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ENVIRONMENTAL BIOTECHNOLOGY

Discovery of commonly existing anode biofilm microbes in two different wastewater treatment MFCs using FLX Titanium pyrosequencing

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Abstract In microbial fuel cells (MFC), wastewater is used as a fuel while organic and nutrient pollution in the wastewater are being treated. In the present study, commonly existing microbial populations in MFC anode biofilms were identified using high throughput FLX Titanium pyrosequencing to provide much more extensive information of anode microbial communities than previously possible. Using 454 FLX Titanium pyrosequencing, 31,901 sequence reads with an average length of 430 bp were obtained from 16S rRNA gene amplicons from different MFC anodes with different substrate exposure and respiration conditions, and microbial community structure and population identification were then analyzed using high-throughput bioinformatics methods. Although community profiles from the four samples were significantly different, hierarchical clustering analysis revealed several bacterial populations that commonly exist in the anode biofilm samples. These bacteria were phylogenetically distributed in Firmicutes and the alpha-, beta-, gamma-, and delta-subclasses of Proteobacteria. In addition, most of these populations were found to be novel anode bacteria and exhibited oligotrophic or substrate-concentration-insensitive growth. These findings suggest that commonly existing anode bacteria may play a key role in the stable operations of MFCs, combined with wastewater treatment plants, under fluctuating substrate and respiration conditions.

Keywords Microbial Fuel Cell (MFC) · Microbial community · Pyrosequencing · Anode biofilm · Renewable energy · Wastewater

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Introduction

A microbial fuel cell (MFC) is an attractive "green" energy technology for the production of electricity from wastewater being used as a fuel while being treated (Liu et al. 2004; Logan and Regan 2006; Lovley 2006). Most MFC studies have focused on increasing the efficiency of electricity generation by testing different reactor types and materials (Kim et al. 2008a, b; Lovley 2006). Although the microbial ecology of anode biofilms is believed to play key roles in improving electricity generation in MFCs (Clauwaert et al. 2008; Kim et al. 2008a, b; Logan and Regan 2006; Logan 2009; Lovley 2008; Rabaey et al. 2007), a few studies have reported that anode-dominant bacteria differed significantly depending upon the biochemical, physiological, and ecological conditions applied in the MFC studies (Jung and Regan 2007; Kim et al. 2008a, b). However, previous MFC anode microbial community analyses (Aelterman et al. 2006; Chae et al. 2009; Chung and Okabe 2009; Ishii et al. 2008; Ki et al. 2008; Kim et al. 2004, 2006, 2007; Lee et

al. 2003; Logan et al. 2005; Phung et al. 2004; Rabaey et al. 2004; Reimers et al. 2007; White et al. 2009) have not identified commonly existing microbial populations in different anode biofilm communities. This observation may suggest that the so-far-known anode-dominant microbes are sensitive to growth/respiration conditions as well as to microbial population dynamics. Such factors would be critical when considering MFC applications in wastewater because in typical wastewater treatment plants, substrate exposure and respiration conditions often fluctuate widely, and the microorganisms in the wastewater could continuously influence the microbial communities on MFC anode.

Previous MFC microbial ecology studies involving the cloning of 16S rRNA gene amplicons and their sequencing by the Sanger method focused mainly on microbial community analysis (Aelterman et al. 2006; Chae et al. 2009; Chung and Okabe 2009; Ishii et al. 2008; Ki et al. 2008; Kim et al. 2004, 2006, 2007; Lee et al. 2003; Logan et al. 2005; Phung et al. 2004; Rabaey et al. 2004; Reimers et al. 2007; White et al. 2009), and the numbers of sequence reads from the 16S rRNA gene amplicons were smaller than 100, which is too small to capture comprehensive and systematic information on wastewater microbial communities. Thus, for the studies cited, the detection limitation of conventional microbial analysis may have been responsible for their lack in identifying commonly existing MFC anode bacteria, rather than due to any ecological causes. This hypothesis has yet to be examined.

