Tetrathibacter mimigardefordensis sp. nov., isolated from compost, a betaproteobacterium capable of utilizing the organic disulfide 3,3'-dithiodipropionic acid

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In this study, a novel betaproteobacterium, strain DPN7^T, was isolated under mesophilic conditions from compost because of its capacity to utilize the organic disulfide 3,3'-dithiodipropionic acid. Analysis of the 16S rRNA gene sequence of strain DPN7^T revealed 98.5 % similarity to that of *Tetrathibacter kashmirensis* LMG 22695^T. Values for sequence similarity to members of the genera *Alcaligenes*, *Castellaniella* and *Taylorella*, the nearest neighbours of the genus *Tetrathibacter*, were about 95 % or less. The DNA G + C content of strain DPN7^T was 55.1 mol%. The level of DNA–DNA hybridization between strain DPN7^T and *T. kashmirensis* LMG 22695^T was 41 %, whereas it was much lower between strain DPN7^T and *Alcaligenes faecalis* LMG 1229^T (7 %) or *Castellaniella defragrans* LMG 18538^T (5 %). This genotypic divergence was supported by differences in biochemical and chemotaxonomic characteristics. For this reason, and because of the differences in the protein and fatty acid profiles, strain DPN7^T should be classified within a novel species of *Tetrathibacter*, for which the name *Tetrathibacter mimigardefordensis* sp. nov. is proposed. The type strain is strain DPN7^T (= DSM 17166^T = LMG 22922^T).

Polythioesters are bacterial polymers containing sulfur in the backbone and are accumulated as hydrophobic inclusions in the cytoplasm (Lütke-Eversloh & Steinbüchel, 2004). The first example of this novel type of biopolymer was found to be synthesized by *Ralstonia eutropha*, a Gram-negative soil bacterium, which naturally accumulates polyhydroxyalkanoates at levels up to more than 90 % of cell dry matter (Pedrós-Alio *et al.*, 1985). *R. eutropha* can produce various polythioester copolymers under specific cultivation conditions in the presence of 3-mercaptopropionic acid, 3-mercaptopaleric acid, 3,3'-thiodipropionic acid or 3,3'-dithiodipropionic acid (DTDP) by employing its polyhydroxyalkanoate synthase (Lütke-Eversloh *et al.*, 2001a, b; Lütke-Eversloh & Steinbüchel, 2003). Polythioester homopolymers were synthesized from 3-mercaptopropionic acid,

3-mercaptopaleric acid or 3-mercaptopaleric acid by a recombinant strain of *Escherichia coli* expressing a non-natural pathway (Lütke-Eversloh *et al.*, 2002).

R. eutropha and *E. coli* are unable to utilize these sulfur-containing substrates as sole carbon sources for growth. In addition, polythioesters currently cannot be synthesized *de novo* from simple carbon sources and sulphate: their biosynthesis depends on the use of the organic sulfur compounds mentioned above. Little is known (or published) about the catabolic pathways of organic sulfur compounds; exceptions are the catabolism of cysteine and methionine as well that as of dimethylsulfoxide, dimethylsulfoniopropionate and dimethylsulfide as intermediates of the sulfur cycle (Kertesz, 2000; Kiene *et al.*, 2000; Lomans *et al.*, 2002; Yoch, 2002) and the biodesulfurization of benzothiophenes (McFarland, 1999) and of the fluorinated organic sulfur compound bis-(3-pentafluorophenylpropyl)-sulfide (Van Hamme *et al.*, 2004).

To investigate the unknown catabolism of DTDP (Fig. 1), which is one of the precursor substrates currently used for the production of 3-mercaptopropionic acid-containing polythioesters (Lütke-Eversloh & Steinbüchel, 2003), bacterial

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Abbreviation: DTDP, 3,3'-dithiodipropionic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DPN7^T is AY880023.

A phase-contrast micrograph of cells of strain DPN7^T is available as supplementary material in IJSEM Online.

Fig. 1. Structural formula of DTDP.

strains capable of utilizing this organic sulfur compound as a carbon source for growth are desirable for the understanding, and potential application, of DTDP-converting enzymes. In this study, we describe the taxonomic and biochemical characterization of a newly isolated bacterium, strain DPN7^T, which is able to grow with DTDP as the sole carbon source.

