Desulfitobacterium aromaticivorans sp. nov. and Geobacter toluenoxydans sp. nov., iron-reducing bacteria capable of anaerobic degradation of monoaromatic hydrocarbons

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Dissimilatory iron reduction plays a significant role in subsurface environments. Currently, it is assumed that members of the genus Geobacter constitute the majority of the iron-reducing microorganisms that oxidize aromatic compounds in contaminated subsurface environments. Here, we report the isolation of two phylogenetically distinct pure cultures of iron-reducing degraders of monoaromatic hydrocarbons, strain TMJ1T, which belongs to the genus Geobacter within the Deltaproteobacteria, and strain UKTLT, belonging to the genus Desulfitobacterium within the Clostridia. Both strains utilize a wide range of substrates as carbon and energy sources, including the aromatic compounds toluene, phenol and p-cresol. Additionally, strain UKTLT utilizes o-xylene and TMJ1T utilizes m-cresol. Anaerobic degradation of toluene in both strains and o-xylene in strain UKTLT is initiated by activation with fumarate addition to the methyl group. The genomic DNA G+C contents of strains TMJ1T and UKTLT are 54.4 and 47.7 mol%, respectively. Based on a detailed physiological characterization and phylogenetic analysis of the 16S rRNA genes of both strains, we propose the names Desulfitobacterium aromaticivorans sp. nov. (type strain UKTLT = DSM 19510T = JCM 15765T) and Geobacter toluenoxydans sp. nov. (type strain TMJ1T = DSM 19350T = JCM 15764T) to accommodate these strains. To the best of our knowledge, strain UKTLT is the first described spore-forming, iron-reducing bacterium that can degrade aromatic hydrocarbons.

INTRODUCTION

Iron reduction by dissimilatory iron-reducing microbes plays an important role as the terminal electron-accepting process in subsurface sedimentary environments (Thamdrup, 2000) and also constitutes a key process in contaminant degradation in polluted groundwater systems, landfill leachates and anoxic sediments (Anderson et al., 1998; Christensen et al., 2001; Lovley & Phillips, 1986). The earliest studies on bacterial iron reduction were performed with a facultatively fermenting bacterium of the genus Bacillus (Roberts, 1947). Later, a non-fermentative, iron-reducing pseudomonad that oxidized H2 with Fe(III) was isolated (Balashova & Zavarzin, 1979) and reports of a number of phylogenetically diverse dissimilatory iron-reducing bacteria and archaea have followed (Weber et al., 2006). In particular, isolates from freshwater sediments have been affiliated to the deltaproteobacterial family Geobacteraceae (Lonergan et al., 1996), with the first species described being Geobacter metallireducens (Lovley et al., 1993). Besides these typical iron reducers, a potential for iron reduction has also been described for several sulfate-reducing micro-organisms and methanogens, or by indirect reduction through sulfur cycling (Bond &
Lovley, 2002; Coleman et al., 1993; Straub & Schink, 2004). Methanogens, however, could not couple iron reduction to growth.

Dissimilatory iron-reducing micro-organisms are important for the degradation of monoaromatic hydrocarbons, polycyclic aromatic hydrocarbons and chlorinated compounds (Anderson & Lovley, 1999; Kunapuli et al., 2007; Sung et al., 2006), with members of the Geobacteraceae identified as very prominent microbes in environmental communities (Cummings et al., 2003; Lin et al., 2005; Rooney-Varga et al., 1999; Sung et al., 2006). Nevertheless, only two species of the genus Geobacter are available in pure culture up to date that degrade aromatic hydrocarbons coupled to iron reduction, G. metallireducens and Geobacter grbiciae (Coates et al., 2001; Lovley et al., 1993). This limited number of isolates certainly does not reflect the significance of iron-reducing micro-organisms involved in aromatic hydrocarbon degradation in the environment.

Thus, in this study, for a better understanding of the diversity and physiology of microbes involved in these processes, we enriched and isolated further iron reducers capable of oxidizing aromatic hydrocarbons from different contaminated groundwater sediments. We describe two novel strains, TMJ1T and UKTLT, which are phylogenetically and metabolically distinct from available pure cultures. As for the species mentioned above, strain TMJ1T is also affiliated to the genus Geobacter. For UKTLT, however, we report here on the first Gram-stain-positive, spore-forming iron-reducer isolated in pure culture that is capable of utilizing aromatic hydrocarbons as sole carbon and energy sources.

