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Geobacter hydrogenophilus, *Geobacter chapellei* and *Geobacter grbiciae*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers

John D. Coates,[1] Vishvesh K. Bhupathiraju,[2] Laurie A. Achenbach,[1] Michael J. McInerney[4] and Derek R. Lovley[3]

Author for correspondence: John D. Coates. Tel: +1 618 453 6132. Fax: +1 618 453 8036. e-mail: jcoates@micro.siu.edu

[1] Department of Microbiology and Center for Systematic Biology, Southern Illinois University, Carbondale, IL 62901, USA

[2] Department of Civil and Environmental Engineering, University of California, Berkeley, CA 94720, USA

[3] Department of Microbiology, University of Massachusetts, Amherst, MA 01003, USA

[4] Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019, USA

Recent studies on the diversity and ubiquity of Fe(III)-reducing organisms in different environments led to the isolation and identification of four new dissimilatory Fe(III)-reducers (strains H-2^T, 172^T, TACP-2^T and TACP-5). All four isolates are non-motile, Gram-negative, freshwater, mesophilic, strict anaerobes with morphology identical to that of *Geobacter metallireducens* strain GS-15^T. Analysis of the 16S rRNA sequences indicated that the new isolates belong to the genus *Geobacter*, in the *δ-Proteobacteria*. Significant differences in phenotypic characteristics, DNA–DNA homology and G+C content indicated that the four isolates represent three new species of the genus. The names *Geobacter hydrogenophilus* sp. nov. (strain H-2^T), *Geobacter chapellei* sp. nov. (strain 172^T) and *Geobacter grbiciae* sp. nov. (strains TACP-2^T and TACP-5) are proposed. *Geobacter hydrogenophilus* and *Geobacter chapellei* were isolated from a petroleum-contaminated aquifer and a pristine, deep, subsurface aquifer, respectively. *Geobacter grbiciae* was isolated from aquatic sediments. All of the isolates can obtain energy for growth by coupling the oxidation of acetate to the reduction of Fe(III). The four isolates also coupled Fe(III) reduction to the oxidation of other simple, volatile fatty acids. In addition, *Geobacter hydrogenophilus* and *Geobacter grbiciae* were able to oxidize aromatic compounds such as benzoate, whilst *Geobacter grbiciae* was also able to use the monoaromatic hydrocarbon toluene.

Keywords: Fe(III)-reduction, *Geobacter*, hydrocarbon oxidation, anaerobic

INTRODUCTION

In the last decade, microbial Fe(III) reduction has been identified as an important process in the mineralization of organic carbon in the environment (Lovley *et al*., 1997, and references therein). During this time, in excess of 40 micro-organisms have been isolated, characterized and identified that couple anaerobic growth to the respiration of Fe(III). Phylogenetically, these organisms are very diverse, including representatives from all subclasses of the *Proteobacteria* (Cummings *et al*., 1999; Lovley *et al*., 1997; and references therein) as well as those forming novel lines of descent in the domain *Bacteria* (Caccavo *et al*., 1996; Coates *et al*., 1999). In addition, a recent study demonstrated that the metabolic ability to grow by dissimilatory Fe(III) reduction is also found amongst the extreme thermophiles in the domain *Archaea* (Vargas *et al*., 1998).

Studies have shown that, of the known Fe(III)-reducing bacteria, members of the genera *Geothrix* and *Geobacter* are the predominant Fe(III)-reducing bacteria found in most environments (Anderson *et al*., 1998; Coates *et al*., 1999). Both of these groups of organisms comprise strict anaerobes that oxidize organic matter completely to carbon dioxide (Coates & Lovley, 2001; Coates *et al*., 1999; Lovley *et al*., 1997). *Geothrix* represents a novel line of descent in the *Bacteria* (Coates *et al*., 1999), whereas *Geobacter* species are members of the family *Geobacteraceae* in

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences described in this work are AF335182 (strain TACP-2^T), AF335183 (strain TACP-5), U28173 (strain H-2^T) and U41561 (strain 172^T).

the *δ-Proteobacteria* (Coates & Lovley, 2001). Of the two genera, species of *Geobacter* are the most readily isolated, especially from hydrocarbon-contaminated environments and freshwater aquatic environments (Anderson *et al*., 1998; Coates & Anderson, 2000; Coates *et al*., 1996, 1998; Lovley & Coates, 2000; Lovley & Phillips, 1988).