Strain DPN7^T was isolated from matured compost after the sieving process from a compost plant in Münster (Germany) under mesophilic and aerobic conditions. An enrichment culture was prepared by inoculating 50 ml mineral salts medium (MSM) (Schlegel *et al.*, 1961) supplemented with 0.5% (w/v) DTDP as the sole carbon source with approximately 2 g compost. The culture was incubated at 30 °C for approximately 1 week. Afterwards, serial dilutions of the enrichment culture were spread onto MSM agar plates containing 0.5% (w/v) DTDP and then incubated for 7 days at 30 °C. Cells from large colonies were repeatedly transferred to identical agar plates until an axenic culture was obtained. Storage was accomplished in screw-capped vials at –70 °C in 20% (v/v) glycerol and as lyophilizates.

Colonies of strain DPN7^T were circular, white to lime-yellowish in colour and were more flat than convex. Older colonies on MSM-DTDP agar developed a more yellowish colour. On complex medium, colonies of DPN7^T grew in 1–2 days and appeared more white than yellow in colour. The cells were cultivated at 30 °C in liquid MSM containing 0.5% (w/v) DTDP and samples were withdrawn from different growth stages of the culture. Cells of DPN7^T were Gram-negative, coccoid rods approximately 1.5–2 µm in length and 1.5 µm in width and often occurred in pairs, as revealed under a Zeiss light microscope (see Supplementary Fig. S1 available in IJSEM Online). Cell motility was observed: after flagella staining performed according to Blenden & Goldberg (1964), one or two flagella could be detected.

Gram-staining performed according to Gerhardt *et al.* (1994) gave a negative reaction. Strain DPN7^T was oxidase-positive, as revealed by using Bactident oxidase test strips (Merck), and was catalase-positive, as revealed by adding 3% (v/v) H₂O₂ to freshly grown colonies. API 20NE (bioMérieux) was used to determine the assimilation of some carbon sources and the presence of important enzymes. The utilization of different carbon sources and the growth at different temperatures were investigated in liquid MSM and on MSM agar plates containing 0.2% (w/v) of the respective carbon source (Table 1), unless

Table 1. Morphological, physiological and biochemical characterization of strain DPN7^T and *Tetrathibacter kashmirensis* LMG 22695^T

Characteristics were determined either by cultivating the cells at 30 °C on MSM agar plates containing the respective substrates as sole carbon sources or by using API 20NE tests. ND, Not detected. Cells of both strains are Gram-negative, coccoid rods that produce circular and flat colonies with yellow pigmentation. Both strains can utilize mercaptosuccinic acid, and neither strain can utilize 3,3'-thiodipropionic acid, 4',4'-dithiodibutyric acid, thioglycolic acid or thiodiglycolic acid. Neither strain can reduce nitrite to dinitrogen gas.

Characteristic	Strain DPN7 ^T	<i>Tetrathibacter kashmirensis</i> LMG 22695 ^T
Cell length (µm)	1.5–2	1.0–1.8
Flagellation	Yes	ND
Utilization of:		
D-Fructose	–	+
DTDP	+	–
3-Mercaptopropionic acid	+*	–
2-Mercaptopropionic acid	+*	–
Dibenzothiophene	+	–
Benzothiophene	+	–
Reduction of nitrate to nitrite	–†	+

*Growth occurred at concentrations of up to 0.1% (w/v).

†Determined using API 20NE tests.

