**Introduction**

Dissimilatory reduction of Fe(III) oxides is an important process for the oxidation of organic matter in terrestrial anoxic soils (Lovley, 1991, 1997; Thamdrup, 2000). This process is dominated by *Geobacter* species (Anderson et al., 1998; Coates et al., 1996; Lovley et al., 2011; Snoeyenbos-West et al., 2000), contributing to the global cycling of metals and carbon (Lovley et al., 2011). The genus *Geobacter* was established by Lovley et al. (1993) with *Geobacter metallireducens* GS-15T isolated from the Potomac River, the first species to be described under the genus, followed by *Geobacter sulfurreducens* PCA1 (Caccavo et al., 1994). Since then, other representatives belonging to the genus *Geobacter* have been described, with 16 valid species already reported at the time of writing.

*Geobacter sulfurreducens* PCA1 is one of the most studied *Geobacter* species as a model organism in various fields of research, such as biochemical and molecular stud-
ies on the respiratory mechanisms of Fe(III) oxide (Kim et al., 2005; Lloyd et al., 2003; Lovley et al., 2011; Rollefson et al., 2009; Yun et al., 2011), microbial fuel cells (MFC) (Bond and Lovley, 2003), development of genetic manipulation techniques (Coppi et al., 2001; Mahadevan et al., 2011; Park and Kim, 2011; Ueki and Lovley, 2010), and the first genome analysis within the genus Geobacter (Methe et al., 2003).

The successful isolation of OSK2A\textsuperscript{T} as a novel Fe(III)-reducing bacterium described as a novel subspecies within the Geobacter sulfurreducens, with 99.6% similarity based on the 16S rRNA gene sequence and the capability of growth with ethanol as a substrate, will supplement the findings and add to the growing list of type strains within the genus Geobacter. Characterization of this strain will also provide insight into features that may lead to improved current production in MFC. Despite the high similarity of OSK2A\textsuperscript{T} to G. sulfurreducens PCA\textsuperscript{T}, there are some distinguishable phenotypic characteristics, such as the ability to utilize ethanol, temperature growth range, and other morphological and physiological differences between them. Here, we report the taxonomic characterization of strain OSK2A\textsuperscript{T} assigned as a novel subspecies belonging to Geobacter sulfurreducens subsp. ethanolicus subsp. nov. (type strain=DSMZ 26126\textsuperscript{T}=JCM 18752\textsuperscript{T}). In addition, new metabolic information obtained from this study and others (Lovley, 2006; Lovley et al., 2011; Speers and Reguera, 2011) validates an emendation to the original description of Geobacter sulfurreducens PCA\textsuperscript{T} which is proposed here.

Materials and Methods

Isolation and growth conditions. Mud samples for enrichment were collected from a lotus field in Japan, and isolation was performed in roll tubes with agar as the solidifying agent. Purity of the isolate was checked by microscopy and the absence of growth in an anaerobic heterotrophic medium (NIH thioglycolate broth, Difco) amended with pyruvate (20 mM).

Geobacter sulfurreducens PCA\textsuperscript{T} (DSM 12127\textsuperscript{T}) purchased from DSMZ was used as the reference strain for all tests under our laboratory conditions.

Morphological and physiological tests. For morphological analysis, strain OSK2A\textsuperscript{T} was grown with acetate and amorphous Fe(III) hydroxide and pieces of glass, washed with PBS, and fixed for scanning electron microscopy using phosphate-buffered saline (pH 7) containing 2% glutaraldehyde. Fixed samples were dehydrated through a stepwise increase in ethanol series (50, 60, 70, 80, 90, and 99.5%) followed by 100% overnight. Ethanol was removed from fixed samples through a paper filter at room temperature, and samples were immediately coated with OsO\textsubscript{4} in an osmium coater (Neoc-ST, Meiwafosis). The OsO\textsubscript{4}-coated plates were observed by SEM at 5 kV (S-4800, Hitachi). Motility was observed for cultures grown on ethanol (20 mM) and amorphous Fe(III) hydroxide (ca. 100 mM) and examined by phase contrast microscopy in accordance with the method described by Childers et al. (2002).

