Appl Microbiol Biotechnol (2016) 100:6055–6068
DOI 10.1007/s00253-016-7401-3

ENVIRONMENTAL BIOTECHNOLOGY

A novel perchlorate- and nitrate-reducing bacterium, *Azospira* sp. PMJ

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Received: 22 October 2015 / Revised: 15 February 2016 / Accepted: 17 February 2016 / Published online: 14 March 2016

Abstract A novel perchlorate-reducing bacterium (PCRB), PMJ, was isolated from the mixed liquor suspended solids in the aerobic tank of a wastewater treatment plant. The 16S ribosomal RNA (rRNA), perchlorate reductase, and chlorite dismutase gene sequences revealed that PMJ belonged to the genus *Azospira*. PMJ was removed high-strength (700 mg/L) perchlorate and also removed low-strength (≤50 mg/L) perchlorate below the detection limit (2 μg/L) when acetate was used as a sole and carbon source. The maximum specific perchlorate utilization rate, q_{max}, was 0.96 mg ClO_4^-/mg dry cell weight day, and the half-saturation constant, K_S, was lower than 0.002 mg ClO_4^-/L. PMJ also utilized inorganic electron donors [(H_2, S^0, and Fe(II)] with perchlorate as an electron acceptor. Perchlorate reduction by PMJ was completely inhibited by oxygen and chlorate but was not inhibited by nitrate. In the presence of similar concentrations (100~140 mg/L) of nitrate and perchlorate, PMJ simultaneously removed both electron acceptors. Therefore, it was concluded that the strains PMJ might possess separate pathways for perchlorate and nitrate reduction. These results indicated that *Azospira* sp. PMJ could be efficiently used for treating perchlorate-contaminated groundwater and wastewater because many of these water bodies are known to contain both perchlorate and nitrate. In addition, low K_S value and autotrophic perchlorate reduction of PMJ might be useful to design the biological treatment systems.

Keywords *Azospira* sp. PMJ · Perchlorate-reducing bacteria (PCRB) · Simultaneous perchlorate and nitrate reduction · Autotrophic perchlorate reduction · Kinetic parameters

Introduction

Perchlorate is known to be used in the manufacture of solid rocket fuels, propellants, fireworks, explosives, and other commercial products (Achenbach et al. 2001; Coates and Achenbach 2004; Gao et al. 2015). Recently, perchlorate (ClO_4^-) contamination of surface water and groundwater has received much attention by researchers and policy makers due to its toxicity, high solubility, and stability in water (Srinivasan and Sorial 2009). Perchlorate is also known to potentially obstruct the function of thyroid hormone by interrupting iodide uptake and its metabolism (Urbansky and Schock 1999). In January 2009, the US EPA announced an interim health advisory level of perchlorate in drinking water to be 15 μg/L (US EPA 2009). US states including New York, Texas, New Mexico, Maryland, California, and Massachusetts set the maximum contaminant level (MCL) of perchlorate in drinking water at 6 μg/L or lower (US EPA 2005; Srinivasan and Sorial 2009). The Korean Ministry of Environment also has been

Electronic supplementary material The online version of this article (doi:10.1007/s00253-016-7401-3) contains supplementary material, which is available to authorized users.

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regulating the perchlorate advisory level in drinking water at 15 μg/L since 2010 (Korea MOE 2010).

Nitrate (NO_3^-) is a frequent co-contaminant with perchlorate in groundwater at military bases that house rockets (Logan et al. 2001). The MCL for nitrate in drinking water is also regulated at 10 mg NO_3^-–N/L (US EPA 2009), because nitrate causes methemoglobinemia in infants. It is also known that nitrate inhibits perchlorate reduction due to competition for the common electron donor particularly when the electron donor is insufficient (Choi and Silverstein 2008; Herman and Frankenberger 1998; Luo et al. 2015; Zhao et al. 2011).

Current technologies for perchlorate removal from perchlorate-contaminated waters include chemical and electrochemical reduction and biological treatment (Srinivasan and Sorial 2009), and microbial reduction of perchlorate to harmless chloride by perchlorate-reducing bacteria (PCRB) is considered as an effective and economic method (Dudley et al. 2008; Urbansky and Schock 1999; Xu et al. 2015). PCRB have been isolated from various environments and are mostly facultatively anaerobic denitrifiers (Coates and Achenbach 2004). Known PCRB belong to α-, β-, and ε-Proteobacteria, and majority of PCRB are found in the two genera of β-Proteobacteria, *Dechloromonas*, and *Azospira* (formerly *Dechlorosoma*) (Achenbach et al. 2001; Bardiya and Bae 2011; Coates and Achenbach 2004; Coates et al. 1999; Wallace et al. 1996). PCRB in the genus *Dechloromonas* is subdivided into RCB-type (*Dechloromonas aromatica* RCB) and CKB-type (*Dechloromonas agitata* CKB) based on 16S ribosomal RNA (rRNA) gene sequence (Coates and Achenbach 2004). Likewise, PCRB in the genus *Azospira* is subdivided into PS-type (*Azospira suillum* PS, *Azospira* sp. KJ, and *Azospira* sp. PDX) and non-PS-type (strain LT-1) (Bardiya and Bae 2011; Coates and Achenbach 2004). The strain LT-1 has recently been re-named as *Dechlorobacter hydrogenophilus* LT-1^T, and novel PCRB *Propionivibrio militaris* MP^T was identified based on 16S rRNA gene sequence and their biochemical characteristics (Thrash et al. 2010). The α-Proteobacteria is represented mainly by *Dechlorospirillum* spp. and *Azospirillum* sp. TT1 (Coates and Achenbach 2004; Waller et al. 2004). The ε-Proteobacteria is exclusively represented by *Wolinella succinogenes* HAP-1 which is most distantly related among reported PCRB (Bardiya and Bae 2011; Coates and Achenbach 2004; Wallace et al. 1996).

The aim of this study was to isolate and characterize a bacterium capable of removing perchlorate below advisory levels of drinking water. In this study, *Azospira* sp. PMJ, a novel heterotrophic PCRB was isolated, identified, and investigated to obtain kinetic parameters for perchlorate and nitrate reduction. Since it is well known that nitrate is frequently found in perchlorate-contaminated water, interaction between these two substances was studied in terms of removal kinetics. The growth of *Azospira* sp. PMJ on a wide range of perchlorate concentration was also monitored. Additionally, the effect of pH and various organic and inorganic electron donors were studied. Moreover, multiple electron acceptors on perchlorate reduction by *Azospira* sp. PMJ was investigated in order to better understand competition behaviors among nitrate, chlorate, and oxygen in perchlorate-contaminated wastewater.

Materials and methods

Isolation and cultivation

In order to isolate PCRB, mixed liquor suspended solids (MLSS) as a source of microorganisms was obtained from an aeration tank of a wastewater treatment plant in Suwon, Korea. The mineral salt medium adopted from Nor et al. (2011) was prepared using analytical-grade chemicals. Then, enrichment was carried out in a serum bottle (160 mL) containing 50 mL of the culture medium and 350 mg/L of MLSS. The culture medium contained a mineral salt medium supplemented with 100 mg/L of perchlorate and 500 mg/L of acetate as the sole electron acceptor and donor, respectively. The bottle was sealed with a butyl rubber stopper and crimped with an aluminum seal. The headspace of serum bottle was repeatedly vacuum-degassed and filled with nitrogen gas, and incubated in a shaker at 30 °C and 120 rpm. The culture became turbid in 7 to 10 days and was transferred (1 %, *v*/*v*) into the fresh medium. This enrichment was repeated for 15 times. Then, culture was streaked on the solid medium containing the same components as the liquid culture medium and 15 g/L of agar (Bacto agar, Difco Laboratories, Detroit, MI, USA) and cultivated for 3 days at 30 °C. The colonies were re-grown in the liquid medium and re-streaked on the solid medium until pure culture was obtained. Morphological characteristics were examined using a light microscope (BX51TR, Olympus, Japan) for cells grown at 30 °C for 2 days in the liquid medium. Growth temperature and initial pH of the medium were examined in the range of 25~35 °C and pH 5.0~8.0, respectively. The denitrification ability was determined by using the alpha-naphthylamine method (Beishir 1996) and by PCR-based analysis of *nirS* which encodes the cytochrome cd_1 nitrite reductase (Throback et al. 2004). Nitrate reduction ability of PMJ was also tested in the medium amended with 50 mg/L of nitrate and 500 mg/L of acetate. In order to determine the optimal ratio of acetate to perchlorate, acetate concentration was varied from 100 to 750 mg/L with perchlorate concentration fixed at 100 mg/L. The effects of perchlorate concentration was investigated in the range of 50~1000 mg/L with the acetate to perchlorate mass concentration ratio of 5.

