**Thauera mechernichensis sp. nov., an aerobic denitrifier from a leachate treatment plant**

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A heterotrophic bacterial strain TL1T capable of aerobic denitrification was previously enriched in continuous culture from a landfill leachate treatment plant and isolated as a pure culture. The taxonomic position of this isolate within the β-subclass of the Proteobacteria was determined by 16S rDNA sequence analysis and by conventional taxonomy including substrate spectrum, quinone type (ubiquinone Q-8) and cellular fatty acid composition. Detection of the specific polyamine 2-hydroxyputrescine supports the membership of strain TL1T in the β-subclass of the Proteobacteria. The results of 16S rDNA sequencing showed that the strain clustered with, but was separate from, *Thauera aromatica* and *Thauera selenatis*. DNA–DNA hybridization experiments indicated that the new isolate represents a new species of the genus, for which the name *Thauera mechernichensis* is proposed; the type strain is DSM 12266T.

**Keywords:** *Thauera mechernichensis* sp. nov., aerobic denitrification

**INTRODUCTION**

Although heterotrophic nitrification has been known for decades (Verstraete, 1975) and aerobic denitrification has been repeatedly reported (Carter et al., 1995a), it was ultimately the work of Robertson & Kuenen (1984) on *Thiosphaera pantotropha* GB17 (now *Paracoccus denitrificans* DSM 2944; renamed as *Paracoccus pantotrophus* according to Rainey et al., 1999) which shed new light on these interesting and unusual conversions of nitrogen compounds (Jetten et al., 1997). The observation of nitrogen losses during a leachate treatment process (Hippen et al., 1997) prompted us to study the bacterial flora in a leachate treatment plant at Mechernich, Germany. Enrichment by continuous culture was successful and led to the isolation of a bacterial strain, TL1T, which proved to be a heterotrophic nitrifier/aerobic denitrifier (Lukow & Diekmann, 1997). Although its metabolism revealed similarities to *Paracoccus denitrificans* DSM 2944, it soon became evident that the strain had peculiar properties.

Partial sequencing of the 16S rDNA indicated a high relationship between strain TL1T and members of the genus *Thauera*. The few known strains of this genus group with the two species *Thauera aromatica* and *Thauera selenatis*, and are well-known for their ability to denitrify (Anders et al., 1995) but have not been reported to denitrify in the presence of oxygen. Results of experiments in conventional taxonomy, as well as chemo- and molecular taxonomy, are reported here which allow the classification of strain TL1T as a novel species.

**METHODS**

**Cultivation of bacterial strains.** Unless otherwise stated, strain TL1T was grown aerobically at 37 °C in 500 ml Erlenmeyer flasks in 100 ml medium containing (l-1): 0.30 g ammonium chloride, 2.93 g sodium acetate and mineral salts at pH 8 (Robertson & Kuenen, 1984; Lukow & Diekmann, 1997). For the preparation of plates, the medium was solidified by addition of 15 g agar l-1. Cells for the detection of N2O formation and nitrate reductase assays were grown in 100 ml mineral medium (Hooijmans et al., 1990) with the addition of 4.0 g potassium nitrate l-1. Cells that were used for the detection of poly-β-hydroxybutyrate were grown in the same medium without nitrate. Cultivation of strain TL1T under anaerobic conditions was performed in serum bottles tightly closed with butyl rubber stoppers with a crimped metal seal and the medium was gassed with O2-free nitrogen.
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gas as described by Miller & Wolin (1974). *T. aromaticum* was grown aerobically on a shaker at 30 °C in *T. aromaticum* medium 586 (DSMZ, 1996).

Strain **m**Xy**N**1 was grown at 30 °C in tightly closed 1 1 glass bottles containing 800 ml medium. The cultures were gently shaken twice a day. The basal medium had the following composition (g deionized water 1-l): NaCl, 1.00; CaCl₂·2H₂O, 0.10; NH₄Cl, 0.25; KH₂PO₄, 0.50; KCl, 0.50; MgSO₄·7H₂O, 0.50; sodium benzoate, 0.29; KNO₃, 0.51 (F. Widdel, personal communication). After autoclaving and cooling, 20 ml NaHCO₃ solution, vitamins, EDTA-chelated mixture of trace elements and selenite and tungstate solution (Widdel & Bak, 1992) were added. The pH of the medium was adjusted to 7-3.

*T. selenatis* was grown aerobically on a shaker at 30 °C in minimal medium containing 0.4% yeast extract, 20 mM nitrate and 20 mM acetate (Macen et al., 1993).

