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

A. Imran,¹ F. Y. Hafeez,^{1†} A. Fröhling,² P. Schumann,² K. A. Malik³ and E. Stackebrandt²

Correspondence

A. Imran

asmaaslam2001@yahoo.com

F. Y. Hafeez

fauzia_y@yahoo.com

¹National Institute for Biotechnology and Genetic Engineering (NIBGE), PO Box 577, Jhang Road, Faisalabad, Pakistan

²DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, Inhoffenstrasse 7B, 38124 Braunschweig, Germany

³Department of Biological Sciences, Forman Christian College University, Ferozepur Road, Lahore, Pakistan

A Gram-staining-negative, aerobic, rod-shaped, non-spore-forming bacterial strain, Ca-34^T, 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-34^T and *Ochrobactrum intermedium* LMG 3301^T are phylogenetic neighbours; the two strains shared DNA–DNA relatedness of 64 %. The fatty acid profile [predominantly C_{18:1}ω7c (67.7 %) and C_{19:0} cyclo ω8c (19.6 %)] also supported the genus affiliation. Metabolically, strain Ca-34^T 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-34^T represents a novel species, for which we propose the name *Ochrobactrum ciceri* sp. nov. (type strain Ca-34^T = DSM 22292^T = CCUG 57879^T).

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 (Lebuhn *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-34^T, 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*.

[†]Present address: Department of Biosciences, COMSATS Institute for Information Technology (CIIT), Chak Shazaad Campus, Park Road, Islamabad, Pakistan.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Ca-34^T is DQ647056.

A dendrogram based on MALDI-TOF MS, ARDRA profiles and Biolog GN utilization data are available as complementary material with the online version of this paper.

Strain Ca-34^T 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 % H₂O₂ 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 QTS-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

(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-34^T 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), aztreonam (30 µg), cefixime (5 µg) and cephadrine (30 µg) (β -lactams). Substrate assimilation (API 20NE; bioMérieux) read after 24 h incubation at 37 °C showed positive reactions of strain Ca-34^T 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-34^T, 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-34^T, was not tested. Most strains were positive in 25–41

reactions. Strain Ca-34^T and *Ochrobactrum intermedium* DSM 17986^T 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-34^T 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-34^T.

The fatty acid pattern of strain Ca-34^T (Table 1) is similar to those of other *Ochrobactrum* type strains. Like these strains, strain Ca-34^T contains C_{18:1} ω 7c (67.7 %) and C_{19:0} cyclo ω 8c (19.6 %) fatty acids, but the amounts detected were larger and smaller, respectively, than those reported for *O. intermedium* LMG 3301^T and *O. anthropi* ATCC 49188^T. In addition, significant amounts of C_{18:0} (4.4 %), C_{16:0} (2.6 %) and C_{18:1} 2-OH (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 7126^T and *Ochrobactrum rhizosphaerae* DSM 19824^T), while others were highly similar (e.g. strain Ca-34^T and *O. intermedium* DSM 17986^T and *O. anthropi*

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

Strains: 1, strain Ca-34^T (data from this study; values in parentheses show results for an independent replicate run); 2, *O. intermedium* DSM 17986^T (data from this study, analysed after 48 h); 3, *O. intermedium* LMG 3301^T; 4, *O. anthropi* ATCC 49188^T; 5, *O. grignonense* DSM 13338^T; 6, *O. tritici* DSM 13340^T; 7, *O. gallinifacies* DSM 15245^T (data in columns 3–7 from Kämpfer *et al.*, 2003); 8, *O. lupini* LMG 22726^T (Trujillo *et al.*, 2005); 9, *O. oryzae* MTCC 4195^T (Tripathi *et al.*, 2006); 10, *O. pseudointermedium* ADV31^T (Teyssier *et al.*, 2007); 11, *O. thiophenivorans* DSM 7216^T; 12, *O. rhizosphaerae* PR17^T (data in columns 11 and 12 from Kämpfer *et al.*, 2008); 13, *O. pseudogrignonense* CCUG 30717^T; 14, *O. haematophilum* CCUG 38531^T (data in columns 13 and 14 from Kämpfer *et al.*, 2007). Values are percentages of total fatty acids. –, Not detected/not reported.