As part of a previous study on the diversity and ubiquity of Fe(III)-reducing bacteria in the environment, several new Fe(III)-reducing isolates were obtained (Coates *et al*., 1996). All of these isolates were strictly anaerobic, Gram-negative, non-motile rods with identical cell shapes and sizes. Phenotypic characterization of each of the isolates revealed that all were capable of oxidizing acetate completely with the concomitant reduction of Fe(III) (Coates *et al*., 1996). Analysis of the 16S rRNA gene sequences indicated that these organisms were all members of the family *Geobacteraceae* in the *δ-Proteobacteria*. Although phenotypic characterization suggested that there were significant differences amongst the isolates, for three of them, the high degree of sequence similarity to *Geobacter metallireducens* made it unclear as to whether these isolates represented new species within the genus *Geobacter*. Here, we report the results of a more extensive analysis of 16S rDNA gene sequences as well as DNA–DNA homology and G+C content analyses. These additional studies support the formation of three new species within the genus *Geobacter*.

METHODS

Sources of organisms. Strain H-2^T was isolated as part of a study on the effects of Fe(III) chelators on the biodegradation of hydrocarbons under Fe(III)-reducing conditions (Coates *et al*., 1996; Lovley *et al*., 1994). Strain 172^T was isolated from acetate-oxidizing, Fe(III)-reducing enrichments of sediments collected from a deep aquifer of the Atlantic Plain in South Carolina, USA (Lovley *et al*., 1990). Strains TACP-2^T and TACP-5 were isolated from a freshwater aquatic sediment collected from the estuary of the Potomac River in Virginia, USA (Coates *et al*., 1996). *Geobacter metallireducens* strain GS-15^T and *Geobacter sulfurreducens* strain PCA^T were obtained from our culture collection of dissimilatory Fe(III)-reducers. *Desulfuromonas acetoxidans* DSM 684^T was obtained from the DSMZ (Braunschweig, Germany). All cultures were maintained in a frozen state at −70 °C after the addition of 10% (v/v) sterile glycerol to an actively growing culture.

Media and growth conditions. Standard anaerobic culture techniques were used throughout (Balch *et al*., 1979; Hungate, 1969; Miller & Wolin, 1974). The medium was boiled under N_2/CO_2 (80:20) to remove dissolved O_2, dispensed into anaerobic pressure tubes or serum bottles under N_2/CO_2, capped with thick butyl-rubber stoppers and then sterilized by autoclaving. The basal medium was the bicarbonate-buffered freshwater medium that was used previously to culture *Geobacter metallireducens* (Lovley & Phillips, 1988). Unless otherwise noted, acetate (10 mM) was the electron donor. Fumarate (50 mM) was supplied as the sole electron acceptor and incubations were done at 30 °C.

