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SHORT COMMUNICATION

Direct enrichment of perchlorate-reducing microbial community for efficient electroactive perchlorate reduction in biocathodes

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Abstract Biological reduction of perchlorate (ClO_4^-) has emerged as a promising solution for the removal of perchlorate in contaminated water and soils. In this work, we demonstrate a simple process to enrich perchlorate-reducing microbial communities separately using acetate as electron donor and the municipal aerobic membrane bioreactor sludge as inoculum. Inoculation of cathodes in microbial fuel cells (MFCs) with these enrichments, and further electrochemical enrichment at constant resistance operation of the MFCs, led to perchlorate-reducing biocathodes with peak reduction rates of 0.095 mM/day (2 mg/m^2/day). Analysis of the microbial diversity of perchlorate-reducing biocathodes using PCR-DGGE revealed unique community profiles when compared to the denitrifying biocathode communities. More importantly, the total time taken for enrichment of the electroactive communities was reduced from several months reported previously in literature to less than a month in this work.

Keywords Perchlorate reduction · Biocathode · Microbial fuel cell · Bioelectrochemical enrichment · Microbial ecology

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Introduction

The perchlorate ion (ClO_4^-), arising largely from man-made perchlorate salts that are used as propellants, is a widespread environmental contaminant found to persist in sources of drinking water [15]. Among the many processes developed to treat perchlorate in water, biological processes are highly effective and economically attractive [10]. Many of the biological processes for perchlorate reduction rely on the ability of the microorganisms to use perchlorate as a terminal electron acceptor while heterotrophically using organic carbon as an electron donor [12]. However, when organic electron donors are used, secondary water quality issues arise due to the presence of residual substrate and fermentation metabolites, causing the water to require further treatment [6]. Early evidence on biological perchlorate reduction pointed to dissimilatory reduction linkages between perchlorate-reducing and nitrate-reducing microbes [5, 18]. Some perchlorate-reducing microbes were found to alternatively, and more favorably, respire nitrate [5]. Additionally, the growth rates when using perchlorate were reported to be much slower than when they used nitrate, prompting suggestions that nitrate presence inhibits perchlorate reduction [2]. Moreover, complete reduction of perchlorate was found to be dependent on the presence of the enzyme, chlorite dismutase, and anaerobic conditions [16].

Among the biological processes, perchlorate reduction using biocathodic microbial fuel cell (MFC) setups have the distinct advantage of decoupling oxidative (anodic)

reactions from reductive (cathodic) reactions across a proton exchange membrane (PEM), thereby minimizing any secondary water quality effects. Biocathodic nitrate reduction in MFCs has been well studied in literature, where an electroactive biofilm closely associated with a cathode utilizes electric current as an electron donor [3]. In contrast, only two groups have so far reported biocathodic perchlorate reduction [1, 18]. The biocathodic reduction of perchlorate was first reported by Thrash et al. [18], who used a poised cathode (−500 to −450 mV against a Ag/AgCl reference electrode) together with pure cultures *Dechloromonas* sp. and *Azospira* sp., and reported perchlorate reduction at a rate of 90 mg/l/day only in the presence of the electron shuttle, anthraquinone-2,6-disulfonate (AQDS). In addition, they reported perchlorate reduction by natural mixed culture again in the presence of AQDS mediator and under poised cathode conditions. A perchlorate-reducing strain VDY, matching *Dechlorospirillum anomalous* by 16S rDNA analysis, was isolated from this natural mixed culture and was tested in batch and continuous MFC configurations without AQDS, and resulted in perchlorate removal rates of 17 mg/l/day and 60 mg/l/day, respectively. However, both systems showed electrolytic molecular hydrogen presence, indicating that electron donor in these systems might have been molecular hydrogen rather than electricity.

