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Bacterial Community Structure of Autotrophic Denitrification Biocathode by 454 Pyrosequencing of the 16S rRNA Gene

Yong Xiao · Yue Zheng · Song Wu · Zhao-Hui Yang · Feng Zhao

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Abstract Few studies have been conducted to explore the community composition in denitrifying biocathode. Herein, the microbial communities of denitrifying biocathodes yielding current of 1 mA (reactor C1) and 1.5 mA (reactor C2) were characterized by 454 pyrosequencing. The nitrate removal efficiencies in C1 and C2 were about 93 and 85 %, respectively. The optimization of data generated high-quality sequences of 18509 in C1 and 14857 in C2. Proteobacteria was the predominant phylum, and Bacteroidetes, Chloroflexi, and Planctomycetes were the subdominant groups. Classes of Alphaproteobacteria, Anaerolineae, and Phycisphaerae may benefit the performance of current production and nitrate removal. Twenty-nine dominant operational taxonomic units (OTUs) accounted for 64 and 65 % of sequences in C1 and C2, respectively. A denitrifying pathway was constructed based on the phylogenetic analysis and function inferring of the dominant OTUs. Obviously, the 454 pyrosequencing provided a high-resolution profile of bacteria community in denitrifying biocathode.

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Y. Xiao (✉) · Y. Zheng · S. Wu · F. Zhao (✉)
Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
e-mail: yxiao@iue.ac.cn
e-mail: fzhao@iue.ac.cn

Y. Zheng · S. Wu · Z.-H. Yang
College of Environmental Science and Engineering, Hunan University, Changsha 410082, China

Introduction

As nitrate can cause eutrophication in water body and be transformed into toxic nitrite in human body, nitrate contamination has become a serious worldwide concern. Nowadays, a number of biological techniques have been developed to remove the nitrate pollution, and most of these techniques use heterotrophic microbes and require organic matter as electron donor for reducing nitrate to nitrogen gas. However, organic matter content is low in some water bodies, e.g., groundwater, and additional organic matter is needed for conventional denitrification techniques. But, the addition of external organic matter may cause secondary pollution and microbial contamination in these water bodies. Compared with heterotrophic denitrification techniques, autotrophic denitrification is a more sustainable and economic solution as organic carbon is not necessary. In autotrophic denitrification, inorganic reductants such as hydrogen are served as electron donors [1]. However, hydrogen is usually yielded from electrolysis which requires a high energy input.

Microbial fuel cell (MFC) can produce electricity from organic matters in domestic or industrial wastewater. In MFCs, exoelectrogenic microbes oxidize organic contaminants in the anodic cells and produce electrons which are transferred to anode and then flow to cathode [2]. On cathode, many oxidants (e.g., oxygen, potassium ferricyanide) can be abiotically or biologically reduced with the electrons. Several studies have showed that a kind of biocathode can efficiently achieve nitrate removal coupling with electricity production, and the biocathode depends on the activity of autotrophic denitrifying microbes [3–6]. Thus, it is important to investigate the community of autotrophic denitrifying microbes for understanding and promoting the nitrate removal.

In previous studies, the communities of autohydrogentrophic denitrifying microbes, using hydrogen as electron donor, have been investigated [7–10]. However, the potentials of autotrophic denitrifying biocathode in MFCs are above 0 V versus normal hydrogen electrode [11, 12], and the hydrogen concentration is too low to be utilized as electron donor for denitrification. Consequently, the autotrophic denitrifying microbes on MFC cathode directly may use the electrons to reduce nitrate to nitrogen gas, and the communities of these microbes may differ from that of autohydrogentrophic denitrifying microbes.

Presently, understanding of autotrophic denitrifying microbial communities on MFC cathodes is limited as only one study has been conducted with phylogenetic microarray to characterize bacterial communities contributing to cathodic denitrification [13]. Next-generation sequencing technology generates a large number of DNA reads, i.e., the 454 pyrosequencing can generate more than 800,000 reads of ~400 bp in a single run, and it has been proved a suitable method for accurate microbial community analysis [14]. Therefore, the main objective of the study was to profile the bacterial community in denitrifying biofilms upon cathode using 454 pyrosequencing. On the other hand, we would like to see whether current/potential had some effects on the community composition.