For investigating the range of energy sources, PMJ was cultivated in the mineral salts medium separately containing 500 mg/L acetate, 435 mg/L butyrate, 1000 mg/L casamino

acid, 230 mg/L ethanol, 340 mg/L formate, 900 mg/L glucose, 160 mg/L methanol, 67.5 mg/L succinate, or 5 g/L yeast extract. In these experiments, the electron acceptor was 100 mg/L of perchlorate. Additionally, inorganic electron donors including hydrogen gas (H_2), elemental sulfur (S^0), and Fe(II) ($FeSO_4{\cdot}7H_2O$) were tested for perchlorate reduction. For autotrophic perchlorate reduction experiments, 2 g/L of sodium bicarbonate ($NaHCO_3$) was added as a carbon source.

Four types of electron acceptors, 100 mg/L perchlorate, 100 mg/L chlorate, 100 mg/L nitrate, and \oxygen filled in the headspace (110 mL) of a serum bottle, were also tested for determining their potential to receive the electrons from 500 mg/L acetate. In addition, PMJ was grown in the medium containing mixed electron acceptors (perchlorate-chlorate, perchlorate-nitrate, perchlorate-chlorate-nitrate) and their concentrations were measured.

PCR, sequencing and phylogenetic analysis

PMJ was grown until optical density at 600 nm (OD_{600}) reached 0.1 in the minimal salt medium supplemented with 100 mg/L of perchlorate and 500 mg/L of acetate, and 20 mg of cells (dry basis) were obtained by centrifugation (Micro 17R, Hanil, Korea). Then, genomic DNA of PMJ was extracted by using a kit (FastDNA® SPIN kit for soil, MP Biomedicals, LLC, Solon, OH, USA) according to manufacturer's instructions. The 16S rRNA gene, perchlorate reductase alpha subunit gene (*pcrA*), chlorite dismutase gene (*cld*), and cytochrome cd_1 nitrite reductase gene (*nirS*) were amplified by PCR using primer sets, 27F/1492R, pcrA 320/pcrA-v2, UCD-238F/UCD-646R, and nirSCd3aF/nirSR3cd, respectively (Supplementary Table S1). PCR reactions were carried out using Ex *Taq* polymerase (Takara Bio, Shiga, Japan) with the following thermal cycles: initial denaturation at 95 °C for 3 min; 30 cycles consisting of 95 °C for 30 s, 50~60 °C for 30 s (see annealing temperature in Supplementary Table S1), and 72 °C for 1 min; and a final extension at 72 °C for 10 min.

The PCR products were purified using a QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced by using an ABI 3730XL Capillary DNA Sequencer (Applied Biosystems, CA, USA) at Bionics (Seoul, Korea). The sequences of the 16S rRNA, *pcrA*, and *cld* genes were analyzed by BLASTN and Classifier services of the National Center for Biotechnology Information (NCBI; http://ncbi.nlm.nih.gov). The sequences of *Azospira* sp. PMJ and the reference sequences of PCRB were aligned with CLUSTALW program (Thompson et al. 1994). The phylogenetic trees were constructed using the MEGA6 program (Tamura et al. 2013). Evolutionary distance matrix were generated from the sequence of 16S rRNA and *cld* genes according to Kimura 2-parameter (Kimura 1980), and phylogenetic tree was constructed using the neighbor-joining method (Nei and Kumar 2000). The *pcrA* gene sequences of PMJ were translated into amino acid sequences using the ExPASy translate tool (http://web.expasy.org/translate/). Maximum likelihood trees were assembled under the Dayhoff and JTT Models using the translated amino acid sequence (Nei and Kumar 2000; Jones et al. 1992). The tree topology was evaluated by bootstrap analysis based on 1000 replicates (Felsenstein 1985).

Estimation of kinetic parameters

For kinetic analysis, *Azospira* sp. PMJ was grown in the mineral salt medium containing 500 mg/L acetate as the electron donor and 100 mg/L perchlorate as the electron acceptor. Cell density was monitored by measuring optical density at 600 nm (OD_{600}) or by measuring the dry cell weight (DCW) on the 0.2-μm membrane filter (Advantec, Japan). Perchlorate was analyzed using an ACQUITY UPLC™ system coupled to a Quattro Premier™ XE tandem quadrupole mass spectrometer (Waters, Manchester, UK). Chromatographic separation was carried out on an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters, Manchester, UK). The flow rate was set at 0.3 mL/min and the column temperature was 25 °C. The injection volume into the UPLC system was 10 μL. A Quattro Premier™ XE tandem quadrupole mass spectrometer was operated in the negative mode with the electrospray-ionization (ESI) source. The operating conditions were as follows: capillary voltage of 3.5 kV, collision voltage of 20 V, cone voltage of 30 V, ion source temperature of 250 °C, desolvation gas flow of 900 L/h, and collision gas flow of 0.3 mL/min. Under these conditions, the detection limit for perchlorate was 2 μg/L. Nitrate nitrogen (NO_3^--N) was analyzed based on the chromotrophic acid method by using the HACH-type HR nitrate Test 'N Tube™ vials (concentration range of 0.2~30.0 mg N/L) and a DR/2500 spectrophotometer (HACH, Colorado, USA). Specific growth rate (μ) and maximum specific growth rate (μ_{max}) were calculated through non-linear regression (SigmaPlot; SPSS Inc., Chicago, IL, USA) using the Monod model and OD_{600} data as Logan et al. (2001) and Waller et al. (2004) did. For utilization rate of an electron acceptor (substrate), the Monod equation was adopted as follows;

$$\frac{dS}{dt} = -q_{\max}\frac{S}{K_S + S}X$$

where, S was the concentration of perchlorate in milligrams per liter, K_S was the half-saturation constant for perchlorate in milligrams per liter, q_{max} was the maximum specific substrate utilization rate for perchlorate in milligrams perchlorate per milligrams $X{\cdot}d$, and X was the biomass concentration measured as DCW in milligrams per liter). In order to obtain the q_{max}, μ_{max}, and K_S, non-linear regression method (SigmaPlot; SPSS Inc., Chicago, IL, USA) was used. The kinetic

experiments were carried out in triplicate and the results were expressed as mean values ± standard deviations (SD).

GenBank sequence accession numbers

Obtained partial sequences of the bacterial 16S rRNA gene, perchlorate reductase (*pcrA*), and chlorite dismutase (*cld*) genes of PMJ were deposited into the GenBank under the name of *Azospira* sp. PMJ (accession no. KT276241~KT276243).