METHODS

Isolation and cultivation. Strains TMJ1T and UKTLT were enriched and isolated in serial liquid dilution-to-extinction cultures with toluene as carbon and energy source and ferricyanide as the electron acceptor in the presence of Amberlite XAD-7 (Morasch et al., 2000). XAD-7 binds aromatic hydrocarbons strongly and was used to maintain toluene in the medium at a constantly low equilibrium concentration to avoid toxicity effects. For the initial enrichments, well sediment from a tar-oil-contaminated site near Stuttgart, Germany (for strain TMJ1T), and soil of a former coal-gasification site in Gliwice, Poland (for strain UKTLT), were used as inocula. Strain UKTLT was isolated on toluene from an iron-reducing, benzene-degrading enrichment that was adapted to mineralize benzene completely to CO2 (Kunapuli et al., 2007). Sediment (5 ml) was added to 120 ml serum bottles, half-filled with anaerobic artificial seawater containing 100 mM NaCl, 20 mM KCl, 5 mM MgCl2, 1 mM FeCl2 (10 μM sodium sulfate as sulfur source) for the cultivation of strain UKTLT and with 0.5 mM Na2S (no additional sulfur source) for the cultivation of strain UKTLT. Strictly anaerobic cultivation techniques were utilized. Toluene was injected with a microlitre syringe (Hamilton Co.) through the butyl-rubber stoppers. After ten transfers (10% inoculum) to fresh medium, the cultures were purified in at least two consecutive liquid dilution series (up to 10-6). The purity of both strains was checked microscopically using a phase-contrast microscope and by growth on alternative substrates such as acetate, lactate, formate, glucose and NB medium.

Growth conditions. Substrate utilization tests with strains TMJ1T and UKTLT were performed in duplicate incubations. Anoxic stock solutions of the substrates were prepared with distilled water, autoclaved and added to the bicarbonate-buffered mineral medium. Pyruvate, lactate and glucose were filter-sterilized. Liquid aromatic compounds were injected directly with a microlitre syringe. Solid substrates were added to the bottles prior to autoclaving and later filled with the freshwater medium. Utilization of individual substrates was monitored as an increase in ferrous iron concentration over time.

Tests for the utilization of different electron acceptors [amorphous Fe(OH)3, as aqueous suspension, powdered S0 and aqueous solutions of sodium sulfate, sodium sulfite, sodium thiosulfite, manganese(IV) chloride, sodium fumarate and sodium nitrate] were performed in either duplicate or triplicate incubations. Electron acceptors were autoclaved and added to the bottles from anoxic stock solutions. Utilization of various electron acceptors was monitored as growth via either microscopy, colour change of the added electron acceptor or visual turbidity (OD578) and sulfide or ferrous iron production (Cline, 1969; Stookey, 1970). Inocula for substrate utilization tests were taken from cultures grown on toluene as substrate and ferric citrate as electron acceptor.

The temperature optimum for growth was determined in triplicate over the range 16–42 °C and optimum pH (pH adjusted with 1 M HCl or 0.5 M Na2CO3) was determined in duplicate incubations between pH 6.5 and 8.0, with toluene as carbon source. To test the dependency of growth on vitamins, a freshwater mineral medium without vitamins was prepared and growth of the strains was monitored by observing iron reduction in triplicate incubations with toluene and ferric citrate over five consecutive transfers.

Electron recoveries for both strains were calculated from three parallel experiments with cultures grown on toluene-ferric citrate medium in the absence of XAD-7, together with sterile (autoclaved three times over three consecutive days) and culture-unamended controls.

