Rhizomicrobium electricum sp. nov., a facultatively anaerobic, fermentative, prosthecae bacterium isolated from a cellulose-fed microbial fuel cell

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A facultatively anaerobic, prosthecae bacterium, strain Mfc52^T, was isolated from a microbial fuel cell inoculated with soil and fed with cellulose as the sole fuel. Cells were Gram-negative, non-spore-forming, straight or slightly curved rods, and some of them had one or two polar prosthecae (stalks). Cells reproduced by binary fission or by budding from mother cells having prosthecae. Strain Mfc52^T fermented various sugars and produced lactate, acetate and fumarate. Ferric iron, nitrate, oxygen and fumarate served as electron acceptors, while sulfate and malate did not. Nitrate was reduced to nitrite. The DNA G + C content was 64.7 mol%. On the basis of 16S rRNA gene sequence phylogeny, strain Mfc52^T was affiliated with the genus *Rhizomicrobium* in the class *Alphaproteobacteria* and most closely related to *Rhizomicrobium palustre* with a sequence similarity of 97 %. Based on these physiological and phylogenetic characteristics, the name *Rhizomicrobium electricum* sp. nov. is proposed; the type strain is Mfc52^T (=JCM 15089^T =KCTC 5806^T).

Microbial fuel cells (MFCs) are devices that exploit micro-organisms to generate electric power from organic matter (Logan *et al.*, 2006; Watanabe, 2008). In our previous study, MFCs were developed with rice-paddy field soil as a source of micro-organisms and crystalline cellulose as the sole fuel, and microbial communities attaching onto graphite anodes in these MFCs were analysed (Ishii *et al.*, 2008a, b). Results showed that rod-shaped members of the class *Alphaproteobacteria* possessing prosthecae-like appendages (represented by phylotype Mfc-9) were abundantly present in anode biofilm communities (Ishii *et al.*, 2008a), suggesting that they may have played important roles in the cellulose/electricity conversion in these MFCs. In order to investigate their ecological roles, we isolated these bacterial strains (including strain Mfc52^T) and examined their ability to generate electric currents (Kodama & Watanabe, 2008). It was revealed that strain Mfc52^T generated electricity in pure-culture MFCs by oxidizing intermediate metabolites (e.g. lactate and acetate) from cellulose degradation, and most cells of strain Mfc52^T had long prosthecae similar to those observed in scanning electron micrographs of the anode biofilm in the cellulose-fed MFC (Kodama & Watanabe, 2008). Strain Mfc52^T is the first isolate of prosthecae

bacteria with the ability to generate electricity in MFCs. In our phylogenetic analysis (Kodama & Watanabe, 2008), strain Mfc52^T was deeply branched within the class *Alphaproteobacteria* and closely related to *Rhizomicrobium palustre* A48^T, a facultatively anaerobic, fermentative stalked bacterium isolated from rice plant roots (Ueki *et al.*, 2010). In the present study, we performed further physiological and taxonomic analyses of strain Mfc52^T. Based on the results, we propose that strain Mfc52^T represents a novel species in the genus *Rhizomicrobium*.

Routine cultivation of strain Mfc52^T was done without shaking at 30 °C in 1/10 PYGV medium (Kodama & Watanabe, 2008) supplemented with 1 g glucose l⁻¹ under oxygen-free N₂ gas in culture bottles capped with Teflon-coated butyl rubber septa and sealed with aluminium crimps. Growth in liquid medium was monitored by measuring optical density at 660 nm (OD₆₆₀) using a spectrophotometer (U-1100, Hitachi), and cell concentration was determined by directly counting 4', 6-diamidino-2-phenylindole (DAPI)-stained cells under an epifluorescence microscope as described elsewhere (Kodama & Watanabe, 2003). Cells of Mfc52^T were stored at -80 °C in liquid 1/10 PYGV medium supplemented with 15 % (v/v) glycerol.

Cell morphology was examined by transmission electron microscopy (Beveridge *et al.*, 1994). Cells were fixed with glutaraldehyde (2.5 %, w/v), negatively stained with

Abbreviation: MFCs, microbial fuel cells.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Mfc52^T is AB365487.

phosphotungstic acid (0.2 %, w/v), and visualized using an H-7000 transmission electron microscope (Hitachi). Motility was checked by phase-contrast microscopy (BX60, Olympus). Gram staining and oxidase/catalase tests were conducted according to standard procedures (Smibert & Krieg, 1994). The presence of heat-tolerant cells was examined as described by Ueki *et al.* (2010).

