Pseudoxanthobacter soli gen. nov., sp. nov., a nitrogen-fixing alphaproteobacterium isolated from soil

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A Gram-type negative or variable, aerobic, rod-shaped, nitrogen-fixing bacterium, designated strain CC4^T, was isolated on nutrient agar from a soil sample collected at a regional agricultural research field station located in Kaohsiung County, Taiwan. 16S rRNA gene sequence analysis demonstrated that this isolate is unique, showing <92.5% similarity with respect to species of the genera *Xanthobacter* (maximum of 92.2% similarity), *Prosthecomicrobium* (92.0%), *Devosia* (91.9%), *Kaistia* (91.9%) and *Methylocystis* (91.9%). The organism utilized acetic acid, formic acid, β -hydroxybutyric acid, DL-lactic acid, succinic acid, bromosuccinic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid and L-proline as substrates, but not methanol or methylamine. Chemotaxonomic data revealed that strain CC4^T contains ubiquinone Q-10 as the major respiratory quinone. The characteristic diamino acid of the peptidoglycan was *meso*-diaminopimelic acid. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, phosphatidylmonomethylethanolamine, phosphatidylmethylethanolamine, an unknown lipid and an unknown aminolipid. The fatty acid profile differed from those of members of the genera *Xanthobacter*, *Prosthecomicrobium*, *Devosia* and *Kaistia*. The predominant fatty acids were C_{18:1} ω 7c, C_{19:0} cyclo and C_{16:0}. On the basis of 16S rRNA gene sequence analysis in combination with chemotaxonomic and physiological data, strain CC4^T represents a novel genus and species, for which we propose the name *Pseudoxanthobacter soli* gen. nov., sp. nov. The type strain of *Pseudoxanthobacter soli* is CC4^T (=DSM 19599^T =CIP 109513^T).

During the characterization of micro-organisms from soil samples from the regional agricultural research station located in Kaohsiung County, Taiwan, strain CC4^T was isolated and maintained on nutrient agar (NA; Oxoid) after incubation at 32 °C for 3 days. Subcultivation was done on NA at 30 °C for 24 h to 3 days. On this medium, strain CC4^T was able to grow at 15–36 °C but not at 40 or 10 °C. The organism was able to grow on NA, tryptone soy agar, yeast extract-mannitol agar and R2A agar (Oxoid). Gram

stain set S (Difco) and the Ryu non-staining KOH method (Powers, 1995) were used to ascertain the Gram reaction. Poly- β -hydroxybutyrate granule accumulation was observed under light microscopy after staining of the cells with Sudan black.

Samples for scanning electron microscopy were prepared according to the method of Hahn *et al.* (1998) except that 4% glutaraldehyde was used to fix the cells. A TOPCON ABT-150S field emission scanning electron microscope was used to examine cells of strain CC4^T (see Supplementary Fig. S1, available in IJSEM Online). The following phenotypic features were investigated: biochemical characteristics, carbon-source utilization (GN2; Biolog), API

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CC4^T is EF465533.

A scanning electron micrograph of strain CC4^T is available as supplementary material with the online version of this paper.

ZYM enzyme profile (bioMérieux) and API 20E profile (bioMérieux). Additionally, antibiotic susceptibilities were tested using ATB STAPH 5 strips (bioMérieux) according to the manufacturer's recommendations. Fluorescence was tested after cells were plated on King's medium B for 48 h.

The diaminopimelic acid isomer in whole-cell hydrolysates (4 M HCl, 100 °C, 16 h) was determined using one-dimensional TLC (Rhuland *et al.*, 1955) on cellulose plates. Polar lipids extracted according to the method of Minnikin *et al.* (1979) were identified using two-dimensional TLC and spraying with specific reagents (Collins & Jones, 1980). Ubiquinones were analysed as described by Tindall (1990a, b). Fatty acid methyl esters were obtained from cells cultivated on tryptic soy agar for 48 h at 28 °C by saponification, methylation and extraction as described previously (Kämpfer & Kroppenstedt, 1996) and were separated in a gas chromatograph (model 5898A; Hewlett Packard). Peaks were automatically integrated and fatty acid names and percentages were determined using the Microbial Identification standard software package (MIDI, Inc.) (Sasser, 1990).

DNA for the determination of the G+C content was isolated by using the UltraClean microbial DNA-isolation kit (MOBIO) according to the manufacturer's instructions. The DNA G+C content was calculated as described previously (Peña *et al.*, 2005). Cell morphology was observed under a Zeiss light microscope at a magnification $\times 1000$, using cells that had been grown for 3 days at 30 °C on NA: the results are given in the species description.