Analytical procedures. Toluene concentrations were determined with GC/MS (Thermo Finnigan GC; 250 μl headspace sample) with split injection (1:10 split ratio) using a fused-silica capillary column (DB-5, 30 m long and 0.25 mm i.d., 0.25 μm film thickness; Agilent). The temperature was held at 40 °C for 1 min, raised to 200 °C at 15 °C min-1 and then to 300 °C at 25 °C min-1 and held there for 1 min. Carrier gas was helium at a flow rate of 1 ml min-1. MS analysis was carried out using a Trace-DSQ MS unit (Thermo Finnigan) in SIM (selective ion monitoring) mode. External standards of toluene were used to calculate concentrations. For the identification of metabolites, culture supernatants were adjusted to pH <2 with 37% HCl and intermediates of toluene and o-xylene degradation were derivatized (silylation) as described elsewhere (Morasch et al., 2004).

Morphological characteristics. For Gram-staining, both strains were grown on toluene-ferric citrate freshwater medium. G. metallireducens DSM 7210T was used as a control for Gram-stain-negative cells and Bacillus subtilis DSM 10T as a control for Gram-stain-positive cells. To check for spore formation, cultures were pasteurized at 80 °C for 10 min in 20 ml glass tubes sealed with butyl-rubber stoppers. The pasteurized cultures were reincubated into triplicate bottles containing 50 ml toluene-ferric citrate freshwater medium and incubated at 30 °C. Utilization of the substrates was monitored as an increase in ferrous iron concentration.

For electron microscopy, the samples were washed twice with 1 x PBS and fixed with 2 ml 5% glutaraldehyde in PBS (pH 7.4) overnight at 4 °C. The fixed cells were dehydrated through a series of ethanol solutions at increasing concentrations (50, 70, 80, 95 and
100 % ethanol). Ethanol was replaced with liquid CO₂ and the samples were dried in a critical-point dryer. After drying, the samples were coated with a 5 nm platinum layer in an Emitech K575X sputter coater (EM technologies) and examined in a scanning electron microscope (JSM 6300F; JEOL).

**Total lipid and respiratory quinone analysis.** Total lipid analysis was performed using previously reported precautions (White & Ringelberg, 1998). Total lipids were extracted from lyophilized cells with the one-phase chloroform/methanol buffer system (Bligh & Dyer, 1959). Medium samples without lyophilized cells served as control blanks. After overnight extraction, equal volumes of chloroform and nanopure water were added to the extract, resulting in a two-phase system. The lower, organic phase (containing lipids) was collected and total lipids were fractionated by silicic acid column chromatography into neutral lipids, glycolipids and polar lipids. Lipopolysaccharide (LPS) hydroxy fatty acids were recovered from the upper aqueous phase of the Bligh and Dyer extraction separately specified by the manufacturer. 16S rRNA gene sequence reads were assembled manually and quality-controlled using SeqMan II (DNASTAR). Closely related 16S rRNA gene sequences were identified using the BLASTN search program (http://ncbi.nlm.nih.gov/blast). For phylogenetic affiliation, sequences were integrated into an ARB database (Ludwig et al., 2004) and aligned using the ARB-EDIT4 alignment editor. A tree showing the phylogenetic affiliation of the two novel isolates was reconstructed using quartet puzzling (Strimmer & von Haeseler, 1996) as implemented in ARB using 10 000 puzzling steps, the TN substitution model and gamma-distributed rate heterogeneities estimated from the dataset. A bacterial 16S rRNA base frequency filter implemented in ARB and inferred to optimally reconstruct clonal phylogeny was used to exclude nucleotide positions with less than 50 % invariance from tree reconstruction. Tree topology and branching order were also verified using maximum-likelihood, maximum-parsimony and neighbour-joining algorithms as implemented in the ARB software package.

**Determination of G+C content.** DNA G+C contents of strains TMJ1<sup>T</sup> and UKTL<sup>T</sup> were measured at the DSMZ. Briefly, DNA was extracted from the cell pellet as described by Cashion et al. (1977). The extracted DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleosides were analysed by HPLC. DNA from *B. subtilis* DSM 402 (43.518 mol% G+C), *Xanthomonas campestris* DSM 3586<sup>T</sup> (65.069 mol%) and *Streptomyces violaceoruber* DSM 40783 (72.119 mol%) was used as references. The HPLC system (Shimadzu) consisted of the following modules: LC-20AD solvent delivery module, DGU-3A online degasser, CTO-10AC column oven, SIL-9A automatic sample injector, SPD-6A UV spectrophotometric detector and a C-R4AX Chromatopac integrator. The analytical column was a Vydac 218TP54, C<sub>18</sub>, 5 μm (250 × 4.6 mm), equipped with a Vydac 210GD54H guard column. The temperature was 45 °C, sample injection volume was 10 μl and the solvent was 0.3 M (NH₄)₂PO₄/acetitonic [40.1 (v/v), pH 4.4] at a flow rate of 1.3 ml min<sup>-1</sup> (adapted from Tamaoka & Komagata, 1984).