Butler et al. [1], observed biocathodic perchlorate reduction at a maximum rate of 24 mg/l/day, using a continuous MFC reactor with granular graphite electrodes. Cathodes were inoculated with a mixed consortia comprised of (1) a culture from a denitrifying biocathode, (2) local activated sludge, (3) a chlorate reducing enrichment from a lake sediment, and (4) the perchlorate-reducing pure culture of *Dechloromonas* sp. PC1. The full experiment lasted for more than 400 days and for the initial 84 days the cathode was fed only with nitrate achieving the biocathodic nitrate reduction. Then perchlorate concentration was increased slowly, reaching a 100 % perchlorate feed by day 303.

In our study, a simplified two-chamber biocathodic MFC with a constant resistance was used. The MFC was operated in fed-batch mode after the cathode chamber was inoculated with perchlorate-reducing precultures. Precultures of unique perchlorate-reducing and nitrate-reducing microbial communities were enriched from a mixed aerobic membrane bioreactor (MBR) sludge sample. The direct selection of electroactive perchlorate-reducing microorganisms led to faster biocathodic perchlorate reduction. Profiles of biocathodic perchlorate-reducing MFC biofilms were compared to the profiles of the preculture enrichments and to electroactive denitrifying MFC biofilms.

Materials and methods

Preculture enrichment

A mixed culture sludge sample from a municipal membrane bioreactor (MBR) plant in Masdar City, Abu Dhabi, UAE, was used as the microbial inoculum source, while acetate was used as the electron donor and perchlorate and nitrate as the electron acceptors for perchlorate- and nitrate-reducing culture enrichment methods, respectively. The cultures were inoculated into 60-ml sealed serum bottles (working volume of 50 ml) with M9 medium [8] and the media for perchlorate-reducing microorganisms was adjusted to pH 8.5 in accordance with previous studies [2]. The mixed cultures were grown anaerobically at room temperature 24 ± 2 °C. The inoculum's volumetric ratio of total culture to media was 1:12 (v/v). A 2-ml sample was removed every other day for chemical analysis and immediately replaced by a 2-ml aliquot of feeding media. Every second day, the denitrifying preculture was fed 41.02 mg sodium acetate and 101.1 mg potassium nitrate in a 50-ml working volume of M9 media. The perchlorate-reducing preculture was fed every second day with 8.203 mg sodium acetate and 24.5 mg sodium perchlorate in a 50-ml working volume of M9 media. The total volume in each bottle was kept constant over the course of the fed-batch experiment. Sample removal and electron acceptor addition controls were fed either 101.1 mg potassium nitrate and 41 mg sodium acetate or 24.5 mg sodium perchlorate and 8.2 mg sodium acetate at day zero. Subsequently, the same sampling and feeding protocol as the biotic enrichment cultures was followed with the controls. All controls were anaerobic and under sterile conditions. All biotic enrichments and abiotic were stirred at the same rate on a multi-point stir plate.

MFC operation and biocathodic enrichment of cultures

The two-chambered MFC used in the experiments are shown in Fig. 1 and S1. The electrodes used consisted of 2.5 × 3.5-cm twisted titanium wire—carbon fiber brushes (specific surface area of 1.07 m^2). The cathode chamber of the MFC reactors was inoculated with 10 ml preculture inoculum and 10 ml of fresh sludge in a final working volume of 230 ml. The reactors operated in batch mode at 24 ± 2 °C, and a pH of 7.0 for the denitrifying MFC, and a pH of 8.5 for the perchlorate-reducing MFC. The perchlorate-reducing MFC was operated in open circuit for a day, before its anode and cathode were connected across a 500-Ω resistor. The anodic chamber media contained (in g/l): 1 NH_4Cl, 0.1 KCl, 5 $NaH_2PO_4{\cdot}H_2O$, 9 Na_2HPO_4, 0.2 yeast extract, 2.5 $NaHCO_3$ [14]. The bioanode chamber was fed with 0.46 g sodium acetate as needed. The

