Sphingobium cupriresistens sp. nov., a copper-resistant bacterium isolated from copper mine soil, and emended description of the genus Sphingobium

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A Gram-negative, aerobic, copper-resistant bacterium, designated strain CU4T, was isolated from copper mine soil in Daye, China. Phylogenetic analysis based on 16S rRNA gene sequences showed highest similarity to Sphingobium rhizovicinum CC-FH12-1T (98.4%), followed by Sphingobium francense Sp+T (97.2%), Sphingobium japonicum UT26T (97.1%), Sphingobium abikonense NBRC 16140T (97.0%), Sphingobium xenophagum DSM 6383T (96.9%) and Sphingobium yanoikuyae DSM 7462T (95.5%). The major fatty acids (>5%) were summed feature 7 (C18:1ω7c, C18:1ω9t and/or C18:1ω12c), summed feature 4 (C16:0ω7c and/or iso-C15:0 2-OH), C16:0 and C14:0 2-OH, and the predominant quinone was ubiquinone Q-10. Spermidine was the major polyamine component. The major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, sphingoglycolipid, phosphatidylcholine. The genomic DNA G+C content of strain CU4T was 64.9 mol%. Comparison of DNA–DNA hybridization, phenotypic and chemotaxonomic characteristics between strain CU4T and phylogenetically related strains revealed that the new isolate represents a novel species of the genus Sphingobium, for which the name Sphingobium cupriresistens sp. nov. is proposed. The type strain is CU4T (=KCTC 23865T=CCTCC AB 2011146T). An emended description of the genus Sphingobium is also proposed.

The genus Sphingobium of the family Sphingomonadaceae was proposed by Takeuchi et al. (2001) with Sphingobium yanoikuyae as the type species (original name Sphingomonas yanoikuyae, Yabuuchi et al., 1990). The genus Sphingobium comprises, at the time of writing, 25 recognized species (www.bacterio.cict.fr/s/sphingobium.html) isolated mostly from contaminated soil and wastewater, and are known for their roles in bioremediation and biodegradation of pollutants, such as carbaryl, hexachlorocyclohexane, pyrethroid and other xenobiotic compounds (Basta et al., 2005; Bala et al., 2010; Yan et al., 2010; Wang et al., 2011). All of the species are strictly aerobic, Gram-negative, chemo-organotrophic and rod-shaped. In the genus description of Sphingobium, it was positive for catalase (Takeuchi et al., 2001), but negative results of the catalase reaction were reported for Sphingobium rhizovicinum DSM 19845T, Sphingobium abikonense NBRC 16140T and Sphingobium lactosutens DS20T (Young et al., 2008; Kumari et al., 2009). All recognized Sphingobium species are negative for nitrate reduction, except for S. rhizovicinum CC-FH12-1T and Sphingobium scionense WP01T (Young et al., 2008; Liang & Lloyd-Jones, 2010). The DNA G+C content of the genus ranges from 62 to 67 mol% (Takeuchi et al., 2001), except that of S. rhizovicinum CC-FH12-1T (59.4 mol%; Young et al., 2008). Members of the genus Sphingobium are characterized as having C18:1ω7c, C16:0 and C14:0 2-OH as the major fatty acids, containing ubiquinone Q-10 as the main respiratory quinone and spermidine as the major polyamine. Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and sphingoglycolipid (SGL) are the major polar lipids in this genus with a few exceptions, phosphatidylmonomethylethanolamine (PME), phosphatidylmonomethylethanolamine (PDE) and phosphatidylglycerol (PC) being predominant in some Sphingobium species (Busse et al., 1999; Pal et al., 2005; Wittich et al., 2007; Young et al., 2008; Dadhwal et al., 2009).

Some copper-resistant bacteria show abilities of biosorption, bioreduction, bioaccumulation and bioleaching of...
copper (Andreazza et al., 2010, 2012), and can be used in combination with plants to phytoremediate copper-contaminated soil (He et al., 2010). In order to isolate copper-resistant bacteria, surface soil was collected from Xinhua copper mine (115° 04′ E 29° 57′ N), in Daye County, Hubei province, central-south China. The soil texture was sandy type with a pH of 8.9 ± 0.10. The total soil copper concentration was 34.1 ± 4.94 mg L⁻¹ (determined by atomic absorption spectrophotometry). The polyphasic taxonomic characterization of a copper-resistant strain, CU4ᵀ, is reported in this study.

