RESEARCH ARTICLE

Effect of nitrate, acetate, and hydrogen on native perchlorate-reducing microbial communities and their activity in vadose soil

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Abstract

The effect of nitrate, acetate, and hydrogen on native perchlorate-reducing bacteria (PRB) was examined by conducting microcosm tests using vadose soil collected from a perchlorate-contaminated site. The rate of perchlorate reduction was enhanced by hydrogen amendment and inhibited by acetate amendment, compared with unamendment. Nitrate was reduced before perchlorate in all amendments. In hydrogen-amended and unamended soils, nitrate delayed perchlorate reduction, suggesting that the PRB preferentially use nitrate as an electron acceptor. In contrast, nitrate eliminated the inhibitory effect of acetate amendment on perchlorate reduction and increased the rate and the extent, possibly because the preceding nitrate reduction/denitrification decreased the acetate concentration that was inhibitory to the native PRB. In hydrogen-amended and unamended soils, perchlorate reductase gene (*pcrA*) copies, representing PRB densities, increased with either perchlorate or nitrate reduction, suggesting that either perchlorate or nitrate stimulates the growth of the PRB. In contrast, in acetate-amended soil *pcrA* increased only when perchlorate was depleted: a large portion of the PRB may have not utilized nitrate in this amendment. Nitrate addition did not alter the distribution of the dominant *pcrA* clones in hydrogen-amended soil, likely because of the functional redundancy of PRB as nitrate-reducers/denitrifiers, whereas acetate selected different *pcrA* clones from those with hydrogen amendment.

Introduction

In the past decade, perchlorate (ClO_4^-) has been increasingly detected both in the environment (US Environmental Protection Agency, 2005a; California Department of Public Health, 2007a) and in food (Kirk *et al.*, 2003; Sanchez *et al.*, 2005; US Food and Drug Administration, 2005) in the United States. Perchlorate salts have been manufactured and used in explosives and pyrotechnics; in particular, ammonium perchlorate has been used as an oxidizer of rocket/missile fuels and disposed in large quantities (Motzer, 2001). Perchlorate is also known to occur naturally in dry regions in the southwestern United States (Dasgupta *et al.*, 2005; Rao *et al.*, 2007) and in Chilean nitrate fertilizer and its deposits (Urbansky *et al.*, 2001). Perchlorate potentially disrupts human thyroid hormone production by interrupting iodide uptake (Urbansky, 1998). An interim health advisory level for perchlorate in drinking water, issued by the US Environmental Protection Agency in January 2009, is 15 $\mu g\,L^{-1}$ (US Environmental Protection Agency, 2008). Some states have established stricter drinking water standards, such as California and Massachusetts, where the adopted maximum contaminant levels (MCLs) are 6 and 2 $\mu g\,L^{-1}$, respectively (Massachusetts Department of Environmental Protection, 2006; California Department of Public Health, 2007b).

High concentrations of perchlorate are often found in soil in the vadose (unsaturated) zone, likely due to land disposal of perchlorate waste(water)s or open burning/detonation of explosives (US Environmental Protection Agency, 2005b). In the US Department of Defense facilities, perchlorate has been detected in soil at concentrations as high as

2100 mg kg^{-1} (US Environmental Protection Agency, 2005a). Gal *et al.* (2008) also reported perchlorate contamination up to 1200 mg kg^{-1} in the deep vadose zone near a manufacturing plant in Israel. Although the concentrations are much lower, widespread naturally occurring perchlorate has also been found in the vadose zone in dry regions in the United States (Rao *et al.*, 2007).

To date, a number of bacterial strains capable of using perchlorate as an electron acceptor and reducing perchlorate to nontoxic chloride have been isolated from various environments (Rikken *et al.*, 1996; Wallace *et al.*, 1996; Bruce *et al.*, 1999; Coates *et al.*, 1999; Logan *et al.*, 2001; Zhang *et al.*, 2002; Waller *et al.*, 2004; Shrout *et al.*, 2005; Wolterink *et al.*, 2005; Nerenberg *et al.*, 2006). These microorganisms are seemingly ubiquitous; therefore, bioremediation is a promising technology to treat perchlorate contamination. However, our knowledge about the ecology of perchlorate-reducing microorganisms in the environment, particularly in the vadose zone, is very limited. It is important to understand the ecology of native perchlorate-reducing microbial communities to design optimal treatment systems appropriate for the particular conditions in the vadose zone.

Most perchlorate-reducing bacteria (PRB) use nitrate in addition to perchlorate as an electron acceptor, and some strains have been confirmed as denitrifiers (Rikken *et al.*, 1996; Coates *et al.*, 1999; Herman & Frankenberger, 1999; Achenbach *et al.*, 2001; Logan *et al.*, 2001; Zhang *et al.*, 2002; Waller *et al.*, 2004; Shrout *et al.*, 2005; Wolterink *et al.*, 2005; Nerenberg *et al.*, 2006). When both perchlorate and nitrate are present, pure cultures of PRB strains reduce nitrate preferentially or concurrently (Herman & Frankenberger, 1999; Chaudhuri *et al.*, 2002). In soil and groundwater, in which nitrate is commonly present, the preferential reduction of nitrate over perchlorate by native microbial communities appeared to be prevalent (Tipton *et al.*, 2003; Tan *et al.*, 2004; Waller *et al.*, 2004; Nozawa-Inoue *et al.*, 2005; Gal *et al.*, 2008), although concurrent reduction has also been observed in some sites (Waller *et al.*, 2004) and due to a repeated exposure to perchlorate (Tipton *et al.*, 2003). Despite the initial inhibitory effect of nitrate (when preferentially reduced) on perchlorate reduction, the rates of the following perchlorate reduction were larger with higher nitrate to perchlorate ratios, suggesting that nitrate could increase the PRB population (Nozawa-Inoue *et al.*, 2005; Gal *et al.*, 2008).