**Phenotypic characterization.** For characterization, bacterial strains were cultivated at 27 °C on agar plates or in LB medium on a shaker at 140 r.p.m. The Gram-reaction was carried out with the test kit Gram colour 2 (Deutsche bioMérieux), and the KOH test for determining the type of cell wall was done according to Gregersen (1978). The presence of catalase and oxidase was examined as detailed by Smibert & Krieg (1994). API 20NE was used as described by the manufacturer (Deutsche bioMérieux). Tests for growth on different substrates were performed aerobically and anaerobically at 37 °C in the medium of Hooijmans et al. (1990) with 4.0 g potassium nitrate 1-l; acetate was replaced by 5 mM of the respective substrate.

For the determination of the optimum temperature for growth, strain **T**1 was grown in 100 ml Erlenmeyer flasks containing 12 ml mineral salt medium with ammonium chloride and acetate (Robertson & Kuenen, 1984). The flasks were not shaken and were incubated in waterbaths at 34, 37, 40 and 44 °C, respectively, with three parallel flasks per temperature. Growth was determined by measurement of OD₅₇₈ after 0, 4, 6 and 8 h.

For the determination of poly-β-hydroxybutyrate, strain **T**1 was grown aerobically at 37 °C. When the cultures had reached the stationary phase, heat-fixed smears were prepared. Staining with Nile Blue A was performed according to the method of Osele & Holt (1982). The preparations were examined with the fluorescence microscope Axioshot (Zeiss) using an excitation filter of 395-440 nm.

**GC detection of N₂O.** Cells of **T**1 were grown aerobically at 37 °C in 500 ml Erlenmeyer flasks and collected by centrifugation. Resuspended cells (5 ml; OD₅₇₈ equivalent to 10) were used to inoculate 50 ml O₂-free mineral medium (Hooijmans et al., 1990) with 1.2 g potassium nitrate 1-l in a 60 ml serum bottle. Incubation was at 37 °C. Samples (50 μl) were drawn from the gas space and applied to a Poraplot Q-column (10 m x 0.32 mm i.d.) in a Chrompack GC model CP 9002. The carrier gas (35 ml min⁻¹) was nitrogen and the N₂O signal was detected by an electron capture detector.

**Assay of nitrate reductase activity.** Cells were grown anaerobically in 125 ml serum bottles and aerobically in 500 ml Erlenmeyer flasks at 37 °C, collected by centrifugation and washed with nitrate-free medium. Protein was estimated by the Lowry method. Nitrate reductase activity was assayed spectrophotometrically at 600 nm in silicon-stoppered cuvettes using benzyll and methyl viologen as described by Jones & Garland (1977).

Chemotaxy. A modified Marmur procedure (Takahashi, 1993) was used to isolate genomic DNA. The G+C content (mol%) was measured by HPLC after hydrolysis as described by Tamaoka & Komagata (1984). Lambda DNA was used as a reference.

For whole-cell fatty acid analysis, the bacteria were grown on TSB agar for 48 h at 28 °C and two to four loopfuls of bacterial cells were scraped from the Petri dish. Fatty acid methyl esters were obtained by saponification, methylation and extraction of the cells as described previously (Kämpfer & Kroppenstedt, 1996). The fatty acid methyl ester mixtures were separated using the model 5898A microbial identification system (Sasser, 1990).

For analysis of polyamines, freeze-dried cultures obtained from bacterial strains grown on LB medium until the late exponential phase were used. Polyamines were dasylylated and extracted with toluene (Scherer & Kneifel, 1993). Separation was performed according to Busse & Auling (1988). Dansylated polyamines were determined by a fluorescence detector (model 420-E/420-AC; Waters) with excitation at 360 nm and a cut-off filter of 450 nm. Quinones were determined by TLC and HPTLC according to Kroppenstedt (1982).

**16S DNA sequence determination.** Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of PCR products were carried out using procedures described previously (Rainey et al., 1996). Purified PCR products were sequenced using the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) as directed in the manufacturer's protocol. The Applied Biosystems 310 DNA Genetic Analyzer was used for electrophoresis of the sequence reaction products.

**Phylogenetic analysis.** The aa2 editor (Maidak et al., 1994) was used to align the 16S rDNA sequence of strains **T**1 and *T. selenatis* strain ATCC 55363 against the 16S rDNA sequences of members of the β subclass of the *Proteobacteria* available from the public databases. Pairwise evolutionary distances were calculated using the correction of De Ley et al. (1970) using the least-squares distance method of De Soete (1983) was used in the construction of the phylogenetic dendrogram from distance matrices.

