Thiopseudomonas denitrificans gen. nov., sp. nov., isolated from anaerobic activated sludge

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A Gram-staining-negative, rod-shaped, motile and facultatively anaerobic bacterial strain, designated X2T, was isolated from the sludge of an anaerobic, denitrifying, sulfide-removal bioreactor, and found to oxidize sulfide anaerobically with nitrate as electron acceptor. The strain grew at salinities of 0–3 % (w/v) NaCl (optimum, 0–1 %). Growth occurred at pH 6.0–10.0 (optimum, pH 8.0) and 10–37 °C (optimum, 30 °C). The genomic DNA G+C content was 59 mol%. Q-8 and Q-9 were detected as the respiratory quinones. The major fatty acids (≥10 %) were C16 : 1ω7c and/or C16 : 1ω6c, C18 : 1ω7c and C16 : 0. The polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and one unidentified phospholipid. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain X2T formed a novel clade within the family Pseudomonadaceae, with the highest sequence similarity to Pseudomonas caeni KCTC 22292T (93.5 %). On the basis of phenotypic, chemotaxonomic and phylogenetic characteristics, it is proposed that this strain represents novel genus and species within the family Pseudomonadaceae, for which the name Thiopseudomonas denitrificans gen. nov., sp. nov. is proposed. The type strain is X2T (=CCTCC M 2013362T =DSM 28679T =KCTC 42076T).

The family Pseudomonadaceae belongs to the class Gamma-proteobacteria and comprises 12 genera at the time of writing (http://www.bacterio.net/-classifgenerafamilies.html - Pseudomonadaceae; Parte, 2014), and the type genus Pseudomonas was first proposed by Migula (1894). During research into the microbial diversity of an anaerobic, denitrifying, sulfide-removal bioreactor, a novel strain, X2T, was isolated. Phylogenetic analysis based on 16S rRNA gene sequences revealed that X2T was closely related to the strain KCTC 22292T in the family Pseudomonadaceae. However, it is clear that the new isolate is distinct from its phylogenetic neighbours by high sequence divergence and significant differences in physiological and chemotaxonomic characteristics. In this study, the results of polyphasic taxonomic analysis of strain X2T are reported.

Strain X2T was isolated from sludge of an anaerobic, denitrifying, sulfide-removal bioreactor by using the anaerobic roll-tube technique and serial dilution until pure culture was obtained. The components of the isolation medium were as follows (g l−1): Na2S.9 H2O, 0.75; CH3COONa . 3H2O, 0.6426; KNO3, 0.7575; NH4Cl, 1.0; KH2PO4, 1.8; Na2HPO4 .12 H2O, 3.0; MgSO4 .7H2O, 0.1. The strain was cultivated, maintained on TSA medium and stored as aqueous glycerol suspensions (20 %, v/v) at −80 °C.

Aerobic growth and colony morphology were observed after incubation for 3 days at 30 °C on tryptic soy agar (TSA; Difco) plates. Gram staining was carried out by using the standard Gram reaction and confirmed by using the KOH lysis test method (Cerny, 1978). Cell morphology
was examined by transmission electron microscopy (model 2100, JEM) after negative staining with 1% (w/v) phosphotungstic acid using cells grown on TSA for 3 days at 30 °C. The temperature range for growth was tested at 4, 10, 20, 25, 30, 37 and 42 °C on TSA plates. TSA plates containing 0, 1, 2, 3, 4 and 5% (w/v) NaCl were used for NaCl tolerance experiments. The pH range for growth was investigated between pH 4.0 and 11.0 in increments of 1 pH unit by using the buffer systems described by Xu et al. (2005) (pH 4.0–5.0, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0, 0.1 M KH2PO4/0.1 M NaOH; pH 9.0–10.0, 0.1 M NaHCO3/0.1 M Na2CO3; pH 11.0, 0.05 M Na3HPO4/0.1 M NaOH) and tryptic soy broth (TSB; Difco) as basal medium. The production of water-soluble fluorescent pigments on King’s B agar was determined as described by King et al. (1954). Catalase activity was determined by bubble production in 3% (v/v) hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% (w/v) tetramethyl-p-phenylenediamine. Anaerobic sulfide oxidation and denitrification were tested by using the following procedure: boil distilled water to remove dissolved oxygen; dissolve the components of the isolation medium mentioned above with oxygen-free distilled water; add 100 ml medium to 250 ml serum bottle; add resazurin (0.1%, w/v) as indicator and bubble the medium with N2 for 30 min; seal the serum bottle and sterile at 121 °C for 15 min; inoculate the sterilized medium with strain X2T (5%, v/v) and shake the serum bottle and sterilize at 121 °C; take samples every 5 h and measure the concentration of compounds by ion chromatography (DIONEX). Additional tests for physiological and biochemical characteristics and enzyme activities were conducted using API 20NE and API ZYM strips (bioMérieux) according to the manufacturer’s instructions.

