Ciceribacter lividus gen. nov., sp. nov., isolated from rhizosphere soil of chick pea (*Cicer arietinum* L.)

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The taxonomic position of strain MSSRFBL1^T, isolated from chickpea rhizosphere soil from Kannivadi, India, was determined. Strain MSSRFBL1^T formed bluish black colonies, stained Gram-negative and was motile, aerobic, capable of fixing dinitrogen, oxidase-negative and catalase-positive. Q-10 was the major respiratory quinone. Major fatty acids of strain MSSRFBL1^T were $C_{18:1}\omega7c$ and $C_{19:0}$cyclo$\omega8c$. Minor amounts of $C_{18:0}$, $C_{12:0}$, $C_{14:0}$ 3-OH, $C_{18:0}$ 3-OH, $C_{16:0}$, $C_{16:1}\omega6c$/$C_{16:1}\omega7c$, $C_{17:0}$ 3-OH and $C_{20:1}\omega7c$ were also present. Polar lipids included diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylcholine and two unidentified glycolipids. Bacteriohopane derivatives (BHD1 and 2), diplopterol, diploptene, bishomohopanediol, adenosylhopane and 2β-methyl bacteriohopanetetrol were the major hopanoids of strain MSSRFBL1^T. The genomic DNA G+C content was 71 mol%. EzTaxon-e-based BLAST analysis of the 16S rRNA gene indicated the highest similarity of strain MSSRFBL1^T to *Ensifer adhaerens* LMG 20216^T (97.3 %) and other members of the genus *Ensifer* (<96.9 %) in the family *Rhizobiaceae* of the class *Alphaproteobacteria*. However, phylogenetic analysis based on 16S rRNA, *recA*, *thrC* and *dnaK* gene sequences showed distinct out-grouping from the recognized genera of the family *Rhizobiaceae*. Based on phenotypic, genotypic and chemotaxonomic characters, strain MSSRFBL1^T represents a novel species in a new genus in the family *Rhizobiaceae* for which the name *Ciceribacter lividus* gen. nov., sp. nov. is proposed. The type strain of *Ciceribacter lividus* is MSSRFBL1^T (=DSM 25528^T=KCTC 32403^T).

While understanding the rhizosphere bacterial diversity of chick pea (*Cicer arietinum* L.) we came across bluish black colonies which happened to be a close relative of the genus *Ensifer* based on 16S rRNA gene sequence analysis. The family *Rhizobiaceae* comprises the genera *Ensifer*, *Shinella*, *Kaistia* and *Rhizobium*. Except for the members of the genus *Kaistia*, all other members of the family *Rhizobiaceae* are dinitrogen fixers and are associated with plants. Through this study, we propose a novel genus in the family *Rhizobiaceae* for strain MSSRFBL1^T isolated from the rhizosphere soil of *C. arietium* L.

Rhizosphere soils of *C. arietium* L. collected from Kannivadi (GPS position of the sample collection site is 10° 22′ 44.40″ N 77° 49′ 48.0″ E), Tamil Nadu, India, were serially diluted and plated on yeast malt agar (no. M424; HiMedia) and incubated at 28 °C for 7 days. Bluish black colonies appeared along with other white colonies and these were of particular interest because of the unique colony colour; they were purified and maintained on yeast malt agar. The purified isolate was designated MSSRFBL1^T and preserved at −80 °C in 20 % (v/v) glycerol.

Genomic DNA was extracted and purified from strain MSSRFBL1^T according to the method of Marmur (1961). G+C content of the DNA as determined by reversed-phase HPLC (Mesbah *et al.*, 1989) was 71±0.5 mol% for strain

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *recA*, *dnaK*, *thrC* and *nifH* gene sequences of strain MSSRFBL1^T are JQ230000, KC189949, KC189950, KC189951 and KC189952, respectively.

Five supplementary figures are available with the online version of this paper.

MSSRFBL1^T. PCR amplification of the 16S rRNA gene was done by using the primer set 27f and 1492r (Lane, 1991). Amplification and sequencing were done as described by Rameshkumar & Nair (2009) using fluorescent terminators (Big Dye; Applied Biosystems) and run in a 3130xl Applied Biosystems ABI prism automated DNA sequencer. Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/).

