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Microbial community analysis of perchlorate-reducing cultures growing on zero-valent iron

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ABSTRACT

Anaerobic microbial mixed cultures demonstrated its ability to completely remove perchlorate in the presence of zero-valent iron. In order to understand the major microbial reaction in the iron-supported culture, community analysis comprising of microbial fatty acids and polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) techniques was performed for perchlorate reducing cultures. Analysis of fatty acid methyl esters (FAMEs) and subsequent principal component analysis (PCA) showed clear distinctions not only between iron-supported perchlorate reducing culture and seed bacteria, but also among perchlorate-reducing cultures receiving different electron donors. The DGGE pattern targeting the chlorite dismutase (*cld*) gene showed that iron-supported perchlorate reducing culture is similar to hydrogen-fed cultures as compared to acetate-fed culture. The phylogenetic tree suggested that the dominant microbial reaction may be a combination of the autotrophic and heterotrophic reduction of perchlorate. Both molecular and chemotaxonomic experimental results support further understanding in the function of zero-valent iron as an adequate electron source for enhancing the microbial perchlorate reduction in natural and engineered systems.

1. Introduction

Perchlorate is a major groundwater contaminant that is characterized by its high solubility and persistence in aquatic environments. Perchlorate contamination may affect the drinking water supplies of at least 15 million people in 35 states within the US [1–4]. Due to the potential health hazard of perchlorate, a number of states have their action guideline for perchlorate. Recently National Academy of Science indicated the reference dose of perchlorate is equivalent to 24.5 ppb in water. And the contaminated level of perchlorate in soil or groundwater ranges from 2 ppb to 500 ppm [5–9]. Although thermodynamics predicted that perchlorate is readily reducible in the presence of reducing agents, chemical reduction in natural environments is found to be insignificant due to a large kinetic barrier under ambient conditions [9,10]. While perchlorate may be difficult to be reduced from water by chemical processes, it is found to be reducible by microorganisms under anaerobic conditions.

In recent years, microbial treatment of perchlorate has been recognized as a promising technology for the removal of perchlorate in water supplies [11–13]. Perchlorate-reducing bacteria (PRB) appear to be ubiquitous [14]. The known PRB isolates represent a broad phylogeny of the *Proteobacteria* [14,15], hence provides an indication that the metabolism of perchlorate reduction is widespread. It has been reported that complete degradation of perchlorate occurs in two steps involving perchlorate reduction and chlorite dismutation [11,16,17]. The first steps in perchlorate reduction, which are mediated by chlorate reductase, include the reduction of perchlorate to chlorate and subsequently chlorate is reduced to chlorite. The dismutation of chlorite into chloride and O_2 is common to all perchlorate-reducing bacteria. It is catalyzed by a conserved enzyme known as chlorite dismutase [14,18]. A number of literature indicated microbial perchlorate reduction may occur through use of hydrogen [1,19,20] and organic substrates (i.e., acetate or ethanol) [6,7,21,22] as electron donors. Zero-valent iron was used successfully as an electron donor for microbial perchlorate reduction as shown in our previous study [23]. Zero-valent iron has also been demonstrated [24,25] as an electron donor for perchlorate reduction by providing hydrogen to a hydrogen-utilizing autotrophs in batch and flow-through reactors. In comparison to hydrogen and acetate, which are commonly used electron donors for biological treatment of perchlorate, zero-valent iron is inexpensive, safe, and/or does not leave any organic residue.

Analysis of fatty acid methyl esters (FAMEs) derived from cellular lipids provides a rapid, nonselective, and unified analysis or fingerprint of an entire microbial community [26–28]. The objec-

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tive of the FAME analysis is to examine the relative abundances of the various fatty acids associated with the organisms within a sample [28]. The whole-community FAME analysis, facilitated with proper multivariate statistic analyses, is ideal for studying mixed microbial populations such as those found in sediments, soil, and wastewater plants [27–32]. The FAME analysis is based on the chemotaxonomic characteristics of microorganisms and therefore it is used for the study. In contrast, the majority of fingerprinting methods such as TRFLP uses the genomic information, which only indicates the potential of microbial reaction. The combination of both functional gene analysis and microbial fatty acid analysis offer a number of advantages in the field of microbial ecology. For example it can be used for identifying microbial populations, characterizing and predicting changes in microbial populations. The iron-supported culture system can also be synergistic in terms of targeted microbial reaction (e.g., perchlorate reduction) due to its more complex and diverse microbial populations as compared to a pure culture enriched system. Given the complex and dynamic nature of the native microbial communities, coupled genetic and chemotaxonomic analysis is essential in order to understand the microbial ecology. Recently the efforts to understand the microbial ecology toward the microbial perchlorate reduction were demonstrated via DGGE and FISH technologies [33–35].