Results

Phenotypic characteristics

Six strains of perchlorate-reducing bacteria were isolated from MLSS in the aerobic tank of a domestic wastewater treatment plant with no known perchlorate exposure. All isolates were Gram negative and were capable of utilizing acetate as an electron donor and perchlorate and oxygen as electron acceptors. Genes of perchlorate reductase (*pcrA*), chlorite dismutase (*cld*), and cytochrome cd_1 nitrite reductase (*nirS*) were detected in these six isolates. Among them, PMJ was selected as the best perchlorate reducer because this isolate removed 125 mg/L of perchlorate to the concentration below the detection limit (2 μg/L) during 3 days of incubation (Fig. 1a). The PMJ strain also appeared to harbor all genes of perchlorate reductase (*pcrA*), chlorite dismutase (*cld*), and cytochrome cd_1 nitrite reductase (*nirS*) (Fig. 1b). The colonies grown for 2~3 days on the mineral salt agar plates containing 100 mg/L perchlorate and 500 mg/L acetate were circular, low-convex, smooth, non-glossy, light apricot-colored and 1 mm in diameter. Cells grew well aerobically and also anaerobically on nutrient agar and trypticase soy agar (TSA) media. Based on the 16S rDNA sequence, PMJ was identified as *Azospira* species (for details, see the "Phylogenetic analysis" section). Table 1 describes physiological characteristics of PMJ and other *Azospira* species in PCRB. Strain PMJ was facultative anaerobic, Gram negative, slightly curved rod and 0.4~0.5 × 1.8~2.0 μm in size. PMJ removed 100 mg/L of perchlorate at the cultivation temperature between 25 and 35 °C with optimum temperature of 30 °C but did not grow at 4 and 45 °C. Perchlorate removal was also observed over a pH range between pH 5.0 and 8.0 with an optimum pH between pH 7.0 and 7.5. Physiological characteristics of isolate PMJ was the most closely related to those of *Azospira* sp. perc1ace. However, perc1ace could not utilize formate, ethanol, and glucose, whereas strain PMJ utilized these substances (Frankenberger and Herman 2000). PMJ utilized glucose, H_2, S^0, and Fe(II) as electron donors for the perchlorate reduction, while most *Azospira* species in PCRB could not. PMJ was deposited at the Korean Collection for Type Cultures (KCTC 12757BP).

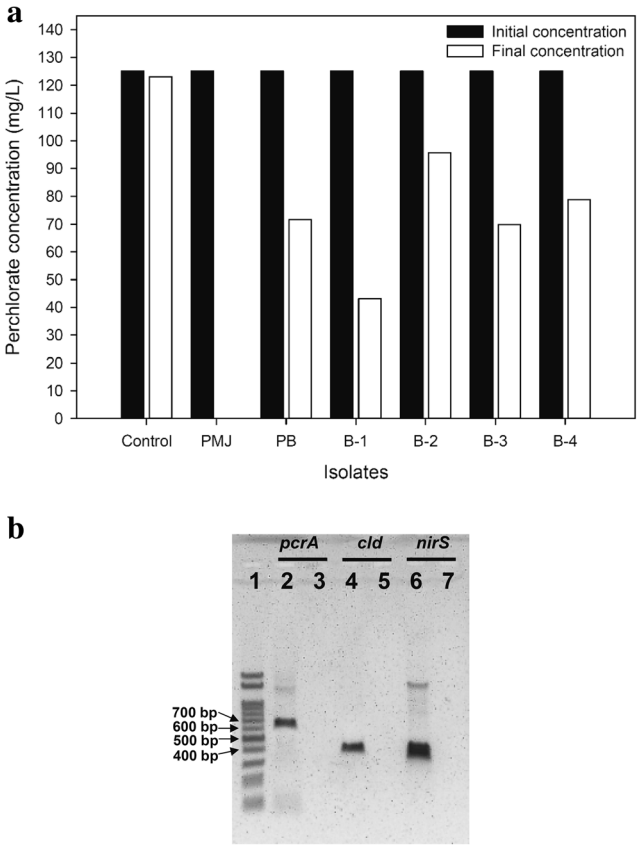

Fig. 1 Isolation and selection of perchlorate-reducing bacteria. **a** Perchlorate removal by six isolates and a control without microbial inoculation for 3 days. **b** The PCR-amplified DNA band of *pcrA*, *cld*, and *nirS* genes of strain PMJ. *Lane 1* 100 bp DNA ladder, *lane 2 pcrA* gene of PMJ, *lane 3* negative control of *pcrA* gene (*Escherichia coli*), *lane 4 cld* gene of PMJ, *lane 5* negative control of *cld* gene (*E. coli*), *lane 6 nirS* gene of PMJ, *lane 7* negative control of *nirS* gene (*E. coli*)

Phylogenetic analysis

The 16S rRNA gene sequence of the strain PMJ determined in this study was a continuous stretch of 1480 bp. Based on the sequence of 16S rRNA gene, strain PMJ was found to be the most closely related to genus *Azospira* of PCRB, showing 99 % identity with *Azospira suillum* PS, *Azospira* sp. HCAP-C, and *Azospira* sp. PDX (Fig. 2a). Most of the known PCRB belong to the β-Proteobacteria and closely related to each other with less than 1 % of divergence in 16S rRNA gene sequences. Especially, the type strain *Azospira suillum* PS and *Azospira oryzae* 6a3 showed 99.9 % similarity in terms of 16S rRNA gene sequences (Bardiya and Bae 2011; Zhao et al. 2011).

The perchlorate reductase gene sequence of the strain PMJ determined in this study was a continuous stretch of 632 bp (210 amino acids). With respect to deduced amino acid sequences of perchlorate reductase, strains PMJ were identical to *Dechloromonas* sp. PC1 and *Azospira* sp. HCAP-C (Fig. 2b). However, isolate PMJ was clearly distinct from the other two PS type of the genus *Azospira* that are known

Table 1 Characteristics of genus *Azospira* in perchlorate-reducing bacteria (PCRB)

Strains/species	*Azospira* sp. PMJ[a]	*Azospira* sp. perc1ace[b]	*Azospira* sp. HCAP-C[c]	*Azospira suillum* PS[d]	*Azospira* sp. GR-1[e]	*Azospira* sp. KJ[f]	*Azospira* sp. PDX[g]
Morphology (size)	Slightly curved rod (1.8~2.0 × 0.4~0.5 μm)	Curved rod	Rod (1.0 × 0.3 μm)	Rod	Rod	Rod (1.6 × 0.74 μm)	Rod (2.1 × 0.55 μm)
Gram stain	Negative	Negative	ND	Negative	Negative	Negative	Negative
Optimum temperature (°C)	30	25~30	22	37	30	25	25
Optimum pH	7.0~8.0	7.0~7.2	7.0	ND	7.0~7.2	8.0	ND
Denitrification	+	+	+	+	+	+	+
Electron acceptor utilized	ClO_4^-, ClO_3^-, NO_3^-, O_2	ClO_4^-, ClO_3^-, NO_3^-, O_2	ClO_4^-, ClO_3^-, NO_3^-, NO_2^-, O_2	ClO_4^-, ClO_3^-, NO_3^-	ClO_4^-, ClO_3^-, NO_3^-, O_2, Mn(IV)	ClO_4^-, ClO_3^-, NO_3^-, O_2	ClO_4^-, ClO_3^-, NO_3^-, O_2
Electron donor utilized	H_2, S^0, Fe(II), acetate, succinate, butyrate, ethanol, formate, yeast extract, casamino acid	Acetate, succinate, propionate, casamino acid, fumarate	H_2, acetate	Acetate, succinate, butyrate, ethanol, propionate, lactate, fumarate	Acetate, succinate, propionate, lactate, malate, caprionate	Acetate, succinate, ethanol, propionate, lactate, Tween 20, glycerol	Acetate, ethanol, lactate, Tween 80
Fermentation of glucose	+	−	ND	−	−	−	ND
Perchlorate and nitrate reduction	Simultaneously ($ClO_4^- \geq NO_3^-$)	Simultaneously ($ClO_4^- \leq NO_3^-$)	ND	NO_3^- preferred ($ClO_4^- \ll NO_3^-$)	NO_3^- preferred ($ClO_4^- \ll NO_3^-$)	ClO_4^- preferred ($ClO_4^- \gg NO_3^-$)	ND
Perchlorate reduction range (mg/L)	<0.002~700	<0.005~100	>(2.2)~1400	500	800	<0.1~500	ND