Physiological properties of strain Mfc52^T were analysed using 10 ml 1/10 PYGV medium (without Na₂CO₃, pH 7.0) or 10 ml BT medium (Kodama & Watanabe, 2008) in test tubes (30 ml capacity) capped with screw caps tightened with butyl rubber stoppers under oxygen-free N₂ gas unless otherwise specified. All following tests were run at least in duplicate. Effects of temperature, pH and salinity (NaCl concentration) on bacterial growth were examined in 1/10 PYGV medium supplemented with 1 g glucose l⁻¹. Aerobic growth was examined on nutrient agar (Difco) and 1/10 PYGV agar plates without cysteine and Na₂CO₃. Fermentative growth was examined in BT medium with substrates added at 10 g l⁻¹ (for sugars and sugar alcohols) or 20 mM (organic acids, amino acids and alcohols). Utilization of electron acceptors was tested in BT medium supplemented with 1 g glucose l⁻¹ as sole carbon source and Na₂SO₄, NaNO₃, ferric citrate, sodium fumarate, sodium DL-malate (each at 10 mM) or O₂ (head-space N₂ gas replaced with air) as sole electron acceptor. The Fe(III)-reducing ability of strain Mfc52^T was examined in BT medium in the presence of 10 mM ferric citrate with glucose (2 g l⁻¹), acetate, pyruvate, lactate, succinate, propionate, formate, ethanol (each at 10 mM) or H₂ [head-space replaced with H₂/CO₂ (80/20 %) gas] as the sole electron donor. Organic acids were analysed by high-performance liquid chromatography (HPLC) as described by Ishii *et al.* (2008a). Glucose concentration was determined using F-kit (R-Biopharm) according to the manufacturer's instructions. H₂, CO₂ and O₂ in the head space were measured by gas chromatography as described elsewhere (Ishii *et al.*, 2005). Concentrations of nitrate, nitrite and sulfate were determined by using an ion chromatograph (ICA-2000, DKK-TOA) equipped with an electrolytic conductivity detector and a spectrophotometer. Ferrous iron concentration was determined by the ferrozine method (Stookey, 1970).

Cellular fatty acids and quinones were analysed by TechnoSuruga Laboratory, Shimizu, Japan. The DNA G+C content was determined by an HPLC method according to Katayama-Fujimura *et al.* (1984). The almost-full-length 16S rRNA gene was sequenced previously (Kodama & Watanabe, 2008). A search of the GenBank database was carried out by using the BLAST program (Altschul *et al.*, 1990). Multiple alignments of the sequences were conducted using the CLUSTAL W program (Thompson *et al.*, 1994). A phylogenetic tree was reconstructed by the neighbour-joining method (Saitou & Nei, 1987) using the MEGA4 program (Tamura *et al.*, 2007). Positions where any sequence had a gap or ambiguous nucleotide were not included in the calculation.

Colonies of strain Mfc52^T on 1/10 PYGV and 1/10 GA agar plates (Kodama & Watanabe, 2008) were translucent and thin with smooth surfaces. Cells were Gram-negative, straight or slightly curved rods (0.9–2.5 µm long and 0.2–0.3 µm wide). They occurred singly or in pairs connected by constrictions, and some of them had one or two polar prosthecae (Fig. 1). Sizes of prosthecae were up to 3 µm in length and 80 to 110 nm in diameter. Some prosthecae had branched structures at the tips (Fig. 1a). Cells reproduced by binary fission or by budding from mother cells with prosthecae. They were motile by means of single polar flagella. Catalase and oxidase activity were not detected. Strain Mfc52^T did not produce heat-tolerant cells. Growth of strain Mfc52^T was observed between 15 °C and 40 °C, with optimum growth at 30 °C, and between pH 5.0 and 7.5 with optimum growth at pH 6.7. Strain Mfc52^T grew with 0 to 1.0 % (w/v) NaCl, optimum 0.5 % (w/v) NaCl. Aerobic growth was observed on agar plates. Strain Mfc52^T also grew fermentatively (see species description). Major fermentation products from glucose were lactate (4.5 mmol l⁻¹), acetate (3.6 mmol l⁻¹) and fumarate (3.4 mmol l⁻¹), and small amounts of CO₂ and H₂ were also produced. We found that oxygen, nitrate, fumarate and Fe(III) were utilized as electron acceptors in the presence of glucose, while sulfate and malate were not. Nitrite was the final product of nitrate reduction. Fe(III) was not reduced with acetate, lactate, succinate, pyruvate, formate, propionate, ethanol or H₂ as sole electron donor. Strain Mfc52^T generated electricity by oxidizing organic acids (lactate and acetate) in an MFC reactor (Kodama & Watanabe, 2008).