Colonies of strain X2T were translucent around the edge and dark yellow in the centre after incubation for 3 days at 30 °C on TSA plates. Cells were Gram-stain negative, facultatively anaerobic and rod-shaped. Cells were 0.4–0.7 μm wide and 1.0–1.5 μm long (Fig. S1, available in the online Supplementary Material) with a polar flagellum. Sulfide was oxidized with nitrate as electron acceptor under anaerobic conditions (Fig. S2). Detailed morphological, physiological and biochemical characteristics of strain X2T, the closest phylogenetic relative and the type strains of type species of genera of the family Pseudomonadaceae are given in Table S1. Strain X2T can be readily differentiated from Pseudomonas caeni KCTC 22292T on the basis of several characteristics (Table 2). The G+C content of genomic DNA of strain X2T was 59 mol%. Differing chemotaxonomic results between strains X2T and P. caeni KCTC 22292T are shown in Table 2, which indicates that the polar lipids, quinones and fatty acids of the two strains are obviously different.

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were performed as described by Li et al. (2007). The identification of phylogenetic neighbours was initially carried out by using the BLASTN (Altschul et al., 1997) program against the database containing type strains with validly published prokaryotic names and representatives of uncultured phyotypes (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The phylogenetic trees were reconstructed by using the software package MEGA version 5.0 (Tamura et al., 2011) according to neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms. Kimura’s two-parameter model was used to calculate evolutionary distance matrices for the neighbour-joining and maximum-likelihood methods (Kimura, 1980). The topology of the phylogenetic trees was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The 16S rRNA gene sequence (1505 bp) of strain X2T was obtained. The BLASTN result indicated that sequence

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>Colony colour</td>
<td>Translucent edge, dark-yellow centre</td>
<td>Translucent, yellowish</td>
</tr>
<tr>
<td>Growth at 4 °C</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Sulfide oxidation</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolysis of gelatin</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Assimilation of caprate</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Enzyme activities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esterase (C4)</td>
<td>W</td>
<td>+</td>
</tr>
<tr>
<td>Esterase lipase (C8)</td>
<td>W</td>
<td>+</td>
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<tr>
<td>Valine arylamidase</td>
<td>–</td>
<td>+</td>
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</table>

Table 1. Differential characteristics of strain X2T and P. caeni KCTC 22292T

Strains: 1, X2T; 2, P. caeni KCTC 22292T. +, Positive; −, negative; w, weakly positive. Data are from this study.

Sherlock Microbial Identification System (MIDI) according to the manufacturer’s instructions. The fatty acid methyl esters were identified by using the Microbial Identification software package (Sherlock version 6.1; MIDI database: TSB6). The G+C content of genomic DNA was calculated from genome sequencing data.

The polar lipids of strain X2T consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and one unidentified phospholipid (Fig. S3). The quinones of the strain were Q-8 and Q-9 (Fig. S4). The major fatty acids (>10%) of strain X2T were C16:1ω7c and/or C16:1ω6c, C18:1ω7c and C16:0 (Table 2). The G+C content of genomic DNA of strain X2T was 59 mol%. Differing chemotaxonomic results between strains X2T and P. caeni KCTC 22292T are shown in Table 2, which indicates that the polar lipids, quinones and fatty acids of the two strains are obviously different.