+ positive, − negative, *ND* not determined

[a] This study

[b] Giblin and Frankenberger 2001; Okeke et al. 2007

[c] Dudley et al. 2008

[d] Chaudhuri et al. 2002

[e] Van Ginkel et al. 1996; Rikken et al. 1996

[f] Logan et al. 2001; Xu et al. 2004; Xu et al. 2015

[g] Logan et al. 2001

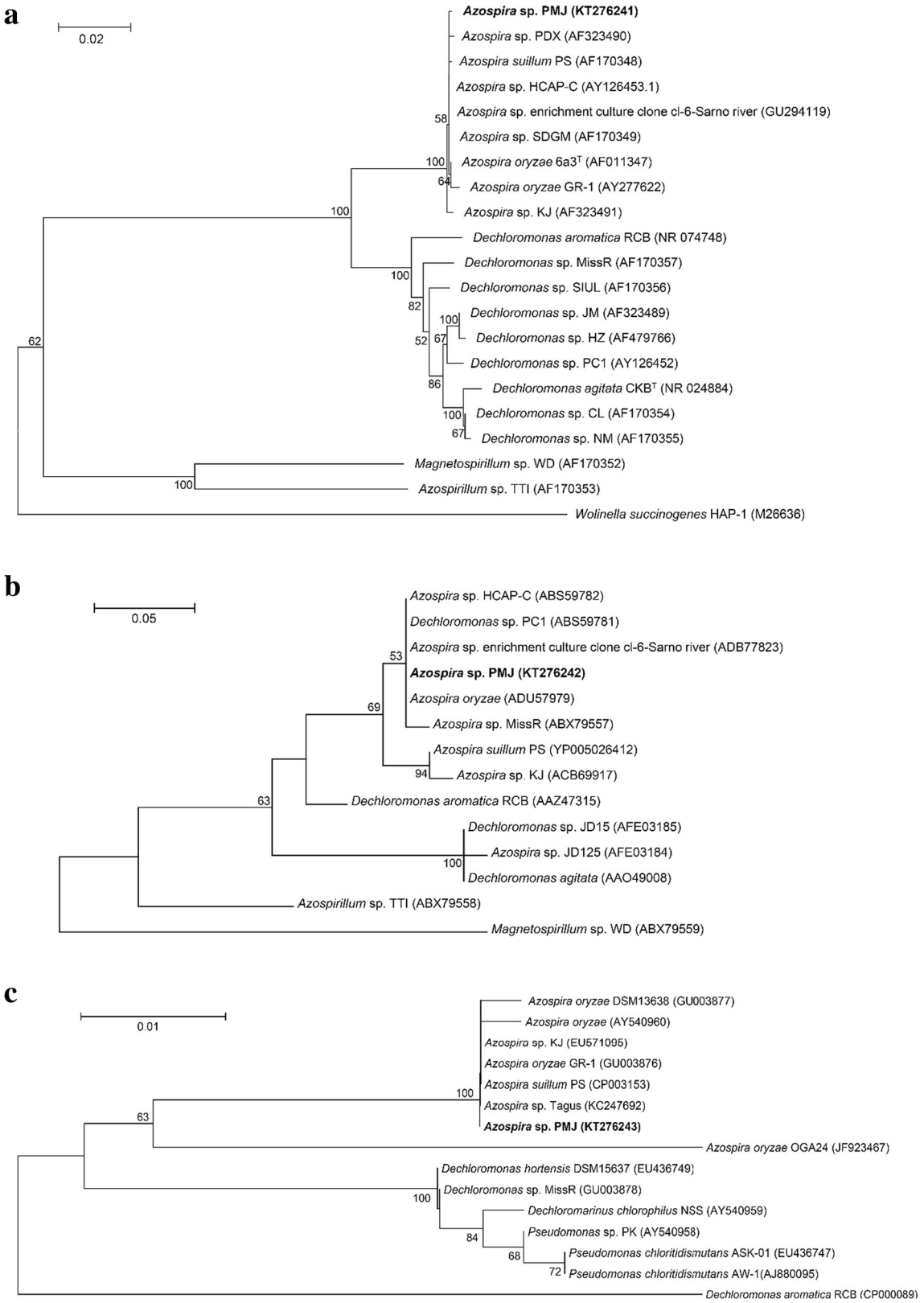

Fig. 2 Phylogenetic tree of isolate PMJ and perchlorate-reducing bacteria (PCRB) based on **a** 16S rRNA gene, **b** *pcrA* amino acid sequence, and **c** *cld* gene sequences. The 16S rRNA and *cld* genes tree were constructed by using the neighbor-joining method. The PcrA tree was constructed by using the maximum likelihood method. The bootstrap values above 50 % are shown at the internal nodes

to de-chlorinate perchlorate, *Azospira suillum* PS and *Azospira* sp. KJ (Bardiya and Bae 2011). PMJ was only 95 % similar to the PS type, and differed by 3 out of 88 amino acids. Strains PS and KJ differed by only one amino acid with 99 % similarity for *pcrA* gene sequences. The chlorite dismutase gene sequence of the strain PMJ determined in this study was a continuous stretch of 412 bp. According to chlorite dismutase gene (*cld*) sequence, strain PMJ was the most closely related to PCRB in the β-proteobacteria, showing 98 % identity with *Azospira suillum* PS, *Azospira* sp. KJ, and *Azospira oryzae* GR-1 (Fig. 2c). In case of *Azospira* sp. perc1ace, sequence of 16S rRNA, *pcrA*, and *cld* genes were not available. The *cld* gene sequence of HCAP-C was also not available. In short, phylogenetic characteristics of isolate PMJ were the most closely related to those of *Azospira* sp. HCAP-C and belonged to the non-PS-type in the genus *Azospira* based on the 16S rRNA and pcrA genes.

Concentration effects of perchlorate and acetate

In order to determine the optimal acetate to perchlorate ratio, the concentration of acetate was varied from 100 to 750 mg/L in the presence of 100 mg/L of perchlorate. The optimal acetate to perchlorate ratio was determined for the highest specific growth rate. As shown in Fig. 3a, PMJ grew well on 100~300 mg/L acetate (equivalent to 1.7~5.1 mM acetate, specific growth rates of 0.051~0.056 h^{-1}) as an electron donor but did not completely remove 100 mg/L perchlorate. The specific growth rates were 0.06 and 0.04 h^{-1} when grown on 500 mg/L (8.5 mM) and 750 mg/L (12.7 mM) acetate, respectively. These results showed that the optimal mass concentration ratio of acetate to perchlorate was approximately 5 (molar ratio of 8.5). This molar ratio of PMJ was much larger than previously-isolated PCRBs (molar ratio of 1~3.2) (Giblin and Frankenberger 2001; Logan et al. 2001; Nerenberg et al. 2006; Rikken et al. 1996; Waller et al. 2004; Xu et al. 2004), but these previously reported acetate concentrations were merely culture conditions. Besides, the results were similar to those of recent reports (Xu et al. 2015; Zhu et al. 2016) in that *Azospira* sp. KJ and a ClO_4^- acclimated mixed culture completely and efficiently reduced perchlorate at the molar ratio of 6.5~8.5. When the mass concentration ratio of acetate to perchlorate was 5, PMJ was able to remove perchlorate from the initial concentrations of 50~210 mg/L to below the detectable limit (<2 μg/L) within 1 day (Fig. 3b). PMJ was also capable of growing on a high-strength perchlorate (700 mg/L) and reduced perchlorate to 2 μg/L in 14 days, but PMJ did not grow on 1000 mg/L of perchlorate. Perchlorate removal rates of PMJ in the range 50~300 mg/L perchlorate were between 4.25 and 8.75 mg ClO_4^-/L/h. (Fig. 4). PMJ showed the highest perchlorate removal efficiency (100 %), removal rate (8.75 mg ClO_4^-/L/h), and specific growth rate (0.10 h^{-1}) when grown on 210 mg/L perchlorate 1000 mg/L acetate. Therefore, PMJ displayed high tolerance against high-strength perchlorate (≥700 mg/L) with high perchlorate reduction rates.

