Alsobacter metallidurans gen. nov., sp. nov., a thallium-tolerant soil bacterium in the order Rhizobiales

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A thallium-tolerant, aerobic bacterium, designated strain SK200a-9T, isolated from a garden soil sample was characterized using a polyphasic approach. Comparative analysis of 16S rRNA gene sequences revealed that strain SK200a-9T was affiliated with an uncultivated lineage within the Alphaproteobacteria and the nearest cultivated neighbours were bacteria in genera in the family Methylocystaceae (93.3–94.4 % 16S rRNA gene sequence similarity) and the family Beijerinckiaeae (92.3–93.1 %) in the order Rhizobiales. Cells of strain SK200a-9T were Gram-stain-negative, non-motile, non-spore-forming, poly-β-hydroxybutyrate-accumulating rods. The strain was a chemo-organotrophic bacterium, which was incapable of growth on C1 substrates. Catalase and oxidase were positive. Atmospheric nitrogen fixation and nitrate reduction were negative. The strain contained ubiquinone Q-10 and cellular fatty acids C18:1ω7c, C16:1ω7c and C18:0 as predominant components. The major polar lipids were diphasphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. The DNA G+C content was 64.8 mol%. On the basis of the information described above, strain SK200a-9T is considered to represent a novel species of a new genus in the order Rhizobiales, for which the name Alsobacter metallidurans is proposed. The type strain of Alsobacter metallidurans is SK200a-9T (=NBRC 107718T=CGMCC 1.12214T).

Thallium (Tl) is a toxic heavy metal found in trace amounts in the earth’s crust, with concentrations ranging from 0.1 to 1.7 mg kg⁻¹ (Peter & Viraraghavan, 2005). In general, pollution with Tl is restricted to specific areas such as in the vicinity of non-ferrous metal mines, smelters and factories using Tl (Wierzbicka et al., 2004; Peter & Viraraghavan, 2005; Yang et al., 2005; Xiao et al., 2012). In our previous study (Bao et al., 2006), soil bacteria tolerant to 0.49 mM Tl (100 mg Tl ⁻¹) had been isolated from a non-polluted garden soil in Japan through a selective cultivation procedure. Phylogenetic analysis revealed that all of the 59 Tl-tolerant strains belonged to the class Alphaproteobacteria and that 17 of these strains constituted a distinct cluster (Group A; Bao et al., 2006) in the order Rhizobiales. One member of this group, strain SK200a-9T, had an MIC of 4.4 mM against Tl. Strain SK200a-9T was also tolerant to other heavy metals such as cadmium (MIC, 0.4 mM), cobalt (1.7 mM), copper (0.8 mM), nickel (1.7 mM), lead (1.4 mM) and zinc (3.1 mM) (Bao et al., 2006). In this paper, we describe strain SK200a-9T phenotypically, chemotaxonomically and phylogenetically and propose that it represents a novel species of a new genus.

Strain SK200a-9T used in this study was isolated on 100-fold-diluted nutrient broth agar supplemented with 0.49 mM Tl nitrate from a 0.98 mM Tl (200 mg Tl ⁻¹)-loaded soil suspension (Bao et al., 2006). In that study, the soil was sampled at a garden in the campus of Ibaraki University College of Agriculture (36° 2’ 7” N 140° 12’ 45” E). Gram staining, motility, oxidase and catalase tests were carried out as described previously (Ohta & Hattori, 1983). Bacterial cells were observed by

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3Abbreviation: PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SK200a-9T is AB231946.