The 16S rRNA gene sequence was analysed as described previously (Young *et al.*, 2005). Analysis of the sequence data was performed by using the software package MEGA

version 2.1 (Kumar *et al.*, 2001), after multiple alignments of the data had been obtained using CLUSTAL_X (Thompson *et al.*, 1997). A distance matrix method (with distance options according to Kimura's two-parameter model), including clustering by neighbour-joining (Fig. 1), and a discrete character-based maximum-parsimony method were used. In each case, bootstrap percentages were calculated on the basis of 1000 replications. The 16S rRNA gene sequence of strain CC4^T was a continuous stretch of 1488 bp. Sequence-similarity calculations indicated that strain CC4^T showed the greatest degree of similarity with respect to *Xanthobacter agilis* DSM 3770^T (92.2 %), *Prosthecomicrobium pneumaticum* MBIC3489^T (92.0 %), *Devosia neptuniae* J1^T (91.9 %), *Kaistia adipata* KCTC 12095^T (91.9 %) and *Methylocystis echinoides* IMET 10491^T (91.9 %). Lower sequence similarities (<91.5 %) were found with respect to all of the other genera shown in Fig. 1. The 16S rRNA gene sequence of strain CC4^T is therefore distinctly different from those of members of related genera and from all other known bacteria in the *Alphaproteobacteria*.

Strain CC4^T contains *meso*-diaminopimelic acid as the characteristic diamino acid of the peptidoglycan. The major fatty acids and ubiquinones of strain CC4^T are listed in Table 1. The polar lipid chromatogram for strain CC4^T is shown in Fig. 2 and the polar lipid components are listed in the species description. Results of the physiological characterization are given in the species description and in Table 1.

The strain was non-fluorescent. It was unable to produce acid from various carbohydrates. Carbon substrate utilization tests with organic acids as substrates showed few positive

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences available from the EMBL database (accession numbers in parentheses), constructed after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura-2 model) and clustering with the neighbour-joining method were obtained using MEGA, version 2.1 (Kumar *et al.*, 2001). Bootstrap percentages (based on 1000 replications) are shown at branching points. Bar, 0.01 substitutions per nucleotide position.

Table 1. Differential characteristics of strain CC4^T and related genera

Taxa: 1, strain CC4^T; 2, *Xanthobacter* (data from Wiegel, 2005; Wiegel & Schlegel, 1984); 3, *Devosia* (data from Yoon *et al.*, 2007); 4, *K. adipata* KCTC 12095^T (data from Im *et al.*, 2004). All the strains are catalase- and oxidase-positive and do not show pleomorphism, prosthecae, gas vacuoles or a biotin requirement. NR, Not reported; –, not detectable; –/+, variable; w, weak.

Characteristic	1	2	3	4
Motility	+	+	–/+	+
Colour of colonies	White	Pale yellow	White	White
Growth on nutrient broth	+	–	+	+
Growth on:				
H ₂ + CO ₂ at 37 °C	–	–	NR	NR
Methanol	–	+	NR	NR
Methylamine	–	–	NR	NR
Sorbitol	+	+	NR	+
Maltose	–	NR	–/+	+
Predominant quinone(s)	Q-10, Q-9 and Q-8	Q-10, Q-9 and Q-8	Q-10 or Q-11	Q-10
Major fatty acids (% of total content)				
C _{16:0}	25.4	1.1–10.3	8.5–25.6	11.0–22.2
C _{18:0}	2.3	1.2–3.2	3.2–9.3	14.8–16.0
Summed feature 7*	35.4	57.4–93.4	21.6–65.6	10.3–47.7
11-methyl C _{18:1} ω7c	0.5	Not separated	11.4–30.8	Not separated
C _{19:0} cyclo	28.2	0–19.7	0–7.8	19.7–42.2
DNA G + C content (mol%)	68.4	65–70	59.9–66.2	67.7

*Summed feature 7 includes one or more of 11-methyl C_{18:1}ω7c, C_{18:1}ω9t, C_{18:1}ω6t, C_{18:1}ω7c and C_{18:1}ω12t.

results. In the API 20E test, CC4^T was positive for urease, in the Voges–Proskauer (acetoin) reaction and for oxidation of arabinose, mannose and glucose. In the API 20NE test (bioMérieux), CC4^T was positive for urease and assimilation

of glucose, arabinose, mannose, mannitol and malate. In the API ZYM enzyme profile, CC4^T was positive for alkaline and acid phosphatase, butyrate esterase (C4), caprylate esterase (C8), myristate lipase (C14), leucine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase.

From the results of the 16S rRNA gene sequencing, it is evident that strain CC4^T differs from members of the genera *Devosia*, *Xanthobacter* and *Kaistia* and from all other genera in the phylogenetic neighbourhood. Phenotypic differentiation from all other genera in the family is possible by means of a combination of the growth characteristics, fatty acid profiles and physiological properties.

