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ORIGINAL ARTICLE

Acetate oxidation by syntrophic association between *Geobacter sulfurreducens* and a hydrogen-utilizing exoelectrogen

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Anodic microbial communities in acetate-fed microbial fuel cells (MFCs) were analyzed using stable-isotope probing of 16S rRNA genes followed by denaturing gradient gel electrophoresis. The results revealed that *Geobacter sulfurreducens* and *Hydrogenophaga* sp. predominated in the anodic biofilm. Although the predominance of *Geobacter* sp. as acetoclastic exoelectrogens in acetate-fed MFC systems has been often reported, the ecophysiological role of *Hydrogenophaga* sp. is unknown. Therefore, we isolated and characterized a bacterium closely related to *Hydrogenophaga* sp. (designated strain AR20). The newly isolated strain AR20 could use molecular hydrogen (H_2), but not acetate, with carbon electrode as the electron acceptor, indicating that the strain AR20 was a hydrogenotrophic exoelectrogen. This evidence raises a hypothesis that acetate was oxidized by *G. sulfurreducens* in syntrophic cooperation with the strain AR20 as a hydrogen-consuming partner in the acetate-fed MFC. To prove this hypothesis, *G. sulfurreducens* strain PCA was cocultivated with the strain AR20 in the acetate-fed MFC without any dissolved electron acceptors. In the coculture MFC of *G. sulfurreducens* and strain AR20, current generation and acetate degradation were the highest, and the growth of strain AR20 was observed. No current generation, acetate degradation and cell growth occurred in the strain AR20 pure culture MFC. These results show for the first time that *G. sulfurreducens* can oxidize acetate in syntrophic cooperation with the isolated *Hydrogenophaga* sp. strain AR20, with electrode as the electron acceptor.
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Introduction

In microbial fuel cells (MFCs), microorganisms (exoelectrogens) in the anode directly convert organic matter to electricity. Anodic microbial community structure is dependent on the operational conditions such as the substrates (Jung and Regan, 2007; Kan *et al.*, 2011) and inoculums (Holmes *et al.*, 2004; Ieropoulos *et al.*, 2010; Yates *et al.*, 2012). The substantial phylogenetic diversity with predominance of well-known electrochemically active bacteria such as *Geobacter* and *Shewanella* species has been frequently reported for MFCs fed with various substrates (for example, acetate, butyrate and glucose) (Jung and Regan, 2007; Kim *et al.*, 2008; Kan *et al.*, 2011; Yates *et al.*, 2012). It has been recognized that *Geobacter* species are acetoclastic exoelectrogens and play a key role in the power generation in MFCs, as *Geobacter* species can directly transfer electrons to electrode via the outer membrane c-type cytochromes and pili (known as microbial nanowires) (Lovley, 2012). *Geobacter* is also capable of direct interspecies electron transfer (DIET) within anaerobic microbial communities (Summers *et al.*, 2010), which may be more effective than interspecies electron transfer via H_2 and formate (Cord-Ruwisch *et al.*, 1998).

When *Geobacter* grow in MFCs where electron acceptor is likely limited (without any dissolved electron acceptors, except for solid anode electrode), they tend to accumulate hydrogen with fatty acids as the electron donor (Cord-Ruwisch *et al.*, 1998). Especially in MFCs fed with acetate as the substrate, *Geobacter*, however, cannot ferment acetate (transfer electrons to protons to form hydrogen as a fermentation product) because the overall reaction ($CH_3COO^- + H^+ + 2H_2O \rightarrow 2CO_2 + 4H_2$) is endergonic under standard thermodynamic conditions. This reaction proceeds only if hydrogen partial pressures are kept low by coupling with hydrogen-consuming partners. Thus, *Geobacter* needs to form syntrophic associations with other microorganisms

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that can utilize hydrogen, which may result in a variety of complex microbial communities. It has been recognized that *Geobacter sulfurreducens* strain PCA (Caccavo *et al.*, 1994) can oxidize acetate in syntrophic cooperation only with *Wolinella succinogenes* or *Desulfovibrio desulfuricans*, with nitrate as the electron acceptor, but not with hydrogenotrophic methanogenic partner (Cord-Ruwisch *et al.*, 1998). These nitrate- and sulfate-reducing bacteria are only the suitable H_2-consuming partners of *G. sulfurreducens* to date. Recently, it has been reported that *Geobacter* species could grow and facilitate methanogenesis under syntrophic association with methanogens (that is, *Methanosarcina*) via DIET with conductive granular-activated carbon (Liu *et al.*, 2012) and (semi)conductive iron-oxide minerals (Kato *et al.*, 2012) as the mediator.

The considerable phylogenetic diversity found in anodic microbial communities in acetate-fed MFCs may suggest that the existence of other candidates besides well-characterized *Geobacter* species contribute to power generation through various ways. However, the functional significance of the associated microbial community members is still unclear.

In this study, anodic microbial communities in acetate-fed MFCs were, therefore, analyzed to investigate the identity and functions of microbial community members associated with the dominated *Geobacter* species. We successfully identified, isolated and characterized one of the dominant bacteria closely related to *Hydrogenophaga* sp. (designated strain AR20) from an acetate-fed MFC. The newly isolated strain AR20 was found to be a hydrogen-utilizing exoelectrogen. To investigate the functional significance of the strain AR20, the strain AR20 was cocultivated with *G. sulfurreducens* strain PCA in the acetate-fed MFC without any dissolved electron acceptors. We report for the first time that *G. sulfurreducens* can oxidize acetate in syntrophic cooperation with a hydrogenotrophic exoelectrogen, *Hydrogenophaga* sp. strain AR20, with current generation.

Materials and methods

MFC configuration, inoculation and electrochemical monitoring

Two two-chamber H-type MFCs were constructed using two glass bottles (each 500 ml in capacity) connected with a glass tubing (φ20 mm) and a pinch-clump assembly. Two chambers were separated by a proton exchange membrane (Nafion 117; DuPont Co., Wilmington, DE, USA) with a cross-sectional area of 3.14 cm^2. Anodes were made from carbon cloth (BASF Japan Ltd., Tokyo, Japan, $2.5 \times 5.0\,cm^2$, 1 mm thick, $255\,g\,m^{-2}$ weight, 30 wt% wet-proofing) and cathodes were made carbon cloth (BASF, $2.5 \times 5.0\,cm^2$, 1 mm thick, $255\,g\,m^{-2}$ weight) containing $4.0\,mg\,cm^{-2}$ of platinum (using 80% Pt on Vulcan XC-72). The cathodic electrolyte was phosphate buffer (80 mM and pH 7.0) and replaced once a week (Chung *et al.*, 2010). The air was continuously supplied to the phosphate buffer during the operation. The MFCs were continuously fed with a synthetic medium consisting of 20 mM sodium acetate as the sole energy source, 0.01% vitamin solution and 0.01% trace element solution in a base medium (Roh and Moon, 2001) at a hydraulic retention time of 2 days and kept at $25 \pm 2\,^{\circ}C$. One MFC was operated in an open-circuit mode (that is, without an external circuit) to determine the effect of open circuit on the microbial community. The MFCs were inoculated with biomass from a two-chamber MFC that has been continuously operated for more than 200 days with acetate as the sole energy source.