16S rRNA gene sequencing and phylogenetic analysis. Cells from 1·5 ml pure culture were harvested and lysed by being boiled in 40 µl sterile water plus 5 µl chloroform. Primers specific to the bacterial 16S rRNA gene (8F, 5′-AGAGTTTGATCCTGGCTCAG-3′; 1525R, 5′-AAGGAGGTGATCCAGCC-3′) were used in a PCR that consisted of 10 mM Tris/HCl (pH 9·0), 50 mM KCl, 0·1% Triton X-100, 1·2 mM $MgCl_2$, 0·2 mM each dNTP, 75 ng each primer, 0·5 µl *Taq* polymerase (Gibco-BRL) and 1 µl lysed cells in a 50 µl reaction. Amplifications were performed as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, followed by a 10 min incubation at 72 °C. Amplification products were gel-purified (GeneClean; Bio 101) and cycle-sequenced (Thermo-Sequenase; Amersham). Sequence entry and manipulation were performed with MACVECTOR 6.0 sequence-analysis software for the Macintosh (IBI). Sequences of selected 16S rRNA genes were obtained from the Ribosomal Database Project (Maidak *et al*., 1999) and GenBank (Benson *et al*., 2000) and were analysed by using the computer program SEQAPP (Gilbert, 1993). The 16S rRNA gene sequences from strains H-2^T, 172^T, TACP-2^T and TACP-5 were aligned manually using secondary-structure information for proper alignment. Only those regions sequenced in all of the organisms (1052 nucleotides) were used in the phylogenetic analyses. Distance-matrix, parsimony and maximum-likelihood analyses of the aligned sequences were performed using PAUP* 4.0d65 (Swofford, 1999). Bootstrap analysis was conducted on 100 replications using a heuristic search strategy to assess the confidence level of various clades. The GenBank accession numbers for the sequences shown in Fig. 1 are as follows (in parentheses): *Desulfuromonas acetexigens* (U23140), *Desulfuromonas acetoxidans* (M26634), *Desulfuromonas chloroethenica* (U49748), *Desulfuromonas palmitatis* (U28172), *Desulfuromonas thiophila* (Y11560), *Desulfomonile tiedjei* (M26635), *Desulfuromusa bakii* (X79412), *Desulfuromusa kysingii* (X79414), *Desulfuromusa succinoxidans* (X79415), '*Geobacter akaganeitreducens*' (U96918), '*Geobacter arculus*' (U96917), *Geobacter chapellei* strain 172^T (U41561), *Geobacter grbiciae* strain TACP-2^T (AF335182), *Geobacter grbiciae* strain TACP-5^T (AF335183), *Geobacter hydrogenophilus* strain H-2^T (U28173), '*Geobacter humireducens*' (AF019932), *Geobacter metallireducens* (L07834), *Geobacter sulfurreducens* (U13928), *Pelobacter acetylenicus* (X70955), *Pelobacter acidigallici* (X77216), *Pelobacter carbinolicus* (X79413), *Pelobacter propionicus* (X70954) and *Pelobacter venetianus* (U41562).

Determination of DNA base composition. DNA was isolated from cell pellets of the individual strains grown in basal medium with acetate as the electron donor (10 mM). DNA isolation was performed using standard procedures (Marmur, 1961). The G+C ratio was determined by using HPLC as described previously (Mesbah *et al*., 1989). Non-methylated λ DNA was used as the standard (Sigma).

DNA–DNA hybridization. DNA isolated from the four new isolates, *Geobacter metallireducens*, *Geobacter sulfurreducens* and *D. acetoxidans* was nick-translated with ^{32}P-labelled dCTP by using a nick translation kit (Boehringer Mannheim) according to the manufacturer's specifications. Labelled DNA preparations were purified with Quick Spin columns (Boehringer Mannheim) and repeated ethanol-precipitation. DNA–DNA reassociation was performed by the free-solution method and analysed by means of the S1-nuclease procedure, as described previously (Johnson, 1994). Reassociation mixtures contained 12·5% formamide. Reassociation was performed at 47 °C.