The strain was isolated by adding 10 g of soil to 100 ml of a 0.85 % sterile NaCl solution, and the sample was then shaken at room temperature for 30 min. The mixed solution was serially diluted tenfold and poured onto 1/5-strength Luria–Bertani (LB) plates (per litre: tryptone, 2 g; yeast extract, 1 g; NaCl, 2 g; agar, 15 g; pH 7.0) supplemented with 0.5 mM CuSO₄. Growth was tested at 4, 10, 15, 28, 30 and 37°C using R2A broth (Difco) plates incubated for 5 days. The growth conditions at different pH (5–10) and NaCl concentrations [1, 2 and 3 % (w/v) and in the absence of NaCl] were tested using R2A broth by incubating at 28°C and shaking at 160 r.p.m. for 5 days. Growth under anaerobic conditions was determined by incubating strains in an anaerobic chamber with an O₂-absorbing and CO₂-generating agent (Anaero-Pack; Mitsubishi Gas Chemical). A motility test was performed using R2A broth with 0.3 % agar. Gram staining was performed using the standard Gram reaction combined with the KOH lysis test method (Ryu, 1938). Morphological observations were carried out using a scanning electron microscope (JSM-6390; JEOL) and transmission electron microscope (H-7650; Hitachi). For analyses of physiological and biochemical characteristics, strain CU4ᵀ and four reference type strains, S. rhizovicinum DSM 19845ᵀ, Sphingobium japonicum DSM 16413ᵀ, Sphingobium xenaphagum DSM 6383ᵀ and S. yanoikuyae DSM 7462ᵀ, were cultivated using R2A agar or broth and incubated under the same condition unless otherwise mentioned. Catalase activity was detected using the standard method (v/v) H₂O₂. The ability of the strain to grow on a range of sole carbon sources was determined using API 20NE and ID 32GN test kits (bioMérieux). The test for acid production from carbohydrates was performed under aerobic conditions (Smibert & Krieg, 1994; Gordon & Mihm, 1957). Degradation of xanthine and hypoxanthine were determined according to Gordon et al. (1974). Hydrolysis of starch was determined as described by Smibert & Krieg (1994). Hydrolysis of Tween 20 and Tween 80 was tested according to Arden-Jones et al. (1979). Sensitivity to antibiotics was determined on R2A agar plates using antibiotic discs (Hangzhou Microbial Reagent). A positive antibiotic sensitivity was considered with an inhibition zone diameter above 10 mm after incubation at 28°C for 2 days. The minimal inhibitory concentration (MIC) for CuSO₄ that completely inhibited the growth of strain CU4ᵀ was determined as described by Lim & Cooksey (1993). Other physiological properties, including enzyme activities, were examined using API 20NE and API ZYM systems (bioMérieux) according to the manufacturer’s instructions.

The nearly full-length 16S rRNA gene sequence of strain CU4ᵀ was amplified by PCR with universal primers Uni-27F and Uni-1492R (Wilson et al., 1990), and the PCR product was ligated into a pGEM-T vector (Promega). DNA sequencing was performed by the Beijing Genomics Institute (Beijing, China). Pairwise sequence similarities were calculated using the EzTaxon server (Kim et al., 2012). Multiple alignments of the sequences were carried out using CLUSTAL X (Thompson et al., 1997). Terminal nucleotides not present in all of the sequences were removed manually from the preliminary alignment file. Neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) trees were constructed using MEGA 4.0 (Tamura et al., 2007), and a maximum-likelihood tree was generated using the PHYML package (Guindon & Gascuel, 2003) with 1000 replicates in a non-parametric bootstrap analysis, the general time-reversible model of nucleotide substitution and four substitution-rate categories.

For whole-cell fatty acid analysis, strain CU4ᵀ and the four reference type strains were grown in trypticase soy broth medium at 28°C until they reached the mid-exponential phase and were analysed by GC (Hewlett Packard 6890) according to the instructions of the Sherlock Microbial Identification System (MIDI Sherlock version 4.5, MIDI database TSBA40 4.10). Polar lipids were extracted as described by Minnikin et al. (1979) and identified by two-dimensional TLC sprayed with specific reagents (Collins & Jones, 1980). Quinones were extracted and purified by the method of Collins et al. (1977) and analysed via HPLC as described by Tamaoka et al. (1983). For polynucleotide analysis, cultures of strain CU4ᵀ were grown in PYE broth (per litre: tryptone, 3 g; yeast extract, 3 g; pH 7.0) for 36 h. After lyophilization, polynucleotides were extracted and analysed as described by Busse & Auling (1988) and Busse et al. (1997, 1999) [Shimadzu HPLC LC-6AD; column: ODS-C18 (4.6 × 250 mm, 5 μm); detector: fluorescence detector RF-20A, Ex: 360 nm, Em: 520 nm]. The G+C content of the genomic DNA was determined by HPLC according to the method of Mesbah et al. (1989). Levels of DNA–DNA relatedness were determined using the thermal denaturation and renaturation method of Huss et al. (1983).

