Microvirga guangxiensis sp. nov., a novel alphaproteobacterium from soil, and emended description of the genus *Microvirga*

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A Gram-negative-staining bacterium, designated strain 25B^T, was isolated from a soil sample from a rice field in Guangxi Province, China, and its taxonomic position was investigated by using a polyphasic approach. Cells were rod-shaped, non-spore-forming, non-motile and strictly aerobic. Strain 25B^T grew optimally at 37 °C and pH 7.0. The predominant fatty acids of this soil isolate were C_{18:1}ω7c, C_{19:0} cyclo ω8c and C_{16:0}. Phylogenetic analysis based on the almost-complete 16S rRNA gene sequence showed that strain 25B^T formed a monophyletic clade with the type strain of *Microvirga subterranea*; the two organisms shared 97.2% 16S rRNA gene sequence similarity. However, the two strains shared low DNA–DNA relatedness. Strain 25B^T was also readily distinguishable from *Microvirga subterranea* DSM 14364^T by various phenotypic characteristics. The combination of genotypic and phenotypic data suggests that the isolate represents a novel species of the genus *Microvirga*, for which the name *Microvirga guangxiensis* sp. nov. is proposed. The type strain is 25B^T (=CGMCC 1.7666^T=JCM 15710^T).

The genus *Microvirga* was established by Kanso & Patel (2003) to accommodate strictly aerobic, Gram-negative, non-sporulating, motile and rod-shaped bacteria from free-flowing geothermal waters of a bore tapping the Great Artesian Basin of Australia, which grew optimally at 41 °C and pH 7.0 and had an absolute requirement for yeast extract. The genus currently encompasses a single species with a validly published name, *Microvirga subterranea*, and is assigned to the class *Alphaproteobacteria*, phylum *Proteobacteria* (Garrity & Holt, 2001; Garrity *et al.*, 2005a), almost equidistant from *Methylobacterium* species (Patt *et al.*, 1976; Urakami *et al.*, 1993), *Chelatococcus asaccharovorans* (Auling *et al.*, 1993; Egli *et al.*, 1988) and *Bosea thiooxidans* (Das *et al.*, 1996), its nearest phylogenetic relatives, with a mean similarity value of 93 %.

A sampling campaign during an ecological survey of soil bacteria in China led to the isolation of a pink-pigmented bacterium, designated strain 25B^T. The soil sample (10 cm in depth, pH 6.2) was collected from a rice field in Guangxi Province. A glucose-yeast extract-malt extract (GYM) agar plate (comprising, l⁻¹: 4 g D-glucose, 4 g yeast extract, 10 g

malt extract, 15 g agar; pH 7.3) was inoculated with a suspension of the soil and incubated at 37 °C for 7 days. The isolate that was obtained, strain 25B^T, was maintained on LB agar (l⁻¹: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar; pH 7.0) at 4 °C and as glycerol suspensions (20%, v/v) at –20 °C. Biomass for the chemotaxonomic and molecular systematic studies was prepared as described previously (Zhang *et al.*, 2002) with the modification that the strain was grown in shake flasks of LB broth.

Micromorphological and colonial properties of strain 25B^T were examined on LB agar, Rourf's agar (Mulder & Deinema, 1992) and GYM agar after incubation at 37 °C for 3–7 days. Phase-contrast and electron microscopy of cells were performed as described by Andrews & Patel (1996). The presence of flagella and spores was assessed by the method of Das *et al.* (1996) and Kanso & Patel (2003). The presence of poly-β-hydroxybutyrate (PHB) was demonstrated by staining lightly heat-fixed cells with Sudan Black B [0.3% (w/v) in 70% (v/v) ethanol] for 10 min followed by decolourizing with xylene for 10 s, drying and counterstaining with safranin [0.5% (w/v) in water] for 1–2 min.

Strain 25B^T was examined for a range of phenotypic properties. Tests for acid production from carbohydrates and utilization of sole carbon sources were carried out according to procedures described elsewhere (Das *et al.*, 1996; Kanso & Patel, 2003; Zhang *et al.*, 2008). Catalase

Abbreviation: PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 25B^T is EU727176.