Materials and Methods

MFC Construction and Operation

Two MFCs, i.e., C1 and C2, were constructed by joining two acrylic reactors (10×10×11 cm) containing a cation exchange membrane (8×8 cm) (QQ-YLM, Qianqiu Group, China) clamped between the two reactors. Each electrode in anode and cathode consisted of three pieces of carbon felt (6×6×0.4 cm each) without any modification, and titanium wires and fixed resistors were used to connect the circuit (Fig. 1).

The two-chamber MFCs were started up by two stages (Fig. 1). Stage 1 was conducted to culture the anode electrogenic microbes. The anode chambers were inoculated with activated sludge (about 5 g dry weight) from a domestic wastewater treatment plant in Jimei, Xiamen City, China. The anode and cathode chambers were filled with 1 L anolyte of 10 mM CH_3COONa, 32 mM K_2HPO_4, and 18 mM KH_2PO_4 and 1 L catholyte of 50 mM $K_3[Fe(CN)_6]$, 32 mM K_2HPO_4, and 18 mM KH_2PO_4, respectively. The MFCs were operated in batch mode, and the external resistors in stage 1 were 1,000 Ω. In each cycle, 60 % of anolyte was replaced with 600-mL fresh medium comprising 16.7 mM CH_3COONa, 32 mM K_2HPO_4, and 18 mM KH_2PO_4, and all the catholyte was replaced with 1-L fresh solution of 50 mM $K_3[Fe(CN)_6]$, 32 mM K_2HPO_4, and 18 mM KH_2PO_4.

After the anode has produced steady current, stage 2 was conducted to culture autotrophic denitrifiers. The anode chamber was still operated in batch mode, and the anolyte was replaced as that in stage 1. Potassium ferricyanide was removed from the cathode chambers, and the cathode was inoculated with the same activated sludge (about 5 g dry weight) as that for anode. The cathode was operated in continuous mode by feeding with a catholyte of 1.5 mM $NaNO_3$, 5 mM Na_2CO_3, 32 mM K_2HPO_4, and 18 mM KH_2PO_4. The effective volume of the cathode chamber was 1 L, and hydraulic retention time of catholyte was 120 h. The pH of anolyte and catholyte was adjusted to about 7.0. The dissolved oxygen in biocathode ranged from 0.2 to 0.5 mg L^{-1}. The external resistors in C1 and C2 were 200 and 500 Ω, respectively, and the current was stabilized after being started up for 1 month. The MFCs were operated at 25±3 °C.

Analysis and Calculations

Water samples from cathode chamber were immediately filtered with a 0.22-μm sterile filter. NO_3^- and NO_2^- were determined according to the standard methods issued by the Ministry of Environmental Protection of China. All analyses were performed in triplicate.

The voltage (E) across the resistor (R_e) and the cathodic half-cell potential (vs. Ag/AgCl) of the MFCs were recorded by an Integra 2700 series equipped with 7700 multiplexer (Keithley Instruments, USA), and the current (I) through the electrical circuit was calculated by $I=E/R_e$. Electrons harvested through the electrical circuit (Q) were determined by integrating current over time ($Q=\int I\mathrm{d}t$).

Sampling and DNA Extraction

After being operated stably for 1 year, biofilm samples were carefully collected from cathode electrodes in the MFCs and stored at −20 °C before the DNA extraction. For each sample, the genomic DNA was extracted using a previously reported protocol using CTAB and proteinase K [15], which has successfully extracted genomic DNA from compost and sludge [16–18]. The extracted genomic DNA was purified with a kit (DP1501, BioTeke, China). DNA quality was assessed by agarose gel electrophoresis and the 260/280- and 260/230-nm absorption ratios on an ND-2000 spectrophotometer (Nanodrop, USA).

Bacterial 16S rRNA Gene Amplification and 454 Pyrosequencing

Before pyrosequencing, the purified DNA was amplified with a set of primers targeting the V1–V3 hypervariable regions of bacterial 16S ribosomal RNA (rRNA) genes. The forward primer was 5′-AGAGTTTGATCCTGGCTCAG-3′ (27 F)