**RESULTS**

**Morphology and physiology**

Strains TMJ1<sup>T</sup> and UKTL<sup>T</sup> were enriched with toluene as growth substrate and ferrihydrite as the electron acceptor in the presence of Amberlite XAD-7. After several transfers, rapid growth of both enrichments was established, and pure cultures were isolated in liquid dilution series.
Morphological characteristics of strains TMJ1ᵀ and UKTLᵀ are depicted in Supplementary Fig. S1 and further described in detail in Supplementary Tables S1 and S2 (available in IJSEM Online). Strains TMJ1ᵀ and UKTLᵀ utilized a wide range of aromatic compounds with ferric citrate as electron acceptor (Supplementary Tables S1 and S2). When acetate was provided as the electron donor, several electron acceptors were used by both strains. Sulfitre, however, was utilized only by UKTLᵀ with pyruvate as electron donor (Supplementary Table S2).

Degradation of aromatic compounds

Electron balances showed that toluene was oxidized completely to CO₂ with an electron recovery of 93 ± 1 % for strain UKTLᵀ (Fig. 1a) and 99 ± 14 % for strain TMJ1ᵀ (Fig. 1b), assuming that the electrons from 1 mmol toluene will reduce 36 mmol iron(III). No decrease in the toluene concentration or increase in the ferrous iron concentration was observed in sterilized or culture-unamended controls (not shown). When the culture supernatants were examined for possible intermediates of toluene and o-xylene degradation, benzylsuccinate was identified in the culture supernatants of both strains grown on toluene and methylbenzylsuccinate in the supernatant of an o-xylene-grown culture of strain UKTLᵀ by GC/MS (not shown).

Similarly, hydroxybenzoate was identified by GC/MS in the culture supernatants of both strains grown on p-cresol and in the supernatant of an m-cresol-grown culture of strain TMJ1ᵀ (not shown).

Phylogenetic analysis

Phylogenetic analysis of 16S rRNA gene sequences revealed that strain TMJ1ᵀ belongs to the genus Geobacter within the Geobacteraceae (Deltaproteobacteria; Fig. 2). The closest relatives of strain TMJ1ᵀ were ‘Geobacter humireducens’ JW3, with a sequence similarity of 96 %, and G. bremensis Dfr1ᵀ, with a sequence similarity of 95.4 %.

BLAST search results with the 16S rRNA gene sequence of strain UKTLᵀ revealed it as a member of the low-G+C-content Gram-positive Clostridia within the family Peptococcaceae, with the closest relatives being the type strains of Desulfosporosinus orientis and Desulfotitobacterium chlororespirans, with sequence similarity of 94.2 and 94 %, respectively. Based on the tree topology (Fig. 2), strain TMJ1ᵀ was clearly affiliated to the genus Geobacter, while strain UKTLᵀ was less directly affiliated to members of the genera Desulfotitobacterium (nearest relatives Desulfotitobacterium chlororespirans and Desulfotitobacterium dehalogenans) and Desulfosporosinus (closest relative Desulfosporosinus orientis). The apparent closer affiliation of UKTLᵀ to members of the genus Desulfotitobacterium indicated in the Puzzle dendrogram in Fig. 2 was not reproduced consistently by the maximum-likelihood, maximum-parsimony and distance-matrix treeing methods applied. This is also reflected by the fact that sequence similarity to the more deeply branching Desulfotitobacterium metallireducens 853-15Aᵀ was only 93.5 %, which is even less than the similarity of 94.2 % to Desulfosporosinus orientis DSM 765ᵀ.

