (Pongsilp and Nuntagij 2009). Partial sequences (approximately 500 bp) of the 16S rDNA were obtained. Analysis of the sequences by BLASTN revealed that the microsymbionts of *I. tinctoria* were related to members of five distinct genera: *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Cupriavidus* and *Pseudoalteromonas*. Among 14 strains, 5 strains—DASA 57010, DASA 57027, DASA 57053, DASA 57065 and DASA 57076—were related to *Rhizobium* with identities ranging from 87% to 99%. The strains DASA 57015 and DASA 57019 were closely related to *Sinorhizobium* (96% identity) and *Bradyrhizobium* (96% identity), respectively. Three strains—DASA 57009, DASA 57020 and DASA 57038—were placed in the *Cupriavidus-Ralstonia* group with 83–84% identity. Four strains—DASA 57003, DASA 57004, DASA 57066 and DASA 57075—showed homology (84–95%) with species of *Pseudoalteromonas*, a genus of marine bacterium including *P. rubra* and *P. viridis*. The partial 16S rRNA gene sequences of root-nodule bacteria isolated from *I. tinctoria* have been deposited with GenBank. Their accession numbers and the closest genera are listed in Table 1.

The results reported here support the hypothesis that root-nodule bacteria associated with leguminous plants in tropical and sub-tropical areas are very diverse. Other similar examples can be found in the literature since the same host plants could be nodulated by bacteria in different genera. Previous studies found that both genera, *Rhizobium* and *Bradyrhizobium*, could nodulate the same host plants (Keyser et al. 1982; Haukka et al. 1998; Fuentes et al. 2002). Similarly, *Acacia* trees could be nodulated by bacteria from three different genera: *Rhizobium*, *Bradyrhizobium* and *Orchrobactrum* (Ngom et al. 2004). The presence of bacteria belonging to different genera in the same host nodule might be a result of genetic diversification and adaptation of the bacteria to their environment (Fuentes et al. 2002). Furthermore, new genera of root nodule symbionts have been proposed recently. Pongsilp and Boonkerd (2007) reviewed 13 genera of root- and stem-nodule bacteria comprising 10 genera of alpha-proteobacteria (*Rhizobium*, *Sinorhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Phyllobacterium*, *Azorhizobium*, *Devosia*, *Methylobacterium* and *Ochrobactrum*), and 3 genera of beta-proteobacteria [*Cupriavidus* (formerly *Ralstonia*), *Burkholderia* and *Herbaspirillum*]. An unidentified microsymbiont that is closely related to three genera (*Chelatococcus*, *Bosea* and *Balneomonas*), of alpha-proteobacteria has been

proposed recently (Andam and Parker 2007). Therefore, it is possible to discover more genera and species in distant taxa from additional hosts and locations. In this study, bacteria related to five generic groups were found to nodulate the same host plant. *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium* have been reported as predominant symbionts that nodulate and fix nitrogen symbiotically with most legume species. *Rhizobium indigoferae* has been proposed as a novel symbiont of other species of *Indigofera* including *I. amblyantha*, *I. carlseii* and *I. pataninii* (Wei et al. 2002). The partial 16S rDNA sequences of *Rhizobium* nodulating *I. tinctoria* are 80–92% similar to that of *R. indigoferae* type strain CCBAU71042 (accession number AF364068). Besides those three genera, bacteria in the *Cupriavidus-Ralstonia* group have been reported to induce nodules with *Dalbergia* (Rasolomampianina et al. 2005) and *Mimosa* (Chen et al. 2001). Interestingly, a bacterium related to *Pseudoalteromonas* from the gamma-class of Proteobacteria was reported here as a legume symbiont.