Previously, Son et al. [23] suggested that iron-supported microbial process is a potential perchlorate treatment technique. It was shown to reduce perchlorate of 15 mg/L to below the detection limit in the column setup. It is important to further understand the metabolic dynamics of microbial populations in the iron-supported culture system in order to design an ideal engineered system for perchlorate removal. It was hypothesized that the following microbial processes are responsible for the reduction of perchlorate in the iron-supported microbial culture. Initially, the anaerobic corrosion of iron particles generates hydrogen gas through the reduction of water. Hydrogen utilization was hypothesized to occur by either direct or indirect metabolic pathways. Autotrophic perchlorate reducers are responsible for direct hydrogen utilization in perchlorate reduction [20,24,25,36]. Alternatively, heterotrophic perchlorate degraders may use the organics (e.g., acetate) transformed by co-existing microorganisms (e.g., homoacetogens) as the electron donor for perchlorate reduction. To characterize the metabolism of perchlorate reducers growing on the iron, a coupled functional gene analysis and chemotaxonomic assay was performed. This paper reports the results of (1) FAME and principal component analysis (PCA) to examine and compare microbial populations enriched by the different electron donors, (2) nested polymerase chain reaction – denaturing gradient gel electrophoresis (PCR–DGGE) and a subsequent phylogenetic analysis to identify the genetic affiliations in the iron-supported perchlorate reducing culture.

2. Materials and methods

2.1. Batch bioreactors for perchlorate reducing culture

A perchlorate reducing seed culture was obtained by mixing biomass samples from the anaerobic digester and the activated sludge aeration basin from the Wilmington wastewater treatment plant (Wilmington, DE, USA). Without performing prior acclimation process, fresh mixed culture was used as the seed culture for batch reactor incubations. Final seed biomass concentration was adjusted to 400–500 mg/L as total suspended solids (TSS) with the culture media. Batch bioreactors were operated for 30 days with a variety of electron donors. Triplicate 125 mL batch reactions were prepared with the following treatments: Fe(0)-cell (i.e., Fe(0) plus microbial cultures), H_2-fed control (5% hydrogen gas plus microbial cultures), Ac-fed control (173 mg/L acetate plus microbial cultures), and cell control (microbial cultures only). The media was prepared as described in the previous study [23] and the pH was maintained around 7 for optimum bacterial growth using a HEPES buffer. In our system, the theoretical total yield of H_2 gas from 2 g Fe(0) in 125 mL batch reaction is 71.6 mg H_2 gas and the solubility of H_2 gas in water is 0.194 mg H_2 gas at ambient temperature.

2.2. FAME analysis

In order to compare microbial communities present in perchlorate reducing cultures fed with various electron donors, FAME analyses were conducted in triplicate with the biomass samples obtained from each batch reactor after 30 day incubation. Fatty acids were also extracted from the seed bacterial culture as a control. The FAME extraction protocol used in this study was a modification of that described by Sasser [37]. Fatty acids were immediately extracted subsequent to the sample collection. Samples were prepared by transferring 10 mL of culture suspension to 40-mL glass tubes and centrifuged at $500 \times g$ for 45 min. After discarding the supernatant, the pellet was resuspended in 15 mL of 0.2 M methanolic potassium hydroxide. Tubes were placed in a 37 °C water bath for 1 h and the contents were resuspended every 10 min. The sample was subsequently neutralized with 1 M acetic acid. Hexane (10 mL) was added to the solutions and centrifuged at $500 \times g$ using the Marathon 22K centrifuge (Fisher Scientific, Pittsburgh, PA) for 30 min to separate the organic and aqueous phases. The overlying hexane was transferred to a glass tube and evaporated under flowing N_2 gas. The extracted FAMEs were redissolved in 0.5 mL of 1:1 hexane:methyl tertiary butyl ether and transferred to a 2 mL vial for analysis by gas chromatograph. FAME profiles were determined using an HP 6890 gas chromatograph (GC, Hewlett Packard, Rolling Meadows, IL) equipped with an HP Ultra 2 capillary column and a flame ionization detector. The standard chromatographic program stipulated by Microbial ID, Inc. (MIDI) for its eukaryotic library was used for all analyses (MIDI, Newark, DE). Data was collected using an interfaced desktop computer running the standard MIDI software and is used to identify FAMEs based on the retention time relative to the commercially prepared FAME calibration mixtures.

2.3. Statistical analysis

PCA [38] and cluster analysis were used to interpret FAME data. PCA are commonly used in conjunction with FAME analysis [39–43]. PCA extracts theoretical principal components (PC) that account for data variation. The scores of PCA were plotted into two dimensions based on the first two principal components (i.e., PC1 and PC2). PCA was used to visualize the data into a lower dimensional space and cluster analysis was used to indicate the similarity among groups. PC directions were standardized to have the unit variances of PC scores (i.e., the standard deviation of PC scores on each direction is 1). The hierarchical cluster analysis was used to generate a tree diagram (i.e., dendrogram) which graphically displays the relevance between samples. Each fatty acid peak area was converted to a percentage of the total peak area for each GC run. The percentages were analyzed by both PCA and cluster analysis using the free R software (http://www.r-project.org).