We examined the effects of adding nitrate (electron acceptor) and acetate or hydrogen (electron donors) on perchlorate reduction and PRB populations in vadose soil by conducting microcosm tests and quantifying functional gene copies for enumerating PRB. While nitrate-reducers/denitrifiers are widely distributed in soil (Tiedje, 1988) and those incapable of reducing perchlorate may also be involved in nitrate reduction, the presence of nitrate likely affects PRB populations and their perchlorate reduction activity. We hypothesized that nitrate would be reduced and delay perchlorate reduction initially and that nitrate would increase the PRB population ultimately. Acetate and hydrogen were commonly used electron donors to isolate PRB (Rikken *et al.*, 1996; Wallace *et al.*, 1996; Bruce *et al.*, 1999; Coates *et al.*, 1999; Logan *et al.*, 2001; Zhang *et al.*, 2002; Waller *et al.*, 2004; Shrout *et al.*, 2005; Wolterink *et al.*, 2005; Nerenberg *et al.*, 2006) or to promote perchlorate bioremediation (US Environmental Protection Agency, 2005b). Acetate and hydrogen were expected to select for different PRB species, because acetate promotes organotrophic conditions and hydrogen promotes lithotrophic conditions.

Materials and methods

Soil samples

The soil used in this study was collected from the vadose zone (15–45 cm below surface grade) at an industrial site in California (hereafter called Industrial Soil). The site has been contaminated with perchlorate, although perchlorate was not detected in the samples collected. The nitrate (NO_3^-) concentration was 0.2 μmol g^{-1} dry soil. The soil is comprised of 66% sand, 14% silt, and 19% clay, and categorized as a sandy loam. The gravimetric moisture content was 12%. The soil pH in water extract, cation exchange capacity, and organic matter was 7.4, 13.9 mEq 100 g^{-1} dry soil, and 1.4%, respectively. The soil was passed through a 2-mm sieve and stored in sealed plastic bags at 4 °C until experiments were performed.

Soil microcosm preparation

Soil microcosms with different treatments (Table 1) were prepared to examine the effect of the electron acceptor nitrate, and the electron donors, acetate or hydrogen, on PRB and their activities; controls with no added electron donor (unamendment) were included. Microcosms treated with ammonium were used to compare with those treated with nitrate. To measure perchlorate and other chemical concentration changes, 10 sets of triplicate microcosm bottles were prepared for each treatment in which perchlorate was added (Table 1), and the three bottles were destroyed at each sampling time. For microbial analysis, a triplicate of each live microcosm series (except microcosms with perchlorate, ammonium, and acetate), including the series in which no perchlorate was added, was prepared.

The nominal perchlorate concentration was 0.2 μmol g^{-1} dry soil (= 20 μg g^{-1} dry soil). Perchlorate was added as a solution of ammonium perchlorate (NH_4ClO_4); other non-gas chemicals were added as solutions of sodium salts

Table 1. Microcosm treatments

Microcosm series	Soil	Perchlorate addition	Nitrogen addition*	Carbon addition†	Headspace gas†
PU	Fresh	+	None	None	Nitrogen
U	Fresh	−	None	None	Nitrogen
PA	Fresh	+	None	**Acetate**	Nitrogen
A	Fresh	−	None	**Acetate**	Nitrogen
PH	Fresh	+	None	Bicarbonate	**Hydrogen**
H	Fresh	−	None	Bicarbonate	**Hydrogen**
cPA	Autoclaved	+	None	**Acetate**	Nitrogen
cPH	Autoclaved	+	None	Bicarbonate	**Hydrogen**
PNU	Fresh	+	**Nitrate**	None	Nitrogen
NU	Fresh	−	**Nitrate**	None	Nitrogen
PNA	Fresh	+	**Nitrate**	**Acetate**	Nitrogen
NA	Fresh	−	**Nitrate**	**Acetate**	Nitrogen
PNH	Fresh	+	**Nitrate**	Bicarbonate	**Hydrogen**
NH	Fresh	−	**Nitrate**	Bicarbonate	**Hydrogen**
cPNA	Autoclaved	+	**Nitrate**	**Acetate**	Nitrogen
cPNH	Autoclaved	+	**Nitrate**	Bicarbonate	**Hydrogen**
PMA	Fresh	+	Ammonium	**Acetate**	Nitrogen
cPMA	Autoclaved	+	Ammonium	Bicarbonate	Nitrogen

*Nitrate (indicated in bold) may serve as an electron acceptor alternative to perchlorate.

†Acetate and hydrogen (indicated in bold) may serve as electron donors for perchlorate reduction.

P, perchlorate added; U, unamended by electron donor; A, acetate amended; H, hydrogen amended; N, nitrate added; M, ammonium added; c, sterilized (autoclaved) control; +, perchlorate added; −, perchlorate not added.

(CH_3COONa, $NaHCO_3$, or $NaNO_3$) or a chloride salt (NH_4Cl). The final concentrations of acetate and bicarbonate in microcosms were approximately 10 μmol g^{-1} dry soil and 20 μmol g^{-1} dry soil, respectively. The nominal concentration of added nitrate and ammonium was 2.1 μmol g^{-1} dry soil. All solutions were either autoclaved or filter sterilized.

