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

Umakanth Kunapuli,¹ Michael K. Jahn,¹ Tillmann Lueders,¹
Roland Geyer,^{2†} Hermann J. Heipieper³ and Rainer U. Meckenstock¹

Correspondence

Rainer U. Meckenstock
rainer.meckenstock@helmholtz-
muenchen.de

¹Institute of Groundwater Ecology, Helmholtz Zentrum München – German Research Center for Environmental Health, Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany

²Department of Environmental Microbiology, Helmholtz Center for Environmental Research – UFZ, Permoserstraße 15, D-04318 Leipzig, Germany

³Department of Bioremediation, Helmholtz Center for Environmental Research – UFZ, Permoserstraße 15, D-04318 Leipzig, Germany

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 micro-organisms 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 TMJ1^T, which belongs to the genus *Geobacter* within the *Deltaproteobacteria*, and strain UKTL^T, 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 UKTL^T utilizes *o*-xylene and TMJ1^T utilizes *m*-cresol. Anaerobic degradation of toluene in both strains and *o*-xylene in strain UKTL^T is initiated by activation with fumarate addition to the methyl group. The genomic DNA G + C contents of strains TMJ1^T and UKTL^T 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 UKTL^T = DSM 19510^T = JCM 15765^T) and *Geobacter toluenoxydans* sp. nov. (type strain TMJ1^T = DSM 19350^T = JCM 15764^T) to accommodate these strains. To the best of our knowledge, strain UKTL^T 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* (Loneragan *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 &

[†]Present address: LC-MS Support, Applera Europe B.V., Rotkreuz, Switzerland.

Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains UKTL^T and TMJ1^T are EU711071 and EU711072.

Electron micrographs of cells of strains TMJ1^T and UKTL^T and detailed comparisons of their properties with their closest relatives are available as supplementary material with the online version of this paper.

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 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, TMJ1^T and UKTL^T, which are phylogenetically and metabolically distinct from available pure cultures. As for the species mentioned above, strain TMJ1^T is also affiliated to the genus *Geobacter*. For UKTL^T, 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 TMJ1^T and UKTL^T were enriched and isolated in serial liquid dilution-to-extinction cultures with toluene as carbon and energy source and ferrihydrite 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 TMJ1^T), and soil of a former coal-gasification site in Gliwice, Poland (for strain UKTL^T), were used as inocula. Strain UKTL^T 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 bicarbonate-buffered freshwater medium (pH 7.2–7.4) prepared under an atmosphere of N₂/CO₂ (80:20, v/v), that were then incubated at 30 °C (Widdel & Bak, 1992). The medium was reduced with 1 mM FeCl₂ (10 µM sodium sulfate as sulfur source) for the cultivation of strain TMJ1^T and with 0.5 mM Na₂S (no additional sulfur source) for the cultivation of strain UKTL^T. 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 TMJ1^T and UKTL^T 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 7210^T was used as a control for Gram-stain-negative cells and *Bacillus subtilis* DSM 10^T 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 reinoculated 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 × 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 and concentrated with a multi-channel evaporator (Büchi Syncore Polyvap). The acyl chains of polar lipids or LPS were transesterified to the fatty acid methyl esters (FAMES) by a mild alkaline methanolysis and analysed by GC/MS with a 21:0 FAME (59 pmol µl⁻¹) as an internal standard (Curtis *et al.*, 2006; Guckert *et al.*, 1985).

The MIDI extraction and derivatization procedure included acid hydrolysis (cleaves ester bonds of acyl chains or vinyl ether bonds of plasmalogens) and derivatization to methyl esters or dimethylacetals, respectively, followed by clean-up steps. Samples were concentrated under nitrogen and resolved in hexane, containing the internal standard.