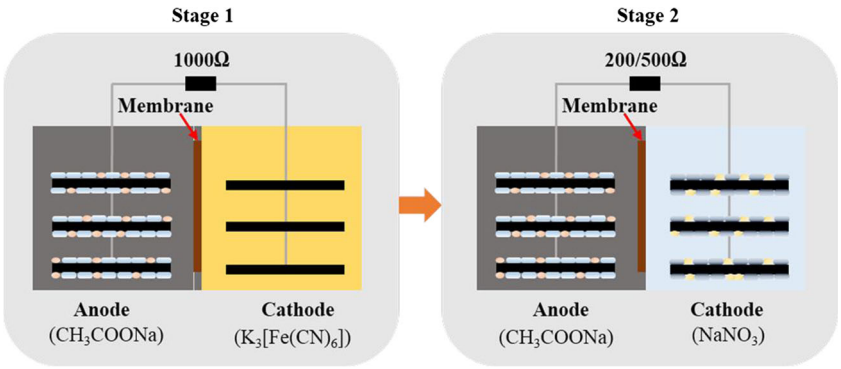

Fig. 1 Construction and operation of two-chamber MFCs

with the Roche 454 "B" adapter, and the reverse primer was 5′-TTACCGCGGCTGCTGGCAC-3′ (533R) which containing the Roche 454 "A" adapter and specific 10-bp barcode. The Roche 454 A/B adapter located on the 5′-end of each primer, respectively. Each 20-μL PCR reaction system contained 4 μL of 5×FastPfu buffer, 2 μL of dNTPs (2.5 mM), 0.8 μL of forward primer (5 μM), 0.8 μL of reverse primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of template DNA (the rest of bulk was add by Milli-Q water). The PCR amplification followed the conditions: 1 cycle of initial denaturation at 95 °C for 2 min; 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min. The PCR products were quantitated by QuantiFluor™ ST and then mixed for pyrosequencing. The high throughput pyrosequencing was processed on Roche GS FLX+ System.

Processing of Pyrosequencing Data

The 16S pyrosequencing data were processed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline [19]. Before the statistical analysis of data, QIIME was used to (1) check the completeness of the barcodes and the primer sequencing, (2) remove reads shorter than 300 bp, (3) and remove reads comprising chimera and quality score below 25. Secondly, the sequences of different samples were exactly assigned using the unique 10-bp barcodes from raw data, and then the barcodes and primer sequences were removed. Only the 97 % identity of the effective sequences were divided into operational taxonomic units (OTUs) for further analysis, and the most abundant sequences from each OTU were selected as the representative sequence. After the representative sequences had were assigned by PyNAST [20], those were used for the classification of taxonomic according the Greengenes database. In order to controlling error as far as possible, we randomly selected 9,000 sequences from per sample to explore the alpha-diversity in each sample and compare the beta-diversity between sample by UniFrac metric. To response the diversity and in every sample, the number of OTUs, Chao1 index, and phylogenetic richness index diversity was calculated from each sample. In the light of the OTU table, we performed principal component analysis by in R v.2.15.0 with the vegan package. OTU abundance is relative abundance determined by dividing the number of sequences for any given OTU by the total number of sequences obtained for that sample.

Twenty-nine OTU sequences with high abundance in samples C1 or C2 have been deposited in the GenBank database under accession numbers KF292180–KF292208.

Results and Discussion

Performance of MFCs

During 1-year operation, MFCs of C1 and C2 showed stable power output and nitrate removal abilities, and performance of voltage, cathodic potential, and nitrogen concentration during the 4 cycles before sample collection was shown in Fig. 2. The maximum voltage outputs in C1 and C2 were about 0.3 and 0.5 V, respectively (Fig. 2a). As the fixed external resistors in C1 and C2 were 200 and 500 Ω, the maximum currents in C1 and C2 were 1.5 and 1 mA, respectively. Therefore, the nitrate removal efficiency in C1 (about 92.5 %) was higher than that in C2 (about 85 %) during the continuous operation (Fig. 2c).

Though C1 and C2 yielded about 1.2 and 0.9 mmol of electrons per day, respectively, the electrons were insufficient to reduce all removed nitrate to nitrogen gas. Nitrite concentration in both MFCs was as low as 0.05 mM (Fig. 2c), and C1 and C2 removed 0.37 and 0.34 mmol of nitrate per day, respectively. Therefore, N_2O may be an important component of reduction products. We used a N_2O microelectrode to detect the concentration of N_2O in catholyte [21]. Due to very low partial pressure of N_2O in the atmosphere, N_2O escaped out of catholyte very fast. Therefore, the collected data from the N_2O microelectrode showed no difference between tests with and without biofilms.

