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Note

qPCR assays to quantify genes and gene expression associated with microbial perchlorate reduction

Susan K. De Long [1], Kerry A. Kinney, Mary Jo Kirisits *

The University of Texas at Austin, Department of Civil, Architectural, and Environmental Engineering, 1 University Station C1786, Austin, TX 78712, United States

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ABSTRACT

Quantitative PCR (qPCR) assays targeting *cld* (developed in this work) and *pcrA* (previously described) were used to quantify these perchlorate-related genes in a perchlorate-reducing enrichment culture. Transcript copies were quantified in perchlorate-reducing *Rhodocyclaceae* strain JDS4. Oxygen and nitrate inhibited expression of *cld* and *pcrA*.

Perchlorate contamination of water is widespread (USEPA, 2005), and perchlorate is known to inhibit thyroid function (Wolff, 1998). Perchlorate-reducing bacteria (PRB) are ubiquitous in the environment (Coates et al., 1999), and biological perchlorate treatment has been demonstrated at bench- and pilot-scale (Brown et al., 2003; Giblin et al., 2000; Hatzinger, 2005; Kim and Logan, 2000; Kim and Logan, 2001; Logan, 2001; Min et al., 2004; Zhang et al., 2005). PRB reduce perchlorate to chlorate and chlorite via perchlorate reductase (encoded by the *pcrABCD* operon) (Bender et al., 2005). Chlorite is toxic, and PRB reduce chlorite to chloride via chlorite dismutase (encoded by *cld*) (Bender et al., 2002).

Molecular tools are becoming increasingly popular for the interrogation of biological treatment processes (Lovley, 2003; Rittmann, 2002; Sharkey et al., 2004). Since PRB are phylogenetically diverse (Coates et al., 1999), tools for interrogating functional genes related to perchlorate reduction (e.g., *cld* and *pcrA*) might be more useful than phylogenetic tools. Bender et al. (2004) developed a set of nested endpoint PCR primers targeting *cld*. The primary PCR primers were highly degenerate and produced spurious by-products in addition to the target amplicon; secondary, more specific PCR primers were used to eliminate by-products. This nested PCR strategy increased the sensitivity of the endpoint PCR assay and allowed for successful detection of *cld* in laboratory cultures and environmental samples. Nozawa-Inoue et al. (2008) developed a quantitative PCR (qPCR) assay targeting *pcrA* and detected it in pure cultures and soil samples. *pcrA* may be an ideal functional gene to monitor in biological perchlorate-reduction processes because it appears to be present solely in PRB and encodes the enzyme subunit that catalyzes the rate-limiting step in perchlorate reduction (Nozawa-Inoue et al., 2008). However, existing *pcrA* qPCR primers are based on the only two *pcrA* sequences from PRB *Dechloromonas agitata* (*D. agitata*) and *Dechloromonas aromatica* (*D. aromatica*) that were available at the time of the primer development (Nozawa-Inoue et al., 2008); thus, the complementarity between these primers and *pcrA* sequences found in other PRB is unknown, and some PRB might not be detected using these primers.

Little is known about the quantity of PRB at field sites and in bioreactors (Nozawa-Inoue et al., 2008), and to our knowledge, no studies have quantified transcripts of perchlorate-related genes (e.g., *pcrA* and *cld*). The objective of our work was to develop or extend molecular tools to quantify PRB and *pcrA* and *cld* transcripts because such tools could aid the development and optimization of biological perchlorate-reduction processes. In this work, we developed a qPCR assay targeting *cld* and extended it to quantify *cld* expression via reverse transcription qPCR (RT-qPCR). The previously developed *pcrA* qPCR assay (Nozawa-Inoue et al., 2008) also was extended to quantify *pcrA* expression via RT-qPCR. These assays were used to quantify gene copies and gene expression in a perchlorate-reducing enrichment culture; they also were used to quantify gene expression in the presence and absence of competing electron acceptors in perchlorate-reducing *Rhodocyclaceae* strain JDS4. The assays targeting *cld* and *pcrA* were run in parallel to provide multiple lines of evidence for the presence and activity of PRB in environmental samples.

