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

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A heterotrophic bacterial strain TL1^T 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 TL1^T 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 12266^T.

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 in Mechernich, Germany. Enrichment by continuous culture was successful and led to the isolation of a bacterial strain, TL1^T, 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 TL1^T 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 TL1^T as a novel species.

METHODS

Cultivation of bacterial strains. Unless otherwise stated, strain TL1^T 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 TL1^T 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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The EMBL accession numbers for the 16S rDNA sequences of strain TL1^T and *T. selenatis* strain ATCC 55363^T are Y17590 and Y17591, respectively.

gas as described by Miller & Wolin (1974). *T. aromatica* was grown aerobically on a shaker at 30 °C in *T. aromatica* medium 586 (DSMZ, 1996).

Strain mXyN1 was grown at 30 °C in tightly closed 1 l glass bottles containing 800 ml medium. The cultures were gently shaken twice a day. The basal medium had the following composition (g deionized water l⁻¹): 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 (Macy *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 Gregsen (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 l⁻¹; acetate was replaced by 5 mM of the respective substrate.

For the determination of the optimum temperature for growth, strain TL1^T 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 detection of poly-β-hydroxybutyrate, strain TL1^T 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 Ostle & Holt (1982). The preparations were examined with the fluorescence microscope Axiophot (Zeiss) using an excitation filter of 395–440 nm.

GC detection of N₂O. Cells of TL1^T 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 l⁻¹ 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 × 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 benzyl and methyl viologen as described by Jones & Garland (1977).

Chemotaxonomy. 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 dansylated and extracted with toluene (Scherer & Kneifel, 1983). 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 rDNA 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 ae2 editor (Maidak *et al.*, 1994) was used to align the 16S rDNA sequence of strains TL1^T and *T. selenatis* strain ATCC 55363^T against the 16S rDNA sequences of members of the β-subclass of the *Proteobacteria* available from the public databases. Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969). 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. aromatica* strain K 172^T, X77118; *T. aromatica* strain mXyN1, X83533; *T. selenatis* strain ATCC 55363^T, X68491; *Azoarcus evansii* strain KB 740^T, X77679; *Azoarcus indigenus* strain BH72^T, L15530; *Azoarcus tolulyticus* strain Tol-4^T, L33694; *Zoogloea ramigera* strain ATCC 19544^T, X74913.

DNA–DNA hybridization. Bacterial cells were harvested by centrifugation at 16000 g for 10 min, washed twice in 0.15 M saline/0.10 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 Gilford model 2600 spectrophotometer as described previously (Wiese *et al.*, 1996).

RESULTS AND DISCUSSION

Phenotypic and physiological characterization

Strain TL1^T was enriched from the rotating biological contactor for nitrification in a leachate treatment plant at Mechernich, Germany, by continuous culture at

Table 1. Characteristics of different species of the genus *Thauera*

+, Positive; (+), weakly positive; —, negative; ND, not determined.

Character	TL1 ^{T*}	<i>T. aromatica</i> K 172 ^{T†}	<i>T. selenatis</i> ‡
Cell morphology	Short rods	Short rods	Rods
Cell size (µm)	0.75 × 1.5–2.0	0.5–1.5 × 1.0–2.5	0.56 × 1.4
Motility	+	(+)	+
Flagella	Single, polar	'Degenerately peritrichous'	Single, polar
Catalase reaction	(+)	+	+
Oxidase reaction	+	+	+
Anaerobic denitrification	+ (mainly N ₂ O)	+ (mainly N ₂ O)	+ (to N ₂ O)
Aerobic denitrification	+	ND	ND
Selenate respiration	ND	—	+
Oxidative metabolism	+	+	+
G + C content (mol %)	65	67	66
Optimum growth pH	ND	7.0–7.4	8.0 (with nitrate), 7.0 (with selenate)
Optimum growth temp. (°C)	40	28	25–30
Growth factors required	—	—	+
Quinone(s)	Ubiquinone Q-8	ND	Ubiquinone Q-8
Fatty acids in lipids (%):§			
10:0 3-OH	4.0	4.9	ND
12:0	5.3	4.9	ND
16:1 ω 7 cis	46.9	40.8	ND
16:0	18.5	32.2	ND
18:1 ω 7 cis	16.6	13.6	ND
Hydrolysis of:			
Gelatin	+	ND	—
Starch	ND	ND	—
Urea	—	ND	ND
Poly- β -hydroxybutyrate	ND	+	+

* Data from Lukow & Diekmann (1997).

