Thauera humireducens sp. nov., a humus-reducing bacterium isolated from a microbial fuel cell

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A Gram-negative, rod-shaped, non-spore-forming bacterium, designated SgZ-1T, was isolated from the anode biofilm of a microbial fuel cell. The strain had the ability to grow under anaerobic condition via the oxidation of various organic compounds coupled to the reduction of anthraquinone-2,6-disulphonate (AQDS) to anthrahydroquinone-2,6-disulphonate (AHQDS). Growth occurred in TSB in the presence of 0–5.5 % (w/v) NaCl (optimum 0–1 %), at 10–45 °C (optimum 25–37 °C) and at pH 6.0–10.0 (optimum 8.0–8.5). Based on 16S rRNA gene sequence similarity, strain SgZ-1T belonged to the genus Thauera. The highest level of 16S rRNA gene sequences similarity (96.7 %) was found to be with Thauera aminoaromatica S2T and Thauera selenatis AXT, and lower values were obtained when compared with other recognized Thauera species. Chemotaxonomic analysis revealed that strain SgZ-1T contained Q-8 as the predominant quinone, and putrescine and 2-hydroxyputrescine as the major polyamines. The major cellular fatty acids (>5 %) were C16 : 1ω6c and/or C16 : 1ω7c (44.6 %), C16 : 0 (18.8 %), and C18 : 1ω6c and/or C18 : 1ω7c (12.7 %). Based on its phenotypic and phylogenetic properties, chemotaxonomic analysis and the results of physiological and biochemical tests, strain SgZ-1T (=KACC 16524T=CCTCC M 2011497T) was designated the type strain of a novel species of the genus Thauera, for which the name Thauera humireducens sp. nov. was proposed.

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Abbreviations: AHQDS, anthrahydroquinone-2,6-disulphonate; AQDS, anthraquinone-2,6-disulphonate.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SgZ-1T is J0038037.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

Humus is a natural organic substance, and its structure is rich in quinone moieties that provide redox activity (Field & Cervantes, 2005). Humus reduction plays a significant role in the anaerobic bio-transformation of both organic and inorganic compounds (Perminova & Hatfield, 2005; Wu et al., 2011). A great deal of evidence has demonstrated that many pollutants could be degraded by microorganisms under anaerobic conditions with humus serving as the terminal electron acceptor (Lovley et al., 1996; Scott et al., 1998; Straub et al., 2005). As such, microbe-mediated humus reductions have been widely recognized, and a large number of humus-reducing micro-organisms have been isolated from a wide range of environments (Field & Cervantes, 2005; Wu et al., 2011).

In 1993, the genus Thauera was proposed, comprised of a single species Thauera selenatis, the type strain of which was capable of selenate reduction (utilizing selenate as electron acceptor) under anaerobic conditions (Macy et al., 1993). Since then, eight more novel species with validly published names in the genus Thauera have been isolated from various environments, and these species were reported to be capable of NO3− reduction and denitrification (utilizing NO3− as an electron acceptor) under anaerobic conditions (Anders et al., 1995; Foss & Harder, 1998; Scholten et al., 1999; Song et al., 2001; Mechichi et al., 2002; Dubbels et al., 2009). However, at the time of writing, no study has reported humus-reduction (utilizing humus as an electron acceptor) by strains in the genus Thauera. In this study, a bacterium designated SgZ-1T, capable of growing under anaerobic conditions via oxidizing various organic compounds coupled to the reduction of humus (quinone), was successfully isolated and investigated. Based on the results of polyphasic taxonomic study, this strain was proposed as a novel species of the genus Thauera.

Strain SgZ-1T was isolated from the anode biofilm of a sediment microbial fuel cell that has been described in
Cell morphology of strain SgZ-1T was determined using a JEM 1400 (JEOL) transmission electron microscope. In preparation for electron microscopy, bacterial cells were suspended in phosphate buffer solution (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 1.8 mM KH2PO4, pH 7.4), dried on a nickel-coated mesh and negatively stained with phosphotungstic acid. The motility of cells was tested using the hanging-drop method. The Gram reaction was determined by the KOH test (Buck, 1982) and further confirmed by the conventional Gram-staining method (Smibert & Krieg, 1994). Utilization of various substrates, enzyme activities and other physiological and biochemical properties were examined using the API 20E, API ID 32GN and API ZYM systems (bioMérieux), respectively, according to the manufacturer's instructions, except for the temperature of incubation. Analysis of starch hydrolysis was conducted as described by Smibert & Krieg (1994). Catalase activity was determined by observing bubble production in 3% (v/v) hydrogen peroxide solution and oxidase activity was determined using an oxidase reagent (bioMérieux). Growth at different temperatures was tested in TSB. The pH range (pH 3.0–10.0 at intervals of 0.5 pH units) for growth was determined in TSB buffered with citrate/phosphate buffer or Tris/hydrochloride buffer (Breznak & Costilow, 1994). The NaCl tolerance for growth was examined in TSB containing 0–10% NaCl (w/v) with increments of 0.5%.

