

## *Delftia tsuruhatensis* sp. nov., a terephthalate-assimilating bacterium isolated from activated sludge

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A terephthalate-assimilating bacterium was isolated from activated sludge collected from a domestic wastewater treatment plant in Japan by enrichment with terephthalate as sole carbon source. The isolate, designated strain T7<sup>T</sup>, was a Gram-negative, short rod-shaped micro-organism. A phylogenetic study based on 16S rRNA gene sequences indicated that strain T7<sup>T</sup> should be placed in the genus *Delftia*. A DNA–DNA hybridization value of 69% was determined between strain T7<sup>T</sup> and *Delftia acidovorans* ATCC 15668<sup>T</sup>. Major cellular fatty acids of strain T7<sup>T</sup> were C<sub>16:0</sub>, C<sub>16:1</sub> and C<sub>18:1</sub>. Substantial amounts of cyclopropanoic acid (C<sub>17:0</sub>), 3-OH C<sub>10:0</sub>, C<sub>12:0</sub>, C<sub>15:0</sub> and C<sub>14:0</sub> were also detected. The total DNA G + C content of strain T7<sup>T</sup> was 66.2 mol%. Strain T7<sup>T</sup> could utilize the following compounds as carbon sources: acetamide, β-alanine, citrate, D-fructose, glycerol, isobutyrate, isophthalate, D(–)-mannitol, maleate, malonate, phenylacetate, propionate, protocatechuate, terephthalate, D-tryptophan and L-tryptophan. Comparisons of phenotypic and genotypic characteristics with other known species belonging to the genus *Delftia* suggest that strain T7<sup>T</sup> represents a novel species, for which the name *Delftia tsuruhatensis* sp. nov. is proposed; strain T7<sup>T</sup> is the type strain (=IFO 16741<sup>T</sup> = ATCC BAA-554<sup>T</sup>).

Terephthalate is an aromatic compound used in the production of polyethylene terephthalate (PET) and is one of the top 50 most abundantly manufactured chemicals in the world (Savostianoff, 1990). A large amount of terephthalate is discharged during production of PET bottles and purification of terephthalic acid. Biological degradation of this compound has therefore been of interest in recent years.

Several micro-organisms have been shown to degrade terephthalate under aerobic conditions, including the Gram-positive bacteria *Nocardia* sp. strain DSM 43251 (Engelhardt *et al.*, 1979), a *Bacillus* species (Karegoudar & Pujar, 1985) and *Dietzia* sp. strain GS-1 (Sugimori *et al.*, 2000). Among the *Proteobacteria*, *Bordetella* sp. strain K1,

*Pseudomonas* sp. strain C4S (Kimura & Ito, 2001) and two *Comamonas testosteroni* strains, T-2 (Schläfli *et al.*, 1994) and YZW-D (Wang *et al.*, 1995), have demonstrated the ability to degrade terephthalate.

Most of these micro-organisms have been reported to produce protocatechuate as an intermediate metabolite. The enzymic system responsible for the degradation of terephthalate has been thoroughly studied for *C. testosteroni* strain T-2. In that strain, terephthalate is degraded to protocatechuate by the terephthalate 1,2-dioxygenase system (TERDOS) (Schläfli *et al.*, 1994) and protocatechuate is then metabolized via the *meta* cleavage pathway. Another *C. testosteroni* strain, YZW-D, reportedly uses a similar enzyme system for degradation of terephthalate (Wang *et al.*, 1995).

Recently, a novel terephthalate-assimilating bacterium, designated strain T7<sup>T</sup>, was isolated from the activated sludge of a domestic wastewater treatment plant. The isolate could utilize terephthalate as sole carbon and energy source. Based on 16S rRNA gene sequences and phylogenetic analyses, strain T7<sup>T</sup> was placed in the genus *Delftia*;

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**Abbreviations:** MLSS, mixed liquor suspended solids; PET, polyethylene terephthalate; TERDOS, terephthalate 1,2-dioxygenase system; TOC, total organic carbon.