The endpoint PCR primers UCD-238F and UCD-646R (Bender et al., 2004) were selected to develop a *cld* qPCR assay. These primers were

Abbreviations: DO, Dissolved oxygen; PRB, Perchlorate-reducing bacteria; qPCR, Quantitative PCR; RT-qPCR, Reverse transcription qPCR.

* Corresponding author. Tel.: +1 512 232 7120; fax: +1 512 471 0592.
E-mail addresses: Susan.De_Long@colostate.edu (S.K. De Long), kakinney@mail.utexas.edu (K.A. Kinney), kirisits@mail.utexas.edu (M.J. Kirisits).
[1] Present address: Department of Civil and Environmental Engineering, Colorado State University, Fort Collins, CO 80523.

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designed to target *cld* in all known PRB and are the secondary, more specific primers of the previously described nested *cld* PCR primer set. qPCR reactions contained 1X Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 0.4 μM of each primer, and 5 ng DNA. qPCR reactions were run on an ABI 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's recommended thermocycling program with the addition of a 45-s annealing step at 50 °C, which was the optimal endpoint PCR annealing temperature for primers UCD-238F and UCD-646R. The thermocycling program was as follows: 50 °C for 2 min; 95 °C for 10 min; 40 cycles: 95 °C for 15 s, 50 °C for 45 s, and 60 °C for 60 s. A final dissociation curve step was used to verify the specificity of the amplified products. The amplicon for this primer set is approximately 400 bp, which is longer than the optimal qPCR amplicon length (typically <200 bp), and, thus, a range of elongation times was tested. Elongation time was varied from 1 to 2.5 min, but increased elongation times did not improve amplification efficiency (data not shown). Therefore, the minimum recommended elongation time of 1 min was selected. Genomic DNA from the completely sequenced PRB *D. aromatica*, which is known to have single copies of *pcrA* and *cld*, was used as a copy number standard. *D. aromatica* genomic DNA was extracted using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA). *cld* copy numbers were calculated for *D. aromatica* using the genome size of 4501 kb (e.g., 5 ng of genomic DNA corresponds to 10^6 *cld* copies). The maximum template mass for the standard curve was 5 ng because higher concentrations inhibited amplification, as observed previously (Nozawa-Inoue et al., 2008). Detection of *cld* was linear ($R^2 = 0.994$) over four orders of magnitude, and the detection limit was approximately 100 copies/reaction (Fig. 1).

The *cld* qPCR assay and a qPCR assay targeting *pcrA* (Nozawa-Inoue et al., 2008) were applied to a perchlorate-reducing enrichment culture that was derived from pilot-scale, biologically active carbon filters treating perchlorate-contaminated groundwater in Rialto, California. Anaerobic modified R2A medium (Fries et al., 1994) was prepared, omitting nitrate and adding perchlorate (5 mg/l) as the electron acceptor. Acetate (10 mM) was provided as the electron donor, and the medium was supplemented with vitamins (Staley, 1968). The culture was fed perchlorate several times per week, and perchlorate degradation was verified using a perchlorate-selective electrode (Thermo Electron Corp., Beverly, MA). The cells were transferred to fresh medium three times over a period of five months to enrich for PRB. DNA was isolated from the enrichment culture using the UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA), and *cld* copies were quantified via the qPCR assay. *pcrA* copies were quantified as described previously (Nozawa-Inoue et al., 2008) but with slight modifications to the thermocycler program to meet the recommendations of the ABI 7900HT Real-Time PCR System used. Thermocycler parameters were as follows: 50 °C for 2 min; 95 °C for 10 min; 40 cycles: 95 °C for 15 s and 60 °C for 60 s. Genomic DNA from *D. aromatica* was used as the copy number standard for both *cld* and *pcrA*.