Determination of the fatty acid composition. Samples containing FAMES were analysed by GC on an Agilent 6890N gas chromatograph with an FID detector. The capillary column was a CP-Sil 88 (Varian Inc.), 50 m in length with an internal diameter of 0.25 mm. The initial oven temperature was 40 °C for 2 min and the final temperature was 220 °C, with a ramp rate of 8 °C min⁻¹. The injector was at 240 °C, the flow was in splitless mode and helium was used as carrier gas. The detector was set at 270 °C. Peak identification was achieved using a qualitative standard of bacterial FAMES from Supelco.

Reference strains for fatty acid analysis were purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *Geobacter bremensis* DSM 12179^T and *Geobacter chapellei* DSM 13688^T were grown with *Geobacter* medium DSM 826. *Desulfotobacterium chlororespirans* DSM 11544^T was grown with *Desulfotobacterium hafniense* medium (DSM 720) and *Desulfosporosinus orientis* DSM 765^T with *Desulfovibrio* medium (DSM 63).

For analysis of respiratory quinones, lyophilized samples were extracted with 5 ml pentane by vigorous shaking for 60 min and five 1 min bursts of sonication. After centrifugation for 10 min at 5000 r.p.m., the supernatant was transferred to a fresh vial and evaporated under a gentle stream of nitrogen. Residues were dissolved in 100 µl methanol and examined by HPLC/APCI/MS/MS (Geyer *et al.*, 2004) using a more-sensitive hybrid quadrupole-linear ion trap mass spectrometer (4000 QTRAP; Applied Biosystems/MDS Sciex) to monitor 44 different quinones (ubiquinones 6–14, menaquinones 6–14, demethylmenaquinones 6–10, thermoplasmamenaquinones, phyloquinones and plastoquinones) with the following HPLC method; a gradient of methanol (solvent A) and tert-methyl butyl ether (TMBE; solvent B) was applied on a DASH-8 column (20 × 2.1 mm, 5 µm; Thermo) at a flow rate of 250 µl min⁻¹ and 15 °C column temperature. The gradient for solvent A was 0.0–0.2 min, 100%; 1.3–5.0 min, 20%; 5.3–8.0 min, 100%. Aliquots of quinone standards

(Q-6 and MK-4) were used to obtain a calibration curve for ubiquinones and menaquinones prior to sample measurements.

DNA extraction and 16S rRNA gene sequencing. DNA was extracted from harvested (~0.5 ml) cell pellets from toluene/ferrocitrate-grown cultures as described previously (Lueders *et al.*, 2004). Full-length 16S rRNA genes were amplified by PCR using primers Ba27f and Ba1492r (Weisburg *et al.*, 1991). The PCR mixture (50 µl) consisted of 1 × PCR buffer (Fermentas), 1.5 mM MgCl₂, 10 µg BSA, 0.1 mM dNTPs, 0.25 µM each primer, 1 U *Taq* DNA polymerase (Fermentas) and 1 µl undiluted or 1/10-diluted DNA extract, depending on yield. The conditions for thermal cycling were initial denaturation at 94 °C for 3 min and 28 cycles of 94 °C for 30 s, 52 °C for 30 s and 70 °C for 60 s followed by a final elongation cycle at 70 °C for 5 min. Direct sequencing using amplicon primers (Ba27f, Ba519f, Ba1492r) was performed on an ABI 3730 sequencer (Applied Biosystems) using the BigDye Terminator version 3.1 chemistry as 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^T and UKTL^T 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^T (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 201SP54, C₁₈, 5 µm (250 × 4.6 mm), equipped with a Vydac 201GD54H guard column. The temperature was 45 °C, sample injection volume was 10 µl and the solvent was 0.3 M (NH₄)H₂PO₄/acetonitrile [40:1 (v/v), pH 4.4] at a flow rate of 1.3 ml min⁻¹ (adapted from Tamaoka & Komagata, 1984).