DISCUSSION

Here, two novel iron-reducing bacterial strains are described that degrade the monoaromatic hydrocarbons toluene (strains TMJ1ᵀ and UKTLᵀ) and o-xylene (strain UKTLᵀ only). Analysis of 16S rRNA gene sequences clearly showed that strains TMJ1ᵀ and UKTLᵀ are phylogenetically distinct from known monoaromatic hydrocarbon-degrading isolates. Strain TMJ1ᵀ is closely related to members of the genus Geobacter, while strain UKTLᵀ is less directly related to members of the genera Desulfosporosinus and Desulfotitobacterium within the Clostridia (Fig. 2).

The closest relatives of strain TMJ1ᵀ, G. bremensis Dfr1ᵀ, which was isolated with biologically produced ferrihydrite from a freshwater ditch (Straub & Buchholz-Cleven, 2001), and ‘Geobacter humireducens’ JW3, isolated from a
hydrocarbon-contaminated wetland with anthraquinone 2,6-disulfonate as electron acceptor (Coates et al., 1998), were reported to utilize various simple organic acids, but not monoaromatic hydrocarbons or other aromatic compounds except benzoate, as carbon and energy sources (Supplementary Table S1). This clearly distinguishes TMJ1T from the other two organisms. With respect to utilization of aromatic compounds, strain TMJ1T shows similarities with G. metallireducens and G. grbicicae (Coates et al., 2001; Lovley et al., 1993), the only known iron-reducing, aromatic hydrocarbon-degraders of the genus Geobacter, but, based on 16S rRNA gene sequencing, it clusters phylogenetically distinctly from both. This is also supported by a slightly more deeply branching benzylsuccinate synthase alpha-subunit gene previously detected in strain TMJ1T (Winderl et al., 2007). A novel feature of strain TMJ1T is its utilization of m-cresol as a carbon and energy source.

For more-detailed classification, we compared the substrate utilization of strain TMJ1T with its closest relative, G. bremensis DSM 12179T, and with G. chapellei DSM 13688T, which clusters a little further away in the 16S rRNA gene phylogenetic tree. The electron donors and acceptors utilized by strain TMJ1T, G. bremensis DSM 12179T and G. chapellei DSM 13688T show differences that suggest a physiological distinction (Supplementary Table S1). Utilization of pyruvate, propionate and butyrate as well as ferric citrate by strain TMJ1T and G. bremensis DSM 12179T distinguish the two from G. chapellei DSM 13688T. On the other hand, strains G. bremensis DSM 12179T and G. chapellei DSM 13688T have in common the utilization of lactate as electron donor and Mn(IV) as electron acceptor, neither of which can be utilized by strain TMJ1T.

The genomic DNA G+C content of strain TMJ1T (54.4 mol%) is comparable to those of its relatives G. bremensis and G. chapellei as well as other members of the genus Geobacter (G. metallireducens GS-15T, 56.6 mol%; Lovley et al., 1993). Whereas menaquinone MK-8 found in strain TMJ1T is typical of members of the Geobacteraceae such as G. metallireducens and Geobacter sulfurreducens (R. Geyer, unpublished results; Lovley et al., 1993), strain TMJ1T is clearly different in total lipid fatty acid content from G. bremensis DSM 12179T and G. chapellei DSM 13688T. Strain TMJ1T contains 15:0 anteiso fatty acids, whereas G. bremensis DSM 12179T does not. In contrast to these two other members of the genus Geobacter, strain TMJ1T does not contain the fatty acid 18:1 o7c. These, along with other discriminative characteristics indicated in Supplementary Table S1, prompt us to propose that strain TMJ1T represents a novel geobacterial species, Geobacter toluenoxydans sp. nov.

For 16S rRNA gene sequencing showed strain UKTLT to cluster together with members of the sulfate-reducing bacterial
genus *Desulfosporosinus* and members of the genus *Desulfotobacterium*, known for reductive dehalogenation (Spring & Rosenzweig, 2006). The closest relatives of strain UKTL\textsuperscript{T} are *Desulfovibrio orientis*, an organic compound-oxidizing sulfate reducer (Stackebrandt *et al.*, 1997), and *Desulfotobacterium chlororespirans*, known for reductive dehalogenation of chlorophenols (Sanford *et al.*, 1996).