Purified water was used to increase the moisture content to 20%. Soil pH was not adjusted: most treatments did not alter the pH from the initial value (pH 7.4), except those with bicarbonate (pH 8.4). A mixture of 12 g of moist soil (10 g dry equivalent) was transferred to a 30-mL serum bottle, and the bottle was sealed with a butyl-rubber septum and an aluminum cap. Sterilized controls were prepared from soil that was autoclaved for one hour each for three consecutive days. The headspace of the bottle was purged with either 100% nitrogen (N_2) or 100% hydrogen (H_2) gas through a 0.2-μm sterile filter. The volume of the gas phase in the microcosm bottle was approximately 32 mL; therefore, estimated H_2 amount was 132 μmol g^{-1} dry soil. The microcosms were incubated at room temperature (23 ± 1 °C).

To reduce 1 mol of ClO_4^- to Cl^-, 1 mol of CH_3COO^- (Rikken *et al.*, 1996) or 4 mol of H_2 (Nerenberg & Rittmann, 2004) is needed, whereas 0.625 mol of CH_3COO^- (Sherwood *et al.*, 1998) or 2.5 mol of H_2 (Nerenberg & Rittmann, 2004) is required to reduce 1 mol of NO_3^- to N_2. The amounts of acetate and hydrogen added to microcosms (10 and 132 μmol g^{-1} dry soil, respectively) were much larger than those stoichiometrically required (up to 1.6 and 6.6 μmol g^{-1} dry soil, respectively), to ensure provision of ample electron donors for the PRB even in the presence of other competing electron acceptors such as nitrate.

Chemical analyses

Perchlorate, chloride, chlorate, nitrate, and nitrite in a microcosm replicate (12 g of moist soil) were extracted by the addition of 20 mL purified water and shaking this suspension in a 50-mL centrifuge tube with a screw cap for 6 h (Nozawa-Inoue *et al.*, 2005). The extract was centrifuged at 10 000 ***g*** for 10 min and the supernatant was filtered with a 0.2-μm membrane filter. The perchlorate concentration in the filtrate, with the addition of an ion strength adjustment buffer [0.04 M $(NH_4)_2SO_4$], was measured using a perchlorate ion-selective electrode (measurable down to 7 μM; Orion 93-81, Thermo Scientific, Beverly, MA) and a reference electrode (Orion 90-02, Thermo Scientific), equipped with an ion analyzer (Orion EA940, Thermo Scientific). pH in the extract was analyzed by a combination pH electrode (Orion 91-07, Thermo Scientific) equipped with a pH meter (Orion 250A, Thermo Scientific). Chloride, chlorate, nitrate, and nitrite were analyzed by ion chromatography using an IonPac AS14 column (Dionex Corp., Sunnyvale, CA). The mobile phase was 2.7 mM Na_2CO_3/1.0 mM $NaHCO_3$ at a flow rate of 1.0 mL min^{-1}.

Soil DNA extraction

For DNA analyses, soil microcosm samples were collected when the perchlorate concentration had decreased to

$< 0.02\,\mu mol\,g^{-1}$ dry soil ($>$ 90% degradation of the initial concentration), or when perchlorate reduction was very slow, as observed in microcosms PA and PNU, samples were collected around 100 days after the incubation started. Percent perchlorate removal in microcosms PA and PNU was approximately 40% at this point. Samples of microcosms with no perchlorate, incubated for the same time periods as those with perchlorate, were also collected for comparison purpose. Samples of microcosms and untreated soil were stored at $-20\,^{\circ}C$ until DNA was extracted. For DNA extraction, soil was prewashed with a buffer containing 0.1% $Na_4P_2O_7$, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA (Rosch *et al*., 2002) to remove free DNA and humic acid, and DNA was extracted using the FastDNA Spin kit for soil (MP Biomedicals, Solon, OH) according to the manufacturer's instruction.

Real-time quantitative PCR (qPCR)

Changes in the population densities of PRB, denitrifying bacteria, and total bacteria were estimated by real-time qPCR targeting perchlorate reductase gene *pcrA*, copper and cytochrome cd_1 nitrite reductase genes *nirK* and *nirS*, and the bacterial 16S rRNA gene, respectively. Five microliters of soil DNA, diluted 100 $\times$ (approximately 2–10 ng DNA per reaction), was subjected to each reaction. The *pcrA* and *nirK* qPCR mixtures (a final volume of 15 μL) consisted of the following (final concentrations): sample DNA, 1 $\times$ SYBR Premix *Ex Taq* (TaKaRa Bio USA, Madison, WI), 1 $\times$ ROX reference dye, 0.2 μM each of pcrA320 and pcrA598 primers (Table 2), and 0.25 μM each of nirK876 and nirK1040 primers (Table 2), respectively. For *nirS* qPCR, sample DNA was added in a mixture (a final volume of 20 μL) with 1 $\times$ Power SYBR green master mix (Applied Biosystems, Foster City, CA) and nirSCd3aF and nirSR3cd primers (Table 2) (0.5 μM each). For total bacteria, the mixture (12.5 μL as a final reaction volume) contained sample DNA, 1 $\times$ Universal TaqMan master mix (Applied Biosystems), 0.8 μM each of primers BACT1369F and PROK1492R, and 0.2 μM of the TM1389 probe (Table 2), respectively.

