Full Paper

Hydrogenophaga electricum sp. nov., isolated from anodic biofilms of an acetate-fed microbial fuel cell

Zen-ichiro Kimura and Satoshi Okabe*

Division of Environmental Engineering, Faculty of Engineering,
Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8628, Japan

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A Gram-negative, non-spore-forming, rod-shaped bacterial strain, AR20^T, was isolated from anodic biofilms of an acetate-fed microbial fuel cell in Japan and subjected to a polyphasic taxonomic study. Strain AR20^T grew optimally at pH 7.0–8.0 and 25°C. It contained Q-8 as the predominant ubiquinone and C_{16:0}, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0}2OH), and C_{18:1}ω7c as the major fatty acids. The DNA G+C content was 67.1 mol%. A neighbor-joining phylogenetic tree revealed that strain AR20^T clustered with three type strains of the genus *Hydrogenophaga* (*H. flava*, *H. bisanensis* and *H. pseudoflava*). Strain AR20^T exhibited 16S rRNA gene sequence similarity values of 95.8–97.7% to the type strains of the genus *Hydrogenophaga*. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain AR20^T is considered a novel species of the genus *Hydrogenophaga*, for which the name *Hydrogenophaga electricum* sp. nov. is proposed. The type strain is AR20^T (= KCTC 32195^T = NBRC 109341^T).

Key Words—*Hydrogenophaga electricum*; hydrogenotrophic exoelectrogen; microbial fuel cell

Introduction

Microbial fuel cells (MFCs) are devices that are able to directly convert the chemical energy of organic compounds into electric energy with microorganisms (Bullen et al., 2006; Davis and Higson, 2007; Kim et al., 1999). Electricity generation in a mediator-less MFC is linked to the ability of certain bacteria, called exoelectrogens, to transfer electrons outside the cell to the anode electrode in the MFC (Logan, 2006). In our previous study, a MFC fed with acetate was constructed with activated sludge as a source of microorganisms, and the microbial community of the anodic biofilm in

the MFC was analyzed. Results showed that bacteria belonging to the genera *Geobacter* and *Hydrogenophaga* were abundantly present in the anodic biofilm community (Kimura and Okabe, 2013). We isolated a bacterium closely related to the genus *Hydrogenophaga* (*H. electricum* strain AR20^T).

The genus *Hydrogenophaga* was proposed by Willemse et al. (1989) with the reclassification of *Pseudomonas* species as *Hydrogenophaga flava*, *H. palleronii*, *H. pseudoflava* and *H. taeniospiralis*. Subsequently, five other species, *Hydrogenophaga intermedia* (Contzen et al., 2000), *H. atypical*, *H. defluvii* (Kämpfer et al., 2005), *H. caeni* (Chung et al., 2007) and *H. bisanensis* (Yoon et al., 2008) have been described. Phylogenetic analysis based on 16S rRNA gene sequences showed that the genus *Hydrogenophaga* falls within the family *Comamonadaceae*, class *Betaproteobacteria* (Anzai et al., 2000; Kämpfer et al., 2005). In this paper, the results of an examination of the phenotypic characteristics of strain AR20^T are described, along with the phy-

* Corresponding author: Satoshi Okabe, Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, North-13, West-8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan.

Tel & Fax: +81-(0)11-706-6266

E-mail: sokabe@eng.hokudai.ac.jp

logenetic placement of the strain. The name *Hydrogenophaga electricum* sp. nov. is proposed with strain AR20^T as the type strain.

Materials and Methods

Test strain and cultivation. Strain AR20^T was isolated by means of the standard dilution plating technique on R2A agar (Difco, Japan BD, Tokyo, Japan) at 25°C. The morphological, physiological and biochemical characteristics of strain AR20^T were investigated by using standard cultivation techniques at 25°C. Cell morphology was examined by transmission electron microscopy (Beveridge et al., 1999). Cells were fixed with glutaraldehyde (2.5%, w/v), negatively stained with phosphotungstic acid (0.2%, w/v), and visualized using a JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan).

Growth at various temperatures (4–50°C) was measured. The pH range for growth was determined in R2A broth that was adjusted to various pH values (pH 4.5–10.5 at intervals of 0.5 pH units) by the addition of HCl and Na₂CO₃. Growth in the various NaCl concentrations (0–5.0% (w/v) at intervals of 1.0%) was investigated by using R2A medium.

