Rhizobium rosettiiformans sp. nov., isolated from a hexachlorocyclohexane dump site, and reclassification of *Blastobacter aggregatus* Hirsch and Müller 1986 as *Rhizobium aggregatum* comb. nov.

Jaspreet Kaur, Mansi Verma and Rup Lal

Correspondence
Rup Lal
ruplal@gmail.com

Molecular Biology Laboratory, Department of Zoology, University of Delhi, Delhi – 110007, India

A Gram-negative, rod-shaped, motile, aerobic bacterial strain, W3^T, was isolated from hexachlorocyclohexane (HCH)-contaminated groundwater from Lucknow, India, and its taxonomic position was determined using a polyphasic approach. Strain W3^T shared highest 16S rRNA gene sequence similarity of 97.8% with *Rhizobium selenitireducens* B1^T, followed by *Rhizobium daejeonense* L61^T (97.7%), *Rhizobium radiobacter* ATCC 19358^T (97.5%) and *Blastobacter aggregatus* IFAM 1003^T (97.2%). Strain W3^T formed a monophyletic clade with *Blastobacter aggregatus* IFAM 1003^T (=DSM 1111^T) in the cluster of species of the genus *Rhizobium*. Phylogenetic analyses of strain W3^T using *atpD* and *recA* gene sequences confirmed the phylogenetic arrangements obtained by using 16S rRNA gene sequences. Hence, the taxonomic characterization of *B. aggregatus* DSM 1111^T was also undertaken. Strains W3^T and *B. aggregatus* DSM 1111^T contained summed feature 8 (18:1 ω 7c and/or 18:1 ω 6c; 65.4 and 70.8%, respectively) as the major fatty acid, characteristic of the genus *Rhizobium*. DNA–DNA relatedness of strain W3^T with *Rhizobium selenitireducens* LMG 24075^T, *Rhizobium daejeonense* DSM 17795^T, *Rhizobium radiobacter* DSM 30147^T and *B. aggregatus* DSM 1111^T was 42, 34, 30 and 34%, respectively. The DNA G + C contents of strain W3^T and *B. aggregatus* DSM 1111^T were 62.3 and 62.7 mol%, respectively. A *nifH* gene encoding dinitrogenase reductase was detected in strain W3^T but not in *B. aggregatus* DSM 1111^T. Based on the results obtained by phylogenetic and chemotaxonomic analyses, phenotypic characterization and DNA–DNA hybridization, it is concluded that strain W3^T represents a novel species of the genus *Rhizobium*, for which the name *Rhizobium rosettiiformans* sp. nov. is proposed (type strain W3^T =CCM 7583^T =MTCC 9454^T). It is also proposed that *Blastobacter aggregatus* Hirsch and Müller 1986 be transferred to the genus *Rhizobium* as *Rhizobium aggregatum* comb. nov. (type strain IFAM 1003^T =DSM 1111^T =ATCC 43293^T).

In the present study, the objective was to classify a bacterial strain, W3^T, which was isolated from a water sample drawn

Abbreviation: HCH, hexachlorocyclohexane.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *nifH*, *atpD* and *recA* gene sequences of strain W3^T are EU781656, GQ241353, GU559878 and GU562963, respectively. Those for the *atpD* gene sequences of *Blastobacter aggregatus* DSM 1111^T, *Rhizobium daejeonense* DSM 17795^T, *Rhizobium radiobacter* DSM 30147^T and *Rhizobium selenitireducens* LMG 24075^T are GU559879–GU559882, respectively, and those for the *recA* gene sequences of *B. aggregatus* DSM 1111^T, *R. daejeonense* DSM 17795^T, *R. radiobacter* DSM 30147^T and *R. selenitireducens* LMG 24075^T are GU559874–GU559877, respectively.

A supplementary figure and a supplementary table are available with the online version of this paper.

from a hand pump situated in a hexachlorocyclohexane (HCH) dumpsite. The water sample was plated on Luria–Bertani (LB) agar. A cream-coloured colony that appeared within 48 h of incubation at 28 °C was picked and purified by repeated streaking on LB agar. Phylogenetic trees reconstructed using 16S rRNA, *atpD* and *recA* gene sequences showed a close relationship of strain W3^T with *Blastobacter aggregatus* IFAM 1003^T in the *Rhizobium* cluster. Thus, the taxonomic position of *B. aggregatus* was also reassessed.

