**Ochrobactrum ciceri** sp. nov., isolated from nodules of *Cicer arietinum*

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A Gram-staining-negative, aerobic, rod-shaped, non-spore-forming bacterial strain, Ca-34T, was isolated from nodules of chickpea (*Cicer arietinum*) in Pakistan and studied for its taxonomic affiliation. The almost full-length 16S rRNA gene sequence showed highest similarities to those of strains of the genus *Ochrobactrum*. Based on results of MALDI-TOF MS and 16S rRNA gene sequence similarity (98.6 %), strain Ca-34T and *Ochrobactrum intermedium* LMG 3301T are phylogenetic neighbours; the two strains shared DNA–DNA relatedness of 64 %. The fatty acid profile [predominantly C<sub>18:1</sub>ω7c (87.7 %) and C<sub>19:0</sub> cyclo ω8c (19.6 %)] also supported the genus affiliation. Metabolically, strain Ca-34T differed from other type strains of *Ochrobactrum* in many reactions and from all type strains in testing positive for gelatin hydrolysis and in testing negative for assimilation of alaninamide and L-threonine. Based on phenotypic and genotypic data, we conclude that strain Ca-34T represents a novel species, for which we propose the name *Ochrobactrum ciceri* sp. nov. (type strain Ca-34T =DSM 22292T =CCUG 57879T).

The genus *Ochrobactrum*, class *Alphaproteobacteria*, was originally described by Holmes et al. (1988) and currently comprises 14 species isolated from different environments including soil and industrial environments (Lebuch et al., 2000; Kämpfer et al., 2008; Huber et al., 2010), the rhizosphere and plants (Ngom et al., 2004; Trujillo et al., 2005; Tripathi et al., 2006; Zurdo-Piñeiro et al., 2007), animals (Kämpfer et al., 2003) and humans (Holmes et al., 1988; Velasco et al., 1998; Teyssier et al., 2005, 2007). Three nodulating species have been described that form nodules on *Acacia* (Ngom et al., 2004), *Lupinus* (Trujillo et al., 2005) and *Cytisus* (Zurdo-Piñeiro et al., 2007). Here, we report a novel strain, Ca-34T, that was isolated in 1996 from nodules of chickpea (*Cicer arietinum*) grown in Pakistan. Its taxonomic affiliation was assessed by a polyphasic approach and the data obtained show that this strain belongs to a novel species of *Ochrobactrum*.

Strain Ca-34T was isolated from root nodules according to Vincent (1970) using yeast mannitol (YEM) agar (Bergersen, 1961). The culture used in further phenotypic and molecular studies was purified from a single colony after overnight incubation at 28 ± 2 °C on nutrient agar (NA). Cells were Gram stained (Doetsch, 1981) and cell morphology was observed under a phase-contrast microscope at ×100 magnification. Aminopeptidase and cytochrome oxidase activities (Bactident; Merck) were tested according to the manufacturer's recommendations. Catalase activity was tested by addition of 3 % H<sub>2</sub>O<sub>2</sub> to bacterial smears on a glass slide. Susceptibility to various antibiotics was examined as described by Valverde et al. (2005) using antibiotic-supplemented discs (Bioanalyse) on antibiotic sulfonamid sensitivity test agar (ASS agar; Merck). The analytical profile index was studied using QT5-24 (DESTO) and API 20NE (bioMérieux) according to the instructions of the manufacturers. Carbon source utilization was studied using the Biolog GN Microplate system and metabolic fingerprints were identified using the MicroLog database software. Indole acetic acid (IAA) production was quantified using HPLC as described by Tien et al. (1979) and the acetylene reduction assay was performed as described by Hafeez et al. (1995). Fatty acids were prepared, separated (Labrenz et al., 1998) and analysed on GC as described by Kämpfer & Kroppenstedt (1996) using the Sherlock MIDI system.

\[ \text{strain sequence similarity} = 98.6\% \]

\[ \text{fatty acid profile} = \text{C}_{18:1}\omega7\text{c (87.7%)} \]

\[ \text{fatty acid profile} = \text{C}_{19:0}\ \text{cyclo} \omega8\text{c (19.6%)} \]

\[ \text{DNA–DNA relatedness} = 64\% \]

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\[ \text{DNA–DNA relatedness} = 64\% \]
(Sasser, 1990). Preparation of cell extracts and handling of samples for MALDI-TOF MS analyses were done as described by Cousin et al. (2008). The 16S rRNA gene was amplified using universal primers P1 and P6 (Tan et al., 1997) and primers F4 and R2 (Romero et al., 1995; Velasco et al., 1998). recA amplification was performed using the primers and amplification conditions described by Scholz et al. (2006, 2008). Sequencing of the latter gene was undertaken by Seqlab (Göttingen, Germany).

