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ORIGINAL PAPER

Burkholderia metalliresistens sp. nov., a multiple metal-resistant and phosphate-solubilising species isolated from heavy metal-polluted soil in Southeast China

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Abstract A metal-resistant and phosphate-solubilising bacterium, designated as strain D414^T, was isolated from heavy metal (Pb, Cd, Cu and Zn)-polluted paddy soils at the surrounding area of Dabao Mountain Mine in Southeast China. The minimum inhibitory concentrations of heavy metals for strain D414^T were 2000 mg L^{-1} (Cd), 800 mg L^{-1} (Pb), 150 mg L^{-1} (Cu) and 2500 mg L^{-1} (Zn). The strain possessed plant growth-promoting properties, such as 1-aminocyclopropane-1-carboxylate assimilation, indole production and phosphate solubilisation. Analysis of 16S rRNA gene sequence indicated that the isolate is a member of the genus *Burkholderia* where strain D414^T formed a distinct phyletic line with validly described *Burkholderia* species. Strain D414^T is closely related to *Burkholderia tropica* DSM 15359^T, *B. bannensis* NBRC E25^T and *B. unamae* DSM 17197^T, with 98.5, 98.3 and 98.3 % sequence similarities, respectively. Furthermore, less than 34 % DNA–DNA relatedness was detected between strain D414^T and the type strains of the phylogenetically closest species of *Burkholderia*. The dominant fatty acids of strain D414^T were $C_{14:0}$, $C_{16:0}$, $C_{17:0}$ cyclo and $C_{18:1}$ $\omega 7c$. The DNA G+C content was 62.3 ± 0.5 mol%. On the basis of genotypic, phenotypic and phylogenetic data, strain D414^T represents a novel species, for which the name *Burkholderia metalliresistens* sp. nov. is proposed, with D414^T (=CICC 10561^T = DSM 26823^T) as the type strain

Keywords *Burkholderia metalliresistens* · Multiple metal resistant · Plant growth promotion · Polyphasic taxonomy · Phylogeny

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain D414^T is KF601211.

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Abbreviation

MIC Minimum inhibitory concentration

Introduction

The genus *Burkholderia*, with *Burkholderia cepacia* as the type species, was separated from the former *Pseudomonas* rRNA homology group II by Yabuuchi et al. 1992. More than 80 *Burkholderia* species with validly published names have been reported to date. *Burkholderia* species were isolated from highly diverse habitats (Coenye and Vandamme 2003; Caballero-Mellado et al. 2004; Reis et al. 2004; Chen et al. 2007). *Burkholderia* is a genus with a steady growth of species numbers. Previously described *Burkholderia* species were classified into two major species clusters and several subgroups on the basis of the phylogenetic analyses of 16S rRNA, *recA*, *gyrB*, *rpoB* and *acdS* gene sequences, as well as genome sequences (Estrada-de los Santos et al. 2013). Group A contains plant-associated species and some saprophytic bacterial species. Group B comprises members of the *B. cepacia* complex (opportunistic human pathogens), the *B. pseudomallei* subgroup (both human and animal pathogens) and the *B. andropogonis* subgroup (including *B. andropogonis*, *B. rhizoxinica*, *B. endofungorum*, *B. caryophylli*, *B. symbiotica* and *B. soli*) (Suárez-Moreno et al. 2012; Estrada-de los Santos et al. 2013; Martínez-Aguilar et al. 2013).

An increasing number of *Burkholderia* species with plant growth-promoting (PGP) traits and legume plant nodulation abilities in legumes have been isolated from soils and plant tissues. *Burkholderia* species with nitrogen-fixing ability were classified under the plant-associated group; these species include *B. unamae*, *B. xenovorans*, *B. kururiensis*, *B. silvatlantica*, *B. tropica*, *B. ferrariae*, *B. bannensis*, *B. heleia*, *B. terrae* and *B. gisengisoli* (Suárez-Moreno et al. 2012). The *B. cepacia* complex species *B. vietnamiensis* is also a nitrogen fixer. Moreover, *B. sabiae*, *B. tuberum*, *B. phymatum*, *B. mimosarum* and *B. nodosa* can fix nitrogen in symbiosis with legumes (Vandamme et al. 2002; Chen et al. 2006, 2007, 2008). Plant growth-promoting rhizobacteria (PGPR) with 1-aminocyclopropane-1-carboxylate (ACC) deaminase promotes plant growth by suppressing the increased level of ethylene content, which causes growth inhibition, in plant tissues under abiotic or biotic stress (Blaha et al. 2006). Plant-beneficial rhizobacteria produce indole-3-acetic acid (IAA) to promote growth. *B. phytofirmans* and *B. unamae* reportedly produce ACC deaminase and IAA (Caballero-Mellado et al. 2007; Onofre-Lemus et al. 2009). Furthermore, plant-associated *Burkholderia* species, such as *B. tuberum* STM678, solubilise phosphate and produce siderophores (Angus et al. 2013). The shoot lengths and dry weights of *Macroptilium atropurpureum* which were inoculated with strain STM678 significantly increased.