2.4. Genomic DNA extraction

Genomic DNAs (gDNA) from the culture samples were extracted following a protocol reported by Wilson [44]. A 50 mL sample of mixed culture was harvested and centrifuged at 5000 rpm for 5 min. The pellet, resuspended in sucrose buffer (40 mM EDTA, 50 mM Tris–HCl, and 750 mM sucrose), was incubated at 37 °C with

lysozyme for 30 min, and 10% SDS and protenase-K for 2 h. The cells were subsequently disrupted using a bead beater (Biospec products, Bartlesville, OK) for 3 min. The gDNA was collected and purified by the standard phenol and chloroform extraction and ethanol precipitation, respectively. The total DNA concentration was determined by using a Nanodrop ND-1000 spectrophotometer (Nanodrop Tech., Wilmington, DE).

2.5. Nested PCR targeting chlorite dismutase gene

A nested PCR approach targeted the cld gene encoding chlorite dismutase for the perchlorate reducing cultures. The nested PCR was used to increase the sensitivity of the molecular detection method [45,46]. The primer sets specific for the cld gene, encoding chlorite dismutase, were (1) DCD-F/DCD-R (F: 5′ GA(A/G)CGCAA(A/G) (A/G)GNGCNGCNG(A/C) NGA(A/G) GT 3′ and R: 5′ TC(A/G)AA(A/G)TANGT(A/T/G)AT (A/G)AA(A/G)TC 3′) and (2) UCD-238F/UCD-646R (F: 5′ T(C/T)GA(A/C/G)AA(A/G) CA(C/T)AAGGA(A/T/C)AA(A/C/G) GT 3′ and R: 5′ GAGTGGT A(A/C/G)A(A/G)(C/T)TT(A/C/G)CG(C/T)TT 3′) [45], synthesized by Integrated DNA Technologies (Coralville, IA). PCRs were performed in a Bio-Rad iCycler (Hercules, CA).

The first reaction of the nested PCR was conducted with 25 ng of gDNA, 1.5 mM $MgCl_2$ (MJ Research, Walthan, MA), 0.2 mM (each) deoxynucleoside triphosphates (MJ Research), 3.0 mg of bovine serum albumin (BSA) per mL (New England Biolabs, Beverley, MA), 1× Mg-free PCR buffer (Qiagen, Valencia, CA), 2.5 U *Taq* polymerase (Qiagen), and 0.8 μM DCD-F/DCD-R primer set. The thermal cycling protocol for touchdown PCR includes the initial denaturation at 94 °C for 2 min, followed by 18 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min (decreased by 1 °C per every two cycles to 50 °C), and elongation at 72 °C for 1 min. After the completion of the initial protocol, additional 20 cycles were implemented at the annealing temperature of 50 °C, and this was followed by a final elongation step consisting of 72 °C for 10 min.

The composition of the subsequent reaction of nested PCR was the same except for a 0.8 μM UCD-238F/UCD-646R primer set and templates that are 1:10 diluted PCR products from the first reaction. The PCR conditions consisted of the initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and elongation at 72 °C for 1 min. The final extension was also 72 °C for 10 min. To verify the accuracy of the amplification, positive (aerobic activated sludge and anaerobic digester sludge cultures) control and negative control (water) were included. PCR products were visualized by gel electrophoresis using a 1% agarose gel with 1× TBE (Tris boric acid EDTA, Promega, Madison, WI) buffer. Gels containing 0.5 μg/mL ethidium bromide were visualized with a UV transilluminator (Fisher Scientific) and photographed. The target DNA sizes were 484-bp and 404-bp for DCD-F/DCD-R and UCD-238F/UCD-646R, respectively.

2.6. DGGE analysis

The DGGE of the PCR products was performed by using a Bio-Rad DCode™ system and a gel that is 16 cm × 16 cm × 1 mm. The nested PCR products amplified with UCD-238F/UCD-646R primers were further analyzed using DGGE technique. The PCR products were purified and concentrated using the QIAquick PCR purification kit (Qiagen). 30 μL PCR products and 10 μL 2× gel loading dye (2% bromophenol blue, 2% xylene cyanol, 100% glycerol, and water) were loaded onto 8% (w/v) polyacrylamide gel in 1× TAE running buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA; pH 8.0). A linear 40–70% denaturing gradient was created by mixing 7 M urea and 40% deionized formamide. All reagents for DGGE were purchased from Bio-Rad except formamide (J.T. Baker, Phillipsburg, NJ). The electrophoresis was performed at 60 °C at 75 V for 12 h. After electrophoresis, the gels were taken out from the DCode™ system and soaked for 1 h in 750 mL of ethidium bromide solution (0.5 μg/mL of 1× TAE buffer). The gel was visualized with a UV transilluminator (Fisher Scientific) and photographed.