Species of the genus *Blastobacter* Zavarzin 1961, belonging to the order *Rhizobiales* and family *Bradyrhizobiaceae*, are rod-shaped, commonly aquatic (especially freshwater), budding, rosette-forming and do not fix nitrogen (Rothe *et al.*, 1987; Sly & Hugenholtz, 2005; Balachandar *et al.*,

2007). The genus is in fact polyphyletic, with a high degree of heterogeneity with respect to its phenotypic (Trotsenko *et al.*, 1989; Sittig & Hirsch, 1992), chemotaxonomic and molecular characteristics (Rothe *et al.*, 1987; Willems & Collins, 1992; Hugenholtz *et al.*, 1994). Two of the five species of the genus *Blastobacter* with validly published names have been transferred subsequently to other genera: *Blastobacter natatorius* to *Blastomonas* (Sly & Cahill, 1997) and *Blastobacter denitrificans* to *Bradyrhizobium* (van Berkum *et al.*, 2006), although the new combination '*Bradyrhizobium denitrificans*' has not been validly published. *B. aggregatus* IFAM 1003^T showed a close relationship with *Rhizobium* species on the basis of 16S rRNA gene cataloguing (Rothe *et al.*, 1987), and the need to revise the taxonomy of genus *Blastobacter* has also been suggested (Sly & Cahill, 1997; Doronina & Trotsenko, 2003). On the other hand, species of the genus *Rhizobium* Frank 1889, belonging to the class *Alphaproteobacteria*, order *Rhizobiales*, family *Rhizobiaceae*, are rod-shaped and include free-living species as well as those that form nitrogen-fixing symbioses with roots of leguminous plants. These symbioses are of considerable importance in agriculture and reduce the need for chemical fertilizers. Rhizobia are commonly found in soil, but have also been isolated from water (Zurdo-Piñeiro *et al.*, 2004), non-legume plants (Peng *et al.*, 2008) and bioreactors (Hunter *et al.*, 2007; Quan *et al.*, 2005). Fatty acids common to all species of the genus *Rhizobium* include 16:0, 18:0, summed feature 2 (one or more of 12:0 aldehyde, iso-16:1 I and 14:0 3-OH), summed feature 3 (16:1 ω 7c and/or 16:1 ω 6c) and summed feature 8 (18:1 ω 7c and/or 18:1 ω 6c) (Tighe *et al.*, 2000).

The reference strains used in this study were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (*Blastobacter aggregatus* DSM 1111^T, *Rhizobium daejeonense* DSM 17795^T and *Rhizobium radiobacter* DSM 30147^T) and the Laboratorium voor Microbiologie, Universiteit Gent (*Rhizobium selenitireducens* LMG 24075^T).

Gram staining was performed using a Gram stain kit (HiMedia). Colony morphology was examined on LB agar plates under a light microscope (Myotic 200) after incubation at 28 °C for 48 h. Cells in the early exponential growth phase were used for detecting flagella using transmission electron microscopy as described by Kumar *et al.* (2008).

Genomic DNA was isolated as described by Sambrook *et al.* (1989). A nearly complete (1406 bp) 16S rRNA gene sequence of strain W3^T was obtained according to the method described by Kumar *et al.* (2008). For phylogenetic analyses, 16S rRNA gene sequences of strains of species with validly published names that are closely related to strain W3^T and *B. aggregatus* IFAM 1003^T (Fig. 1) were retrieved from the GenBank database at NCBI (<http://blast.ncbi.nlm.nih.gov/blast.cgi>; Altschul *et al.*, 1997). The selected sequences were aligned using the CLUSTAL_X

software, version 1.81b (Thompson *et al.*, 1997), and terminal nucleotides not common to all sequences were trimmed. The evolutionary distance matrix was calculated using the distance model of Jukes & Cantor (1969) within the TREECONW software package, version 1.3b (Van de Peer & De Wachter, 1994). A phylogenetic tree (Fig. 1) was reconstructed using the neighbour-joining method of Saitou & Nei (1987) and the resultant tree topology was evaluated using bootstrap analysis of 1000 replications. Similar tree topologies were obtained using Kimura's two-parameter model in TREECONW and a DNA parsimony method using PHYLIP, version 3.5 (Felsenstein, 1993) (not shown). The results indicated that strain W3^T and *B. aggregatus* IFAM 1003^T grouped with representatives of the genus *Rhizobium*, with clear separation of *B. aggregatus* IFAM 1003^T from other species of the genus *Blastobacter* with validly published names. Strain W3^T shared 97.8 % 16S rRNA gene sequence similarity with *R. selenitireducens* B1^T, followed by *R. daejeonense* L61^T (97.7 %), *R. radiobacter* ATCC 19358^T (97.5 %) and *B. aggregatus* IFAM 1003^T (97.2 %). Likewise, *B. aggregatus* IFAM 1003^T shared 95.6 % 16S rRNA gene sequence similarity with *R. daejeonense* L61^T and *Blastobacter capsulatus* IFAM 1005^T, followed by *R. selenitireducens* B1^T (95.3 %) and *R. radiobacter* ATCC 19358^T (95.0 %).