A table showing the fatty acid contents of strain 25B^T and the type strain of *Microvirga subterranea* is available as supplementary material with the online version of this paper.

activity was determined on freshly grown colonies using 3% (v/v) hydrogen peroxide solution. Determination of starch and casein hydrolysis and oxidative or fermentative catabolism followed the methods described by Smibert & Krieg (1994) and La Scola *et al.* (2003). Growth with various pH, temperature and sodium chloride regimes was determined on LB agar plates incubated for up to 3 days. Anaerobic growth was tested by using TYEG medium (Wynter *et al.*, 1996). Resistance to lysozyme was determined by using the method of Gordon *et al.* (1974). Resistance to antibiotics was examined by placing impregnated filter-paper discs (Goodfellow & Orchard, 1974) on LB agar; the results were recorded following incubation at 37 °C for up to 3 days. *Microvirga subterranea* DSM 14364^T was used as the control.

Genomic DNA preparation, PCR-mediated amplification of the 16S rRNA gene sequence and purification of the PCR product from strain 25B^T were carried out after Rainey *et al.* (1996). The PCR product was sequenced directly using the method of Lu *et al.* (2001). Sequence gel electrophoresis was performed and the nucleotide sequences were obtained automatically by using an Applied Biosystems DNA Sequencer (model 377) with the software provided by the manufacturer. The resultant 16S rRNA gene sequence was aligned manually against corresponding sequences of representatives of genera classified in the order *Rhizobiales* (Kuykendall, 2005) that had been retrieved from the GenBank/EMBL/DDBJ databases using CLUSTAL X 1.8 software (Thompson *et al.*, 1997). Phylogenetic trees were inferred using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Saitou & Nei, 1987) algorithms. Evolutionary distance matrices were generated after Kimura (1980). The resultant unrooted topologies were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining dataset, based on 1000 resamplings, using the SEQBOOT and CONSENSE options. The PHYLIP package (Felsenstein, 1993) was used for all phylogenetic analyses.

Respiratory lipoquinones were extracted and purified after Das *et al.* (1996) and determined by using reversed-phase HPLC (Wu *et al.*, 1989). The fatty acids were extracted, purified, methylated and quantified by GC using the standard Microbial Identification System (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). The G+C content of the DNA was determined using the thermal denaturation method (Marmur & Doty, 1962) with *Escherichia coli* AS 1.365 as the standard. DNA–DNA relatedness between strain 25B^T and *Microvirga subterranea* DSM 14364^T was determined using a thermal denaturation procedure (De Ley *et al.*, 1970; Huß *et al.*, 1983) with a UV-1206 spectrophotometer (Shimadzu) fitted with a TB-85 thermostath and standard software (Jahnke, 1992).

An almost-complete 16S rRNA gene sequence (1397 nt) was obtained for strain 25B^T. The phylogenetic analysis showed that the sequence contained all of the signature

nucleotides that are expected for members of the order *Rhizobiales* and, more specifically, those that are characteristic of members of the family *Methylobacteriaceae* (Garrity *et al.*, 2005b) and the genus *Microvirga* (Kanso & Patel, 2003). The high 16S rRNA gene sequence similarity between strain 25B^T and *Microvirga subterranea* DSM 14364^T (97.2%) provides further support for the isolate's assignment to the genus *Microvirga*.

The classification of strain 25B^T in the genus *Microvirga* was supported by morphological and phenotypic characteristics. The strain was a strictly aerobic, Gram-negative-staining bacterium. Colonies (0.5–1.5 mm) were light pink, circular and convex with entire smooth edges and shiny surfaces, thin in texture and easily emulsifiable. The rod-shaped cells (0.6–0.8 × 1.3–2.1 µm) with round ends occurred singly and as pairs, tetrads and packets. Large intracellular granules in cells from ageing cultures were observed with phase-contrast microscopy and staining revealed darkly stained PHB granules. Spores were not observed and the notion that strain 25B^T was a non-spore-former was supported by its sensitivity to heat (70 °C for 10 min). The organism did not grow anaerobically in TYEG medium. The doubling time of strain 25B^T under optimal growth conditions was 230 min. Ubiquinone 10 was the major ubiquinone. The non-hydroxylated fatty acid profile of strain 25B^T consisted of straight chain and cyclo saturated, unsaturated and 11-methyl-branched components, mainly containing C_{18:1}ω7c (41.8%), C_{19:0} cyclo ω8c (32.1%), C_{16:0} (9.0%), C_{18:0} (2.8%), C_{18:1}ω7c 11-methyl (1.8%), C_{17:0} (1.6%), C_{17:0} cyclo (1.4%), C_{20:2}ω6,9c (1.2%), C_{18:0} 3-OH (1.1%). The fatty acid pattern was similar to that of *Microvirga subterranea* DSM 14364^T but there were notable differences in the levels of C_{18:1}ω7c 11-methyl and C_{17:0} (see Supplementary Table S1, available in IJSEM Online). In addition, the DNA of strain 25B^T was rich in guanine and cytosine (G+C content 64.3 mol%).