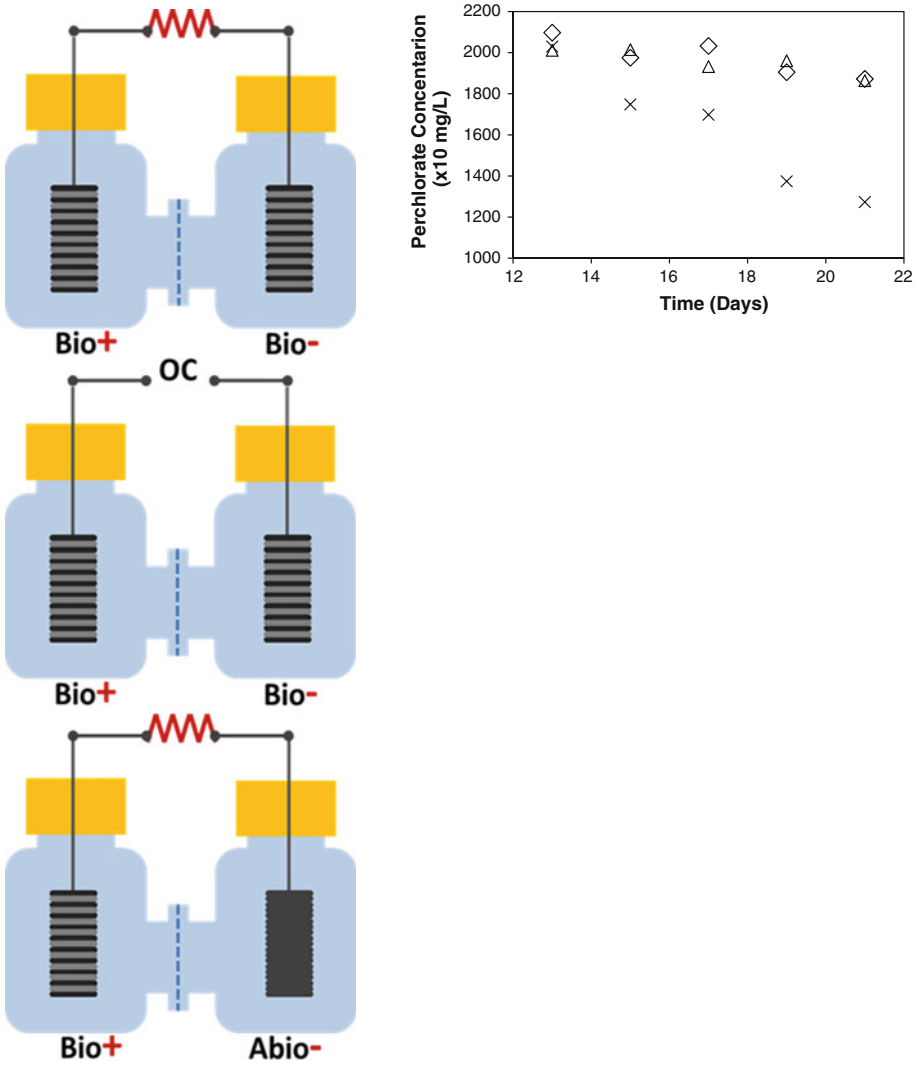

Fig. 1 Figure on the *left* shows a cartoon depiction of the experimental design for microbial fuel cell (MFC) runs. All MFCs were run with acetate-oxidizing bioanodes (Bio+). The *top figure* depicts an MFC with a biocathode (Bio−) potentially capable of reducing perchlorate (*multiplication sign*) connected with the bioanode across a 500-Ω resistance. The *middle figure* (control A) depicts the same setup as the *top figure* but with an open circuit (OC) or infinite resistance control (*diamond*), while the *bottom figure* (control B) depicts an abiotic cathode (Abio−) control (*triangle*). The *graph* above exhibits the variation of bulk perchlorate concentration (in mg/l) in the biocathode chamber over time with respect to controls A and B. A late (day 15) but significant onset of perchlorate reduction was observed in comparison to the controls

bioanode consisted on an electroactive heterotrophic acetate oxidizing biofilm grown from previous MFC experiments that utilized the same inoculum and were run with an open-air cathode. After inoculation, both chambers were purged with argon for 30 min.