On basis of these results, we propose that strain CC4^T represents a novel genus and species, for which the name *Pseudoxanthobacter soli* gen. nov., sp. nov. is proposed.

Description of *Pseudoxanthobacter* gen. nov.

Pseudoxanthobacter (Pseu.do.xan.tho.bac'ter. Gr. adj. *pseudes* false; N.L. masc. n. *Xanthobacter* a bacterial genus name; N.L. masc. n. *Pseudoxanthobacter* the false *Xanthobacter*).

Cells are Gram-type negative or variable, aerobic, motile, short rod-shaped and non-spore-forming. Cells accumulate poly-β-hydroxybutyrate acid as polar inclusion bodies. Q-10 is the predominant ubiquinone and *meso*-diaminopimelic acid is the characteristic diamino acid of the peptidoglycan. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, phosphatidylmonomethylethanolamine, phosphatidyldi-

Fig. 2. Two-dimensional TLC of polar lipids of strain CC4^T. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PME, phosphatidylmonomethylethanolamine; PDE, phosphatidyldimethylethanolamine; AL, unknown aminolipid; L, unknown lipid.

methylethanolamine, an unknown lipid and an unknown aminolipid occur as polar lipids. The predominant fatty acids are C_{18:1}ω7c, C_{19:0} cyclo and C_{16:0}. The DNA G+C content of strain CC4^T is 68 mol%. The type species is *Pseudoxanthobacter soli*.

Description of *Pseudoxanthobacter soli* sp. nov.

Pseudoxanthobacter soli (so'li. L. neut. gen. n. *soli* of soil, the source of the type strain).

Positive for oxidase, acetoin and catalase; shows aerobic metabolism. Reproduction occurs by asymmetric division (budding) of the mother cell to produce a shorter daughter cell. Growth is visible after 48 h incubation on NA at 30 °C. Cells are 0.2 × 2.0 μm. Colonies on complex standard media at 37 °C are whitish, circular, smooth and convex with entire edges. In glucose medium, acid and gas are not produced. Grows at 10–37 °C but not at 42 °C. The optimum temperature for growth is 37 °C. Grows at pH 5.5–10.0 (optimally at pH 7.0). The fatty acid profile of the type strain comprises C_{18:1}ω7c (34.8%), C_{19:0} cyclo (28.2%), C_{16:0} (25.4%), C_{18:0} (2.3%), C_{14:0} 3-OH (2.3%), C_{20:0} (1.9%), C_{18:0} 3-OH (1.2%), C_{16:1}ω7c (1.1%), C_{16:0} 3-OH (0.7%), 11-methyl C_{18:1}ω7c (0.5%), C_{14:0} (0.5%), C_{20:2}ω6,9c (0.3%) and C_{17:0} cyclo (0.2%). Shares the quinone system and polar lipids listed in the genus description. Fixes nitrogen when grown in nitrogen-deficient media. In addition, methanol, methylamine and thiosulphate are not utilized. Aesculin and gelatin are not hydrolysed. Non-fluorescent. On the basis of the method described by Kämpfer *et al.* (1991), the type strain utilizes D-ribose, D-xylose, 4-aminobutyrate, fumarate and L-malate but not L-arbutin, mesaconate, 2-oxoglutarate, maltitol, putrescine, adipate, azelate, suberate, L-tryptophan, 3-hydroxybenzoate or phenylacetate. On the basis of the method described by Kämpfer *et al.* (1991), L-alanine *p*-nitroanilide (pNA) is hydrolysed. The following compounds are not hydrolysed: *p*-nitrophenyl (pNP) β-D-galactopyranoside, pNP β-D-glucuronide, pNP α-D-glucopyranoside, pNP β-D-glucopyranoside, pNP β-D-xylopyranoside, bis-pNP phosphate, bis-pNP phenylphosphonate, bis-pNP phosphorylcholine, L-aniline pNA, γ-L-glutamate pNA and L-proline pNA. No acids are produced from glucose, lactose, sucrose, D-mannitol, dulcitol, salicin, adonitol, inositol, sorbitol, L-arabinose, raffinose, rhamnose, maltose, D-xylose, trehalose, cellobiose, methyl D-glucoside, erythritol, melibiose, D-arabitol or D-mannose. The following carbon sources are utilized (positive with the Biolog GN2 system): adonitol, L-arabinose, D-arabitol, D-fructose, L-fucose, D-galactose, α-D-glucose, *myo*-inositol, D-mannitol, D-mannose, D-psicose, L-rhamnose, D-sorbitol, xylitol, succinic acid monomethyl ester, acetic acid, formic acid, β-hydroxybutyric acid, DL-lactic acid, succinic acid, bromosuccinic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, L-proline and glycerol. Weakly positive reactions are observed for D-alanine, α-hydroxybutyric acid, L-aspartic acid and L-