Electron donor

Most PCRBs are known to utilize a wide variety of organic and inorganic electron donors (Bardiya and Bae 2011). Particularly, all reported PCRBs used acetate as a single organic electron donor except *Moorella perchloratireducens* (Balk et al. 2008; Bardiya and Bae 2011). It has also been

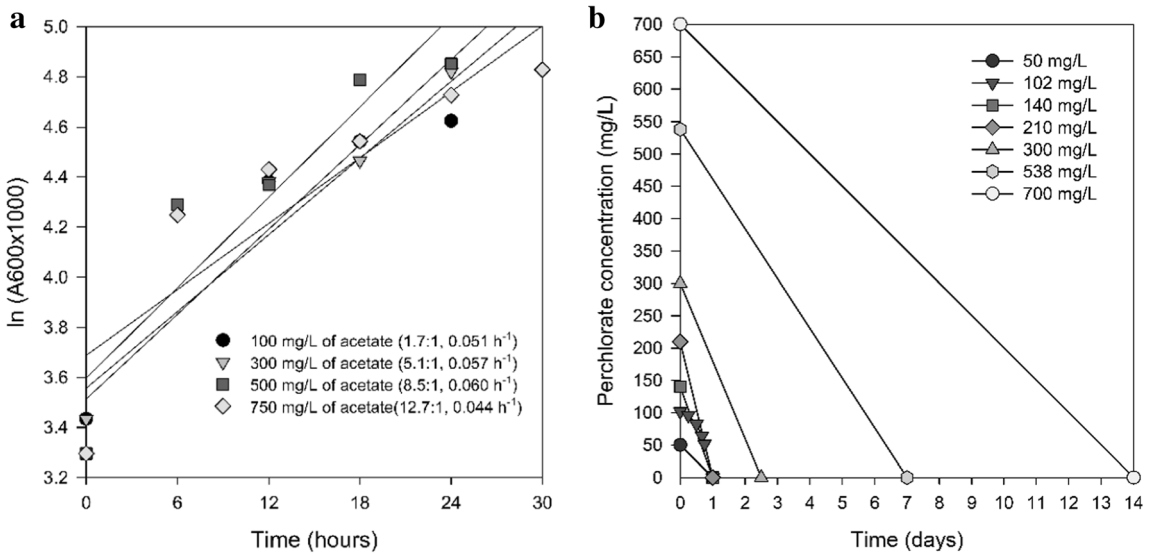

Fig. 3 Optimum perchlorate removal condition of PMJ. **a** Effects of molar ratios of perchlorate (100 mg/L) to acetate (100~750 mg/L) on specific growth rates. **b** Perchlorate removal according to various initial concentrations of perchlorate (containing 250~3500 mg/L of acetate satisfying perchlorate to acetate mass concentration ratio of 1:5)

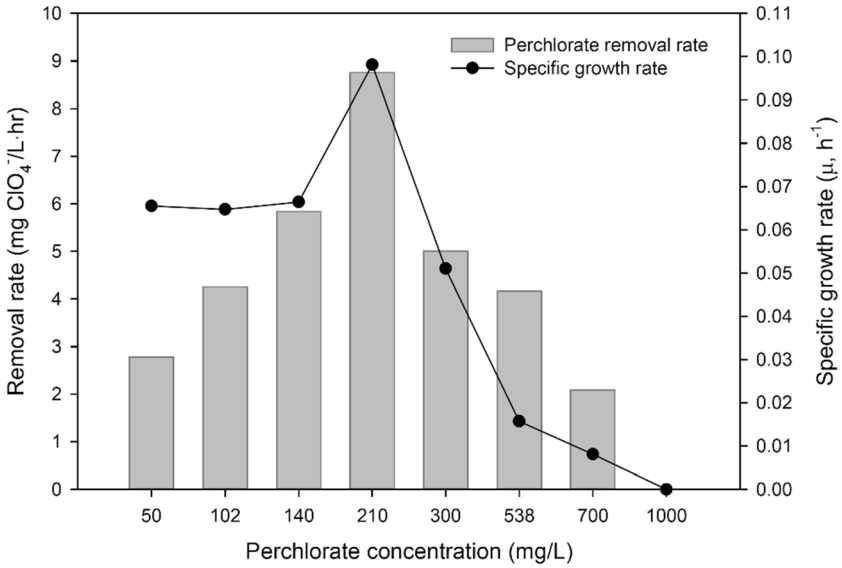

Fig. 4 Perchlorate reduction rate and specific growth rate of *Azospira* sp. PMJ in the batch cultures containing various initial concentrations of perchlorate and 250~3500 mg/L of acetate. The mass ratio of perchlorate to acetate concentration was 1:5

reported that some PCRB can utilize H_2 (Dudley et al. 2008; Shrout et al. 2005; Zhang et al. 2002), H_2S (Gregoire et al. 2014), elemental sulfur (S^0, Gao et al. 2015; Ju et al. 2007; Ju et al. 2008), $S_2O_3^{2-}$ (Ju et al. 2008), ferrous [Fe(II)] iron (Bruce et al. 1999; Lack et al. 2002; Gregoire et al. 2014), and zero-valent iron (Ju et al. 2008; Yu et al. 2007). PMJ used acetate as an organic electron donor for perchlorate reduction. Besides acetate, PMJ used heterotrophic electron donors, including carboxylic acids (butyrate, formate, and succinate); proteineous carbon sources (casamino acid and yeast extract); sugar (glucose); and alcohols (ethanol) (Table 1, Supplementary Fig. S1). The maximum specific growth rates for casamino acid (0.14 h^{-1}), yeast extract (0.06 h^{-1}), acetate (0.06 h^{-1}), and succinate (0.05 h^{-1}) were obtained within 48 h. PMJ utilized glucose as well with the specific growth rate of 0.045 h^{-1}, but a long lag phase (48 h) appeared. The strain also slowly utilized ethanol with the specific growth rate as low as 0.015 h^{-1} but did not utilize methanol.

PMJ was also capable of autotrophic perchlorate reduction using various inorganic electron donors including H_2, S^0, and Fe(II) (Tables 1 and 2). Strain PMJ exhibited perchlorate reduction rate of 0.37~1.11 mg ClO_4^-/L/h. when utilizing inorganic electron donors [H_2, S^0, and Fe(II)] with 140 mg/L of perchlorate. Table 2 shows that PMJ preferred electron donors in order of acetate, H_2, Fe(II), and S^0 in terms of the specific growth rate and perchlorate removal efficiency. Furthermore, perchlorate removal rate on S^0 and Fe(II) were significantly lower than acetate and H_2. During the cultivation, cell density was not measured due to insoluble powdery S^0 and green rust on Fe(II).