A phylogenetic tree based on the 16S rDNA sequences was constructed (Fig. 1). The strain DASA 57019 was closely related to *Bradyrhizobium elkanii* under a bootstrap supported value of 85%. The strain DASA 57015 was placed in the *Sinorhizobium* clade. *Sinorhizobium terangae*

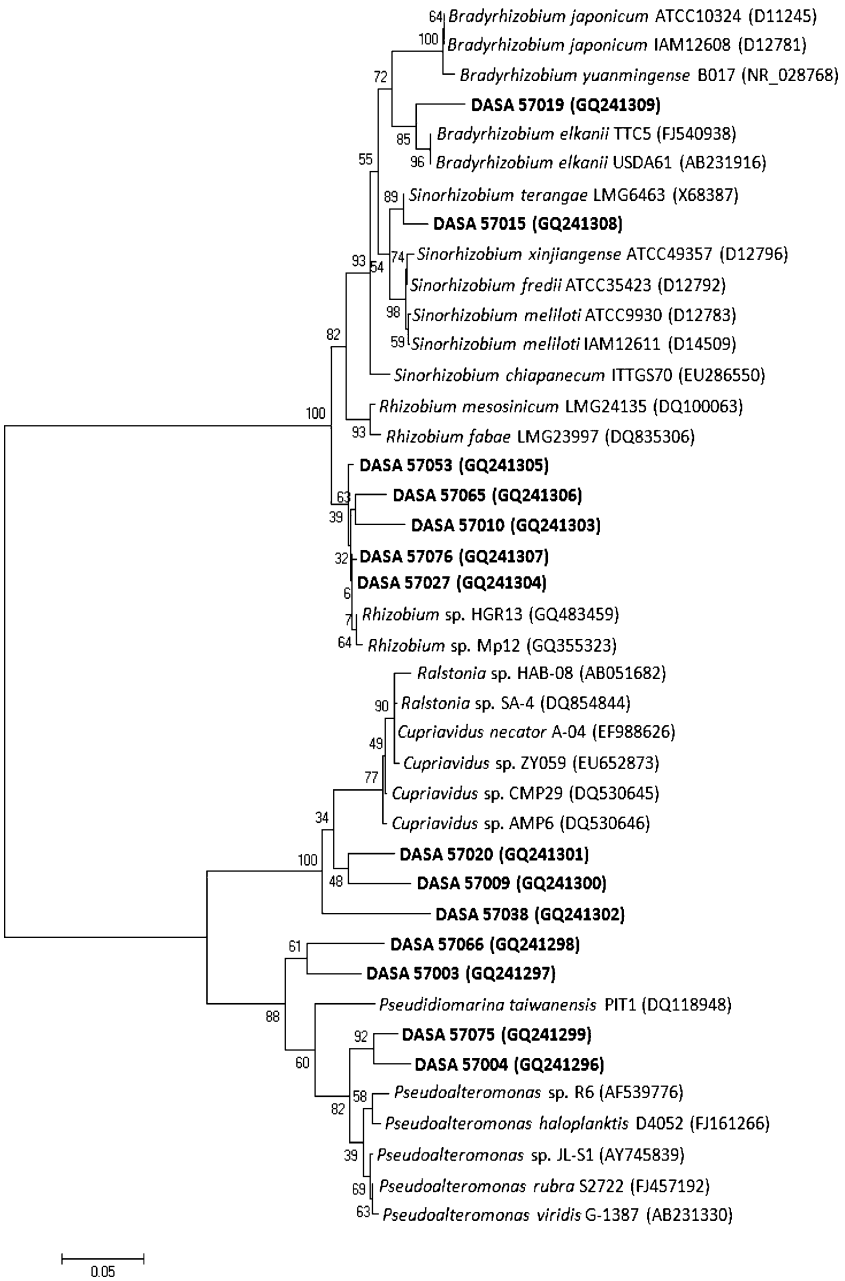


**Fig. 1** Phylogenetic tree based on 16 S rDNA sequences. The numbers at the node are bootstrap values based on 1,000 re-samplings. *Bar* Mutations per sequence position