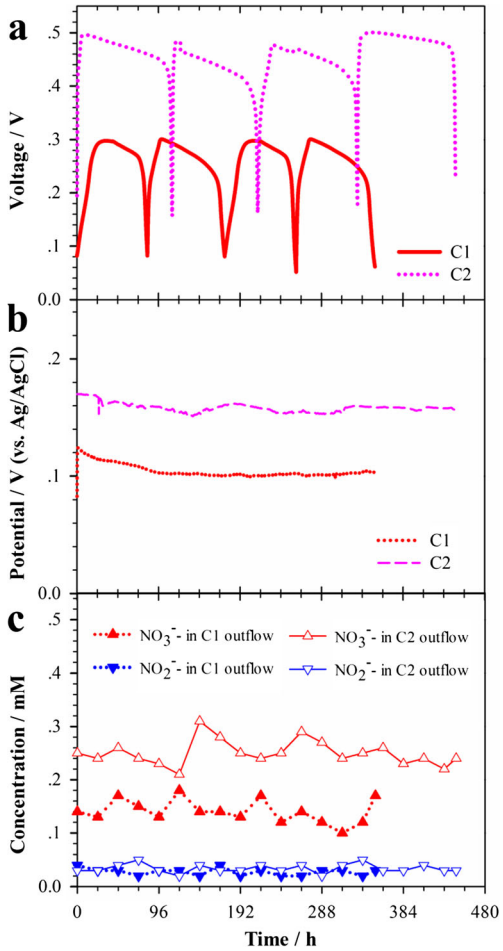

Fig. 2 Performance of the MFCs and their nitrate removal abilities during 4 cycles before sample collection. **a** Voltage as a function of time in MFCs of C1 and C2, **b** cathodic potential as a function of time for C1 and C2, and **c**: average concentration of NO_3^- and NO_2^- in cathodic effluent

The cyclic voltammetry analysis of cathodic biofilm showed reductive peak potentials at 0.12 and 0.16 V (vs. Ag/AgCl) in C1 and C2, respectively, and the peak current in C1 was significantly higher than that in C2 (Fig. A1). The stable cathodic potentials in C1 and C2 were at about 0.1 and 0.16 V (vs. Ag/AgCl) (Fig. 2b), respectively. The cathodic potential was also in accordance with previous reports [11, 12]. The results indicated that the electrochemically active cathodic biofilm might adopt different microbial communities. Comparing to MFC C2, C1 seemed to have higher current, possess lower potential, and perform a higher ability of denitrifying.

Overall Pyrosequencing Information

In this study, 28,945 and 23,654 valid reads were produced from samples C1 and C2 by means of Roche GS FLX+ System, respectively (Table A1). The optimization of data generated high-quality sequences of 18,509 in C1 and 14,857 in C2 (Table A1). The goods coverage was 92.5 for C1 and 92.2 % for C2. The average length of sequences is 482.8 bp. The OTU numbers increased with the number of sequences. A plot of OTUs vs. the number of 16S rRNA gene sequences resulted in rarefaction curves which did not approach a plateau (Fig. 3), in spite of a large number of sequences. At 3 % distance, the maximum OTU numbers of 4854 and 4559 for C1 and C2, respectively, were predicted by the nonparametric Chao1 estimator (Table A1). This study showed a rich bacterial diversity in autotrophic denitrifying biofilm.

Phylogenetic Identification in Bacterial Community

Figure 4 showed the relative abundances on phylum and class levels in the bacterial communities. Proteobacteria was the predominant phylum, constituting 40 % of all sequences in C1 and 49 % of all sequences in C2 (Fig. 4a). Bacteroidetes, Chloroflexi, and Planctomycetes were the subdominant groups, comprising 14 and 24, 13 and 5, and 25 and 14 % of the detections in C1 and C2, respectively (Fig. 4a). The four phyla represented more than 90 % of sequences within both samples. In contrast, other phyla of Acidobacteria, Actinobacteria, and Chlorobi showed similar low abundances (0.8 to 1.4 %) in both samples, suggesting that bacteria from these phyla were not dominant species regardless of MFC operation method.

In previous reports, Proteobacteria is usually detected as the dominant phylum in autohygrogentrophic denitrifying biofilm [7, 9]. A study using PhyloChip also shows that Proteobacteria dominates some cathodic denitrifying biofilms [13]. Though Firmicutes was reported as the predominant phylum in those biofilms achieving better performance [13], our results showed less than 0.1 % of all sequences belonging to Firmicutes in samples C1 and C2. According to previous report, most Firmicutes denitrifies heterotrophically [22]. So, we thought that our study showed reasonable results of bacterial diversity. However, we did notice a decrease of Proteobacteria in reactor C1 which performed better in current production and nitrogen removal.