The reactions were performed using a 7300 real-time PCR system (Applied Biosystems). The *pcrA* gene fragments were amplified by a thermal cycling program of 95 °C for 1 min, followed by 35 cycles of 95 °C for 5 s and 60 °C for 31 s. The *nirK* fragments were amplified by a program of 95 °C for 30 s, followed by five touchdown cycles of 95 °C for 15 s, 63–59 °C (decreased by 1 °C per cycle) for 30 s, and 72 °C for 30 s, and 30 cycles of 95 °C for 15 s, 63 °C for 30 s, and 72 °C for 30 s. For amplification of *nirS* fragments, after initial denaturing at 95 °C for 10 min, five touchdown cycles as in the *nirK* qPCR program were performed, followed by 35 cycles of 95 °C for 15 s, 63 °C for 30 s, 72 °C for 30 s, and 80 °C for 30 s as the data acquisition stage. For the qPCR samples of *pcrA*, *nirK*, and *nirS*, the absence of nonspecific PCR products was confirmed both by dissociation curve analysis and by 1.5% agarose gel electrophoresis. The bacterial 16S rRNA gene fragments were amplified by a program of 50 °C for 2 min for uracil *N*-glycosylase activation and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 56 °C for 60 s.

The plasmid standard curves, for calculating gene copy numbers in samples, were generated by plotting the qPCR threshold cycle (C_T) against the gene copy numbers of plasmids containing the target genes amplified with qPCR primers. To generate these plasmids, the *pcrA*, *nirK*, *nirS*, and 16S rRNA gene fragments were PCR-amplified from *Dechloromonas agitata* CKB (ATCC 700666), *Sinorhizobium meliloti*, *Pseudomonas fluorescens*, and *Escherichia coli* K-12, respectively, and cloned into a plasmid and sequenced as described below. The copy numbers of the plasmids were calculated based on the DNA concentrations determined by measuring absorbance at 260 nm. Each plasmid standard was strongly linear ($R^2 > 0.99$) over eight orders of magnitude.

Cell ratios were estimated from the gene copy numbers using the following assumptions: one *pcrA* copy and two *nirS* copies per cell, as carried in the genome of perchlorate- and nitrate-reducing *Dechloromonas aromatica* RCB (Coates *et al*., 2001; Bender *et al*., 2005); one *nirK* copy per copper

Table 2. Oligonucleotide primers and probe used in this study

Target gene	Primer/probe	Sequence (5′–3′)	Reference
pcrA	pcrA320	GCGCCCACCACTACATGTAYGGNCC	Nozawa-Inoue *et al*. (2008)
	pcrA598	GGTGGTCGCCGTACCARTCRAA	
nirK	nirK876	ATYGGCGGVCAYGGCGA	Henry *et al*. (2004, 2005)
	nirK1040	GCCTCGATAGRTTRTGGTT	
nirS	nirSCd3aF	AACGYSAAGGARACSGG	Throback *et al*. (2004), Kandeler *et al*. (2006)
	nirSR3cd	GASTTCGGRTGSGTCTTSAYGAA	
Bacterial 16S rRNA gene	BACT1369F	CGGTGAATACGTTCYCGG	Suzuki *et al*. (2000)
	PROK1492R	AAGGAGGTGATCCRGCCGCA	
	TM1389*	CTTGTACACACCGCCCGTC	

*Fluorogenic probe, labeled with FAM and BHQ1 at the 5′- and 3′-ends, respectively.

denitrifier cell (Philippot, 2006); and an average of four copies of 16S rRNA genes per bacterial cell (Klappenbach *et al.*, 2001).

Cloning and sequencing

The *pcrA* gene fragments obtained from microcosms treated with perchlorate and hydrogen, with perchlorate, nitrate, and acetate, and with perchlorate, nitrate, and hydrogen (Table 1) were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clones were screened by PCR with M13 universal primers; about 20 positive clones were randomly picked from each sample and were subjected to restriction fragment length polymorphisms (RFLP) using the restriction endonuclease HhaI. The digestion patterns were examined as described previously (Nozawa-Inoue *et al.*, 2008). The plasmids of the *pcrA* clones with distinct RFLPs were extracted using the Plasmid Mini kit (Qiagen, Valencia, CA).

The inserts of the plasmids were sequenced at the UC Davis DNA sequencing facility (Davis, CA). One or two clones with the same RFLP were subjected to sequencing. The deduced PcrA protein sequences (92 amino acids), including *pcrA* clones from Yolo silt loam soil enriched with perchlorate and either acetate or hydrogen (Nozawa-Inoue *et al.*, 2005, 2008) and five perchlorate-reducing isolates (*D. agitata* CKB, *D. aromatica* RCB, *Dechloromonas* sp. MissR, *Azospirillum* sp. TTI, and *Dechlorospirillum* sp. WD), were aligned by CLUSTALW (Thompson *et al.*, 1994). A phylogenetic tree was constructed using the neighbor-joining method (Saitou & Nei, 1987) and visualized with NJ plot (Perriere & Gouy, 1996).

The sequences of *pcrA* clones (PH1–PH3, PNH1–PNH2, and PNA1–PNA5), obtained in this study (Table 1), have been deposited in the GenBank database under accession numbers FJ602703–FJ602712.

Results

Effect of acetate, hydrogen, and nitrate on perchlorate reduction

Perchlorate was degraded by native soil microorganisms in Industrial Soil (Fig. 1a and b). When 0.24 ± 0.02 μmol g^{-1} dry soil perchlorate was degraded to < 0.02 μmol g^{-1} dry soil, the chloride concentrations increased in the range of 0.20–0.22 μmol g^{-1} dry soil. As high as 0.01 μmol g^{-1} dry soil chlorate (ClO_3^-), an intermediate of perchlorate reduction, was detected transiently in a few samples (data not shown).

