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Characterization of Two Novel Biovar of *Agrobacterium tumefaciens* Isolated from Root Nodules of *Vicia faba*

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Abstract A total of eight strains of bacteria were isolated from the root nodule of *Vicia faba* on the selective media of *Rhizobium*. Two of these strains produced phenotypically distinct mucoid colonies (one slow growing and the other fast growing) and were examined using a polyphasic approach for taxonomic identification. The two strains (MTCC 7405 and MTCC 7406) turned out to be new strains of biovar 1 *Agrobacterium* rather than *Rhizobium*, as they showed growth on alkaline medium as well as on 2% NaCl and neither catabolized lactose as the carbon source nor oxidized Tween-80. The distinctness between the two strains was marked with respect to their growth on dextrose and the production of lysine dihydrolase, ornithine decarboxylase and DNA G + C content. 16S rDNA sequencing and their comparison with the 16S rDNA sequences of previously described agrobacteria as well as rhizobia strains confirmed the novelty of the two strains. Both of the strains clustered with strains of *Agrobacterium tumefaciens* in the 16S rDNA-based phylogenetic tree. The phenotypic and biochemical properties of the two strains differed from those of the recognized biovar of *A. tumefaciens*. It is proposed that the strains MTCC 7405 and MTCC 7406 be classified as novel biovar of the species *A. tumefaciens* (Type strains MTCC 7405 = DQ383275 and MTCC 7406 = DQ383276).

Keywords *Agrobacterium tumefaciens* · Biovar · Root nodule · *Vicia faba* · Diversity

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Introduction

The genus *Agrobacterium*, a member of family Rhizobiaceae has been included in the α-2 subclass of *Proteobacteria* mainly on the basis of ribosomal characteristics [16, 27]. Agrobacteria are soil microorganisms, some of which induce crown gall tumors primarily at the crown or on roots [4, 5]. The ability of strains to produce crown gall depends on the presence of a fragment called T-DNA present on the Ti plasmid, which is stably integrated in the nuclear genome during infection in wounded plant cells [26]. The expression of T-DNA leads to synthesis of plant hormones and some unusual compounds called opines, which play a significant role in the epidemiology of crown gall [7, 26]. Although tumor-inducing agrobacteria are common, some non-tumorogenic strains have also been reported from aerial tumors and root nodules [19, 28].

There has been a great deal of dispute over the classification and nomenclature of *Agrobacterium* and *Rhizobium* because of a number of characteristics they share in common [8, 29, 30]. Two major lineages were distinguished: One included *Agrobacterium rhizogenes* along with most of the *Rhizobium* sp. and the other included *A. tumefaciens* [29]. A polyphasic approach, which includes both phenotypic as well as phylogenetic parameters such as 16S rDNA sequence data, has, nevertheless, been

useful in the delimitation of *Agrobacterium* from that of the genus *Rhizobium* [8].

Previous findings suggest that the genus *Agrobacterium* is polyphyletic [12]. Keane et al. [15], based on some specific phenotypic and biochemical characteristics, suggested that the genus *Agrobacterium* be subdivided into two biovar, 1 and 2. An additional biovar 3 was subsequently described from the isolates from grapevine [21]. Whereas biovar 1 contains several strains of *A. radiobacter* and *A. tumefaciens*, biovar 2 contains many strains of *A. rhizogenes* and biovar 3 corresponds to *A. vitis* [11, 21]. Different strains of *A. tumefaciens* have been further classified in biovar 1, 2, and 3 on the basis of characteristic physiological and biochemical differences [13]. This implies that *A. tumefaciens* alone shows a great deal of diversity within species and isolation of novel strains of this bacterium cannot be ruled out.

During routine screening for studying bacterial diversity in the fertile cropland located south of the river Ganges in the state of Bihar, India, for testing the potential of selected strains as a biofertilizer/biomineralizer, we isolated two strains of *A. tumefaciens* from root nodules of *Vicia faba* when plants had just started flowering. Because these strains differed mainly with respect to morphological (colony configuration and cell size) and biochemical characteristics, they were subjected to a polyphasic taxonomic study including partial 16S rRNA (*rrs*) gene sequence analysis. A phylogenetic tree based on the results thus obtained conclusively suggests that the two isolates are novel strains/biovar of *A. tumefaciens*.[1]

Materials and Methods

Isolation and Culturing of Strains

The root nodule of *V. faba* was used as the starting material. It was surface sterilized using 0.1% mercuric chloride for 5 min, followed by washing with sterile distilled water three times. The nodule was crushed in sterile normal saline [prepared by dissolving 0.87% (w/v) NaCl in distilled water, followed by autoclaving at 121°C for 15 min]. 0.1 mL of serially diluted suspension (10^{-5}) was plated over yeast extract mannitol agar (YEMA) containing (per liter) 0.5 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 10 g mannitol, 1 g yeast extract, and 1.5% (w/v) agar, a nutrient medium commonly used for selective isolation of rhizobia [25]. Colonies were randomly selected after 36 h of incubation at 30 ± 2°C based on differences in margin; they were then purified and maintained on YEMA slants at 4°C. Growth was studied spectrophotometrically [optical density at 600 nm (OD_{600})] using broth culture at an interval of 2 h up to 24 h. All subsequent growth conditions were the same as above.