To clarify further the taxonomic status of strain W3^T and *B. aggregatus* DSM 1111^T, two housekeeping genes, *atpD* and *recA*, were amplified and sequenced as described by Gaunt *et al.* (2001). Partial *atpD* and *recA* gene sequences of strain W3^T, *B. aggregatus* DSM 1111^T, *R. selenitireducens* LMG 24075^T, *R. daejeonense* DSM 17795^T and *R. radiobacter* DSM 30147^T were obtained. Phylogenetic trees based on *atpD* (Fig. 2) and *recA* (Fig. 3) gene sequences were reconstructed by using the neighbour-joining method of Saitou & Nei (1987). Strain W3^T and *B. aggregatus* DSM 1111^T exhibited 88.6–94.5 % *atpD* gene sequence similarity and 84.5–89.2 % *recA* gene sequence similarity to the reference *Rhizobium* strains used in this study.

To sum up, phylogenetic analyses based on ATP synthase β (*atpD*) and DNA recombinase A (*recA*) gene sequences corroborated the 16S rRNA gene sequence-based phylogeny, thereby providing strong evidence that strain W3^T and *B. aggregatus* DSM 1111^T belong to the genus *Rhizobium*.

Symbiotic properties are important characteristics of rhizobia; hence the presence of symbiotic genes in strain W3^T and *B. aggregatus* DSM 1111^T was examined by amplifying *nifH* (Poly *et al.*, 2001), *nodA* (Haukka *et al.*, 1998), *nodC* (Laguerre *et al.*, 2001) and *nodD* (Rivas *et al.*, 2002). A partial *nifH* gene sequence (344 bp) from strain W3^T showed highest sequence similarity with that of *Azohydromonas lata* IAM 12599^T (93.8 %) followed by *Sinorhizobium* sp. TJ170 (91.8 %) and *Azohydromonas australica* IAM 12664^T (91.0 %). However, no *nifH* gene could be detected in *B. aggregatus* DSM 1111^T. None of the

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain W3^T and *B. aggregatus* IFAM 1003^T and strains of related species. The tree was constructed using the neighbour-joining method and rooted by using *Rhodanobacter lindanoclasticus* RP5557^T as an outgroup. Numbers at nodes represent bootstrap values based on 1000 resamplings. Bar, 0.02 substitutions per nucleotide position.

three *nod* genes could be detected in strain W3^T or *B. aggregatus* DSM 1111^T. Fatty acid methyl ester analysis of strain W3^T, *B. aggregatus* DSM 1111^T, *R. selenitireducens* LMG 24075^T, *R. daejeonense* DSM 17795^T and *R. radiobacter* DSM 30147^T was performed at Disha Institute of Biotechnology Pvt. Ltd (Ahmedabad, India) using the method described by Miller (1982) and Kuykendall *et al.* (1988). The major fatty acid in strain W3^T and *B. aggregatus* DSM 1111^T was summed feature 8 (18:1 ω 7c and/or 18:1 ω 6c). Overall, the fatty acid profiles of strain W3^T and *B. aggregatus* DSM 1111^T (Supplementary Table S1, available in IJSEM Online) were in accordance with those of members of the genus *Rhizobium* (Hunter *et al.*, 2007; Tighe *et al.*, 2000), further supporting the placement of strain W3^T and *B. aggregatus* DSM 1111^T in the genus *Rhizobium*.

DNA–DNA hybridization is considered to be a standard method to affirm species delineation (Wayne *et al.*, 1987). Hence, DNA–DNA hybridization studies were conducted between strain W3^T, *B. aggregatus* DSM 1111^T, *R. selenitireducens* LMG 24075^T, *R. daejeonense* DSM 17795^T

and *R. radiobacter* DSM 30147^T as described by Tourova & Antonov (1987) and Bala *et al.* (2004). DNA (10 μ g) from each strain was transferred onto a positively charged nylon membrane (Hybond-N; Amersham) using a dot-blot apparatus (Bio-Rad). The membrane was air-dried and cross-linked and the DNA probe for each strain was labelled with [α -³²P]ATP (BRIT) using a nick-translation kit (Amersham Pharmacia). Hybridization was performed overnight at 65 °C. After hybridization, the filter was washed with SSC and SDS to remove unbound probe. The amount of probe bound to the DNA was estimated using a β -scintillation counter (Perkin Elmer) and the hybridization values obtained were expressed as percentages of the amount of probe bound relative to the homologous reaction. Since low 16S rRNA gene sequence similarities (<97 %) were found between *B. aggregatus* IFAM 1003^T and type strains of other species of the genera *Rhizobium* and *Blastobacter* with validly published names, DNA–DNA hybridization studies were not performed between *B. aggregatus* DSM 1111^T and closely related type strains. The DNA–DNA relatedness of strain W3^T with *R. selenitireducens* LMG 24075^T, *R. daejeonense* DSM