To address the issues raised above, we sought to determine if common anode biofilm-forming microbes, stable under different substrate exposure and respiration conditions, existed. To find such microbes, we screened for bacteria that commonly exist in differently operated anode biofilms by using a novel and more sensitive microbial community-profiling method named FLX Titanium pyrosequencing for targeting 16S rRNA genes. Compared with the current pyrosequencing methods (average lengths shorter than 250 bp), titanium-based pyrosequencing increased the read length of individual sequences to over 400 bp (Sogin et al. 2006; Wolcott et al. 2009). The expanded read length provides a higher degree of resolution for bacterial identification at the species level (Wolcott et al. 2009). While pyrosequencing has become a general molecular method for detecting rare microbial populations in environmental samples (Hamp et al. 2009; McLellan et al. 2009; Parameswaran et al. 2010; Sanapareddy et al. 2009; Zhang et al. 2009), there are a very limited number of studies that have used titanium-based pyrosequencing. One study used the novel titanium-based technique in microbial community analysis for medical purposes (Wolcott et al. 2009). This study was the first to attempt the use of titanium-based pyrosequencing in microbial community analysis of environmental samples, including wastewater and its related microbes.

Materials and methods

MFC operation and sampling

The anode biofilm samples were obtained from two different MFCs (an MFC from Jeonju Univ. [JM] and an MFC from Pusan National Univ. [PM]), among which the highest levels of generated electricity were 462 mW/m^2 for JM and 29 W/m^2 for PM. The main characteristics of JM and PM MFC operation are summarized in Table 1. JM was constructed as a single chamber with an air cathode and membraneless microbial fuel reactor; the space between anode and cathode was separated by a sponge-like material (S. Figure 1). PM was constructed as a single chamber with membrane electrode at one side and was submerged into the aeration chamber filling with distilled water and 100 mM phosphate buffer (S. Figure 2). Both reactors were continuously fed by synthetic wastewater solutions in up-flow modes. For the PM reactor, acetate was contained in a synthetic wastewater solution (Chen et al. 2008). For the JM reactor, glucose was contained in other synthetic wastewater solution (He et al. 2005). The JM MFC was inoculated with denitrifying activated sludge from a municipal wastewater treatment plant near Jeonju, while the PM MFC was seeded with activated sludge from a municipal wastewater treatment plant near Pusan. After the reactors reached steady state, biofilm samples were collected from the surface of each anode. For JM, biofilms were scraped out of the submerged anode surface, in which denitrification occurred under substrate sufficient conditions. For PM, biofilm samples were collected from separate parts of anode after the anode was carefully taken out of the reactor and then cut into three different pieces (0–10 cm [PM1], 10–20 cm [PM2], 20–30 cm [PM3] from the influent). Since the chemical oxygen demand (COD) concentrations varied depending upon depth, the effect of substrate exposure level on anode microbial community could be examined.

DNA extraction, PCR, and FLX Titanium pyrosequencing

Genomic DNA was extracted using the PowerSoil™ DNA Isolation Kit (MoBio, CA, USA) according to the manufacturer's instructions. Fragments of 16S rRNA genes containing variable V3 regions were amplified from the extracted DNA with primer sets, 27F (GAGTTTGATCMTGGCTCAG) and 518R (WTTACCGCGGCTGCTGG) with four different barcodes (AGTCACTGAC for JM, GTCACAGTAC for PM1, TAGCGCATAC for PM2,

Table 1 The main characteristics of JM and PM MFC operations

Characteristic	JM	PM
Respiration condition	Facultative denitrification	Strict anaerobic
Substrate	Glucose	Acetate
Membrane	Membraneless	Cation exchange membrane (CEM)
Cathode	Dried air cathode	Wetted air cathode
Mode	Up-flow	Up-flow
Influent COD	550±15 mg/L	151±16 mg/L
Effluent COD	50±7 mg/L	8±2 mg/L

TATAGCGCAC for PM3) to sort each sample from the mixed pyrosequencing outcomes. Each PCR reaction was performed in two of 25 μl reaction mixtures containing 15–25 ng (8–10 ng/μl) of DNA, 10 μM of each primer (BIONEER, Seoul, South Korea), 120 ng/μl of bovine serum albumin (New England BioLabs, MA, USA), and AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen, WI, USA) to give the following final concentrations: 1.25 U of *Taq* polymerase, 50 mM of $MgSO_4$, and 10× of PCR buffer. A C1000TM Thermal Cycler (BIO-RAD, CA, USA) was used for the PCR as follows: (1) an initial denaturation step at 94°C for 3 min, (2) 25 cycles of annealing and extending (each cycle occurred at 94°C for 1 min followed by 54°C for 30 s then an extension step at 72°C for 2 min), and (3) final extension at 72°C for 5 min. After this PCR amplification, the amplicons were purified by one-time gel electrophoresis/isolation and two-times purifications using a QIAquick Gel extraction kit (Qiagen, CA, USA) and a QIAquick PCR purification kit (Qiagen, CA, USA). To recover sufficient amount of purified amplicon from the purification steps, two of 25 μl reaction mixtures were combined into one mixture prior to the amplicon purification. Amplicon pyrosequencing was performed by Macrogen Inc. (Seoul, South Korea) using a 454/Roche GS-FLX Titanium instrument (Roche, NJ, USA). Four samples were pooled and their sequences separated according to barcodes. Low-quality sequences were filtered out using an algorithm to remove sequences of length shorter than 200 nucleotides and with an average quality score of lower than 20 (Huse et al. 2007; Brockman et al. 2009).