Cells of strain Ca-34T are Gram-negative (aminopeptidase-positive), aerobic (oxidase- and catalase-positive), motile, dumbbell-shaped short rods, commonly observed as single cells under the microscope. Growth occurs on YEM agar, LB agar and NA at 28–37 °C. Colonies (2–3 mm diameter) are circular, off-white, mucoid and translucent with entire margins. Resistant to ampicillin (10 μg), ceftimoxime (5 μg) and cephradine (30 μg) (β-lactams). Substrate assimilation (API 20NE; bioMérieux) read after 24 h showed positive reactions of strain Ca-34T for o-nitrophenyl β-galactopyranoside (ONPG), sodium malonate, urea hydrolysis, gelatin hydrolysis, tryptophan deaminase and nitrate reduction. Acid production was observed from glucose, maltose, mannose, arabinose, rhamnose, sorbitol, inositol and melibiose. Gelatin hydrolysis is a unique characteristic of strain Ca-34T, not reported for any of the described Ochrobactrum type strains. In the Biolog analysis (MicroPlate system), Ochrobactrum pseudogrignonense, which is phylogenetically distinct from strain Ca-34T, was not tested. Most strains were positive in 25–41 reactions. Strain Ca-34T and Ochrobactrum intermedium DSM 17986T shared 58 positive reactions and differed in 11 reactions, including utilization of certain lipids, sugars, acids and amino acids (see Supplementary Table S1, available in IJSEM Online), which is similar to the level of similarity between the phylogenetically closely related type strains of Ochrobactrum anthropi, O. lupini, O. cytisi and O. tritici. IAA was produced up to 6.5 p.p.m. Although nodule occupancy by strain Ca-34T was confirmed using transmission electron microscopy and immunogold labelling (A. Imran and others, unpublished data), nitrogen fixation could not be confirmed in vitro. Moreover, primers reported to amplify nif and nod genes from other Ochrobactrum strains (Trujillo et al., 2005; Zurdo-Piñeiro et al., 2007) were negative for strain Ca-34T.

The fatty acid pattern of strain Ca-34T (Table 1) is similar to those of other Ochrobactrum type strains. Like these strains, strain Ca-34T contains C18:1ω7c (67.7 %) and C19:0 cyclo ω8c (19.6 %) fatty acids, but the amounts detected were larger and smaller, respectively, than those reported for O. intermedium DSM 3301T and O. anthropi ATCC 49188T. In addition, significant amounts of C18:0 (4.4 %), C16:0 (2.6 %) and C18:1 ω9c (2.6 %) were present.

As judged from the MALDI-TOF MS dendrogram, the spectra of some Ochrobactrum type strains differed significantly from each other (e.g. Ochrobactrum thiophenivorans DSM 7126T and Ochrobactrum rhizosphaerae DSM 19824T), while others were highly similar (e.g. strain Ca-34T and O. intermedium DSM 17986T and O. anthropi).

Table 1. Fatty acid methyl ester compositions of Ochrobactrum type strains

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<tr>
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<td>2.6</td>
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<tr>
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<td>–</td>
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<tr>
<td>C20:1ω9c</td>
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DSM 6882ᵀ, O. cytisi DSM 19778ᵀ and O. lupini DSM 16930ᵀ) (Supplementary Fig. S1). The lack of congruence in dendrogram topology between 16S rRNA gene sequences and MALDI-TOF MS profiles has been noted previously in studies on members of Flavobacterium (Cousin et al., 2008; Ali et al., 2009).

Sequencing of the nearly complete 16S rRNA gene of strain Ca-34ᵀ indicated the presence of a 46 nt insertion at Escherichia coli 16S rRNA position 187 that folds into a stem–loop structure when analysed through MFOLD (http://www.mfold.burnet.edu.au/). This insertion has already been described in clinical isolates of O. anthropi including strain ADV1 (Teyssier et al., 2003) and O. intermedium isolates including strains CCUG 1838 and CCUG 44770 (Scholz et al., 2008) and has been reported to prolong helix H184 when placed on the Brucella suis 16S rRNA gene sequence secondary structure. Three O. anthropi isolates (GenBank accession numbers AM114410, AM11409 and AM490614), showing the highest 16S rRNA gene sequence similarity to strain Ca-34ᵀ, also possess this insertion. Most of the strains with this insertion are either clinical isolates or uncultured bacterial clones isolated from the environment. To our knowledge, this is the first report of this insertion in any plant-associated bacterium.