Voltage (E) and current (I) were measured every 3 min across an external resistor ($R = 100\,\Omega$) using a multimeter connected to a data acquisition system (Agilent HP 34970, Agilent-technologies Inc, Santa Clara, CA, USA). Power ($P = IE$) and current were normalized by the anode projected surface area (12.5 cm^2). The Coulombic efficiency (CE) was determined based on the total current generation and change in acetate concentrations in the reactor influent and effluent as described previously (Liu and Logan, 2004; Chung and Okabe, 2009a).

Microbial community analysis by DNA-SIP

For DNA stable-isotope probing (DNA-SIP) analysis, the MFCs were continuously fed with the synthetic medium containing ^{12}C-labeled acetate (20 mM) for 13 days. The medium feeding was ceased on the 13th day, and then ^{13}C-labeled acetate solution ($^{13}CH_3^{13}COONa$, >99% ^{13}C; Wako, Saitama, Japan) was added to the anode chambers, resulting in final concentrations of 14 mM and remaining ^{12}C-labeled acetate (6 mM). The external circuit of one MFC was opened. The MFCs were operated in a batch mode for 5 days for DNA-SIP analysis. Anodic biofilm samples (ca. 0.1 g (wet weight) of anode electrode with biofilms) and the anode culture fluid (1 ml) were collected after 8 and 85 h, respectively. The anode electrode samples were cut with a flame-sterilized scissors.

Total DNA (light and heavy DNA) was extracted from each sample using a Fast DNA spin kit (Bio 101; Qbiogene Inc., Carlsbad, CA, USA) as described in the manufacturer's instruction. DNA gradients were prepared as described by Neufeld *et al.* (2007) and Ito *et al.* (2011). Briefly, 1 g of cesium chloride (CsCl) was added to 2 ml of the extracted DNA (5 μg), and 100 μl of ethidium bromide ($10\,mg\,ml^{-1}$) was added to the DNA + CsCl solution in an ultracentrifuge tube ($11 \times 32\,mm^2$, Quick-Seal PA tube; Beckman Coulter, Fullerton, CA, USA). Heavy and light DNAs were separated by centrifugation with Optima TLX (Beckman Coulter, Inc., Brea, CA, USA) at 201 458 *g* (68 000 r.p.m. using a Beckman rotor TLA-120.2 (Beckman Coulter, Fullerton, CA, USA)) for 36 h at 20 °C (Ito *et al.*, 2011). Centrifuged gradients were fractionated into 19 fractions (100 μl each fraction) with a fraction recovery system

(Beckman Coulter) at a flow rate of 200 μl min^{-1} by displacement with dH_2O using a syringe pump (Neufeld *et al.*, 2007). The amount of DNA of each fraction was quantified fluorometrically by RiboGreen assay (Invitrogen, Carlsbad, CA, USA), showing that the heavy ^{13}C-labeled DNA was enriched in the fraction no. 10. The heavy ^{13}C-labeled DNA was retrieved from the fraction no. 10. Ethidium bromide was extracted from the retrieved DNA with an equal volume of chloroform (repeated two times), and DNA was precipitated for 2 h at room temperature with two volumes of polyethylene glycol solution (30% polyethylene glycol, 1.6 M NaCl) and 3 μl of glycogen. After centrifugation for 30 min at 16 000 *g* at 4 °C, pellets were washed with 70% (v/v) ice-cold ethanol. After centrifugation for 15 min at 9000 *g* at 4 °C, pellets were air dried for 10–20 min and then dissolved in 40 μl dH_2O (Chen *et al.*, 2008).

PCR–DGGE analysis

Bacterial and archaeal community structures were analyzed by PCR–denaturing gradient gel electrophoresis (PCR-DGGE) targeting 16S rRNA genes. Variable V3 region of bacterial 16S rRNA genes was amplified by using a forward primer 341f with a GC clamp on the 5′ terminus (Sheffield *et al.*, 1989) and a reverse primer 534r (Muyzer *et al.*, 1993). Archaeal 16S rRNA gene was amplified by using a forward primer 348f with a GC clamp and a reverse primer 691r (Watanabe *et al.*, 2004).

DGGE was performed with a Dcode DGGE system (Bio-Rad, Richmond, CA, USA). PCR-amplified products (about 50 ng) were subjected to electrophoresis in a 10% (wt/vol) polyacrylamide gel in 1 × TAE at 100 V for 12 h. DGGE gels were prepared as described previously (Miller *et al.*, 1999; Miura *et al.*, 2007). The denaturant gradient range of the gel was 30% to 70%. After electrophoresis, the gels were stained with ethidium bromide for 30 min.

The major DGGE bands were excised and washed in 200 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)) and directly used as the template for PCR as described above excluding the GC clamp. The PCR products were purified and cloned as described previously (Okabe *et al*, 2007; Satoh *et al.*, 2007). Partial sequencing of 16S rRNA gene inserts was performed using an automatic sequencer (ABI Prism 3100-Adant Genetic Analyzer; Applied Biosystems Japan, Tokyo, Japan) with a Bigdye terminator Ready reaction kit (Applied Biosystems) as described previously (Ariesyady *et al*, 2007). All sequences obtained were compared with the reference 16S rRNA gene sequences available in the GenBank/EMBL/DDBJ databases using the Ribosomal Database Project (RDP) search (Cole *et al.*, 2005).

Isolation and characterization of a hydrogenotrophic exoelectrogen, strain AR20

Anodic biofilms were collected from the closed-circuit MFC, homogenized and subjected to the standard dilution plating with the R2A medium (Difco Japan BD. Co. Ltd., Tokyo, Japan). After 1–2 weeks of incubation, several colonies appeared, which were further purified by restreaking on the R2A medium several times. For further confirmation, a few colonies were subjected to PCR amplification using a forward primer 27f and a reverse primer 1492r (Turner *et al.*, 1999), and the amplified PCR products were purified as described above. The nearly full-length of 16S rRNA gene sequences were compared with that of DGGE band B-3 obtained from the anodic biofilm of the closed-circuit MFC at 85 h. The sequences obtained were also compared with those available in public databases (GenBank and DDBJ) with the BLAST system (Altschul *et al.*, 1990). Multiple alignments of sequence data and construction of phylogenetic tree using the neighbor-joining algorithms (Saitou and Nei, 1987) were performed using the CLUSTAL W package ver. 1.83 (Thompson *et al.*, 1994). Bootstrap resampling analysis for 1000 replicates of the neighbor-joining data was performed to estimate the confidence of tree topologies.