indicated otherwise. Besides having the physiological characteristics shown in Table 1, both strains were positive for utilization of D-glucose, sodium gluconate, acetic acid, propionic acid, butyric acid and succinic acid by conventional cultivation and according to the API 20NE system both strains were positive for utilization of L-arabinose, adipic acid, citric acid and malic acid and negative for D-mannose, D-mannitol, D-maltose, N-acetylglucosamine, capric acid and phenylacetic acid. Additional biochemical characteristics (shown in Table 1) according to the API 20NE system were as follows: both strains were positive for urease activity and negative with regard to the fermentative degradation of glucose, indole production and activity of arginine dihydrolase, β-glucosidase, protease and β-galactosidase. This strain had no specific requirement for vitamins and also showed no enhanced growth in the presence of vitamin solution (Mohn, 1995) in defined medium. Growth was typically observed after 2–4 days on MSM agar plates containing 0.5% (w/v) DTDP when incubated at 20–37 °C. In liquid MSM containing 0.2–0.5% (w/v) DTDP as the sole carbon source, strain DPN7^T exhibited a characteristic lag phase of approximately 48 h. After that period of time, the exponential phase followed, with a reproducible duration of more than 50 h. To the best of our knowledge, this is the first bacterial isolate found to utilize DTDP as the sole carbon and energy source for growth. Whereas good growth

occurred with DTDP, no growth was observed with the C₄ analogue 4,4'-dithiodibutyric acid or with 3,3'-thiodipropionic acid. The putative cleavage product of DTDP, 3-mercaptopropionic acid, could be utilized only at concentrations of less than 0.2 %, because of its toxicity (Table 1).

The polyester content of the cells, and its composition, was determined upon methanolysis of 7–10 mg lyophilized cells in the presence of 15 % sulfuric acid by gas chromatographic analysis of the resulting methyl esters, as described previously (Brandl *et al.*, 1988; Timm *et al.*, 1990). The cells were capable of synthesizing poly(3-hydroxybutyrate) as a storage compound when they were cultivated in MSM containing gluconate as the sole carbon source. When DTDP or 3-mercaptopropionic acid was provided as an additional carbon source, the cells accumulated a copolymer (up to 20 mol%), consisting of 3-hydroxybutyrate and 3-mercaptopropionic acid.

Antibiotic-susceptibility testing on nutrient broth agar (Sambrook *et al.*, 1989) containing different concentrations of antibiotics showed good growth of strain DPN7^T on plates containing 5 µg chloramphenicol ml⁻¹ and poor growth with 50 µg chloramphenicol ml⁻¹, but no growth with 500 µg chloramphenicol ml⁻¹. Growth also occurred in the presence of 50 µg thiostrepton ml⁻¹. The strain showed no growth on plates containing 5 µg kanamycin ml⁻¹, 5 µg gentamicin ml⁻¹, 6.25 µg tetracycline ml⁻¹ or 50 µg streptomycin ml⁻¹ (the lowest concentrations tested). Strain DPN7^T was able to grow slowly on agar plates containing ampicillin at concentrations up to the standard concentration for *E. coli* (75 µg ml⁻¹).

To determine the sequence of the 16S rRNA gene, genomic DNA was extracted according to Marmur (1961). The gene was amplified by a PCR using *Pfx* DNA polymerase (MBI Fermentas) and total genomic DNA as template for the oligonucleotide primers 27f and 1525r, which were complementary to conserved regions of the 16S rRNA gene sequence of *E. coli*. The 1502 bp PCR product was purified using the NucleoTrap kit (Macherey-Nagel) and then sequenced. The PCR product was also ligated to *EcoRV*-linearized pBluescript II SK(–) (Stratagene) DNA and transformed into competent cells of *E. coli* TOP10. Plasmid DNA was isolated as described by Birnboim & Doly (1979).

DNA sequencing was performed by applying the Sequi-Therm long-read cycle sequencing kit (Epicenter Technologies), with the following oligonucleotides as sequencing primers: 27f (5'-GAGTTTGATCCTGGCTCAG-3'), 343r (5'-CTGCTGCCTCCCGTA-3'), 357f (5'-TACGGGAGGC-AGCAG-3'), 519r [5'-G(T/A)-ATTACCGCGGC(T/G)GC-TG-3'], 536f [5'-CAGC(C/A)GCCGCGGTAAT(T/A)C-3'], 803f (5'-ATTAGATACCCTGGTAG-3'), 907r (5'-CCGT-CAATTCATTTGAGTTT-3'), 1114f (5'-GCAACGAGCGC-AACCC-3'), 1385r [5'-CGGTGTGT(A/G)CAAGGCC-3'] and 1525r (5'-AGAAAGGAGGTGATCCAGCC-3'), as well as the universal primer 5'-GTAAACGACGGCCAGT-3' and the reverse primer 5'-CAGGAAACAGCTATGAC-3'