Comparative physiological characteristics revealed important differences between strain UKTL\textsuperscript{T} and its nearest relatives (Spring & Rosenzweig, 2006). Strain UKTL\textsuperscript{T} resembles members of the genera *Desulfovibrio* and *Desulfotobacterium* in pyruvate fermentation, utilization of sulfite and thiosulfate and its spore-forming ability but differs in the absence of lactate utilization, sulfate and sulfur reduction and autotrophic growth. The ability of strain UKTL\textsuperscript{T} to use acetate as sole electron donor with ferric iron as the electron acceptor (Supplementary Table S2) clearly distinguishes it from other members of the genera *Desulfotobacterium* and *Desulfovibrio*, which do not utilize acetate as a carbon and energy source and perform incomplete oxidation of organic compounds to acetate (Robertson *et al.*, 2001; Spring & Rosenzweig, 2006; Villemur *et al.*, 2006). Most *Desulvosporosinus* strains are known to utilize sulfite and thiosulfate in addition to sulfide as electron acceptors, whereas members of the genus *Desulfotobacterium* were reported to utilize sulfide and thiosulfate but not sulfate, with one known exception (Robertson *et al.*, 2001; Villemur *et al.*, 2006). Strain UKTL\textsuperscript{T} does not reduce sulfate but utilizes sulfide and thiosulfate as electron acceptors in the presence of pyruvate. Strain UKTL\textsuperscript{T} additionally utilizes ferric iron as an electron acceptor, whereas *Desulfovibrio orientis* cannot (Stackebrandt *et al.*, 1997). None of the members of the genus *Desulfotobacterium* isolated so far has been reported to use monoaromatic compounds as carbon and energy sources (Robertson *et al.*, 2001; Villemur *et al.*, 2006). Furthermore, *Desulvosporosinus* strain Y5, a distant relative of strain UKTL\textsuperscript{T}, was shown to couple the oxidation of toluene, but not xylene, to the reduction of As(V) (Liu *et al.*, 2004).

Strain UKTL\textsuperscript{T} stains Gram-positive, in contrast to its nearest relatives, which stain Gram-negative (Supplementary Table S2). The G+C content (47.4 mol%) of strain UKTL\textsuperscript{T} is within the range of 45–49 mol% reported for the genera *Desulvosporosinus* and *Desulfotobacterium* (Spring & Rosenzweig, 2006). Strain UKTL\textsuperscript{T} has MK-7 as the major respiratory quinone, which is generally found in the genera *Desulvosporosinus* and *Desulfotobacterium* (Imachi *et al.*, 2002; Ramamoorthy *et al.*, 2006; Spring & Rosenzweig, 2006; Stackebrandt *et al.*, 1997).

Of the 11 specific 16S rRNA gene signature nucleotides defined by Stackebrandt *et al.* (2003) to discriminate between the genera *Desulvosporosinus* and *Desulfotobacterium*, strain UKTL\textsuperscript{T} shares three signatures with the former and six with the latter. The two remaining signature positions are distinct from both (not shown). This substantiates the classification of strain UKTL\textsuperscript{T} within a novel species in this phylogenetic neighbourhood. In summary, the large phylogenetic and physiological differences between members of the genera *Desulfovibrio* and *Desulfotobacterium* and the new iron-reducing isolate UKTL\textsuperscript{T} allow strain UKTL\textsuperscript{T} to be classified within a separate species of the genus *Desulfotobacterium* (Stackebrandt *et al.*, 1997; Villemur *et al.*, 2006).