Comparative analysis of anode community profiles

Multiple sequence alignment and complete linkage clustering were used to cluster the sequences from 0% to 10% dissimilarity using Ribosomal Database Project (RDP)'s pyrosequencing pipeline (Cole et al. 2008). These clusters served as Operational Taxonomic Units (OTUs) for generating rarefaction curves and for calculating the richness and diversity indexes. Representative sequences from each OTU were phylogenetically assigned with taxonomic classifications obtained from the RDP-II Classifier (Cole et al. 2008; Wang et al. 2007), the National Centre for Biotechnology Information (NCBI) BLAST (Johnson et al. 2008), and the Greengenes databases (DeSantis et al. 2006). Hierarchical clustering based on 5% dissimilarity clustering data from RDP's pyrosequencing pipeline and visualization of hierarchical clustering data were performed using Cluster 3.0 and Java Treeview, respectively (Cardenas et al. 2009; Eisen et al. 1998; Saldanha 2004). The sequences determined to belong to commonly existing anode biofilm microbes were aligned using MUSCLE (Edgar 2004) and a phylogenetic tree was constructed using MEGA4 (Kumar et al. 2008) with a neighbor-joining algorithm employing a similarity matrix of pairwise comparisons with 1,000 replicates of bootstrap.

The representative nucleotide sequences obtained in this study (Table 3) were deposited in GenBank under accession numbers GU594281–GU594292.

Results

Bacterial diversity

The four anode biofilm samples (PM1, PM2, PM3, and JM) yielded 31,901 16S rRNA sequence reads with an average length of 430±65 bp (11,025 reads for PM1, 5,494 reads for PM2, 2,387 reads for PM3, and 12,995 reads for JM) following the sequence quality filtering steps. The number of sequence reads in the present study is one order of magnitude greater than the number (1.5 thousand) of anode 16S rRNA sequences reported in previous studies (Clauwaert et al. 2008). These data illustrate the greatly improved efficiency of gaining environmental sequences using high-throughput pyrosequencing.

Bacterial community structures (at class level) of the four different anode samples are shown in Fig. 1. Actinobacteria class population was the most abundant in the JM (28.8% of total bacteria) followed by Gammaproteobacteria (18.0%), unclassified class populations (11.2%), Bacilli (9.7%), and Clostridia (8.8%), while Deltaproteobacteria was in PM1 (60.4%), PM2 (39.9%), and PM3 (15.8%).

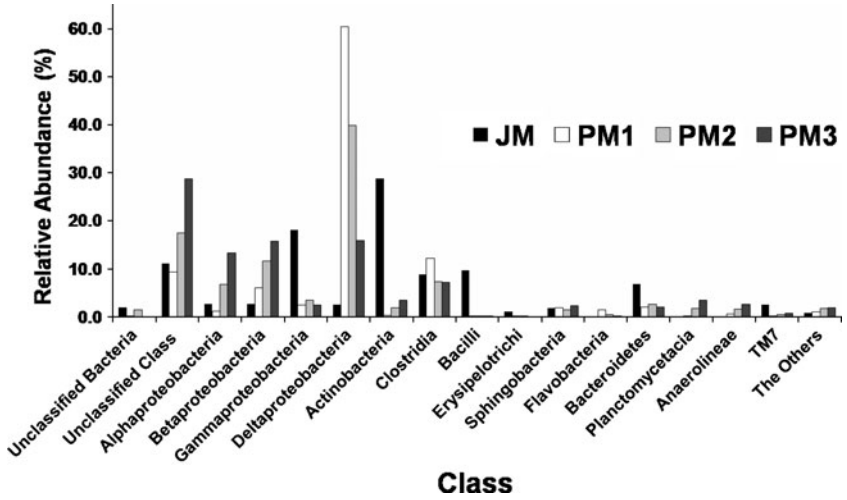

Fig. 1 Composition of class level bacterial groups in the library of 16S rRNA sequences in this study. The *unclassified bacteria* and *unclassified class* indicates the population which is less than the bootstrap cut-off of 50% after RDP classifier in kingdom level and class level, respectively. *The others* indicates populations were the other classified classes are less than 1% of total composition