Based on the 1392 bp 16S rRNA gene sequence BLAST search analysis, *Ensifer adhaerens* LMG 20216^T was the nearest phylogenetic neighbour of strain MSSRFBL1^T with 97.3 % sequence similarity. The CLUSTAL W algorithm of MEGA 4.0 software (Tamura *et al.*, 2007) was used for sequence alignments and for phylogenetic analysis of the individual sequences. Distances were calculated by using the Kimura correction in a pairwise deletion manner (Kimura, 1980). Neighbour-joining (NJ), maximum-parsimony (MP) and minimum-evolution (ME) methods in the MEGA 4.0 software were used to reconstruct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure. Phylogenetic trees showed that the novel isolate (MSSRFBL1^T) branched separately from other members of the genus *Ensifer* (Fig. 1). The highest sequence similarities for strain MSSRFBL1^T were found with the type strain of *E. adhaerens* LMG 20216^T (97.3 %) and a few other members of the genera *Ensifer*, *Rhizobium*, *Shinella* and *Kaistia* (<96.9 %); Fig. 1 shows the NJ tree, while the ME and MP trees show similar tree topology (data not shown). Such phylogenetic distinction of strain MSSRFBL1^T from the rest of the genera in the family *Rhizobiaceae* was also observed when all the type species were considered for tree reconstruction (Fig. S1, available in IJSEM Online). For a more reliable classification of strain MSSRFBL1^T, we have considered three housekeeping genes, *recA*, *dnaK* and *thrC*, which were amplified as described by Martens *et al.* (2007). The NJ dendrograms of *recA* (Fig. S2), *dnaK* (Fig. S3) and *thrC* (Fig. S4) support the 16S rRNA gene sequence-based distinction of strain MSSRFBL1^T from the rest of the taxa in the family *Rhizobiaceae*.

E. adhaerens LMG 20216^T (=A^T; representing the type species of the genus *Ensifer* and also phylogenetically closely related to strain MSSRFBL1^T), *Ensifer kostiensis* DSM 13372^T (=TTR 15^T; an additional strain representing the genus *Ensifer*) and *Sinorhizobium americanum* DSM 15007^T (=CFNEI 156^T; a representative of the genus *Ensifer*) were used for comparative taxonomic studies along with strain MSSRFBL1^T. Colonies of strain MSSRFBL1^T were bluish black, while those of both members of the genus *Ensifer* were white. Morphological properties (cell shape, size and cell division) were observed under an Olympus model BH-2 phase-contrast light microscope. Cells of strain MSSRFBL1^T were rod-shaped, 1–2 μm long and 0.3–0.5 μm wide, motile (confirmed through hanging drop method) and multiplied by binary fission. The colour of the cell suspension was bluish black. *In vivo* absorption spectra as measured with a Spectronic Genesys 2 spectrophotometer in sucrose solution (Trüper & Pfennig, 1981) exhibited maxima at 286 nm; however, the λ_{max} indicated that the pigment was not indigoidine, which has absorption maxima at 300, 350 and 395 nm, found in *Vogesella indigofera* (Kuhn *et al.*, 1965) and *Vogesella alkaliphila* (Subhash *et al.*, 2013). While indigoidine is easily extractable in methanol, the pigment of strain MSSRFBL1^T was not extractable with most of the common organic solvents and hence could not be characterized in this study.

Strain MSSRFBL1^T grew within a pH range of 6.0–8.5 (optimum pH 7). The temperature range for growth was determined using yeast malt broth under optimum pH. The broth was incubated at different temperatures ranging from 20 to 45 °C for 3 days and the growth was measured turbidometrically at 660 nm. Strain MSSRFBL1^T was able to grow well from 25 to 37 °C. A test for utilization of electron acceptors in the presence of yeast extract (0.03 %, w/v) was carried out. Propionate, methanol and ethanol were used as electron donors at concentrations of 0.1 % (v/v) in the presence of $NaHCO_3$ (0.1 %, w/v) and media without electron donor served as control. Strain MSSRFBL1^T was able to utilize propionate but not methanol or ethanol. Chemolithoautotrophy using $Na_2S_2O_3$ (1 mM) or Na_2S (0.5 mM) as electron donor and $NaHCO_3$ (0.1 %, w/v) as carbon source could not be

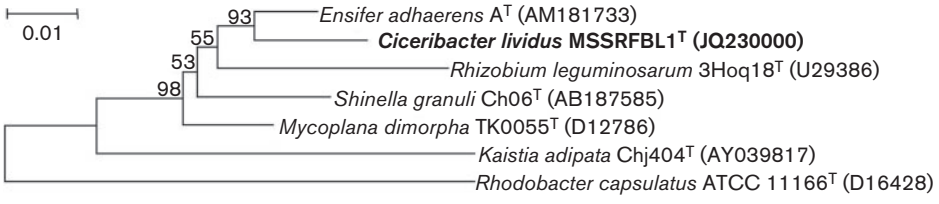

Fig. 1. Phylogenetic tree based on 16S rRNA sequences available from the EMBL database (accession numbers in parentheses), reconstructed after multiple alignment of data by CLUSTAL W (Thompson *et al.*, 1994) as described. Bootstrap values based on 1000 replications are given as percentages at branching points. Bar, 0.01 substitutions per nucleotide position by using the neighbour-joining method.