Fig. 2. Phylogenetic tree based on *atpD* gene sequences showing relationships between strain W3^T and *B. aggregatus* IFAM 1003^T and strains of related species. The tree was constructed using the neighbour-joining method and rooted by using *Bradyrhizobium japonicum* USDA 6^T as an outgroup. Bar, 0.02 substitutions per nucleotide position. Bootstrap values (from 1000 replications) >400 are shown at branching points.

17795^T, *R. radiobacter* DSM 30147^T and *B. aggregatus* DSM 1111^T was 42, 34, 30 and 34 %, respectively. These values are clearly below the recommended threshold value for delineation of genospecies (Stackebrandt & Goebel, 1994), indicating that strain W3^T represents a novel species of the genus *Rhizobium*.

The DNA G + C contents of strain W3^T, *R. selenitireducens* LMG 24075^T, *R. daejeonense* DSM 17795^T, *R. radiobacter* DSM 30147^T and *B. aggregatus* DSM 1111^T were estimated using the procedure of Gonzalez & Saiz-Jimenez (2002) (Table 1). The DNA G + C contents of strain W3^T and *B. aggregatus* DSM 1111^T were found to be 62.3 and

Fig. 3. Phylogenetic tree based on *recA* gene sequences showing relationships between strain W3^T and *B. aggregatus* IFAM 1003^T and strains of related species. The tree was constructed using the neighbour-joining method and rooted by using *Bradyrhizobium japonicum* DSM 30131^T as an outgroup. Bar, 0.02 substitutions per nucleotide position. Bootstrap values (from 1000 replications) >400 are shown at branching points.

Table 1. Differential characteristics of strain W3^T, *B. aggregatus* DSM 1111^T and type strains of related *Rhizobium* species

Strains: 1, strain W3^T; 2, *B. aggregatus* DSM 1111^T (isolated from lake water); 3, *R. selenitireducens* LMG 24075^T (laboratory bioreactor); 4, *R. daejeonense* DSM 17795^T (bioreactor treating cyanide); 5, *R. radiobacter* DSM 30147^T (not known; strains of the species have been isolated from soil, plant rhizospheres, young gall tissues and hairy roots of many plant species); 6, *Blastobacter capsulatus* IFAM 1004^T (pond water; unless indicated, data from Hirsch & Müller, 1985). Data were obtained in this study unless indicated. All strains were positive for catalase and oxidase and utilization of D-glucose and maltose and negative for utilization of cellulose, indole production and hydrolysis of starch and gelatin. +, Positive; –, negative; (+), weakly positive; ND, no data available. DNA G+C contents given in parentheses are values reported in the original species descriptions; ranges represent values for several strains including the type strain.

Characteristic	1	2	3	4	5	6
Cell morphology	Rod	Rod*	Rod	Rod	Rod	Rods, often bent
Flagellation/motility	+	+	+	+	+	–
Colony colour	Cream	Cream	Opaque white	Cream	Light beige	White ^{a†}
Growth in:						
2 % NaCl	+	+	–	+	+	+
3 % NaCl	+	+	–	–	+	+
Citrate utilization	+	–*	–	–	+	–
Nitrate reduction	+	–*	+	–	+	+
Urease	+	+	–	+	+	–
β-Galactosidase	+	+	+	+	–	ND
Utilization as carbon source of:						
Lactose	–	+	+	–	+	+
D-Fructose	–	–*	–§	+	+	+
Raffinose	–	–	–	–	+	ND
D-Mannitol	(+)	+	–	+	+	+
Adonitol	–	+	–	+	+	ND
D-Galactose	–	–*	–§	+	+	+
Utilization as nitrogen source of:						
L-Ornithine	+	+	+	–	+	ND
L-Proline	+	–*	+	+	+	–
L-Serine	+	–*	+	+	+	–
Reduction of methylene blue	+	–	+	+	+	ND
Presence of <i>nifH</i>	+	–	ND	+	ND	ND
DNA G+C content (mol%)	62.3	62.7 (60.4±0.4) ^d	62.9 (60.8) ^e	63.6 (60.1–60.9) ^c	64.3 (57–63) ^f	58.9

*Data in agreement with results reported by Hirsch & Müller (1985).

†Data taken from: a, Sly (1985); b, Sly & Hugenholz (2005); c, Quan *et al.* (2005); d, Hirsch & Müller (1985); e, Hunter *et al.* (2007); f, Sawada *et al.* (1993).