is the closest neighbor to strain DASA 57015 with a bootstrap supported value of 89%. Strains DASA 57010, DASA 57027 DASA 57053, DASA 57065 and DASA 57076 were closely related to *Rhizobium* spp. The strains DASA 57009, DASA 57020 and DASA 57038 formed a distinct branch, most related to *Ralstonia* and *Cupriavidus*. They might be classified with other species in the *Ralstonia-Cupriavidus* group or they might belong to other genera. Although the strains DASA 57003, DASA 57004, DASA 57066 and DASA 57075 fall within the *Pseudoalteromonas* cluster, these *I. tinctoria* symbionts formed a lineage separated from members of *Pseudoalteromonas* and *Pseudidiomarina taiwanensis*. These taxa are not recognized as legume symbionts, therefore these symbionts remain unidentified. Further characterization will be required to identify the exact genus of these *Pseudoalteromonas*-like strains. This is the first report to note that the same species of legume can be nodulated by bacteria from up to five different genera in three distinct classes. This finding supports the hypothesis that the genes responsible for symbiosis with legumes are transmissible horizontally and function in a relatively wide range of bacterial genera (Fuentes et al. 2002).

## Studies on phenotypic features

Growth curves of bacteria related to the various generic groups (*Pseudoalteromonas*; the *Cupriavidus-Ralstonia* group, *Sinorhizobium* sp. and *B. elkanii*; and *Rhizobium* spp.) are presented in Fig. 2, and the phenotypic characteristics of 14 strains of root-nodule symbionts isolated from *I. tinctoria* are summarized in Table 2.

The strains in different genera exhibited particular features regarding utilization of carbon and nitrogen sources. All strains were able to utilize DL-alanine, glycerol, L-asparagine, lithium lactate, L-alanine, proline, mannitol, L-ornithine, D-fructose, sodium tartrate, L-arabinose, casein, D-glucose, D-xylose, L-glutamine, D-mannose, D-galactose, dextrose and ammonium citrate as sole carbon sources. None could utilize 4-phenylphenol, tannic acid and benzoic acid as sole carbon source. All strains used glycine, DL-phenylalanine, DL-lysine, DL-valine, DL-aspartic acid, urea, L-ornithine, proline, ammonium dihydrogen orthophosphate and ammonium molybdate-4-hydrate but not sodium nitrite, diphenyl amine or trimethyl ammonium bromide as sole nitrogen source.

Even though *Rhizobium*-, *Sinorhizobium*- and *Bradyrhizobium*-like strains could be distinguished from each other based on their utilization of carbon and nitrogen sources, *Pseudoalteromonas*- and *Cupriavidus*-like strains exhibited more diversity in their utilization of carbon and nitrogen sources. Among *Rhizobium*-, *Sinorhizobium*- and *Bradyrhizobium*-like strains, *Rhizobium*-like strains could be distinguished from