Fatty acid	1		2	3	4	5	6	7	8	9	10	11	12	13	14
	24 h	48 h													
C _{13:1} at 12–13	–	–	–	–	0.6	0.7	–	–	–	–	–	–	–	–	–
C _{14:0}	–	–	–	–	–	–	–	0.7	–	0.1	–	–	–	–	–
C _{16:0}	3.4	2.6 (2.5)	3.6	3.7	6.6	2.9	3.7	8.9	4.2	5.1	12.0	7.3	8.3	9.7	10.8
C _{17:0}	–	1.9 (1.8)	2.1	3.1	1.4	1.7	0.9	–	1.6	0.3	3.1	1.4	2.8	1.6	2.5
C _{17:0} cyclo	2.4	–	–	–	0.8	–	–	2.9	–	<0.1	1.4	–	–	1.0	1.3
C _{17:1} ω 6c	1.2	–	–	1.1	–	0.5	–	–	1.4	–	–	–	–	–	–
C _{18:0}	3.4	4.4 (4.3)	4.4	4.1	8.8	7.2	9.6	3.7	3.0	5.6	4.2	2.9	7.6	4.9	7.7
C _{18:1} ω 7c	67.6	67.7 (67.3)	72.6	25.8	45.6	31.6	77.9	28.8	70.8	65.2	29.3	40.9	61.5	19.2	32.7
11-Methyl C _{18:1} ω 7c	0.6	–	–	–	–	1.0	–	–	–	–	–	1.5	–	1.7	–
C _{19:0} cyclo ω 8c	15.6	19.6 (19.4)	10.4	57.4	32.7	50.2	5.9	47.2	4.3	16.5	39.8	34.9	11.2	57.0	43.2
C _{18:1} 2-OH	4.7	2.6 (2.6)	4.4	1.8	0.6	0.5	1.4	1.5	6.2	–	3.5	1.5	4.7	1.8	–
C _{18:0} 3-OH	0.5	–	–	–	–	0.5	–	–	1.0	–	1.1	–	–	0.5	–
C _{20:2} ω 6,9c	–	–	–	–	–	0.8	–	–	–	–	–	1.8	–	0.9	1.2

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain Ca-34^T, neighbouring *Ochrobactrum* type strains and *Brucella melitensis* biovar *melitensis* ATCC 23456^T. 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 46016^T served as an outgroup. Bar, 2% sequence divergence.

DSM 6882^T, *O. cytisi* DSM 19778^T and *O. lupini* DSM 16930^T) (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-34^T 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-34^T, 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* and some partially characterized *O. tritici* isolates.

Omitting the insert, BLASTN analysis (Altschul *et al.*, 1997) was followed by a thorough phylogenetic analysis by aligning the sequence of strain Ca-34^T 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-34^T 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* SCII24^T, 97.0%; *O. anthropi* LMG 3331^T, 96.8%), ranging between 94.6 and 97%. Distances were

Fig. 2. Representative RAPD profiles of strain Ca-34^T and some strains of *Ochrobactrum* generated with primer OPC-13. Lanes: 1, DNA marker (100 bp); 2, strain Ca-34^T; 3, *O. tritici* DSM 13341; 4, *O. tritici* DSM 13340^T; 5 and 7, *O. lupini* LMG 22726^T; 6, *O. oryzae* DSM 17471^T; 8, *O. gallinifacis* DSM 15295^T; 9, *O. intermedium* DSM 17986^T; 10, *O. anthropi* DSM 6882^T; 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 23456^T 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-34^T and *O. intermedium* LMG 3301^T 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 are recommended (Stackebrandt & Ebers, 2006),

hybridization analysis was performed between strain Ca-34^T and the type strains *O. intermedium* DSM 17986^T and *O. anthropi* DSM 6882^T. 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 17986^T and *O. anthropi* DSM 6882^T. For G+C content determination, DNA was analysed by HPLC (Shimadzu) according to Mesbah *et al.* (1989). The G+C content of strain Ca-34^T was 58.8 mol%, which is within the range 54.5–59.0 mol% reported for other *Ochrobactrum* type strains (Lebuhn *et al.*, 2000; Teyssier *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-34^T and reference *Ochrobactrum* type strains

Strains: 1, strain Ca-34^T; 2, *O. intermedium* DSM 17986^T; 3, *O. anthropi* DSM 6882^T; 4, *O. lupini* DSM 16930^T; 5, *O. cytisi* DSM 19778^T; 6, *O. tritici* DSM 13340^T; 7, *O. grignonense* DSM 13338^T; 8, *O. rhizosphaerae* DSM 19824^T; 9, *O. thiophenivorans* DSM 7216^T; 10, *O. gallinifacis* DSM 15295^T; 11, *O. pseudintermedium* DSM 17490^T; 12, *O. oryzae* DSM 17471^T. The table is arranged according to phylogenetic clustering. All data are from this study (for a detailed comparison, see Supplementary Table S1). +, Positive; –, negative; w, weakly positive.