MFC chambers were separated by a CMI-7000 cation exchange membrane (Membranes International Inc., Ringwood, NJ, USA). Each chamber had a total working volume of 230 ml. Both carbon fiber brushes electrodes were pretreated using a method described in the literature [8]. Both electrodes were connected by a resistor and to a datalogger (Keithley Instruments, 2701 Ethernet Multimeter, USA) to monitor the voltage generated between the two chambers (Fig. 1). Polarization curves were run on the perchlorate-reducing MFC periodically using variable resistance values in order to ensure stability of the electrochemical operation. MFC controls were run such that control A had the bioanode and biocathode set up in the same manner as the contaminant bioelectrochemical reduction experiment, but with an open circuit (i.e., infinite resistance or no current flow), and a bioanode coupled to an abiotic un-inoculated cathode in control B. Samples were taken every other day after the first day of MFC operation.

Chemical analysis

Samples obtained from the MFCs and pre-cultures were collected every other day and filtered through 0.2-μm nylon, multigrade glass-fiber syringe filters (Millipore, Bedford, MA, USA) and stored at 4 °C for no more than 2 days, or −20 °C for a longer duration. An ion chromatography system (Dionex ICS-3000, Sunnyvale, CA, USA) coupled to an electrospray ionization-tandem mass spectrometer (Applied Biosystems SCIEX API 2000, Foster City, CA, USA) or an IC-ESI-MS/MS, was used to measure nitrate and perchlorate. Details on the analytical methodology for perchlorate quantification are provided

below while the details for nitrate and nitrite quantification can be found in Farhat et al. [7].

The multiple reaction monitoring (MRM) transition technique [7] was used in this study to uniquely identify and quantify each contaminant. For quantifying perchlorate, MRM transitions of both of its chlorine isotopomers were used. The first isotopomer, MRM transitions employed were *m/z* 99 ($^{35}ClO_4^-$) → *m/z* 83 ($^{35}ClO_3^-$) and *m/z* 99 ($^{35}ClO_4^-$) → *m/z* 67 ($^{35}ClO_2^-$) transitions representing the transformation of perchlorate anions into chlorate and chlorite, respectively. Similarly, the two MRM transitions designed for the identification and quantification of the second chlorine isotopomer of perchlorate ($^{37}ClO_4^-$) were *m/z* 101 ($^{37}ClO_4^-$) → *m/z* 85 ($^{37}ClO_3^-$) and *m/z* 101 ($^{37}ClO_4^-$) → *m/z* 69 ($^{37}ClO_2^-$). The total perchlorate concentration was obtained by the summation of all four MRM transitions.

DNA extraction and DGGE analysis

Upon completion of the respective experiments, total DNA extraction was carried out from precultures and MFC samples using the UltraClean Soil DNA Isolation Kit (MO-BIO, Carlsbad, CA, USA). Primer 341f with a GC clamp and 534r [13] were used in the DNA amplification for the DGGE gel. DGGE was performed as described previously [13] using the C.B.S. Scientific (Del Mar, CA, USA) with 1 mm thick gels. The gradient was mixed between low 40 % denaturant gel solution (1 ml TAE (50×), 10 ml polyacrylamide (40 %; 37.5:1), 8 ml formamide, 8.4 g urea, 1 ml glycerol, 5 µl 10 % APS and 50 µl TEMED, to 50 ml with water) and high 60 % denaturant gel solution of (1 ml TAE (50×), 10 ml polyacrylamide (40 %; 37.5:1), 12 ml formamide, 12.6 g urea, 1 ml glycerol, 5 µl 10 % APS and 50 µl TEMED, to 50 ml with water). PCR products (50 ng) were mixed with 5× loading dye, applied directly onto gel then developed at 100 V and 60 °C for 16 h. The DGGE gel was stained with ethidium bromide and photographed using Bio-Rad's Image analyzer (Bio-Rad, Hercules, CA, USA). Prominent bands (selected on the basis of intensity and uniqueness) from the DGGE gel containing the 16S rRNA gene PCR product were extracted and the DNA was purified from the gel pieces by adding 50 µl of MilliQ water and incubating overnight at 4 °C. The eluted DNA was reamplified in a PCR step using Primer 341f and 534r and the DNA amplicons were stored at −20 °C. These DNA samples were selected for DNA sequencing on an ABI 3730xl capillary sequencer using Big Dye terminator chemistry v3.1 (Applied Biosystems) by Source Bioscience (Nottingham, UK). Sequencing was carried out bi-directionally using two primers 341f and 534r.

