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Quantitative Detection of Perchlorate-Reducing Bacteria by Real-Time PCR Targeting the Perchlorate Reductase Gene▽

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A quantitative real-time PCR assay targeting the *pcrA* gene, encoding the catalytic subunit of perchlorate reductase, detected *pcrA* genes from perchlorate-reducing bacteria in three different genera and from soil microbial communities. Partial *pcrA* sequences indicated differences in the composition of perchlorate-reducing bacterial communities following exposure to different electron donors.

Perchlorate (ClO_4^-) is a widespread environmental contaminant that disrupts thyroid gland function (11, 21). According to a recent U.S. Environmental Protection Agency report, contamination of groundwater, surface water, and soil by perchlorate has been detected in 35 states, with California reporting the largest number of detections (22). Bacterial strains capable of respiratory perchlorate reduction, a process that produces innocuous chloride, have been isolated from a variety of sources (4–6, 9, 15, 17, 24, 25, 28). Although these perchlorate-reducing bacteria (PRB) appear to be ubiquitous (5), our knowledge of their population dynamics in the environment is very limited.

Estimating the abundance and growth of PRB can be helpful in assessing the potential for and optimization of biological treatment, a promising technology for perchlorate remediation. Quantitative information, however, is limited mostly to pure-culture studies (9, 15, 25), with few techniques available for enumerating PRB within larger microbial communities. Existing culture-dependent most-probable-number methods require a several-month incubation time to develop estimates of numbers of PRB in soil and water samples (5, 27).

More rapid detection could be achieved by targeting functional genes common to this bacterial group, such as genes encoding perchlorate reductase (*pcrABCD*) (3) and chlorite dismutase (*cld*) (1, 2). These two enzymes catalyze reactions of perchlorate to chlorite (ClO_2^-) (8) and chlorite to chloride (23), respectively. A nested PCR assay targeting the *cld* gene has been developed and applied to environmental samples (2). However, the *cld* gene is not specific to PRB, because non-PRB such as chlorate (ClO_3^-)-reducing bacteria also possess *cld* genes (18, 26). Targeting the perchlorate reductase is more appropriate for detecting PRB, because the *pcr* gene appears to be present exclusively in PRB, and the enzyme catalyzes the rate-limiting step in perchlorate reduction (15). A slot-blot hybridization probe has been designed for the *pcrA* gene, which encodes the catalytic subunit of perchlorate reductase (3), but the method has not been applied to environmental samples.

This study aimed to design a real-time quantitative PCR (qPCR) assay, based on the *pcrA* gene, for quantitatively detecting PRB in environmental samples. To our best knowledge, this is the first report of qPCR assay developed to detect PRB. Partial *pcrA* sequences for PRB isolates and enrichment cultures were determined to collect more information about *pcrA* sequences, as well as to examine the members of communities associated with reduction of perchlorate.

Primer design. To identify conserved regions, deduced PcrA protein sequences from *Dechloromonas agitata* strain CKB (GenBank accession AAO49008) and *Dechloromonas aromatica* strain RCB (AAZ47315; http://genome.jgi-psf.org/finished_microbes/decar/decar.home.html) were aligned using Clustal W (19) (Fig. 1). Several other molybdoenzyme sequences from the dimethyl sulfoxide (DMSO) reductase family were included in order to identify unique PcrA sequence regions (Fig. 1). This enzyme group includes those with important roles in anaerobic respiration, specifically, respiratory reduction of oxyanions, such as nitrate, selenate, arsenate, and chlorate, in addition to perchlorate (10). Studies of the diversity and abundance of respiratory nitrate-reducing bacteria possessing *narG* (13, 14) suggest that specific molybdoenzyme sequences can be selectively detected despite being members of a larger, broader superfamily of sequences. Similar approaches could be applied to studying the diversity and abundance of PRB.

A primer pair, pcrA320F (5′-GCGCCCACCACTACATGT AYGGNCC-3′) and pcrA598R (5′-GGTGGTCGCCGTACC ARTCRAA-3′), was selected using CODEHOP (20) along with inspection of the sequences and the degrees of genetic code degeneracy. The primer sequences correspond to nucleotide positions 320 to 344 and 577 to 598 of the *D. agitata* CKB *pcrA* gene.