‡Reported as negative by Hunter *et al.* (2007).

§Reported as positive by Hunter *et al.* (2007).

62.7 mol%, respectively, well within the range reported for other *Rhizobium* species (Jordan, 1984).

All phenotypic tests for strain W3^T and *B. aggregatus* DSM 1111^T were performed at 28 °C unless stated otherwise. The ability of the strains to grow at 4–45 °C, pH 4–12 and 0–5 % (w/v) NaCl was determined as described by Arden Jones *et al.* (1979). Strain W3^T showed rich growth on LB agar and tryptic soy agar (TSA) but slow growth was seen on nutrient agar (NA), whereas *B. aggregatus* DSM 1111^T showed good growth on LB agar but slow growth on TSA and NA. Motility of the two cultures was checked on motility agar (Farmer, 1999). Like *B. aggregatus* DSM 1111^T, strain W3^T was found to form rosettes (Supplementary Fig. S1). The two strains were tested for

oxidase activity using Oxidase discs (HiMedia). Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution (Merck) as described by McCarthy & Cross (1984). The ability of the strains to produce melanin was checked as described by Cubo *et al.* (1988). Urease activity was tested using the method of Christensen (1946). Utilization of different carbohydrates was detected by using the HiCarbohydrate kit (HiMedia). Tests for indole production, nitrate reduction and the Voges–Proskauer reaction were performed as described by Smibert & Krieg (1994) and Prakash *et al.* (2007). Hydrolysis of gelatin, starch and aesculin was assessed as described by Cowan & Steel (1965). Citrate utilization was tested on Simmons' citrate agar (Simmons, 1926). Cellulase activity was checked on CEA medium (0.7 %

CM-cellulose, 0.3 % yeast extract, 2.5 % agar) after 5 days of incubation at 28 °C (García-Fraile *et al.*, 2007). The ability of the strains to reduce methylene blue was tested as described by Gao *et al.* (1994). The ability of the strains to produce H₂S was tested using TSI medium (1⁻¹: 3 g beef extract, 3 g yeast extract, 20 g meat peptic digest, 1 g glucose, 10 g lactose, 10 g sucrose, 0.3 g ferric citrate, 5 g NaCl, 0.3 g sodium sulfate, 12 g agar and 0.025 g phenol red; pH 7.4). Differential results of biochemical tests with the closest relatives of strain W3^T and *B. aggregatus* DSM 1111^T are given in Table 1.

While strain W3^T was found to be positive for utilization of citrate, reduction of nitrate and methylene blue, *B. aggregatus* DSM 1111^T was negative for these tests. Both strains were found to be positive for hydrolysis of aesculin and activities of catalase, oxidase and urease. Based on the results obtained, it is proposed that strain W3^T represents a novel species of the genus *Rhizobium*, for which the name *Rhizobium rosettiformans* sp. nov. is proposed. It is also proposed that *Blastobacter aggregatus* be transferred to the genus *Rhizobium* as *Rhizobium aggregatum* comb. nov.

Description of *Rhizobium rosettiformans* sp. nov.

Rhizobium rosettiformans (ro.set.ti.for'mans. N.L. n. *rosetta* (from L. n. *rosa* rose) rosette; L. part. adj. *formans* forming; N.L. part. adj. *rosettiformans* rosette-forming, referring to the ability of the organism to form rosette-shaped structures).

Gram-negative, aerobic, motile, rod-shaped (0.6–0.7 × 1.3–1.5 µm) and rosette-forming. Colonies are cream-coloured, circular with entire margins and 0.5–1.2 mm in diameter when grown on LB agar at 28 °C for 48 h. The presence of a single flagellum is revealed by transmission electron microscopy. Rich growth on TSA but slow growth on NA. Grows at 25–40 °C, pH 5–9 and 0–3 % (w/v) NaCl. Optimum conditions are 28 °C, pH 7.0 and 1.0 % NaCl. Catalase- and oxidase-positive. Positive for hydrolysis of urease, β-galactosidase activity and utilization of D-glucose, maltose, D-mannitol, L-ornithine, L-proline and L-serine. Utilizes citrate and nitrate is reduced. Can grow in medium supplemented with 0.1 % methylene blue. Does not hydrolyse starch or gelatin. Negative for cellulase activity, indole and melanin production and utilization of lactose, D-fructose, raffinose, adonitol and D-galactose. Voges–Proskauer reaction is negative. H₂S is not produced. Sensitive to chloramphenicol (30 µg), gentamicin (10 µg), rifampicin (5 µg) and tetracycline (30 µg). Less sensitive to vancomycin (30 µg) and resistant to kanamycin (30 µg). The major cellular fatty acid is summed feature 8 (18:1ω7c and/or 18:1ω6c), followed by summed feature 2 (one or more of 12:0 aldehyde, iso-16:1 I and 14:0 3-OH), 18:0 3-OH, 18:0, 16:0, 11-methyl 18:1ω7c, summed feature 3 (16:1ω7c and/or 16:1ω6c), iso-18:0 and 17:0. Traces (<1 %) of the following fatty acids are also present: 12:0, 14:0, 17:1ω6c, 17:1ω8c, 18:1ω5c, 20:1ω7c, iso-14:0 3-OH, 15:0 3-OH, 16:0 3-OH, 17:0 3-OH and summed