In this study, phylum of Proteobacteria was mainly consisted of alpha, beta, and gamma classes (Fig. 4b), which was in accord with previous report [13]. Alpha and beta classes of Proteobacteria seemed to be easily affected by operation conditions like current and potential because alpha and beta classes accounted for 22 and 10 % of sequences in C1 and 16 and 24 % of sequences in C2, respectively. In contrast, gammaproteobacteria seemed to be not affected by current or potential as its relative abundance was the similar 6.5 % in both MFCs.

Flavobacteriia and Sphingobacteriia, Anaerolineae, Phycisphaerae, and Planctomycetia were the main classes of Bacteroidetes, Chloroflexi, and Planctomycetes, respectively.

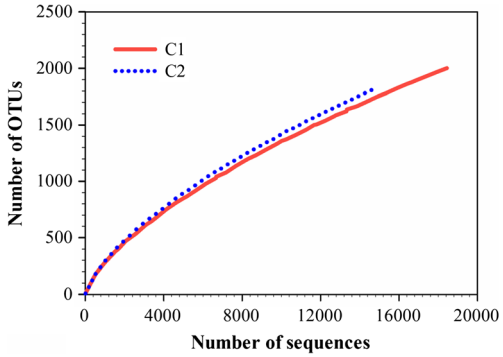

Fig. 3 Rarefaction curves depicting the effect of 16S rRNA gene sequence numbers on the number of operational taxonomic units (*OTUs*) (3 % distance) identified from the cathodic biofilm samples of C1 and C2

Sphingobacteriia (1.2 vs. 1.4 %) and Planctomycetia (4.3 vs. 3.9 %) had similar abundance in C1 and C2, suggesting that current or potential had limited effect on these bacteria (Fig. 4b). The abundance of Flavobacteriia in C2 was notably higher than that in C1 (C1 12.5 %, C2 22.6 %). However, the abundances of Anaerolineae and Phycisphaerae in C1 (11.7 and 20.6 %, respectively) were significantly higher than that in C2 (4.7 and 10.0 %, respectively). Since the performance of C1 in terms of current production and nitrate removal was significantly better than C2, the results meant that bacteria from classes Anaerolineae and Phycisphaerae might benefit the current production and nitrate removal of biocathode.

Dominant Members and Their Relationship to Autotrophic Denitrification

For the 33,366 high-quality sequences obtained from C1 and C2, a total number of 3,115 OTUs were classified with 3 % distance (Table A2). The relative abundance of most of OTUs was less than 0.1 %; however, there were 29 OTUs whose relative abundance was higher than 0.5 % in C1 or C2 (Fig. 5). The 29 OTUs accounted for 64 and 65 % of sequences in C1 and C2, respectively. Simpson's evenness was calculated for these dominant OTUs in each sample, and C1 had greater evenness (0.90) than that of C2 (0.83). The results indicated that different bacteria species might be selected as dominant population by specific operation method, and reactor with greater evenness seemed to yield higher current and possess higher nitrogen removal ability. Moreover, there were three most dominant OTUs, i.e., OTUs of 1837, 2039, and 2950 in C1 and OTUs of 1526, 1837, and 2950 in C2, which accounted for more than 5 % of the sequences. The three most dominant OTUs accounted for 31 and 41 % of sequences in C1 and C2, respectively. These results indicated that only several bacterial species dominated the denitrifying biocathode despite of high diversity in the bacterial communities.

In order to further compare the microbial communities of two samples, sequences of the 29 dominant OTUs were extracted from the pyrosequencing data, and a neighbor-joining phylogenetic tree was derived by analyzing the OTU sequences (Fig. 5). While most of OTUs in Proteobacteria showed very high similarity of 16S rRNA genes to known genus or species, only 2 of 11 OTUs in other classes had above 95 % similarity to 16S rRNA genes of known genus/species. It could be inferred that bacteria in Proteobacteria have been widely recognized, but little was known about denitrifiers in other classes.