Planctomycetacia and Anaerolinea were not detected in JM, but Erysipelotrichi existed only in JM. A certain portion (11.2%) of bacteria from JM was unclassified at the class level. The increasing patterns from PM1 to PM3 were detected in the class groups of TM7, Alphaproteobacteria, Betaproteobacteria, Planctomycetacia, Anaerolineae, and Actinobacteria, but the portions of Deltaproteobacteria, Clostridia, and Flavobacteria decreased. A large fraction of unclassified bacteria at the class level for PM tends to increase by COD concentration (9.4%, 17.4%, and 28.7% for PM1, PM2, and PM3, respectively).

The shape of the rarefaction curve and the Shannon index value for the denitrifying and glucose-fed JM MFC (5.00) were closer to that for PM1 than for PM2 and PM3 (Fig. 2). In the anode biofilms exhibiting steeper slopes (PM3>PM2>PM1 or JM), there are higher percents of unclassified populations at the class level (Fig. 1), indicating that there may be more populations to assign in the PM3 and PM2 than PM1 and JM. In the PM samples, as the level of primary substrate (acetate) decreased from 151 ppm COD at the influent [PM1] to 8 ppm COD at the effluent [PM3], the slopes of the rarefaction curves became steeper

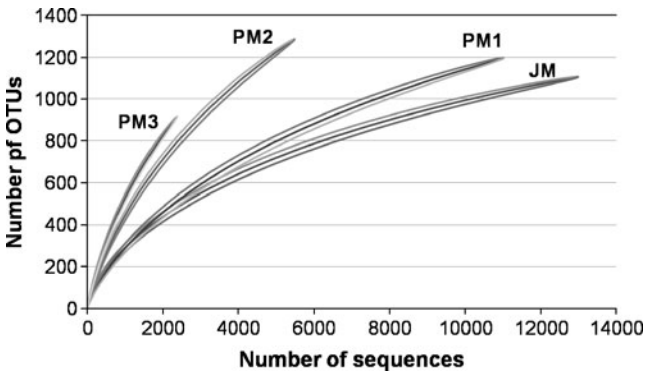

Fig. 2 Rarefaction curves. The *y*-axis indicates the number of OTUs with a dissimilarity cut-off <5%. Each line for samples contains 95% confidence intervals

(Fig. 2), and their Shannon index values increased (i.e., 4.55, 5.60, and 6.11 for PM1, PM2, and PM3, respectively). These results suggest that primary substrate limitation increased the degree of microbial diversity in the PM anode biofilm communities.

Comparative analysis of MFC communities

With the grouped OTUs, a two-dimensional hierarchy analysis was performed (Fig. 3). The microbial community profiles showed significantly different patterns among the anode biofilm samples. According to our *x*-axis hierarchical clustering analysis of the community profile patterns, the community structure of JM was obviously different from those of the PM samples. This was supported by the following identification of dominant populations, i.e., a *Pseudomonas* population was the most dominant in the JM community (11.72% of total), while *Geobacter* populations were the most dominant for the PM communities. These findings may be attributed to differences in respiration conditions and growth substrate compounds between the JM and PM MFCs.

Among the PM samples, community structures changed in response to the levels of primary substrate exposure (i.e., substrate sufficient versus limiting conditions). According to our *x*-axis hierarchical clustering analysis of community profile patterns (Fig. 3), the community structures of PM1 and PM2 were closer than that of PM3. This statistical estimation was supported by the following identification of dominant populations for the PM communities. In PM1 and PM2, only two genera were identified as dominant populations (dominant population cut-off >1% relative abundance of total), i.e., *Geobacter* (46.89 % for PM1 and 26.46 % for PM2) and *Spoanaerobacter* (1.23 % for PM1 and 1.42% for PM2). In PM3, however, a wider range of genus groups were identified as being dominant populations, i.e., *Geobacter* (6.38 %), *Levilinea* (4.06 %),