The findings of benzylsuccinate and methylbenzylsuccinate as major intermediates in toluene- and o-xylene-grown cultures of strains TMJ1\textsuperscript{T} and UKTL\textsuperscript{T} indicated that degradation of toluene and o-xylene proceeds via fumarate addition. The benzylsuccinate synthase pathway has been intensively studied and elucidated for *Thauera* and *Azorarcus* species (Beller & Spormann, 1997; Biegert *et al.*, 1996; Leuthner *et al.*, 1998). The benzylsuccinate synthase pathway has been found in every anaerobic, toluene-degrading micro-organism investigated to date (Chakraborty & Coates, 2004), and bssA genes coding for the alpha subunit of the benzylsuccinate synthase have been established as an environmental marker gene for hydrocarbon degraders (Winderl *et al.*, 2007). The same authors also reported a closely geobacterial bssA gene for strain TMJ1\textsuperscript{T}, but no homologue has been detected to date in strain UKTL\textsuperscript{T}. Activation by benzylsuccinate synthase is not only observed in aromatic hydrocarbon-degrading pure-cultured proteobacteria, but was also observed in the spore-forming, Gram-positive *Desulfotomaculum* sp. OX39, which is able to degrade several monoaromatic hydrocarbons (Morasch *et al.*, 2004). Following *Desulfotomaculum* sp. OX39 and *Desulvosporosinus* sp. strain Y5, strain UKTL\textsuperscript{T} is the third pure culture of a non-proteobacterial isolate capable of degrading aromatic hydrocarbons.

Enrichment and isolation of strain TMJ1\textsuperscript{T} with ferric iron as the electron acceptor from a tar-oil-contaminated site with aromatic non-aqueous-phase liquids (NAPLs) (Zamfirescu & Grathwohl, 2001) further supports the importance of *Geobacter* species in natural attenuation of aromatic hydrocarbons. Members of this genus have been observed to be dominant at several sites contaminated with organic compounds (Lovley & Anderson, 2000). Moreover, clostridia of the genera *Desulvosporosinus* and *Desulfotobacterium*, family *Peptococcaceae*, have also often been isolated from sites contaminated with BTEX (benzene, toluene, ethylbenzene and xylenes) and chlorinated hydrocarbons. Several of these species were reported to be capable of dissimilatory ferric iron reduction, although they are classically considered to be involved in sulfate reduction or dehalogenation (Robertson *et al.*, 2000; Villemur *et al.*, 2006). Recently, as-yet uncultured spore-forming members of the *Peptococcaceae* in a benzene-degrading enrichment culture were reported to utilize ferric iron as an electron acceptor (Kunapuli *et al.*, 2007). Furthermore, two 16S rRNA clones from an iron-reducing enrichment utilizing structural ferric iron in smectite with acetate as carbon source were reported to be closely related to *Desulfotubacterium chlororespirans* (Kostka *et al.*, 2002). These findings further support the
almost-overlooked environmental importance of members of the Peptococcaceae in dissimilatory iron reduction in subsurface environments (Kunapuli et al., 2007; Zavarzina et al., 2007). The isolation of strains TMJ\textsuperscript{T} and UKTL\textsuperscript{T} also indicates that a wider range of phylogenetically diverse micro-organisms within and outside the Proteobacteria are responsible for degradation of aromatic hydrocarbons with ferric iron as the terminal electron acceptor and that iron-reducing micro-organisms can also oxidize monoaromatic compounds other than toluene.

**Description of Geobacter toluenoxydans sp. nov.**

*Geobacter toluenoxydans* [to.lu.e.noxy.dans. N.L. n. tolue-num toluene; N.L. v. oxydo (from Gr. adj. oxus sharp, acid) to oxidize; N.L. part. adj. *toluenuxydans* oxidizing toluene].

Gram-stain-negative, non-spore-forming, non-motile, straight rods, 2.1–3.8 \( m \) long and 0.4 \( m \) wide. Obligate anaerobe. Utilizes ferrihydrite, ferric citrate and fumarate as electron acceptors with acetate as electron donor. Does not reduce Mn(IV), nitrate, sulfate, sulfite, thiosulfate or sulfur. Electron donors used include toluene, benzyl alcohol, benzyldiethanol, phenol, \( m \)-cresol, \( p \)-cresol, acetate, butyrate, formate, propionate, pyruvate and benzoate. Does not utilize fumarate, lactate, malate, succinate or o- cresol. pH range for growth is pH 6.6–7.5 (optimum pH 6.6–7.0). Optimum growth temperature is 25–32 \( ^\circ \)C. Contains 15:0 anteiso but not 18:1\( \text{o7c} \) fatty acids. Characteristics useful to differentiate the type strain from other members of the genus *Geobacter* are depicted in Table 1. The \( G+C \) content of the type strain is 54.4 mol%.