feature 7 (one or more of 19:1ω6c, 19:1ω7c and 19:0 cyclo). A *nifH* gene encoding a component of the nitrogenase complex is detected. The DNA G+C content of the type strain is 62.3 mol%.

The type strain is W3^T (=CCM 7583^T =MTCC 9454^T), isolated from an HCH dump site in India.

Description of *Rhizobium aggregatum* comb. nov.

Rhizobium aggregatum (ag.gre.ga'tum. L. neut. adj. *aggregatum* joined together, referring to the frequent formation of rosettes).

Basonym: *Blastobacter aggregatus* Hirsch and Müller 1986.

Cells are Gram-negative, aerobic, motile, rod-shaped (0.5–0.6 × 1.8–2.1 µm) and rosette-forming. Colonies are cream-coloured, circular with entire margins and 0.5–1.0 mm in diameter when grown on LB agar at 28 °C for 48 h. The presence of a single flagellum is revealed by transmission electron microscopy. Good growth occurs on LB agar but growth is slow on TSA and NA. Grows at 25–40 °C, pH 6–10 and 0–3 % (w/v) NaCl. Optimum conditions are 28 °C, pH 7.0 and 1.0 % NaCl. Positive for β-galactosidase, urease, catalase and oxidase activities. Positive for utilization of D-glucose, maltose, lactose, D-mannitol, adonitol and L-ornithine. Does not hydrolyse starch or gelatin and nitrate is not reduced. Negative for cellulase activity, indole and melanin production and utilization of D-fructose, raffinose, D-galactose, L-proline and L-serine. Negative for utilization of citrate. Cannot grow in medium supplied with 0.1 % methylene blue. Voges–Proskauer reaction is negative. H₂S is not produced. Sensitive to chloramphenicol (30 µg), rifampicin (5 µg) and tetracycline (30 µg). Less sensitive to gentamicin (10 µg) and vancomycin (30 µg) and resistant to kanamycin (30 µg). The major cellular fatty acid is summed feature 8 (18:1ω7c and/or 18:1ω6c), followed by summed feature 2 (one or more of 12:0 aldehyde, iso-16:1 I and 14:0 3-OH), 11-methyl 18:1ω7c, 16:0, 18:0, 18:0 3-OH, summed feature 3 (16:1ω7c and/or 16:1ω6c) and iso-18:0. Traces (<1 %) of the following fatty acids are also present: 12:0, 14:0, 17:0, 17:1ω6c, 17:1ω8c, 18:1ω5c, 20:1ω7c, iso-14:0 3-OH, 15:0 3-OH, 16:0 3-OH, 17:0 3-OH and summed feature 7 (one or more of 19:1ω6c, 19:1ω7c and 19:0 cyclo). The *nifH* gene cannot be detected in the type strain. The DNA G+C content of the type strain is 62.7 mol%.

The type strain is IFAM 1003^T (=DSM 1111^T =ATCC 43293^T), isolated from Lake Hötsee (Holstein, Germany).

Acknowledgements

This work was supported by grants from the Department of Biotechnology (DBT) and the National Bureau of Agricultural Important Microorganisms (NBAIM), Government of India. J.K. and M.V. acknowledge DBT and CSIR, Government of India, respectively, for providing research fellowships. We gratefully

acknowledge the Department of Anatomy, AIIMS, India, for providing the transmission electron microscopy facility. We would also like to thank J. P. Euzéby for etymological advice and Lisa Leukel for providing the paper containing the original description of *B. aggregatus*.