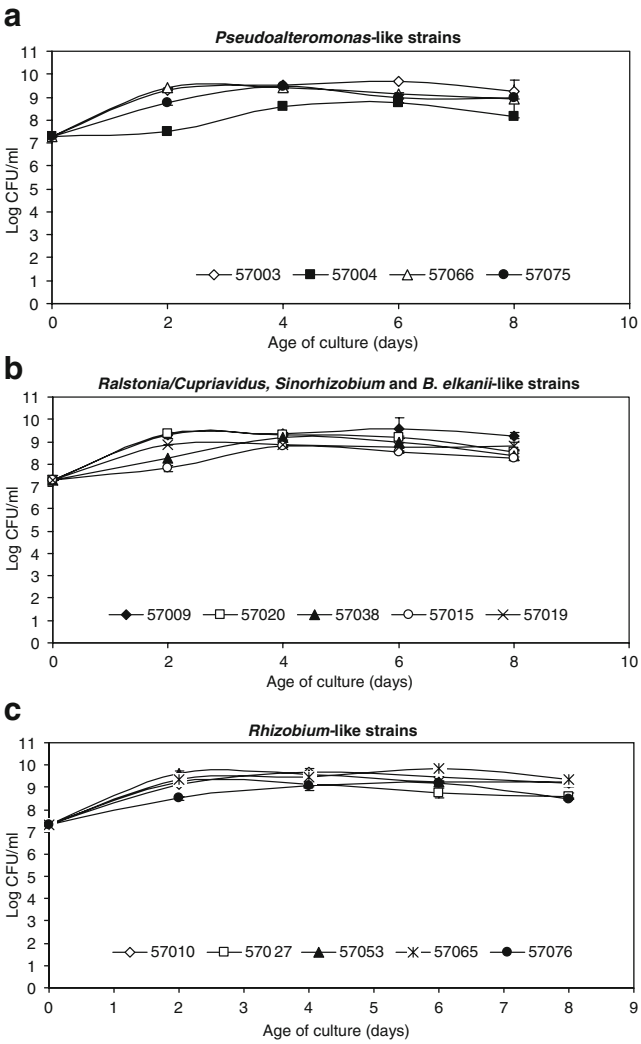


**Fig. 2a–c** Growth curves of root-nodule symbionts. **a** Strains related to *Pseudoalteromonas* spp. **b** Strains related to *Cupriavidus-Ralstonia* spp., *Sinorhizobium* sp. and *Bradyrhizobium elkanii*. **c** Strains related to *Rhizobium* spp. Values shown are the mean of three replicates. *Error bars* Standard deviation

the other two genera based on their inability to utilize sodium acetate and D-serine as sole carbon source, as well as their ability to use ammonium oxalate as a sole nitrogen source.

The *Sinorhizobium*-like strain was different from 2 other genera based on the disability to utilize D-raffinose, citric acid and phthalic acid as sole carbon sources. The *Bradyrhizobium*-like strain could be distinguished from the other two genera based on the ability to utilize sodium benzoate as sole carbon source. Overall the ability to utilize citric acid and phthalic acid as sole carbon sources was the only discriminating feature between the *Sinorhizobium*-like strain and strains in the other four genera. *Rhizobium*-like strains nodulating *I. tinctoria* were similar to *R. indigoderae* CCBAU71042 (Wei et al. 2002), which nodulates

**Table 2** Summary of characteristics of root-nodule symbionts of *Indigofera tinctoria*

| Characteristic | *Pseudo-alteromonas*-like strains (*n*=4) | *Ralstonia-Cupriavidus*-like strains (*n*=3) | *Rhizobium*-like strains (*n*=5) | *Sino-rhizobium*-like strain (*n*=1) | *Bradyrhizobium elkanii*-like strain (*n*=1) |
|---|---|---|---|---|---|
| Maximum colony size (cm) | 0.20–1.30 | 0.40–1.30 | 0.70–1.30 | 1.4 | 0.1 |
| Acid/alkaline production | Acid | Acid | Acid | Acid | Neutral |
| Utilization of C sources:[a] | | | | | |
| A | +[c] | + | + | + | + |
| B | + / – | + / – | – | – | + |
| C | + / – | + | – | + | + |
| D | + / – | + / - | + | – | + |
| E | + | – | + / – | + | + |
| F | + | + / – | + / – | + | + |
| G | + | + | + / – | + | + |
| H | + | + / – | – | + | + |
| I | + | + / – | + / – | – | + |
| J | + | + / – | + / – | + | – |
| K | + / – | + / – | + / – | + | + |
| L | + / – | – | + / – | + | – |
| M | + / – | + / – | + / – | + | – |
| N | + / – | – | – | + | – |
| O | + / – | + / – | + / – | – | – |
| P | + / – | + / – | – | – | – |
| Q | – | – | + / – | + | – |
| R | – | – | – | + | – |
| S | – | – | – | – | – |
| Utilization of N sources:[b] | | | | | |
| AA | + | + | + | + | + |
| BB | + | + | + / – | + | + |
| CC | + | + / – | + / – | + | + |
| DD | + | + / – | + / – | – | – |
| EE | + / – | + | + | + | + |
| FF | + / – | + | + | – | – |
| GG | + / – | – | + / – | + | – |
| HH | + / – | + / – | + / – | + | – |
| II | + / – | + / – | + / – | + | + |
| JJ | + / – | – | – | – | – |
| KK | + / – | + / – | – | – | – |
| LL | – | – | – | – | – |
| Tolerance to antibiotics (μg): | | | | | |
| Tetracycline (30), spectinomycin (10), streptomycin (10) and novobiocin (30) | + / – | + / – | + / – | – | – |
| Ceftazdime (30) and ampicillin (10) | + / – | + / – | + / – | + | + |
| Chloramphenicol (30) | – | + / – | + / – | – | + |
| Cefotaxine (30) | + / – | + / – | – | – | – |
| Gentamycin (120) and kanamycin (30) | + / – | – | + / – | – | – |
| Vitamin requirement: | | | | | |
| Myo-inositol (vitamin B8) and biotin (vitamin B7) | + / – | + / – | – | – | – |
| Aminobenzoic acid (vitamin B) | + / – | – | + / – | – | + |
| Thiamine hydrochloride (vitamin B1) | – | + / – | + / – | – | + |
| Riboflavin (vitamin B2) | + / – | – | – | – | + |