The cell morphology of strain CU4ᵀ grown in R2A medium is shown Supplementary Fig. S1 (available in IJSEM Online). Detailed results of the polyphasic taxonomic characterization of strain CU4ᵀ are given in the species description. Strain CU4ᵀ showed characteristics typical of Sphingobium species, but there were some clear differences compared with recognized species of the genus Sphingobium, especially as regards nitrate reduction. The main differential phenotypic characters of strain CU4ᵀ compared with S. rhizovicinum DSM 19845ᵀ, S. japonicum DSM 16413ᵀ, S. xenaphagum DSM 6383ᵀ, S. yanoikuyae DSM 7462ᵀ and six other closely
related *Sphingobium* species are shown in Table 1. In addition, strain CU4\(^T\) displayed resistance to CuSO\(_4\) with an MIC of 0.9 mM in R2A medium.

The 16S rRNA gene sequence of strain CU4\(^T\) (1430 bp) showed highest pairwise similarity to *S. rhizovicinum* CC-FH12-1\(^T\) (98.4 %), followed by *Sphingobium francense* Sp\(^+\)\(^T\) (97.2 %), *S. japonicum* UT26\(^T\) (97.1 %), *S. abikonense* NBRC 16140\(^T\) (97.0 %), *S. xenophagum* DSM 6383\(^T\) (96.9 %) and *S. yanoikuyae* DSM 7462\(^T\) (95.5 %). Phylogenetic analyses using the neighbour-joining, maximum-parsimony and maximum-likelihood methods all showed that strain CU4\(^T\) fell in the same cluster with *S. rhizovicinum* CC-FH12-1\(^T\) (=DSM 19845\(^T\)) with high bootstrap values (Fig. 1).

The assignment of strain CU4\(^T\) was further confirmed by chemotaxonomic and DNA–DNA relatedness. Major fatty acids (>5 %) were summed feature 7 (consisting of C\(_{16:1}\) 3\(\omega\), C\(_{16:0}\) 3\(\omega\) and/or iso-C\(_{15:0}\) 2\(\omega\)-OH), C\(_{16:0}\) and C\(_{14:0}\) 2\(\omega\)-OH, which were similar to the other *Sphingobium* species. The detailed fatty acid profile of strain CU4\(^T\) in comparison with those of the other ten recognized *Sphingobium* species is shown in Table S1. The major polyaromatics were DPG, PE, PG, PDE, SGL and PC (Fig. S2).

The polar lipid profile shared the majority of characteristics with recognized *Sphingobium* species. However, the presence of PC together with the absence of PME differentiated strain CU4\(^T\) from its most closely related species, *S. rhizovicinum* (Young et al., 2008). Also, the presence of PDE differentiated strain CU4\(^T\) from *S. japonicum* DSM 16413\(^T\) and *S. francense* Sp\(^+\)\(^T\) (Pal et al., 2005). The respiratory quinones consisted of ubiquinone Q-10 (83.8 %) as the major compound and a small amount of Q-9 (16.2 %), and the major polyamine was spermidine (40.9 \(\mu\)mol (g dry weight)\(^{-1}\)), data which matched those for the type species (Takeuchi et al., 2001) and other *Sphingobium* species. Mean (± SD of three independent determinations) DNA–DNA hybridization values between strain CU4\(^T\) and *S. rhizovicinum* DSM 19845\(^T\), and between strain CU4\(^T\) and *S. japonicum* DSM 16413\(^T\) were 57.2 ± 0.6 and 48.2 ± 2.8 %, respectively. These values were well below the 70 % cut-off point for species classification recommended by Wayne et al. (1987). The mean G+C content of the genomic DNA of strain CU4\(^T\) was 64.88 ± 0.02 mol% (Fig. S3).