Phylogenetic analysis of microbial communities from precultures and MFCs

Sequences were checked for chimeras and trimmed using BioEdit software version 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; Ibis Biosciences, Carlsbad, CA, USA). Valid sequences were compared to the GenBank database using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences (>150 bp) determined in this study have been deposited in GenBank.

Results and discussion

Preculture enrichment

Initial inocula from the MBR were grown using acetate as electron donor and either nitrate or perchlorate as electron acceptor. Both cultures displayed biomass growth after 3 days (Fig. S4). The initial nitrate concentration at day zero was 1,100 mg/l, which was completely reduced by the second day and nitrate was below detectable limit by IC-ESI-MS/MS. The removal of media for sampling was supplemented with fresh acetate and nitrate, which got completely reduced before the next sampling, leaving no measurable nitrate in the inoculum and for the remainder of the experiment. Less than 5 % of total nitrate was reduced in the abiotic control indicating that the acetate-fed denitrifying preculture) was active right away and was able to remove 6,400 mg of nitrate over the 10-day duration of the enrichment.

The perchlorate-reducing preculture inoculum showed results similar to the denitrifying preculture (Fig. S2). There was a measurable rate of perchlorate reduction within 2 days and the sampled volume was replaced with additional perchlorate and the data showed that there was a continual reduction of excess perchlorate by the perchlorate-reducing microorganisms in the preculture. The rate of reduction increased over time, with a maximum removal of perchlorate achieved after 8 days of growth when 100 % of the perchlorate fed into the system was reduced within the 48-h interval between consecutive sampling events. Again, the abiotic degradation of perchlorate was monitored as previously described. However, perchlorate removal was slower (1,900 mg of perchlorate over the 10 days) compared to cultures fed with nitrate. The nitrate-reducing and perchlorate-reducing cultures were directly used as inoculum for nitrate-reducing and perchlorate-reducing MFC, unlike the previous research in which they used nitrate-reducing enrichments perchlorate-reducing MFC [1].

MFC operation and biocathodic enrichment of cultures

Perchlorate-reducing precultures were used for the inoculation and polarization curves of the perchlorate-reducing MFC at days 14 and 17 indicated its stability over time (Fig. S3). One feeding batch for the perchlorate-reducing MFC and its controls over a period of 22 days is shown in Fig. 1. The initial and final concentrations in the biocathode chamber were 203 and 127.3 mg/l, respectively (37.3 % removal efficiency), with a late onset of reduction by day 15 (Fig. 1). No significant reduction could be measured in control A (the open circuit control) or control B (abiotic cathode control), suggesting that perchlorate removal was a bioelectrochemically mediated reaction. Peak perchlorate removal rate of 9.46 mg/l/day (2 mg/m^2/d, normalized by liquid volume and electrode surface area) or 0.095 mM/day, upon onset of reduction. This biocathodic reduction rate was lower than the previously observed maximum perchlorate reduction rate of 24 mg/l/day (0.24 mM/day) in a MFC with similar electrical set up of a constant resistance, albeit a continuous rather than fed-batch operation and a granular packed-bed electrode of a much larger surface area, achieved after over 9 months of operation [1]. In contrast, the MFC operation in this study lasted only 21 days.

As a comparison, nitrate-reducing preculture was used for the inoculation of a nitrate-reducing biocathode in a dual-chamber MFC with a 500-Ω external resistance. The MFC reactor operated for approximately 33 days and the nitrate concentration in the anaerobic biocathode chamber was monitored over this time. Batch feeding with 8 mg sodium acetate as the sole electron donor was conducted in the anodic chamber once a week. The nitrate concentration in the biocathode rapidly decreased to less than 200 mg/l from an initial concentration of 1,000 mg/l by day 6 (not shown). This peak reduction rate of 133 mg/l/day (29 mg/m^2/day, normalized by liquid volume and electrode surface area) or 2.15 mM/day in denitrifying MFCs was faster than perchlorate-reducing MFCs. Re-spiking the cathode chamber with nitrate to the initial concentration again produced 80 % removal in less than 5 days with an average removal rate of 88.8 mg/l/day (1.43 mM/day).