For cellular fatty acid analysis, cells grown on TSA at 30 °C for 48 h to exponential phase were saponified, methylated and extracted using the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.0B). The fatty acids were analysed with GC (Agilent Technologies 6850) and identified by using the TSBA6.0 database of the Microbial Identification System (Sasser, 1990). For analysis of the polyamine pattern, cells were grown in TSB at 30 °C. Polyamines were extracted according to the protocol of Busse & Auling (1988) from biomass that had been harvested at approximately 70% of the maximum optical density and analysed by HPLC [Waters 1525 equipped with a XTerra MS C18 column (250 mm × 4.6 mm, 5 μm particles)] as described by De Mey et al. (2012). Quinones were extracted according to the protocol of Collins et al. (1977) and analysed with HPLC as described by Tamaoka et al. (1983).

To confirm the AQDS-reducing capacity of strain SgZ-1T, batch experiments were conducted. Cells grown aerobically in TSB to late-exponential phase were harvested by centrifugation (6000 r.p.m. at 4 °C for 10 min), washed twice and resuspended in sterilized MSM. And the resulting bacterial cell suspension (OD600=1.0) was used as seed solution for further studies. To determine alternative electron donors for AQDS reduction, 1 ml of the seed solution was introduced into bottles, each containing 20 ml MSM supplied with 1 mM AQDS and one of the following substrates (5 mM): acetate, glucose, lactate, propionate and pyruvate. For the treatment using glucose as electron donor, cell number was determined by direct colony counts on TSA. Strict anaerobic and sterile techniques were used throughout the reduction experiments as described by Li et al. (2009). All treatments were tested in triplicate in the dark at 30 °C with two control assays: an abiotic set without cells and a biotic set without electron donor or acceptor. At an interval of 2 days, triplicate cultures were used for determining the quantities of reduced AQDS [i.e. anthrahydroquinone-2,6-disulphonate (AHQDS)], and the samples were filter-sterilized (PVDF, 0.22 μm; Millipore). The concentration of AHQDS was quantified with a UV–Vis spectrophotometer (TU1800-PC; Beijing) at a wavelength of 450 nm compared with the controls using Na2S2O3 as chemical reduc tant (OD sample/OD Na2S2O3) (Liu et al., 2007).

Genomic DNA was extracted according to standard procedures (Sambrook & Russell, 2001). The 16S rRNA gene was PCR-amplified from genomic DNA using two bacterial universal primers (27f and 1492r; Baker et al., 2003). The PCR product was gel purified using Gel Extraction kit D2500-01 (Omega Bio-tek) and sequenced using an automated sequencer. Pairwise sequence similarity was calculated using a global alignment algorithm implemented at the EzTaxon server (http://www.eztaxon.org; Chun et al., 2007). Phylogenetic analysis was carried out using the software package MEGA version 4.0 (Tamura et al., 2007) after multiple alignment of the sequence data with CLUSTAL_X (Thompson et al., 1997). Distances were calculated using distance options according to Kimura's two-parameter model (Kimura, 1980) and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987) and maximum-parsimony method (Eck & Dayhoff, 1966). Statistical support for the branches of phylogenetic trees was determined using bootstrap analysis (based on 1200 resamplings) (Felsenstein, 1985). The G+C content of the genomic DNA was determined by thermal denaturation (Mandel & Marmur, 1968).