The substrate utilization ability of strain AR20 was investigated by batch experiments using the basal medium (Chung and Okabe, 2009b), with various combinations of electron donors and acceptors. Sodium nitrite (10 mM), sodium nitrate (10 mM), sodium sulfate (10 mM), ferric citrate (10 mM), oxygen or electrode (carbon cloth) was used as an electron acceptor. Yeast extract (0.1 g l^{-1}), glucose (10 mM), acetate (10 mM), succinate (10 mM), formate (10 mM) or H_2 (the headspace of the Hungate tube was pressurized to 150 kPa with 80% N_2 + 10% CO_2 + 10% H_2) was used as an electron donor. When an electron acceptor is an electrode (carbon cloth), H-type MFC reactors were used for batch experiment as described above. The strain AR20 was preincubated in the R2A medium under oxic conditions for 24 h with shaking, harvested by centrifugation (5000 *g* for 3 min) and washed two times with phosphate-buffered saline. The obtained biomass was inoculated in the tubes and H-type MFCs, and incubated at 25 °C for 96 h; OD_{600} was measured by a spectrophotometer (Novaspec Plus; GE Healthcare, Tokyo, Japan).

Electron transfer mechanism of isolated strain AR20

To investigate electron transfer mechanism of the strain AR20, current generation was measured in H-type MFCs containing the medium with various electron transport chain inhibitors (see Supplementary Materials).

Cyclic voltammetry

Cyclic voltammetry was conducted to determine the electrochemical activity of strain AR20 using a three-electrode electrochemical cell (50 ml in capacity) equipped with a platinum working

electrode (1.6 mm Pt; BAS, Warwickshire, UK), a platinum-wire counter electrode (BAS) and an Ag/AgCl reference electrode (RE-1B; BAS) as described previously (Chung and Okabe, 2009b). All three electrodes were inserted into the electrochemical cell by avoiding any contact between the electrodes. The supernatant of anodic culture fluid (without biomass) was prepared by centrifugation (5000 *g* for 3 min) and was transferred into the electrochemical cell containing 80 mM phosphate buffer. For biofilm samples, an anode electrode with attached biofilms was taken from the H-type MFC and used as a working electrode. Cyclic voltammetry was performed by starting from −600 mV and going up to 600 mV and back. The data were logged in a personal computer that connected to the potentiostat (PC 4/750 potentiostat; Gamry, Instruments, Warminster, PA, USA). All solutions were purged with N_2 gas for 10 min before electrochemical measurements and the headspace gas was replaced with N_2 gas.

Cocultivation of G. sulfurreducens PCA and Hydrogenophaga sp. strain AR20

To investigate the possibility of interspecies hydrogen transfer between *G. sulfurreducens* strain PCA and *Hydrogenophaga* sp. strain AR20, two-chamber H-type MFCs (500 ml each camber) were inoculated with pure cultures of *G. sulfurreducens* PCA, *Hydrogenophaga* sp. strain AR20 or both the strains, respectively. As for inoculums, the strain AR20 was grown in the liquid R2A medium for 48 h, harvested by centrifugation at 5000 *g* and washed with sterilized saline (0.85% NaCl) solution, which was purged with N_2 gas for 10 min in an anaerobic chamber. Similarly, the strain PCA was grown in the liquid DSM826 medium, harvested and washed like the strain AR20. The washed cells were inoculated to the anode chamber.

For all runs, the anode chambers were filled with 250 ml of a sterilized medium containing 20 mM acetate as the sole electron donor and carbon electrode as the sole electron acceptor. The anode potential was controlled at −300 mV vs Ag/AgCl (KCl sat., +206 mV vs a standard hydrogen electrode, 25 °C) by a potentiostat (HA-151B; Hokutodenko, Tokyo, Japan) for initial 35 h. The inocula were added to all anode chambers after 25 h. The anode potential control was stopped at 35 h, and then the current was measured up to 120 h. The total amount of electrons accepted by the anode electrode was calculated by integrating the current measured during the incubation. The culture fluid samples were taken from the anode chambers at 0, 25, 35, 55, 80 and 120 h. Acetate and quinone concentrations were measured.

Chemical analyses

The volatile fatty acid concentrations were determined using an ion chromatograph (HIC-6Aj; Shimadzu, Kyoto, Japan). The samples were filtered with 0.2-μm cellulose acetate membrane filters before analyses. Gas concentrations including CH_4 in the headspace were determined using a gas chromatograph (GC-8A; Shimadzu) equipped with the thermal conductivity detector. $^{12}CH_4$ and $^{13}CH_4$ were separately quantified using a gas chromatography–mass spectrometry (QP5050; Shimadzu) as described previously (Sugimoto and Wada, 1993). For quantification of quinone concentrations, 0.1 g (wet weight) of anodic biofilms and 10 ml of anode culture fluid were collected from MFCs. The biomass were harvested by centrifugation (9000 *g*, 10 min) and washed two times with 50 mM phosphate buffer (pH 6.8) containing 1 mM ferricyanide. Quinones were extracted from these samples and quantified as described previously (Hiraishi *et al.*, 1998). The quinone concentrations were converted to the cell numbers of corresponding strains, as the quinones are quantitative biomarkers of active bacterial populations (Hiraishi *et al.*, 2003). *G. sulfurreducens* strain PCA has menaquinone (MK)-8 as a main quinone, whereas *Hydrogenophaga* sp. strain AR20 has ubiquinone (UQ)-8 (Urakami *et al.*, 1995; Mikoulinskaia *et al.*, 2001).

Accession number

The 16S rRNA gene sequence of strain AR20 has been deposited under DDBJ accession number AB746948.

Results and discussion

DNA-SIP-DGGE analysis

The current density began to increase 3 days after biomass inoculation (Figure 1). After 8 days, the current density was above 1.4 A m^{-2}, acetate removal ratio was about 70% and CE was about 10%. This low CE was probably due to high methane conversion ratio (ca. 31%). Continuous acetate supply was stopped on the 13th day, ^{13}C-labeled acetate ($^{13}CH_3^{13}COONa$) was added to both the closed- and open-circuit MFCs (the final concentration was 20 mM) and then MFCs were operated in a batch mode. Acetate degradation was promoted in the closed-circuit MFC. Microbial community members that actively utilized ^{13}C-labeled acetate after 8 and 85 h were identified by DGGE analysis, followed by sequencing major DGGE bands (Figure 2 and Supplementary Table S1).

The anodic biofilm contained about 30 times more biomass than the culture fluid as shown by quinone concentrations (Supplementary Figure S1), indicating that the anodic biofilm was mainly responsible for acetate degradation and current generation. For anodic biofilms in the closed-circuit MFC, three major bands (B-2, B-4 and B-6) were detected at 8 h, which were closely related to uncultured *Bacteroidetes* clone 22e05 (100% similarity), *G. sulfurreducens* strain PCA (100%) and