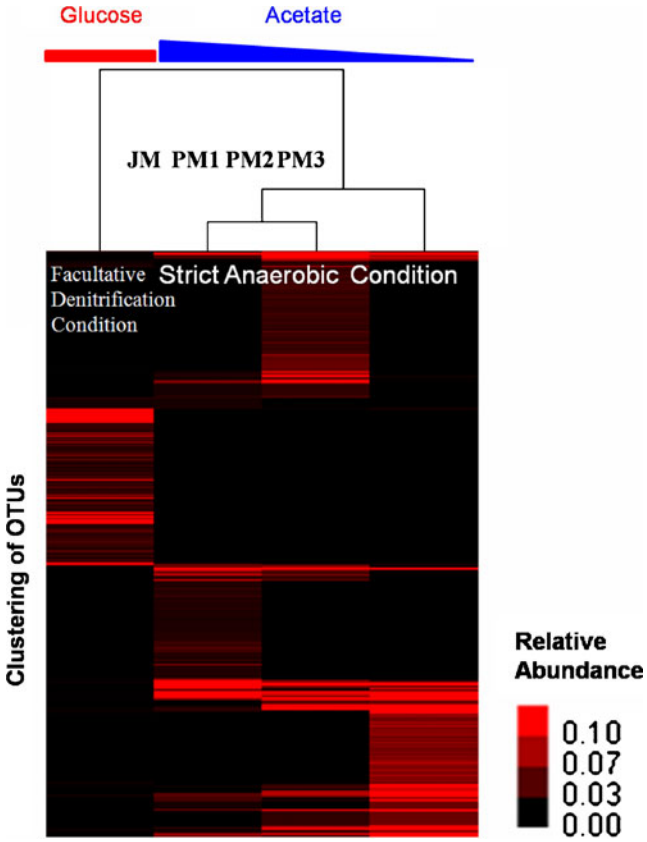

Fig. 3 Hierarchical clustering analysis for microbial community profiles of the tested anode biofilm samples. The *y*-axis indicates a dissimilarity cut-off <5%. The *color scale* indicates relative abundance (the *brightest red* indicates higher than 0.1, while *black* indicates 0.0)

Spoanaerobacter (2.43 %), *Peredibacter* (1.80 %), and *Rhodobacter* (1.01 %).

Commonly existing anode biofilm populations

Although the microbial diversity and community structures differed between the anode samples, pyrosequencing revealed 12 OTU groups that commonly existed in the different microbial communities (Table 2). To ensure statistical significance, the relative abundance cut-off values for screening the commonly existing groups were set at two times higher than the sequencing detection limits, i.e., one read per the number of total sequence reads for each sample. Since the number of sequence reads varied for each sample, different cut-off values were applied, 0.02 % for JM, 0.02 % for PM1, 0.04% for PM2, and 0.08% for PM3.

Most of the commonly existing anode biofilm (CEAB) groups may have been rare populations in the communities since their relative abundances were smaller than 1% (Table 2). One exception was CEAB17 in JM, which was the predominant population for the JM anode biofilm community. Although the individual CEAB populations seem to be rare, their overall relative abundances were not

Table 2 Relative abundances of the commonly existing anode microbes detected in this study

OTU ID	Unit (percent of total)			
	JM	PM1	PM2	PM3
CEAB3	0.02	0.29	0.75	0.21
CEAB6	0.04	0.05	0.04	0.17
CEAB8	0.02	0.24	0.71	0.84
CEAB9	0.02	0.10	0.16	0.17
CEAB11	0.02	0.05	0.16	0.25
CEAB13	0.02	0.05	0.04	0.08
CEAB14	0.02	0.05	0.11	0.34
CEAB15	0.03	0.02	0.04	0.08
CEAB17	11.72	0.24	0.07	0.08
CEAB18	0.02	0.06	0.60	1.17
CEAB19	0.03	0.32	0.53	0.17
CEAB21	0.02	0.02	0.07	0.08
Overall	11.98	1.49	3.28	3.64

negligible in each community (i.e., greater than 1%). In the JM sample, the overall relative abundance (11.98%) of CEAB groups was greater than in the PM samples (i.e., 1.49%, 3.28%, and 3.64%, respectively, for PM1, PM2, and PM3), and 97.8% of the JM CEAB members belonged to the CEAB17 group.

In the PM samples, the overall relative population abundances of total community increased when primary substrate concentration decreased. The relative abundances of individual CEAB groups differed in response to changes in the primary substrate exposure levels. In the case of CEAB17, the highest relative abundance was observed at the PM1 location, where primary substrate was sufficiently present. This suggests that CEAB17 may be a copiotrophic population. In the case of the CEAB6, CEAB8, CEAB11, CEAB14, and CEAB18 groups, the highest relative abundance was observed at the PM3 location, where primary substrate exposure was limited. This suggests that these CEAB populations may have preferred oligotrophic conditions. CEAB3, CEAB9, and CEAB19 grew in both oligotrophic and copiotrophic conditions, indicating their stable growth under varying primary substrate exposure levels. In the case of CEAB13, CEAB15, and CEAB21 groups, however, no definitive conclusions can be drawn regarding their growth response since their relative abundance values were not significantly different from the detection limits.