A nearly complete 16S rRNA gene sequence (1444 nt) was determined for strain SgZ-1T. Phylogenetic analysis based on 16S rRNA gene sequence revealed that strain SgZ-1T...
could be grouped into the genus Thauera. Comparison between the 16S rRNA gene sequence of strain SgZ-1T and those of the species of the genus Thauera with validly published names showed that, the closest relatives of strain SgZ-1T were Thauera aminoaromatica S2T (96.7 %) and Thauera selenatis AXT (96.7 %), followed by Thauera phenylacetica B4PT (96.6 %). The type strains of all other recognized species of the genus Thauera showed lower levels of 16S rRNA gene sequence similarity to strain SgZ-1T. Given the low levels of 16S rRNA gene sequence similarity (<97 %) between strain SgZ-1T and all the species with validly published names of the genus Thauera, DNA–DNA hybridization studies were not carried out. A sequence alignment was created with sequences from all type strains of the genus Thauera, and the neighbour-joining and maximum-parsimony trees depicting the phylogenetic relationships between strain SgZ-1T and its nearest phylogenetic neighbours are shown in Fig. 1 and supplementary Fig. S2, respectively. It was clear from the phylogenetic analysis based on nearly complete 16S rRNA gene sequences that the new isolate was grouped as a Thauera species and represented a distinct phyletic line that can be considered as a separate genomic species (Stackebrandt & Goebel, 1994).

Strain SgZ-1T was examined for a range of characteristics. This strain was facultatively anaerobic and cells of strain SgZ-1T were Gram-negative, motile and rod-shaped (0.6–0.8 × 1.8–2.5 μm) (Fig S1, available at IJSEM online). Colonies of this strain were light brown, convex and circular with entire margins. Growth occurred in TSB at 10–45 °C (optimum 25–37 °C) and at pH 6.0–10.0 (optimum 8.0–8.5). Growth occurred in the presence of 0–5.5 % (w/v) NaCl with optimal growth at 0–1 % (w/v) NaCl. This strain was able to grow on MacConkey agar.

The DNA G+C content of strain SgZ-1T was 65.8 mol%, which fell within the range observed for the other members of the genus Thauera (64.2–70.6 mol%). Detailed morphological, physiological and biochemical characteristics of strain SgZ-1T are summarized in the species description below and in Table 1.

The quinone system of strain SgZ-1T contained ubiquinone Q-8 as the predominant component, and the main cell polyamines were putrescine and 2-hydroxyputrescine. Cellular fatty acid analysis of strain SgZ-1T revealed that C12:0 (4.6 %), C16:0 (18.8 %), summed feature 3 (44.6 %) and summed feature 8 (12.7 %) were the major non-hydroxylated fatty acids, and that C10:0 3-OH (4.8 %) was the major hydroxylated fatty acid. The complete fatty acid profile is presented in Table S1, and this profile was consistent with that of the genus Thauera. However, strain SgZ-1T could be distinguished from its closest phylogenetic neighbours, both qualitatively and quantitatively, with regard to certain fatty acids and by the unique fatty acid proportion of summed feature 7 (1.3 %). In view of the above, strain SgZ-1T had chemotaxonomic characteristics typical of members of the genus Thauera (Macy et al., 1993).

The substrate spectra for strain SgZ-1T under aerobic and denitrifying conditions are shown in Table S2. Species in the genus Thauera have been reported as being capable of NO3− reduction, and most of them were able to grow under denitrifying conditions utilizing aromatic or terpenoid hydrocarbons as the sole carbon and energy sources, which was considered as a major characteristic of the recognized species of the genus Thauera (Macy et al., 1993; Anders et al., 1995; Foss & Harder, 1998; Scholten et al., 1999; Song et al., 2001; Mechichi et al., 2002; Dubbels et al., 2009).

![Fig. 1. Phylogenetic tree constructed using the neighbour-joining method based on 16S rRNA gene sequences of strain SgZ-1T. Thio bacter subterraneus C55T (the type species of its genus) was used as outgroup. Bootstrap values, generated from 1200 resamplings, at or above 50 % are indicated at the branching points. Bar, 0.01 nt substitutions per nucleotide position.](http://ijs.sgmjournals.org)
Table 1. Differential characteristics of strain SgZ-1T and closely related species of the genus Thauera

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (μm)</td>
<td>0.6–0.8×1.8–2.5</td>
<td>0.5–0.75×2–3</td>
<td>1.4×0.56</td>
<td>3.0–3.7</td>
</tr>
<tr>
<td>Flagella</td>
<td>Monotrichous</td>
<td>ND</td>
<td>Monotrichous</td>
<td>Peritrichous</td>
</tr>
<tr>
<td>Optimum pH for growth</td>
<td>8.0–8.5</td>
<td>7.5–8.6</td>
<td>8.0 with nitrate, 7.0 with selenate</td>
<td>7.5–8.0</td>
</tr>
<tr>
<td>Optimum temperature for growth (°C)</td>
<td>25–37</td>
<td>28</td>
<td>25–30</td>
<td>30</td>
</tr>
<tr>
<td>Growth factors required</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Growth on nutrient agar</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Electron acceptors</td>
<td>O2, NO3, AQDS</td>
<td>O2, NO3</td>
<td>O2, NO3, Se (VI), Se (IV)</td>
<td>O2, NO3</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>65.8</td>
<td>68.4</td>
<td>66</td>
<td>68.6</td>
</tr>
</tbody>
</table>

Strain SgZ-1T was also capable of growing on various organic substrates when NO3 was supplied as the terminal electron acceptor, and its substrate spectra were distinct from those of the other species of the genus Thauera.