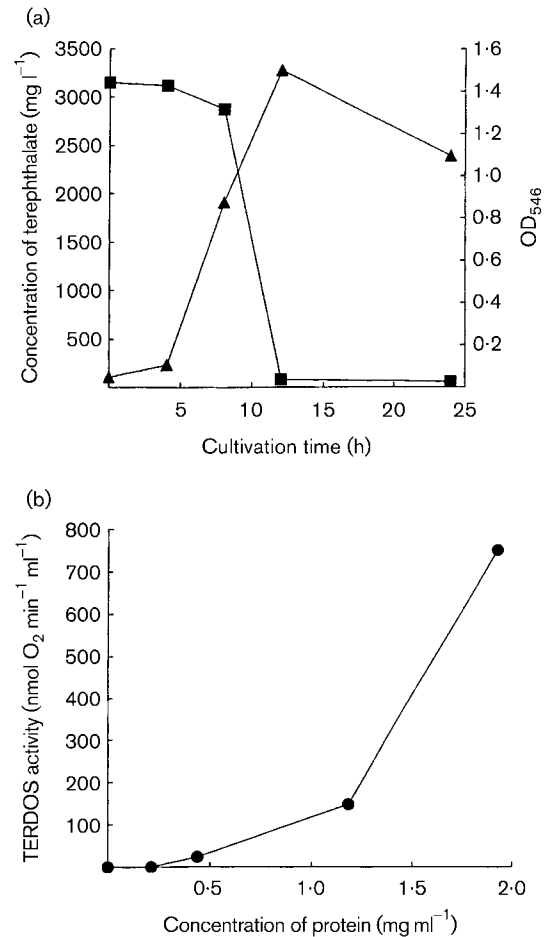
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain T7<sup>T</sup> is AB075017.

no members of this genus have previously demonstrated the ability to degrade terephthalate. Based on phenotypic and phylogenetic characterizations, it is suggested that strain T7<sup>T</sup> constitutes a novel species, for which the name *Delftia tsuruhatensis* sp. nov. is proposed.

For enrichment of terephthalate-assimilating bacteria, a laboratory-scale activated sludge process was constructed. The aeration tank with a working volume of 3 l was maintained at 30 °C and air was supplied at 3 l min<sup>-1</sup>. A 2.8 l aliquot of activated sludge, which was provided by Kumamoto Hokubu sewage works (Tsuruhata, Kumamoto, Japan), was used as the initial reactor seed. The mixed liquor suspended solids (MLSS) concentration of the activated sludge, determined according to the Japanese standard method (Japanese Industrial Standards Committee, 1986), was 1620 mg l<sup>-1</sup>. Concentrations of total organic carbon (TOC) and inorganic carbon in the activated sludge supernatant, obtained after centrifugation at 10 000 r.p.m. for 10 min, were respectively 11.3 and 49.1 mg l<sup>-1</sup>. The pH of the supernatant was 6.86. A basal salt medium supplemented with sodium terephthalate was fed continuously into the reactor at a rate of 1.2 l day<sup>-1</sup>. The basal salt medium (pH 7.6) contained the following (l<sup>-1</sup>): K<sub>2</sub>HPO<sub>4</sub>, 3.48 g; KH<sub>2</sub>PO<sub>4</sub>, 0.435 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.02 g; NaCl, 0.1 g; and CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.1 g. Concentrations of sodium terephthalate were increased stepwise as follows: 200 mg l<sup>-1</sup> for days 1–12; 500 mg l<sup>-1</sup> for days 12–21; and 1000 mg l<sup>-1</sup> for days 21–26. After 26 days continuous cultivation, the MLSS concentration of the culture broth in the reactor increased to 2800 mg l<sup>-1</sup> and the concentrations of TOC and terephthalate in the reactor supernatant were respectively 15 and <1 mg l<sup>-1</sup>. After 26 days continuous cultivation, the culture broth was used for isolation of terephthalate-assimilating bacteria with the basal salt medium containing sodium terephthalate at a concentration of 500 mg l<sup>-1</sup> supplemented with 1.5% agar. A 1 ml portion of the culture broth was diluted with sterilized water, inoculated onto agar plates and incubated at 30 °C. Following the first screening using an agar plate, positive colonies were cultivated in the basal salt medium containing sodium terephthalate at a concentration of 1000 mg l<sup>-1</sup> at 30 °C with continuous shaking. An isolate with a specific growth rate of 0.65 h<sup>-1</sup> was obtained and designated strain T7<sup>T</sup>.