DGGE profiling of microbial communities

Differences in microbial communities in the original MBR inoculum, enriched precultures, and biocathode biofilm of working MFCs were analyzed by comparing their respective DGGE profiles (Fig. 2). The banding pattern shifted from the initial four prominent bands in the inoculum to more varied prominent band patterns for each step of the enrichment (i.e., preculture and biocathodic enrichment). DGGE banding patterns (Fig. 2) were sample-specific, and

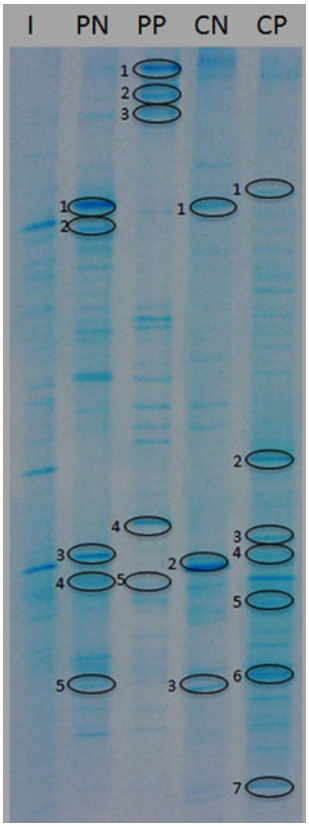

Fig. 2 DGGE analysis for the comparison between inoculum (MBR sludge) and different perchlorate and nitrate-reducing culture, *I* MBR inoculum, *PN* preculture on nitrate, *PP* preculture on perchlorate, *CN* biocathode on nitrate, *CP* biocathode on perchlorate

there were differences observed even in the profiles of precultures and biocathodic cultures supplied with the same electron acceptor. This result further supported the strategy of enriching perchlorate-removing microorganisms separately, instead of using nitrate-reducing cultures in perchlorate-fed MFC.

Phylogenetic analysis of microbial communities

The identity and phylogenetic affiliations (Table 1) of the microbial communities in four of the five different biological samples (see Fig. 2) was revealed by sequencing 18 of the most prominent DGGE bands in precultures and MFCs fed with nitrate and perchlorate. Since most of the known perchlorate-reducing bacteria are closely related to each other and to the bacterial species *Rhodocyclus tenuis* and *Ferribacterium limneticum* [4], the 16S rRNA sequences of these two species were taken for phylogenetic analysis (Fig. S5). Two further sequences, *Magnetospirillum bellicus* Strain VDY and *Dechloromonas agitates* Strain CKB, from two isolated dissimilatory chlorate reducers were also used [17]. It should be noted that approximately 150–200 bp 16S rRNA gene sequences of

Table 1 Identification of excised and sequenced DGGE bands (see Fig. S5)