The type strain, TMJ\textsuperscript{T} (=DSM 19350\textsuperscript{T} =JCM 15764\textsuperscript{T}), was isolated from sludge of a monitoring well at a tar-oil-contaminated aquifer near Stuttgart, Germany.

**Description of Desulfitobacterium aromaticivorans sp. nov.**

*Desulfitobacterium aromaticivorans* [ar.o.ma.ti’ci.vo’rans. L. adj. aromaticus aromatic, fragrant; L. part. adj. vorans devouring; N.L. part. adj. *aromaticivorans* devouring aromatic (compounds)].

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### Table 1. Characteristics useful for differentiating strain TMJ\textsuperscript{T} from closely related members of the genus *Geobacter*

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<td>( p )-Cresol (0.5 mM)</td>
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<td>NR</td>
<td>–</td>
<td>+</td>
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<td>NR</td>
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<tr>
<td>Butyrate (10 mM)</td>
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<td>–</td>
<td>+</td>
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<tr>
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<tr>
<td>Lactate (10 mM)</td>
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<td>+</td>
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<td>+</td>
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<tr>
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<td>+</td>
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<td>Mn(IV) (10 mM)</td>
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<td>+</td>
<td>+</td>
<td>–</td>
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<td>+</td>
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<td>Sulfur (1 g L\textsuperscript{–1})</td>
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<td>NR</td>
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<td>+</td>
<td>NR</td>
<td>+</td>
<td>U</td>
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<td>3.9*</td>
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<td>NR</td>
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<tr>
<td>18:1( \text{o7c} )</td>
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<td>3.7</td>
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*Data for the type strain from this study.
†Data from Shelobolina et al. (2008).
Motile, slightly curved rods, 2.3–4.0 μm long and 0.5 μm wide. Strictly anaerobic, Gram-stain-positive, spore-forming organism that oxidizes acetate totally to CO₂. Utilizes ferrirhydrite and ferric citrate as electron acceptors with acetate as electron donor and ferrirhydrite, ferric citrate, sulfate and thiosulfate with pyruvate as electron donor. Does not reduce sulfate or sulfur. Ferments pyruvate but does not grow with hydrogen or with butyrate, propionate, lactate, succinate, m-cresol or o-cresol as electron donors. Electron donors used include toluene, o-xylene, benzyl alcohol, benzoaldehyde, phenol, p-cresol, acetate, fumarate, formate, succinate, malate and benzoate. Contains 15:0 iso but not 18:1o7c fatty acids. The major menaquinone is MK-7. The pH range for growth is pH 6.5–7.5 (optimum pH 6.6–7.0). Optimum growth temperature is 30 °C. Characteristics that are useful to differentiate the type strain from other members of the genus Desulfotobacterium are depicted in Table 2. The G+C content of the type strain is 47.7 mol%.

The type strain, UKTLT (=DSM 19510T =JCM 15765T), was isolated from soil of a former coal-gasification site in Gliwice, Poland.

**ACKNOWLEDGEMENTS**

The authors thank Bernhard Schink for stimulating discussions. Sabine Schäfer is acknowledged for assistance with gene sequencing. Helga Wehnes is thanked for electron microscopy and Angelika Wichmann for technical assistance with lipid analysis.

**REFERENCES**


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**Table 2.** Characteristics useful for differentiating strain UKTLT from related species of the genera Desulfotobacterium and Desulfosporosinus

<table>
<thead>
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<th>5</th>
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<td>Hydrogen</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
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<td>+</td>
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<td>–</td>
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<tr>
<td>Butyrate (10 mM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fumarate (10 mM)</td>
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<td>+</td>
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<td>–</td>
</tr>
<tr>
<td>Malate (10 mM)</td>
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<td>Succinate (10 mM)</td>
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<tr>
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<td>Fe(III) citrate (50 mM)</td>
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<td>Mn(IV) (10 mM)</td>
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<td>+</td>
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<td>None</td>
<td>E, T</td>
<td>E, ST</td>
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</table>

*With sulfite as electron acceptor, strain UKTLT grew with pyruvate only as electron donor, not with acetate.
†Data for the type strain from this study.
‡E, Ellipsoidal; ST, subterminal; T, terminal.