Preliminary Characterization of Strains

The pattern of growth of both the strains was studied on Hofer's alkaline medium (pH 11.0) described for differentiating *Rhizobium* from *Agrobacterium* [1]. Gram staining was performed as described elsewhere [10]. Cell morphology was studied under an Olympus CX41 research microscope at ×1000, with cells grown for 48 h at 30°C. Growth of strains was studied at a temperature range between 4°C and 65°C. Tolerance of the strains to NaCl was determined on YEMA supplemented with different concentrations of the salt between 2% and 10% (w/v). The pH requirements of the strains were tested in the range from 4.0 to 12.0.

Biochemical Tests

MTCC 7405 and MTCC 7406 were further examined for biochemical characteristics such as hydrolysis of casein, gelatin, starch, urea, and Tween-20. Growth and acid production were studied in basal synthetic media containing (per liter) 2 g $(NH_4)_2SO_4$, 0.24 g K_2HPO_4, 0.24 g $MgSO_4$ $7H_2O$, 0.1 g KCl, 0.1 g yeast extract, and 8–10 drops of 0.2% aqueous solution of bromocresol purple (pH 7.2). Tests were conducted using disks of different carbon sources in duplicate (Table 1) as described in the standard protocol for taxonomic characterization of bacteria [16, 17].

Test for Resistance to Antibiotics

The antibiotic resistance profile was studied in the presence of a fixed concentration (15 μg) of six different antibiotics (erythromycin, ofloxacin, norfloxacin, tetracycline, gatifloxacin, and clarithromycin) at 30 ± 2°C on YEMA by the disk diffusion method [3].

PCR Amplification and Sequencing of 16S rDNA

Genomic DNA from strains MTCC 7405 and MTCC 7406 was isolated using a genomic DNA isolation kit (Qiagen) and was quantified spectrophotometrically (Lambda 35, Perkin-Elmer). Polymerase chain reaction (PCR) amplification of 16S rDNA was performed with universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) of the *Escher-*

[1] The GenBank accession numbers for the 16S rRNA gene sequences of the two biovar deposited in NCBI, Bethesda, MD, USA is DQ383275 for MTCC7405 and DQ383276 for MTCC 7406, which canbe found online at http://www.ncbi.nlm.nih.gov. The sequence is also available in EMBL in Europe and the DNA Data Bank of Japan.

Table 1 Biochemical characterization of strains

Property	Strain MTCC 7405	Strain MTCC 7406
Biochemical tests		
Growth on MacConkey agar	NLF	NLF
Indol test	–	–
Voges–Proskauer test	–	–
Citrate utilization	–	–
Gas production from glucose	–	–
Nitrate reduction	–	–
H_2S production	–	–
Casein hydrolysis	–	–
Gelatin hydrolysis	+	+
Starch hydrolysis	–	–
Urea hydrolysis	+	(W)
Catalase	–	–
Oxidase	+	+
Lysin dihydrolase	–	+
Ornithine decarboxylase	–	+
Tween-20 hydrolysis	–	–
Lipase test (Tween-80)	–	–
Utilization of sole carbon sources		
Arabinose	+	+
Cellobiose	+	+
Dextrose	–	+
Innulin	+	+
Inositol	+	+
Lactose	–	–
Maltose	+	+
D-Manitol	+	+
Melibiose	+	+
Rhamnose	+	+
Sucrose	–	–

NLF = nonlactose fermenters; (W) = poor growth

ichia coli 16S rDNA numbering system as described by Brosius et al. [6]. The reaction mixture of 25 μL contained 70 ng of chromosomal DNA, 1 U of Deep Vent DNA polymerase, 1X Thermopol reaction buffer, 200 μM of each deoxynucleoside triphosphate (New England Biolabs), and 20 pmol of each primer (BioBasic Inc). PCR cycling parameters included an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 75°C for 2 min, and a final extension for 10 min at 75°C. An ~1.5-kb amplicon was separated by gel electrophoresis, eluted by a Qiaquick gel extraction kit (Qiagen), and sequenced using 27F and 1492R primers. The 16S rDNA sequence was determined following the dideoxy chain-termination method using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit as directed in the manufacturer's protocol. Sequence reactions were electrophoresed and analyzed by an ABI 310 Genetic Analyzer (Applied Biosystems, USA). The complete sequences were submitted to GenBank.