2.7. Cloning and sequencing analysis

The selected DGGE bands were excised from the polyacrylamide gel using a sterile blade and the DNA was extracted using a QIAquick gel extraction kit from Qiagen. Since the gel extraction kit is designed for low-melting agarose gel, the extraction procedure for polyacrylamide gel was modified by the extension of the elution time. The excised gels were placed in an elution buffer at 4 °C for 4 h before proceeding to gel extraction. The eluted DNAs were reamplified using UCD-238F/UCD-646R primers and the reamplifed PCR products were purified and concentrated using the QIAquick PCR purification kit (Qiagen) prior to ligation into cloning vectors. Reamplification was confirmed by agarose gel electrophoresis and DGGE for the next steps. The purified PCR products for reamplification were ligated into the pCR 2.1-TOPO vectors using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The following transformation was implemented by using One shot® chemically competent *E. coli* (Invitrogen). The successful transformation was selected by Luria–Bertain (LB) plate with 50 μg/mL kanamycine. The ligation of *cld* gene fragments to vectors was confirmed by EcoR1 restriction enzyme digestion at 37 °C. Sequences of the *cld* gene inserts were identified using vector primer (M13R) and ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA) by BioDye™ terminator cycle sequencing.

2.8. Phylogenetic analysis

The *cld* sequences generated in this study or recovered from the GenBank database were aligned and then used to create a phylogenetic tree by using ClustalW1.8 (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Therefore a neighbor-joining tree was generated by inserting the 22 sequences obtained in the sequencing experiment. Similarities to *cld* gene sequences from known perchlorate reducing bacteria in GenBank were determined by BLAST 2 analyses [47], (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) and represented in sequence identities (%). The similarity table was used for the subsequent statistical analysis (i.e., multidimensional scaling).

2.9. Multidimensional scaling analysis

Multidimensional scaling was employed to represent the phylogenetic relation among the *cld* clones including the known perchlorate reducers. Multidimensional scaling (also known as principal coordinate analysis) is a technique employed to construct a new configuration of objects using the information of distances (e.g., dissimilarity) between objects. Multidimensional scaling has been popularly used in the visualization for exploring similarities or dissimilarities in data in several fields such as psychology, physics, and biology [48–50]. The similarity table obtained from BLAST 2 analyses was subsequently converted to multidimensional scaling plot based on Kruskal's non-metric method [51]. This method chooses a configuration such that the points of larger dissimilarity would be farther away. The distance presented in the multidimensional scaling plot is the Euclidean distance transformed from matching percentage [52].

2.10. Nucleotide sequence accession numbers

Representative novel sequences of *cld* gene encoding chlorite dismutase generated from this study have been

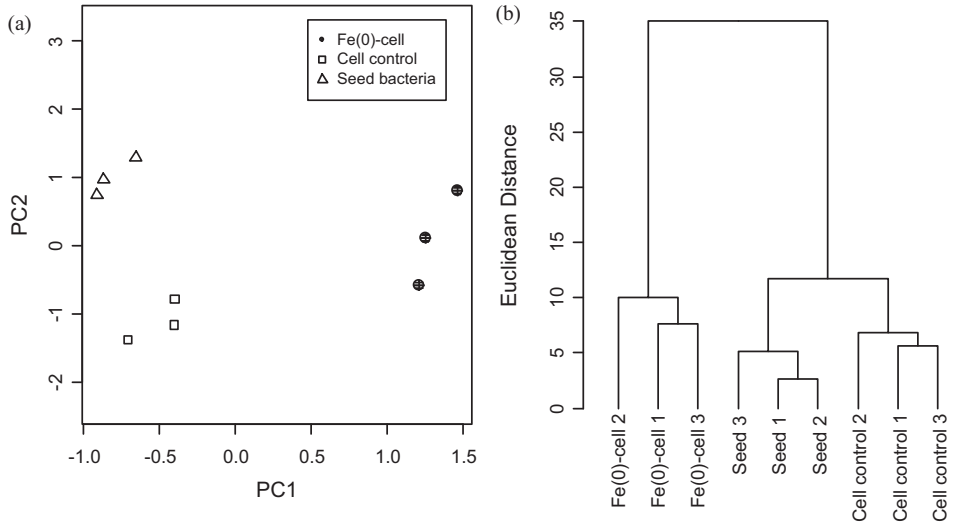

Fig. 1. Statistical analyses of microbial fatty acid data. (a) Principal component analysis (PCA). *X*-axis: PC1, 90.8% explained variance, *Y*-axis: PC2, 5.6% explained variance. The scatter along the horizontal axis is more significant than the scatter in the vertical axis. The Fe(0)-fed population structure significantly deviates from that of the seed culture, but similar to that for H_2-fed culture. (b) The dendrogram constructed by cluster analysis. The numbers after the sample name indicated the triplicate batch reactors. Seed culture and cell control were clustered together indicating their similarity as compared to Fe(0)-cell.

deposited in the GenBank database under accession numbers DQ151561–DQ151581.