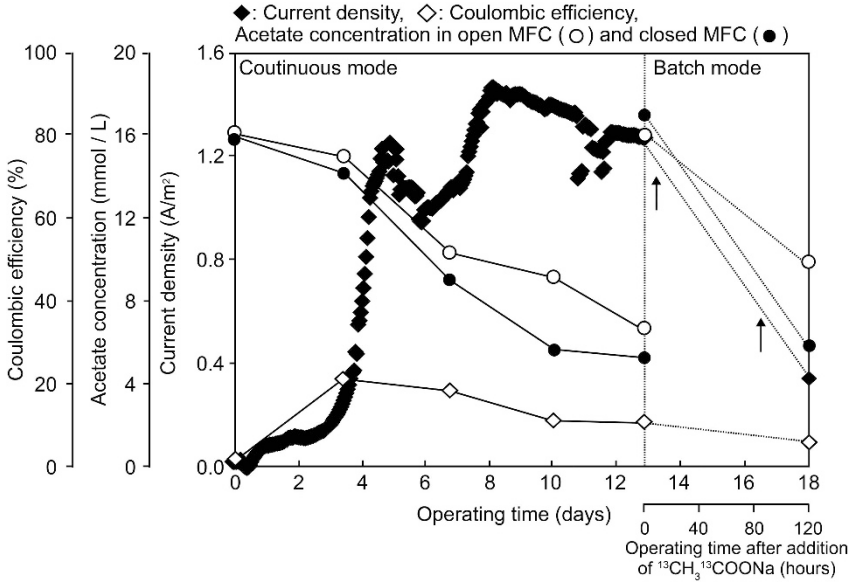

Figure 1 Current density and effluent acetate concentration of the closed- and open-circuit MFCs continuously fed with acetate (20 mM). The external resistance of 100 Ω was applied. On the 13th day, ^{13}C-labeled acetate ($^{13}CH_3{}^{13}COONa$) was added to both MFCs (the final concentration was 20 mM), and then the MFCs were operated in a batch mode for DNA-SIP followed by DGGE analysis. Anodic biofilms and culture fluid samples were taken 8 and 85 h after the addition of ^{13}C-labeled acetate as indicated by arrows.

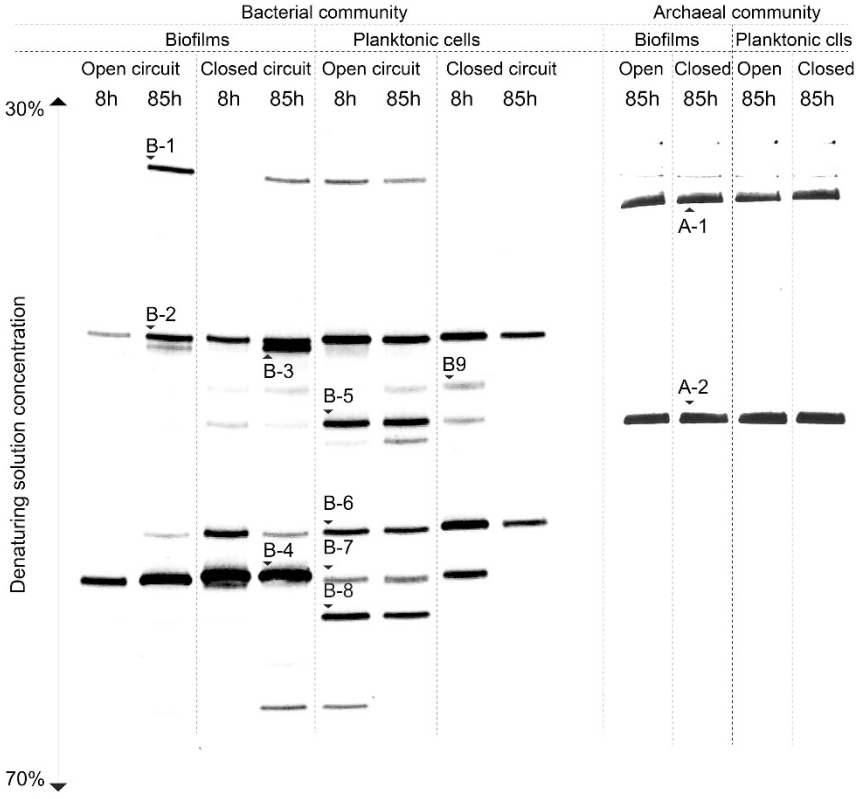

Figure 2 DGGE profiles of PCR products, amplified by bacteria- and archaea-specific primers, of ^{13}C-labeled (heavy) 16S rRNA genes derived from anodic biofilms and culture fluid (planktonic cells) of the closed- and open-circuit MFCs, showing metabolically active bacterial and archaeal communities after 8 and 85 h addition of ^{13}C-labeled acetate. Bands with numbers were excised and identified (B = bacteria and A = archaea) (see Supplementary Table S1).

uncultured *Veillonellanceae* clone AA0f (100%), respectively (Supplementary Table S1). After 85 h, an additional major band (B-3) appeared, which was closely related to *Hydrogenophaga* sp. strain BAC20 (100%). The most dominant population in the closed MFC biofilm was obviously a bacterium

closely related to *G. sulfurreducens* strain PCA, represented by the band B-4 (Figure 2), suggesting that *G. sulfurreducens* could be responsible for current generation. This outcome is consistent with the previous studies showing that *Geobacter* is the most abundant exoelectrogen in acetate-fed MFCs (Lee *et al.*, 2003; Kim *et al.*, 2008; Yates *et al.*, 2012). Comparison of the band patterns of biofilm samples between the closed-circuit MFC and the open one indicated that the major difference was the appearance of band B-3, closely related to *Hydrogenophaga* sp., after 85 h. Although the presence of *Hydrogenophaga* sp. in MFCs fed with short-chain fatty acids is sometimes reported (Teng *et al.*, 2010; Xing *et al.*, 2010), their ecophysiological roles in the MFCs are largely unknown. In general, members of the genus *Hydrogenophaga* are known to be aerobic bacteria that can oxidize hydrogen but not acetate (Kämpfer *et al.*, 2005).

For archaeal community, only two same bands (A-1 and A-2) were detected from all samples of anodic biofilms and culture fluid, both of which are closely related to *Methanosarcina* sp. *Methanosarcina* could produce methane from both acetate and hydrogen + CO_2.

Isolation and characterization of Hydrogenophaga sp. strain AR20

It is interesting to know why hydrogen-utilizing bacteria were present actively in the MFC fed with only acetate as the sole energy source. We, therefore, attempted to isolate a bacterium (designated strain AR20) that is closely related to *Hydrogenophaga* sp., represented by the DGGE band B-3, to investigate their ecophysiological roles in the acetate-fed MFC. We could successfully isolate the strain AR20 by repeating the standard dilution plating with the R2A medium (Difco). The identity of the isolated strain was checked by sequencing the nearly full-length 16S rRNA gene. The result indicated that the strain AR20 was identical to that of DGGE band (B-3) and was affiliated with members of the genus *Hydrogenophaga* with sequence similarity of 97.7%, 97.5% and 97.6% to the type strains of *H. flava*, *H. pseudoflava* and *H. bisanensis*, respectively, but it was distantly related to other known species of the genus (95.8–96.9% identity) (Figure 3). These results indicated that the strain AR20 was successfully isolated in pure culture.

The strain AR20 was electrochemically active and could generate high current density (up to 1.5 A m^{-2}) only when molecular hydrogen (H_2) was supplied as the sole electron donor (Figure 4a). However, this strain could not use acetate as the electron donor with any electron acceptors tested (Figure 4b and Table 1). These results imply that the strain AR20 is a chemoautotrophic hydrogenotrophic exoelectrogen. Although there are only a few reports on the presence and ability of hydrogenotrophic exoelectrogens (Pham *et al.*, 2003; Chung *et al.*, 2010), the strain AR20 has the highest ability to generate current to date.