Phylogenetic Analysis

The 16S rDNA sequences of closely related validly published taxa were retrieved from the GenBank database using BLASTN [2] and aligned using the CLUSTAL X program [23]. For the neighbor-joining analysis [22], the distances between the sequences were calculated based on the Juke and Cantor method [14] and the phylogenetic tree was constructed using the TREECON program [24]. Bootstrap analysis of 1000 replications was performed to assess the confidence limits of the branching [9].

Estimation of Genomic G + C Content

The G + C content of genomic DNA was determined spectrophotometrically (Lambda 35, Perkin-Elmer) using the thermal denaturation method [18].

Culture Collection and Gene Bank ID for the Strains

The two strains of *A. tumefaciens* after identification were deposited in MTCC and Gene Bank, IMTECH, Chandigarh, India with the ID MTCC 7405 and MTCC 7406. The Accession Numbers of the complete 16S rRNA gene sequence available at NCBI, Maryland are DQ383275 for MTCC7405 and DQ383276 for MTCC 7406.

Results and Discussion

Isolation and Growth Characteristics of Strains

Two (MTCC 7405 and 7406) out of eight isolates obtained from surface-sterilized root nodules on selective media appeared visibly distinct with respect to the margin of the colonies and size of the cells (MTCC 7405: margin irregular, size 1.0–2.0 μm; MTCC 7406: margin entire, size 1.5–2.5 μm). On YEMA plates, MTCC 7405 produced slow-growing, small colonies, whereas MTCC 7406 showed very fast growth and large colonies. When growing in liquid medium, the two strains produced a large amount of slime, making the cultures very viscous. The growth curve profile of the two strains also differed significantly.

Interestingly both of the isolates produced mucoid colonies like most of the strains of *Rhizobium*. However, growth of these isolates on Hofer's alkaline medium (pH

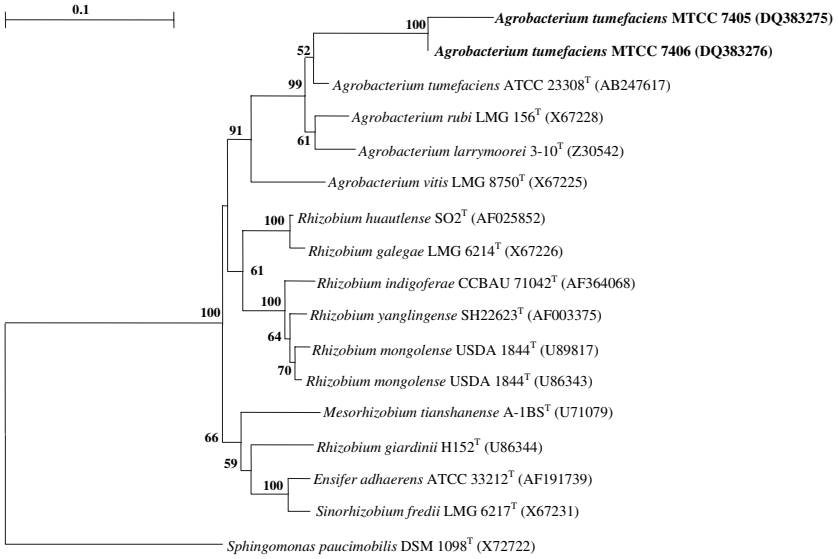

Fig. 1 Phylogenetic neighbor-joining tree based on 16S rDNA sequences showing relationship between *A.tumefaciens* MTCC 7405 and *A.tumefaciens* MTCC 7406 and other type strains of related taxa. *Sphingomonas paucimobilis* DSM 1098^T was used as the outgroup. Numbers at nodes indicate levels of bootstrap support ≥ 50% based on a neighbor-joining analysis of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar = 10 nucleotides substitution per 100 nucleotides.

11.0) and 2% NaCl as well as the inability to catabolize lactose, unlike most of the strains of *Rhizobium* and biovar 1 *Agrobacterium* [28, 29], led to a suspicion that the two isolates under investigation could be different strains of *Agrobacterium*. The identity of each of the suspected *Agrobacterium* isolates was determined by the appropriate physiological and biochemical tests [12, 19, 20]. Both of the strains were Gram-negative, none spore-forming motile rods producing circular, creamy, raised, opaque, and smooth mucoid colonies. None of them grew under anaerobic conditions. The growth of both the strains occurred well between 15°C and 42°C and from pH 5.0 to 12.0. Analysis of the carbon sources oxidized by the strains revealed that both could oxidize a wide range of carbon compounds similar to those reported previously for other strains of *Agrobacterium* [4]. However, they were differentiated from known agrobacteria by their inability to oxidize Tween-80 and by being nonlactose fermenters (NLFs) (Table 1). In addition, a marked difference was also observed in the ability to oxidize dextrose by MTCC 7405 (no growth) and MTCC 7406 (normal growth) as the sole source of carbon. These findings clearly indicated that the two strains were distinct from hitherto described biovar of *A. tumefaciens*.