Growth under anaerobic conditions was determined after incubation in an anaerobic chamber in R2A broth supplemented with potassium nitrate and nitrite (0.1%, w/v), both of which had been prepared anaerobically under a nitrogen atmosphere. Nitrate and nitrite reduction were studied as described by Lanyi (1988). Electrochemical activity of the AR20^T was evaluated using H-type MFCs that consisted of 500 ml anode and cathode glass chambers separated by a proton exchange membrane (PEM, NafionTM 117, Dupont Co., Fayetteville, NC, USA) (Chung et al., 2011). The anode chamber was filled with 500 ml of a sterilized mineral medium (Chung and Okabe, 2009b) containing H₂ gas as the sole electron donor and a carbon electrode as the sole electron acceptor. The cathode chamber was filled with 500 ml of phosphate buffer (80 mM and pH 7.1) containing 50 mM ferricyanide. The H-type MFCs were operated at 25 ± 2°C. Utilization of thiosulfate was tested in R2A broth supplemented with 10 mM Na₂S₂O₃ · 5H₂O as described by Spring et al. (2004). The concentrations of nitrate, nitrite and thiosulfate in R2A broth were measured by ion chromatograph (ICS; Dionex, Osaka, Japan). Chemolithoautotrophic growth of strain AR20^T with hydrogen gas was tested on a liq-

uid medium (Chung and Okabe, 2009a) under the conditions described by Malik and Schlegel (1981). Utilization of various substrates, enzyme activities and other physiological and biochemical properties were tested by using the API 20NE and API 50CH (bioMérieux, Marcy l'Etoile, France); utilization of various substrates was determined by inoculating the API 50CH strip with cells suspended in AUX medium (bioMérieux). Utilization of various substrates was also tested as described by Kämpfer et al. (1991).

Minimum inhibitory concentrations (MIC) were determined in R2A broth containing the following antibiotics: chloramphenicol, ampicillin, tetracycline, kanamycin and neomycin.

Chemotaxonomic analyses. Total cellular fatty acid analysis of cells grown on YPG agar (Japan BD, Tokyo, Japan) for 48 h at 30°C was performed by Gas Liquid Chromatography (GLC) according to the instructions of the Microbial Identification System (MIDI) Sherlock version 6.0 (Sasser, 1990) with the RTSBA6 MIDI database (MIDI Inc., Newark, DE, USA). Quinone was extracted with a chloroform-methanol mixture, purified by thin layer chromatography and analyzed by reversed-phase HPLC (L-7000, Hitachi, Tokyo, Japan) as described previously (Hiraishi, 1988).

16S rRNA gene sequencing and phylogenetic analysis. The 16S rRNA gene of strain AR20^T was PCR amplified, purified and sequenced directly with an automated DNA sequencer (ABI PRISM 3100-Avant Genetic Analyzer; Life Technologies, Grand Island, NY, USA). Sequence data were compiled with the BioEdit program. Multiple alignment of sequence data, calculation of the corrected evolutionary distance (Kimura, 1980), and construction of neighbor-joining phylogenetic tree (Saitou and Nei, 1987) were performed using the CLUSTAL W program ver. 1.83 (Thompson et al., 1994). The topology of the tree was evaluated by bootstrapping with 1,000 resamplings (Felsenstein, 1985).

DNA base composition. Genomic DNA was extracted and purified according to the method of Marmur (1961). The guanine plus cytosine (G+C) ratio of genomic DNA was determined by the HPLC/PDA method with external nucleotide standards as described by Mesbah (1989). DNA-DNA hybridization were performed using the quantitative dot-blot hybridization with the Alkphos Direct Labelling and Detection System with CDP-star (GE Healthcare Japan, Tokyo, Japan) as described previously (Kubota et al., 2005).

Results and Discussion

Morphology and cultural characteristics

Morphological, cultural, physiological and biochemical characteristics of strain AR20^T are shown in Fig. 1, Fig. 2 and Table 1. The cells were Gram-negative, non-spore-forming, motile, and rod-shaped organisms. Strain AR20^T grew under anoxic conditions in R2A broth with nitrate or nitrite as a terminal electron acceptor. This strain grew well in a temperature range from 20 to 30°C, but did not grow at 4°C on R2A agar or YPG agar (Difco) (Table 1). Strain AR20^T could generate an electrical current via oxidation of hydrogen in a pure culture MFC (Fig. 2). Although there are only a

Fig. 1. Transmission electron microscopic (TEM) image of the cell morphology of the isolated strain AR20^T.

few reports on the presence and ability of hydrogenotrophic exoelectrogens (Chung et al., 2009b; Pham et al., 2003), strain AR20^T has the highest ability to generate current to date. The cultural, physiological and biochemical characteristics of strain AR20^T are apparently different from all other nine *Hydrogenophaga* species and *Xenophilus azovorans* strain KF46F^T, for example

Fig. 2. Characterization of the electrochemical activity of strain AR20^T in a two-chamber MFC.