Four supplementary figures are available with the online version of this paper.
phase-contrast microscopy with an Olympus BX-51 microscope and by transmission electron microscopy [JEM-2000EX (JEOL) at TechnoSuruga Laboratory (Shizuoka, Japan)]. To examine growth and morphological characteristics, cells were grown in peptone-yeast extract (PY) liquid medium at 30 °C for 3 days (late exponential phase). PY medium contained (w/v): 1% polypeptone (Nihon Seiyaku), 0.2% yeast extract (Difco Laboratories) and 0.1% MgSO₄·7H₂O. Poly-β-hydroxybutyrate (PHB) granule accumulation was observed under light microscopy after staining of cells with Sudan Black B-safranin. Colony morphology was observed on PY agar medium using an Olympus SZ61 stereoscopic microscope. Growth at different temperatures (5–45 °C at intervals of 5 °C) was assessed in PY liquid medium adjusted to pH 7.0 and growth at different pH (5.0–9.5 at intervals of 0.5 pH units) was monitored at 30 °C. Tolerance to NaCl was tested in liquid PY medium containing 0.25, 0.5, 1, 1.5, 2, 3, 4 and 5% (w/v) NaCl. Growth rate in the exponential growth phase was determined by measuring the optical densities (OD₆₆₀) of broth cultures as described previously (Ohara, 2001). Anaerobic culturing was performed using an anaerobic jar with an O₂-absorbing and CO₂-generating compound, 5% (w/v) NaCl. Growth was monitored for 7 days and the utilization of each substrate was assessed by comparing growth in the presence and absence of the substrate. Ability to utilize methane was tested using nitrate mineral salts (NMS) medium (Whittenbury et al., 1970) with a mixture of methane and air (1:1, v/v) according to the protocol of Dianou et al. (2012). Cellulose decomposition was determined using MS medium with filter paper for 1 month. Nitrogen fixation was assessed based on growth at 30 °C for 2 weeks on a nitrogen-free mineral salts (M52N) medium (Whittenbury et al., 1970) with a mixture of methane and air (1:1, v/v) according to the protocol of Dianou et al. (2012). Colony morphology was observed on PY agar medium using phase-contrast microscopy with an Olympus BX-51 microscope and by transmission electron microscopy [JEM-2000EX (JEOL) at TechnoSuruga Laboratory (Shizuoka, Japan)]. To examine growth and morphological characteristics, cells were grown in peptone-yeast extract (PY) liquid medium at 30 °C for 3 days (late exponential phase). PY medium contained (w/v): 1% polypeptone (Nihon Seiyaku), 0.2% yeast extract (Difco Laboratories) and 0.1% MgSO₄·7H₂O. Poly-β-hydroxybutyrate (PHB) granule accumulation was observed under light microscopy after staining of cells with Sudan Black B-safranin. Colony morphology was observed on PY agar medium using an Olympus SZ61 stereoscopic microscope. Growth at different temperatures (5–45 °C at intervals of 5 °C) was assessed in PY liquid medium adjusted to pH 7.0 and growth at different pH (5.0–9.5 at intervals of 0.5 pH units) was monitored at 30 °C. 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Growth was monitored for 7 days and the utilization of each substrate was assessed by comparing growth in the presence and absence of the substrate. Ability to utilize methane was tested using nitrate mineral salts (NMS) medium (Whittenbury et al., 1970) with a mixture of methane and air (1:1, v/v) according to the protocol of Dianou et al. (2012). Cellulose decomposition was determined using MS medium with filter paper for 1 month. Nitrogen fixation was assessed based on growth at 30 °C for 2 weeks on a nitrogen-free mineral salts (M52N) medium (pH 7.1) composed of (w/v): 0.1% K₂HPO₄, 0.02% MgSO₄·7H₂O, 0.01% CaCl₂, 0.02% NaCl and 0.002% Na₃MoO₄·2H₂O. Filter-sterilized glucose, sodium succinate or sodium acetate was added to the medium at 0.2% (w/v) as growth substrate. An acetylene reduction assay was also performed by using washed cells grown on the 0.2% (w/v) succinate-containing M52N medium supplemented with 0.01% (w/v) Difco yeast extract. The procedure for the assay was essentially the same as described previously (Sato et al., 2009). Acid production from glucose was tested using the OT test medium described by Hugh & Leifson (1953): glucose was added at three concentrations (0.13, 0.25 and 0.5%) and anaerobic cultivation was performed using the Anaero Pack system. API20 NE and API ZYM kits (bioMérieux) were used to examine biochemical characteristics of the strain according to the manufacturer’s instructions.

