Desulfotignum toluenicum sp. nov., a novel toluene-degrading, sulphate-reducing bacterium isolated from an oil-reservoir model column

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A Gram-negative, sulphate-reducing bacterium (strain H3^T) was isolated from an oil-reservoir model column. The new isolate was able to oxidize toluene coupled to hydrogen sulphide production. For growth, the optimum salt concentration was 1.5 % (w/v), the optimum pH was 7.2 and the optimum temperature was 34 °C. The cells were straight to slightly curved rods, 0.6–1.0 µm in diameter and 1.4–2.5 µm in length. The predominant fatty acids were C_{16:0}, C_{16:1ω7c} and C_{17:0} cyclo, and the cells also contained dimethylacetals. Cloning and sequencing of a 1505 bp long fragment of the 16S rRNA gene showed that strain H3^T is a member of the *Deltaproteobacteria* and is related closely to *Desulfotignum balticum* DSM 7044^T. The G + C content of the DNA was 52.0 mol% and the DNA–DNA similarity to *D. balticum* DSM 7044^T was 56.1 %. Based on differences in DNA sequence and the unique property of toluene degradation, it is proposed that strain H3^T should be designated a member of a novel species within the genus *Desulfotignum*, for which the name *Desulfotignum toluenicum* sp. nov. is proposed. The type strain is H3^T (=DSM 18732^T=ATCC BAA-1460^T).

The genus *Desulfotignum* was established when the mesophilic, sulphate-reducing strain SAX (Drzyzga *et al.*, 1993) was described as *Desulfotignum balticum* (Kuever *et al.*, 2001). The type strain, *Desulfotignum balticum* DSM 7044^T, has the ability to oxidize aromatic compounds such as benzoic acid and 4-hydroxybenzoate, but does not oxidize toluene. The genus also contains the sulphate-reducing bacterium *Desulfotignum phosphitoxidans*, which grows lithoautotrophically with phosphite as electron donor and sulphate as electron acceptor (Schink *et al.*, 2002).

Strain H3^T was isolated from an anaerobic enrichment culture inoculated with bacteria from an oil-reservoir model column (Myhr *et al.*, 2002), supplied with 0.8 % (v/v) crude oil as the only carbon source. The enrichment culture was diluted serially with crude oil as carbon source in reduced bicarbonate-buffered seawater medium (Widdel & Pfennig, 1981) modified to 1.2 g KH₂PO₄ l⁻¹ and 0.25 g NH₄Cl l⁻¹ (W20). The 10⁻⁷ dilution was submitted to an

anoxic agar dilution series (Widdel & Bak, 1992) with 10 mM sodium caproate. After four consecutive agar dilution series of bacteria originating from the 10⁻⁷ dilution described above, three beige-coloured colonies (strains H1, H2 and H3^T) were picked and propagated in W20 with caproate (10 mM). Strain H3^T was characterized fully. *Desulfotignum balticum* DSM 7044^T was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany).

Growth was monitored by light microscopy in a Zeiss phase-contrast microscope and by hydrogen sulphide measurements (Cord-Ruwisch, 1985). Cells were counted in a Thoma chamber, but cell counts were difficult in cultures supplied with crude oil, due to disturbances from oil in the preparations. Transmission electron microscopy (TEM) of unstained preparations of strain H3^T was done at the electron microscope facilities at the University of Bergen, Norway. TEM images with size bars were used to determine the cell size. Salt requirement for growth was tested in DSMZ medium 383a (<http://www.dsmz.de/media/med383a.htm>) in triplicate with 0–60 g NaCl l⁻¹ in intervals of 5.0 g l⁻¹ (pH 7.0, 30 °C). The pH range for growth was tested in triplicate as described by Knoblauch *et al.* (1999) in medium 383a adjusted to a pH value between 4.94 and 9.06 (30 °C) and the temperature optimum was tested in duplicate in medium 383a in a temperature-gradient block with temperatures between 9.9 and 48.2 °C (pH 7.0).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences determined in this study are EF207157–EF207159 for strain H3^T clone sequences 1, 4 and 7, respectively.

A supplementary table showing the varying bases in the analysed *Desulfotignum* sp. 16S rRNA gene sequences, and supplementary figures showing a transmission electron micrograph of unstained cells of *Desulfotignum toluenicum* H3^T and rep-PCR profiles for the analysed strains of *Desulfotignum*, are available with the online version of this paper.

Gram typing was determined by thorough mixing of cells of strain H3^T in a drop of 3 % (w/v) KOH on a glass slide, followed by incubation at room temperature for 1 min. Catalase activity was tested in 3 % (v/v) hydrogen peroxide.

The fatty acid composition, DNA base composition and DNA–DNA hybridization value to *Desulfotignum balticum* DSM 7044^T were determined at the DSMZ by standard protocols.