Physiological tests on growth ranges for temperature, pH, and NaCl concentration were performed with acetate (10 mM) and fumarate (20 mM), in a basal medium slightly modified compared with that of Widdel and Pfennig (1977), used previously to culture “Geobacter luticola” OSK6\textsuperscript{T} (Viulu et al., 2012). Tests for all growth ranges and optima for strain OSK2A\textsuperscript{T} and the reference strain PCA\textsuperscript{T} were performed in duplicate and the growth was monitored using a spectrophotometer by measuring OD at 560 nm.

Fe(III) utilization and growth tests. Fe(III) utilization and growth of the strain were observed at 30°C with acetate as an electron donor and Fe(III)-NTA as an electron acceptor. Cell growth was determined by measuring protein concentrations and direct cell counting with SYBR Gold fluorescent dye with a Bicinchoninic Acid (BCA) Protein Assay kit (Pierce Biotechnology) according to the manufacturer’s instructions, with slight modifications compared with a previously described method (Viulu et al., 2012).

The same composition of bicarbonate-buffered basal medium described above was used for all phenotypic characterizations in which acetate or Fe(III)-citrate was used as the electron donor or acceptor, respectively. Utilization of electron donors was examined at 30°C in the presence of Fe(III)-citrate (50 mM) with the following substrates (concentrations in mM given in parentheses, unless otherwise stated): formate (10), propionate (10), butyrate (10), pyruvate (10), lactate (10), fumarate (10), succinate (10), ethanol (10), butanol (10), glucose (10), phenol (1), benzoate (1), toluene (1), methanol (5), propanol (10), isopropanol (10), H\textsubscript{2} (ca. 62 kPa), and CH\textsubscript{4} (ca. 62 kPa). Utilization of electron acceptors was examined with nitrate (20), malate (20), fumarate (40), Fe(III)-NTA (10), amorphous Fe(III) hydroxide (50), sulfate (20), el-
 elemental sulfur (3.0 g/L), sulfite (20), and thiosulfate (20) in the presence of acetate (10).

16S rRNA gene amplification and sequence analyses. The 16S rRNA gene of strain OSK2A<sup>T</sup> was amplified by PCR using primers EU10F and U1500R. Sequencing was performed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems), according to previously described procedures (Nakamura et al., 2011). For phylogenetic analyses, 16S rRNA gene sequences of the type strains of the genus Geobacter and other related taxa were aligned using the online alignment tool SINA (Pruesse et al., 2007) and manually edited in ARB (Ludwig et al., 2004). Aligned sequences were imported into MEGA 5.0 and PHYML 3.0.1 (Guindon and Gascuel, 2003; Tamura et al., 2011) to estimate phylogenetic trees using neighbor-joining (Saitou and Nei, 1987) and maximum likelihood (Felsenstein, 1981) methods, respectively, with the Jukes-Cantor model (Jukes and Cantor, 1969). Reliabilities of the bootstrap values were inferred by both methods in accordance with Hall (2008).

Analyses of respiratory quinone and cellular fatty acids. Cells for respiratory quinone analysis were extracted in chloroform/methanol, purified by TLC in hexane/benzene/chloroform mixture, and determined by HPLC according to a previously described procedure (Collins et al., 1982). Analyses of cellular fatty acids were carried out by the Identification Service of the DSMZ, Braunschweig, Germany. G. sulfurreducens PCA<sup>T</sup> was used as the reference strain for both respiratory quinone and fatty acid analyses. Cells for both strains were cultured on acetate (20 mM) and Fe(III)-citrate (100 mM) until early stationary phases for chemotaxonomic analyses.