Test for Antibiotic Resistance

The antibiotic-resistance profile in the presence of a fixed amount (15 μg) of six different antibiotics revealed that strain MTCC 7405 was more resistance to all antibiotics tested compared to MTCC 7406. Although the two strains were significantly resistant to norfloxacin, a marked difference was observed in terms of zones of inhibition in the presence of erythromycin and ofloxacin.

16S rRNA Gene Sequencing and Phylogenetic Tree

The distinct nature of MTCC 7405 and MTCC 7406 was further confirmed by 16S rDNA sequencing and comparing it to the 16S rRNA gene sequences of previously described biovar and strains of *A. tumefaciens* and *Rhizobium.* It is evident from the phylogenetic tree (Fig. 1) that strains MTCC 7405 (1440 nt; DQ383275) and MTCC 7406 (1432 nt; DQ383276) were distinct but closely resembled the *A. tumefaciens* ATCC 23308^T (AB247617) with a 16S rDNA sequence homology of 99.0% and 98.5%, respectively. However, neither of the two strains had resemblances with either the strains of other biovar of *Agrobacterium* or those of *Rhizobium* used in comparison.

Genomic GC Content

The DNA G + C contents of strains MTCC 7405 and MTCC 7406 were estimated to be 62.6 and 63.0 mol% (mean of three replications), respectively—values within the range (57–63 mol%) known in *A. tumefaciens* [17] but different from their closest phylogenetic neighbor *A. tumefaciens* ATCC 23308^T (61.0 mol%) [21]. Based on the data obtained from phylogenetic analysis and the genomic GC ratio, both of the strains represented novel biovar of *A. tumefaciens.*

Comparison of Characteristics of Different Biovar of *Agrobacterium*

Almost all of the strains of *A. tumefaciens* described so far were isolated from either crown gall or root nodules of dicotyledonous angiosperms or from soil [28–30]. We

Table 2 Comparison of properties of new strains with existing biovar of *A. tumefaciens*

Property	Existing *A. tumefaciens*[a]			New strains	
	Biovar 1	Biovar 2	Biovar 3	MTCC7405	MTCC7406
Growth on 2% NaCl	+	–	+	+	+
Growth at 35°C	+	–	d	+	+
Lactose catabolism	+	–	+	–	–
Acid from dextrose	NT	NT	NT	–	+
Catalase	+	+	+	–	–
Lysin dihydrolase	NT	NT	NT	–	+
Ornithine decarboxylase	NT	NT	NT	–	+
Urease	+	+	+	+	(W)

+ = positive reaction; – = negative reaction, d =11–89% strains are positive; (W) = weak reaction; NT= not tested

[a] Described in [13]

report the isolation of two new strains of *A. tumefaciens* from the root nodule of the cultivated leguminous plant of *V. faba.* Table 2 summarizes the characteristic differences of these strains and their comparison with the existing biovar of *A. tumefaciens* [13]. Growth on alkaline medium (pH 11.0) and 2% NaCl differentiated these strains from the known strains of *Rhizobium*. The ability to grow at 35°C (up to 42°C), the failure to oxidize lactose as the sole carbon source, and being catalase negative suggested the novelty of the two strains within the species of *A. tumefaciens*. The inability to utilize dextrose as the carbon source by MTCC 7405 and the ability of MTCC 7406 to use this monosaccharide as the energy source apart from some biochemical parameters (lysine dihydrolase and ornithine decarboxylase) as well as genomic G + C content differentiated the two strains. Moreover, both of the strains showed resistance to norfloxacin.

The presence of nontumorogenic agrobacteria in crown gall or root nodules has been reported earlier [19, 28]. The isolated strains have, however, been shown to be incapable of nodulating on their original or alternate host [28]. Such strains are thought to be opportunistic bacteria from soil invading the nutrient-rich tumor environment. Our finding that *A. tumefaciens* might inhabit root nodules of legumes itself points toward an important question pertaining to the role of this soil bacterium. Do some novel biovar play some critical role in nodulation and/or nitrogen fixation in association with *Rhizobium*? Our assumption is that, if not all, at least some nonpathogenic biovar of *A. tumefaciens* might be involved in the process of root nodule or tumor formation, the induction of which requires some component, yet unknown, produced by this bacterium as a symbiont.

The type of strains have been identified as two novel biovar of *A. tumefaciens:* MTCC 7405 (DQ383275) and MTCC 7406 (DQ383276).

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