Strain T7<sup>T</sup> was cultivated in liquid basal salt medium containing sodium terephthalate at a concentration of 15 mM and the terephthalate in the culture broth was quantified according to Yumihara *et al.* (2002). The initial terephthalate concentration of 15 mM was degraded completely within 12 h (Fig. 1a). TERDOS activity of the cell lysate of the isolate was determined using a Clarke-type oxygen electrode according to the method of Schläfli *et al.* (1994) (Fig. 1b). The result suggests that the isolate degrades terephthalate by TERDOS, as described previously for *C. testosteroni* strain T-2 (Schläfli *et al.*, 1994).

To investigate the effect of substrate concentration on the



**Fig. 1.** (a) Utilization of terephthalate during growth of strain T7<sup>T</sup> using basal salt medium containing sodium terephthalate (3000 mg l<sup>-1</sup>) (■, terephthalate concentration; ▲, OD<sub>546</sub>). (b) TERDOS activity (Schläfli *et al.*, 1994) determined as O<sub>2</sub> consumption in the cell lysate of strain T7<sup>T</sup> at protein concentrations of 0–2 mg ml<sup>-1</sup>.

respiration activity of the isolate, oxygen consumption rates in liquid basal salt medium were determined at sodium terephthalate levels between 0.2 and 10.0 g l<sup>-1</sup>, according to Morimura *et al.* (1998). With sodium terephthalate concentrations between 0.5 and 4.0 g l<sup>-1</sup>, the oxygen consumption rate of the isolate was constant, at approximately 1.0 µl min<sup>-1</sup>. At sodium terephthalate concentrations below 0.2 g l<sup>-1</sup> or between 6.0 and 10.0 g l<sup>-1</sup>, a lower oxygen consumption rate of approximately 0.8 µl min<sup>-1</sup> was observed. These results indicated that sodium terephthalate concentrations below 4.0 g l<sup>-1</sup> had no inhibitory effect on respiration of the isolate.

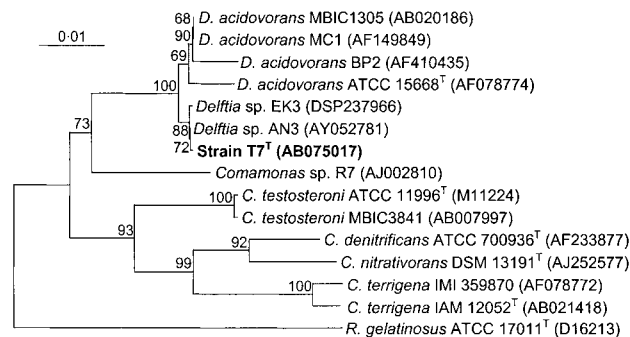
Colonies of strain T7<sup>T</sup> on nutrient broth plates were white. The cells did not produce water-soluble pigments or fluorescent pigments on King A or King B media. Cells appeared as slightly curved, short rods, 0.7–1.2 × 2.4–4.0 µm, and occurred singly or in pairs. They were motile.

Gram-staining, performed according to standard microbiological procedures (Gerhardt *et al.*, 1994), was negative. The isolate accumulated poly- $\beta$ -hydroxybutyrate, determined according to Stanier *et al.* (1966).

Strain T7<sup>T</sup> grew at 10–40 °C, with optimum growth at 35 °C. No growth was detected after 10 days incubation at 7 or 42 °C. A pH range of 5 to 9 was suitable for growth, with an optimum response at pH 7.0. The doubling time was approximately 1.06 h under optimum growth conditions in liquid basal salt medium containing 1000 mg sodium terephthalate l<sup>-1</sup>. The isolate could utilize the following compounds as sole energy and carbon sources under aerobic conditions: acetamide,  $\beta$ -alanine, citrate, D-fructose, glycerol, isobutyrate, isophthalate, D(-)-mannitol, maleate, malonate, phenylacetate, propionate, protocatechuate, terephthalate, D-tryptophan and L-tryptophan. The isolate could not utilize the following compounds: DL-3-aminobutyrate, D(+)-arabitol, catechol, 2,3-dihydroxybenzoate, D-glucose, lactose, phthalate, L-serine, starch, sucrose, L-valine and D(+)-xylose. Arginine dihydrolase, catalase, oxidase, lipase (Tween 80 hydrolysis) and urease activities, evaluated according to standard microbiological procedures (Gerhardt *et al.*, 1994), were positive. The isolate performed *meta* cleavage of protocatechuate, determined according to Hugh & Gilardi (1974). It could not denitrify, although the strain reduced nitrate to nitrite, as determined by the method of Stanier *et al.* (1966). The isolate could not hydrolyse starch. The isolate demonstrated no fermentative response, determined by the O/F reaction (Hugh & Leifson, 1953), using D-glucose, D(-)-fructose or terephthalate as substrates in OF basal medium (Difco).