To analyse the fatty acid profile, strain SK200a-9T was grown in PY liquid medium at 30 °C for 3 days. Cellular fatty acid methyl esters were prepared by heating dried cells in anhydrous methanolic HCl at 100 °C for 3 h (Ikemoto et al., 1978) and then analysed by GLC with a Shimadzu GC-14A and a ULBON HR-SS-10 capillary column (0.23 mm × 50 m; Shimadzu). Fatty acid methyl ester peaks were identified with a bacterial acid methyl ester mix (Supelco) by using retention-time comparison against standard compounds. Polar lipids were extracted using the procedure described by Komagata & Suzuki (1987) and identified by two-dimensional TLC followed by spraying with the molybdenum blue reagent (Dittmer & Lester, 1964) for phospholipids, ninhydrin reagent for lipids containing amino groups and ethanolic phosphomolybdic acid reagent for total polar lipids. Isoprenoid quinones were extracted and analysed by HPLC as described by Komagata & Suzuki (1987). Details of HPLC conditions and the preparation of standard ubiquinones were as described previously (Ohara et al., 2003). The G+C content was determined by hydrolysis the DNA enzymically and quantifying the nucleotides by HPLC (Tamaoka & Komagata, 1984).

Genomic DNA of strain SK200a-9T was extracted previously according to the method of Wang & Wang (1997) and the 16S rRNA gene sequence was determined according to a conventional protocol (Lu et al., 2011). The sequence was compared with available 16S rRNA gene sequences in GenBank using the program BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment with sequences from related species and clones was performed by using the program CLUSTAL W (Thompson et al., 1994) in MEGA5 (Tamura K et al., 2011). Evolutionary distances were calculated using Kimura’s two-parameter model. Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA5. Topologies of the resultant trees were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Presence of the nitrogenase gene (nifH) was examined by PCR using four different sets of primers and reaction conditions as described by Ueda et al. (1995), Poly et al. (2001a, b) and Rösch & Bothe (2005). The DNA extracted from Bradyrhizobium japonicum NBRC 14792T was used as a positive control.

Cells of strain SK200a-9T were Gram-stain-negative, non-motile rods (0.8–1.0×1.4–2.8 μm; see Figs S1 and S2 available in IJSEM Online). As judged by microscopic investigations, strain SK200a-9T did not form spores. White inclusion bodies were often present, which were stained black with Sudan Black B, suggesting the presence of PHB. The colonies were punctiform, white to cream in colour and reached 0.2–0.3 mm in diameter after 1 week at 30 °C on PY medium. The maximum specific growth rate of strain SK200a-9T reached 0.12 h⁻¹ when repeatedly
subcultured in liquid PY cultures at 30 °C and at pH 7.0. The strain was able to grow at 10–40 °C (optimum, 25–30 °C) and at pH 5.5–9.0 (optimum, pH 7.0) in liquid PY cultures within 3 days (Fig. S3). No visible growth occurred at 5 or 45 °C and at pH 5.0 or 9.5 after prolonged incubation for 7 days. NaCl concentrations ≥0.25 % in PY medium suppressed growth (Fig. S3). Strain SK200a-9T was strictly aerobic, positive for catalase and oxidase, and did not reduce nitrate to nitrite. Glucose was used but acid production from glucose was negative. Methanol and methane were not used. Strain SK200a-9T was not able to grow in the nitrogen-free medium and was negative for nitrogenase activity by washed cells grown on the 0.01 % yeast extract-containing MS2N medium. The nifH gene was not detected by PCR amplification using the four tested primer sets. More detailed results of the phenotypic tests for strain SK200a-9T are given in the species description. The major cellular fatty acids (>5.0 %) of strain SK200a-9T were C18:1ω7c (53.8 %), C18:0 (14.1 %), C16:1ω7c (11.3 %) and C16:0 (10.5 %). Other minor fatty acids (<5.0 %) were C17:0 cyclo (2.2 %), C19:0 cyclo (2.2 %), C19:0 (1.3 %) and C17:0 (0.9 %). The polar lipid profile of strain SK200a-9T comprised diphostatidyglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and three minor unknown lipids (Fig. S4). The predominant quinone of strain SK200a-9T was ubiquinone Q-10 and the DNA G+C content was 64.8 mol%.