that hybridize to pBluescript II SK(–). Sequence reactions were accomplished by using the GeneReadIR 4200 DNA analyser (LI-COR). 16S rRNA gene sequences were analysed using the program BLAST (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>) by running the BLASTN program. The nucleotide sequence of the 16S rRNA gene of strain DPN7^T and of related type strains belonging to the same phylogenetic group and also to well-known representatives of the *Betaproteobacteria* were aligned using CLUSTALX (Thompson *et al.*, 1997). 16S rRNA gene sequences were retrieved from the EMBL database and from the Ribosomal Database Project (Maidak *et al.*, 1997). The resulting trees, displayed with TreeView (Page, 1996), were calculated by using the neighbour-joining method (Saitou & Nei, 1987).

The nucleotide sequence of the 16S rRNA gene of strain DPN7^T shows high levels of sequence similarity (98.6–98.0 %) to several *Alcaligenes* sp. strains (Hiraishi *et al.*, 2003; Boivin-Jahns *et al.*, 1996), but these strains could not be analysed further since they are no longer available (R. Christen, personal communication). A sequence similarity of 98.5 % with respect to the type strain of the recently isolated and classified species *Tetrathibacter kashmirensis* (Ghosh *et al.*, 2005) was found, whereas lower similarity values were found with respect to the type strains of *Castellaniella defragrans* (strain 54Pin^T) (95.1 %), *Alcaligenes faecalis* (the type species of the genus *Alcaligenes*) (94.0 %), *Alcaligenes latus* (88.9 %) and *Taylorella equigenitalis* (94.7 %). The phylogenetic position of strain DPN7^T is illustrated in a neighbour-joining dendrogram with *Burkholderia kururiensis* as the outgroup (Fig. 2).

For determination of the G + C content, DNA was enzymically degraded into nucleosides, as described by Mesbah *et al.* (1989), which were then separated by HPLC using a Waters Symmetry Shield C8 column thermostatted at 37 °C. The solvent used was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated λ phage DNA (Sigma) was used as the calibration reference. The DNA G + C content of strain DPN7^T was 55.1 mol%, which is the same as that reported for *Tetrathibacter kashmirensis* LMG 22695^T. The separated phylogenetic position of strain DPN7^T was confirmed by DNA–DNA hybridization. Experiments were performed at 42 °C with photobiotin-labelled probes in microplate wells as described previously (Goris *et al.*, 1998), using an HTS7000 Bio Assay Reader (PE Applied Biosystems) for the fluorescence measurements. The value for DNA–DNA hybridization to *Tetrathibacter kashmirensis* LMG 22696 was only 41.4 %, whereas the values obtained with *A. faecalis* subsp. *faecalis* LMG 1229^T and with *C. defragrans* LMG 18538^T were low (7 and 5 %, respectively).

Agarose gel electrophoresis of crude extracts of cells of strain DPN7^T obtained by the method of Birnboim & Doly (1979) revealed the presence of a 22 kbp plasmid. In preliminary experiments, this plasmid could not be connected with the capacity to utilize DTDP or other organic sulfur

Fig. 2. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain DPN7^T within the *Betaproteobacteria*; *Burkholderia kururiensis* LMG 19447^T was used as the outgroup. Bootstrap values exceeding 60 % are shown at branch points. Accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position.

compounds. No plasmids were detected in *Tetrathlobacter kashmirensis* LMG 22695^T or *Tetrathlobacter kashmirensis* LMG 22696.