Cells of strain H3^T were 0.6–1.0 µm in diameter and 1.4–2.5 µm in length. The cell shape varied from short blunt rods, almost cigar-shaped in the early growth phase, to long and slightly curved rods in late exponential phase. The cells were covered with an extracellular layer that appeared like a halo around the cells. Turbid, well-grown cultures had a strong tendency to attach to the inner walls of the growth tubes. A TEM image of strain H3^T is available as Supplementary Fig. S1 in IJSEM Online. Growth occurred between 0.5 and 5.5 % (w/v) NaCl (optimum, 1.5 %), between pH 6.5 and 9.0 (optimum, 7.2) and between 16 and 35 °C (optimum, 34 °C). Strain H3^T was Gram-negative and catalase-positive.

The predominant fatty acids were 16:0 (20.3 %), 16:1ω7c (14.0 %) and 17:0 cyclo (13.4 %) and, in addition, four different dimethylacetals were found that were unique to strain H3^T (Table 1). The DNA G+C content was determined as 52.0 mol% and the DNA–DNA hybridization value to *Desulfotignum balticum* DSM 7044^T was 56.1 %.

Growth on crude oil as carbon source was tested in W20 or in medium 383a with 1.4 or 5.6 % (v/v) anoxic sterile crude oil, respectively, supplied through an N₂-flushed syringe. *n*-Alkanes (*n*C₇–*n*C₉ and *n*C₁₂), toluene, ethylbenzene and *o*-, *m*-, or *p*-xylene were diluted 2 % (v/v) in heptamethylnonane (Harms *et al.*, 1999) before 0.5 ml sterile and anoxic hydrocarbon solution was added per 16 ml culture. The culture tubes were incubated in a near-horizontal position with the orifices below the liquid surface (Widdel *et al.*, 2006). In addition to strain H3^T, *Desulfotignum balticum* DSM 7044^T was tested with toluene and crude oil as carbon sources. Strain H3^T grew with toluene and crude oil (Fig. 1a, b), whereas *Desulfotignum balticum* DSM 7044^T did not, and whole-oil GC (Skaare, 2007) showed that 4.4 mM of the toluene naturally present in crude oil was depleted from the oil when 8.1 mM H₂S was produced after 50 days incubation (Fig. 1b). Strain H3^T is the first member of the genus *Desulfotignum* with the ability to grow solely on a monoaromatic hydrocarbon, i.e. toluene. Intermediate products from incomplete hydrocarbon oxidation were not detected by GC, which indicated complete oxidation of toluene to CO₂. The H₂S: toluene molar ratio during growth on toluene was approximately 2, which was lower than the 4.12 ratio shown for *Desulfobacula toluolica* DSM 7467^T (Rabus *et al.*, 1993). The results from the complete substrate analysis for strain H3^T are listed in Table 2.

A reverse primer (5'-CTTCACCCCAGTTATCAACC-3') was constructed for PCR amplification of the 16S rRNA

Table 1. Fatty acid content of strain H3^T and closely related species

Percentage of total fatty acids is shown. Taxa: 1, strain H3^T; 2, *Desulfotignum balticum* DSM 7044^T; 3, *Desulfobacula toluolica* DSM 7467^T; 4, *Desulfobacula phenolica* DSM 3384^T; 5, *Desulfospira joergensenii* DSM 10085^T. Data for taxa 2–5 are from Kuever *et al.* (2001). Abbreviations: OH, hydroxy; Me, methyl; DMA, dimethylacetal; i, iso; cyc, cyclopropane.

Fatty acid	1	2	3	4	5
14:0	8.5	3.7	8.3	8.7	13.9
15:0	6.3	0.2	1.6	2.0	1.3
16:0	20.3	24.2	31.2	20.4	28.4
17:0	6.2	0.4	0.7	0.7	0.6
18:0	4.6	9.8	3.1	2.1	0.7
i15:0	—	—	—	—	1.8
i19:0	—	1.7	2.5	—	—
14:1	—	—	—	1.4	—
15:1ω6c	2.2	—	—	—	—
15:1ω9c	—	0.8	—	—	—
16:1ω5c	1.0	—	—	—	—
16:1ω7c	14.0	—	—	—	—
16:1ω9c	—	8.5	19.7	16.5	38.9
18:1ω9c	1.7	3.7	—	—	—
18:1ω11c	—	7.4	6.0	3.9	5.3
3-OH 14:0	—	—	—	1.3	1.6
3-OH 15:0	1.4	—	—	—	—
3-OH 16:0	2.1	—	—	—	2.3
10-Me 16:0	—	14.0	17.6	17.2	—
14:0 DMA	2.7	—	—	—	—
16:1ω7c DMA	0.6	—	—	—	—
16:0 DMA	4.0	—	—	—	—
17:0 DMA	0.7	—	—	—	—
17:0 cyc	13.4	19.5	2.6	3.9	2.4
19:0 cyc	—	1.1	—	—	—
16:0 aldehyde	1.2	—	—	—	—