The almost-complete 16S rRNA gene sequence of strain SK200a-9T (1448 bp) had been determined in our previous study (Bao et al., 2006). Sequence comparisons with representative bacteria differentiated this bacterium from all currently described members of the families

![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain SK200a-9T and closely related taxa within the order Rhizobiales. Filled circles indicate that the corresponding nodes were also recovered with the maximum-likelihood algorithm. Numbers at nodes are bootstrap values (%) based on 1000 replicates (neighbour-joining/maximum-likelihood); values lower than 50 % are not shown. Sphingomonas paucimobilis ATCC 29837T was used as an outgroup. Bar, 0.02 nt substitutions per position.](http://ijs.sgmjournals.org)
Methylocystaceae and Beijerinckiaceae (Fig. 1). Levels of 16S rRNA gene sequence similarity with its closest relatives were 93.3–94.4% (members of the genera Methylocystis and Methylosinus in the family Methylocystaceae) and 92.3–93.1% (members of the genera Beijerinckia, Methylocapsa, Methylocella, Methyleneferula and Methylenevirgula in the family Beijerinckiaceae). The results of further 16S rRNA gene sequence comparisons with environmental clones revealed that the sequence of strain SK200a-9T was highly similar to those of clone 031 (99.4%; GenBank accession number AB252932) recovered from an iron-oxidation biofilm, clone SuR49 (98.5%; AB608698) from a rice paddy soil (Ishii et al., 2011) and clone BF0001B047 (97.2%; AM697021) from indoor dust (Rintala et al., 2008). As shown in the phylogenetic tree (Fig. 1), strain SK200a-9T formed a monophyletic clade with these three clones (SuR49, 031 and BF0001B047), distinct from members of the families Methylocystaceae and Beijerinckiaceae.

The major characteristics differentiating strain SK200a-9T from phylogenetically related genera are summarized in Table 1. Strain SK200a-9T was unable to grow on methanol and methane, and it could be differentiated from methanotrophic or methylotrophic members of the families Methylocystaceae and Beijerinckiaceae based on the use of sugars. Strain SK200a-9T was not capable of atmospheric nitrogen fixation, which also distinguished it from members of the methanotrophic or methylotrophic genera and the genus Beijerinckia. The inability to grow below pH 5.0 and the ability to grow at 40 °C distinguished the strain from species of the genus Beijerinckia. Based on these results and the above-mentioned phylogenetic characterization, we conclude that strain SK200a-9T should be placed in a novel species of a new genus in the order Rhizobiales, for which the name Alsobacter metallophilus gen. nov., sp. nov. is proposed.

### Table 1. Differential characteristics between strain SK200a-9T and related genera in the order Rhizobiales

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Beijerinckiaceae</th>
<th>Methylocystaceae</th>
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<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6</td>
<td>7 8</td>
</tr>
<tr>
<td>Motility</td>
<td>−</td>
<td>+/−</td>
</tr>
<tr>
<td>Nitrogen fixation</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Growth on methanol</td>
<td>−</td>
<td>+/−</td>
</tr>
<tr>
<td>Growth on methane</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Growth on sugars</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at pH 5.0</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 40 °C</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Major cellular fatty acid</td>
<td>C_{18,10}7c</td>
<td>C_{18,10}97c</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>64.8</td>
<td>54.7−60.7</td>
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arylamidase, acid phosphatase and naphth phosphohydrolase activities are present. Valine arylamidase is weakly present. N-Acetyl-β-glucoaminidase, arginine dihydrolase, α-chymotrypsine, cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-fucosidase, lipase (C14), α-mannosidase, trypsin and urease activities are absent. Able to use acetate, L-arabinose, L-ascorbic acid, citrate, D-fructose, D-galactose, D-glucose, L-glutamate, DL-lactic acid, L-rhamnose, D-mannose, pyruvate, succinate and D-xylene. Unable to use benzoate, p-coumarate, ethanol, formate, lactose, L-malate, maltose, D-mannitol, raffinose, proteocatechuate or succinate. No acid is produced from D-glucose. The major cellular fatty acids are C₁₈:1ω7c, C₁₈:0, C₁₆:1ω7c and C₁₆:0.

The type strain, SK200a-9T (=NBRC 107718T=CGMCC 1.12214T), was isolated from a garden soil sample in Ami, Ibaraki, Japan. The DNA G+C content of the type strain is 64.8 mol%.

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References


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