Six OTUs showed high similarity to bacteria in Betaproteobacteria. OTUs 1526 and 3380 showed high similarity to *Thauera* sp. As reported by a previous study [23], *Thauera* sp. was enriched in autotrophic denitrifying microbial communities. Thus, we inferred that the two OTUs might be important for autotrophic denitrification in C2 as their relative abundance in C2 (as a sum) was about 15 %. However, the bacteria might not adapt to a larger current since their abundance in C1 (as a sum) was about 2 %. OTU 901 was not classified to specific genus, and the bacterium seemed to prefer C2 rather than C1 as the abundance in C2 was twice higher than that in C1. OTU 141 was 100 % similar to a strain of *Nitrosomonas europaea*, a typical ammonia-oxidizing bacterium [24]. Though the major form of nitrogen in influent was nitrate, the degradation of dead cells might release some ammonia feeding them. OTU 1516 showed 96 % identity to a *Ralstonia* sp. which was considered as a H_2 oxidizing

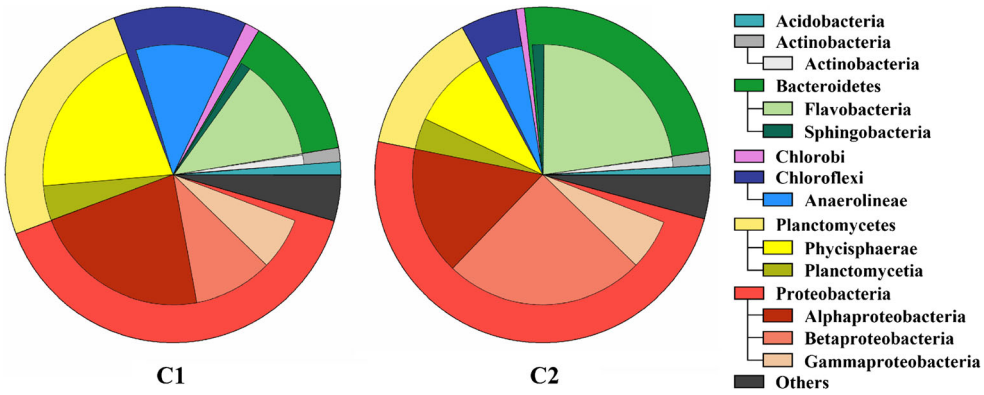

Fig. 4 Relative abundance of different bacterial phyla and classes in the sequenced 16S rRNA gene clone libraries (3 % distance)