demonstrated as no growth was observed. Utilization of various organic/inorganic compounds as a carbon/electron donor by strain MSSRFBL1^T is described in the species description. Chemoorganoheterotrophic growth occurred with a number of organic carbon substrates (Table 1). Fermentative growth [anaerobically in fully filled screw-cap test tubes, in dark, pyruvate or glucose (0.3 %, w/v) as fermentable sugars] occurred in strain MSSRFBL1^T. Vitamin requirement was tested by replacing yeast extract with single and also combinations of vitamins as growth factors and strain MSSRFBL1^T had no requirement for added vitamins.

Strain MSSRFBL1^T grew well in a medium devoid of combined nitrogen on repeated subculturing and its *nifH* gene was amplified using the primers PolF (5′-TGCG-AYCCSARRGCBGGYATCGG-3′) and PolR (5′-ATSGCC-ATCATYTCRCCGGA-3′) (Poly *et al.*, 2001). The expected amplification of 360 bp was observed in MSSRFBL1^T. The amplified 360 bp stretch was sequenced and showed 93 and 89 % similarity to the *nifH* sequences of *Rhizobium daejeonense* L61^T and *Rhizobium leguminosarum* Qtx-10-1, respectively. However, the *nifH* sequence similarities to other strains from the NCBI database ranged from 75 to 85 %. Strain MSSRFBL1^T was grown on nitrogen-free plates and the ability to fix nitrogen was determined by acetylene reductase activity. The amount of ethylene produced was measured using (v/v) acetylene according to the method of Li & MacRae (1992) in a Hewlett Packard 4890 GC equipped with a poropack N column. Strain MSSRFBL1^T reduced acetylene to ethylene and fixed 35.7 nmol ethylene ml^{-1} h^{-1}. The results of the *nifH* gene sequence comparison and acetylene reductase assay confirmed the nitrogen fixing ability of strain MSSRFBL1^T.

Various biochemical tests such as hydrolysis of starch, casein, pectin and gelatin, indole production from L-tryptophan, denitrification, and chitinase, oxidase and

Table 1. Differentiating characteristics of strain MSSRFBL1^T

Taxa: 1, strain MSSRFBL1^T (data from this study); 2, *Ensifer* (data from this study based on *E. adhaerens* LMG 20216^T; 3, *Ensifer* (data from this study based on *S. americanum* DSM 15007^T); 4, *Rhizobium* (Turdahon *et al.*, 2012); 5, *Kaistia* (Kim, *et al.*, 2010); 6, *Shinella* (An *et al.*, 2006). Cells of all taxa are motile rods. All taxa are positive for growth on mannitol and negative for hydrolysis of gelatin. All taxa contain $C_{16:0}$, $C_{18:0}$, $C_{18:1}\omega7c$ and $C_{19:0}$cyclo$\omega8c$ but none contain $C_{14:0}$.+, Positive reaction: −, negative reaction; −/+, some strains positive and others negative; ND, not determined.

Characteristic	1	2	3	4	5	6
Size (length × width)	1–2 × 0.3–0.5	1.5–2.5 × 0.5–1.3	1.5–2.5 × 0.5–1	1.0–1.5 × 0.5–1	1–1.2 × 0.8–0.9	1.1–1.5 × .4–0.9
Slime formation	−	+	+	+	ND	ND
Colony colour	Bluish black	White	White	White	Ivory	Pale yellow
Growth on NaCl (%)						
2	+	+	+	−	−	+
3	−	+/−	−	−	−	+
4	−	−	−	−	−	+
Hydrolysis of starch	−	−	−	ND	−	ND
Growth on:			−			
Arabinose	+	+	−	+	+	+
Sorbitol	−	+	−	−	+	+
Fructose	+	−	+	+	+	ND
Mannose	+	+	−	+	+	+
Succinate	−	−	+	ND	ND	ND
Maltose	+	+	+	−	+	+
Sucrose	+	+/−	−	+	+	+
Ribose	+	+	+	−	+	+
Urease	+	−	−	+	ND	+
Citrate	+	+	−	−	ND	ND
Presence of *nif*H	+	+	+	+	ND	−
Fatty acid composition						
$C_{12:0}$	+	+	+	−	−	−
$C_{14:0}$ 3-OH	+	+	+	−	−	−
$C_{16:1}\omega6c/C_{16:1}\omega7c$	+	+	+	+	−	−
$C_{17:0}$ 3-OH	+	−	−	−	−	−
$C_{18:0}$ 3-OH	+	+	+	+	−	−
11-Methyl $C_{18:1}\omega7c$	−	+	−	−	−	−
$C_{20:1}\omega7c$	+	−	+	−	−	−
DNA G+C content (mol%)	71.0	62.8	63.34	57–63	59–64	63–66