3. Results and discussion

3.1. FAME analysis

As previously described [23], perchlorate (~65 mg/L) in the bioreactors fed with Fe(0), H_2, and acetate was completely removed below the detection limit (<20 μg/L) at approximately the same rate. The bacterial cultures growing on different electron donors were used for the following microbial community analysis. The first FAME analysis resulted in the extraction of 21 significant fatty acids, which do not have zero value of relative fatty acid amount (%) from the MIDI output, from Fe(0)-cell, cell control, and seed bacteria samples. Biomass samples from cell control and seed bacteria had similar types and amount of fatty acids. Based on the comparison of individual fatty acids, many of the peak areas in Fe(0)-cell sample were either substantially greater or lower than that in the seed culture. PCA and cluster analysis, which were performed for better visualization of FAME data, were presented in Fig. 1. The horizontal axis of Fig. 1a (PC1), explains 90.8% of the total variance in the original data and the vertical axis (PC2) represents an additional 5.6% of the total variance. According to the PC plot, the microbial community established in the Fe(0)-cell was different from that in the cell control and seed bacteria samples (Fig. 1a).

The original data was analyzed with cluster analysis based on the distances among the objects. The dendrogram presented in Fig. 1b confirmed the grouping information derived from the PC plot, indicating that the iron-treated microbial community is very different from the two control microorganisms (i.e., cell control and seed culture). Both PCA and cluster analysis of FAME data illustrated a shift in fatty acid profiles of mixed culture grown on the iron as the primary electron donor. The result suggests that iron granules are likely to have enriched populations that were initially present in the seed culture.

Twenty six fatty acids were selected for the further analysis to evaluate the effects of various electron donors due to their significant proportion. The perchlorate-reducing cultures with various electron donors include Fe(0)-cell, acetate-fed control, H_2-fed control and seed bacteria samples. The PC plot of FAMEs extracted from these cultures is presented in Fig. 2a. Principal components elucidated 66.3% (PC1) and 21.5% (PC2) of the variance. The PC plot indicates that significant changes occurred in the microbial populations in Fe(0)-cell reactors as compared to the seed bacteria. The fatty acid composition of H_2-fed control was similar to that of the Fe(0)-cell samples. This suggested that microbial populations of the iron-supported culture may be similar to that of the hydrogen-fed culture. The similarity in fatty acid compositions between the iron-treated and the hydrogen-treated microbial communities was also shown in the dendrogram obtained from the cluster analysis (Fig. 2b). Even though the hydrogen sources are different, the same populations of bacteria may be involved in perchlorate reduction. However the fatty acid composition data of the acetate-fed culture clustered further away from both Fe(0)-cell and H_2-cell bottles, indicating that the addition of acetate supported different types of microbial populations than those found in Fe(0)-cell system. These results suggest that the cathodic hydrogen derived from iron corrosion is the primary electron donor for the perchlorate reduction in the iron-supported culture system.

Interestingly, similar perchlorate reduction rates were observed with three different electron donors (i.e., Fe(0), H_2, and acetate) based on perchlorate analysis [23]. However FAME and subsequent multivariate statistical analyses were able to indicate that different microbial communities may be responsible for the reduction activity with the same rates (Fig. 2). Since heterotrophic microorganisms are most likely to be responsible for perchlorate reduction in the acetate-cell system, results from the FAME analysis suggests that Fe(0)-cell systems may be predominated by non-heterotrophic PRB populations.

3.2. PCR–DGGE analysis

Nested PCR results for four perchlorate-reducing were presented in Fig. 3a. No amplification occurred in water (lane 8) while nested PCR products of 408-bp were clearly visible in every sample. The band of PCR product was thicker in perchlorate reducing culture (lanes 2 through 5 in Fig. 2a), compared to seed sludge cultures (i.e., activated sludge and anaerobic digester sludge) (lanes 6 and 7 in Fig. 3a), suggesting the enrichment for organisms containing the *cld* gene in the perchlorate reducing cultures.