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were performed as described by Li et al. (2007). The identification of phylogenetic neighbours was initially carried out by using the BLASTN (Altschul et al., 1997) program against the database containing type strains with validly published prokaryotic names and representatives of uncultured phyotypes (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The phylogenetic trees were reconstructed by using the software package MEGA version 5.0 (Tamura et al., 2011) according to neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms. Kimura’s two-parameter model was used to calculate evolutionary distance matrices for the neighbour-joining and maximum-likelihood methods (Kimura, 1980). The topology of the phylogenetic trees was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The 16S rRNA gene sequence (1505 bp) of strain X2T was obtained. The BLASTN result indicated that sequence
The clade was grouped into another larger, compact cluster (70 % bootstrap value support) with other species were less than 93.6 %. The neighbour-joining phylogenetic tree showed that the novel isolate formed a stable clade with the members of the genus *Pseudomonas*, the relatively high sequence divergence value (>6.4 %) showed that the isolate was distantly related to the described taxon. Besides the phylogenetic analysis based on 16S rRNA gene sequences, strain X2T was also clearly distinguishable from *P. caeni* KCTC 22292T based on physiology, biochemistry, chemotaxonomy and genomic DNA G+C content (Tables 1 and 2). In addition, strains of the species of the genus *Pseudomonas* include in their compositions the hydroxylated fatty acids C₁₀:0 3-OH and C₁₂:0 2-OH, and C₁₂:0 and ubiquinone Q-9 (Brenner et al., 2005). But the fatty acid C₁₂:0 2-OH (0.07 %) was almost absent from strain X2T and the major ubiquinone of strain X2T was Q-8 (90.38 %) (Fig. S4). These results further differentiate strain X2T from the species of genus *Pseudomonas*.

On the basis of the results of physiological, biochemical, chemotaxonomic and phylogenetic analysis, strain X2T should be classified as a representative of a novel species within a new genus, for which the name *Thiopseudomonas denitrificans* gen. nov., sp. nov. is proposed.

**Description of Thiopseudomonas denitrificans** gen. nov., sp. nov.

*Thiopseudomonas* [thi.o.pseudo.monas. Gr. n. theion (Latin transliteration thium) sulfur; N.L. n. *Pseudomonas* from Gr. adj. pseudo false; Gr. n. monas a unit, monad; *Thiopseudomonas* an organism with a false single unit and sulfur].

Cells are Gram-stain-negative, rod-shaped and facultatively anaerobic. Catalase- and oxidase-positive. The predominant respiratory quinone is Q-8. The major fatty acids are C₁⁶:₁₀₇c and/or C₁₆:₁₀₆c, C₁₈:₁₀₇c and C₁₆:₁₀. The polar lipids consist of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and one unidentified phospholipid. The genomic DNA G+C content of the type strain of the type species is 59 mol%. Phylogenetically, the genus is affiliated to the family *Pseudomonadaceae*. The type species is *Thiopseudomonas denitrificans*.

**Description of Thiopseudomonas denitrificans** sp. nov.


Cells are Gram-stain-negative, facultatively anaerobic, rod-shaped and approximately 0.4–0.7 × 1.0–1.5 μm in size. Cells are motile by means of a polar flagellum. Colonies are translucent at the edge and dark yellow in the centre after
incubation for 3 days at 30 °C on TSA plates. Growth occurs at 10–37 °C (optimum, 30 °C). The pH range for growth is pH 6.0–10.0 (optimum, pH 8.0). Growth occurs in the presence of 0–3 % (w/v) NaCl (optimum, 0–1 %), but not in the presence of 4 % (w/v) NaCl. Cells do not produce fluorescent pigment on King's B media. Catalase-positive. Positive for activities of esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase. Negative for activities of alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, insulin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-fucosidase, α-mannosidase and N-acetyl-β-glucosaminidase (API ZYM test strip). Positive for aerobic and anaerobic denitrification, and assimilation of malate. Negative for indole production, acidification of D-glucose, activities of arginine dihydrolase and urease, hydrolysis of aesculin. Negative for assimilation of D-glucose, arabinose, mannose, mannnitol, N-acetylglucosamine, maltose, gluconate, caprate, adipate, citrate and phenylacetate (API 20NE test strip). Sulfide is oxidized anaerobically with nitrate as electron acceptor. The predominant respiratory quinone is Q-8. The major fatty acids (>10 %) are C₁₆;₁ω7c and/or C₁₆;₁ω6c, C₁₈;₁ω7c and C₁₆;₀. The polar lipids consist of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycolinositol and one unidentified phospholipid.

The type strain is X2T (=CCTCC M 2013362T=DSM 28679T =KCTC 42076T) was isolated from the sludge of an anaerobic, denitrifying, sulfide-removal bioreactor. The genomic DNA G+C content of the type strain is 59 mol%.

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**References**