gene from strain H3^T, as the normally used 1525r primer (Lane, 1991) did not have a complementary sequence in the 16S rRNA gene of the reference strain *Desulfotignum balticum* DSM 7044^T. Combined with the primer A8f (Edwards *et al.*, 1989), a 1505 bp long DNA fragment was PCR-amplified by standard protocols, cloned into the pCR4-TOPO vector (Invitrogen) and sequenced by using the primers T3, T7 (Invitrogen) and 338f (Lane, 1991). The partial sequences were assembled to a consensus sequence by using the Institut Pasteur CAP alignment tool (<http://www.pasteur.fr/english.html>). Cloning and sequencing of the 16S rRNA gene resulted in seven distinct sequences, with reciprocal sequence similarity between 98.6 and 99.9 %. Three clone sequences, 1, 4 and 7, were chosen to represent strain H3^T in GenBank and submitted under the accession numbers EF207157 (clone 1), EF207158 (clone 4) and EF207159 (clone 7), as demonstrated previously for novel *Clostridium* species (Spring *et al.*, 2003). All variable nucleotides are presented in Supplementary Table S1 in IJSEM Online.

Fig. 1. Growth of strain H3^T on hydrocarbons. (a) Growth with toluene as carbon source. The H₂S concentration curve (◆) and cell counts (■) are shown. (b) H₂S concentrations produced with crude oil (■) and toluene (▲) as carbon sources.

CAP-assembled clone sequences were aligned in CLUSTAL_X (Thompson *et al.*, 1997) to 16S rRNA gene sequences of members of the *Deltaproteobacteria* available in GenBank (Benson *et al.*, 2004), obtained by BLAST search (Wheeler *et al.*, 2003). Similarity values between sequences were calculated by using the Matrix Global Alignment Tool (MatGAT; Campanella *et al.*, 2003). The phylogenetic relationships were presented graphically in a neighbour-joining tree (Saitou & Nei, 1987) based on the CLUSTAL_X alignment of the 16S rRNA gene sequences, with *Escherichia coli* as outgroup (Fig. 2). The tree was bootstrapped by 1000 recalculations and evaluated by the maximum-parsimony and maximum-likelihood algorithms (PHYLIP software package; Felsenstein, 2001). The 16S rRNA gene sequences of clones of strain H3^T were 98.7–99.9% similar to that of *Desulfotignum balticum* DSM 7044^T, and placed strain H3^T within the genus *Desulfotignum*. Still, the DNA–DNA hybridization value of 56.1% to *Desulfotignum balticum* DSM 7044^T showed that the novel strain H3^T did not belong to the species

Table 2. Physiological characterization of the type strains of species within the genus *Desulfotignum*

Taxa: 1, strain H3^T; 2, *Desulfotignum balticum* DSM 7044^T; 3, *Desulfotignum phosphitoxidans* FiPS-3^T. Data for taxon 2 were obtained from Kuever *et al.* (2001) and Drzyzga *et al.* (1993) and those for taxon 3 from Schink *et al.* (2002). +, Positive; (+), weakly positive; –, negative; ND, not determined. All taxa were positive for pyruvate, fumarate, sulphate and sulphite tests.

Test	1	2	3
Crude oil	+	–*	ND
C ₇ –C ₉ <i>n</i> -alkanes	–	ND	ND
C ₁₂ <i>n</i> -alkanes	–	ND	ND
Toluene	+	–*	(+)
Ethylbenzene	–	ND	ND
<i>m</i> -Xylene	–	ND	ND
<i>p</i> -Xylene	–	ND	ND
<i>o</i> -Xylene	–	ND	ND
Phenol	ND	(+)	+
Phenylacetate	ND	(+)	ND
Benzoate	+	+	ND
4-hydroxybenzoate	+	+	ND
H ₂ /CO ₂	+	(+)	+
Formate	+	(+)	+
Acetate	+	(+)	–
Lactate	–	+	–
Butyrate, C ₄	+	+	–
Valerate, C ₅	–	ND	ND
Caproate, C ₆	+	ND	ND
Heptanoate, C ₇	+	ND	ND
Nonate, C ₉	+	ND	ND
Decanoate, C ₁₀	+	(+)	ND
Undecanoate, C ₁₁	–	ND	ND
Dodecanoate, C ₁₂	–	(+)	ND
Tetradecanoate, C ₁₄	(+)	ND	ND
Hexadecanoate, C ₁₆	+	(+)	ND
Octadecanoate, C ₁₈	–	ND	ND
Ethanol	–	–	ND
Succinate	+	(+)	ND
Malate	–	+	+
Fermentation of pyruvate	+	+	–
Vitamins	+	+	ND
Thiosulphate	–	+	+
Elementary sulphur	–	ND	–

*Growth on crude oil and toluene for *Desulfotignum balticum* DSM 7044^T was tested in this study.