**Nucleotide sequence accession numbers.** The strain designations and accession numbers of the reference strains used in the phylogenetic analyses are as follows: *T. aromaticum* strain K 172, X77118; *T. aromaticum* strain **m**Xy**N**1, X83533; *T. selenatis* strain ATCC 55363, X68491; *Azoarcus evansii* strain KB 740, X77679; *Azoarcus indigens* strain BH72, L15530; *Azoarcus toluolyticus* strain Tol-2, L33694; *Zoogloea ramigera* strain ATCC 19544, X74913.

DNA-DNA hybridization. Bacterial cells were harvested by centrifugation at 16000 g for 10 min, washed twice in 0.15 M saline/0.1 M EDTA pH 8.0 and, if necessary, stored at −20 °C. DNA was prepared and DNA-DNA hybridization was done by the thermal renaturation rate method of De Ley et al. (1970) using a Giford model 2600 spectrophotometer as described previously (Wiese et al., 1996).

**RESULTS AND DISCUSSION**

Phenotypic and physiological characterization

Strain **T**1 was enriched from the rotating biological contactor for nitrification in a leachate treatment plant at Meckernich, Germany, by continuous culture at
Table 1. Characteristics of different species of the genus Thauera

<table>
<thead>
<tr>
<th>Character</th>
<th>TL1*</th>
<th>T. aromatica K 172T†</th>
<th>T. selenatis‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Short rods</td>
<td>Short rods</td>
<td>Rods</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>0.75 x 1.5-2.0</td>
<td>0.5-1.5 x 1.0-2.5</td>
<td>0.56 x 1.4</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flagella</td>
<td>Single, polar</td>
<td>‘Degenerately peritrichous’</td>
<td>Single, polar</td>
</tr>
<tr>
<td>Catalase reaction</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase reaction</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic denitrification</td>
<td>+ (mainly N₂O)</td>
<td>+ (mainly N₂O)</td>
<td>+ (to N₂O)</td>
</tr>
<tr>
<td>Aerobic denitrification</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Selenate respiration</td>
<td>ND</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Oxidative metabolism</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>65</td>
<td>67</td>
<td>66</td>
</tr>
<tr>
<td>Optimum growth pH</td>
<td>ND</td>
<td>7-0-7-4</td>
<td>8-0 (with nitrate), 7-0 (with selenate)</td>
</tr>
<tr>
<td>Optimum growth temp. (°C)</td>
<td>40</td>
<td>28</td>
<td>25-30</td>
</tr>
<tr>
<td>Growth factors required</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Quinone(s)</td>
<td>Ubiquinone Q-8</td>
<td>ND</td>
<td>Ubiquinone Q-8</td>
</tr>
<tr>
<td>Fatty acids in lipids (%)§</td>
<td>10:0 3-OH</td>
<td>4-0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>12:0</td>
<td>5:3</td>
<td>4-9</td>
</tr>
<tr>
<td></td>
<td>16:1o7cis</td>
<td>46:9</td>
<td>40:8</td>
</tr>
<tr>
<td></td>
<td>16:0</td>
<td>18:5</td>
<td>32:2</td>
</tr>
<tr>
<td></td>
<td>18:1o7cis</td>
<td>16:6</td>
<td>13:6</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Starch</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Urea</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Poly-β-hydroxybutyrate</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Data from Lukow & Diekmann (1997).
† Data from Anders et al. (1995), Tschech & Fuchs (1987) and Song et al. (1998).
‡ Data from Rech & Macy (1992) and Macy et al. (1993).
§ Abbreviations: 10:0 3-OH, 3-hydroxy decanoic acid; 16:1o7cis, cis-9-hexadecenoic acid; 18:1o7cis, cis-9-octadecenoic acid.

pO₂ of 50% and dilution rate of 0.04 h⁻¹ in an acetate/ammonium chloride-containing medium with a C/N ratio of 10:5. Upon a nitrate pulse, a high denitrification rate was observed (Lukow & Diekmann, 1997). The Gram-negative strain was isolated as a pure culture by repeated streaking on agar plates with a mineral medium (Robertson & Kuenen, 1984). The cells of strain TL1T are slightly curved rods of 1.5-2.0 μm length and 0.75 μm width. Acetate can be replaced by propionate, butyrate or ethanol, but not by methanol (Lukow & Diekmann, 1997). A single polar flagellum (2 μm long; determined by scanning electron microscopy) is observed, which is similar to that of T. selenatis (Macy et al., 1993), but different from the 'degenerately peritrichous' type found in T. aromatica K 172T (Song et al., 1998). When grown in the absence of nitrate, cells accumulated large amounts of storage materials. Cells exhibited a bright orange fluorescence in the test for the presence of poly-β-hydroxybutyrate indicating a positive result. We tried to identify the strain by performing the API 20NE test. The code obtained, 1004667, did not point to any of the bacterial species listed in the Analytical Profile Index. Some characters pointed to the genus Zoogloea, but only the chemotaxonomic data and the partial sequencing of the 16S rDNA gave evidence for its relation to the genus Thauera. The characters of strain TL1T were compared to other strains in this genus and are described in Table 1. The optimum growth temperature was 40 °C. Growth rates between 6 and 8 h of cultivation at 40 °C were twice as high as those at 37 °C, and the growth rate fell sharply as the temperature increased beyond this point, eventually reaching zero.
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Under aerobic conditions, strain TLIT used acetate, 2-aminobenzoate, benzoate, L-leucine, phenyl acetate and DL-phenyl-aminobenzoate, benzoate, L-glutamate, 4-hydroxy-alanine (weak growth), but not phenol. All these substances, including phenol, were used anaerobically as a substrate.