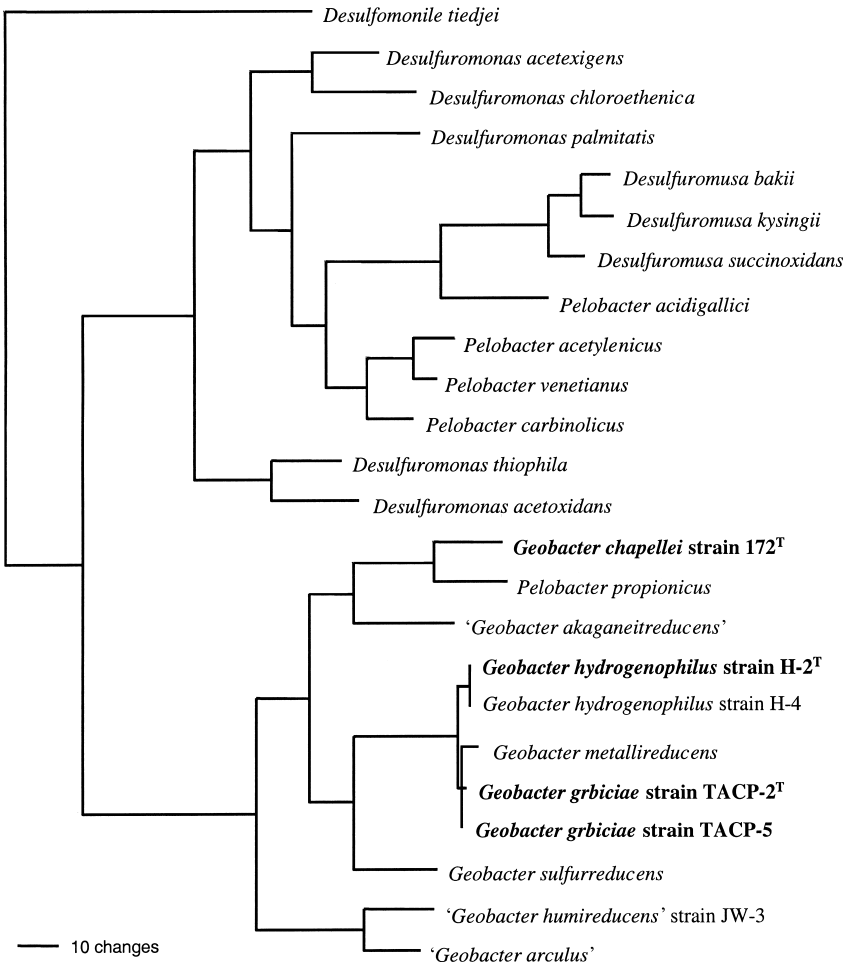

Fig. 1. Phylogenetic tree of the 16S rDNA sequence dataset resulting from parsimony analysis. Branch lengths are from a branch-and-bound analysis. Tree length, 722; consistency index minus uninformative sites, 0·504; retention index, 0·722. Bar, 10 changes.

RESULTS

Strains H-2^T, 172^T, TACP-2^T and TACP-5 were all Gram-negative, non-motile, non-fermenting, non-spore-forming, strict anaerobes (Coates *et al*., 1996). All of the strains were morphologically identical (Coates *et al*., 1996). In addition, all of the strains coupled the complete oxidation of organic carbon compounds to the reduction of Fe(III) (Coates *et al*., 1996). Phenotypic and morphological characterization demonstrated that the characteristics were most similar to those of *Geobacter metallireducens* strain GS-15^T, the type species of the genus *Geobacter* (Lovley *et al*., 1993) within the family *Geobacteraceae* (see Table 3).

Analyses of the 16S rRNA gene sequence

Analysis of the almost complete sequences of the 16S rRNA genes of all of the strains revealed that the four new isolates were related to members of the genus *Geobacter* in the *δ-Proteobacteria* (Fig. 1). The 16S rRNA gene sequence of strain 172^T differed from those of other described *Geobacter* species and from those of strains H-2^T, TACP-2^T and TACP-5 by more than 5% (Table 1). Strain 172^T appears to be most closely related to *P. propionicus* (3·4% difference), which is also a member of the *Geobacteraceae*. The 16S rRNA gene sequences of strains H-2^T, TACP-2^T and TACP-5 were closely related to each other (distance values ranged from 0·097 to 0·49%) and to that of *Geobacter metallireducens* strain GS-15^T (distance values ranged from 0·39 to 0·78%). The close similarity between the 16S rRNA gene sequences of strains H-2^T, TACP-2^T and TACP-5 and *Geobacter metallireducens* renders speciation based on 16S rRNA gene sequences alone ambiguous.