Many *Burkholderia* species play important roles in pollution remediation (Lim et al. 2008; Otsuka et al. 2011; Lu et al. 2012). Jiang et al. (2008) isolated the heavy metal-tolerant *Burkholderia* sp. J62 from heavy metal-contaminated soils and found that this strain promotes growth through IAA production, siderophore synthesis and phosphate solubilisation. Moreover, the Cd and Pb contents in the biomasses and tissues of *Zea mays* L. and *Lycopersicon esculentum* increase upon inoculation with *Burkholderia* sp. J62 in heavy metal-contaminated soils. Some scholars isolated *Burkholderia* species which exhibit heavy metal resistance, promote plant growth and enhance metal uptake from heavy metal-polluted soils (Li et al. 2007; Jiang et al. 2008; Guo et al. 2011; Martínez-Aguilar et al. 2013). The present study aims to investigate the taxonomy of an isolate which belongs to a new *Burkholderia* species. The name *Burkholderia metalliresistens* sp. nov. is proposed for this new species.

Materials and methods

Isolation and screening of isolate

Strain D414^T was isolated from arable layers (0–20 cm) of heavy metal-polluted paddy soils at the surrounding area of Dabao Mountain Mine, Guangdong, Southeast China. The basic properties of the soil samples are shown in Supplementary Table 1. Ten grams of soils that were collected from the heavy metal-polluted paddy fields were added to 100 ml sterile modified SMN mediums without agar in a clean bench. The enrichment mediums were incubated at 200 rpm for 72 h at 30 °C in a rotary shaker. The

selective medium used was modified SMN medium [2 g L^{-1} $(NH_4)_2SO_4$, 5 g L^{-1} mannitol, 0.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $MgSO_4$, 0.1 g L^{-1} NaCl, 0.05 g L^{-1} $FeSO_4$, 0.05 g L^{-1} $MnSO_4$, 20 g L^{-1} agar and 1000 mL of distilled water; pH 6.8 ± 0.2] supplemented with 50 mg L^{-1} Cd as $CdCl_2$. Single colonies which grew well were selected and streaked on other modified SMN agar plates supplemented with 0.5 % ACC as the sole carbon source. The isolates were purified by streaking in three other modified SMN agar plates. Finally, the pure isolates were stored in 30 % v/v glycerol at −80 °C for long-term storage and on modified SMN slants for short-term storage.

Phenotypic characteristics

Morphological properties were examined by light microscopy (Carl Zeiss Axioskop, Germany) after 3 days of culture on modified SMN agar at 30 °C. After serial dehydration in 50, 60, 70, 80, 90 and 100 % ethanol solutions (three times for 10 min at each stage), the critical-point dried samples were sputter-coated with gold and viewed under a scanning electron microscope (FEI Quanta 200, Netherlands). The Gram reaction was performed following the method described by Palinska and Marquardt (2008).

Strain D414^T and reference type strains were inoculated with a suspension of late exponential growth phase cells in 0.7 % (w/v) YNB minimal growth medium (Difco) adjusted to pH 7.0, and their utilisation of different substrates and enzyme activities was determined with the API 20NE and API 50CH microtest systems in accordance with the manufacturers' instructions (bioMérieux, France). The effects of temperature and pH on the growth of strain D414^T were determined in modified SMN medium. The growth of the strain on LB broth (Difco), yeast nitrogen base agar (Difco) and trypticase soy agar (Difco) was also evaluated.