Special attention was given to the denitrification abilities under aerobic conditions and the formation of N₂O under anaerobiosis. Tests were carried out as described by G. Fuchs (personal communication; volume ratio gas phase/liquid phase was 1/11, KNO₃ concentration was 1.20 g l⁻¹). Three hours after inoculation, N₂O was identified in the gas space. It was observed that N₂O peaks diminished with time and were not detectable after 18 h.

Micro-organisms showing aerobic nitrate respiration have been characterized by the investigation of levels of membrane-bound and periplasmic nitrate reductases (Bell et al., 1990; Carter et al., 1995a). Tests following the protocol of Jones & Garland (1977) revealed that in strain TLIT, the ratios of nitrate reduction using benzyl and methyl viologen as electron donors do not differ significantly when the cells were grown either aerobically or anaerobically. This indicates that the nitrate reductase is of the periplasmic type and no additional cytoplasmic nitrate reductase is induced under anaerobic growth conditions as has recently been shown for the newly isolated Pseudomonas putida 2.9 (Carter et al., 1995b). The ability of strain TLIT to carry out heterotrophic nitrification was calculated from balances in continuous culture (Lukow & Diekmann, 1997).

Chemotaxonomic characterization

Major cellular non-polar fatty acids detected in strain TLIT are 16:1 3-OH and 12:0 3-OH. Similar data were found for T. aromatica 6964T and A. evansi DSM 6898T. Existing quantitative differences do not seem to be valid for differentiation within the group. Ubiquinone Q-8 was detected, but not rhodoquinone. Polyamine analysis revealed 60.5 µmol hydroxy-

putrescine (g dry wt)⁻¹, 11.1 µmol spermidine (g dry wt)⁻¹, 4.7 µmol cadaverine (g dry wt)⁻¹ and 4.4 µmol putrescine (g dry wt)⁻¹.

Phylogenetic analyses

The almost complete 16S rDNA sequences of strains TLIT and Thauera selenatis strain ATCC 55363T, comprising 1491 nucleotides (>95% of the Escherichia coli sequence; Brosi et al., 1978), were determined in this study. The phylogenetic dendrogram shown in Fig. 1 was reconstructed from evolutionary distances (Jukes & Cantor, 1969) by the least-squares distance method (De Soete, 1983). A total of 1428 nucleotides present in all strains between positions 38 and 1482 (E. coli positions) were used for these analyses. The sequence of T. selenatis strain ATCC 55363T was re-determined in this study since the original sequence (X68491) was not complete and comprised only 1336 nucleotide positions between 33 and 1376 (E. coli positions). The original sequence of T. selenatis strain ATCC 55363T (X68491) had 99.0% similarity when compared to the sequence of T. selenatis strain ATCC 55363T determined in this study (Y17591), the original sequence was not used in the analyses presented in this study.

The 16S rDNA sequence-based phylogenetic tree shown in Fig. 1 indicates the position of strain TLIT within the radiation of the species of the genera Thauera and Azoarcus. Z. ramigera was used as an outgroup in these analyses. 16S rDNA similarity values between the sequences included in the analyses (Fig. 1) and that of strain TLIT indicate that strain TLIT is most closely related at the 16S rDNA level to members of the genus Thauera. The similarity values of the sequence of strain TLIT to the species of the genus Thauera are as follows: 98.5% similarity to T. aromatica strain K 172T; 98.7% similarity to T. aromatica strain mXYN1; and 98.5% similarity to T. selenatis strain ATCC 55363T. The 16S rDNA sequence similarity values between strain TLIT and species of the genus Azoarcus were in the range 94.7-95.0%.