Detection of *pcrA* genes in perchlorate-reducing cultures. Detection of PRB by qPCR using the designed *pcrA* primers was confirmed with DNA from pure cultures of five PRB strains from four genera, *Dechloromonas*, *Azospira*, *Azospirillum*, and *Dechlorospirillum*, representing most of the previously identified PRB (4, 5). The assay was also tested with DNA extracted from Yolo silt loam soil enriched with 0.25 mM

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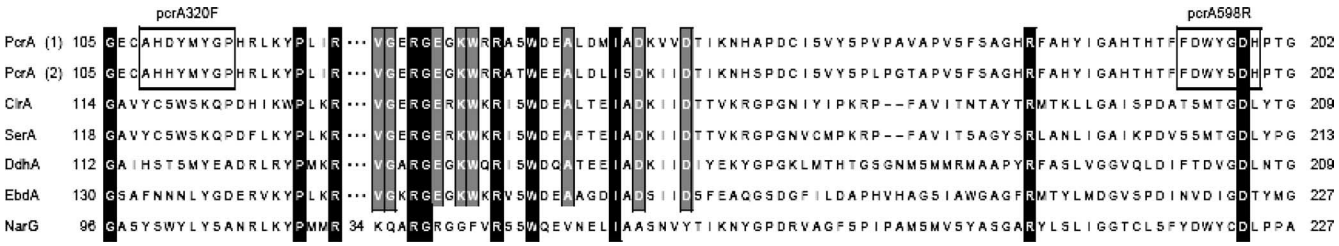

FIG. 1. Multiple-sequence alignment for perchlorate reductase and related molybdoenzymes. The region encompassed by the primers used for the qPCR assay is shown, and the residues within the PcrA protein sequences corresponding to the primer DNA sequences are boxed. Positions with identical residues in all seven sequences are shaded black, and positions with identical residues in all sequences except NarG are shaded gray. The conserved aspartyl residue that provides a side chain ligand to Mo in the molybdenum cofactor corresponds to NarG residue Asp-223 (7). The NarG sequence contains a 34-residue region that is not present in the other sequences (indicated by three dots). PcrA, perchlorate reductase (sequence 1, *D. agitata* CKB [GenBank accession number AY180108]; sequence 2, *D. aromatica* RCB [AAZ47315]); ClrA, chlorate reductase (*Ideonella dechloratans* ATCC 51718; CAD97447); SerA, selenate reductase (*Thauera selenatis* AX; AJ007744); DdhA, dimethylsulfide dehydrogenase (*Rhodovulum sulfidophilum* SH1; AF453479); EbdA, ethylbenzene dehydrogenase (*Azoarcus* sp. strain EB1; AF337952); and NarG, nitrate reductase (*E. coli* K-12; NP 415742).

perchlorate and either acetate (YA) or hydrogen (YH), provided as electron donors (12). Two non-PRB were also tested, including the chlorate-reducing *Pseudomonas* sp. strain PK (presumably containing the *clrA* gene, encoding the molybdoenzyme chlorate reductase) (3, 5) and the nitrate-reducing *Escherichia coli* strain K-12 (which produces several molybdoenzymes).

Pure culture and soil enrichment DNA was extracted using an UltraClean microbial DNA kit (MoBio laboratories, Carlsbad, CA) and a FastDNA spin kit for soil (using 0.5 g soil) (MP Biomedicals, Solon, OH), respectively. Five nanograms of each DNA sample was added to a qPCR mixture (15 μl as a final reaction volume) containing 1× SYBR Ex *Taq* premix (TaKaRa Bio USA, Madison, WI) and primers (0.2 μM each). PCR was performed with a 7300 real-time PCR system (Applied Biosystems, Foster City, CA) with thermal cycling of 95°C for 1 min followed by 35 cycles of 95°C for 5 s and 60°C for 31 s. The absence of nonspecific PCR products was confirmed both by dissociation curve analysis and by 1.5% agarose gel electrophoresis.

Although the primers were designed using the only two available *pcrA* sequences, both from the genus *Dechloromonas*, substantial amplifications were also observed with three other PRB, including *Azospirillum* sp. strain TTI and *Dechlorospirillum* sp. strain WD (Table 1). The primer pair did not detect *Azospira suillum* PS (Table 1), which appears to possess the *pcrA* gene (3); therefore, the primer sequences will be improved when the *pcrA* sequence of this strain is available. No amplification was observed with the negative controls.

TABLE 1. qPCR detection of *pcrA* genes in PRB strains, soil enriched with perchlorate, and non-PRB strains

Source	Perchlorate reduction[b]	qPCR detection[c]
Dechloromonas agitata strain CKB	+	+
Dechloromonas sp. strain MissR	+	+
Azospira suillum strain PS	+	−
Azospirillum sp. strain TTI	+	+
Dechlorospirillum sp. strain WD	+	+
Pseudomonas sp. strain PK	−	−
E. coli K-12	−	−
YA[a]	+	+
YH[a]	+	+

[a] Yolo silt loam soil enriched in a mineral liquid medium containing 2.5 mM ClO_4^- with either 10 mM acetate under N_2 headspace (YA) or 10 mM bicarbonate under H_2 headspace (YH) for 4 to 5 months (12).
[b] Ability to reduce perchlorate to chloride.
[c] Detection above the detection limit (nine copies/reaction).