Assimilation of:	1	2	3	4	5	6	7	8	9	10	11	12
Tween 80	+	–	w	+	+	+	+	+	–	+	+	+
N-Acetyl-D-galactosamine	+	+	+ ^{a†}	+	+	+ ^a	+ ^a	+	+	– ^a	–	+
Adonitol	+	+ ^b	+	+	+	+ [*]	– [*]	–	–	–	+	+
D-Arabitol	+	+	+	+	+	+	+	+	+	–	+	+
Cellobiose	+	+ ^a	+ ^a	+‡	+	– ^a	+‡	–	+	+‡	+	–
Gentiobiose	+	+	+	+	+	– ^b	+ [*]	–	–	–	+	– [*]
L-Fucose	+	+	+	+ ^b	+	+	+	+	+	+	+	+ [*]
myo-Inositol	+	+ [*]	+	+‡	+	+	+	–	+	–	+	+ [*]
D-Mannitol	+	+ [*]	+	+ ^b	+	+	+	+	+	–	–	+ [*]
L-Rhamnose	+	– [*]	+	–‡	+	+	+	+	–	–	+	+ [*]
D-Sorbitol	+	+	+	+	+	+	w	+	–	–	+	+ [*]
Raffinose	–	+	–	–	–	–	–	–	–	–	–	–
Sucrose	+	+	+	+	+	+	–	+	–	–	+	+ [*]
Trehalose	–	+ ^b	+ ^a	+	+	+ [*]	– [*]	+	–	– ^a	+	+
Succinamic acid	–	+	–	w	+	–	–	–	w	w	–	+
Citric acid	+	+ [*]	+	+ ^b	+	+	+	+	–	–	–	– [*]
D-Glucosaminic acid	+	+ ^b	+	+	+	+ [*]	– [*]	–	–	–	+	+
γ-Hydroxybutyric acid	+	+ ^b	+	+	+	– [*]	+ [*]	+	–	–	+	+
p-Hydroxyphenylacetic acid	–	–	–	–	–	–	–	–	+	–	–	+
α-Ketobutyric acid	–	+	–	+	–	w	+	–	–	–	+	+
Sebacic acid	–	– ^b	–	–	–	+ [*]	– [*]	–	–	–	–	+
Glucuronamide	–	+	–	–	–	–	+	–	–	–	–	+
Alaninamide	–	+	+	+	+	+	+	+	+	w	+	+ [*]
L-Threonine	–	+	+	+	+	+	+	+	+	+	+	+ [*]
Uridine	+	–	+	+	–	–	+	+	–	–	+	+
Glycerol	–	– ^b	+	– ^b	–	–	+	+	–	+	–	–

*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).

ing the protocol of Scholz *et al.* (2006, 2008). However, primers reported to amplify more than 100 *Ochrobactrum* strains were unable to give any amplification with DNA from strain Ca-34^T, suggesting that this strain is not closely related to *O. intermedium* DSM 17986^T or *O. anthropi* DSM 6882^T, placed adjacent in the 16S rRNA gene sequence-based dendrogram. Even primers F4 and R2, originally described to be specific for *Brucella* species and strains of *O. intermedium* (Romero *et al.*, 1995; Velasco *et al.*, 1998), did not result in positive amplification with Ca-34^T.

The differences between strain Ca-34^T and *O. intermedium* LMG 3301^T were further confirmed by amplified rDNA restriction analysis (ARDRA) of 16S rRNA genes digested with restriction enzymes *Mbo*I, *Hpa*I, *Bam*HI, *Eco*RI, *Bsu*RI, *Taq*I, *Nci*I, *Pst*I, *Apa*I and *Alu*I (Supplementary Fig. S2) and RAPD fingerprinting (Fig. 2). In each experiment, strain Ca-34^T gave a unique pattern. Two-primers 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. anthropi*, *O. lupini* and *O. tritici* (Trujillo *et al.*, 2005).

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

Description of *Ochrobactrum ciceri* sp. nov.