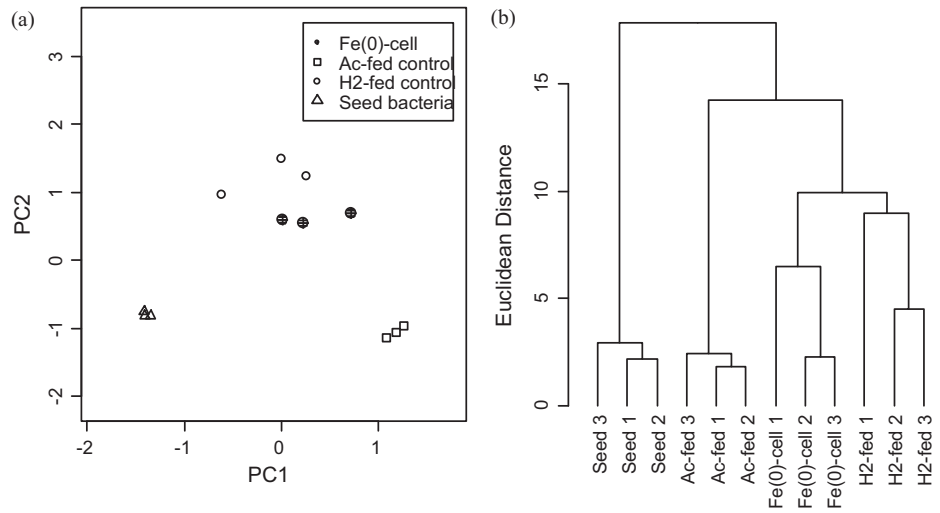

Fig. 2. Multivariate statistical analyses of microbial fatty acid data. (a) PCA. *X*-axis: PC1, 66.3% explained variance, *Y*-axis: PC2, 21.5% explained variance. The Fe(0)-fed population structure significantly deviates from that of the seed culture, but similar to that for H_2-fed culture. (b) The dendrogram constructed by cluster analysis. Fe(0)-fed cultures and H_2-fed controls were closely clustered compared to other controls (i.e., seed cultures and Ac-fed controls).

DGGE result (Fig. 3b) yielded the differences in the positions of bands between the perchlorate reducing samples and the seed sludge cultures. The dsDNA fragments of the Fe(0)-supported culture and H_2-fed control displayed the same pattern in DGGE gel (lanes 3 and 5 in Fig. 3b). However, Ac-fed control shows a marked difference in band intensities, which imply that they have a different amount of template for PCR amplification [53]. This finding is similar to the DGGE analysis for the soil microcosms enriched with acetate and hydrogen [54].

3.3. Sequences analysis

Selected bands excised from the DGGE gel (Fig. 3b) generated 22 clones by transformation and the plasmids were purified for the sequencing. Sequenced genes were used for multiple alignment and phylogenic tree construction. It is evident that the chlorite dismutase gene involved in the perchlorate reduction exhibits a diversity of gene sequences in the phylogenetic tree (Fig. 4) and two sequences comparative analysis (table not shown). Since the bar distance in the phylogenetic tree indicates a 10% sequence variation in Fig. 4, the four groups in the tree have different genetic affiliations with each other.

In the upper part of the tree, the known sequences of uncultured clones grouped with *Dechlorospirillum* sp. (Group 1) and none of our clones clustered with this group. The uncultured clones in the Group 1, LfsedA and LH12m-A were selected from the lake sediment [45] and Vida-B in the same group was originated from the diesel-contaminated site [55]. The clone sequences derived from cultures with different electron donors (i.e., Fe(0), acetate, and H_2) are clustered together in Group 2, indicating the gene cluster in Group 2 shares the significant similarities among H_2, Ac, and Fe cultures. Those clones were also clustered with *Dechloromonas* sp. LT-1 in Group 2. However, the two sequences analysis shows 73–79% identities between the clones in Group 2 and *Dechloromonas* sp. LT-1, suggesting that the clones in Group 2 represent distinct species from *Dechloromonas* sp. LT-1. More significantly, H8 and H11 formed a small cluster with Ac4 as the closest relatives and Ac2 clustered with H6 in the same manner. Since there is no input-source