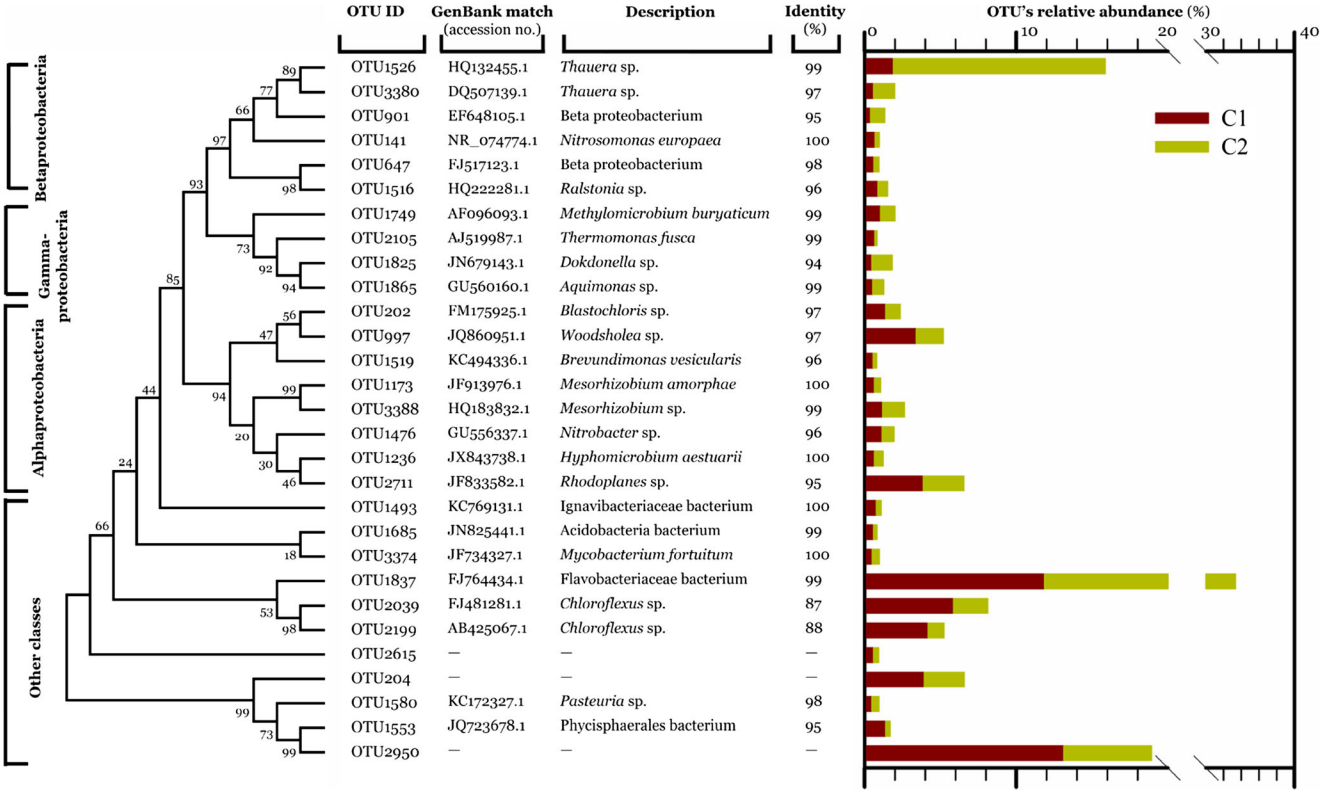

Fig. 5 Relative abundance of the dominant OTUs in the sequenced 16S rRNA gene sequences (3 % distance). Abundance of any OTU is >0.5 % in C1 or C2. A phylogenetic tree was also constructed based on 16S rRNA gene sequences of dominant OTUs. The tree was constructed using neighbor-joining algorithm. The percentage shown at each branch is gained from 1,000 bootstrap resamplings. The *scale bar* represents 2 % sequence divergence

denitrifier [25]. It is known that syntrophic anaerobic biodegradation can produce a big amount of hydrogen which might be used by *Ralstonia* for denitrification.

Compared with beta and gamma proteobacteria, Alphaproteobacteria had the richest diversity as eight OTUs showed high similarity to species in this class. Except for OTU 3388, the other seven OTUs had higher relative abundance in C1 than that in C2 and showed high identities to known genera. OTUs 997 and 2711 were two most abundant Alphaproteobacteria in C1 (3.4 and 3.9 %, respectively), and they were highly similar to sequences of known genera of *Woodsholea* (97 %) and *Rhodoplanes* (95 %), respectively. Few studies have focused on genus *Woodsholea*, and a report has indicated that the genus has no activity on nitrate reduction [26]. Though genus of *Rhodoplanes* previously has been reported as heterotrophic denitrifiers [27], no paper discusses its autotrophic denitrifying mechanisms. Thus, this strain might use dead cells or degradation products as carbon sources for heterotrophic denitrification. OTU 1173 related to a strain of *Mesorhizobium amorphae*, a nitrogen fixing bacterium [28]. OTU 1476 showed 96 % identity to *Nitrobacter* sp., a genus showing a unique ability to convert nitrite to nitrate [29]. It is surprising as nitrite concentration in these MFCs was lower than 0.05 mM. OTU 202 showed 97 % identity to *Blastochloris* sp., a genus photoheterotrophic bacteria [30]. OTU 1236 was 100 % similar to strains of