Hydrogen amendment enhanced the perchlorate degradation rate (> 90% was degraded in 7 days) compared with unamendment (37 days) (Fig. 1a). With acetate amendment, only about 22% of perchlorate was reduced in 43 days (Fig. 1a): even after 100 days of incubation, about 0.14 ± 0.08 μmol g^{-1} dry soil perchlorate remained in soil (data not shown).

The addition of nitrate substantially prolonged the lag period of perchlorate biodegradation in unamended soil and slightly in hydrogen-amended soil (Fig. 1b). Perchlorate

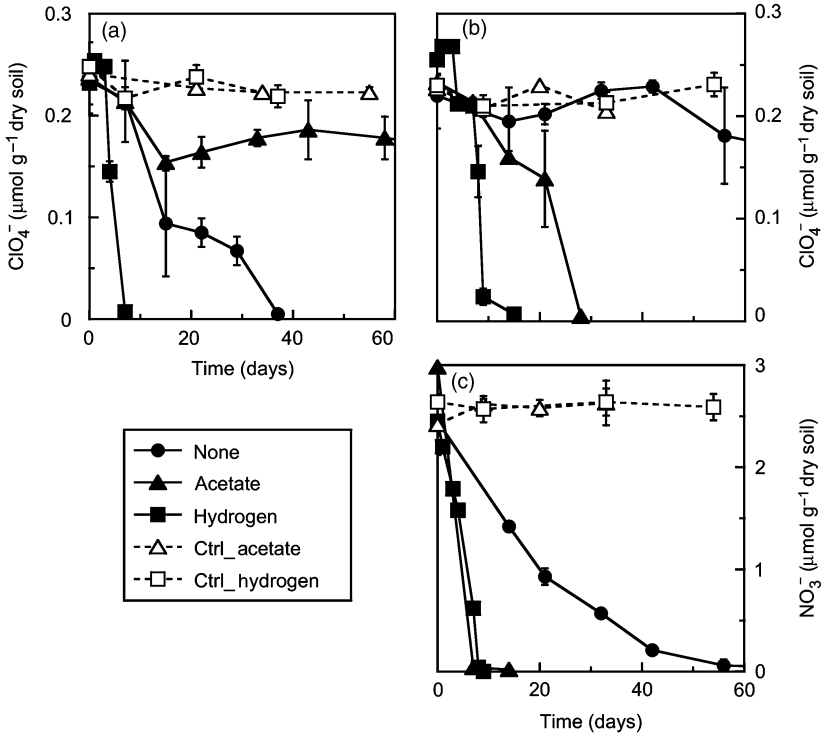

Fig. 1. Changes in perchlorate concentrations without (a) and with (b) nitrate addition, and nitrate concentrations with nitrate addition (c) in soil microcosms amended with different electron donors (none, acetate, or hydrogen) (mean ± SD; $n = 3$). Ctrl, autoclaved control.

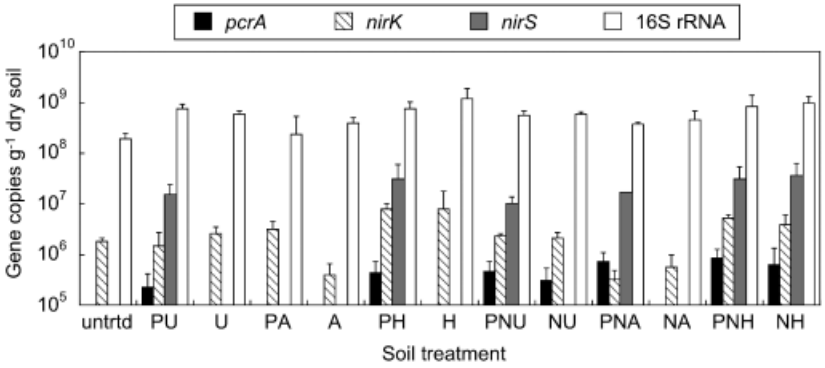

Fig. 2. Changes in the copy numbers of *pcrA*, *nirK*, *nirS*, and bacterial 16S rRNA genes in untreated soil (= untrtd) and soil microcosms (mean ± SD; $n = 3$). Each microcosm name indicates treatment types: P, perchlorate added; U, unamended with an electron donor; A, acetate amended; H, hydrogen amended; N, nitrate added.

could not be completely reduced in unamended soil with nitrate addition even after 100 days ($0.13 \pm 0.08\,\mu mol\,g^{-1}$-dry soil perchlorate remained; data not shown). Nitrate was reduced before perchlorate without a substantial lag period (Fig. 1c). Nitrate was also depleted in non-perchlorate-added microcosms (data not shown). A transient slight increase in the nitrite concentrations was observed during nitrate reduction in hydrogen-amended soil, although the concentrations remained $< 0.2\,\mu mol\,g^{-1}$ dry soil (data not shown).

Nitrate was also reduced before perchlorate in acetate-amended soil (Fig. 1c). Compared with no nitrate addition, nitrate addition enhanced the rate and the extent of perchlorate reduction (over 90% degradation in 28 days of incubation) in acetate-amended soil (Fig. 1a and 1b). To test whether this was due to the effect of nitrate providing an N source rather than serving as a terminal electron acceptor, ammonium was used instead of nitrate with perchlorate and acetate. Perchlorate was also degraded over 90% with the addition of ammonium, but most of the reduction occurred beyond 50 days (data not shown).