catalase activity were carried out in the recommended media as mentioned by Cappuccino & Sherman (1998). Strain MSSRFBL1^T was catalase-positive but was negative for oxidase, chitinase and tryptophanase, starch and gelatin hydrolysis and denitrification.

Cellular fatty acid, polar lipid and hopanoid analysis was done from cells grown in yeast malt broth and harvested when growth of the cultures was around 70 % of its maximal optical density (exponential growth phase). For fatty acid analysis, 40 mg of bacterial cells was subjected to a series of four different reagents followed by saponification and methylation of fatty acids thus enabling their cleavage from lipids. The fatty acid methyl esters (FAME) thus obtained were analysed by GC with the Sherlock MIS software (Microbial ID; MIDI version 6.0; Agilent 6850; peak identification was based on the RTSBA6 database) (Sasser, 1990; revised http://www.midi-inc.com). The peaks obtained were then labelled and the equivalent chain-length (ECL) values were computed by the Sherlock software (FAME analysis was outsourced through Royal Research Laboratories, Secunderabad, India). Major fatty acids of strain MSSRFBL1^T included $C_{18:1}\omega7c$ (58 %) and $C_{19:0}$cyclo$\omega8c$ (14.2 %), minor amounts of $C_{18:0}$ (82 %), $C_{12:0}$ (4.9 %), $C_{14:0}$ 3-OH (4.8 %), $C_{18:0}$ 3-OH (4.4 %), $C_{16:0}$ (2.0 %), $C_{16:1}\omega6c$/$C_{16:1}\omega7c$ (1.0 %), $C_{17:0}$ 3-OH (1.0 %) and $C_{20:1}\omega7c$ (1.0 %) were also present (Table 1). While most of the major and minor fatty acids of strain MSSRFBL1^T were in line with other taxa of the family *Rhizobiaceae* (Table 1), the presence of $C_{17:0}$ 3-OH differentiates it from the rest.

Polar lipids were extracted with methanol/chloroform/saline (2 : 1 : 0.8, by vol.) from 1 g freeze-dried cells as described by Kates (1986). Separation and identification of lipids was done by two-dimensional chromatography on a silica gel TLC plate (Kieselgel 60 F254; Merck) as described by Raj *et al.* (2012). Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylmethylethanolamine (PME), phosphatidylcholine (PC) and two unidentified glycolipids (GL1, 2) were the polar lipids identified in strain MSSRFBL1^T (Fig. S5a). The polar lipid profile of strain MSSRFBL1^T differed from the type strain of *E. adhaerens* LMG 20216^T (Fig. S5b) by the presence of two unidentified glycolipids (GL1, 2). While *E. kostiensis* DSM 13372^T (Fig. S5c) and *S. americanum* DSM 15007^T (Fig. S5d) shared a near similar polar lipid profile with *E. adhaerens* LMG 20216^T indicating that they belong to the same genus, *Ensifer*. Such heterogeneity in polar lipids allows the separation of strain MSSRFBL1^T from the genus *Ensifer*. Q-10 (>99 %) was the major quinone of strain MSSRFBL1^T as analysed by HPLC after extraction with a chloroform/methanol (2 : 1, v/v) mixture and purification by TLC (Hiraishi *et al.*, 1984).