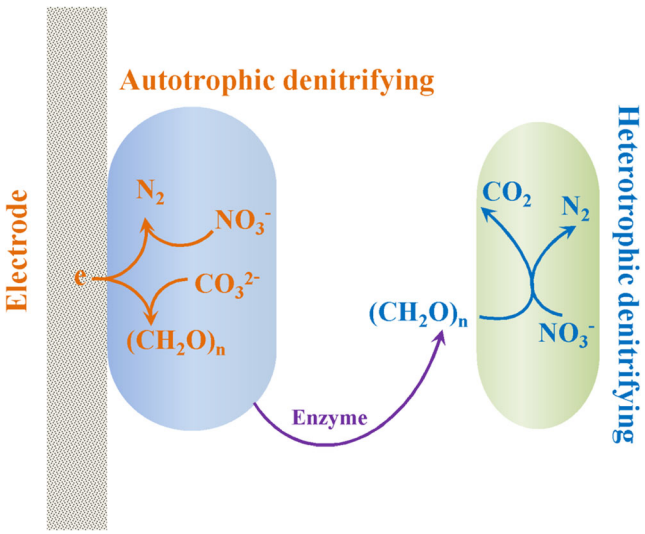

Fig. 6 Proposed pathways of nitrate reduction in the biocathode. Autotrophic denitrifying bacteria use electron from electrode to reduce nitrate and synthesis organics for growth. Dead cells are degraded to available carbon sources by enzymes from some heterotrophic bacteria. The degraded cells could also be used by heterotrophic denitrifying bacteria for nitrate reduction

Hyphomicrobium zavarzinii and *Hyphomicrobium aestuarii* that belong to a genus of heterotrophic denitrifiers [31], which means that the strain may use dead cells or degradation products as carbon sources for denitrifying. OTU 1519 had 96 % identity to *Brevundimonas olei*, a species not reducing nitrate [32]. As shown above, dominant OTUs belong to Alphaproteobacteria that showed a variety of functions, and no direct relationship between these OTUs and autotrophic denitrifying could be built.

Eleven OTUs were classified to cluster "Other classes" in the phylogenetic tree. OTUs 1580 and 3374 showed 98 and 100 % identity to genus *Pasteuria* and a strain of *Mycobacterium fortuitum*, respectively. Though, both of *Pasteuria* and *Mycobacterium fortuitum* had been not reported in autotrophic denitrifying reactor, and the function of them in biotcathode was uncertain. OTUs 204, 1493, 1553, 1685, 2615, and 2950 showed high similarity (>95 %) to unclassified sequences. It is interesting to find that the abundance of these unclassified OTUs in C1 was significantly higher than that in C2 ($p<0.01$), showing their higher adaptability to operation method of C1. Among these six OTUs, OTU 2950 was the most abundant one in C1, accounting for more than 13 % of sequences. The OTU also accounted for about 6 % of sequences in C2. The OTU had 100 % identity to a sequence reported in the granular sludge bed reactor for autotrophic nitrogen removal [33]. The result meant that this OTU might be very important for autotrophic denitrification in biocathode, and its abundance was positively correlated to current production and denitrification. OTUs of 2039 and 2199 were only 88 % similar to sequences that belong to *Chloroflexus* sp., suggesting that these OTUs might belong to unknown bacterial species. Since the abundance of these OTUs in C1 was 2.5 to 4 times higher than that in C2, they might also benefit the performance of biocathode. OTU 1837 was the most abundant one in C2 (>21 %), and its abundance was also very high in C1 (about 12 %), which indicated that the OTU was very important to the biocathode. However, it is difficult to investigate the OTU's function in the biofilm as the OTU showed only 94 % identity to *Flavobacterium* sp.

As shown above, we have investigated the OTUs with high abundance in communities. Though previous studies have reported autotrophic denitrifier dominant species in autotrophic denitrification systems [7–10], the results from this study showed that only a few OTUs directly contributed to autotrophic denitrification, and many other dominant OTUs showed high identity to heterotrophic bacteria. Based on sequence alignment and function analysis of the dominant OTUs, we proposed a pathway of nitrate reduction in the biocathode (Fig. 6). In the framework, autotrophic denitrifying bacteria (i.e., OTUs 1526 and 3380) use electrons from electrode to reduce nitrate and synthesis organics for growth. Some heterotrophic bacteria may release some enzyme to degrade dead cells as available carbon sources which could be used by heterotrophic denitrifying bacteria for nitrate reduction (i.e., OTUs 1236 and 2711).

Conclusions

The study shows that MFC with smaller external resistor had increased performance of current production and rates of nitrate removal. Pyrosequencing analysis using 16S rRNA genes indicated that autotrophic denitrifying biocathode in MFCs can sustain a great number of active bacterial OTUs and bacteria from Proteobacteria, Bacteroidetes, Chloroflexi, and Planctomycetes were dominant members in the autotrophic denitrifying biofilms. Classes of Alphaproteobacteria, Anaerolineae, and Phycisphaerae seemed to benefit the performance of current production and nitrate removal. Twenty-nine OTUs dominated the cathodic biofilms, and both autotrophic and heterotrophic denitrification seemed to be very important processes for nitrate removal after constructing a relationship between some dominant OTUs and their functions based on existent literature.

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