Cyclic voltammograms of the isolated strain AR20 samples (the biofilm and the supernatant of the AR20 culture fluid) showed that both the samples exhibited electrochemical activity (the oxidation–reduction peaks at about −230 and 50 mV (vs Ag/AgCl), respectively) (Supplementary Figure S2), suggesting that the soluble electron carriers could be excreted by this strain and involved in current generation (electron transfer to the anode). The midpoint potential of a redox compound produced by the strain AR20 was about −90 mV (vs Ag/AgCl; +116 mV vs standard hydrogen electrode), which is close to one of the ubiquinone (UQ10; +90 mV vs standard hydrogen electrode) (Takamiya and Dutton, 1979) and higher than one of the c-type cytochromes (Qian *et al.*, 2011).

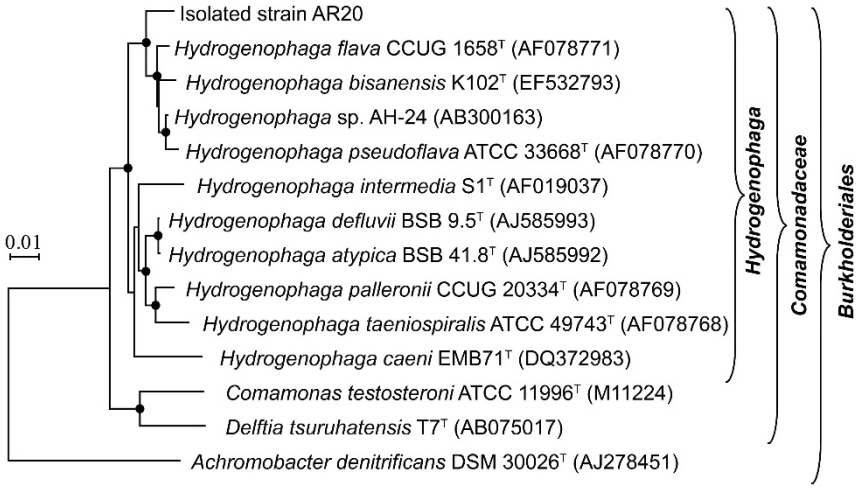

Figure 3 Neighbor-joining phylogenetic tree of the newly isolated *Hydrogenophaga* sp. strain AR20, showing the phylogenetic position of strain AR20. The phylogenetic tree was constructed based on available 16S rRNA gene sequences. The nodes supported by a bootstrap value of 80% were marked with solid circles. Scale bar represents 1.0% sequence divergence.

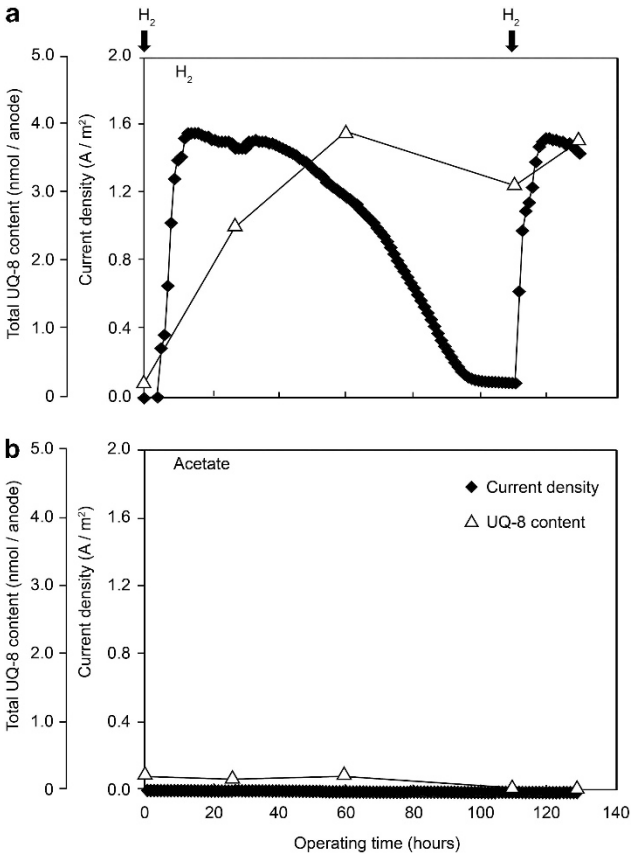

Figure 4 Characterization of the electrochemical activity of *Hydrogenophaga* sp. strain AR20 in a two-chamber MFC. Changes in current density and total UQ-8 content with time were shown when hydrogen (**a**) or acetate (20 mM) (**b**) was used as the sole electron donor without any other dissolved electron acceptors. Hydrogen gas (100% H_2) was bubbled directly into the anode culture fluid for 10 min as indicated by arrows. Hydrogen-dependent power generation by strain AR20 is shown in (**a**), but no power was generated with acetate (**b**). UQ-8 is a biomarker of active of *Hydrogenophaga* sp. strain AR20.

Table 1 Growth characteristics of the newly isolated *Hydrogenophaga* sp. strain AR20

Electron acceptors	*Electron donors*					
	Yeast extract	*Glucose*	*Acetate*	*Succinate*	*Formate*	*Hydrogen*
Electrode	+ + +	−	−	+	+	+ +
Oxygen	+ + +	−	−	+	+	+ +
Nitrate	+ + +	−	−	+	+	+ +
Nitrite	+ +	−	−	−	−	+
Sulfate	−	−	−	−	−	−
Fe(III)	+	−	−	−	−	+

Abbreviation: OD, optical density.
Growth was determined as OD_{600} after 96-h incubation.
−, OD_{600} <0.01; +, OD_{600} >0.01; + +, OD_{600} >0.10; + + +, OD_{600} >0.30.

Electron transfer mechanisms of strain AR20

The effect of various electron transfer chain inhibitors on current generation was investigated to study the electron transfer mechanism of strain AR20 (Supplementary Figure S3). The current generation was reduced by the addition of rotenone (0.1 mM) and thenoyltrifluoroacetone (TTFA) (0.1 mM), which inhibit the complex I and II of the electron transport chain (Supplementary Figure S4), respectively, while antimycin A (0.12 mM) showed no effect (Supplementary Figure S3). These results suggest that the strain AR20 utilizes nicotinamide adenine dinucleotide and flavin adenine dinucleotide as the electron transport carriers, but does not use the complex III of the electron transport chain. Furthermore, as the strain AR20 could produce UQ-8 in anaerobic condition (Supplementary Figure S1), electrons are probably transferred to UQ-8, and then the reduced UQ-8 or unknown molecule might be excreted, which carries the electrons to the anode.