RESULTS

Morphology and physiology

Strains TMJ1^T and UKTL^T 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 TMJ1^T and UKTL^T are depicted in Supplementary Fig. S1 and further described in detail in Supplementary Tables S1 and S2 (available in IJSEM Online). Strains TMJ1^T and UKTL^T 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. Sulfite, however, was utilized only by UKTL^T 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 UKTL^T (Fig. 1a) and 99 ± 14 % for strain TMJ1^T (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 UKTL^T 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 TMJ1^T (not shown).

Phylogenetic analysis

Phylogenetic analysis of 16S rRNA gene sequences revealed that strain TMJ1^T belongs to the genus *Geobacter* within the *Geobacteraceae* (*Deltaproteobacteria*; Fig. 2). The closest relatives of strain TMJ1^T were '*Geobacter humireducens*' JW3, with a sequence similarity of 96 %, and *G. bremsensis* Dfr1^T, with a sequence similarity of 95.4 %.

BLAST search results with the 16S rRNA gene sequence of strain UKTL^T 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 *Desulfitobacterium chlororespirans*, with sequence similarity of 94.2 and 94 %, respectively. Based on the tree topology (Fig. 2), strain TMJ1^T was clearly affiliated to the genus *Geobacter*, while strain UKTL^T was less directly affiliated to members of the genera *Desulfitobacterium* (nearest relatives *Desulfitobacterium chlororespirans* and *Desulfitobacterium dehalogenans*) and *Desulfosporosinus* (closest relative *Desulfosporosinus orientis*). The apparent closer affiliation of UKTL^T to members of the genus *Desulfitobacterium* 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

Fig. 1. Electron balance experiments with strain TMJ1^T (a) and strain UKTL^T (b) in batch culture with toluene as growth substrate and ferric citrate as electron acceptor. Concentrations of toluene (diamonds) and ferrous iron (squares) were monitored over time. Error bars represent standard deviations of three parallel incubations.

sequence similarity to the more deeply branching *Desulfitobacterium metallireducens* 853-15A^T was only 93.5 %, which is even less than the similarity of 94.2 % to *Desulfosporosinus orientis* DSM 765^T.

DISCUSSION

Here, two novel iron-reducing bacterial strains are described that degrade the monoaromatic hydrocarbons toluene (strains TMJ1^T and UKTL^T) and *o*-xylene (strain UKTL^T only). Analysis of 16S rRNA gene sequences clearly showed that strains TMJ1^T and UKTL^T are phylogenetically distinct from known monoaromatic hydrocarbon-degrading isolates. Strain TMJ1^T is closely related to members of the genus *Geobacter*, while strain UKTL^T is less directly related to members of the genera *Desulfosporosinus* and *Desulfitobacterium* within the *Clostridia* (Fig. 2).

The closest relatives of strain TMJ1^T, *G. bremsensis* Dfr1^T, which was isolated with biologically produced ferrihydrite from a freshwater ditch (Straub & Buchholz-Cleven, 2001), and '*Geobacter humireducens*' JW3, isolated from a

Fig. 2. Phylogenetic tree reconstructed from 16S rRNA gene sequences showing the placement of strains TMJ1^T and UKTL^T among closely related members of the *Geobacteraceae* and the *Peptococcaceae*, respectively. GenBank accession numbers are given for each strain. The tree was constructed with the quartet PUZZLE method. Percentages at nodes show branching confidence values deduced from 10 000 intermediate trees. Bar, 10% sequence divergence.

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 TMJ1^T from the other two organisms. With respect to utilization of aromatic compounds, strain TMJ1^T shows similarities with *G. metallireducens* and *G. grbiciae* (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 TMJ1^T (Winderl *et al.*, 2007). A novel feature of strain TMJ1^T is its utilization of *m*-cresol as a carbon and energy source.