† Data from Anders *et al.* (1995), Tschsch & 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; 16:1 ω 7 cis , *cis*-9-hexadecenoic acid; 18:1 ω 7 cis , *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 TL1^T 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 172^T (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 TL1^T 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.

Fig. 1. Phylogenetic position of strain TL1^T among members of the β -subclass of the Proteobacteria.

Under aerobic conditions, strain TL1^T used acetate, 2-aminobenzoate, benzoate, L-glutamate, 4-hydroxybenzoate, L-leucine, phenyl acetate and DL-phenylalanine (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 TL1^T, 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 TL1^T 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 TL1^T are 16:1, 16:0 and 18:1 ω 7 cis , and hydroxylated fatty acids are 10:0 3-OH and 12:0 3-OH. Similar data were found for *T. aromatica* 6964^T and *A. evansii* DSM 6898^T. 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 μ mol hydroxy-

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

Phylogenetic analyses

The almost complete 16S rDNA sequences of strains TL1^T and *Thauera selenatis* strain ATCC 55363^T, comprising 1491 nucleotides (>95% of the *Escherichia coli* sequence; Brosius *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 55363^T 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 55363^T (X68491) had 99.0% similarity when compared to the sequence of *T. selenatis* strain ATCC 55363^T 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 TL1^T 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 TL1^T indicate that strain TL1^T is most closely related at the 16S rDNA level to members of the genus *Thauera*. The similarity values of the sequence of strain TL1^T to the species of the genus *Thauera* are as follows: 98.5% similarity to *T. aromatica* strain K 172^T; 98.7% similarity to *T. aromatica* strain mXyN1; and 98.5% similarity to *T. selenatis* strain ATCC 55363^T. The 16S rDNA sequence similarity values between strain TL1^T and species of the genus *Azoarcus* were in the range 94.7–95.0%.

Table 2. DNA–DNA hybridization studies

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

Strain	Reassociation (%) with:			
	DSM 12266 ^T	DSM 6984 ^T	Strain mXyN1	ATCC 55363 ^T
<i>T. mechernichensis</i> DSM 12266 ^T	100			
<i>T. aromatica</i> DSM 6984 ^T	27 (4)	100		
Strain mXyN1	39 (2)	90 (3)	100	
<i>T. selenatis</i> ATCC 55363 ^T	25 (2)	43 (2)	27 (2)	100

Fig. 2. Phylogenetic position of all known members of the genus *Thauera*.

DNA–DNA hybridization experiments within the genus *Thauera* compared strain TL1^T 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 6984^T 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 TL1^T 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 TL1^T 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 TL1^T 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 TL1^T. The finding that strain TL1^T has an active periplasmic nitrate reductase deserves further investigation.

Description of *Thauera mechernichensis* sp. nov.

Thauera mechernichensis (me.cher.ni.chen'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 μ m 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. N_2O is formed anaerobically but can then be used as electron acceptor. Phylogenetically, *T. mechnichensis* 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 TL1^T (= DSM 12266^T). 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 TL1^T to the new *Thauera* species are as follows: 96.9% similarity to *Thauera linaloolentis* strain 47Lo1^T; and 96.7% similarity to *Thauera terpenica* strain 58Eu^T. 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. linaloolentis* strain 47Lo1^T, AJ005816; *T. terpenica* strain 58Eu^T, AJ005817, as well as *T. aromatica* strain T1, U95176 (Song *et al.*, 1998).

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