Electron equivalent balance

On the basis of these experimental data, possible electron sinks such as residual acetate, H_2, CH_4, biomass and soluble microbial product in the acetate-fed MFC were assessed (Supplementary Figure S5) as demonstrated previously by Lee and Rittmann (2010). Supplementary Figure S5 shows the distribution of electron equivalent balance for the acetate-fed MFC. Only 11.1% (among which 8.9 ± 0.4% were generated by *Geobacter* sp. directly from acetate oxidation and 2.2 ± 0.3% were generated by *Hydrogenophaga* sp. from H_2 that is produced by *Geobacter* sp.) of the electrons of acetate utilized ($\Delta e^-_{acetate}$) was recovered as current, that is, CE is ca. 11%. This low CE is probably because CH_4 production was consistently observed owing to a long-term continuous operation of MFC, and its electron equivalent was 36.4 ± 0.4% of $\Delta e^-_{acetate}$, in which acetoclastic methanogenisis dominated (Supplementary Table S2). Total biomass (*Geobacter* sp., *Hydrogenophaga* sp. and others) and soluble microbial product synthesis comprised 19.3 ± 0.2% and 15.3 ± 0.9% of $\Delta e^-_{acetate}$. The electron equivalent of soluble microbial product is slightly higher than the value reported in the previous study (11%; Lee and Rittmann, 2010). It should be noted that the electron balance was not completely closed, and the fate of 17.9 ± 0.3% of $\Delta e^-_{acetate}$ was unidentified in this study.

Coculture study of G. sulfurreducens strain PCA and Hydrogenophaga sp. strain AR20

Now the question is why and/or how the hydrogenotrophic exoelectrogen, strain AR20, could gain energy for growth in the MFC that was fed with only acetate as the sole energy source. It could be hypothesized that under electron acceptor-limiting conditions, *Hydrogenophaga* sp. strain AR20 use hydrogen that is produced by *G. sulfurreducens*, that is, *G. sulfurreducens* oxidize acetate in syntrophic cooperation with a hydrogen-consuming partner strain AR20 with current generation.

The effect of hydrogen partial pressure on hydrogen production by *G. sulfurreducens* strain PCA was investigated. In the absence of any dissolved electron acceptors and electrode, *G. sulfurreducens* strain PCA could produce H_2 up to hydrogen partial pressure of 150 Pa under the following conditions: 10 mM acetate and 30 mM HCO_3^- (Supplementary Figure S6).

To confirm the interspecies hydrogen transfer between *G. sulfurreducens* strain PCA and *Hydrogenophaga* sp. strain AR20, individual cell growth, current generation and acetate consumption of coculture MFC and individual pure culture MFCs were determined and compared (Figure 5). Only acetate was used as the sole electron donor without any dissolved inorganic electron acceptor in all the MFCs. No current generation, acetate consumption and cell growth occurred in the strain AR20 pure culture MFC, indicating that strain AR20 cannot use acetate with anode electrode as the electron acceptor. This result is consistent with metabolic characteristics of the strain AR20 shown in Figure 4b and Table 1. Current generation occurred concomitantly with acetate consumption and cell growth in both the strain PCA pure culture MFC and coculture MFC. The integrated current generation of the coculture MFC during the incubation was 12.1 mmol-e eq, which was about 72% higher than one of the strain PCA pure culture MFC (6.7 mmol-e eq) (Figure 5a). Accordingly, more acetate was consumed in the coculture MFC than the strain PCA pure culture MFC (Figure 5b). The cell number of strain AR20 increased only in the coculture MFC fed with only acetate as the sole electron donor (Figure 5c). These results indicate that strain AR20 utilizes metabolites produced by the strain PCA for cell growth, as strain AR20 cannot use acetate and grow in the pure culture MFC (Figure 4b). The hydrogen partial pressures in the anode chambers were undetectable levels in all the MFCs.

To confirm the syntrophic acetate oxidation by *G. sulfurreducens* strain PCA with *Hydrogenophaga* sp. strain AR20 via the interspecies hydrogen transfer, the hydrogen partial pressure in the anode chamber in the mixed population MFC was elevated by bubbling H_2 gas. Increasing hydrogen partial pressure decreased acetate degradation rate and the population size of *Geobacter* sp. but increased *Hydrogenophaga* sp. strain AR20, suggesting the disruption of syntrophic acetate oxidation possibly occurring in the MFC (Supplementary Figure S7). However, this experiment needs further investigation