The reduction of AQDS by strain SgZ-1T with different electron donors was determined and the results are shown in Fig. 2. A considerable amount of AHQDS was produced with glucose or pyruvate as electron donor, and a lesser amount of AHQDS was produced with acetate or lactate, while almost no AHQDS was detected with propionate. Significant microbial growth was observed with glucose as electron donor over 10 days incubation when AQDS reduction and increase of cell numbers occurred concurrently (data not shown). Cell growth was negligible in the controls without AQDS or glucose, which indicated that strain SgZ-1T could not ferment glucose and only grew in the presence of both AQDS and glucose. This is the first report, to our knowledge, of a member of the genus Thauera that can reduce extracellular quinones and harvest energy for microbial growth through quinone reduction.

The phylogenetic tree based on 16S rRNA gene sequences, G+C content and the chemotaxonomic characteristics clearly suggested that strain SgZ-1T was a member of the genus Thauera. However, besides the low 16S rRNA gene similarities, comparison of physiological and biochemical characteristics between strain SgZ-1T and its neighbouring strains demonstrated significant differences, suggesting that strain SgZ-1T was evidently distinct from all recognized species of the genus Thauera. On the basis of these data, strain SgZ-1T represents a novel species of the genus Thauera, and for this novel species the name Thauera humireducens sp. nov. was proposed.

**Description of Thauera humireducens sp. nov.**

Thauera humireducens [hu.mi.re.duc.en’s. L. n. humus soil, used to refer to humic substances; L. part. adj. reducens leading back (to a reduced oxidation state); N.L. part. adj. humireducens converting humic substances to a reduced oxidation state].

Cells are facultative anaerobic, Gram-negative, catalase- and oxidase-positive, motile, rods with rounded ends, approximately 0.6–0.8 μm wide and 1.8–2.5 μm long. After 24 h of aerobic growth on TSA at 30 °C, colonies were small (0.8–1.2 mm in diameter), circular with entire edges and convex. Growth occurred in TSB containing up to 5.5% (w/v) NaCl (optimum 0–1%), at 10–45 °C (optimum 25–37 °C) and at pH 6.0–10.0 (optimum 8.0–8.5). Growth occurred on MacConkey agar. Gelatin and starch were not hydrolysed. Cells could grow with AQDS or NO3 as the sole terminal electron acceptor under anaerobic conditions. With the API 20E kit, results were positive for arginine dihydrolase, hydrolysis of urea, Voges–Proskauer reaction and indole production, and

**Fig. 2.** Characteristics of AQDS reduction by strain SgZ-1T with various electron donors. All treatments were tested in triplicate; error bars, SE.
negative for β-galactosidase, lysine decarboxylase, ornithine decarboxylase, gelatinase, tryptophan deaminase and H₂S production. Acid was not produced from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin or arabinose. Activities of C4 esterase, C8 lipase, leucine arylamidase and naphthol-AS-BI-phosphohydrolyase were positive, while activities of alkaline phosphatase, C14 lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and β-fucosidase were negative. In API ID 32GN tests, the following substrates were utilized: propionic acid, valeric acid, 3-hydroxybutyric acid, 4-hydroxybenzoic acid, L-proline, sodium acetate, lactic acid, L-alanine, 3-hydroxybenzoic acid and L-serine. The predominant quinone was ubiquinone Q-8. The main polyamines were putrescine and 2-hydroxyputrescine. The major cellular fatty acids (>5%) were C₁₆:₁ω₆c and/or C₁₆:₁ω₇c (44.6 %), C₁₆:₀ (18.8 %) and C₁₈:₁ω₆c and/or C₁₈:₁ω₇c (12.7 %). The DNA G+C content of the type strain was 65.8 mol%.

The type strain SgZ-1ₜ (=KACC 16524ₜ=CCTCC M 2011497ₜ) was isolated from a microbial fuel cell in our laboratory in Guangdong Province, PR China.

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References