Soil pH increased slightly as nitrate was reduced, from the initial 7.4 to up to 7.9 in unamended and acetate-amended soil, and from the initial 8.4 to up to 8.7 in hydrogen-amended soil (data not shown). The high pH in hydrogen/bicarbonate-amended soil did not appear to inhibit perchlorate reduction substantially.

Changes in the abundance of *pcrA*, *nirS*, *nirK*, and bacterial 16S rRNA genes

Although the *pcrA* gene was not initially detectable in untreated soil, the gene was detected in the unamended and hydrogen-amended microcosms (Fig. 2), in which about $0.2\,\mu mol\,g^{-1}$ dry soil perchlorate was degraded (Fig. 1a). The *pcrA* copies remained at undetectable levels in the acetate-amended microcosm without nitrate, in which perchlorate degradation was limited (Fig. 1a), and in microcosms to which neither perchlorate nor nitrate was added (Fig. 2). With nitrate addition, in hydrogen-amended and unamended soil, *pcrA* copies increased regardless of perchlorate addition (Fig. 2). In acetate-amended soil, however, the *pcrA* gene was detected only when both nitrate and perchlorate were added (Fig. 2). When perchlorate was completely reduced, substantial differences were not observed in *pcrA* copy numbers among different electron donor treatments (Fig. 2).

The *nirK* gene, but not the *nirS* gene, was detected in untreated soil ($1.8 \pm 0.4 \times 10^6$ copies g^{-1} dry soil). About 10^7 copies of *nirS* genes were detected in soil microcosms in which *pcrA* genes were also detected, except unamended microcosms treated with nitrate only (Fig. 2). Although minor changes were observed, *nirK* copies remained in the range of 10^5–$10^6\,g^{-1}$ dry soil (Fig. 2). Nitrate addition did not appear to increase the total copy numbers of *nirK* and *nirS*.

There were only minor changes in bacterial 16S rRNA gene copies after incubation: the copy numbers remained in the range of 10^8–10^9 copies g^{-1} dry soil (Fig. 2). Before treatment, the ratio of *nirK* to bacterial 16S rRNA gene copies was $1.0 \pm 0.4\%$, corresponding to the estimated cell ratio of $3.9 \pm 1.7\%$. After perchlorate and/or nitrate reduction, the ratios of *pcrA* and *nirK*+*nirS* (when both genes were detected) to bacterial 16S rRNA gene copies were $0.1 \pm 0.2\%$ and $3.8 \pm 2.4\%$, corresponding to the estimated cell ratios of $0.4 \pm 0.5\%$ and $8.7 \pm 5.2\%$, respectively.

Changes in perchlorate-reducing bacterial communities

Five distinct PcrA sequences were detected in acetate-amended soil to which both perchlorate and nitrate were added, whereas three and two different sequences were found in hydrogen-amended soil treated with perchlorate only and with both perchlorate and nitrate, respectively. In hydrogen-amended soil, the most abundant *pcrA* clones, PH1 and PNH1 (accounting for 15 and 17 out of 20 recovered clones, respectively), had sequences 100% identical to one another (Fig. 3). An identical clone was also detected in acetate-amended soil, but not in as high a

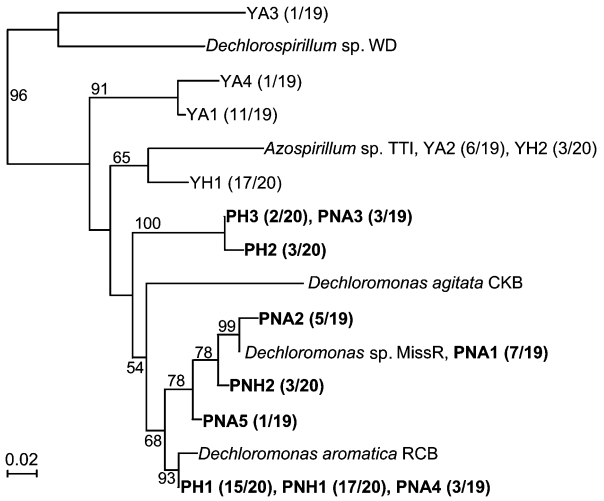

Fig. 3. Phylogenetic tree of deduced PcrA amino acid sequences of clones and perchlorate-reducing isolates. The sequences PH, PNH, and PNA (indicated in bold type) are *pcrA* clones obtained from soil microcosms in this study. P, perchlorate added; A, acetate amended; H, hydrogen amended; N, nitrate added. The numbers in parentheses, *n*/*m*, indicate *n* clones with identical sequences in the total *m* clones screened from each soil microcosm sample. Other PcrA sequences included in the tree are those of known PRB [*Dechloromonas agitata* CKB (GenBank accession number AY180108, *Dechloromonas aromatica* RCB (AAZ47315), *Dechloromonas* sp. MissR (EU273890), *Azospirillum* sp. TTI (EU273891), and *Dechlorospiriilum* sp. WD (EU273892)] and the *pcrA* clones from Yolo silt loam soil enriched in a medium containing perchlorate and either acetate (YA) or hydrogen (YH) (EU273893–EU273898). Bootstrap values above 50 from 100 resampling are shown at each node. The scale bar indicates 0.02 changes per amino acid.

proportion (3 out of 19 clones). In addition, the PcrA sequences of 13 out of 19 clones recovered from acetate-amended soil were distinct from those from hydrogen-amended soils. The PcrA sequences of the majority of PH and PNA clones, and all PNH clones, were closely related to the PcrA sequences of perchlorate-reducing *Dechloromonas* sp. strain MissR or *D. aromatica* strain RCB (Fig. 3).