Kinetic parameters of PMJ

Several researchers have determined kinetic parameters for PCRB (Bardiya and Bae 2011; Dudley et al. 2008; Logan et al. 2001; Nerenberg et al. 2006; Rikken et al. 1996; Xu

Table 2 Growth of PMJ on organic and inorganic electron donors

Electron donor	Electron acceptor	Specific growth rate (μ, h^{-1})	Removal efficiency of perchlorate (%)	Removal rate of perchlorate (mg ClO_4^-/L/h)
500 mg/L acetate	140 mg/L ClO_4^-	0.066	99.99	5.83
H_2[a]	140 mg/L ClO_4^-	0.017	38.07	1.11
50 g/L S^0	140 mg/L ClO_4^-	ND	25.60	0.37
1.4 g/L Fe(II)	140 mg/L ClO_4^-	ND	33.08	0.48

PMJ was incubated in a mineral salts medium containing 140 mg/L of perchlorate as the electron acceptor and four different electron donors at 30 °C for 2 to 3 days. In case of inorganic electron donors [H_2, S^0, and Fe(II)], 2 g/L of $NaHCO_3$ was added as a carbon source

ND not determined

[a] For hydrogen gas experiments, the culture medium was purged with 100 % H_2 gas through a 0.2 μm sterile filter for 20 min to remove oxygen, and then 110 mL of the headspace of a serum bottle was sparged with H_2 gas for 5 min at atmospheric pressure

Table 3 Kinetic parameters of perchlorate-reducing bacteria from literatures and this study

PCRB	Perchlorate conc. mg/L	μ_{max} h^{-1}	q_{max} mg ClO_4^-/mg DCW/day	K_S mg ClO_4^-/L	Reference
Vibrio dechloratans			1.68	–	Korenkov et al. 1976
Wolinella succcinogenes			2.57	–	Wallace et al. 1996; Wallace et al. 1998
Azospirillum sp. SN1A	100	0.069	4.60	2.2	Waller et al. 2004
Azospirillum sp. ABL1	100	0.086	5.43	4.8	Waller et al. 2004
Dechloromonas sp. INS	100	0.067	4.35	18	Waller et al. 2004
Dechloromonas sp. RC1	100	0.085	6.00	12	Waller et al. 2004
Dechloromonas sp. PC1	100	–	3.1	0.14	Nerenberg et al. 2006
Azospira sp. GR-1	–	0.1	5.65	–	Rikken et al. 1996
	–	–	(3.8)	2.7 (27)	Okeke and Frankenberger
Azospira sp. Perc1ace	–	0.07	–	–	Herman and Frankenberger 1998
	–	–	(4.8)	3.45 (34.5)	Okeke and Frankenberger
Azospira sp. KJ	100	0.20	24	33	Logan et al. 2001
Azospira sp. PDX	100	0.24	7.5	12	Logan et al. 2001
Azospira sp. HCAP-C	200	–	4.4	76.6	Dudley et al. 2008
Azospira sp. PMJ	100	0.07 ± 0.003	0.96 ± 0.16	<0.002	This study

All kinetic parameters were obtained based on simple Monod equation neglecting competitive inhibition Numbers in parentheses refer to V_{max} (μmol/min/mg protein) and K_m (μm) reported by Okeke and Frankenberger (2003). Following conversion for K_S was used: 1 μM of K_m = 0.1 mg ClO_4^-/L

et al. 2015), which would be needed to predict the rates of perchlorate degradation in natural and engineered systems. PMJ completely removed 100 mg/L of perchlorate and grew to 115 mg/L of dry cell weight in 24 h (Supplementary Fig. S2). These batch data were subdivided over time interval and specific growth rates and specific utilization rates for perchlorate different concentration of were obtained and fitted into the Monod equation. The determined kinetic parameters for PMJ and other PCRB are summarized in Table 3. Maximum anaerobic growth rates on perchlorate (μ_{max}) have been reported to be in a range of 0.07 and 0.28 h^{-1} under heterotrophic conditions (Bardiya and Bae 2011; Logan et al. 2001; Rikken et al. 1996; Waller et al. 2004). The μ_{max} of PMJ was 0.07 ± 0.003 h^{-1} on perchlorate and acetate, which was in the range of those of other *Azospira* species (0.07~0.24 h^{-1}) and was similar to that of perc1ace (0.07 h^{-1}).

The maximum specific substrate utilization rate for perchlorate (q_{max}) and the half-saturation constant for perchlorate (K_S) values of PMJ, 0.96 ± 0.16 mg ClO_4^-/mg DCW/day and below 0.002 mg ClO_4^-/L, respectively, were much lower than other strains. The K_S values of other PCRB ranged from 0.14 (Nerenberg et al. 2006) to 76.6 mg/L (Dudley et al. 2008). Low K_S could particularly be effective in removing perchlorate at low concentrations (Bardiya and Bae 2011; Nerenberg et al. 2006). On the contrary, high K_S indicates low affinity for perchlorate, and thus removal efficiency of low concentrations of perchlorate might be poor. In the other sense, strains with high K_S such as HCAP-C could play an effective role for treating perchlorate at high concentrations (Bardiya and Bae 2011; Dudley et al. 2008). Of course, as Xu et al. (2015) reported, the kinetic parameters should not be simply compared unless experimental methods and conditions are completely uniform. However, the kinetic constant data suggested that PMJ possesses an advantage over other PCRB for treating perchlorate at low concentrations.

Competition among electron acceptors

Most PCRB are known to utilize inorganic electron acceptors such as O_2, chlorate (ClO_3^-), and nitrate (NO_3^-) in preference to perchlorate (Bardiya and Bae 2011). Specific growth rates of PMJ on 500 mg/L of acetate decreased for four electron acceptors in the order of O_2, ClO_3^-, NO_3^-, and ClO_4^- (Table 4). Similar results were also found in many PCRB (Bardiya and Bae 2011; Zhao et al. 2011). The O_2 and ClO_3^- supported higher specific growth rates (0.20~0.40 h^{-1}) than NO_3^- and ClO_4^- (0.07~0.10 h^{-1}). When perchlorate was used as a single electron acceptor, *Azospira* sp. PMJ removed perchlorate below the detection limit (2 μg/L) within 24 h without any lag phase. However, perchlorate reduction was significantly delayed in the presence of multiple electron acceptors (Fig. 5). A short lag phase for cell growth was found in ClO_4^-, ClO_3^-, NO_3^-, and ClO_4^- + O_2 systems, but an 18 h lag phase was observed in ClO_4^- + ClO_3^-, ClO_4^- + NO_3^-, and

Table 4 Growth of PMJ on various electron acceptors in the presence of 500 mg/L of acetate as an electron donor

Electron acceptor	Specific growth rate μ, h^{-1}	Perchlorate removal			
		Concentration, mg/L		Removal efficiency %	Removal rate mg/L/h
		Initial	Final		
140 mg/L ClO_4^-	0.066	140	BD	100	5.83
100 mg/L ClO_3^-	0.20	–	–	–	–
92 mg/L NO_3^-	0.104	(92)	(42)	(54.4)	(2.08)
O_2[a]	0.40	–	–	–	–
140 mg/L ClO_4^- + O_2[a]	0.391	140	104	25.7	3.0
140 mg/L ClO_4^- + 100 mg/L ClO_3^-	0.199	140	87.3	37.64	2.20
140 mg/L ClO_4^- + 100 mg/L NO_3^-	0.098	140 (100)	0.03 (52)	99.98 (48.0)	3.89 (1.33)
140 mg/L ClO_4^- + 100 mg/L ClO_3^- + 134 mg/L NO_3^-	0.122	140 (134)	102 (106)	27.14 (20.9)	0.79 (0.58)

Numbers in parentheses refer to concentration, removal rate, and removal efficiency of nitrate

BD below the detection limit (2 μg/L)

[a] PMJ was aerobically incubated at 30 °C with shaking (120 rpm)

ClO_4^- + ClO_3^- + NO_3^- systems. In particular, little perchlorate were reduced in ClO_4^- + O_2 system, which indicated that perchlorate reduction was severely inhibited by oxygen (Table 4). Other studies have also shown that oxygen inhibited perchlorate reduction (Bardiya and Bae 2011; Xu et al. 2015). The reason is generally attributed to a higher energy yield of oxygen than perchlorate (Choi and Silverstein 2008).