Chemotaxonomic properties

For chemotaxonomic property studies, strain D414^T and reference type strains (*B. tropica* DSM 15359^T, *B. bannensis* NBRC E25^T and *B. unamae* DSM 17197^T) were cultured in modified SMN medium at 30 °C for 48 h. The strains in the late exponential growth phase were harvested by centrifugation at 10,000×*g* for 10 min and washed with distilled water several times for further study. The whole-cell fatty acids were saponified, methylated and then extracted using the standard protocol of the MIDI Sherlock Microbial Identification System (version 6.0). Fatty acids were analysed by GC (Agilent Technologies 6850) and identified using the TSBA6 database of the Microbial Identification System (Sasser 1990).

The growth and nitrogen-fixing ability of strain D414^T were evaluated in Ashby's nitrogen-free medium and serials of other test media (Jones 1970). The nitrogenase activity of strain D414^T was determined by acetylene reduction assay (ARA) (Hardy et al. 1968). Flasks which contain 20 mL of Ashby's nitrogen-free semi-solid medium (with 5 g L^{-1} agar) were inoculated with the isolate at a concentration of 1×10^8 cfu mL^{-1}. After incubation at 30 °C for 48 h, 10 % of the gas volume in each flask was replaced with acetylene. The flasks were incubated for 1 h at 30 °C and analysed for ethylene production by GC with an FID detector (Agilent 7890A). *B. tropica* DSM 15359^T and *B. unamae* DSM 17197^T served as positive controls. Uninoculated flasks served as negative controls. The experiment was carried out thrice.

Whole-cell protein SDS-PAGE analyses were performed as described by Laemmli (1970). Bacterial cultures (1.0 mL) in the late exponential growth phase were harvested and washed as described above. The pellets were resuspended in 100 µL of 0.125 M Tris–HCl, 4 % SDS, 20 % glycerol and 10 % mercaptoethanol at pH 6.8. The mixtures were heated at 100 °C for 4 min. The samples were cooled on ice and centrifuged at 10,000×*g* for 10 min, and the concentration was recovered and stored at −20 °C until use. The lysate solutions (10 µL) were subjected to SDS-PAGE electrophoresis on vertical slabs at 50 mA and 120 V. The samples were fixed by immersing the gels in a 10 % (v/v) trichloroacetic solution for 1 h and then stained overnight with Coomassie blue [0.25 % (w/v) Coomassie blue R-250, 50 % (v/v) methanol and 10 % (v/v) acetic acid]. Excess stain was removed by rinsing with a solution of 25 % (v/v) methanol and 10 % (v/v) acetic acid (Ghazi et al. 2009).

Phylogenetic analysis

Genomic DNA extraction and 16S rRNA gene PCR were carried out following the procedures by Chun and Goodfellow (1995). The universal bacterial 16S

rRNA gene primers (the forward primer P1and the reverse primer P6) were used (Wei et al. 2002). The PCR product was purified and directly sequenced by an automated DNA Sequencing System (ABI 3730XL, USA) with the forward primer P1 and the reverse primer P6 as described above. Two sequencing reactions with the forward primer P1 and the reverse primer P6 were carried out. Sequence fragments were then assembled using Contig Express (Vector NTI Suite 8; InforMax, Bethesda, MD, USA). The almost complete 16S rRNA gene sequence of the strain was aligned by ClustalX version 1.8 (Thompson et al. 1997) with almost complete 16S rRNA gene sequences of validly described type strains obtained from the GenBank/EMBL/DDBJ databases and EzTaxon-e (Kim et al. 2012). A phylogenetic tree was constructed by neighbour-joining (Saitou and Nei, 1987), minimum-evolution (Rzhetsky and Nei 1993) and maximum-parsimony (Fitch 1971) in TREECON 1.3b (Van de Peer and De Wachter. 1994) and MEGA 3.1 (Kumar et al. 2004). The genetic distance matrices were estimated by the Kimura two-parameter model (Kimura 1980). The topology of the tree was evaluated by bootstrap analysis based on 1000 replicates (Felsenstein 1985).

DNA–DNA hybridisation and G+C content

DNA–DNA relatedness between strain D414^T and its most closely related type strain described by De Ley et al. (1970) was determined by thermal denaturation with a spectrophotometer (Beckman DU 800, USA). The DNA G+C content was determined through HPLC (Mesbah et al. 1989). All analyses on DNA–DNA relatedness and G+C content were carried out in triplicates.