DNA base composition

The G+C content of DNA from strain 172^T was 50·2 mol%, which is markedly different from that of the other species in the genus *Geobacter* (Table 2) and

Table 1 Evolutionary distance matrix of 16S rDNA sequences, including strains H-2^T, 172^T, TACP-2^T and TACP-5

Taxon	1	2	3	4	5	6	7	8
1. Strain H-2^T								
2. Strain 172^T	5·3							
3. Strain TACP-2^T	0·49	5·5						
4. Strain TACP-5	0·39	5·4	0·097					
5. *Geobacter metallireducens*	0·78	5·7	0·49	0·39				
6. '*Geobacter akaganeitreducens*'	5·8	5·3	6·0	5·9	6·2			
7. *Pelobacter propionicus*	6·7	3·4	6·6	6·5	6·8	6·0		
8. *Geobacter sulfurreducens*	4·6	6·3	4·6	4·5	4·8	5·7	5·8	
9. '*Geobacter humireducens*'	6·8	6·7	6·8	6·7	7·0	6·7	7·3	6·8

Table 2 DNA base composition and levels of DNA–DNA homology between strains H-2^T, 172^T, TACP-2^T, TACP-5 and related bacteria

Taxon	G+C content (mol%)	DNA hybridization (%) with ^{32}P-labelled DNA from:		
		Strain H-2^T	Strain 172^T	Strain TACP-2^T
Geobacter metallireducens	58·9±0·3	54	30	30
Geobacter sulfurreducens	60·6±0·4	26	32	46
Desulfuromonas acetoxidans	52·2±0·3	13	33	22
Strain H-2^T	58·4±0·2	100	15	40
Strain 172^T	50·2±0·3	14	100	35
Strain TACP-2^T	57·4±0·3	43	18	100
Strain TACP-5	57·3±0·2	39	20	86

is consistent with the analysis of the 16S rRNA gene sequence information. The values for strain H-2^T and *Geobacter metallireducens* were almost identical (Table 2), supporting their close phylogenetic relationship. The G+C contents of strains TACP-2^T and TACP-5 were also almost identical to each other, but differed from that of *Geobacter metallireducens* by 1·5% and from that of strain H-2^T by about 1%. It should be noted that, although the G+C content determined here for *D. acetoxidans* was similar to values published previously (Finster *et al.*, 1997), the value obtained for *Geobacter metallireducens* strain GS-15^T was slightly higher than that published previously. This difference was probably a function of the different method employed in this study (HPLC, as opposed to the thermal denaturation method employed previously by Lovley *et al.*, 1993).

DNA–DNA hybridization

In support of the close similarities in the 16S rRNA gene sequences and G+C content, there was also a high percentage of DNA–DNA homology between strains TACP-2^T and TACP-5 (86%). Little or no DNA–DNA homology was observed between strain TACP-2^T and any of the other tested members of the genus *Geobacter*, which suggests that these two organisms are representative strains of the same species within the genus *Geobacter*. Strains 172^T and H-2^T exhibited little or no DNA–DNA homology with each other or with any other organisms tested, suggesting that these strains each represent new species in the genus *Geobacter*.

DISCUSSION

Previous phenotypic characterization studies (Coates *et al.*, 1996) of strains H-2^T, 172^T, TACP-2^T and TACP-5 (Table 3) demonstrated that all of the strains were similar to many species of the known genera in the family *Geobacteraceae*; however, incomplete genotypic analysis rendered taxonomic description of these organisms ambiguous. The genotypic analyses presented in this study suggest that significant differences exist between these strains and previously described Fe(III)-reducing bacteria. The analysis of 16S rRNA gene sequences and DNA–DNA hybridization studies further support the general conclusion that strains with 16S rRNA gene-sequence similarities of less than 97% do not exhibit DNA–DNA homologies of 70% or more (Stackebrandt & Goebel, 1994). It is also interesting to note that organisms such as strain TACP-2^T and *Geobacter metallireducens*, which have greater than 99% similarity in terms of 16S rRNA gene sequence, exhibit DNA–DNA homology of only 30%, which is well below the minimum percentage

Table 3 Phenotypic differences amongst strains H-2^T, 172^T, TACP-2^T and TACP-5 and related bacteria

The properties of strain TACP-5 were identical to those listed for strain TACP-2^T. Abbreviations: AQDS, 2,6-Anthraquinone disulfonate, a humic-substance analogue; *G.*, *Geobacter*.