Change in power density was shown when hydrogen was used as the sole electron donor without any other dissolved electron acceptors. Hydrogen gas (100% H₂) was bubbled directly into the anode culture fluid for 10 min as indicated by arrows. Hydrogen-dependent power generation by strain AR20 is shown.

Table 1. Comparison of characteristics of strain AR20 and some related species.

Characteristics	1	2	3	4	5	6	7	8	9	10	11
Optimum temperature	25°C	30°C	35–38°C	30–37°C	25°C	25°C	30°C	33–37°C	30°C	30°C	30°C
Denitrification	+	–	+	+	–	–	–	+	+	+	–
Nitrite reduction	+	+	+	+	+	+	–	+	+	+	–
Colony pigmentation	–	+	+	+	+	+	+	+	–	–	+
Chemolithotrophic growth with H ₂	+	+	+	–	+	–	–	+	–	–	ND
Oxidation of thiosulfate to sulfate	–	–	–	+	–	–	+	–	+	+	–
Utilization of											
Acetate	w	+	w	–	–	–	–	–	+	+	+
L-Arabinose	–	+	+	–	–	–	+	–	–	–	–
D-Fructose	–	+	+	+	–	–	+	–	–	–	+
Sorbitol	–	+	+	–	–	–	+	–	+	+	–
Mannitol	–	+	+	–	–	–	–	+	–	–	+
Maltose	–	+	+	–	–	–	–	–	+	+	–
L-Histidine	–	+	+	–	+	–	–	–	+	+	+
Major quinone	UQ-8	UQ-8	UQ-8	UQ-8	UQ-8	UQ-8	UQ-8	UQ-8	UQ-8	UQ-8	UQ-8
G+C content (mol%)	67.1	66.7	66.0	64.8	65.0	64.0	67.3	64.8	61.6	61.6	70.4

Strains: 1, *H. electricum* AR20^T; 2, *H. flava* CCUG 1658^T; 3, *H. pseudoflava* ATCC 33668^T; 4, *H. bisanensis* K102^T; 5, *H. defluvii* BSB 9.5^T; 6, *H. atypica* BSB 41.8^T; 7, *H. palleronii* CCUG 20334^T; 8, *H. taeniospiralis* ATCC 49743^T; 9, *H. intermedia* S1^T; 10, *H. caeni* EMB71^T; 11, *Xenophilus azovorans* KF46F^T. +, positive; –, negative; w, weakly positive; ND, not determined.

The phylogenetic tree was constructed based on available 16S rRNA gene sequences. The nodes supported by a bootstrap value of 80% were marked with solid circles. The scale bar represents 2.0% sequence divergence.

20NE and API 50CH systems, adipate is utilized, but glycerol, glucose, gluconate, caprate, malate, citrate, phenylacetate, erythritol, D-arabinose, ribose, L-xylose, adonitol, methyl β -D-xyloside, sorbose, rhamnose, dulcitol, inositol, methyl α -D-mannoside, methyl α -D-glucoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, lactose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, 2-ketogluconate and 5-ketogluconate are not utilized. Strain AR20^T is susceptible to chloramphenicol, ampicillin, tetracycline, kanamycin, and neomycin.

The predominant ubiquinone is Q-8. The major components of fatty acid (10% of the total) are C_{16:0}, C_{18:1 ω 7c} and summed feature 3 (C_{16:1 ω 7c} and/or iso-C_{15:0}2OH), minor components (0.1% of the total) are C_{14:0}, C_{18:0}, C_{16:1 ω 5c}, and 3-OH fatty acids are not included.

The DNA G+C content is 67.1 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1. The type strain, AR20^T (= KCTC 32195^T = NBRC 109341^T), was isolated from anodic biofilms of a microbial fuel cell that was fed with acetate as an electron donor at Hokkaido University, Sapporo, Hokkaido, Japan.

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