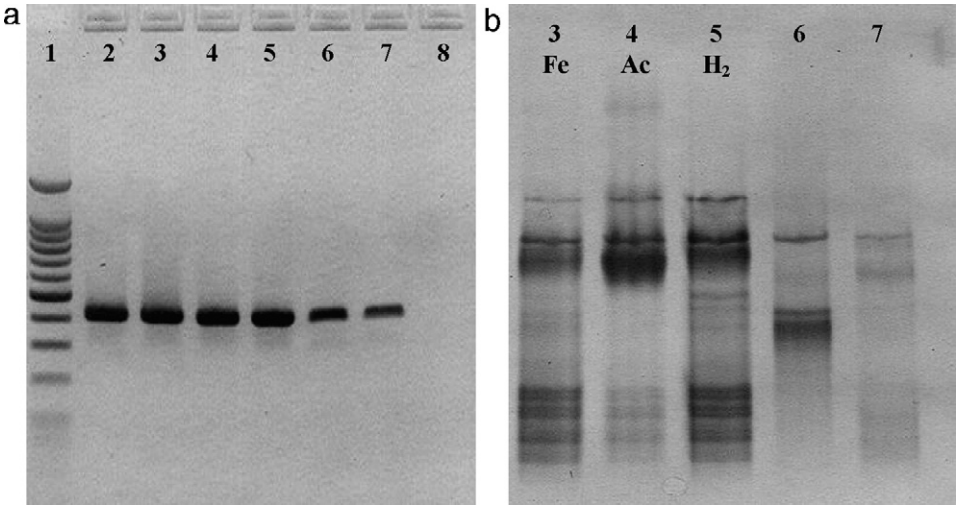

Fig. 3. (a) Nested PCR amplification of a 408-bp internal region of the *cld* gene using the DCD-F/DCD-R and UCD- 238F/UCD-646R primers sets. (b) DGGE patterns for the nested PCR amplifications of the *cld* gene using the UCD- 238F/UCD-646R primer set. The denaturing gradient was 40% (upper) to 70% (lower). All of DGGE bands were excised for the subsequent sequence analysis. Lane 1, 100-bp ladder; lane 2, Cell control: cells only; lane 3, Fe(0)-cell: Fe(0) plus cultures; lane 4, Ac-fed control: acetate plus cultures; lane 5, H_2-fed control: H_2 (5%) plus cultures; lane 6, activated sludge culture (seed cultures); lane 7, anaerobic digester culture (seed cultures); lane 8, Water: a negative control.

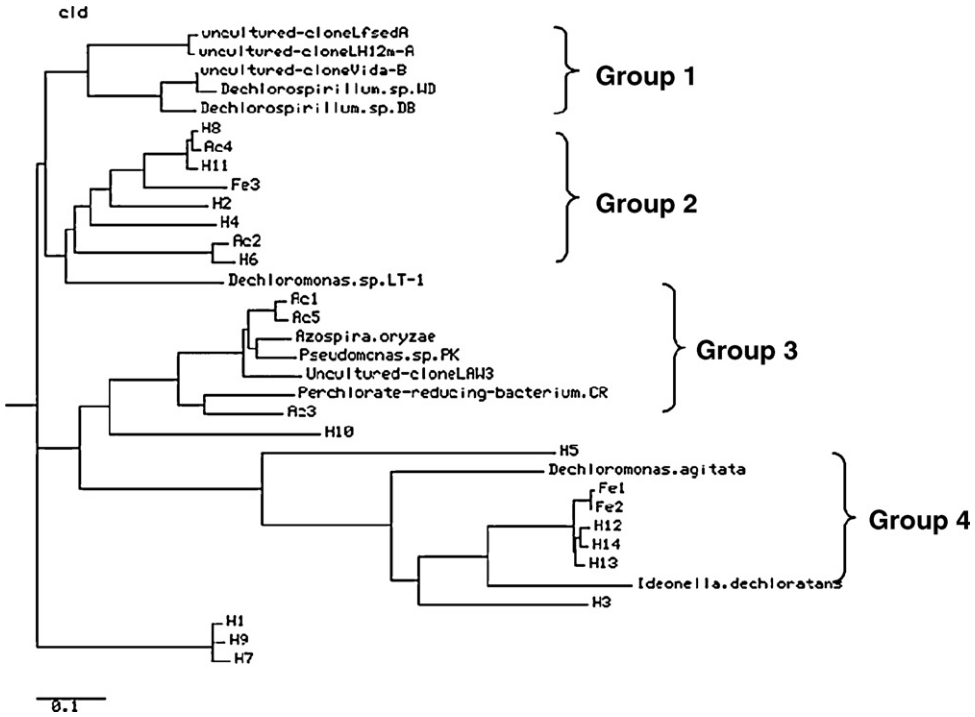

Fig. 4. Phylogenetic tree (neighbor-joining tree) showing the genetic affiliations between the known *cld* gene and the sequences obtained from DGGE bands in this study. The tree was constructed based on the multiple alignments of sequences by using the neighbor-joining method in ClustalW1.8. The bar indicates 10% sequence variation.

inducing autotrophic perchlorate reduction such as hydrogen, the acetate-fed control culture is dominated by heterotrophic perchlorate reduction. Therefore, the Group 2 cluster among the electron donors including acetate, H_2, and Fe(0), suggests that it may be involved in heterotrophic perchlorate reduction.