For more-detailed classification, we compared the substrate utilization of strain TMJ1^T with its closest relative, *G. bremensis* DSM 12179^T, and with *G. chapellei* DSM 13688^T, which clusters a little further away in the 16S rRNA gene phylogenetic tree. The electron donors and acceptors utilized by strain TMJ1^T, *G. bremensis* DSM 12179^T and *G. chapellei* DSM 13688^T show differences that suggest a physiological distinction (Supplementary Table S1). Utilization of pyruvate, propionate and butyrate as well

as ferric citrate by strain TMJ1^T and *G. bremensis* DSM 12179^T distinguish the two from *G. chapellei* DSM 13688^T. On the other hand, strains *G. bremensis* DSM 12179^T and *G. chapellei* DSM 13688^T have in common the utilization of lactate as electron donor and Mn(IV) as electron acceptor, neither of which can be utilized by strain TMJ1^T.

The genomic DNA G+C content of strain TMJ1^T (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-15^T, 56.6 mol%; Lovley *et al.*, 1993). Whereas menaquinone MK-8 found in strain TMJ1^T is typical of members of the *Geobacteraceae* such as *G. metallireducens* and *Geobacter sulfurreducens* (R. Geyer, unpublished results; Lovley *et al.*, 1993), strain TMJ1^T is clearly different in total lipid fatty acid content from *G. bremensis* DSM 12179^T and *G. chapellei* DSM 13688^T. Strain TMJ1^T contains 15:0 anteiso fatty acids, whereas *G. bremensis* DSM 12179^T does not. In contrast to these two other members of the genus *Geobacter*, strain TMJ1^T does not contain the fatty acid 18:1 ω 7c. These, along with other discriminative characteristics indicated in Supplementary Table S1, prompt us to propose that strain TMJ1^T represents a novel geobacterial species, *Geobacter toluenoxydans* sp. nov.

16S rRNA gene sequencing showed strain UKTL^T to cluster together with members of the sulfate-reducing bacterial

genus *Desulfosporosinus* and members of the genus *Desulfitobacterium*, known for reductive dehalogenation (Spring & Rosenzweig, 2006). The closest relatives of strain UKTL^T are *Desulfosporosinus orientis*, an organic compound-oxidizing sulfate reducer (Stackebrandt *et al.*, 1997), and *Desulfitobacterium chlororespirans*, known for reductive dehalogenation of chlorophenols (Sanford *et al.*, 1996).

Comparative physiological characteristics revealed important differences between strain UKTL^T and its nearest relatives (Spring & Rosenzweig, 2006). Strain UKTL^T resembles members of the genera *Desulfosporosinus* and *Desulfitobacterium* 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^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 *Desulfitobacterium* and *Desulfosporosinus*, 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 *Desulfosporosinus* strains are known to utilize sulfite and thiosulfate in addition to sulfate as electron acceptors, whereas members of the genus *Desulfitobacterium* were reported to utilize sulfite and thiosulfate but not sulfate, with one known exception (Robertson *et al.*, 2001; Villemur *et al.*, 2006). Strain UKTL^T does not reduce sulfate but utilizes sulfite and thiosulfate as electron acceptors in the presence of pyruvate. Strain UKTL^T additionally utilizes ferric iron as an electron acceptor, whereas *Desulfosporosinus orientis* cannot (Stackebrandt *et al.*, 1997). None of the members of the genus *Desulfitobacterium* isolated so far has been reported to use monoaromatic compounds as carbon and energy sources (Robertson *et al.*, 2001; Villemur *et al.*, 2006). Furthermore, *Desulfosporosinus* strain Y5, a distant relative of strain UKTL^T, was shown to couple the oxidation of toluene, but not xylene, to the reduction of As(V) (Liu *et al.*, 2004).