Heavy metal resistance and phosphate solubilisation properties

The minimum inhibitory concentrations (MICs) of the metals (Pb, Cd, Cu and Zn) for the strain D414^T were determined by plate dilution as described by Aleem et al. (2003). The modified SMN agar without the metals served as the control. The cultures were incubated at 30 °C for 7 days, and the experiments were carried out in triplicate.

The phosphate solubilisation ability of strain D414^T was determined in Pikovskays's medium (Zaidi et al. 2006) with 0.5 % tricalcium phosphate. When the bacterial suspension reached 1.0 (1×10^8 cfu mL^{-1}) of optical density (600 nm), 1 mL of the suspension was added into 250 mL conical flasks which contain 100 mL of the medium. After incubation at 200 rpm for 72 h at 30 °C in a rotary shaker, the supernatants were collected by centrifugation at $8000 \times g$ for 10 min. The soluble phosphate in the supernatants was estimated by Mo-blue (Watanabe and Olsen 1965). Each experiment was performed in triplicates.

Results and discussion

In the present study, strain D414^T with the ability to assimilate ACC was isolated from a heavy metal-polluted paddy soil. Glick et al. (2007) reported that the plant-associated strains with the ability to assimilate ACC can prevent ethylene-induced growth inhibition by decreasing the amount of ACC, the immediate precursor of ethylene, through hydrolysis. ACC assimilation is a key plant-beneficial trait of PGPR (Blaha et al. 2006). Strain D414^T was found to be 0.5–0.7 μm × 0.8–2.0 μm in cell size, Gram negative, aerobic and non-motile (Fig. 1). After 3 days of cultivation at 30 ± 1 °C, milk-like, yellowish-white, convex and circular colonies with clear margins were observed on the modified SMN agar plate. A diffusible pigment was not observed in the modified SMN medium. Strain D414^T grew within the

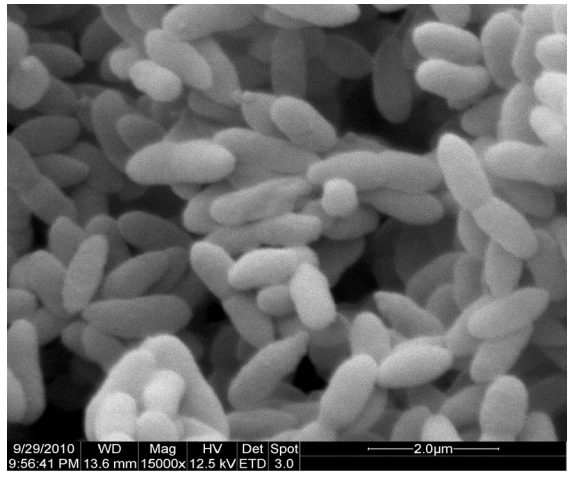

Fig. 1 Scanning electron micrograph of *Burkholderia metalliresistens* sp. nov. strain D414^T after 3 days culture on modified SMN agar. *Bar* 2 μm

Table 1 Differential of phenotypic characters of *Burkholderia metalliresistens* sp. nov. strain D414^T and closely related type strains of the genus *Burkholderia*

Character	1	2	3	4
Colony colour	Yellowish-white	Yellowish-white	Yellowish-white	Pale yellowish-white
Growth at				
10	+	−	−	−
50	+	+	−	±
pH range				
2	−	±	±	−
8	+	+	±	±
Erythritol	−	−	+	−
D-arabinose	+	+	+	−
D-ribose	+	+	+	−
L-xylose	−	−	+	−
L-sorbose	−	−	−	+
Arbutin	−	−	+	−
Salicin	+	−	+	−
D-maltose	−	−	+	−
D-lactose	−	+	+	−
D-melibiose	+	+	+	−
D-trehalose	−	+	−	+
Xylitol	−	−	+	+
D-gentiobiose	+	−	±	−
D-lyxose	+	+	+	±
D-fucose	+	+	−	−
L-fucose	+	+	−	−
L-arabitol	−	−	−	−