The sequences of clones Fe1, Fe2, H12, H13 and H14 are clustered (noted as Group 4) with the sequences of *Dechloromonas agitata* (AY124796) and *Ideonella dechloratans* (AJ296077). The Fe1, Fe2, H12, H13, and H14 sequences Exhibit 97–98% identity, suggesting that they represent variants of a single species. It indicates that the *cld* genes derived from Fe(0)-cell are almost identical to those from the H_2-fed control. The monophyletic nature of Group 4 suggests that Group 4 may be the gene cluster related to autotrophic perchlorate reduction. Based on the findings from Groups 2 and 4, the Fe(0)-cell culture may contain both autotrophic and heterotrophic reactions that simultaneously reduce perchlorate.

The multidimensional scaling drawn from the two sequence comparison was presented in Fig. 5. The plot (Fig. 5) showed the similar grouping as the phylogenetic tree (Fig. 4) drawn from the multiple alignments. The known heterotrophic perchlorate reducers were clustered distantly with Ac-fed clones as indicated with two circles in both center and right side of the plot. This tendency was similarly found in Group 1 and Group 3 in Fig. 4. At the left side of the plot in another circle it shows the main constituents that are clones from H_2-fed control and Fe(0)-cell. This observation indicates the similarity of the H_2-fed control and the Fe(0)-cell as compared to the Ac-fed controls.

From the results of phylogenetic analysis above, we have concluded that the possible pathway for the perchlorate reduction in the presence of Fe(0) is the combination of (1) the direct coupling of hydrogen oxidation and perchlorate reduction by hydrogen utilizing perchlorate reducers, and (2) the indirect utilization of hydrogen gas by heterotrophic perchlorate reducers via possibly homoacetogens. Note that the organisms used for known chlorite dismutase gene sequences were cultivated heterotrophically in order to be isolated as a pure culture [45]. However the culturing method does not have an effect on the gene-level analysis. Unlike the reduction of perchlorate and chlorate, the activity of chlorite dismutase was not affected by the addition of acetate as the main electron donor for heterotrophic perchlorate reduction [16]. Therefore, heterotrophic cultivation of a pure culture may not be equivalent to the heterotrophic mechanism for perchlorate reduction.

Zero-valent iron produces the hydrogen gas that is required to fuel the autotrophic reduction of perchlorate by hydrogen-utilizing perchlorate degraders. The heterotrophic perchlorate degraders

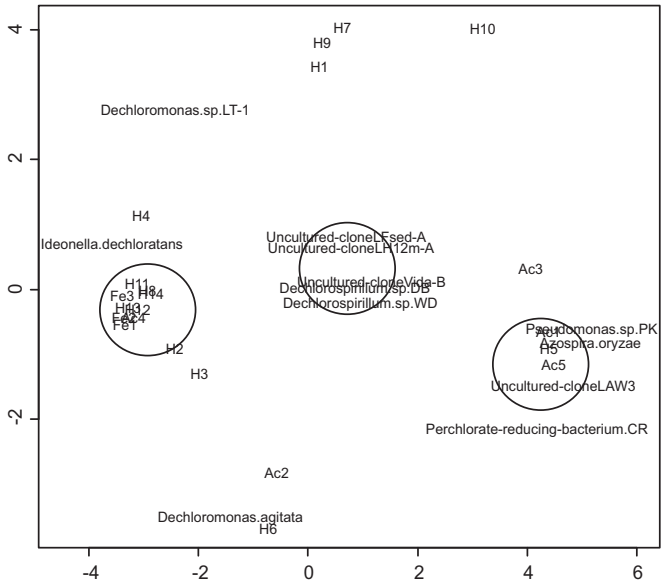

Fig. 5. Multidimensional scaling plot drawn from two sequences comparison by BLAST 2 analysis. Three circled groupings indicate the majority constituents are: the clones from both hydrogen and iron fed reactors; the known heterotrophic perchlorate reducers only; and Ac-fed clones with heterotrophic perchlorate reducers (from left to right). This grouping shares the similarity with the findings from the phylogenetic tree as presented in Fig. 4.

may however use organic substances such as acetate transformed from hydrogen gas via homoacetogenic reaction. Even though this study can potentially permit the understanding of the complicated microbial ecology associated with the perchlorate reduction, further work will be required to elucidate the role of homoacetogens as well as to quantify the microbial dominance. This will enable better engineering of perchlorate removal system with the zero-valent iron as an electron donor.

4. Conclusion

We have characterized the dominant microbial populations of perchlorate reducing culture growing on zero-valent iron. Specifically, we have shown that (1) iron-supported perchlorate reduction is almost identical with that by H_2-fed control, (2) the dominant microbial reaction may be the combination of autotrophic and heterotrophic reduction of perchlorate, and (3) zero-valent iron can be an adequate electron donor for the microbial perchlorate reduction. This study can help to further understand the metabolic dynamics of microbial populations in the iron-supported culture system and it will facilitate the design of an ideal engineered system for perchlorate removal. Furthermore, the microbial ecological techniques presented here can be extended to understand the microbial reduction of other contaminants using zero-valent iron as the electron donor.

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