PCR-based fingerprinting analyses of repetitive extragenic palindromic (rep) elements. Genomic DNA for rep-PCR was obtained for strain OSK2A<sup>T</sup> from cells grown with ethanol (10 mM) and Fe(III)-citrate (50 mM), while strain PCA<sup>T</sup> cells were obtained from acetate (10 mM) and Fe(III)-citrate (50 mM). Rep-PCR amplifications were performed with REP2-I and REP1R-I primers according to PCR conditions described by Versalovic et al. (1991) and Sung et al. (2006). The rep-PCR products were electrophoresed in 1.5% agarose gel with 1× TAE buffer at 18 V for 5 h. Resulting band patterns were obtained using a UV trans-illuminator equipped with a digital camera (GelDoc XR, Bio-Rad).

G+C content and DNA-DNA hybridization analyses. For determination of the G+C content (mol%), cells of strain OSK2A<sup>T</sup> and the reference strain PCA<sup>T</sup> were grown with either ethanol (20 mM) or acetate (20 mM) and Fe(III)-citrate (100 mM) and were extracted according to the protocol described by Versalovic et al. (1991). The G+C content was determined by HPLC according to previously described procedures of Tamaoka and Komagata (1984). DNA-DNA hybridization analyses (Cashion et al., 1977; De Ley et al., 1970; Huss et al., 1983) were performed by the Identification Service of the DSMZ, Braunschweig, Germany.

Results and Discussion

Characteristics of the strain

Strain OSK2A<sup>T</sup> was isolated from lotus field sediments by a roll tube method with agar as the solidifying agent, with colonies appearing red and spherical in shape. No growth was observed upon cultivation in the medium for heterotrophic anaerobes. Further microscopy observation of uniformity in cell morphology showed that strain OSK2A<sup>T</sup> was eventually purified. The original description of strain PCAT also lacked motility (Caccavo et al., 1994) and genomic information for strain PCAT indeed indicates that its flagellum system is impaired (Ueki et al., 2012). Similar to the reference strain, OSK2A<sup>T</sup> thrives in 0-1.0% NaCl and grows at an optimum temperature of 30-37°C with a growth range of 20-40°C, while the
closest relative grows at a similar optimum temperature but with a significant difference in temperature range of 10–45°C. On the other hand, strain OSK2AT grows at a pH range of 6.0–8.0, which is comparable to that of its closest relative. The lack of motility of its most closely related taxon, in contrast to OSK2AT, and the different temperature growth range observed clearly differentiate OSK2AT from PCA T as two separate type strains. All the morphological and physiological results are summarized in Table 1.

In accordance with the hallmark of all other Geo-
bacter species, strain OSK2AT thrives by coupling the reduction of Fe(III) oxides (Fe(III)-NTA, Fe(III)-citrate, and amorphous Fe(III) hydroxide) to the oxidation of acetate, coinciding with an increase in cell number and protein concentration (Fig. 2). Both the novel strain OSK2AT and the reference strain PCA T utilized the following electron donors with Fe(III)-citrate: acetate, lactate, pyruvate, and formate, while only the novel strain could utilize ethanol. The original description of strain PCA T (Caccavo et al., 1994) indicated that it did not utilize carboxylic acids; however, this study and others (Lovley, 2006; Lovley et al., 2011; Speers and Reguera, 2011) found otherwise. Acetate or ethanol as the substrate with the following electron acceptors was utilized by the novel strain OSK2AT: amorphous Fe(III) hydroxide, Fe(III)-NTA, malate, fumarate, and elemental sulfur (Table 1).

Ethanol as the distinguishing substrate showed growth when measured at OD 560 (Fig. S1), unlike the