Perchlorate reduction by PMJ was also inhibited until chlorate was completely removed. When perchlorate was used as a single electron acceptor, PMJ's reduction rate was 5.83 mg ClO_4^-/L/h. However, in the multiple ClO_4^-/ClO_3^- and ClO_4^-/ClO_3^-/NO_3^- systems, perchlorate reduction rates decreased to 0.79~2.20 mg ClO_4^-/L/h. Bardiya and Bae (2011) reported that most PCRB utilized chlorate in preference to perchlorate. Nerenberg et al. (2006) and Dudley et al. (2008) observed that even a small amount of chlorate caused intense inhibitory effects on perchlorate reduction. Xu et al. (2015) suggested that the reduction of chlorate to chlorite by perchlorate reductase proceeded more efficiently than the reduction of perchlorate to chlorate.

In the multiple ClO_4^-/NO_3^- system, PMJ's perchlorate reduction was not severely inhibited by nitrate (Table 4). The presence of nitrate decreased the rate of perchlorate reduction rate as compared to the case of sole perchlorate reduction, from 5.83 to 3.89 mg/L/h. The reduction of 140 mg/L perchlorate to the concentration below 2 μg/L required 24 h in the single electron acceptor system. However, reduction of perchlorate below 0.03 mg/L required 36 h in the multiple ClO_4^-/NO_3^- system (Table 4 and Fig. 6). Nitrate reduction also showed an identical tendency with perchlorate reduction. At almost equal concentrations of nitrate (92~100 mg/L) and perchlorate (107~140 mg/L), PMJ simultaneously removed perchlorate and nitrate (Fig. 6). Similar results were reported in that *Azospira* sp. per1ace simultaneously reduced NO_3^- and ClO_4^-, but most studies on genus *Azospira* in PCRB have shown that perchlorate reduction was inhibited by nitrate (Bardiya and Bae 2011; Chaudhuri et al. 2002; Giblin and Frankenberger 2001; Xu et al. 2015). On the contrary, perchlorate was more rapidly reduced than nitrate by PMJ, which suggested that preferred electron acceptor was perchlorate rather than nitrate (Fig. 6). When μ_{max} was calculated, it was

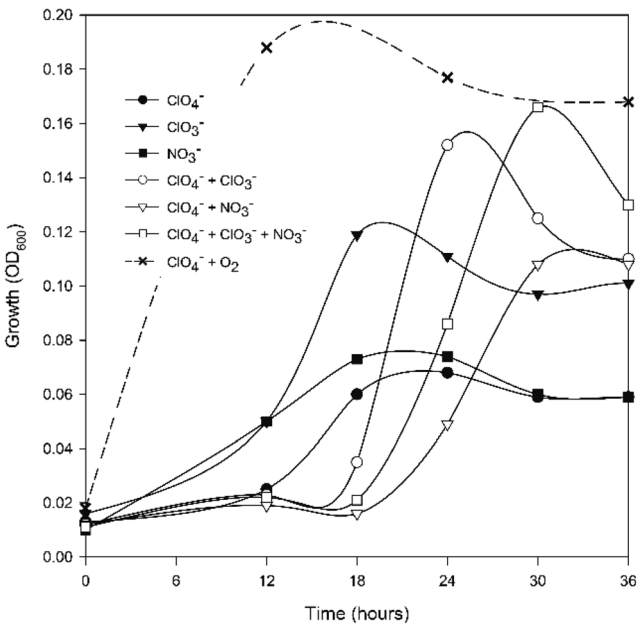

Fig. 5 Growth of PMJ on multiple electron acceptor systems. Concentration of electron acceptors were almost equal at 100 mg/L except oxygen, and acetate concentration was fixed at 500 mg/L as the electron donor

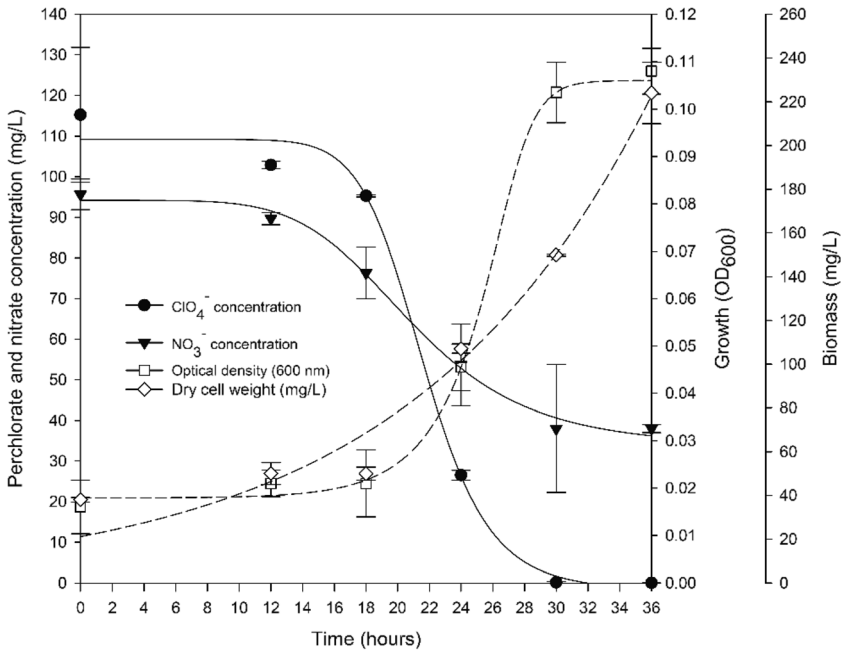

Fig. 6 Cell growth and consumption of perchlorate and nitrate by PMJ at pH 7.0 and 30 °C. The medium contained 115 mg/L of perchlorate, 96 mg/L of nitrate, and 500 mg/L of acetate. The *error bars* represent the standard deviation of three replicates

found that μ_{max} (0.09 h^{-1}) of multiple ClO_4^-/NO_3^- system was slightly higher than that (0.07 h^{-1}) of single electron acceptor (ClO_4^-) system. Furthermore, q_{max} for ClO_4^- of multiple ClO_4^-/NO_3^- system was calculated to be 1.2 mg ClO_4^-/mg DCW/day, which was higher than sole system of ClO_4^- (0.96 mg ClO_4^-/mg DCW/day). The higher q_{max} indicated that PMJ grew faster by using two electron acceptors together and nitrate did not inhibit perchlorate removal. This could be an important consideration for designing perchlorate removal processes.

Discussion

The primary objective of a ground water treatment system is to remove perchlorate to less than a detectable level. Another purpose of the research has been the removal of perchlorate at high concentrations which are associated with wastewater generated in industries. To be an effective remediation strategy, biological treatment should ensure the removal of perchlorate to concentrations less than the current advisory level of 15 μg/L, and preferably, to below the limit of detection. *Azospira* sp. HCAP-C could be effective for reducing perchlorate at high concentrations, e.g., 1360 mg ClO_4^-/L, but would not be effective at low concentrations (below 200 mg ClO_4^-/L) (Dudley et al. 2008). *Dechlorobacter hydrogenophilus* LT-1^T and *Propionivibrio militaris* MP^T were utilized for treating perchlorate in the range of 300~1000 and 400~1100 mg ClO_4^-/L, respectively (Thrash et al. 2010). In contrast, *Azospira* sp. perc1ace grew on perchlorate between 0.1 and 100 mg/L and reduced perchlorate to levels below 5 μg/L (Frankenberger and Herman 2000). *Azospira* sp. KJ utilized 500 mg/L of perchlorate (Xu et al. 2015). The perchlorate-reducing activity of isolate PMJ was more robust than other PCRBs because it was capable of growing on high-strength perchlorate (≥700 mg/L) and reduced 700 mg/L of perchlorate to a non-detectable level (2 μg/L) using acetate as the electron donor and carbon source. In addition, the significant difference in PMJ's K_S value might be useful to design biological treatment systems.