The fatty acids (>0.1 %) of strain Mfc52^T were C_{18:1}ω7c (47.3 %), anteiso-C_{15:0} (14.0 %), C_{16:0} (8.6 %), iso-C_{15:0} (7.6 %), C_{16:0} 3-OH (3.0 %), C_{14:0} (2.7 %), 11-methyl C_{18:1}ω7c (2.7 %), C_{18:0} (2.6 %), iso-C_{14:0} (2.2 %), iso-C_{17:0} 3-OH (1.2 %), C_{17:0} 2-OH (1.1 %), iso-C_{15:0} 2-OH and/or C_{16:1}ω7c (0.8 %), iso-C_{17:0} (0.7 %), C_{18:1}ω9c (0.7 %), iso-C_{16:0} 3-OH (0.6 %), iso-C_{16:1} I and/or C_{14:0} 3-OH (0.6 %), anteiso-C_{17:0} (0.5 %), iso-C_{16:0} (0.5 %), iso-C_{15:0} 3-OH (0.5 %), C_{13:1} AT 12-13 (0.4 %), C_{20:1}ω7c

Fig. 1. Transmission electron micrographs of strain Mfc52^T cultivated for 4 days on 1/10 PYGV medium, showing a polar prostheca with a tip-branched structure (a) and the development of two polar prosthecae (b). Bars, 1 µm.

(0.4 %), 10-methyl $C_{19:0}$ (0.3 %), $C_{18:0}$ 3-OH (0.3 %), 10-methyl $C_{16:0}$ (0.3 %), $C_{18:1}$ 2-OH (0.3 %) and $C_{17:0}$ (0.2 %). The predominant respiratory quinone was ubiquinone Q-10. The genomic DNA G + C content of strain Mfc52^T was 64.7 mol%.

A database search based on the 16S rRNA gene sequence of strain Mfc52^T revealed that it was most closely related to environmental clone RB521 (97.8 % sequence similarity) that was retrieved from rhizosphere biofilm in a reed bed reactor. The second closest relative was *Rhizomicrobium palustre* A48^T (97.3 %), isolated from roots of rice plants grown in flooded rice field soil (Ueki *et al.*, 2010). Other sequences in the databases were not highly similar; e.g. the third and fourth closest relatives were *Pleomorphomonas oryzae* strain DSM 16300^T (87.8 %), a nitrogen-fixing bacterium isolated from rice (Xie & Yokota, 2005) and *Methylocystis parvus* NCIMB 11129^T (87.6 %), a methanotroph belonging to the family *Methylocystaceae* in the order *Rhizobiales* (Bowman *et al.*, 1993). 16S rRNA gene sequence similarities with other bacteria in the class *Alphaproteobacteria* were less than 85 %; e.g. *Sphingomonas paucimobilis* ATCC 29837^T (84.9 %), *Caulobacter vibrioides* DSM 9893^T (84.6 %), *Kordiimonas gwangyangensis* JCM 12864^T (84.7 %) and *Hyphomonas polymorpha* DSM 2665^T (81.3 %). Strain Mfc52^T and *R. palustre* A48^T clustered together to form a distinct branch within the class *Alphaproteobacteria* (Fig. 2). They were most closely related to the order *Rhizobiales* but seemed not to be affiliated with any established order in the class *Alphaproteobacteria*. We also found that some important characteristics of strains A48^T and Mfc52^T were different from those of the related genera *Pleomorphomonas* and *Methylocystis* in the order *Rhizobiales*; for instance, members of these genera are aerobic and do not have prostheca (Xie & Yokota, 2005; Bowman *et al.*, 1993). Further studies will be

needed to identify whether a novel order should be proposed for strains A48^T and Mfc52^T.