Character	*G. metallireducens* GS-15^T	*G. hydrogenophilus* H-2^T	*G. grbiciae* TACP-2^T	*G. sulfurreducens*	*G. chapellei* 172^T
Source	Aquatic sediments	Contaminated aquifer	Aquatic sediments	Contaminated ditch	Deep subsurface sediments
Electron donors oxidized with Fe(III):					
H_2	No	Yes	Yes	Yes	No
Formate	No	Yes	Yes	No	Yes
Acetate	Yes	Yes	Yes	Yes	Yes
Propionate	Yes	Yes	Yes	No	No
Ethanol	Yes	Yes	Yes	No	Yes
Lactate	No	No	No	No	Yes
Benzoate	Yes	Yes	Yes	No	No
Other electron donors used	Butyrate, valerate, isovalerate, toluene, phenol, *p*-cresol, benzaldehyde, pyruvate	Butyrate, pyruvate, succinate	Toluene, pyruvate, butyrate	None	None
Electron acceptors	Mn(IV), nitrate, U(VI), AQDS, humic substances	Fumarate, U(VI)	AQDS	S^0, fumarate, malate, Co(III)	Mn(IV), U(VI), fumarate

homology (70%) required for two strains to be accepted as being representatives of a single species (Johnson, 1994).

Taxomonic status of strain 172^T

On the basis of physiological differences with respect to other described Fe(III)-reducing bacteria (Table 3) and a low level of 16S rRNA sequence similarity, strain 172^T was recently described as a new species of the genus *Geobacter* (Coates *et al*., 1996; Lonergan *et al*., 1996) and was tentatively named *Geobacter chapellei*. The facts that strain 172^T exhibited very low levels of DNA–DNA homology with the other organisms examined in this study and that it had significantly lower G+C content than other Fe(III)-reducers confirm this conclusion. It is proposed that strain 172^T is the type strain of a new species, *Geobacter chapellei* sp. nov. In contrast to other Fe(III)-reducing bacteria, *Geobacter chapellei* cannot use Fe(III) chelated with citrate as an electron acceptor.

Taxonomic status of strain H-2^T

In addition to having similar morphologies, strain H-2^T and *Geobacter metallireducens* also have several similar phenotypic traits. Both organisms oxidize simple fatty acids and aromatic compounds coupled to Fe(III) reduction (Coates *et al*., 1996; Lovley *et al*., 1993) and reduce S^0, although S^0 reduction does not provide energy to support the growth of either organism (Coates *et al*., 1996). Although there was a high degree of 16S rDNA sequence similarity between *Geobacter metallireducens* and strain H-2^T, other significant physiological differences (Table 3) led to the suggestion that these organisms were, in fact, different species (Coates *et al*., 1996; Lonergan *et al*., 1996). The low DNA–DNA homology between strain H-2^T and the other *Geobacter* species tested shows that strain H-2^T represents a new species in the genus *Geobacter*. In view of the ability of this strain to use H_2 (Coates *et al*., 1996), the species name *Geobacter hydrogenophilus* sp. nov. is proposed.

Taxonomic status of strains TACP-2^T and TACP-5

Strains TACP-2^T and TACP-5 are almost identical morphologically, phylogenetically and in DNA base composition; only slight phenotypic differences are observed between the two strains (Coates *et al*., 1996), which suggests that they are representatives of the same species. Although there is a high degree of similarity between *Geobacter metallireducens* and these strains, both physiologically and in terms of 16S rRNA gene sequence, the low DNA–DNA homology that exists between strain TACP-2^T and *Geobacter metallireducens* suggests that strains TACP-2^T and TACP-5 represent a different species from *Geobacter metallireducens*. On the basis of DNA–DNA homology studies, we propose that strains TACP-2^T and TACP-5 are two representative strains of a new species in the genus *Geobacter*, *Geobacter grbiciae* sp. nov.

Description of *Geobacter chapellei* sp. nov.