Bacterial classification and identification were performed for the screened CEAB populations. The CEAB populations were found to belong to alpha-, beta-, delta-, and gamma-subclasses of Proteobacteria and Firmicutes (Table 3). Except for CEAB11 and CEAB18, at least two different database searches showed consistent best-match

Table 3 Bacterial identification of the commonly existing anode microbes detected in this study (the identified strain is the best match with each representative population from each OTU cluster [dissimilarity cut-off<5%])

OUT ID	Accession number	RDP-II		Greengenes		NCBI BLAST	
CEAB3	GU594281	(D) *Geobacter psychrophilus* P39	81%	(D)*Geobacter psychrophilus* P35	95%	(D) *Geobacter psychrophilus* P35	95%
CEAB6	GU594282	(B) *Thauera* sp. 27	100%	(B) *Thauera* sp. 27	100%	(B) *Thauera* sp. 27	100%
CEAB8	GU594283	(B) *Variovorax limosa* EMB320	96%	(B) *Variovorax limosa* EMB320	96%	(B) *Polynucleobacter* sp. Fw70s-77	96%
CEAB9	GU594284	(G) *Thioflavicoccus mobilis* 83	92%	(G) *Thiococcus* sp. AT2204	95%	(G) *Thiococcus pfennigii* 4252	95%
CEAB11	GU594285	Unclassified bacteria	N.A.	(A) *Methylocystis* sp. KS7	81%	(A) *Oceanibaculum indicum* P24	81%
CEAB13	GU594286	(A) *Novosphingobium lentum* W-51	97%	(A) *Sphingomonas* sp. MT1	99%	(A) *Sphingomonas* sp. MT1	99%
CEAB14	GU594287	(A) *Afipia* sp. SP17	100%	(A) *Afipia* sp. SP17	100%	(A) *Afipia* sp. SP17	100%
CEAB15	GU594288	(F) *Fusibacter* sp. SA1	95%	(F) *Fusibacter* sp. SA1	95%	(F) *Fusibacter* sp. SA1	95%
CEAB17	GU594289	(G) *Pseudomonas mendocina PC12*	99%	(G) *Pseudomonas mendocina* PC12	99%	(G) *Pseudomonas mendocina* PC12	99%
CEAB18	GU594290	Unclassified bacteria	N.A.	(C) *Dehalococcoides* sp. BHI80-15	75%	(D) *Sorangium cellulosum* M5	85%
CEAB19	GU594291	(G) *Acinetobacter* sp. DZ0503SBS4	98%	(G) *Acinetobacter* sp. DZ0503SBS4	98%	(G) *Acinetobacter* sp. DZ0503SBS4	98%
CEAB21	GU594292	(F) *Clostridium lituseburense*	93%	(F) *Clostridium* sp. C01-2409	97%	(F) *Clostridium lituseburense*	96%

The letter in parenthesis indicates the identified phylum for each OUT group. The percent number indicates sequence similarity between the best-matched database sequence and the representative sequence of each OUT group

A alpha-Proteobacteria, *B* beta-Proteobacteria, *D* delta-Proteobacteria, *G* gamma-Proteobacteria, *C* Chloroflexi, *F* Firmicutes

results at the genus or species levels (Table 3), confirming reliability of our classification and identification. At the species level (dissimilarity cut-off <3%), the CEAB8, CEAB13, CEAB14, CEAB17, and CEAB19 groups were identified as *Thauera* sp., *Sphingomonas* sp., *Afipia* sp., *Pseudomonas mendocina*, *Acinetobacter* sp., respectively. At the genus level (dissimilarity cut-off <5%), CEAB3, CEAB8, CEAB9, CEAB15, and CEAB21 were identified as *Geobacter*, *Variovorax*, *Fusibacter*, and *Clostridium*, respectively. It is necessary to note that RDP-II classified the CEAB3 group as non-*Geobacter*, while Greengenes and NCBI BLAST classified it as a *Geobacter* genus group. For CEAB11 and CEAB18, no close relatives were found in the databases. Their sequence similarity with the best match was lower than 85%, indicating the possibility that they are novel bacteria.

To compare these commonly existing anode microbes with previously known anode bacteria, phylogenetic analysis was performed for the identified CEABs together with representative anode bacteria from previous studies (Aelterman et al. 2006; Back et al. 2004; Clauwaert et al. 2008; Kim et al. 2004, 2006; Lee et al. 2003; Logan et al. 2005; Phung et al. 2004; Rabaey et al. 2004). CEAB3, CEAB8, CEAB9, CEAB11, CEAB15, and CEAB18 were found to be phylogenetically distant (dissimilarity >7%) from previously identified anode bacteria (Fig. 4).