**Table 2** (continued)

| Characteristic | *Pseudo-alteromonas*-like strains ($n$=4) | *Ralstonia-Cupriavidus*-like strains ($n$=3) | *Rhizobium*-like strains ($n$=5) | *Sino-rhizobium*-like strain ($n$=1) | *Bradyrhizobium elkanii*-like strain ($n$=1) |
|---|---|---|---|---|---|
| Nicotinic acid (vitamin B3) and ascorbic acid (vitamin C) | + / − | – | – | – | – |
| Calcium panthothenate (vitamin B5) and pyridoxine hydrochloride (vitamin B6) | + / − | + / − | + / − | – | + |
| Folic acid (vitamin B9) | + / − | + / − | + / − | – | – |
| Cyanocobalamin (vitamin B12) | + / − | + / − | – | – | + |

+, all strains are positive; −, all strains are negative; +/−, some strains are positive

[a] *A* DL-Alanine, glycerol, L-asparagine, lithium lactate, L-alanine, proline, mannitol, L-ornithine, D-fructose, sodium tartrate, L-arabinose, casein, D-glucose, D-xylose, L-glutamine, D-mannose, D-galactose, dextrose and ammonium citrate; *B* sodium benzoate; *C* D-serine; *D* sodium acetate; *E* DL-lysine; *F* potassium sodium tartrate, isopropyl alcohol, D-maltose and lactose; *G* D-cellobiose and DL-aspartic acid; *H* DL-valine; *I* sucrose; *J* ammonium oxalate; *K* glycine, trehalose and galacturonic acid; *L* α-cellulose, anthrone, urea and adonitol; *M* D-sorbitol, xylitol, inulin and myo-inositol; *N* D-raffinose; *O* potassium acetate; *P* sorbose; *Q* malic acid; *R* citric acid and phthalic acid; *S* 4-phenylphenol, tannic acid and benzoic acid

[b] *AA* Glycine, DL- phenylalanine, DL-lysine, DL-valine, DL-aspartic acid, urea, L-ornithine, proline, ammonium dihydrogen orthophosphate and ammonium molybdate-4-hydrate; *BB* L-glutamine; *CC* DL-threonine, DL- alanine, L-alanine, ammonium nitrate, sodium nitrate and L-asparagine; *DD* L-tyrosine; *EE* ammonium citrate tribasic; *FF* ammonium oxalate; *GG* D-serine; *HH* L-aspartic acid; *II* L-arginine, ammonium chloride and calcium nitrate; *JJ* sodium barbitone; *KK* galactonoric acid; *LL* sodium nitrite, diphenyl amine and trimethyl ammonium bromide

other species of *Indigofera* based on the ability to use galactose, mannose, xylose and asparagine as sole carbon source, although they differed in their utilization of arabinose as a sole carbon source as well as the utilization of glycine and valine as sole nitrogen sources.