Discussion

As hypothesized, nitrate was reduced before perchlorate reduction in all soil treatments, and perchlorate reduction was delayed in unamended and hydrogen-amended soil. While the negligible lag periods of nitrate reduction may indicate that there is a relatively large preexistent nitrate-reducing population including those not capable of utilizing perchlorate, the large portion of the native PRB also appeared to utilize nitrate preferentially, as inferred from the delayed perchlorate reduction and the growth of PRB by nitrate. Incomplete reduction of perchlorate in unamended soil with nitrate addition probably resulted from the depletion of naturally occurring electron donors by nitrate reduction. The increase in *pcrA* copy numbers, reflecting the PRB densities, in unamended and hydrogen-amended soils with nitrate addition even in the absence of perchlorate, suggests that the PRB populations also use nitrate as an electron acceptor, as observed in PRB isolates (Rikken *et al.*, 1996; Coates *et al.*, 1999; Herman & Frankenberger, 1999; Achenbach *et al.*, 2001; Logan *et al.*, 2001; Zhang *et al.*, 2002; Waller *et al.*, 2004; Shrout *et al.*, 2005; Wolterink *et al.*, 2005; Nerenberg *et al.*, 2006). This functional redundancy may have also resulted in the lack of a substantial difference in the PRB community compositions, based on the recovered *pcrA* sequences, in hydrogen-amended soils between treatments without and with nitrate addition.

The sandy loam soil investigated in this study, Industrial Soil, and an agricultural soil we tested previously, Yolo silt loam (Nozawa-Inoue *et al.*, 2005), responded differently to acetate and hydrogen. Acetate shortened the lag period of perchlorate reduction more than hydrogen in Yolo loam soil, whereas acetate, without nitrate addition, appeared to inhibit perchlorate reduction in Industrial Soil. The present result also apparently conflicts with a previous study of aquifer materials from 12 different locations, in which perchlorate was degraded to an undetectable level in all acetate-amended microcosms (Waller *et al.*, 2004). Differences may result from a high concentration of acetate: while up to 10 mM acetate was used in the previous study (Waller *et al.*, 2004), the 10 μmol g^{-1} dry soil acetate corresponds to 50 mM in the aqueous phase in our unsaturated soil microcosms. The responses of PRB population to acetate, therefore, cannot be generalized across soils, but may be specific to the microbial populations and other characteristics of a particular soil. This suggests the importance of preliminary studies to test which electron donors/carbon sources and what range of concentrations are suitable for a particular system before bioremediation technologies are applied.

In acetate-amended soil, nitrate facilitated perchlorate reduction. A possible explanation is that Industrial Soil was too deficient in N to support acetate-utilizing PRB in perchlorate reduction. Providing an alternative N source to nitrate, in this case ammonium, in fact did support the reduction of perchlorate. However, the reaction was much slower with ammonium than with nitrate; supplementing an N source for PRB, therefore, does not seem to be the major cause of the enhanced perchlorate reduction with nitrate. Another possible explanation is that the consumption of acetate by heterotrophic nitrate-reducers/denitrifiers led to a decrease in an inhibitory concentration of acetate [10 μmol g^{-1} dry soil or 50 mM in aqueous phase] to a level at which more oligotrophic PRB in this soil could become active and use perchlorate. Up to 1.9 μmol g^{-1} dry soil of acetate was presumably consumed by reduction/denitrification of all nitrate (3.0 ± 0.0 μmol g^{-1} dry soil) in acetate-

amended soil, corresponding to the change in aqueous concentration to 41 mM.

Without perchlorate, nitrate did not appear to support the growth of PRB in acetate-amended soil, in contrast to unamended and hydrogen-amended soil. This may be because a large portion of these two populations, PRB and nitrate-reducing/denitrifying bacteria, are different in acetate-amended soil. There is also a possibility, however, that the PRB density decreased in acetate-amended soil without perchlorate after most nitrate was reduced, due to the delay in sampling (3 weeks later).

We used nitrite reductase genes as a marker for quantifying denitrifying bacteria. Although nitrate reduction is the first step in denitrification, the ability to reduce nitrate is widespread, even in nondenitrifying bacteria (Philippot, 2005). Two types of dissimilatory nitrate reductase genes, *narG* (membrane bound) and *napA* (periplasmic bound), are present (Philippot, 2005), and a qPCR assay for *narG* has been developed previously (Lopez-Gutierrez *et al.*, 2004); however, probably due to the large sequence diversity of *narG*, the assay only targeted an uncultured group of nitrate reducers. The next step in denitrification, nitrite reduction by either NirK or NirS, distinguishes denitrifiers from nondenitrifying nitrate reducers (Hallin & Lindgren, 1999). Therefore, we adopted qPCR methods that were previously developed for *nirK* and *nirS* and applied to the estimation of denitrification populations in soil samples (Henry *et al.*, 2004; Kandeler *et al.*, 2006), with slight modifications.