Strains: 1, *B. metalliresistens* D414^T; 2, *B. tropica* DSM 15359^T; 3, *B. bannensis* NBRC E25^T; 4, *B. unamae* DSM 17197^T

+ good growth; ± poor growth; − no growth; Data for reference strains were obtained in this study

temperature range of 10–50 °C (optimum 30 °C) and pH range of 3.0 to 8.0 (optimum 7.0) (Table 1). The strain grew well on LB broth (Difco), yeast nitrogen base agar (Difco) and trypticase soy agar (Difco) but weakly on Ashby's nitrogen-free medium. Furthermore, the result of the ARA showed that the nitrogenase activity of strain D414 was ranged from 87.2 to 105.6 nmol C_2H_4 h^{-1} mL^{-1}.

The major fatty acids of strain D414^T were $C_{14:0}$, $C_{16:0}$, $C_{17:0}$ cyclo and $C_{18:1}$ $\omega 7c$ (Supplementary Table 2). The results demonstrate that the fatty acid characteristics of strain D414^T are consistent with those of the genus *Burkholderia* (Coenye et al. 2000). However, strain D414^T differed from closely related *Burkholderia* species, namely, *B. tropica* DSM 15359^T, *B. bannensis* NBRC E25^T and *B. unamae* DSM 17197^T, by having higher concentrations of $C_{16:0}$ (25.1 %), $C_{17:0}$ cyclo (25.2 %) and $C_{18:1}$ ω7c (6.1 %) and lower concentration of the Sum In Feature 2 ($C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I; 8.3 %) (Reis et al. 2004; Caballero-Mellado et al. 2004; Aizawa et al. 2011). Moreover, the results of API 20NE and API 50CH microtests showed that the physiological characters of type strain D414^T can be distinguished from closely related type strains of *Burkholderia* species, such as *B. tropica* DSM 15359 ^{T}, *B. bannensis* NBRC E25^T and *B. unamae* DSM 17197^T (Table 1). Furthermore, bacteria with highly similar protein patterns possess high genome similarity (Vandamme et al. 1996). In this study, the SDS-PAGE results strongly suggest that strain D414^T can be distinguished from its closely related type strains as shown in Supplementary Fig. 1.

Based on 16S rRNA gene sequence analysis, the strain D414^T separated from other closely-related species of the *Burkholderia* in the phylogenetic tree (Supplementary Fig. 2). The results revealed that the novel strain D414^T was integrated into the group A (plant-associated and saprophytic *Burkholderia* lineage) described by Suárez-Moreno et al. (2012) and

Estrada-de los Santos et al. (2013). The strain is closely related to *B. tropica* DSM 15359^T, *B. bannensis* NBRC E25^T and *B. unamae* DSM 17197^T with similarities of 98.5, 98.3 and 98.3 %, respectively (Fig. 2).

The DNA–DNA relatedness values of strain D414^T with the three aforementioned reference type strains were 22.7 to 33.9 %, 25.4 to 27.2 % and 32.9 to 33.7 %, respectively. These values are significantly lower than the 70 % cut-off point recommended for the delineation of genomic species (Wayne et al. 1987). The results of the DNA–DNA relatedness study confirm that strain D414^T belongs to a novel *Burkholderia* species. The DNA G+C content is range from 61.8 to 62.8 %, which is within the G+C content range of group A (61.6 to 64.2 %) but different from the G+C content of group B (66.1 to 68.1 %), *B. andropogonis* (59.0 %) and *B. rhizoxinica/B. endofungorum* group (60.7 %) as described by Estrada-de los Santos et al. (2013).

Strain D414^T showed high MIC values for Cd (2000 mg L^{-1}), Pb (800 mg L^{-1}), Cu (150 mg L^{-1}) and Zn (2500 mg L^{-1}). The order of toxicity of metals to strain D414 was Cu > Pb > Cd > Zn. Rhizosphere bacteria and endophytes, which were isolated from heavy metal-contaminated soils or plants, often exhibit considerably high heavy metal resistance to adapt to such environments (Pal et al. 2005). In the present study, the average content of soluble phosphate was estimated to be ranged from 154.3 to 161.0 mg L^{-1}. Phosphorus is an essential macronutrient for plants; thus, phosphate solubilisation is an important trait of PGPR (Guo et al. 2011). Therefore, *Burkholderia* species could potentially be employed for the phytoremediation of multiple metal-contaminated soils.