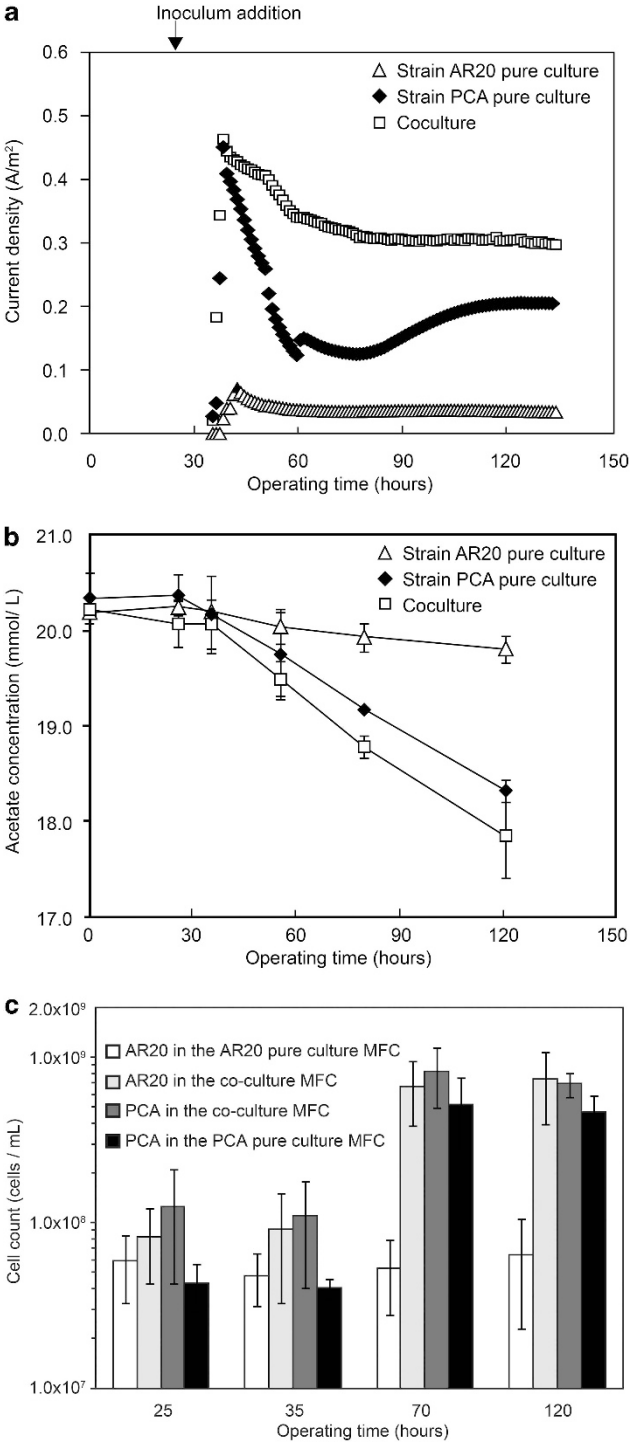

Figure 5 Performance of the cocultured MFC with *G. sulfurreducens* strain PCA and the isolated *Hydrogenophaga* sp. strain AR20, which was compared with those of pure cultures of individual strains. In this study, only acetate was used as the sole electron donor. Time courses of current density (**a**), acetate degradation (**b**) and population sizes of *G. sulfurreducens* strain PCA and *Hydrogenophaga* sp. strain AR20 (**c**) during incubation were shown. Experiments were performed under each MFC condition in triplicate, and reproducibility was confirmed. A typical time course of current density is presented. The data on acetate concentrations and population sizes of each strain are the means of three independent experiments, and error bars represent standard deviations. The anode potential in each MFC was continuously controlled at −300 mV (vs Ag/AgCl) by a potentiostat (PS) before inoculation. The PS was switched off after about 35 h. Cell numbers of *Hydrogenophaga* sp. strain AR20 and *G. sulfurreducens* strain PCA were estimated from UQ-8 and menaquinone (MK)-8 concentrations, respectively. No power generation and cell growth were observed in the pure culture MFC of *Hydrogenophaga* sp. strain AR20.

because increasing the hydrogen concentration downregulates the expression of genes for acetate metabolism in *Geobacter* (Ueki and Lovley, 2010) and increases acetate production by acetogens.

Syntrophic acetate degradation by *G. sulfurreducens* has been reported to occur only when good hydrogen-consuming partners such as *W. succinogenes* or *D. desulfuricans*, but not hydrogenotrophic methanogens, were present with nitrate as the electron acceptor (Cord-Ruwisch *et al.*, 1998). The isolated *Hydrogenophaga* sp. strain AR20 can preferentially utilize hydrogen, with electrode as the electron acceptor. On the basis of these results, the metabolite is thought to be hydrogen. These results show for the first time that *G. sulfurreducens* strain PCA can oxidize acetate in syntrophic cooperation with the newly isolated *Hydrogenophaga* sp. strain AR20, a hydrogenotrophic exoelectrogen, with electrode as the electron acceptor.

Electron transfer in the coculture MFC in this study may not only proceed exclusively via interspecies hydrogen transfer but may also proceed through an alternative carrier system. Unknown soluble electron carriers could be excreted by *Hydrogenophaga* sp. strain AR20 (Supplementary Figures S2 and S5), which might be involved in electron transfer between *G. sulfurreducens* and strain AR20. Recently, DIET was discovered in cocultures (aggregates) of *G. sulfurreducens* and *G. metallireducens*, which grew with ethanol as the electron donor and fumarate as the electron acceptor (Summers *et al.*, 2010). The DIET could be an efficient alternative strategy for microorganisms to cooperate in anaerobic degradation of organic substrates, in which *Geobacter* dominated (Summers *et al.*, 2010; Morita *et al.*, 2011). A recent publication shows the ability of a pure culture of *Geobacter* to grow in syntrophy with methanogens via DIET with conductive granular-activated carbon as the mediator (Liu *et al.*, 2012). *G. sulfurreducens* could also produce electrically conductive type IV pili that enable long-range electron transfer in biofilms (Malvankar *et al.*, 2011; Morita *et al.*, 2011). The further study is required to investigate the contribution of these alternative electron transfer mechanisms in the coculture of between *G. sulfurreducens* and *Hydrogenophaga* sp. strain AR20.

In summary, this study shows for the first time that *G. sulfurreducens* can oxidize acetate in syntrophic association with a hydrogenotrophic exoelectrogen, *Hydrogenophaga* sp. strain AR20, with current generation. This implies that other exoelectrogens besides well-characterized *Geobacter* species are present in anode microbial communities in the MFC fed with non-fermentable substrate (that is, acetate), which contributes to current generation.

Conflict of Interest

The authors declare no conflict of interest.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Ariesyady HD, Ito T, Okabe S. (2007). Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester. *Wat Res* **41**: 1554–1568.

Caccavo F Jr, Lonergan DJ, Lovley DR, Davis M, Stolz JF, McInerney MJ. (1994). *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. *Appl Environ Microbiol* **60**: 3752–3759.

Chen Y, Dumont MG, Neufeld JD, Bodrossy L, Stralis-Pavese N, McNamara NP *et al.* (2008). Revealing the uncultivated majority: combining DNA stable-isotope probing, multiple displacement amplification and metagenomic analyses of uncultivated *Methylocystis* in acidic peatlands. *Environ Microbiol* **10**: 2609–2622.

Chung K, Okabe S. (2009a). Continuous power generation and microbial community structure of the anode biofilms in a three-stage two-chamber microbial fuel cell. *Appl Microbiol Biotechnol* **83**: 965–977.

Chung K, Okabe S. (2009b). Characterization of electrochemical activity of a strain ISO2-3 phylogenetically related to *Aeromonas* sp. isolated from a glucose-fed microbial fuel cell. *Biotechnol Bioeng* **104**: 901–910.

Chung K, Fujiki I, Okabe S. (2010). Effect of formation of biofilms and chemical scale on the cathode electrode on the performance of a continuous two-chamber microbial fuel cell. *Bioresour Technol* **102**: 355–360.

Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM *et al.* (2005). The Ribosomal Database Project (RDP-II): sequence and tools for high-throughput rRNA analysis. *Nucleic Acids Res* **33**: D294–D296.

Cord-Ruwisch R, Lovley DR, Schink B. (1998). Growth of *Geobacter sulfurreducens* with acetate in syntrophic cooperation with hydrogen-oxidizing anaerobic partners. *Appl Environ Microbiol* **64**: 2232–2236.

Hiraishi A, Ueda Y, Ishihara J. (1998). Quinone profiling of bacterial communities in natural and synthetic sewage activated sludge for enhanced phosphate removal. *Appl Environ Microbiol* **64**: 992–998.

Hiraishi A, Iwasaki M, Kawagishi T, Yoshida N, Narihiro T, Kato K. (2003). Significance of lipoquinones as quantitative biomarkers of bacterial populations in the environment. *Microbes Environ* **18**: 89–93.

Holmes DE, Bond DR, O'Neil RA, Reimers CE, Tender LR, Lovley DR. (2004). Microbial communities associated with electrodes harvesting electricity from a variety of aquatic sediments. *Microb Ecol* **48**: 178–190.

Ieropoulos I, Winfield J, Greenman J. (2010). Effects of flow-rate, inoculum and time on the internal resistance of microbial fuel cells. *Bioresour Technol* **101**: 3520–3525.

Ito T, Yoshiguchi K, Ariesyady HD, Okabe S. (2011). Identification of a novel acetate-utilizing bacterium belonging to *Synergistes* group 4 in anaerobic digester sludge. *ISME J* **5**: 1844–1856.

Jung S, Regan JM. (2007). Comparison of anode bacterial communities and performance in microbial fuel cells with different electron donors. *Appl Microbiol Biotechnol* **77**: 393–402.