Discussion

In this work, we used FLX Titanium pyrosequencing in microbial community analysis of MFC anode biofilms and to determine CEAB populations under different respiration and substrate exposure conditions. While the pre-existing pyrosequencing methods have been successfully used to identify microbial communities from wastewater treatment sludge (Hamp et al. 2009; McLellan et al. 2009; Parameswaran et al. 2010; Sanapareddy et al. 2009; Szczepanowski et al. 2008; Zhang et al. 2009), to the best of our knowledge, the present study represents the first case where Titanium pyrosequencing has been used in microbial exploration of wastewater-treating microbial sludge and MFC electrode biofilms. The previous MFC studies were limited in that they could not capture information on anode bacterial populations with a relative abundance of less than 1% due to the limitations of the sequencing detection methods they used (Aelterman et al. 2006; Chae et al. 2009; Chung and Okabe 2009; Ishii et al. 2008; Ki et al. 2008; Kim et al. 2004, 2006, 2007; Lee et al. 2003; Logan et al. 2005; Phung et al. 2004; Rabaey et al. 2004; Reimers et al. 2007; White et al. 2009). This limitation was successfully circumvented herein with use of Titanium pyrosequencing, which allows for high-throughput sequencing of bacterial 16S rRNA gene ampli-

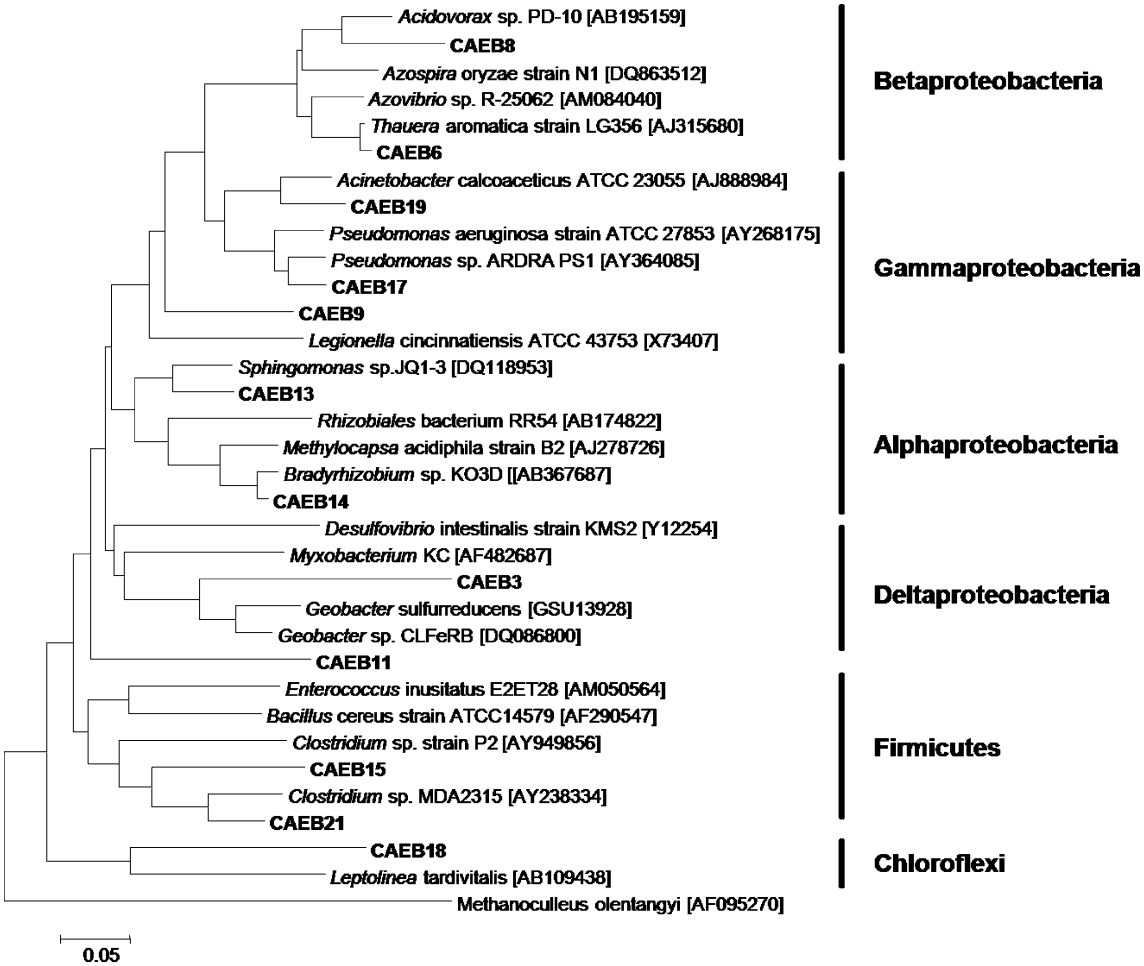

Fig. 4 Phylogenetic tree illustrating the relationship between the commonly existing anode isolates (from this study) and previously known anode-dominant relatives (references). The *scale bar* represents 5% difference in nucleotide sequences

cons. Furthermore, the extended read length (~400 bp) allowed by Titanium pyrosequencing improved the resolution of bacterial identification at the species level. This conclusion is consistent with a previous study that used Titanium pyrosequencing for medical purposes (Wolcott et al. 2009). In addition, this study showed that the RDP's pyrosequencing pipeline can be successfully used in high-throughput bacterial community analysis for sequence reads generated by titanium-based pyrosequencing. Indeed, our results yielded bacterial identifications that were consistent with the other two database search methods (Table 3). This supports the validity of use of the RDP's pyrosequencing pipeline for analyzing Titanium pyrosequencing results. This finding is significant because the high-throughput pyrosequencing pipeline method, which was originally developed for non-titanium amplicon pyrosequencing (Cole et al. 2008), had never been used to analyze the massive sequence reads generated by titanium-based pyrosequencing prior to this study.