Hopanoids were extracted according to Rohmer *et al.* (1984). Separation of hopanoids was done by two-dimensional chromatography on a silica gel TLC plate (Kieselgel 60 F254; Merck) using dichloromethane (DCM) in the first dimension and DCM/CH_3OH (99 : 1, v/v) in the second dimension. Total hopanoids were detected by spraying with 0.1 % solution of barberine chlorohydrate in ethanol and visualizing the plates at 366 nm (Rohmer *et al.*, 1984). Hopanoids were identified by eluting the spots using dichloromethane and derivatizing with benzoyl chloride according to Barrow & Chuck (1990). The benzoated derivatives were identified by LC-MS/MS using a Phenomenex C18 column (150 mm × 4.6 μm) with a flow rate of 0.4 ml min^{-1} using the following solvent system: A (acetonitrile) and B (0.1 % acetic acid in 80 % methanol). Separation was started using 40 % of solvent A and in 15 min it reached 98 %. In the next 13.5 min it reached 100 % and then reduced to 40 % in 1 min. LC-MS/MS was performed using a quadrupole-time-of-flight (Q-TOF) mass spectrometer (6500; Agilent) with electrospray ionization (ESI) probe, which was operated in positive ion mode (auxiliary gas flow, 10 ml min^{-1}; collision energy 40; scan from *m*/*z* 50 to 1700). Strain MSSRFBL1^T had bacteriohopane derivatives (BHD1 and 2), diplopterol (DPL), diploptene (DPE), bishomohopanediol (BiHD), adenosylhopane (ADH) and 2β-methyl bacteriohopanetetrol (MBT) as the major hopanoids (Fig. S5A). Hopanoids of strain MSSRFBL1^T share similarity with *S. americanum* DSM 15007^T and differ from *E. adhaerens* LMG 20216^T and *E. kostiensis* DSM 13372^T by the presence of BiHD (Fig. S5B–D).

The phylogenetic distinction of strain MSSRFBL1^T based on 16S rRNA, *recA*, *dnaK* and *thrC* gene sequences was supported by phenotypic traits like colony colour, absence of oxidase, presence of $C_{17:0}$ 3-OH fatty acid, and polar lipid and hopanoid profiles, which allowed a clear separation from the genus *Ensifer*. Since the delineation of strain MSSRFBL1^T is at the level of a genus, DNA–DNA hybridization with *E. adhaerens* LMG 20216^T becomes redundant even though they share 16S rRNA gene sequence similarity of 97.2 %. Strain MSSRFBL1^T also differed from other closely related genera of the family *Rhizobiaceae* (Table 1). Based on morphological, physiological and genotypic differences we suggest a novel genus and species to accommodate strain MSSRFBL1^T, with the name *Ciceribacter lividus* gen. nov., sp. nov.

Description of *Ciceribacter* gen. nov.

Ciceribacter [Ci.ce.ri.bac′ter. L. n. *cicer –eris* the chickpea and also a botanical genus name (*Cicer* L.); N.L. masc. n. *bacter* a rod; N.L. masc. n. *Ciceribacter* a rod isolated from *Cicer*].

Aerobic, Gram-negative-staining rods, forming bluish black colonies on yeast malt agar. Mesophilic, grow at neutral pH and fix dinitrogen. Catalase-positive and oxidase-negative. Major fatty acids are $C_{18:1}\omega7c$ and $C_{19:0}$cyclo$\omega8c$. Q-10 is the major quinone. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylcholine and two unidentified glycolipids (GL1, 2) are the polar lipids. Bacteriohopane derivatives (BHD1 and 2), diplopterol, diploptene, bishomohopanediol, adenosylhopane and 2β-methyl bacteriohopanetetrol are the

hopanoids. The G+C content of genomic DNA of the type strain of the type species is 71 mol% (HPLC). Delineation of the genus was determined primarily by the phylogenetic information from 16S rRNA, *recA*, *dnaK* and *thrC* gene sequences. The type species is *Ciceribacter lividus*.

Description of *Ciceribacter lividus* sp. nov.

Ciceribacter lividus (li′vi.dus. L. masc. adj. *lividus* bluish, blue).

Much of the information is provided in the genus description. In addition, a wide range of sugars like glucose, mannose, sucrose, mannitol, trehalose and maltose supported growth. NaCl is not required for growth but growth is observed with 1 % NaCl and not with 2 % NaCl. Growth occurs between 25 and 37 °C, optimal at 28 °C. Lipase and amylase are not produced. Chemoautotrophy is not observed in the presence of sodium thiosulfate and sodium sulphide. Ferments and produces acids from lactose, xylose, maltose, fructose, glucose, galactose, raffinose, sucrose, L-arabinose, rhamnose and D-arabinose. Minor amounts of $C_{18:0}$, $C_{12:0}$, $C_{14:0}$ 3-OH, $C_{18:0}$ 3-OH, $C_{16:0}$, $C_{16:1}\omega6c$/$C_{16:1}\omega7c$, $C_{17:0}$ 3-OH and $C_{20:1}\omega7c$ are present.

The type strain, MSSRFBL1^T (=DSM 25528^T=KCTC 32403^T), was isolated from chickpea (*Cicer arietinum* L.) rhizosphere soil, Kannivadi, Tamil Nadu, India.

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