Teyssier et al. (2003) reported that the 46 bp insertion showed no significant similarity with genome sequence data, but BLAST analysis of this 46 bp sequence showed 100 % similarity to the 16S rRNA gene sequences of some uncultured bacterial clones (GenBank accession numbers EU769179, EU149209 and DQ917822), a few isolates of O. anthropi and O. intermedium isolates including strains CCUG 1838 and CCUG 44770 (Scholz et al., 2008) and has been reported to prolong helix H184 when placed on the Brucella suis 16S rRNA gene sequence secondary structure. Three O. anthropi isolates (GenBank accession numbers AM114410, AM11409 and AM490614), showing the highest 16S rRNA gene sequence similarity to strain Ca-34ᵀ, also possess this insertion. Most of the strains with this insertion are either clinical isolates or uncultured bacterial clones isolated from the environment. To our knowledge, this is the first report of this insertion in any plant-associated bacterium.

Omitting the insert, BLASTN analysis (Altschul et al., 1997) was followed by a thorough phylogenetic analysis by aligning the sequence of strain Ca-34ᵀ to the database of the ‘All-species living tree’ (http://arb-silva.de/projects/living-tree/). Binary comparisons showed 99 % similarity to a cloned sequence of a phenol-degrading uncultured Ochrobactrum sp. P3 and to two reclassified O. intermedium strains, CCUG 44770 (GenBank accession no. AM114410) and CCUG 1838 (AM114409, AM490614) (Scholz et al., 2008), which are not closely related to the type strain of this species (not shown).

Strain Ca-34ᵀ showed the highest 16S rRNA gene sequence similarity to the type strain of O. intermedium (98.6 %), while similarity to other type strains of the genus was lower (e.g. O. tritici SCI24ᵀ, 97.0 %; O. anthropi LMG 3331ᵀ, 96.8 %), ranging between 94.6 and 97 %. Distances were

![Fig. 1.](#)

**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain Ca-34ᵀ, neighbouring Ochrobactrum type strains and Brucella melitensis biovar melitensis ATCC 23456ᵀ. Branching points that are identical to those of the maximum-likelihood tree are indicated by filled circles. Numbers at branching points are bootstrap values (>35 % (percentages of 1000 resamplings). Pseudochrobactrum asaccharolyticum CCUG 46016ᵀ served as an outgroup. Bar, 2 % sequence divergence.

![Fig. 2.](#)

**Fig. 2.** Representative RAPD profiles of strain Ca-34ᵀ and some strains of Ochrobactrum generated with primer OPC-13. Lanes: 1, DNA marker (100 bp); 2, strain Ca-34ᵀ; 3, O. tritici DSM 13341; 4, O. tritici DSM 13340; 5 and 7, O. lupini LMG 22726ᵀ; 6, O. oryzae DSM 17471ᵀ; 8, O. gallinicae DSM 15295ᵀ; 9, O. intermedium DSM 17986ᵀ; 10, O. anthropi DSM 6882ᵀ; 11, negative control; 12, DNA marker (1 kb).
calculated according to Kimura’s two-parameter model (Kimura, 1980). A phylogenetic tree (Fig. 1) was inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The topology of the phylogenetic tree, especially the relatedness of *O. intermedium* with *Brucella* species (represented by *Brucella melitensis* ATCC 23456T in Fig. 1) and the polyphyletic origin of several species assigned to the genus *Ochrobactrum*, is in accord with previously published studies on *Ochrobactrum* (Scholz et al., 2006, 2008).

Phylogenetic analysis (with and without the 46 bp insertion; not shown) clearly showed that strain Ca-34T and *O. intermedium* LMG 3301T form a separate group with some uncultured species and misclassified strains of *O. anthropi*, *O. intermedium* and *Ochrobactrum* sp. DASA 35030. As the binary 16S rRNA gene sequence similarities were at the borderline at which DNA–DNA reassociation values were recommended (Stackebrandt & Ebers, 2006), hybridization analysis was performed between strain Ca-34T and the type strains *O. intermedium* DSM 17986T and *O. anthropi* DSM 6882T. DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion et al. (1977) which was carried out as described by De Ley et al. (1970) with the modifications described by Huß et al. (1983) using a model Cary 100 Bio UV/Vis spectrophotometer. DNA–DNA relatedness values were 64.0 and 52.1%, respectively, with *O. intermedium* DSM 17986T and *O. anthropi* DSM 6882T. For G+C content determination, DNA was analysed by HPLC (Shimadzu) according to Mesbah et al. (1989). The G+C content of strain Ca-34T was 58.8 mol%, which is within the range 54.5–59.0 mol% reported for other *Ochrobactrum* type strains (Lebuhn et al., 2000; Teyszier et al., 2007).