Geobacter chapellei (cha.pel′le.i. N.L. gen. masc. n. *chapellei* of Chapelle, named after Frank Chapelle, who contributed to our knowledge of subsurface biogeochemistry).

Rod-shaped, non-motile, Gram-negative bacterium with cell dimensions of 1–2 × 0·6 μm. Does not form spores. Strictly anaerobic chemo-organotroph that oxidizes acetate with the concomitant reduction of Fe(III). Other electron donors used in addition to acetate include ethanol, lactate and formate. Electron acceptors used include Fe(III), Mn(IV), fumarate and the humic-substance analogue 2,6-anthraquinone disulfonate; it does not use Fe(III) chelated with citrate. Whole-cell suspensions reduce U(VI), although it is not known whether this type of metabolism can supply energy for growth. *Geobacter chapellei* is redox sensitive and its growth rate is improved significantly by the presence of a reducing agent, such as Fe(II), in the medium. The cells contain *c*-type cytochromes. The optimum temperature for growth is 25 °C. *Geobacter chapellei* strain 172^T was obtained from Fe(III)-reducing enrichments of subsamples from deep aquifer sediments of the Atlantic Coastal Plain in South Carolina, USA. The type strain is strain 172^T (= ATCC 51744^T = DSM 13688^T).

Description of *Geobacter hydrogenophilus* sp. nov.

Geobacter hydrogenophilus (hy.dro.ge.no′phi.lus. N.L. n. *hydrogenium* hydrogen; Gr. adj. *philos* friendly to; N.L. adj. *hydrogenophilus* liking hydrogen, referring to the ability of the organism to grow by oxidation of hydrogen).

Non-motile, non-spore-forming, rod-shaped, Gram-negative organism with cell dimensions of 1–2 × 0·6 μm. A strictly anaerobic chemo-organotroph that oxidizes acetate, formate, propionate, butyrate, ethanol, pyruvate, succinate and benzoate with the concomitant reduction of Fe(III). It also grows by the oxidation of H_2 with the reduction of Fe(III) when citrate is provided as a carbon source. Growth is also possible with fumarate or the humic-substance analogue 2,6-anthraquinone disulfonate as the electron acceptor. S^0 is reduced, but S^0 reduction does not yield energy to support growth. Cell suspensions reduce U(VI). The temperature and pH optima are 35 °C and 6·5. *Geobacter hydrogenophilus* can grow in medium containing 1% (w/v) NaCl, but grows optimally in freshwater medium. The cells contain *c*-type cytochromes. *Geobacter hydrogenophilus* was enriched from samples taken from a hydrocarbon-contaminated aquifer at the Defense Fuel Supply Center, Hanahan, SC, USA, using acetate as the electron donor and Fe(III)-nitrilotriacetic acid as the electron

acceptor. The type strain is strain H-2^T (= ATCC 51590^T = DSM 13691^T).

Description of *Geobacter grbiciae* sp. nov.

Geobacter grbiciae (grb.i′ci.ae. N.L. gen. fem. n. *grbiciae* of Grbic, named in honour of Dunja Grbic-Galic for her significant contributions to the field of anaerobic aromatic hydrocarbon oxidation).

Cells are rod-shaped, Gram-negative, 1–2 × 0·6 μm, non-motile and do not form spores. Strictly anaerobic chemo-organotroph that oxidizes acetate and other simple fatty acids or ethanol with the concomitant reduction of Fe(III). Strain TACP-5 can also oxidize monoaromatic compounds, including toluene, as alternative electron donors. Strain TACP-5 can use H_2. Strain TACP-2^T can be grown with the various forms of soluble Fe(III) as well as with poorly crystalline Fe(III) oxide. In contrast, strain TACP-5 does not grow with Fe(III) chelated with citrate. Strains TACP-2^T and TACP-5 were isolated from freshwater aquatic sediment taken from the estuary of the Potomac River in Virginia, USA, at the same site that previously yielded *Geobacter metallireducens*. The type strain is strain TACP-2^T (= ATCC BAA-45^T = DSM 13689^T). Strain TACP-5 (= ATCC BAA-46 = DSM 13690) is a reference strain.

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