*Pseudoalteromonas*-like strains differed from *Pseudoalteromonas* described by Gauthier et al. (1995) since all *Pseudoalteromonas*-like strains used L-ornithine and some strains used adonitol. Strains belonging to the same genus group varied in their antibiotic resistance and vitamin

**Table 3** Growth of root-nodule symbionts of *I. tinctoria* at different conditions. The values shown are the mean values of three replicates ± standard deviations. *CFU* Colony forming units

| Strains | Growth under different conditions (log CFU/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Temperature | | | pH | | | NaCl | | |
| | 20°C | 30°C | 40°C | 5.0 | 6.5 | 8.0 | 0M | 0.2M | 1.0M |
| *Pseudoalteromonas*-like strains | | | | | | | | | |
| DASA 57003 | 8.52±0.07 | 8.22±0.13 | 8.88±0.04 | 8.17±0.11 | 8.22±0.13 | 9.67±0.10 | 8.52±0.16 | 10.33±0.01 | 7.73±0.13 |
| DASA 57004 | 8.05±0.09 | 8.17±0.10 | 8.32±0.11 | 5.88±0.06 | 8.17±0.10 | 8.62±0.17 | 8.37±0.22 | 7.73±0.04 | 4.46±0.15 |
| DASA 57066 | 9.34±0.09 | 8.87±0.07 | 5.82±0.05 | 8.82±0.06 | 8.87±0.07 | 8.50±0.12 | 9.05±0.09 | 9.16±0.03 | 5.49±0.04 |
| DASA 57075 | 8.80±0.05 | 8.26±0.08 | 8.46±0.12 | 7.72±0.01 | 8.26±0.08 | – | 8.81±0.05 | 7.80±0.12 | 5.54±0.05 |
| *Ralstonia-Cupriavidus*-like strains | | | | | | | | | |
| DASA 57009 | 10.00±0.00 | 8.44±0.03 | 6.15±0.03 | 7.91±0.05 | 8.44±0.03 | 5.25±0.14 | 8.88±0.07 | 5.49±0.09 | 3.00±0.00 |
| DASA 57020 | 8.87±0.09 | 9.06±0.07 | 8.26±0.03 | 7.60±0.00 | 9.06±0.07 | 8.34±0.06 | 8.30±0.00 | 9.01±0.04 | 4.65±0.06 |
| DASA 57038 | 8.45±0.05 | 9.02±0.01 | 4.52±0.04 | 7.53±0.04 | 9.02±0.01 | 4.00±0.00 | 8.90±0.05 | 4.55±0.14 | – |
| *Rhizobium*-like strains | | | | | | | | | |
| DASA 57010 | 9.17±0.00 | 9.18±0.06 | 9.12±0.02 | 6.61±0.40 | 9.18±0.06 | 8.97±0.03 | 9.09±0.10 | 9.07±0.06 | 5.22±0.65 |
| DASA 57027 | 5.00±0.00 | 8.94±0.01 | 8.16±0.02 | 6.67±0.16 | 8.94±0.01 | 9.12±0.05 | 8.48±0.12 | 8.33±0.09 | 1.36±0.17 |
| DASA 57053 | 9.20±0.07 | 9.52±0.15 | 7.33±0.06 | 8.00±0.00 | 9.52±0.15 | 8.94±0.02 | 6.48±0.00 | 5.90±0.00 | 3.54±0.06 |
| DASA 57065 | 7.43±0.20 | 7.30±0.00 | 6.18±0.06 | 7.05±0.04 | 7.30±0.00 | – | 8.37±0.22 | 7.60±0.24 | 1.12±0.16 |
| DASA 57076 | 8.98±0.02 | 8.86±0.02 | 8.42±0.00 | 8.52±0.52 | 8.86±0.02 | 8.17±0.06 | 9.40±0.23 | 9.13±0.03 | 5.17±0.03 |
| *Sinorhizobium*-like strain | | | | | | | | | |
| DASA 57015 | 6.94±0.07 | 8.69±0.15 | 8.20±0.07 | 4.30±0.18 | 8.69±0.15 | 8.34±0.11 | 9.30±0.00 | 9.02±0.03 | 5.14±0.05 |
| *Bradyrhizobium elkanii*-like strain | | | | | | | | | |
| DASA 57019 | 8.68±0.10 | 8.90±0.03 | 3.69±0.14 | 8.50±0.08 | 8.90±0.03 | 8.26±0.08 | 8.89±0.24 | 4.50±0.01 | 1.48±0.00 |