The position of strain 25B^T in the unrooted 16S rRNA gene sequence tree, based on three algorithms, is shown in Fig. 1. Strain 25B^T was most closely related to and formed a monophyletic clade with *Microvirga subterranea* DSM 14364^T, and bootstrap analysis gives a high degree of confidence to this relationship (100%). The 16S rRNA gene similarity value between strain 25B^T and *Microvirga subterranea* DSM 14364^T was 97.2% (35 differences over 1397 nucleotide positions). Strain 25B^T was also closely related to *B. thiooxidans* BI 42^T (93.5% similarity; 87 differences), *Methylobacterium suomiense* F20^T (93.3%; 88 differences) and *C. asaccharovorans* TE2^T (93.1%; 90 differences). The mean DNA–DNA relatedness value between the novel isolate and the type strain of *Microvirga subterranea* was 21.7%, a value well below the 70% cut-off point recommended by Wayne *et al.* (1987) for the delineation of genomic species. A range of phenotypic properties separated strain 25B^T from *Microvirga subterranea* DSM 14364^T (Table 1), in particular with its ability to utilize arabinol, (+)-D-glucose, (+)-

Fig. 1. An unrooted neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the position of strain 25B^T. Percentages at nodes (>50%) are levels of bootstrap support based on 1000 resamplings. *, Node recovered using the least-squares and maximum-likelihood algorithms; f, node recovered using the least-squares method. Bar, 0.02 substitutions per nucleotide position.

D-mannitol, (+)-melezitose, (+)-D-sorbitol and malonate as carbon sources as well as its inability to hydrolyse gelatin. *Microvirga subterranea* DSM 14364^T had a restricted substrate range for growth in comparison with strain 25B^T and, unlike *Microvirga subterranea* DSM 14364^T, strain 25B^T did not have an absolute requirement for yeast extract. Salt tolerance was another characteristic feature for phenotypic differentiation: strain 25B^T grew in the presence of 2% (w/v) NaCl and *Microvirga subterranea* DSM 14364^T tolerated 1% NaCl and did not grow in the presence of 2% NaCl.

The genotypic and phenotypic data show that strain 25B^T merits recognition as a representative of a novel species of the genus *Microvirga*. It is, therefore, proposed that the isolate be classified as *Microvirga guangxiensis* sp. nov.

Emended description of the genus *Microvirga* Kanso and Patel 2003

Strictly aerobic, small, rod-shaped cells that stain Gram-negative and produce light-pink colonies. The optimum temperature for growth is 37–41 °C, with a temperature range for growth between 16 and 45 °C. Grows with or without glucose but uses xylose. Reduces nitrate to nitrite. Cells are susceptible to antibiotics that inhibit members of domain *Bacteria*. Bacteriochlorophyll is absent but carotenoids are present. PHB is produced. Thiosulfate does not stimulate growth. The G+C content of the DNA is 63–65 mol%. 16S rRNA gene sequence analysis indicates that the genus is a member of the family *Methylobacteriaceae*, class *Alphaproteobacteria*, phylum *Proteobacteria*, placed almost equidistantly between *C. asaccharovorans* and *B.*

thiooxidans, members of the families *Beijerinckiaceae* and *Bradyrhizobiaceae*, respectively, which are its nearest phylogenetic relatives. The type species is *Microvirga subterranea*.

Description of *Microvirga guangxiensis* sp. nov.

Microvirga guangxiensis (gu.ang.xi.en'sis. N.L. fem. adj. *guangxiensis* referring to Guangxi Province, China, the source of the soil from which the species was first isolated).