Fig. 1. Phylogenetic position of strain TLIT among members of the β-subclass of the Proteobacteria.
**Table 2.** DNA–DNA hybridization studies

Mean values are given; numbers of determinations are shown in parentheses.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reassociation (%) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSM 12266T</td>
</tr>
<tr>
<td><em>T. mechernichensis</em> DSM 12266T</td>
<td>100</td>
</tr>
<tr>
<td><em>T. aromatica</em> DSM 6984T</td>
<td>27 (4)</td>
</tr>
<tr>
<td>Strain mXyN1</td>
<td>39 (2)</td>
</tr>
<tr>
<td><em>T. selenatis</em> ATCC 55363T</td>
<td>25 (2)</td>
</tr>
</tbody>
</table>

DNA–DNA hybridization experiments within the genus *Thauera* compared strain TL1T to *T. selenatis* (Macy *et al.*, 1993), *T. aromatica* (Anders *et al.*, 1995) and strain mXyN1 (Rabus & Widdel, 1995). The data presented in Table 2 show that the toluene-degrading, denitrifying strain mXyN1 hybridized well with *T. aromatica* DSM 6984T and is therefore identified as belonging to that species according to Wayne *et al.* (1987). On the other hand, a low degree of similarity (25–39%) between strain TL1T and the three other members of the genus *Thauera* was observed. Since it is generally accepted that among strains of the same species the levels of DNA similarity should be equal to or greater than 70%, strain TL1T cannot be allocated to any of the three known species of the genus *Thauera* and constitutes a new species in the genus *Thauera* on the basis of DNA relatedness.

**Comparison with species belonging to the genus *Thauera***

While the presently recognized species of the genus *Thauera* are characterized either by their abilities to reduce selenate or to degrade aromatic compounds anaerobically, the new species proposed here was enriched and characterized by its ability for heterotrophic nitrification and aerobic denitrification (Lukow & Diekmann, 1997). Further investigation of physiological, chemotaxonomic and phylogenetic characters revealed (as shown in Table 1) that there are few differences, but the distinctness of the 16S rDNA sequence of strain TL1T from those of the two previously described species of the genus *Thauera* are in congruence with the results of the DNA–DNA experiments indicating its novel species status. A distinction from other species of the genus is possible due to its optimal temperature for growth (40 °C), which may reflect an adaptation to the special conditions of the natural habitat of strain TL1T. The finding that strain TL1T has an active periplasmic nitrate reductase deserves further investigation.

**Description of *Thauera mechernichensis* sp. nov.**

*Thauera mechernichensis* (*me.cher.nich.en'sis. M.L. adj. mechernichensis* pertaining to Mechernich, because the organism was isolated from the leachate treatment plant at Mechernich, Germany).

Gram-negative, slightly curved, rod-shaped cells are 1·5–2·0 μm long and 0·75 μm wide, have rounded ends.
and contain granules of poly-β-hydroxybutyrate in the stationary growth phase. Cells are highly motile, having one polar flagellum 2 μm long. The optimum temperature of growth is 40 °C. Cells are oxidase-positive and weakly catalase-positive. Translucent, white-yellow, slimy colonies on mineral salts medium containing acetate and ammonium chloride are 1 mm in diameter after growth at 37 °C for 48 h. Preferred growth substrates are acetate, propionate, butyrate and ethanol. It cannot use methanol. Many aromatic compounds as well as malate and citrate are used as sole carbon sources under denitrifying conditions. N₂O is formed anaerobically but can then be used as electron acceptor. Phylogenetically, T. meckernichensis is a member of the β-subclass of the Proteobacteria, ubiquinone Q-8 being the major quinone and 2-hydroxyputrescine the specific polyamine. This organism was isolated from the nitrification step in a leachate treatment plant. The type strain is TL1T (= DSM 12266T). The DNA G+C composition is 64.9 mol%.

NOTE ADDED IN PROOF
After submission of the manuscript, a publication by S. Foss & J. Harder [Syst Appl Microbiol 21, 365-373 (1998)] appeared describing two new species of the genus Thauera. The 16S rDNA sequence similarity values of the sequence of strain TL1T to the new Thauera species are as follows: 96.9% similarity to Thauera linaloolensis strain 47Lo1T; and 96.7% similarity to Thauera terpenica strain 58EuT. To evaluate the position of all known Thauera species, a new phylogenetic dendrogram was constructed (Fig. 2) using the nucleotide sequence accession numbers as follows: T. linaloolensis strain 47Lo1T, AJ005816; T. terpenica strain 58EuT, AJ005817, as well as T. aromaticum strain T1, U95176 (Song et al., 1998).

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REFERENCES