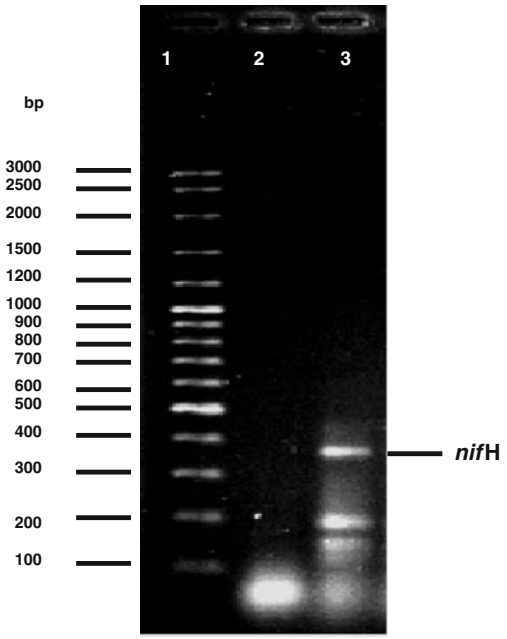


**Fig. 3** A 360-bp portion of the *nif*H gene amplified from *Pseudoalteromonas*-like strain DASA 57075. Lanes: *1* 100 bp ladder, *2* negative control, *3* DASA 57075

requirements, and in fact strains in five genera groups could not be distinguished from each other based on their antibiotic resistance and vitamin requirement. Six strains—DASA 57066, DASA 57075, DASA 57020, DASA 57027, DASA 57076 and DASA 57015—did not required vitamins for growth. Strain DASA 57009 required other factors besides the ten vitamins tested for its growth. Strain DASA 57053 required each of the 10 vitamins tested for its growth. Strain DASA 57004 required vitamin B2. The strains DASA 57038, DASA 57010 and DASA 57019 required one of several B vitamins. Strain DASA 57003 required one of several B vitamins or vitamin C for its growth. The growth behavior of the strains at different temperature, pH and concentrations of NaCl is presented in Table 3. Even among the same genus, the tested strains varied in their response to levels of temperature, pH and NaCl. For example, *Pseudoalteromonas*-like strains DASA 57003, DASA 57004 and DASA 57066 as well as *Rhizobium*-like strains DASA 57010, DASA 57027, DASA 57053 and DASA 57076 were tolerant to alkalinity at pH 8.0, while growth of *Pseudoalteromonas*-like strain DASA 57075 and *Rhizobium*-like strain DASA 57065 was totally inhibited at pH 8.0. Of the five generic groups of *I. tinctoria* symbionts, four have been reported as nodule symbionts for other legume species, while the *Pseudoalteromonas*-like strains are not currently known symbionts. The results of these phenotypic features and 16S rDNA sequence analysis leave the *Pseudoalteromonas*-like strains unidentified. Further studies on both phenotypic and genotypic characterization are required to identify the exact genera of these strains.

## Sequence analysis of partial *nif*H and *nod*C genes

A 360-bp portion of the *nif*H gene could be amplified from *Pseudoalteromonas*-like strain DASA 57075 (Fig. 3). BLASTN analysis of the sequences indicated that the partial *nif*H gene of DASA 57075 had 96% similarity with the *nif*H gene of *Bradyrhizobium yuanmingense*. The partial *nif*H gene of DASA 57075 has been deposited with GenBank under accession number GQ241310.