R. palustre A48^T was isolated from rice plant roots (Ueki *et al.*, 2010), and closely related sequences were also retrieved from the anode of a sediment MFC submerged in a rice rhizosphere (Kaku *et al.*, 2008), suggesting that the bacterial group represented by strain A48^T (*R. palustre*) thrives in rhizospheres. In contrast, strain Mfc52^T was isolated from an MFC bioreactor (Kodama & Watanabe, 2008). In addition to their habitats, we also found several distinct features that could clearly differentiate strain Mfc52^T from *R. palustre* (Table 1); these features include the number and tip structure of prosthecae, DNA G + C content, abilities to utilize nitrate and fumarate as electron acceptors, sugar-utilization pattern, fermentative products from glucose, $C_{18:1\omega7c}$ content and minor fatty-acid composition. From these results and the 16S rRNA gene sequence comparison, strain Mfc52^T is considered to represent a novel species in the genus *Rhizomicrobium*, for which the name *Rhizomicrobium electricum* sp. nov. is proposed.

Description of *Rhizomicrobium electricum* sp. nov.

Rhizomicrobium electricum [e.lec'tri.cum. L. n. *electrum* amber; L. suff. *-icus -a -um* suffix used with the sense of pertaining to; N.L. neut. adj. *electricum* referring to generation of electricity (a physical force so called because it first was generated by rubbing amber)].

Cells are Gram-negative, non-spore-forming, straight or slightly curved rods, 0.9–2.5 µm long and 0.2–0.3 µm wide. Some cells have one or two polar prosthecae with tip-branched structures. Prosthecae are ~3 µm in length and 80–110 nm in diameter. Cells reproduce by budding from

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing the position of strain Mfc52^T in the class *Alphaproteobacteria*. *Escherichia coli* was used as the outgroup. Accession numbers of sequences retrieved from the databases are given in parentheses. Numbers at branch nodes are bootstrap values (per 100 trials); only values greater than 50 are shown. Bar, 0.02 substitutions per site.

Table 1. Differences in characteristics of strain Mfc52^T and *R. palustre* A48^T

+, Positive; –, negative; A, acetate; E, ethanol; F, fumarate; L, lactate.

Characteristic	Mfc52 ^T	<i>R. palustre</i> A48 ^T *
Tip structure of prosthecae	Straight or branched	Straight or holdfast-like
Number of prosthecae	One or two	One
DNA G + C content (mol%)	64.7	55.4
Anaerobic growth on glucose by reduction of:		
Nitrate	+	–
Fumarate	+	–
Fermentative growth on:		
Ribose	+	–
Mannose	+	–
Trehalose	+	–
Raffinose	+	–
Pyruvate	+	–
Xylan	–	+
Fermentation products (mol l ⁻¹)	L (4.5), A (3.6), F (3.4)†	L (10.4), E (5.1), A (4.1)†
Major fatty acids (%)	C _{18:1ω7c} (47.3), anteiso-C _{15:0} (14.0), C _{16:0} (8.6)	C _{18:1ω7c} (29.4), anteiso-C _{15:0} (16.2), C _{16:0} (13.2)

*Data from Ueki *et al.*, 2010.†Small amounts of CO₂ and H₂ were also produced.

a mother cell having prosthecae. Motile by single polar flagella. Catalase- and oxidase-negative. Facultatively anaerobic. The optimum growth temperature is 30 °C (range 15–40 °C), the optimum pH is 6.7 (range 5.0–7.5), and the optimum NaCl concentration for growth is 0.5 % (w/v) (range 0–1.0 %, w/v). Aerobic growth is observed on nutrient agar and 1/10 PYGV agar plates. Utilizes glucose, galactose, arabinose, xylose, cellobiose, maltose, lactose, sucrose and soluble starch as fermentative substrates. Slow growth (lag phase continues for 10 days or longer) is observed on rhamnose, raffinose, mannose, fructose, ribose, trehalose and pyruvate. Major fermentation products of glucose are lactate, acetate and fumarate. Does not utilize lactate, malate, fumarate, succinate, citrate, glycine, serine, alanine, glutamate, aspartate, xylan, sorbose, cellulose, glycerol, inositol, mannitol, sorbitol, ethanol, methanol or propanol. Utilizes oxygen, nitrate, Fe(III) and fumarate as electron acceptors in the presence of glucose. Nitrate is reduced to nitrite. Sulfate and malate are not utilized as electron acceptors. The major cellular fatty acids are C_{18:1ω7c}, anteiso-C_{15:0}, C_{16:0} and iso-C_{15:0}. The predominant respiratory quinone is ubiquinone Q-10. The genomic DNA G + C content of the type strain is 64.7 mol%.

The type strain, Mfc52^T (=JCM 15089^T =KCTC 5806^T), was isolated from biofilm attached to anode graphite of a cellulose-fed microbial fuel cell.

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