The species description is based on a single strain. In addition to the properties described for the genus, the following properties apply. Cells are non-motile rods, 0.6–0.8 × 1.3–2.1 µm, occurring singly, and as pairs, tetrads and packets. Spores are not formed. Cells from old cultures are pleomorphic and contain large intracellular granules (PHB). Colonies are light pink, convex, smooth, mucoid and circular with entire margins, 0.5–1.5 mm in diameter after 3 days at 37 °C on LB agar. Grows well on Rourf's agar and GYM agar. Diffusible pigments are not formed. Catalase-, lipase- and oxidase-positive. Negative for arginine dihydrolase and lysine decarboxylase. Grows at 16–42 °C (optimum, 37 °C) and pH 5.0–9.5 (optimum, pH 7.0). Urea is hydrolysed, but not aesculin, casein, gelatin or starch. Nitrate is reduced. Indole, methyl red and Voges–Proskauer reactions are negative. Hydrogen sulfide is not produced. Very weak acid production is detected from (+)-D-arabinose but not from (+)-D-glucose, (+)-maltose, (+)-D-mannitol, peptone or (+)-D-xylose. Arabitol, (+)-D-glucose, *myo*-inositol (weak), (+)-maltose (weak), (+)-D-mannitol, (+)-melezitose, (+)-melibiose (weak), peptone, (+)-D-sorbitol, (+)-D-xylose,

Table 1. Characteristics that distinguish strain 25B^T (*Microvirga guangxiensis* sp. nov.) from the type strain of *Microvirga subterranea*

Data are from this study and Kanso & Patel (2003). Both strains are rod-shaped and positive for utilization of D-xylose and acetate as sole carbon sources. Both strains are negative for hydrolysis of casein and starch, stimulation of growth by thiosulfate and utilization of *N,N*-dimethylformamide, ethanol, D-fructose, glycerol and citrate as sole carbon sources. +, Positive; –, negative; w, weak.

Characteristic	Strain 25B ^T	<i>Microvirga subterranea</i> DSM 14364 ^T
Isolation source	Soil	Water
Cell dimensions (µm)	0.6–0.8 × 1.3–2.1	1.0 × 1.5–4.0
Conditions for growth		
Temperature range (°C)	16–42	25–45
Optimum temperature (°C)	37	41
NaCl tolerance (% w/v)	<2	<1
Enzyme activities		
Oxidase	+	–
Urease	+	–
Hydrolysis of gelatin	–	+
Carbon source utilization		
Arabitol	+	–
D-Glucose	+	–
myo-Inositol	w	–
Malonate	+	–
D-Mannitol	+	–
Melezitose	+	–
D-Sorbitol	+	–
Acid production from D-glucose	–	w
DNA G + C content (mol%)	64.3	63.5 ± 0.5

acetate, fumarate, gluconate (weak), malate and malonate are used as sole carbon and energy sources, but not (+)-D-arabinose, (+)-cellobiose, *N,N*-dimethylformamide, ethanol, (+)-D-fructose, (+)-D-galactose, methyl α-D-glucopyranoside, glycerol, (+)-lactose, α-L-rhamnose, (+)-trehalose, benzoate, citrate, oxalate or tartrate. Growth occurs in the presence of sodium chloride at 2 %, but not at 2.5 % (w/v). Resistance is shown to (µg per disc unless otherwise stated) aztreonam (30), erythromycin (15) and kanamycin sulphate (30). Susceptible to amikacin (30), amoxicillin plus clavulanic acid (10), ampicillin (10), cefotaxime (30), ciprofloxacin (5), clindamycin hydrochloride (2), mezlocillin (75), ofloxacin (5), penicillin G (10 IU), rifampicin (5), streptomycin sulphate (10), tetracycline hydrochloride (30), tobramycin sulphate (10) and lysozyme (0.005 %, w/v). Major fatty acids are C_{18:1}ω7c, C_{19:0} cyclo ω8c and C_{16:0}. Ubiquinone 10 is the major ubiquinone. The DNA G + C content of the type strain is 64.3 mol%.

The type strain, 25B^T (=CGMCC 1.7666^T=JCM 15710^T), was isolated from a rice-field soil sample collected in Guangxi Province, China.

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