Ochrobactrum ciceri [ci'ce.ri. L. gen. n. *ciceri* of chickpea (*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. intermedium*, are as follows: glycogen, Tween 80, raffinose, L-rhamnose, trehalose, α -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 *Ochrobactrum* type strains. Resistant to ampicillin (10 µg), aztreonam (30 µg), cefixime (5 µg) and cephradine (30 µg) (β -lactams). The fatty acid profile is composed largely of C_{18:1}ω7c and C_{19:0} cyclo ω8c. In addition, significant amounts of C_{18:0}, C_{16:0} and C_{18:1} 2-OH are present. The DNA G+C content of

the type strain is 58.8 mol%. PCR with *Brucella*- and *O. intermedium*-specific primers F2 and R4 is negative.

The type strain, Ca-34^T (=DSM 22292^T =CCUG 57879^T), was isolated at NIBGE in 1996 from chickpea nodules.

References

- Ali, Z., Cousin, S., Frühling, A., Brambilla, E., Schumann, P., Yang, Y. & Stackebrandt, E. (2009). Description of *Flavobacterium rivuli* sp. nov., *Flavobacterium subsaxonicum* sp. nov., *Flavobacterium swingsii* sp. nov. and *Flavobacterium reichenbachii* sp. nov., isolated from a hard water rivulet. *Int J Syst Evol Microbiol* **59**, 2610–2617.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Bergersen, F. J. (1961). The growth of *Rhizobium* in synthetic media. *Aust J Biol* **14**, 349–360.
- Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.
- Cousin, S., Brambilla, E., Yang, J. & Stackebrandt, E. (2008). Culturable aerobic bacteria from the upstream region of a karst water rivulet. *Int Microbiol* **11**, 91–100.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Doetsch, R. N. (1981). Determinative methods of light microscopy. In *Manual of Methods for General Bacteriology*, pp. 21–33. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Philips. Washington, DC: American Society for Microbiology.
- Hafeez, F. Y., Asad, S., Ahmad, T. & Malik, K. A. (1995). Host specificity and characterization of fast growing rhizobia from *Macroptilium atropurpureum* cv. Siratro in Pakistan. *Soil Biol Biochem* **27**, 729–733.
- Holmes, B., Popoff, M., Kiredjian, M. & Kersters, K. (1988). *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimens and previously known as Group Vd. *Int J Syst Bacteriol* **38**, 406–416.
- Huber, B., Scholz, H. C., Kämpfer, P., Falsen, E., Langer, S. & Busse, H.-J. (2010). *Ochrobactrum pituitosum* sp. nov., isolated from an industrial environment. *Int J Syst Evol Microbiol* **60**, 321–326.
- Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.
- Kämpfer, P., Buczolits, S., Albrecht, A., Busse, H.-J. & Stackebrandt, E. (2003). Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifaecis* sp. nov. *Int J Syst Evol Microbiol* **53**, 893–896.
- Kämpfer, P., Scholz, H. C., Huber, B., Falsen, E. & Busse, H.-J. (2007). *Ochrobactrum haematophilum* sp. nov. and *Ochrobactrum pseudogrignoneense* sp. nov., isolated from human clinical specimens. *Int J Syst Evol Microbiol* **57**, 2513–2518.
- Kämpfer, P., Sessitsch, A., Schlöter, M., Huber, B., Busse, H. J. & Scholz, H. C. (2008). *Ochrobactrum rhizosphaerae* sp. nov. and