The inorganic electron donors [S^0, S^{2-}, Fe^0, Fe(II), and H_2] showed a slower perchlorate reduction rate. Even so, the capacity of autotrophic perchlorate reduction could be advantageous for designing a biological treatment system because heterotrophic perchlorate reduction requires continuous feeding of organic substrate and removing of byproduct (overflowing biomass) (Gao et al. 2015; Ju et al. 2008). Inorganic electron donors can overcome disadvantages of organic electron donors and thus are currently drawing a great deal of attention for biological treatment of perchlorate. H_2 is a good energy substrate, and perchlorate reduction using H_2 as the electro donor has been successfully used in bioreactors (Ju et al. 2008; Nerenberg et al. 2002; Van Ginkel et al. 2010; Zhang et al. 2002). However, H_2 handling and storage might raise a safety issue (Gao et al. 2015; Ju et al. 2008). S^0 and Fe(II) are insoluble and thus provide slow release of electron on demand, offering advantages of low maintenance, low cost, and less byproducts. PMJ utilized the reduced S^0 and Fe(II) to support perchlorate removal albeit removal rates were lower than H_2 (Table 2). The perchlorate removal rate of PMJ was 0.37 mg/L/h. (equivalent to 0.089 mM/day, Table 2) on S^0 as an electron donor. Ju et al. (2007, 2008) reported similar results for perchlorate removal rate on variety types of S^0 as electron donors (0.084~0.119 mM/day).

Therefore, it was expected that PMJ could use a variety of organic and inorganic electron donors in wastewater treatment system to remove perchlorate.

Groundwater contaminated with perchlorate often contains appreciable amounts of nitrate. When attempting bioremediation of perchlorate-contaminated groundwater, it is important that the presence of nitrate should not interfere with the perchlorate reduction. The Gibbs free energy changes (ΔG) for the acetate oxidation with redox potential of ClO_4^-/ClO_2^- and NO_3^-/N_2 are −792 and −801 kJ/mol, respectively (Xu et al. 2015). Close similarity in the ΔG values makes nitrate a strong competitor of perchlorate (Bardiya and Bae 2011; Xu et al. 2015). As a consequence, several PCRB differ significantly in their responses towards the two electron acceptors. The previous studies have revealed that most of the known PCRB utilized nitrate in preference to perchlorate except *Azospira* sp. KJ (Bardiya and Bae 2011; Chaudhuri et al. 2002; Xu et al. 2015) in which nitrate reduction was inhibited by the perchlorate. For another case, Giblin and Frankenberger (2001) reported that *Azospira* sp. per1ace reduced NO_3^- and ClO_4^- simultaneously when the acetate (electron donor) was not limiting. Zhao et al. (2011) suggested that the responses of PCRBs and denitrifiers to ClO_4^- and NO_3^- were species-specific. In case of *Azospira* sp. PMJ, perchlorate and nitrate were simultaneously removed (Fig. 6). Moreover, PMJ reduced perchlorate more rapidly than nitrate, suggesting a slight preference for this electron acceptor, while strain per1ace decreased perchlorate slowly (Giblin and Frankenberger 2001). Because most PCRBs preferred nitrate over perchlorate as an electron acceptor, complete removal of nitrate was achieved in the multiple ClO_4^-/NO_3^- systems (Bardiya and Bae 2011; Chaudhuri et al. 2002; Xu et al. 2015). Strain perc1ace completely removed 130 μg/L of perchlorate and simultaneously removed more than 95 % of the nitrate (20 mg/L) in a sand-packed bioreactor (Herman and Frankenberger 1999). In contrast, PMJ removed 100 % of the perchlorate and simultaneously reduced about 50 % of the nitrate which indicated that perchlorate was preferred than nitrate. Similar results were reported by Xu et al. (2015), who showed that *Azospira* sp. KJ grown in equal concentration of multiple ClO_4^-/NO_3^- and $ClO_4^-/ClO_3^-/NO_3^-$ systems removed 100 % of the perchlorate (500 mg/L) and approximately 50 % of the nitrate (260~280 mg/L) until cell growth reached stationary phase. They also observed that nitrate in multiple electron acceptor systems was rapidly removed during stationary and death phases. In addition, Xu et al. (2015) suggested that the activity of nitrate reductase was probably weakened in mixed electron acceptor systems.

Recent studies reported that the perchlorate and nitrate were reduced by separate pathways, and the presence of nitrate rendered an extended lag phase in perchlorate reduction in most PCRB (Bardiya and Bae 2011; Chaudhuri et al. 2002; Giblin and Frankenberger 2001; Xu et al. 2015). Chaudhuri et al. (2002) found that perchlorate-grown *Azospira suillum* PS preferentially reduced nitrate and did not reduce perchlorate until nitrate was completely removed when transferred to the medium containing equal molar amounts of perchlorate and nitrate. In contrast, an extended lag phase was observed if a similar nitrate-perchlorate medium was inoculated with a nitrate-grown culture. Xu et al. (2004) also reported that nitrate reduction by the *Azospira* sp. KJ grown on ClO_4^- medium was weak. Perchlorate reduction was also inhibited by the nitrate-grown *Azospira* sp. KJ cells. In addition, Giblin and Frankenberger (2001) found that nitrate and perchlorate reductases were located in different cell fractions (membrane and periplasmic fractions, respectively), indicating that these two enzymes were separate in *Azospira* sp. perc1ace. It was suggested that the perchlorate and nitrate were reduced by separate pathways which were individually catalyzed only by the respective reductases, perchlorate and nitrate reductases, in most *Azospira* species of PCRB. In addition, Xu et al. (2015) suggested that efficiency of perchlorate and nitrate reduction may be changed by the electron acceptor. We found that there was no difference in the perchlorate reduction whether PMJ was previously grown on nitrate or on perchlorate (data not shown). However, an extended lag phase and slow ClO_4^-/NO_3^- reduction were observed in multiple ClO_4^-/NO_3^- system as compared to sole electron acceptor system. Xu et al. (2004) reported that the perchlorate reduction became fast when cells was acclimated to the mixed ClO_4^-/NO_3^- medium, but nitrate reduction became slow. Based on perchlorate reductase gene sequencing and analysis, strains PMJ was different from *Azospira suillum* PS and *Azospira* sp. KJ, and closely related to *Azospira* sp. HCAP-C and *Dechloromonas* sp. PC1 (Dudley et al. 2008; Nerenberg et al. 2006). HCAP-C and PC1 were shown to grow on nitrate as a single electron acceptor, but experiments were not conducted to further clarify the role of nitrate reduction in those bacteria. In addition, the use of perchlorate and other physiological characteristics significantly distinguished PMJ from *Azospira* sp. HCAP-C (Table 1). Based on physiological characteristics of perchlorate reduction, PMJ was very similar to *Azospira* sp. KJ and *Azospira* sp. perc1ace. Therefore, we concluded that the strain PMJ might possess separate pathways for perchlorate and nitrate reduction that might be catalyzed by the different reductases. More detailed studies are needed to further explain the PMJ behavior and to expand the information on perchlorate-nitrate reductase in the genus *Azospira* of PCRB.

Taken together, differences in the kinetic parameters and phenotypic, genotypic, and metabolic data between strain PMJ and known PCRB indicate that strain PMJ represents a novel strain of genus *Azospira* in perchlorate-reducing bacteria. These results could extend the current understanding on PCRBs isolated from diverse environments. As a feature of the present study, PMJ may be well suited for use in a wide

variety of anaerobic bioreactors which are presently in use for removing contaminants. It was also expected that PMJ could be used for efficiently treating perchlorate-contaminated waters considering many water bodies are known to contain both perchlorate and nitrate.

Acknowledgments This subject was supported by Korea Ministry of Environment (MOE) as "Advanced Technology Program for Environmental Industry" under grant No. 2013001340002.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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