Fatty acid analysis was carried out with a loopful of well-grown cells after an incubation period of 48 h. Fatty acid methyl esters were prepared, separated and identified using the Microbial Identification System (Microbial ID) as described previously (Vandamme *et al.*, 1992). Strain DPN7^T contained the following fatty acid components: 12:0 (4.5 %), 16:0 (18.2 %), 17:0 cyclo (4.8 %), 16:0 3-OH (3.3 %), 18:1 ω 7c (26.5 %), 18:0 (2.1 %), 19:0 ω 8c cyclo (2.6 %), summed feature 2 (13.3 %, comprising 12:0 ALDE, 16:1 iso I and/or 14:0 3-OH) and summed feature 3 (23.3 %, comprising 16:1 ω 7c and/or iso-15:0 2-OH) (summed features are groups of two or three fatty acids that cannot be distinguished using the Microbial Identification System). Members of this phylogenetic lineage typically contain 14:0 3-OH and 16:1 ω 7c; therefore, the peaks designated as summed features 2 and 3 probably corresponded to these fatty acids. A comparison with the fatty acid profile of *Tetrathlobacter kashmirensis* LMG 22695^T (data available in Ghosh *et al.*, 2005) revealed only small variations in composition.

The whole-cell protein profile of strain DPN7^T was determined by using SDS-PAGE (Pot *et al.*, 1994) and was compared with those of reference taxa belonging to this bacterial lineage. Data for reference strains were taken from previous studies (Coenye *et al.*, 2003; Jang *et al.*, 2001). The protein profile of strain DPN7^T was clearly different from

those of *Alcaligenes*, *Achromobacter*, *Bordetella*, *Castellaniella* and *Taylorella* reference strains, as well as from the type species of the genus *Tetrathlobacter* (Fig. 3).

The phylogenetic classification of strain DPN7^T resulted largely from the analysis of sequence similarities of the 16S rRNA gene. The similarity values with respect to the taxonomically closest type strains were about 95 % or less, with the exception of that for *Tetrathlobacter kashmirensis* LMG 22695^T (98.5 %). Both the colony morphology and the cell morphology of these two strains on MSM, MSTY (Mukhopadhyaya *et al.*, 2000) and complex media appeared to be identical. In addition, the DNA G + C contents of the two strains were the same (55.1 mol%), but the DNA–DNA hybridization value was relatively low (only 41.4 %); much lower values (7 and 5 %, respectively) were obtained with *A. faecalis* subsp. *faecalis* LMG 1229^T and *C. defragrans* LMG 18538^T. Furthermore, the physiological and biochemical characteristics of strain DPN7^T (Table 1), particularly with regard to utilization of the organic disulfide DTDP, led to the phylogenetic differentiation of strain DPN7^T and *Tetrathlobacter kashmirensis*. Therefore, strain DPN7^T should be classified as the type strain of a novel species of the genus *Tetrathlobacter*, for which the name *Tetrathlobacter mimigardefordensis* sp. nov. is proposed.

Description of *Tetrathlobacter mimigardefordensis* sp. nov.

Tetrathlobacter mimigardefordensis (mi.mi.gar.de.for.den'sis. M.L. masc. adj. *mimigardefordensis* of Mimegardefordum,

Fig. 3. Digitized representation of normalized whole-cell protein profile of strain DPN7^T compared with the whole-cell protein profiles of reference strains. The dendrogram was derived from unweighted pair group average linkage of correlation coefficients between the protein patterns of the strains studied. The correlation coefficient, *r*, is expressed as percentage similarity for convenience.

a medieval name of Münster, where the type strain was isolated).

Cells are motile, Gram-negative, non-spore-forming coccoid rods (approx. 1.5–2 µm in length). They are oxidase- and catalase-positive, showing an oxidative metabolism. The temperature range for growth is 15–40 °C and the best growth occurs at 30–37 °C. Colonies on DTDP-containing agar plates are more flat than convex, up to 3 mm in diameter and white to lime-yellowish in colour, showing an increase in yellow colour with culture age. On complex medium, colonies grow in 1–2 days and appear more white than yellow and are up to 5 mm in diameter. Susceptible to kanamycin, gentamicin, tetracycline and streptomycin, with no significant antibiotic resistance. Fatty acid profile largely comprises 18:1ω7c (26.5%), 16:0 (18.2%) and of the two fatty acids 16:1ω7c and/or iso-15:0 2-OH (together constituting 23.3%), which cannot be distinguished using the Microbial Identification System. The DNA G+C content of the type strain is 55.1 mol%. Carbon sources utilized are indicated in Table 1.

The type strain, DPN7^T (=DSM 17166^T=LMG 22922^T), was isolated from a sample of matured compost from the compost plant in Münster (Germany).

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