References

- 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.
- Arden Jones, M. P., McCarthy, A. J. & Cross, T. (1979). Taxonomic and serological studies on *Micropolyspora faeni* and *Micropolyspora* strains from soil bearing the specific epithet *rectivirgula*. *J Gen Microbiol* **115**, 343–344.
- Bala, S., Khanna, R., Dadhwal, M., Prabakaran, S. R., Shivaji, S., Cullum, J. & Lal, R. (2004). Reclassification of *Amycolatopsis mediterranei* DSM 46095 as *Amycolatopsis rifamycinica* sp. nov. *Int J Syst Evol Microbiol* **54**, 1145–1149.
- Balachandrar, D., Raja, P., Kumar, K. & Sundaram, S. P. (2007). Non-rhizobial nodulation in legumes. *Biotechnol Mol Biol Rev* **2**, 49–57.
- Christensen, W. B. (1946). Urea decomposition as a means of differentiating *Proteus* and *Paracolon* cultures from each other and from *Salmonella* and *Shigella* types. *J Bacteriol* **52**, 461–466.
- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.
- Cubo, M. T., Buendia-Claveria, A. M., Beringer, J. E. & Ruiz-Sainz, J. E. (1988). Melanin production by *Rhizobium* strains. *Appl Environ Microbiol* **54**, 1812–1817.
- Doronina, N. V. & Trotsenko, Y. A. (2003). Reclassification of '*Blastobacter viscosus*' 7d and '*Blastobacter aminooxidans*' 14a as *Xanthobacter viscosus* sp. nov. and *Xanthobacter aminooxidans* sp. nov. *Int J Syst Evol Microbiol* **53**, 179–182.
- Farmer, J. J., III (1999). Enterobacteriaceae: introduction and identification. In *Manual of Clinical Microbiology*, 7th edn, pp. 442–458. Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover & R. H. Tenover. Washington, DC: American Society for Microbiology.
- Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Department of Genome Sciences, University of Washington, Seattle, USA.
- Frank, B. (1889). Über die Pilzsymbiose der Leguminosen. *Ber Dtsch Bot Ges* **7**, 332–346. (in German).
- Gao, J. L., Sun, J. G., Li, Y., Wang, E. T. & Chen, W. X. (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province, China. *Int J Syst Bacteriol* **44**, 151–158.
- García-Fraile, P., Rivas, R., Willems, A., Peix, A., Martens, M., Martínez-Molina, E., Mateos, P. F. & Velázquez, E. (2007). *Rhizobium cellulosilyticum* sp. nov., isolated from sawdust of *Populus alba*. *Int J Syst Evol Microbiol* **57**, 844–848.
- Gaunt, M. W., Turner, S. L., Rigottier-Gois, L., Lloyd-Macgilp, S. A. & Young, J. P. (2001). Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *Int J Syst Evol Microbiol* **51**, 2037–2048.
- Gonzalez, J. M. & Saiz-Jimenez, C. (2002). A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. *Environ Microbiol* **4**, 770–773.
- Haukka, K., Lindström, K. & Young, J. P. W. (1998). Three phylogenetic groups of *nodA* and *nifH* genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. *Appl Environ Microbiol* **64**, 419–426.
- Hirsch, P. & Müller, M. (1985). *Blastobacter aggregatus* sp. nov., *Blastobacter capsulatus* sp. nov., and *Blastobacter denitrificans* sp. nov., new budding bacteria from freshwater habitats. *Syst Appl Microbiol* **6**, 281–286.
- Hugenholtz, P., Stackebrandt, E. & Fuerst, J. A. (1994). A phylogenetic analysis of the genus *Blastobacter* with a view to its future reclassification. *Syst Appl Microbiol* **17**, 51–57.
- Hunter, W. J., Kuykendall, L. D. & Manter, D. K. (2007). *Rhizobium selenireducens* sp. nov.: a selenite-reducing alpha-Proteobacteria isolated from a bioreactor. *Curr Microbiol* **55**, 455–460.
- Jordan, D. C. (1984). Family III. *Rhizobiaceae* Conn 1938, 321^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–242. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kumar, M., Verma, M. & Lal, R. (2008). *Devosia chinhatensis* sp. nov., isolated from a hexachlorocyclohexane (HCH) dump site in India. *Int J Syst Evol Microbiol* **58**, 861–865.
- Kuykendall, L. D., Roy, M. A., O'Neill, J. J. & Devine, T. E. (1988). Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Bacteriol* **38**, 358–361.
- Laguerre, G., Nour, S. M., Macheret, V., Sanjuan, J., Drouin, P. & Amarger, N. (2001). Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. *Microbiology* **147**, 981–993.
- McCarthy, A. J. & Cross, T. (1984). A taxonomic study of *Thermomonospora* and other monosporic actinomycetes. *J Gen Microbiol* **130**, 5–25.
- Miller, L. T. (1982). Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *J Clin Microbiol* **16**, 584–586.
- Peng, G., Yuan, Q., Li, H., Zhang, W. & Tan, Z. (2008). *Rhizobium oryzae* sp. nov., isolated from the wild rice *Oryza alta*. *Int J Syst Evol Microbiol* **58**, 2158–2163.
- Poly, F., Monrozier, L. J. & Bally, R. (2001). Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol* **152**, 95–103.
- Prakash, O., Kumari, K. & Lal, R. (2007). *Pseudomonas delhiensis* sp. nov., from a fly ash dumping site of a thermal power plant. *Int J Syst Evol Microbiol* **57**, 527–531.
- Quan, Z. X., Bae, H. S., Baek, J. H., Chen, W. F., Im, W. T. & Lee, S. T. (2005). *Rhizobium daejeonense* sp. nov. isolated from a cyanide treatment bioreactor. *Int J Syst Evol Microbiol* **55**, 2543–2549.
- Rivas, R., Velázquez, E., Willems, A., Vizcaino, N., Subba-Rao, N. S., Mateos, P. F., Gillis, M., Dazzo, F. B. & Martínez-Molina, E. (2002). A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f.) Druce. *Appl Environ Microbiol* **68**, 5217–5222.
- Rothe, B., Fischer, A., Hirsch, P., Sittig, M. & Stackebrandt, E. (1987). The phylogenetic position of the budding bacteria *Blastobacter aggregatus* and *Gemmobacter aquatilis* gen. nov. sp. nov. *Arch Microbiol* **147**, 92–99.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