Partial *nod*C genes were amplified from *Pseudomonas*-like strains DASA 57075 and DASA 57038 in the *Ralstonia-Cupriavidus* group, *Sinorhiozobium*-like strain DASA 57015 and the *B. elkanii*-like strain DASA 57019 (Fig. 4). BLASTN analysis of the sequences indicated that the *nod*C gene of DASA 57075 exhibited 88% similarity with the *nod*C gene of *Bradyrhizobium japonicum*. The *nod*C gene of DASA 57038 showed high homology (85%) with the *nod*C gene of *B. yuanmingense*. The *nod*C gene of DASA 57015 and DASA 57019 showed the highest homology with the *nod*C gene of *Sinorhizobium meliloti* (87% similarity) and *Bradyrhizobium* sp. (85% similarity), respectively. The partial *nod*C genes of these strains have been deposited with GenBank under the accession numbers GQ241311–GQ241314.

These two major symbiotic genes, *nif*H and *nod*C, were characterized in this study to confirm the symbiotic

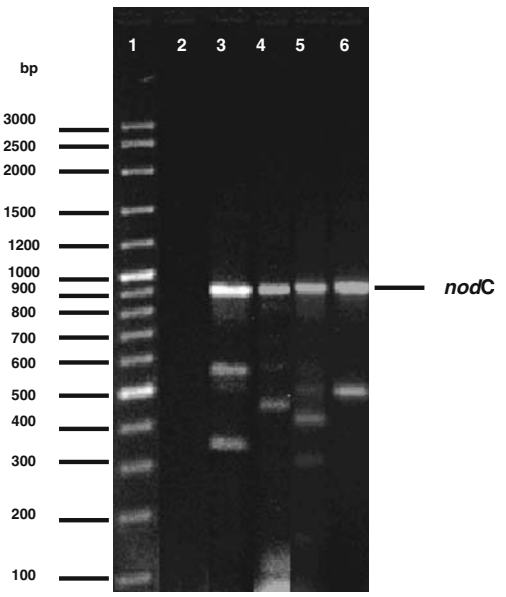


**Fig. 4** A 930-bp fragment of the *nod*C gene amplified from root-nodule symbionts of *Indigofera tinctoria*. Lanes: *1* 100 bp ladder, *2* negative control, *3* *Sinorhizobium*-like strain DASA 57015, *4* *Bradyrhizobium elkanii*-like strain DASA 57019, *5* *Ralstonia-Cupriavidus*-like strain DASA 57038, *6* *Pseudoalteromonas*-like strain DASA 57075

properties of *I. tinctoria* symbionts at the molecular level. Both *nif*H and *nod*C are well conserved among symbiotic nitrogen-fixing bacteria (Ruvkun and Ausubel 1980; Geremia et al. 1994; Zhang et al. 2000). The *nif*H gene encodes an Fe-containing protein—the dinitrogenase reductase of the nitrogenase enzyme complex. Dinitrogenase reductase transfers electrons to dinitrogenase, which binds and reduces dinitrogen to ammonia (Tate 2000). The *nod*C gene encodes chitin synthase, which is responsible for synthesis of lipochitooligosaccharide molecules called Nod factors (Geremia et al. 1994). Nod factors induce several steps in nodule formation including root cortical cell division, root hair deformation and transcription of nodulation-related genes in root hairs (Angelini et al. 2003). The presence of *niff*H and *nod*C genes in *Pseudoalteromonas*-like strain DASA 57075 supports the hypothesis of Fuentes et al. (2002) that the genes essential for rhizobia-legume symbiosis are transmissible horizontally in a relatively wide range of bacterial genera. The results presented here indicate that the novel gamma-proteobacterium related to *Pseudoalteromonas* harbors *nif*H and *nod*C—two major genes essential for the symbiotic relationship. Therefore, we proposed a bacterium related to *Pseudoalteromonas* as a new symbiont of the medicinal legume *I. tinctoria*.

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