Amplification was also observed in DNA extracted from YA and YH soil enrichment cultures (Table 1), in which 16S rRNA genes identical to *Dechlorospirillum* sp. and *Azospirillum* sp. were previously detected (12).

Partial *pcrA* gene sequences. *pcrA* amplicons from *Dechloromonas* sp. strains CKB and MissR, *Dechlorospirillum* sp. strain WD, *Azospirillum* sp. strain TTI, and the soil enrichment cultures, YA and YH, were cloned by using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Positive clones were identified following screening with M13 universal primers. For the enrichment cultures that likely contained multiple strains of PRB, the M13 PCR products of 20 positive clones were subjected to restriction fragment length polymorphisms using the restriction endonuclease HhaI. The digestion patterns were examined by performing gel electrophoresis with 3% low-melting-point agarose gel (Fisher Scientific, Fair Lawn, NJ) in 1× Tris-borate-EDTA buffer at 6 V/cm and 4°C. Plasmids were extracted from the *pcrA* clones with distinct restriction fragment length polymorphisms and from those of PRB pure cultures by using a Plasmid Minikit (Qiagen, Valencia, CA). Inserts were sequenced at the University of California, Davis, DNA sequencing facility (Davis, CA).

A phylogenetic tree of the deduced PcrA protein sequences (92 amino acids), and the corresponding sequences of the enzymes in the DMSO reductase family, was constructed by using the neighbor-joining method (16) (Fig. 2). The PcrA sequences of PRB isolates and soil enrichments were closely related to each other but distinct from other molybdoenzymes in the DMSO reductase family. Within the PcrA cluster, the three *Dechloromonas* sp. PcrA sequences formed a tight group, indicating that they are closely related.

The enrichment culture PcrA sequences YA3 and YH2 were closely related to those from the *Dechlorospirillum* and *Azospirillum* pure cultures, respectively, as anticipated from the previous identification of the corresponding 16S rRNA gene sequences in these samples (12). In contrast, the YA2 PcrA sequence was identical to that from the *Azospirillum* sp. pure culture, although we did not detect *Azospirillum* sp. 16S rRNA

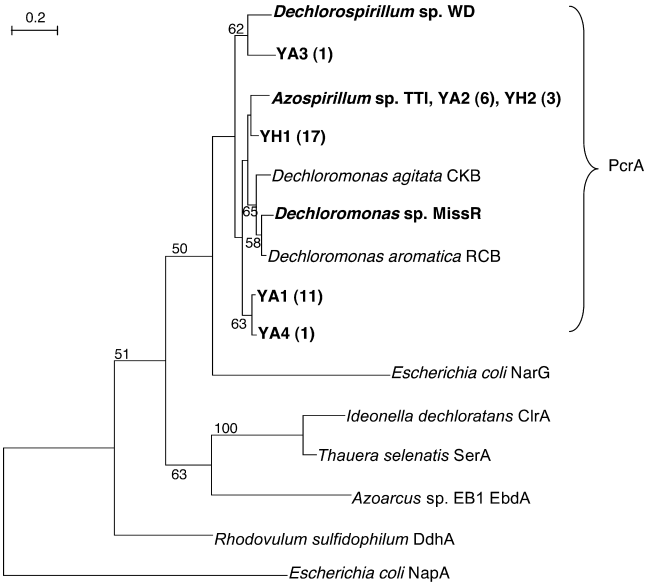

FIG. 2. Phylogenetic tree of deduced PcrA protein sequences and reference sequences. Bootstrap values above 50 obtained from 100 resamplings are shown at each node. The sequences obtained in this study are in bold type. The numbers of clones with identical sequences, in total 19 and 20 clones from the soil enrichments YA and YH, respectively, are in parentheses. The GenBank accession numbers for reference sequences are in the legend to Fig. 1, except for *E. coli* NapA (AAC75266). Bar, 0.2 changes per amino acid.

gene sequences in the YA enrichment (12). More sequence data are needed to verify the similarity and disparity of sequence phylogenies between PcrA and16S rRNA genes.

A total of five and two different PcrA sequences were obtained from the YA and YH enrichments, respectively. Only one *pcrA* clone sequence was identical between the YA and YH enrichments, suggesting that different electron donors may enrich different PRB.