Based on its distinct phylogenetic position, DNA–DNA relatedness data, chemotaxonomic traits and physiological properties, it is concluded that strain CU4\(^T\) represents a

Table 1. Comparison of the phenotypic characteristics of strain CU4\(^T\) with closely related members of the genus *Sphingobium*

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* Y, yellow; LY, light yellow; CY, cream-yellow.
† Data from Garg et al. (2012).
‡ Data from Dadhwal et al. (2009).
novel species of the genus *Sphingobium*, for which the name *Sphingobium cupriresistens* sp. nov. is proposed.

**Emended description of the genus Sphingobium**

This emended description is based on that given by Takeuchi et al. (2001), with the following changes and additions. Most species are positive for catalase and negative for nitrate reduction. The major polar lipids are DPG, PE, PG and SGL with a few exceptions. PME, PDE and PC are also found among species of the genus. The DNA G+C content is 59–67 mol%. The other characteristics conform to the original description for the genus. The type species is *Sphingobium yanoikuyae* (Takeuchi et al., 2001).

**Description of Sphingobium cupriresistens sp. nov.**

*Sphingobium cupriresistens* (cu.pri.re.sist’en s. L. n. cuprum copper; L. part. adj. resistens resisting; N.L. part. adj. cupriresistens copper resisting).
Cells are Gram-negative and rod-shaped (0.3–0.5 × 1.2–2.0 μm). Colonies (1.5–3.0 mm in diameter) are pale yellow, smooth, circular and slightly elevated on R2A plates. Aerobic, non-motile, catalase-positive but oxidase-negative. Temperature range for growth is 4–30 °C (optimum, 28 °C). Growth occurs with NaCl concentrations in the range 0–2% (optimum, 0.5%) and at pH 5–9 (optimum, pH 7.0). Tween 80 is hydrolysed, but not xanthine, hypoxanthine, Tween 20 or casein. H₂S is not produced. Acid is produced from glucose, xylose, maltose and cellobiose, but not from ribitol or sucrose. In API ZYM tests, positive for alkaline phosphatase, acid phosphatase, butyrate esterase (C4), caprylate esterase (C8), leucine arylamidase, valine arylamidase, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and β-glucosidase, but negative for myristate lipase (C14), cystine arylamidase, β-fucosidase, trypsin, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-galactosidase. In API 20NE tests, it is positive for nitrate reduction, acetoin hydrolysis, β-galactosidase and assimilation of glucose, arabinoose, maltose and malic acid, but negative for indole production, arginine dihydrolase, urease, glucose fermentation, gelatin hydrolysis and assimilation of mannose, mannotriose, N-acetylglucosamine, gluconate, capric acid, adipic acid, trisodium citrate and phenylacetic acid. Sensitive to (μg per disc): chloramphenicol (30), novobiocin (5), tetracycline (30), kanamycin (30), neomycin (30), nitrofurantoin (300), tobramycin (10), cefoxitin (30), fluprediacid (10), teicoplanin (30), erythromycin (15), rifampicin (5), vancomycin (30), nalidixic acid (30), cefotaxime (30), streptomycin (10) and amoxicillin (10), but not to cephalothin IV (30), cephalothin V (30), penicillin (10), lincomycin (2), cephalo-lothin (20), ampicillin (10), carbenicillin (100), oxazoline (1) and trimethoprim (5). Major fatty acids are summed feature 7 (C₁₂:0 16:0 17:0 ω7c, C₁₈:0 9c and/or C₁₈:1ω9c), summed feature 4 (C₁₆:0 10:0 12:0 12:0 3-OH, C₁₆:0 10:0 12:0 2-OH). Polar lipid profile consists of DPG, PE, PG, PDE, SGL and PC. The predominant ubiquinone is Q-10. Spermidine is the major polyamine component. A copper-resistant bacterium which is able to grow with 0.9 mM CuSO₄ in R2A broth.

The type strain, Cu4ᵀ (KCTC 23865ᵀ = CCTCC AB 2011146ᵀ), was isolated from copper mine soil in Daye, central-south China. The DNA G+C content of the type strain is 64.9 mol%.

Acknowledgements

We are grateful to DSMZ for providing S. rhizophicicum DSM 19845ᵀ, S. yunokiyaue DSM 7462ᵀ, S. japonicum DSM 16413ᵀ and S. xenophagum DSM 6383ᵀ, Dr Jean Eureby (École National Vétentaire) for etymological advice, and Dr Wenjun Li and Ms Lingling Yang (Yunnan University) for polar lipid and quinone analyses. This work was supported by the National Natural Science Foundation of China (31010103903).

References