To better resolve the phylogeny between strains highly related by 16S rRNA gene sequence similarities, *recA* amplification and sequence analysis was attempted, follow-

**Table 2.** Differential biochemical properties of strain Ca-34T and reference *Ochrobactrum* type strains

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*Result confirms data from the original species description.
†Result confirms data reported by: a, Kämpfer et al. (2003); b, Lebuhn et al. (2000).
‡Reported as variable by Kämpfer et al. (2003) or Lebuhn et al. (2000).
The differences between strain Ca-34\textsuperscript{T} and \(O.\) \textit{intermedium} LMG 3301\textsuperscript{T} were further confirmed by amplified \textit{rDNA} restriction analysis (ARDRA) of 16S \textit{rRNA} genes digested with restriction enzymes \textit{MboI}, \textit{HpaI}, \textit{BanHI}, \textit{EcoRI}, \textit{BsrRI}, \textit{TaqI}, \textit{NciI}, \textit{PstI}, \textit{ApaI} and \textit{Alul} (Supplementary Fig. S2) and RAPD fingerprinting (Fig. 2). In each experiment, strain Ca-34\textsuperscript{T} gave a unique pattern. Two-primer RAPD (TP-RAPD) patterns analysed according to the method described by Rivas et al. (2002) confirmed these differences (not shown). TP-RAPD patterns of strains from the same species are always identical (Rivas et al., 2002, 2004), while they differ between species, e.g. \(O.\) \textit{anthropi}, \(O.\) \textit{lupini} and \(O.\) \textit{triticum} (Trujillo et al., 2005).

These results confirmed the phylogenetic distinctiveness of strain Ca-34\textsuperscript{T}, which can be differentiated metabolically, chemotaxonomically and genomically from the type strains of \textit{Ochrobactrum}, specially from the phylogenetically related \(O.\) \textit{intermedium} (Table 2). We therefore propose a novel species \textit{Ochrobactrum ciceri} sp. nov. to accommodate strain Ca-34\textsuperscript{T}.

**Description of \textit{Ochrobactrum ciceri} sp. nov.**

\(Ochrobactrum ciceri\) \textsuperscript{ciceri} L. \textit{gen. n. ciceri} of chickpea \textit{(Cicer arietinum)}, pertaining to the habitat from which the type strain was isolated.

Cells are Gram-staining-negative (aminopeptidase-positive), aerobic (oxidase- and catalase-positive), highly motile, dumbbell-shaped short rods, commonly observed as single cells. Growth occurs on YEM agar, LB agar and NA at 28–37 °C. Colonies (2–3 mm diameter) are circular, off-white, mucoid and translucent with entire margins. No colony pigment development is observed on any medium. Of the 95 carbon sources of the Biolog GN system, 35 carbon sources show strong oxidation reactions. Biolog reactions that differentiate the type strain from its closest relative, \(O.\) \textit{intermedium}, are as follows: glycogen, Tween 80, raffinose, L-rhamnose, trehalose, \(\alpha\)-ketobutyric acid, succinamic acid, glucuronamide, alaninamide, L-threonine and uridine. Positive reaction for gelatin hydrolysis can be used as a distinguishing character for rapid identification of the type strain among other \textit{Ochrobactrum} type strains. Resistant to ampicillin (10 µg), aztreonam (30 µg), cefixime (5 µg) and cephradine (30 µg) (\(\beta\)-lactams). The fatty acid profile is composed largely of \(C_{18:1}\text{ω}7c\) and \(C_{19:0}\text{cyco}\). In addition, significant amounts of \(C_{18:0}\text{C}_{16:0}\) and \(C_{18:1}\text{2-}OH\) are present. The DNA G+C content of the type strain is 58.8 mol%. PCR with \textit{Brucella}- and \(O.\) \textit{intermedium}-specific primers F2 and R4 is negative.

The type strain, Ca-34\textsuperscript{T} (=DSM 2229\textsuperscript{T} =CCUG 57879\textsuperscript{T}), was isolated at NIBGE in 1996 from chickpea nodules.

### References