Plasmid standard curve for quantification. A standard curve was constructed relating gene copy numbers to qPCR threshold cycle using a plasmid with the cloned *D. agitata* CKB (ATCC 700666) *pcrA* gene. The copy number of the plasmid was calculated based on the DNA concentration determined by measuring absorbance at 260 nm. Five microliters of 10-fold serial dilutions of the plasmid solution was added to a qPCR mixture, and qPCR was performed as described above. The curve relating gene copy numbers and qPCR threshold cycles was strongly linear ($R^2 = 0.99$) over 9 orders of magnitude (Fig. 3). The detection limit was approximately nine copies/reaction.

Quantification of *pcrA* genes in soil samples. The *pcrA* genes were amplified in samples of a previously unexposed Yolo silt loam soil and in an undescribed soil collected from a perchlorate-contaminated site in California (soil B). In addition, two sets of anaerobic unsaturated microcosms using these two soils were exposed to perchlorate and amended with either acetate or hydrogen, as a commonly used organic electron donor and an inorganic electron donor for PRB, respectively. Approximately 1.0 and 0.2 μmol/g dry soil^{-1} of perchlorate was reduced in the Yolo soil microcosms and the soil B microcosms, respectively, in the presence of either acetate and nitrogen gas

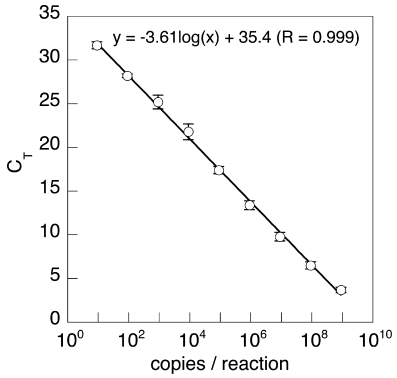

FIG. 3. Standard curve relating *pcrA* gene copy numbers and qPCR threshold cycles (C_T) using a plasmid containing the *D. agitata pcrA* gene (means ± standard deviations; $n = 6$).

(Yolo soil only) or bicarbonate and hydrogen gas (both Yolo soil and soil B) (12; M. Nozawa-Inoue, M. Jien, K. Yang, D. E. Rolston, K. R. Hristova, and K. M. Scow, unpublished data). Five microliters of 100×-diluted soil DNA, corresponding to 2 to 10 ng DNA (a semiquantitative estimate on agarose gel in comparison with serial dilutions of a known concentration of lambda DNA), was analyzed by qPCR. Prior to the analysis, several dilution rates were compared for each soil. The 100× dilution was the lowest dilution that exhibited the least inhibition while producing the most consistent results between triplicate reactions; this dilution was used for quantification. The *pcrA* copy numbers were calculated based on the standard curve described above, assuming 100% of DNA recovery from soil DNA extraction and no inhibition from the soil matrices. Although *pcrA* genes were not detected in any of the soils before treatment, 10^4 to 10^5 copies of *pcrA* genes per gram dry soil were successfully detected in samples after perchlorate reduction (Table 2), presumably due to the growth of PRB. Though a smaller amount of perchlorate was reduced, soil B had a larger copy number of *pcrA* genes than did Yolo loam soil. On possible explanation for this difference is that the PRB in Yolo soil had a higher level of perchlorate reduction activity on a per-cell basis.

The developed qPCR assay targeting *pcrA* genes was able to quantify the abundance of *pcrA* genes, presumably reflecting the PRB population, in environmental samples. The sequence information collected using the *pcrA* gene primers and also different environmental conditions enriched for sequences associated with different types of perchlorate-reducing bacteria. The assay may help to estimate cell densities of naturally occurring organisms potentially involved in perchlorate reduc-

TABLE 2. *pcrA* copy numbers in soil microcosm samples

Soil	Treatment	No. of *pcrA* copies/g dry soil[a]
Yolo silt loam	None	<DL
	ClO_4^-/acetate	$(3.4 \pm 2.1) \times 10^4$
	ClO_4^-/H_2	$(9.6 \pm 6.0) \times 10^4$
Soil B	None	<DL
	ClO_4^-/H_2	$(4.5 \pm 2.7) \times 10^5$

[a] <DL, under the detection limit.

tion if the copy numbers of *pcrA* genes in PRB strains are known. Estimates of cell densities can be useful for optimizing biological treatment of perchlorate, including in bioreactors and in situ bioremediation. More information on the diversity and abundance of these microorganisms possessing or expressing *pcr* may also provide new insights into the PRB ecology of the environment, information not readily available from culture-based studies.

Nucleotide sequence accession numbers. The sequences in this study have been deposited in the GenBank database under accession numbers EU273890 to EU273898.

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