To assess detection ability of FLX Titanium pyrosequencing comparing with the clone library analysis, we compared with the survey of previously identified anode populations (Clauwaert et al. 2008). Our results showed a different composition of anode biofilm populations, i.e., delta-subclass of Proteobacteria and Actinobacteria were the major groups, while gamma- and beta-subclasses of Proteobacteria were the major groups identified by Clauwaert et al. 2008, and significant portions of 16S rRNA sequence reads from the JM and PM anodes were not classified to even class level, indicating that numerous bacteria in the anode biofilms are novel.

Using Titanium pyrosequencing in combination with the high-throughput bacterial classification and identification methods, we have revealed the existence of some commonly occurring bacterial populations in the anode biofilm communities under different substrate exposure and respiration conditions (Fig. 3 and Table 3). We have also shown that most of the CEAB members were rare in the MFC anode biofilm communities (Table 2). Although the CEAB members were not dominant microorganisms in the anode biofilm communities, the current findings have led us to speculate that the CEAB members may be actively involved in anode electricity generation. The first reason

for this speculation is that in nature, a rare microbial population often plays a critical role in the eco-physiology of an entire community (Musat et al. 2008). Secondly, the steady growth of CEAB populations, under different eco-physiological conditions (primary substrate and respiration conditions), suggests that the CEAB populations have obtained certain benefits from interacting with the electricity-generating anodes. However, whether the steady growth of CEAB populations was due to their preference to electrode biofilm formation, their preference for respiring on anode or other unknown mechanisms have yet to be determined (Rabaey et al. 2007; Lovley 2008).

Most of the CEAB populations (more than 75%) exhibited oligotrophic growth or growth that was insensitive to primary substrate exposure levels. Five CEAB populations (CEAB6, CEAB8, CEAB11, CEAB14, and CEAB18) showed oligotrophic responses. This was confirmed by the following bacterial identification. Except for the two unclassified groups (CEAB11 and CEAB18), the majority of the CEAB populations (CEAB6, CEAB8, and CEAB14) were identified as typical oligotrophs, such as *Thauera* (beta-Proteobacteria), *Variovorax* (beta-Proteobacteria), and *Afipia* (alpha-Proteobacteria) genus populations (Kamagata et al. 1997; Moosvi et al. 2005). In addition, three CEAB populations (CEAB3, CEAB9, and CEAB19) exhibited growth that was insensitive to primary substrate exposure levels and were identified as *Geobacter* (delta-Proteobacteria), *Thiococcus* (gamma-Proteobacteria), and *Acinetobacter* (gamma-Proteobacteria), respectively. Due to their steady growth under varying substrate exposure conditions, such anode biofilm populations exhibiting oligotrophic and substrate-concentration-insensitive growth may prove useful microbial resources for MFCs integrated with wastewater treatment plants, in which organic substrate concentrations widely fluctuate.

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