Whole-cell fatty acids were extracted as methyl esters according to Komagata & Suzuki (1987) and analysed by GC-MS. The most abundant fatty acids in strain T7<sup>T</sup> were C<sub>16:0</sub> (33.2% total fatty acid methyl esters), C<sub>16:1</sub> (31.1%) and C<sub>18:1</sub> (24.6%). In addition, significant amounts of cyclopropanoic acid (C<sub>17:0</sub>; 3.6%), 3-OH C<sub>10:0</sub> (2.6%), C<sub>12:0</sub> (2.0%), C<sub>15:0</sub> (1.6%) and C<sub>14:0</sub> (1.3%) were detected. 3-OH C<sub>8:0</sub> was not detected. The DNA G+C content of strain T7<sup>T</sup>, measured according to Kamagata & Mikami (1991), was 66.2 mol%.

For determination of the 16S rRNA gene sequence of strain T7<sup>T</sup>, cells were lysed according to Hiraishi (1992). The 16S rDNA fragment was amplified by PCR (Hiraishi *et al.*, 1994) using the following universal primers: forward, 5'-AGAGTTTGGATCATGGCTCGA-3' (positions 8–27 of the *Escherichia coli* 16S rRNA gene); and reverse, 5'-GGCTACCTTGTACGACTT-3' (positions 1510–1492). The sequence of the amplified 16S rDNA fragment (1456 bp) was aligned with reference sequences using CLUSTAL X, version 1.8 (Thompson *et al.*, 1997), and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the Kimura two-parameter model (Kimura, 1980) using MEGA version 2.1 (Kumar *et al.*, 2001) (Fig. 2). Sequence similarities between strain T7<sup>T</sup> and relatives were as follows: *Delftia* sp.



**Fig. 2.** Phylogenetic tree showing the relationship between strain T7<sup>T</sup> and related *Delftia* and *Comamonas* strains in the  $\beta$ -Proteobacteria based on 16S rDNA sequences. CLUSTAL X version 1.8 (Thompson *et al.*, 1997) was used to align the sequences. The tree was constructed using the neighbour-joining method as outlined in the text. All gaps in the alignment were excluded; 1542 bases were compared. The sequence of *Rubrivivax gelatinosus*, family *Comamonadaceae*,  $\beta$ -Proteobacteria, was used to root the tree. Bootstrap probabilities (Felsenstein, 1985) are indicated at branch points. DDBJ/EMBL/GenBank accession numbers for reference strains are shown in parentheses. Bar, 1 nt substitution per 100 nt.

EK3, 99.8%; *Delftia* sp. AN3, 99.7%; *Delftia acidovorans* MBIC1305, 99.4%; *D. acidovorans* MCI1, 99.2%; *D. acidovorans* BP2, 98.7%; *D. acidovorans* ATCC 15668<sup>T</sup>, 98.6%. The tree indicated that the isolate is apparently affiliated with the genus *Delftia*, which was introduced by the relocation of [*Comamonas*] *acidovorans* on the basis of phylogenetic and phenotypic analyses (Wen *et al.*, 1999). The sequences of strains belonging to the genus *Delftia* form two distinct clusters, both with significant bootstrap values (69 and 88). One cluster, with a bootstrap value of 69, includes the sequences of *D. acidovorans* strains ATCC 15668<sup>T</sup>, MBIC1305, BP2 and MCI1. The sequence of strain T7<sup>T</sup> and two *Delftia* strains, AN3 and EK3, were assigned to the other cluster (bootstrap value of 88). Therefore, the sequences of strains belonging to the genus *Delftia* can be divided in two distinct phylogenetic groups. DNA–DNA hybridization was performed according to Willems *et al.* (2001) at a hybridization temperature of 52 °C using total DNAs of strain T7<sup>T</sup> and *D. acidovorans* ATCC 15668<sup>T</sup>. Hybridization values were calculated as means of four duplicate hybridizations, with a mean standard deviation of  $\pm 5.2$ %. As an internal control, reciprocal hybridizations were carried out. Using DNA from either strain T7<sup>T</sup> or ATCC 15668<sup>T</sup> as the labelled DNA, hybridization values with DNA from ATCC 15668<sup>T</sup> or strain T7<sup>T</sup> were respectively 68.7 and 68.6%. The DNA–DNA hybridization value between the two strains was thus not significantly below the threshold value of 70% used for species delineation (Stackebrandt & Goebel, 1994), and therefore does not provide a clear indication of the taxonomic placement of strain T7<sup>T</sup> within the genus *Delftia*.