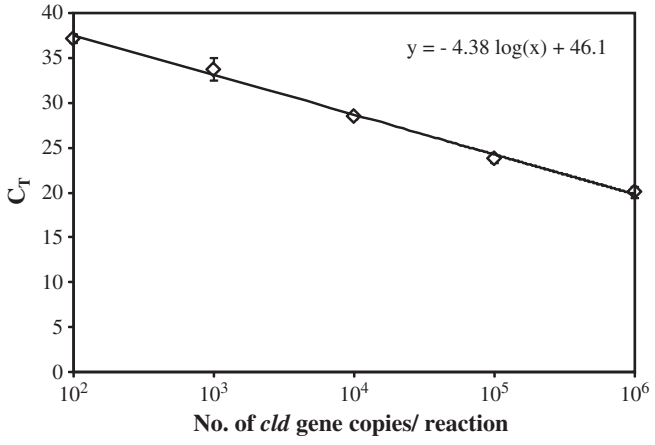

Fig. 1. Standard curve for qPCR assay targeting *cld*. The number of qPCR cycles to reach the quantification threshold (C_T) as a function of the number of *cld* gene copies in *D. aromatica* genomic DNA is shown. Means and standard deviations represent six qPCR replicates.

In the enrichment culture, 9.26×10^7 *cld* copies/μg DNA and 5.91×10^6 *pcrA* copies/μg DNA were detected. This result suggests that *cld* was present in higher copy number as compared to *pcrA* (e.g., ~15 copies of *cld* per copy of *pcrA*); however, this was not observed previously for the completely sequenced *D. aromatica* (http://genome-sonline.org-). Alternatively, this might indicate that the enrichment culture includes microorganisms that possess *cld* but not *pcrA*, such as chlorate-reducing bacteria. It seems unlikely that the latter explanation can fully account for the 15-fold higher number of *cld* copies given that the enrichment culture was grown with perchlorate as the electron acceptor for five months. Another possibility is that the *pcrA* assay might not have accurately quantified all *pcrA* sequences in the enrichment culture due to primer mismatches. To examine this possibility, the *pcrA* and *cld* copy numbers were used to estimate the percentage of PRB in the enrichment culture. Percent PRB was estimated according to the following equation:

$$P = \frac{Q}{\left(\frac{M^*C}{G}\right)} * 100$$

P indicates the percent PRB. Q is the number of *pcrA* or *cld* copies detected per qPCR reaction, which was used to estimate the number of PRB detected per qPCR reaction assuming that *pcrA* and *cld* are present as a single copy in each PRB. M is the mass of genomic DNA (ng) from the enrichment culture per qPCR reaction. C is a constant equal to 9.13×10^{11} base pairs/ng DNA, and G is the assumed average bacterial genome size of 4501 kb (*D. aromatica* genome size). According to the *cld* qPCR assay, the percent PRB was 46%, and according to the *pcrA* qPCR assay, the percent PRB was only 2.9%. It is possible that the enrichment culture contained aerobes because PRB evolve molecular oxygen when using perchlorate as the electron acceptor. However, the strong selection pressure for perchlorate-reducers in the culture suggests that the *pcrA* qPCR assay might have undercounted *pcrA*.

The accuracy of qPCR assays for environmental applications is affected by the ability of the primers to anneal to the target gene sequences in a mixed microbial community. Even single primer mismatches can affect primer annealing, thereby impacting the accuracy of quantification (Boyle et al., 2009). For some target genes, primer mismatches are difficult to avoid even when primers contain degenerate bases. Table 1 shows the number of primer mismatches between publically available gene sequences from known PRB and the *pcrA* and *cld* primers used in this study. Although the *pcrA* sequences from *D. aromatica* and *D. agitata* were considered in the design of the degenerate *pcrA* primers, there are four total mismatches between the primers and *pcrA* for both strains (Table 1). To examine the impact of the mismatches on the accuracy of quantification, we quantified *pcrA* copies in *D. agitata* genomic DNA using the *pcrA* qPCR assay and a *pcrA* copy number plasmid as the standard. Nozawa-Inoue et al. (2008) generated the *pcrA* copy number plasmid by amplifying *pcrA* from *D. agitata* with primer set pcrA320F/pcrA598R and cloning into a standard vector; therefore, the *pcrA* sequence in this copy number plasmid is perfectly complementary to those primers. We prepared qPCR reactions containing 10^2 to 10^6 copies of *pcrA* from *D. agitata* genomic DNA, assuming that there is one *pcrA* copy per genome. The measured number of copies was approximately two orders of magnitude smaller than the theoretical number of copies (data not shown). If multiple copies of *pcrA* are present in the *D.*