Strain UKTL^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^T is within the range of 45–49 mol% reported for the genera *Desulfosporosinus* and *Desulfitobacterium* (Spring & Rosenzweig, 2006). Strain UKTL^T has MK-7 as the major respiratory quinone, which is generally found in the genera *Desulfosporosinus* and *Desulfitobacterium* (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 *Desulfosporosinus* and *Desulfitobacterium*, strain UKTL^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^T within a novel species in this phylogenetic neighbourhood. In summary, the large phylogenetic and physiological differences between members of the genera *Desulfosporosinus* and *Desulfitobacterium* and the new iron-reducing isolate UKTL^T allow strain UKTL^T to be classified within a separate species of the genus *Desulfitobacterium* (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^T and UKTL^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 *Azoarcus* 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 clearly geobacterial *bssA* gene for strain TMJ1^T, but no homologue has been detected to date in strain UKTL^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 *Desulfosporosinus* sp. strain Y5, strain UKTL^T is the third pure culture of a non-proteobacterial isolate capable of degrading aromatic hydrocarbons.

Enrichment and isolation of strain TMJ1^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 *Desulfosporosinus* and *Desulfitobacterium*, 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 *Desulfitobacterium 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 TMJ1^T and UKTL^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.nox'y.dans. N.L. n. *toluenum* toluene; N.L. v. *oxydo* (from Gr. adj. *oxus* sharp, acid) to oxidize; N.L. part. adj. *toluenoxydans* oxidizing toluene].

Gram-stain-negative, non-spore-forming, non-motile, straight rods, 2.1–3.8 µm long and 0.4 µm 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, benzaldehyde, 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 °C. Contains 15:0 anteiso but not 18:1 ω 7c 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, TMJ1^T (=DSM 19350^T =JCM 15764^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)].

Table 1. Characteristics useful for differentiating strain TMJ1^T from closely related members of the genus *Geobacter*

Taxa: 1, strain TMJ1^T (data from this study); 2, *G. bremerensis* (Straub *et al.*, 1998; Straub & Buchholz-Cleven, 2001); 3, *G. chapellei* (Coates *et al.*, 2001); 4, *G. metallireducens* (Lovley *et al.*, 1993); 5, *G. sulfurreducens* (Caccavo *et al.*, 1994); 6, *G. pelophilus* (Straub & Buchholz-Cleven, 2001); 7, *G. grubiaceae* (Coates *et al.*, 2001); 8, *G. argillaceus* (Shelobolina *et al.*, 2007); 9, *G. pickeringii* (Shelobolina *et al.*, 2007); 10, *G. hydrogenophilus* (Coates *et al.*, 2001); 11, *G. bemidjensis* (Nevin *et al.*, 2005); 12, *G. psychrophilus* (Nevin *et al.*, 2005); 13, *G. uranireducens* (Shelobolina *et al.*, 2008); 14, *G. lovleyi* (Sung *et al.*, 2006). Unless indicated, data were determined in the studies listed above using identical methods. Characteristics in bold differentiate strain TMJ1^T from all other tested members of the genus *Geobacter*. +, Utilized; –, not utilized; u, utilization but no growth; UFE, utilization not coupled to iron reduction; NR, not reported in the original literature; tr, present at less than 1 % of total fatty acids.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Substrate utilization														
Toluene (0.5 mM)	+	–	–	+	NR	NR	+	NR	NR	NR	NR	NR	NR	–
Phenol (1 mM)	+	NR	–	+	–	NR	NR	NR	NR	NR	NR	NR	NR	NR
<i>p</i> -Cresol (0.5 mM)	+	NR	–	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
<i>m</i>-Cresol (0.5 mM)	+	NR	NR	–	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Benzaldehyde (0.5 mM)	+	NR	–	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Benzoate (0.5 mM)	+	+	–	+	–	NR	+	NR	NR	+	+	–	NR	–
Pyruvate (10 mM)	+	+	–	+	–	+	+	+	+	+	+	+	NR	+
Butyrate (10 mM)	+	+	–	+	–	NR	+	+	+	+	+	–	–	–
Propionate (10 mM)	+	+	–	+	–	+	+	NR	NR	+	+	–	–	–
Fumarate (10 mM)	–	+	NR	–	–	+	NR	NR	NR	NR	NR	NR	NR	NR
Malate (10 mM)	–	+	NR	–	+	+	NR	NR	NR	NR	+	+	NR	NR
Lactate (10 mM)	–	+	+	–	+ UFE	NR	–	+	+	–	NR	+	+	–
Electron acceptors used														
Fe(III) citrate (50 mM)	+	+	–	+	+	+	+	+	+	+	+	+	–	+
Mn(IV) (10 mM)	–	+	+	+	–	+	NR	+	+	NR	+	+	NR	+
Sulfur (1 g l ^{–1})	–	+	NR	–	+	+	NR	+	+	U	–	–	–	+
Motility	–	+	–	–	–	–	–	+	+	–	–	+	+	+
Total lipid fatty acids (mol%)														
15:0 anteiso	4.6	0*	3.9*	NR	NR	NR	NR	NR	NR	NR	tr†	NR	tr	NR
18:1 ω 7c	0	0.8*	0.4*	3.7	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