- Ochrobactrum thiophenivorans* sp. nov., isolated from the environment. *Int J Syst Evol Microbiol* **58**, 1426–1431.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Labrenz, M., Collins, M. D., Lawson, P. A., Tindall, B. J., Braker, G. & Hirsch, P. (1998). *Antarctobacter heliothermus* gen. nov., sp. nov., a budding bacterium from hypersaline and heliothermal Ekho Lake. *Int J Syst Bacteriol* **48**, 1363–1372.
- Lebuhn, M., Achouak, W., Schlöter, M., Berge, O., Meier, H., Barakat, M., Hartmann, A. & Heulin, T. (2000). Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov. *Int J Syst Evol Microbiol* **50**, 2207–2223.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Ngom, A., Nakagawa, Y., Sawada, H., Tsukahara, J., Wakabayashi, S., Uchiumi, T., Nuntagij, A., Kotepong, S., Suzuki, A. & other authors (2004). A novel symbiotic nitrogen-fixing member of the *Ochrobactrum* clade isolated from root nodules of *Acacia mangium*. *J Gen Appl Microbiol* **50**, 17–27.
- Rivas, R., Velázquez, E., Palomo, J. L., Mateos, P., García-Benavides, P. & Martínez-Molina, E. (2002). Rapid identification of *Clavibacter michiganensis* subspecies *sepedonicus* using two primers random amplified polymorphic DNA (TP-RAPD) fingerprints. *Eur J Plant Pathol* **108**, 179–184.
- Rivas, R., Willems, A., Palomo, J. L., García-Benavides, P., Mateos, F. P., Martínez-Molina, E., Gillis, M. & Velázquez, E. (2004). *Bradyrhizobium betae* sp. nov., isolated from roots of *Beta vulgaris* affected by tumour-like deformations. *Int J Syst Evol Microbiol* **54**, 1271–1275.
- Romero, C., Gamazo, C., Pardo, M. & Lopez-Goni, I. (1995). Specific detection of *Brucella* DNA by PCR. *J Clin Microbiol* **33**, 615–618.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*. MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Scholz, H. C., Tomaso, H., Dahouk, S. A., Witte, A., Schlöter, M. & Kämpfer, P. (2006). Genotyping of *Ochrobactrum anthropi* by *recA*-based comparative sequence, PCR-RFLP, and 16S rRNA gene analysis. *FEMS Microbiol Lett* **257**, 7–16.
- Scholz, H. C., Al Dahouk, S., Tomaso, H., Neubauer, H., Witte, A., Schlöter, M., Kämpfer, P., Falsen, E., Pfeffer, M. & Engel, M. (2008). Genetic diversity and phylogenetic relationships of bacteria belonging to the *Ochrobactrum-Brucella* group by *recA* and 16S rRNA gene-based comparative sequence analysis. *Syst Appl Microbiol* **31**, 1–16.
- Stackebrandt, E. & Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **33**, 152–155.
- Tan, Z.-Y., Xu, X.-D., Wang, E.-T., Gao, J.-L., Martínez-Romero, E. & Chen, W.-X. (1997). Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. *Int J Syst Bacteriol* **47**, 874–879.
- Teyssier, C., Marchandin, H., Siméon de Buochberg, M., Ramuz, R. & Jumas-Bilak, E. (2003). Atypical 16S rRNA gene copies in *Ochrobactrum intermedium* strains reveal a large genomic rearrangement by recombination between *rrn* copies. *J Bacteriol* **185**, 2901–2909.
- Teyssier, C., Marchandin, H., Jean-Pierre, H., Darbas, H., Siméon de Buochberg, M., Diego, I., Gouby, A. & Jumas-Bilak, E. (2005). Molecular and phenotypic features for identification of the opportunistic pathogens *Ochrobactrum* spp. *J Med Microbiol* **54**, 945–953.
- Teyssier, C., Marchandin, H., Jean-Pierre, H., Masnou, A., Dusart, G. & Bilak, E. J. (2007). *Ochrobactrum pseudintermedium* sp. nov., a novel member of the family *Brucellaceae*, isolated from human clinical samples. *Int J Syst Evol Microbiol* **57**, 1007–1013.
- Tien, T. M., Gaskins, M. H. & Hubbel, D. H. (1979). Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Appl Environ Microbiol* **37**, 1016–1024.
- Tripathi, A. K., Verma, S. C., Chowdhury, S. P., Lebuhn, M., Gättinger, A. & Schlöter, M. (2006). *Ochrobactrum oryzae* sp. nov., an endophytic bacterial species isolated from deep-water rice in India. *Int J Syst Evol Microbiol* **56**, 1677–1680.
- Trujillo, M. E., Willems, A., Abril, A., Planchuelo, A. M., Rivas, R., Ludena, D., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2005). Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. *Appl Environ Microbiol* **71**, 1318–1327.
- Valverde, A. M., Benito, M. & Lorenzo, M. (2005). The brown adipose cell: a model for understanding the molecular mechanisms of insulin resistance. *Acta Physiol Scand* **183**, 59–73.
- Velasco, J., Romero, C., López-Gofí, I., Leiva, J., Díaz, R. & Moriyón, I. (1998). Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. *Int J Syst Bacteriol* **48**, 759–768.
- Vincent, J. M. (1970). *A Manual for the Practical Study of the Root Nodule Bacteria*. IBP Handbook no. 15. Oxford: Blackwell Scientific.
- Zurdo-Piñero, J. L., Rivas, R., Trujillo, M. E., Vizcaino, N., Carrasco, J. A., Chamber, M., Palomares, M., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2007). *Ochrobactrum cytisi* sp. nov., isolated from nodules of *Cytisus scoparius* in Spain. *Int J Syst Evol Microbiol* **57**, 784–788.