**Table 1.** Features that can be used to differentiate between strain T7<sup>T</sup> and *D. acidovorans*

Abbreviations: +, present in all strains; -, absent in all strains; d, variable reaction in different strains; NA, no data available. Data for *D. acidovorans* were taken from Wen *et al.* (1999) and Willems *et al.* (1991).

Characteristic	Strain T7 <sup>T</sup>	<i>D. acidovorans</i>
Cell size (µm)	0.7–1.2 × 2.4–4.0	0.4–0.8 × 2.5–4.1
Carbon source used for growth:		
β-Alanine	+	d
3-Aminobutyrate	–	d
Glycerol	+	d
Phthalate	–	d
Terephthalate	+	–
Isophthalate	+	–
Protocatechuate	+	NA
Major 3-OH acid(s)	10:0	10:0, 8:0
G+C content (mol%)	66.2	66.6–68.7

Table 1 shows comparative phenotypic properties for strain T7<sup>T</sup> and *D. acidovorans*. The isolate could not utilize 3-aminobutyrate, whereas most *D. acidovorans* strains (20 of 21 strains tested), including the type strain, can utilize this substrate (Willems *et al.*, 1991). 3-OH C<sub>8:0</sub>, which is characteristic of members of the genus *Delftia*, was not detected among the cellular fatty acids, and the G+C content of the isolate (66.2 mol%) was slightly lower than those of other strains belonging to the genus. On the basis of the phenotypic and phylogenetic analysis above and of the DNA–DNA hybridization value of around 70%, it is proposed that strain T7<sup>T</sup> should be classified as a novel species in the genus *Delftia*, for which the name *Delftia tsuruhatensis* sp. nov. is proposed.

### Description of *Delftia tsuruhatensis* sp. nov.

*Delftia tsuruhatensis* (tsu.ru.ha.ten'sis. N.L. fem. adj. *tsuruhatensis* referring to Tsuruhata, Kumamoto Prefecture, Japan, where the type strain was isolated).

Gram-negative, motile, slightly curved, short rods (0.7–1.2 × 2.4–4.0 µm). Cells occur individually or in pairs. Growth occurs between 10 and 40 °C; optimum growth at 35 °C. Growth occurs at pH 5.0–9.0; optimum growth at pH 7.0. Doubling time is approximately 1.06 h under optimum growth conditions. Non-fermentative. Positive for arginine dihydrolase, catalase, oxidase, lipase (Tween 80 hydrolysis) and urease activities and *meta* cleavage of protocatechuate. Denitrification ability is not observed, although nitrate reduction is found. Does not hydrolyse starch. The following compounds can be utilized as energy and carbon sources: acetamide, β-alanine, citrate, D-fructose, glycerol, isobutyrate, isophthalate, D(–)-mannitol, maleate, malonate, phenylacetate, propionate, protocatechuate, terephthalate, D-tryptophan and L-tryptophan. The following

compounds cannot be utilized: DL-3-aminobutyrate, D(+) -arabitol, catechol, 2,3-dihydroxybenzoate, D-glucose, lactose, phthalate, L-serine, starch, sucrose, L-valine and D(+) -xylose. The most abundant fatty acids are C<sub>16:0</sub>, C<sub>16:1</sub> and C<sub>18:1</sub>. Cyclopropanoic acid (C<sub>17:0</sub>), 3-OH C<sub>10:0</sub>, C<sub>12:0</sub>, C<sub>15:0</sub> and C<sub>14:0</sub> are detected in smaller amounts. 3-OH C<sub>8:0</sub> is not detected. The total DNA G+C content of the type strain is 66.2 mol%.

The type strain, T7<sup>T</sup> (=IFO 16741<sup>T</sup> = ATCC BAA-554<sup>T</sup>), was isolated from activated sludge in Tsuruhata, Kumamoto Prefecture, Japan.

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