Cultures	Band #	Identification with closes species sequence		Phylum	Similarity (%)
		Closest species and strain	Accession no.		
Nitrate-reducing precultures	1	Uncultured proteobacterium, 196-ATA-Chax-AsIII	FR839366.1	Epsilon-Proteobacteria	100
	2	Uncultured bacterium	JF692622.1	Firmicutes	99
	3	Uncultured Firmicutes bacterium	JQ433805.1	Firmicutes	93
	4	Uncultured bacterium	JF692622.1	Firmicutes	97
	5	Uncultured bacterium clone BP_U1B_2b01	AY711062	Chlorobi	98
Perchlorate-reducing precultures	1	Uncultured *Thauera* sp. clone MBfR_NSP-152	JN125306.1	Beta-proteobacteria	100
	2	Uncultured *Bacteroides* sp. clone NSBac28	JX462546	Bacteroidetes	89
	3	Uncultured *Dysgonomonas* sp. clone OTU16	JQ328210.1	Bacteroidetes	97
	4	*Aquiflexum* sp. LW9	FN393747	Bacteroidetes	96
Bio-cathode nitrate	1	Uncultured Firmicutes bacterium clone S3B_98	JQ433805	Firmicutes	93
	2	Uncultured bacterium clone ncd1615c06c1	JF139156	Firmicutes	85
Biocathode perchlorate	1	Uncultured bacterium clone ICBAF7	AF390935.1	Bacteroidetes	100
	2	Uncultured *Bacteroides* sp. clone NSBac2	JX462546	Bacteroidetes	99
	3	Uncultured bacterium clone 4-1	JQ768750	Bacteroidetes	100
	4	Uncultured bacterium clone CC12H10	KC208355	Gamma-proteobacteria	90
	5	*Clostridium thermopalmarium* strain NMY	JF747199	Firmicutes	92
	6	*Streptococcus gallolyticus* 16S ribosomal RNA gene	AF323911	Firmicutes	99
	7	Uncultured bacterium clone inf68	JN245819	Firmicutes	93

the DGGE bands can only provide limited evidence about the function of the microbial members.

The nitrate preculture yielded DNA band (PN1) having sequences closely affiliated to the class epsilon proteobacteria. It is well known that the members belonging to this class are potential nitrate reducers that use inorganic electron donors such as hydrogen and sulfur compounds [9], and were also isolated and tested for denitrification in activated anaerobic sludge [11].

The next interesting result was obtained in perchlorate-reducing culture, where band PP1 yielded sequences closely affiliated with *Thauera* sp. of the class beta proteobacteria. The closest species was previously identified in hydrogen-based membrane bioreactor, where microbial reduction of nitrate, sulfate, and *p*-chloronitrobenzene was reported (unpublished). In addition, it was also evolutionarily related to perchlorate-reducing strains *M. bellicus* and *D. agitates* [17] indicating their potential role in the perchlorate reduction. Perchlorate reduction by the co-consortium of epsilon and beta proteobacteria in a bench scale bioreactor was reported previously [6], which is in line with our results. Similarly, band CP4 in MFC fed with perchlorate yielded sequences affiliated to gamma proteobacterium *Klebsiella* sp.

Other than the above-mentioned sequences belonging to proteobacteria members, many DGGE bands (PN2, PN3, and PN4) in the precultures and MFC fed with nitrate yielded sequences affiliated to the phylum Firmicutes. In line with enrichment cultures, three analyzed bands from the biocathodic MFC fed with nitrate showed affiliation to the members of the phylum Firmicutes. Band CN1 shows a high similarity to PN3 and since the bands show the same migration pattern in the DGGE gel (Fig. 2), it can be assumed that these Clostridia are the same and are found in both heterotrophic and electrochemical denitrifying systems. CN2 and CN3 could also be identified as Firmicutes/Clostridia and the analyzed sequence shows high similarities to uncultured bacterial strains (Table 1).

Only one DNA band in each of the perchlorate-reducing preculture (PP5) was identified as Clostridia sp., very similar to *Clostridium butyricum* (KC195777.1). Most of the bacteria identified in the perchlorate-reducing preculture and MFC belong to the phylum of Bacteroidetes (Table 1). The members belonging to the phylum Firmicutes and Bacteroidetes, are common in anaerobic microbial communities as fermentative chemoorganoheterotrophs. Identification of these organotrophs in the biocathode chambers that should encourage lithotrophic organisms could be due

to the presence of organic substrates and biomass in sludge samples added along with the precultures as inoculum. Some researchers have also reported their denitrification ability by isolating and testing with pure cultures [14].

The above analysis using PCR-DGGE indicated that nitrate-fed precultures and MFC had very distinct populations compared to perchlorate-reducing precultures and MFC. This specific enrichment procedure can be adopted for processes removing perchlorate using inorganic and heterogeneous electron donors. It is important to note that there was a significant time reduction compared to previous published growth times lasting 10 months to less than 1 month.

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