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
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<th>4</th>
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</thead>
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<tr>
<td>Cell width (µm)</td>
<td>0.28–0.45</td>
<td>0.5</td>
<td>0.6</td>
<td>0.5</td>
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<td>Cell length (µm)</td>
<td>0.76–1.65</td>
<td>2–3</td>
<td>1.0–2.0</td>
<td>2–4</td>
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<tr>
<td>Motility</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Temperature range (°C)</td>
<td>20–40</td>
<td>10–45*</td>
<td>30 †</td>
<td>30–35 ‡</td>
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<tr>
<td>pH range</td>
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<td>5.6–8.0</td>
<td>ND</td>
<td>6.7–7.0 ‡</td>
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<td>Electron acceptor usage</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Nitrate</td>
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<td>– *</td>
<td>ND</td>
<td>+</td>
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<td>Fumarate</td>
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<td>ND</td>
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<tr>
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<td>+ *</td>
<td>ND</td>
<td>ND</td>
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<td>Elemental sulfur</td>
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<td>Propionate</td>
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<td>Butyrate</td>
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<td>– *</td>
<td>+</td>
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<td>+ *</td>
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<td>61.9 LC*</td>
<td>57.3 LC</td>
<td>56.6 TM</td>
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<td>16S rRNA gene similarity against strain OSK2AT (%)</td>
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<td>99.6</td>
<td>95.6</td>
<td>95.6</td>
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</table>

Table 1. Characteristics of strain OSK2AT differentiating it from closely related members of the genus Geobacter.

Taxa: 1, strain OSK2AT (data from this study); 2, G. sulfurreducens PCA T and DSM 12127 T (Caccavo et al., 1994, and this study); 3, G. grbiciae TACP-5 T (Coates et al., 2001); 4, G. metallireducens GS-15 T (Lovley et al., 1993). All strains utilize acetate and pyruvate as an electron donor. +, positive; –, negative; ND, no data available in the original reference. *, data from this study using DSM 12127 T; †, optimum; ‡, determined by HPLC (LC) or melting temperature (TM).
Novel subsp. of *Geobacter sulfurreducens*

Reference strain with no growth after 10 days of cultivation. Strain OSK2AT has phenotypic characteristics almost identical to those of the phylogenetically closest relative (*G. sulfurreducens* PCAT), except for ethanol, which the novel strain can utilize but which strain PCAT cannot. The majority of *Geobacter* species can utilize ethanol as a substrate for growth with concomitant reduction of Fe(III); however, PCAT does not have this ability and this seems to be indicative of the whole genome sequence of PCAT, which does not possess the genes for ethanol utilization (genes of neither NAD⁺ nor NADP⁺-dependent aldehyde dehydrogenase were found, although those of alcohol dehydrogenase exist), distancing OSK2AT from PCAT. Other comparisons of phenotypic characteristics between the novel strain and its most closely related strain are given in Table 1.

Our preliminary comparisons of current production between strain OSK2AT and PCA T in microbial fuel cells (MFC) in the presence of acetate as the substrate showed the former producing a significantly higher maximum current density than the latter, namely, 6.5 times higher (7.1 and 1.1 A/m², respectively). Previous studies conducted with an isolate named KN400 and the wild-type strain of *G. sulfurreducens* also showed similar results to this study (Yi et al., 2009). In the previous study, strain KN400 produced higher current than the wild-type PCA T strain. Furthermore, strain KN400 has 100% similarity to *G. sulfurreducens* PCAT, based on the 16S rRNA gene, but a comparison of the complete genomes of KN400 and PCA T strains showed divergence of genes involved in current production (Butler et al., 2012). Similarly, the difference in current production between OSK2AT and PCA T strains may support strain OSK2AT as a separate strain from PCAT, albeit belonging to the same species. On the other hand, although current production has not been officially recognized as a standard criterion for the taxonomic classification of *Geobacter* species, there is an increasing number of studies on their promise for current production in MFCs, and it is becoming an integral component of their physiological description (Lovley et al., 2011, and references therein).

**Respiratory quinone and fatty acid compositions**

The novel strain has MK-8 as the major respiratory quinone, as commonly reported for the genus *Geobacter* (Hedrick et al., 2009; Kunapuli et al., 2010; Lovley et al., 2011; Viulu et al., 2012). Unlike the respiratory quinones, fatty acids are specific for each strain. Strain OSK2AT contains almost the same major fatty acids (≥ 1 mol%), having just one less than its closest relative: 16 : 1 ω7c, 16 : 0, 14 : 0, 15 : 0 iso, 16 : 1 ω5c, and 18 : 1 ω7c (Table 2), in agreement with the findings of Hedrick et al. (2009). Other fatty acids absent in the novel strain but present in the closely related strain PCA T, albeit in tiny proportions (< 1 mol%), are 17 : 1 iso ω9c, 17 : 0 iso, 15 : 1 iso F, and 13 : 0 iso. On the other hand, 18 : 1 ω7c 11-methyl (< 1 mol%) is found in the novel strain but is absent in the reference strain PCA T. Thus, fatty acid composition could not clearly differentiate the novel strain from its most closely related strain PCA T; rather, the findings of