Kan J, Hsu L, Cheung ACM, Pirbazari M, Nealson KH. (2011). Current production by bacterial communities in microbial fuel cells enriched from wastewater sludge with different electron donors. *Environ Sci Technol* **45**: 1139–1146.

Kämpfer P, Schulze R, Jäckel U, Malik KA, Amann R, Spring S. (2005). *Hydrogenophaga defluvii* sp. nov. and *Hydrogenophaga atypica* sp. nov., isolated from activated sludge. *Int J Syst Evol Microbiol* **55**: 341–344.

Kato S, Hashimoto K, Watanabe K. (2012). Methanogenesis facilitated by electric syntrophy via (semi)conductive iron-oxide minerals. *Environ Microbiol* **14**: 1646–1654.

Kim IS, Chae K-J, Choi M-J, Verstraete W. (2008). Microbial fuel cells: recent advances, bacterial communities and application beyond electricity generation. *Environ Eng Res* **13**: 51–65.

Lee J, Phung NT, Chang IS, Kim BH, Sung HC. (2003). Use of acetate for enrichment of electrochemically active microorganisms and their 16S rDNA analyses. *FEMS Microbiol Lett* **223**: 185–191.

Lee H-S, Rittmann B. (2010). Significance of biological hydrogen oxidation in a continuous single-chamber microbial electrolysis cell. *Environ Sci Technol* **44**: 948–954.

Liu F, Rotaru A-E, Shrestha PM, Malvankar NS, Nevin KP, Lovley DR. (2012). Promoting direct interspecies electron transfer with activated carbon. *Energy Environ Sci* **5**: 8982–8989.

Liu H, Logan BE. (2004). Electricity generation using an air-cathode single chamber microbial fuel cell in the presence and absence of a proton exchange membrane. *Environ Sci Technol* **38**: 4040–4046.

Lovley DR. (2012). Electromicrobiology. *Annu Rev Microbiol* **66**: 391–409.

Malvankar NS, Vargas M, Nevin KP, Franks AE, Leang C, Kim B-C *et al.* (2011). Tunable metallic-like conductivity in microbial nanowire networks. *Nat Nanotechnol* **6**: 573–579.

Miller KM, Ming T, Schulze A, Withler RE. (1999). Denaturing gradient gel electrophoresis (DGGE): a rapid and sensitive technique to screen nucleotide sequence variation in populations. *BioTechniques* **27**: 1016–1031.

Mikoulinskaia O, Akimenko V, Galouchko A, Thauer RK, Hedderich R. (2001). Cytochrome *c*-dependent methacrylate reductase from *Geobacter sulfurreducens* AM-1. *Eur J Biochem* **263**: 346–352.

Miura Y, Hiraiwa MN, Ito T, Itonaga T, Watanabe Y, Okabe S. (2007). Bacterial community structures in MBRs treating municipal wastewater: relationship between community stability and reactor performance. *Water Res* **41**: 627–637.

Morita M, Malvankar NS, Franks AE, Summers ZM, Giloteaux L, Rotaru AE *et al.* (2011). Potential for direct interspecies electron transfer in methanogenic wastewater digester aggregates. *mBio* **2**: e00159–11.

Muyzer G, De Waal EC, Uitterlinden AG. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**: 695–700.

Neufeld JD, Dumont MG, Vohra J, Murrell JC. (2007). Methodological considerations for the use of stable isotope probing in microbial ecology. *Microb Ecol* **53**: 435–442.

Okabe S, Odagiri M, Ito T, Satoh H. (2007). Succession of sulfur-oxidizing bacteria in the microbial community on corroding concretes in sewer systems. *Appl Environ MIcrobiol* **73**: 971–980.

Pham CA, Jung SJ, Phung NT, Lee J, Chang IS, Kim BH *et al.* (2003). A novel electrochemically active and Fe (III)-reducing bacterium phylogenetically related to *Aeromonas hydrophila*, isolated from a microbial fuel cell. *FEMS Microbiol Lett* **223**: 129–134.

Qian X, Mester T, Morgado L, Arakawa T, Sharma ML, Inoue K *et al.* (2011). Biochemical characterization of purified OmcS, a *c*-type cytochrome required for insoluble Fe(III) reduction in *Geobacter sulfurreducens*. *Biochem Biophys Acta* **1807**: 404–412.

Roh Y, Moon HS. (2001). Iron reduction by a psychrotolerant Fe (III)-reducing bacterium isolated from ocean sediment. *Geosci J* **5**: 183–190.

Saitou N, Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.

Satoh H, Miura Y, Tsushima I, Okabe S. (2007). Layered structure of bacterial and archaeal communities and their *in situ* activities in anaerobic granules. *Appl Environ Microbiol* **73**: 7300–7307.

Sheffield VC, Cox DR, Lerman LS, Myers RM. (1989). Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc Natl Acad Sci USA* **86**: 232–236.

Sugimoto A, Wada E. (1993). Carbon isotopic composition of bacterial methane in a soil incubation experiment: contributions of acetate and CO_2/H_2. *Geochim Cosmochim Acta* **57**: 4015–4027.

Summers ZM, Fogarty HE, Leang C, Franks AE, Malvankar NS, Lovley DR. (2010). Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. *Science* **330**: 1413–1415.

Takamiya KI, Dutton PL. (1979). Ubiquinone in *Rhodopseudomonas sphaeroides*. Some thermodynamic properties. *Biochim Biophys Acta* **546**: 1–16.

Teng SX, Tong ZH, Li WW, Wang SG, Sheng GP, Shi XY *et al.* (2010). Electricity generation from mixed volatile fatty acids using microbial fuel cells. *Appl Microbiol Biotechnol* **87**: 2365–2372.

Thompson JD, Higgins DG, Gibson TJ. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.

Turner S, Pryer KM, Miao VPW, Palmer JD. (1999). Investigating deep phylogenetic relationships among Cyanobacteria and plastids by small subunit rRNA sequence analysis. *J Eukaryotic Microbiol* **46**: 327–338.

Ueki T, Lovley DR. (2010). Genome-wide gene regulation of biosynthesis and energy generation by a novel transcriptional repressor in *Geobacter* species. *Nucleic Acids Res* **38**: 810–821.

Urakami T, Araki H, Komagata K. (1995). Characteristics of newly isolated *Xanthobacter* strains and fatty acid compositions and quinone systems in yellow-pigmented hydrogen-oxidizing bacteria. *Int J Syst Evol Microbiol* **45**: 863–867.

Watanabe T, Asakawa S, Nakamura A, Nagaoka K, Kimura M. (2004). DGGE method for analyzing 16S rDNA of methanogenic archaeal community in paddy field soil. *FEMS Microbiol Lett* **232**: 153–163.

Xing D, Cheng S, Logan BE, Regan JM. (2010). Isolation of the exoelectrogenic denitrifying bacterium *Comamonas denitrificans* based on dilution to extinction. *Appl Microbiol Biotechnol* **85**: 1575–1587.

Yates MD, Kiely PD, Call DF, Rismani-Yazdi H, Bibby K, Peccia J *et al.* (2012). Convergent development of anodic bacterial communities in microbial fuel cells. *ISME J* **6**: 1–12.

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