- Sawada, H., Ieki, H., Oyaizu, H. & Matsumoto, S. (1993).** Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. *Int J Syst Bacteriol* **43**, 694–702.
- Simmons, J. S. (1926).** A culture medium for differentiating organisms of typhoid-colon aerogenes groups and for isolating of certain fungi. *J Infect Dis* **39**, 209–241.
- Sittig, M. & Hirsch, P. (1992).** Chemotaxonomic investigation of budding and hyphal bacteria. *Syst Appl Microbiol* **15**, 209–222.
- Sly, L. I. (1985).** Emendation of the genus *Blastobacter* Zavarzin 1961 and description of *Blastobacter natatorius* sp. nov. *Int J Syst Bacteriol* **35**, 40–45.
- Sly, L. I. & Cahill, M. M. (1997).** Transfer of *Blastobacter natatorius* (Sly 1985) to the genus *Blastomonas* gen. nov. as *Blastomonas natatoria* comb. nov. *Int J Syst Bacteriol* **47**, 566–568.
- Sly, L. I. & Hugenholtz, P. (2005).** Genus IV. *Blastobacter* Zavarzin 1961, 962^{AL} emend. Sly 1985, 44. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2C, pp. 452–459. Edited by D. R. Boone, R. W. Castenholz, D. H. Bergey, G. M. Garrity & J. T. Staley. New York: Springer.
- Smibert, R. M. & Krieg, N. R. (1994).** Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Tighe, S. W., de Lajudie, P., Dipietro, K., Lindström, K., Nick, G. & Jarvis, B. D. (2000).** Analysis of cellular fatty acids and phenotypic relationships of *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* species using the Sherlock Microbial Identification System. *Int J Syst Evol Microbiol* **50**, 787–801.
- Tourova, T. P. & Antonov, A. S. (1987).** Identification of microorganisms by rapid DNA-DNA hybridization. *Methods Microbiol* **19**, 333–355.
- Trotsenko, Y. A., Doronina, N. V. & Hirsch, P. (1989).** Genus *Blastobacter*. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 1963–1968. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.
- van Berkum, P., Leibold, J. M. & Eardly, B. D. (2006).** Proposal for combining *Bradyrhizobium* spp. (*Aeschynomene indica*) with *Blastobacter denitrificans* and to transfer *Blastobacter denitrificans* (Hirsch and Muller, 1985) to the genus *Bradyrhizobium* as *Bradyrhizobium denitrificans* (comb. nov.). *Syst Appl Microbiol* **29**, 207–215.
- Van de Peer, Y. & De Wachter, R. (1994).** TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**, 569–570.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Willems, A. & Collins, M. D. (1992).** Evidence for a close genealogical relationship between *Afipia* (the causal organism of cat scratch disease), *Bradyrhizobium japonicum* and *Blastobacter denitrificans*. *FEMS Microbiol Lett* **96**, 241–246.
- Zavarzin, G. A. (1961).** [Budding bacteria]. *Mikrobiologiya* **30**, 952–975 (in Russian).
- Zurdo-Piñero, J. L., Velázquez, E., Lorite, M. J., Brelles-Mariño, G., Schröder, E. C., Bedmar, E. J., Mateos, P. F. & Martínez-Molina, E. (2004).** Identification of fast-growing rhizobia nodulating tropical legumes from Puerto Rico as *Rhizobium gallicum* and *Rhizobium tropici*. *Syst Appl Microbiol* **27**, 469–477.