Desulfotignum balticum (Wayne *et al.*, 1987) and defined strain H3^T as a member of a novel species within the genus *Desulfotignum*. Strain H3^T was also related to *Desulfobacula toluolica* DSM 7467^T (Rabus *et al.*, 1993) and *Desulfobacula phenolica* DSM 3384^T (Kuever *et al.*, 2001), which have been shown to degrade toluene. Sulphate-reducing bacteria able to degrade aliphatic hydrocarbons, *Desulfoglaeba alkanexedens* ALDC^T (Davidova *et al.*, 2006), strain Hxd3 (Aeckersberg *et al.*, 1991), *Desulfatibacillum aliphaticivorans* CV2803^T (Cravo-Laureau *et al.*, 2004), strain Pnd3

Fig. 2. Phylogenetic tree showing the affiliation of the 16S rRNA gene sequences of *Desulfotignum toluenicum* H3^T (shown in bold) within the Deltaproteobacteria, with *Escherichia coli* as outgroup. GenBank accession numbers are shown in parentheses. The tree was calculated by the neighbour-joining method. Asterisks show branches also resolved by the maximum-likelihood and maximum-parsimony algorithms (Einen & Ovreas, 2006). Bootstrap values (1000 recalculations) >50 % are shown. Bar, 0.02 substitutions per nucleotide.

(Aeckersberg *et al.*, 1998) and strain AK-01 (So & Young, 1999), were affiliated to remote clusters.

Strain-specific genomic fingerprint analyses by repetitive PCR (rep-PCR) (Stern *et al.*, 1984; Versalovic *et al.*, 1991) were performed with whole cells; extracted DNA was obtained by using a FastDNA SPIN kit for soil (Qbiogene). Strains H3^T and *Desulfotignum balticum* DSM 7044^T had very similar genomic fingerprints, but differed by the lack of bands of approximately 450 and 900 bp in the fingerprint of *Desulfotignum balticum* DSM 7044^T. A picture showing the rep-PCR patterns for the analysed *Desulfotignum* species is available as Supplementary Fig. S2 in IJSEM Online.

Based on the DNA–DNA hybridization value of 56.1 % to *Desulfotignum balticum* DSM 7044^T, the fatty acid composition and the ability to degrade toluene, we conclude that strain H3^T should be assigned to a novel species within the genus *Desulfotignum*, for which the name *Desulfotignum toluenicum* sp. nov. is proposed.

Description of *Desulfotignum toluenicum* sp. nov.

Desulfotignum toluenicum (tol.u.en'i.cum. N.L. n. *toluenum* toluene; L. suff. *-icus -a -um* suffix used with the sense of belonging to; N.L. neut. adj. *toluenicum* pertaining to toluene).

Rod-shaped, mesophilic, Gram-negative and sulphate-reducing. Cells are 0.6–1.0 µm in diameter and 1.4–2.5 µm in length, and have a tendency to curve with ageing. Motility is not observed and there are no flagella. Growth occurs at NaCl concentrations between 0.5 and 5.5 % (v/w), with optimum growth at 1.5 % NaCl, at temperatures between 16 and 35 °C (optimum, 34 °C) and at pH values between 6.5

and 9.0 (optimum, 7.2). The predominant fatty acids are C_{16:0}, C_{16:1ω7c} and C_{17:0} cyclo and the cells also contain dimethylacetals. Catalase-positive. The following are used as substrates for growth: toluene, crude oil, formate, benzoate, 4-hydroxybenzoate, acetate, butyrate, caproate, heptanoate, nonate, decanoate, tetradecanoate, hexadecanoate, pyruvate, fumarate, succinate and H₂/CO₂. Vitamins are required for growth. *n*-Alkanes (*n*C₇–*n*C₉ and *n*C₁₂), ethylbenzene, *m*-, *p*-, or *o*-xylene, lactate, valerate, undecanoate, dodecanoate, octadecanoate, ethanol and malate are not used as substrates. Electron acceptors are sulphate and sulphite. No growth was observed with thiosulphate or elemental sulphur as electron acceptor or with caproate as electron donor. The DNA G + C content is 52.0 mol%.

The type strain, H3^T (=DSM 18732^T=ATCC BAA-1460^T), was isolated from an oil-reservoir model column at the University of Bergen, Norway.

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