*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 ferrihydrite and ferric citrate as electron acceptors with acetate as electron donor and ferrihydrite, ferric citrate, sulfite 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, benzaldehyde, phenol, *p*-cresol, acetate, fumarate, formate, succinate, malate and benzoate. Contains 15:0 iso but not 18:1 ω 7c 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 *Desulfitobacterium* are depicted in Table 2. The G+C content of the type strain is 47.7 mol%.

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

Table 2. Characteristics useful for differentiating strain UKTL^T from related species of the genera *Desulfitobacterium* and *Desulfosporosinus*

Taxa: 1, strain UKTL^T (data from this study); 2, *Desulfitobacterium chlororespirans* (Sanford *et al.*, 1996); 3, *Desulfitobacterium dehalogenans* (Spring & Rosenzweig, 2006); 4, *Desulfitobacterium metallireducens* (Spring & Rosenzweig, 2006); 5, *Desulfitobacterium hafniense* (Spring & Rosenzweig, 2006); 6, *Desulfosporosinus orientis* (Devereux *et al.*, 1989; Stackebrandt *et al.*, 1997). Unless indicated, data were determined in the studies listed above using identical methods. +, Utilized; –, not utilized; NR, not reported in the original literature; d, different reaction among strains. Characteristics in bold differentiate strain TMJ1^T from all other tested species of the genera *Desulfitobacterium* and *Desulfosporosinus*.

Characteristic	1	2	3	4	5	6
Substrate utilization						
Hydrogen	–	+	+	–	–	+
Acetate (10 mM)	+	–	–	–	–	–
Butyrate (10 mM)	–	+	+	+	d	+
Fumarate (10 mM)	+	–	NR	NR	NR	+
Malate (10 mM)	+	NR	NR	NR	NR	–
Succinate (10 mM)	+	–	NR	NR	d	–
Lactate (10 mM)	–	+	+	+	+	+
Ethanol (5 mM)	–	NR	+	+	NR	+
Methanol (5 mM)	–	NR	NR	NR	NR	+
Electron acceptors used						
Fe(III) (50 mM)	+	NR	NR	NR	NR	–
Fe(III) citrate (50 mM)	+	NR	NR	+	NR	–
Mn(IV) (10 mM)	–	NR	NR	+	NR	–
Sulfite (5 mM)	+	+	+	–	+	+
Sulfur (1 g l⁻¹)	–	+	+	+	+	+
Nitrate (10 mM)	–	–	+	–	–	–
Fumarate (40 mM)	–	–	+	–	–	+
Total lipid fatty acids (mol%)						
15:0 iso	1.9	0†	–	+	–	0†
18:1ω7c	0	7.1†	NR	NR	NR	1.4†
Motility	+	+	+	–	d	+
Spores‡	E, T	E, T	None	None	E, T	E, ST

*With sulfite as electron acceptor, strain UKTL^T grew with pyruvate only as electron donor, not with acetate.

†Data for the type strain from this study.

‡E, Ellipsoidal; ST, subterminal; T, terminal.

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