The qPCR methods for *nirK* and *nirS* appeared to have a difference in the detection limits, of nearly two orders of magnitude: *nirK* qPCR was detectable down to 20 copies per reaction using the plasmid standard, whereas *nirS* qPCR could only be detected down to 1000 copies per reaction. These detection limits were slightly better than or similar to the previously reported values, 10^2 *nirK* gene copies per reaction (Henry *et al.*, 2004) and 1.25×10^3 *nirS* gene copies per reaction (Kandeler *et al.*, 2009). The sensitivity difference may be one of the reasons why *nirS* was detected only in six treatments, whereas *nirK* was detected in all microcosms including untreated soil.

nirS was detected in most cases when *pcrA* was detected. In non-nitrate-added soil (with hydrogen or unamendment), the growth of a *nirS*-harboring population was likely supported by reduction of perchlorate (i.e. the PRB likely harbored *nirS*) in addition to indigenous nitrate, because denitrification of indigenous nitrate requires less electron donor than perchlorate reduction (e.g. 0.5 and 0.8 $\mu mol\ g^{-1}$ dry soil H_2, respectively). In nitrate-added soil, nitrate likely contributed to the increases in *nirS* denitrifier densities, as *nirS* was detected with hydrogen amendment regardless of perchlorate addition. No detection of *nirS* in acetate amendment and unamendment, even with nitrate addition, may have been due to declines in *nirS*-harboring populations after nitrate reduction was completed, because sampling was conducted much later than nitrate was depleted. In particular, excess acetate may have promoted further anaerobic respiration such as sulfate reduction, as observed in a previous microcosm study (Waller *et al.*, 2004), creating an unfavorable redox condition for denitrifiers and causing decreases in their densities.

No substantial increase in bacterial 16S rRNA gene copies may reflect the fact that only a small portion of total bacteria were selected by the electron acceptors (perchlorate and nitrate) and donors in soil. The densities of PRB and denitrifying bacteria appeared to have increased in some treatments; however, their changes were too small to be detected as the increase in the total bacterial densities. The estimated densities of PRB remained $< 1\%$ to those of total bacteria. The increases in the gene ratios of *nirK*+*nirS* to bacterial 16S rRNA were only up to 3% (the increases in the estimated cell ratios of denitrifiers to total bacteria were up to 8%). Consequently, any changes in bacterial 16S rRNA gene copies caused by the growth of PRB and denitrifying bacteria were not easily detectable.

Acetate and hydrogen appeared to select for different PRB. The result was similar to what we observed in enrichment cultures of Yolo silt loam soil: the majority of *pcrA* clones (Nozawa-Inoue *et al.*, 2008), as well as the known PRB 16S rRNA gene sequences recovered from the selected denaturing gradient gel electrophoresis (DGGE) bands (Nozawa-Inoue *et al.*, 2005), were different between acetate and hydrogen amendments. In contrast, the same 16S rRNA gene sequences, closely related to known PRB, were recovered from soil batch cultures grown on lactate or root extract, by DGGE analysis (Shrout *et al.*, 2006). Lactate, root extract, and acetate could provide organoheterotrophic environments, whereas hydrogen/bicarbonate amendment could change the soil environment to more lithoautotrophic; lactate, root extract, and acetate probably select for different PRB than hydrogen.

Although differences were observed in PRB compositions between acetate and hydrogen amendments, the majority of the *pcrA* clones from Industrial Soil fell within the tight *Dechloromonas* spp. *pcrA* cluster (Fig. 3). In contrast, many of the *pcrA* clones from Yolo soil enrichments were closely related to *pcrA* of *Azospirillum* sp. TTI and *Dechlorospirillum* sp. WD, previously isolated PRB (Fig. 3) (Nozawa-Inoue *et al.*, 2008). 16S rRNA gene sequences closely related to *Dechlorospirillum* spp. and *Azospirillum* spp., both being members of *Alphaproteobacteria*, were also recovered in the same Yolo soil enrichments (Nozawa-Inoue *et al.*, 2005). The correlation between *pcrA* and 16S rRNA phylogenies is still not known, but genus- or subclass-level congruency may be possible as observed between 16S rRNA gene and NarG, another DMSO reductase (Philippot, 2002). *Dechloromonas* spp., the most frequently isolated PRB and

belonging to *Betaproteobacteria*, have also been detected in other rRNA gene-based perchlorate-reducing microbial community studies, such as in acetate-fed (Zhang *et al.*, 2005; Choi *et al.*, 2008) or hydrogen-fed (Nerenberg *et al.*, 2008) bioreactors or lactate-fed soil enrichments (Shrout *et al.*, 2006). While Yolo soil is the only example of molecular-based detection of *Azospirillum* sp. in perchlorate-reducing communities, perchlorate-reducing *Azospirillum* sp. have been isolated from various perchlorate-contaminated sites (Waller *et al.*, 2004). The differences in the detected known PRB species were likely because of the native PRB compositions in soil and groundwater.

Investigating the site-specific biodegradation potential/process is important when *in situ* bioremediation strategies are applied to a contaminated field site (Bombach *et al.*, 2010). For perchlorate remediation, although electron donors creating organotrophic conditions such as acetate and lactate are used frequently, those promoting lithotrophic conditions may enhance perchlorate reduction more depending on the structure of the native PRB community. In other words, optimal electron donors of use for *in situ* biostimulation of perchlorate reduction are likely to be specific to the soil/site. Therefore, conducting a potential study before a full-scale operation could prevent costly mistakes and save time. The effect of nitrate also needs to be taken into consideration. The effect is not necessarily negative: nitrate may increase PRB populations in some cases. Thus, combining the measurements of perchlorate reduction potential with the molecular characterization of the types of PRB present in the soil can be useful in designing and assessing effective *in situ* biostimulation strategies.

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