ornithine. The following substrates are not utilized as carbon sources: dextrin, α-cyclodextrin, glycogen, Tweens 40 and 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, cellobiose, i-erythritol, gentiobiose, α-D-lactose, lactulose, maltose, melibiose, methyl β-D-glucoside, raffinose, sucrose, trehalose, turanose, pyruvic acid methyl ester, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebamic acid, succinamic acid, glucuronamide, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyrroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL-α-glycerol phosphate, α-D-glucose 6-phosphate and D-glucose 1-phosphate. Positive for the following enzyme activities: alkaline and acid phosphatases, butyrate esterase (C4), caprylate esterase (C8), myristate lipase (C14), leucine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase. Negative for the following enzyme activities: valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The type strain is sensitive to cotrimoxazole, gentamicin, tetracycline, minocycline, rifampicin, norfloxacin, levofloxacin and nitrofurantoin but is resistant to penicillin, clindamycin, erythromycin, fusidic acid, plindamycin, vancomycin, teicoplanin, quinupristin-dalfopristin, coag(–) oxacillin and oxacillin. Additional physiological features are given in Table 1.

The type strain, CC4^T (=DSM 19599^T =CIP 109513^T), was isolated from a soil sample collected at a regional agricultural research field station located in Kaohsiung County, Taiwan.

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References

- Collins, M. D. & Jones, D. (1980). Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2,4-diaminobutyric acid. *J Appl Bacteriol* **48**, 459–470.
- Hahn, T.-W., Willby, M. J. & Krause, D. C. (1998). HMW1 is required for cytoadhesin P1 trafficking to the attachment organelle in *Mycoplasma pneumoniae*. *J Bacteriol* **180**, 1270–1276.

- Im, W. T., Yokota, A., Kim, M. K. & Lee, S. T. (2004). *Kaistia adipata* gen. nov., sp. nov., a novel alpha-proteobacterium. *J Gen Appl Microbiol* **50**, 249–254.
- 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., Steiof, M. & Dott, W. (1991). Microbiological characterization of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microb Ecol* **21**, 227–251.
- Kumar, S., Tamura, K., Jakobsen, I.-B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.
- Minnikin, D. E., Collins, M. D. & Goodfellow, M. (1979). Fatty acid and polar lipid composition in the classification of *Cellulomonas*, *Oerskovia* and related taxa. *J Appl Bacteriol* **47**, 87–95.
- Peña, A., Valens, M., Santos, F., Buczolits, S., Antón, J., Kämpfer, P., Busse, H.-J., Amann, R. & Rosselló-Mora, R. (2005). Intraspecific comparative analysis of the species *Salinibacter ruber*. *Extremophiles* **9**, 151–161.
- Powers, E. M. (1995). Efficacy of the Ryu nonstaining KOH technique for rapidly determining gram reactions of food-borne and waterborne bacteria and yeasts. *Appl Environ Microbiol* **61**, 3756–3758.
- Rhuland, L. E., Work, E., Denman, R. F. & Hoare, D. S. (1955). The behavior of the isomers of α,ϵ -diaminopimelic acid on paper chromatograms. *J Am Chem Soc* **77**, 4844–4846.
- Sasser, M. (1990). Identification of bacteria by gas chromatography of cellular fatty acids. *USFCC Newsl* **20**, 16.
- 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.
- Tindall, B. J. (1990a). A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.
- Tindall, B. J. (1990b). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.
- Wiegel, J. K. W. (2005). Genus XX. *Xanthobacter* Wiegel, Wilke, Baumgarten, Opitz and Schlegel 1978, 573^{AL}. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, part C, pp. 555–566. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer.
- Wiegel, J. K. W. & Schlegel, H. G. (1984). Genus *Xanthobacter* Wiegel, Wilke, Baumgarten, Opitz and Schlegel 1978, 573^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 325–333. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Yoon, J.-H., Kang, S.-J., Park, S. & Oh, T. K. (2007). *Devosia insulae* sp. nov., isolated from soil of Dokdo in Korea, and emended description of the genus *Devosia*. *Int J Syst Evol Microbiol* **57**, 1310–1314.
- Young, C.-C., Kämpfer, P., Shen, F.-T., Lai, W.-A. & Arun, A. B. (2005). *Chryseobacterium formosense* sp. nov., isolated from the rhizosphere of *Lactuca sativa* L. (garden lettuce). *Int J Syst Evol Microbiol* **55**, 423–426.