![Fig. 2. Growth of strain OSK2AT with acetate and Fe(III)-NTA as electron donor and acceptor, respectively.](image-url)
both the novel strain and the reference strain PCAT revealed distinguishable fingerprints between the two, indicating that they are indeed separate strains (Fig. 3). On the basis of all the similarities and differentiating characteristics observed between strain OSK2AT and its closest relative (strain PCAT), it is proposed that strain OSK2AT be classified as a novel subspecies of Geobacter sulfurreducens with the name Geobacter sulfurreducens subsp. ethanolicus, subsp. nov.

**Emended description of Geobacter sulfurreducens** (Caccavo et al., 1994)

The description of Geobacter sulfurreducens is based on the data from Caccavo et al. (1994), with the following modifications and additional features: utiliz-
Novel subsp. of Geobacter sulfurreducens

Description of Geobacter sulfurreducens subsp. ethanolicus, subsp. nov.

**Geobacter sulfurreducens** subsp. *ethanolicus* (etymology: e.tha.no.li.cus. N.L. n. *ethanol* ethanol; L. suff. -icus suffix used with various meanings; N.L. masc. adj. *ethanolicus* belonging to ethanol, in reference to the ability of the species to utilize ethanol as a substrate for growth).

The cells are Gram-negative, motile, rod-shaped, strictly anaerobic, 0.76–1.65 µm long, and 0.28–0.45 µm wide. Growth occurs at 20–40°C with an optimum of 30–37°C, pH 6.0–8.1 (optimum pH 7.0) and can tolerate up to 1% NaCl. Electron donors utilized in the presence of Fe(III)-citrate include H₂, ethanol, acetate, lactate, pyruvate, and formate. Other electron donors tested but not utilized are propionate, butyrate, succinate, malate, fumarate, benzoate, butanol, methanol, propanol, iso-propanol, phenol, toluene, glucose, and yeast extract. Amorphous Fe(III) hydroxide, Fe(III)-citrate, Fe(III)-NTA, fumarate, malate, and elemental sulfur are utilized as electron acceptors with either ace-
tate or ethanol as substrates, while nitrate, sulfate, sulfite, and thiosulfate are not utilized. The major respiratory quinone is MK-8. The major fatty acids are 16 : 1 ω7c, 16 : 0, 14 : 0, 15 : 0 iso, 16 : 1 ω5c, and 18 : 1 ω7c. The G+C content of the genomic DNA is 61.2 mol%.

The type strain, OSK2A\textsuperscript{T} (=DSMZ 26126\textsuperscript{T}=JCM 18752\textsuperscript{T}), was isolated from lotus field sediments in Aichi Prefecture, Japan.

Geobacter sulfurreducens subsp. sulfurreducens (Caccavo et al., 1994) is hereby created based on the description of Geobacter sulfurreducens reported by Caccavo et al. (1994).

Acknowledgments

We thank Prof. Jean P. Euzéby at École Nationale Vétérinaire de Toulouse for cross-checking the nomenclature of the strain. This work received private financial support from Mr. Kiyomi Yoshizaki and also from the JST A-step feasibility study program (AS242Z02501N).

Supplementary Materials

Fig. S1. Growth of strain OSK2A\textsuperscript{T}, observed at 30°C with ethanol and fumarate as electron donor and acceptor, respectively.

Data are presented as means of OD\textsubscript{560} in triplicate incubations and error bars represent standard deviation values.

Supplementary figures are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

References


crobioL., 60, 686–695.