Based on a combination of phenotypic and genotypic studies, strain D414^T represents a novel species of the genus *Burkholderia,* for which the name *Burkholderia metalliresistens* sp. nov. is proposed.

Description of *Burkholderia metalliresistens* sp. nov

Burkholderia metalliresistens (metalliresistens: me.tal.li.re.sis'tens.L. n. metallum, metal; L. part. adj. resistens, resisting; N.L. part. adj. *metalliresistens*, metal resisting, referring to the ability of the organism to resist metal).

The cells of this species are 0.5–0.7 μm (width) × 0.8–2.0 μm (length) in size, Gram negative, aerobic and non-motile. The colonies are milk-like, yellowish-white, convex and circular with clear margins on modified SMN agar after 3 days of cultivation at 30 °C. Grows within the temperature range of 10–50 °C (optimum 30 °C) and pH range of 3.0–8.0 (optimum 7.0). Grows well on LB broth (Difco), yeast nitrogen base agar (Difco) and trypticase soy agar (Difco) but weakly on Ashby's nitrogen-free medium. Positive for the production of indole; activity of urease, oxidase, catalase and nitro-D-methyl galactose; and assimilation of ACC, esculine, glucose,

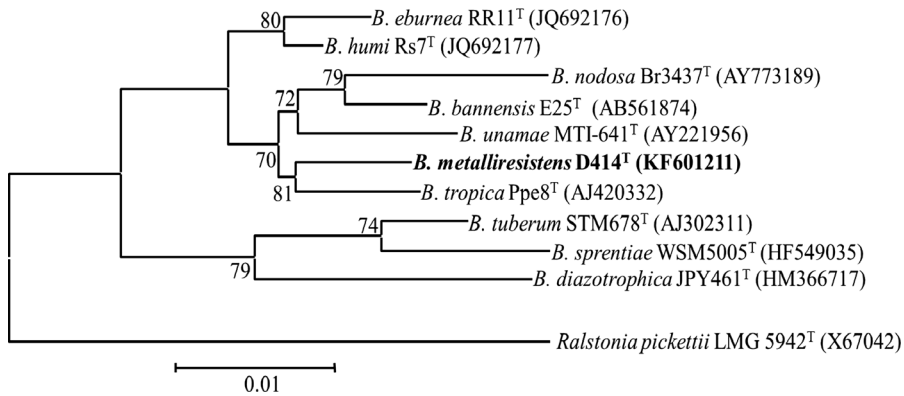

Fig. 2 Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing relationships between *Burkholderia metalliresistens* sp. nov. strain D414^T and the most closely related type strains of the genus *Burkholderia*. *Numbers* at branching nodes indicate bootstrap percentages (based on 1000 replications). *Bar* 0.01 substitutions per nucleotide position

arabinose, mannose, mannitol, *N*-acetylglucosamine, malate, gluconate, capric acid, adipate, citrate, phenylacetate, D-galactose, D-ribose, D-xylose, D-adonitol, D-fructose, L-rhamnose, inositol, sorbitol, salicin, D-cellobiose, D-melibiose, D-gentiobiose, D-lyxose, fucose and D-arabitol. Negative for nitrate reduction, glucose fermentation, assimilation of arginine and gelatin hydrolysis, erythritol, L-xylose, methyl-β-D-pyranoid xyloside, L-sorbose, dulcitol, methyl-α-D-pyranoid mannoside, methyl-α-D-pyranoid glucoside, amygdalin, arbutin, D-lactose, D-saccharose, inulin, D-melezitose, D-raffinose, amylum, glycogen, xylitol, D-tagatose, L-arabitol, potassium gluconate, 2-ketogluconate and 5-ketogluconate. The tricalcium phosphate solubilisation ability is positive in Pikovskays's medium. The major fatty acids are $C_{14:\ 0}$, $C_{16:\ 0}$, $C_{17:0}$ cyclo and $C_{18:\ 1}$ $\omega 7c$. The DNA G+C content of the type strain is 62.3 ± 0.5 mol%.

The type strain D414^T (=CICC 10561^T = DSM 26823^T) was isolated from multiple metal-polluted paddy soil at the surrounding area of Dabao Mountain Mine, Guangdong, Southeast China.

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