Journal of Microbiological Methods 88 (2012) 263–270

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Quantitative real-time PCR (qPCR) detection chemistries affect enumeration of the *Dehalococcoides* 16S rRNA gene in groundwater

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ARTICLE INFO

Article history:
Received 28 October 2011
Received in revised form 8 December 2011
Accepted 8 December 2011
Available online 19 December 2011

Keywords:
Quantitative real-time PCR (qPCR)
SYBR Green
TaqMan
Dehalococcoides
Chlorinated solvents
Bioremediation

ABSTRACT

Quantitative real-time PCR (qPCR) commonly uses the fluorogenic 5′ nuclease (TaqMan) and SYBR Green I (SG) detection chemistries to enumerate biomarker genes. *Dehalococcoides* (*Dhc*) are keystone bacteria for the detoxification of chlorinated ethenes, and the *Dhc* 16S ribosomal RNA (rRNA) gene serves as a biomarker for monitoring reductive dechlorination in contaminated aquifers. qPCR enumeration of *Dhc* biomarker genes using the TaqMan or SG approach with the same primer set yielded linear calibration curves over a seven orders of magnitude range with similar amplification efficiencies. The TaqMan assay discriminates specific from nonspecific amplification observed at low template concentrations with the SG assay, and had a 10-fold lower limit of detection of ~3 copies per assay. When applied to *Dhc* pure cultures and *Dhc*-containing consortia, both detection methods enumerated *Dhc* biomarker genes with differences not exceeding 3-fold. Greater variability was observed with groundwater samples, and the SG chemistry produced false-positive results or yielded up to 6-fold higher biomarker gene abundances compared to the TaqMan method. In most cases, the apparent error associated with SG detection resulted from quantification of nonspecific amplification products and was more pronounced with groundwater samples that had low biomarker concentrations or contained PCR inhibitors. Correction of the apparent error using post-amplification melting curve analysis produced 2 to 21-fold lower abundance estimates; however, gel electrophoretic analysis of amplicons demonstrated that melting curve analysis was insufficient to recognize all nonspecific amplification. Upon exclusion of nonspecific amplification products identified by combined melting curve and electrophoretic amplicon analyses, the SG method produced false-negative results compared to the TaqMan method. To achieve sensitive and accurate quantification of *Dhc* biomarker genes in environmental samples (e.g., groundwater) and avoid erroneous conclusions, the analysis should rely on TaqMan detection chemistry, unless additional analyses validate the results obtained with the SG approach.

1. Introduction

Excessive use of tetrachloroethene (PCE) and trichloroethene (TCE) as dry cleaning and metal degreasing agents, respectively, resulted in widespread aquifer contamination, where abiotic and biotic processes lead to the formation of dichloroethenes (DCEs) and vinyl chloride (VC) (see U.S. Environmental Protection Agency Superfund Site Information at http://www.epa.gov/superfund/sites/siteinfo.htm; Moran et al., 2007). Under anoxic conditions, diverse groups of bacteria contribute to PCE and TCE reductive dechlorination to *cis*-1,2-DCE (reviewed in Smidt and de Vos, 2004), but only members of the *Dhc* group are capable of complete reductive dechlorination of *cis*-DCE and VC to ethene (Cupples et al., 2003; He et al., 2003b; Maymó-Gatell et al., 1997; Sung et al., 2006). Several studies established a link between the presence of *Dhc* and ethene formation at contaminated sites (Fennell et al., 2004; Hendrickson et al., 2002; Lendvay et al., 2003; Major et al., 2002; van der Zaan et al., 2010). Therefore, the accurate identification and quantification of *Dhc* biomarker genes has become increasingly important for site assessment and the implementation of bioremediation using naturally occurring bacteria in the treatment and cleanup of sites impacted by chlorinated pollutants.

Quantitative real-time PCR (qPCR) has emerged as the method of choice for environmental monitoring of *Dhc* biomarker genes. To this end, several groups have developed qPCR primers that target the *Dhc* 16S rRNA gene (Cupples, 2008; He et al., 2003a; Ritalahti et al., 2006). The known *Dhc* genomes harbor a single copy of the 16S rRNA gene indicating that the enumeration of this target allows for estimates of *Dhc* cell abundance. qPCR enumeration of the *Dhc* 16S rRNA gene established a relationship between *Dhc* population abundances and remedial

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doi:10.1016/j.mimet.2011.12.005

success (i.e., ethene formation and detoxification) (Lee et al., 2008; Lendvay et al., 2003) and is useful to predict dechlorinating potential at contaminated sites (Lendvay et al., 2003; Rahm et al., 2006; Ritalahti et al., 2010a).

The two most commonly used detection chemistries for qPCR assays are the TaqMan chemistry and the SG chemistry. When surveyed in 2005, the TaqMan assay was the most used (72%) (Bustin, 2005) but a later survey estimated that the SG chemistry gained ground and was used in 85% of qPCR assays (Colborn et al., 2008). The ease and somewhat lower cost of qPCR assays using the SG chemistry compared to the TaqMan method, which requires the design and purchase of a fluorogenic, linear hybridization probe, are most often cited as the reasons for favored usage of the SG method.

The SG approach uses two PCR primers and the SG dye that is included in the amplification mix. The SG dye binds nonspecifically to all double-stranded nucleic acid molecules including specific and nonspecific amplification products generated during PCR, as well as primer-dimers (Higuchi et al., 1993; Wittwer et al., 1997). For the TaqMan assay, target gene quantification not only requires the two amplification primers, but also a fluorogenic probe binding between the amplification primers. Following target amplification, the TaqMan probe anneals to the single-stranded amplicon. The probe is then displaced and cleaved by the 5′→3′ exonuclease activity of the Taq polymerase during the next round of amplification. The fluorescent signal is thus produced by the release of the fluorophore on the 5′ end of the probe from the quencher on the 3′ end (Holland et al., 1991; Wittwer et al., 1997). Only amplicons to which the TaqMan probe hybridizes contribute to the fluorescent signal. While qPCR assays utilizing the SG chemistry can rival the sensitivity of the TaqMan method, a distinct advantage of the TaqMan chemistry is higher target specificity due to the requirement of a matching probe binding between the amplification primers (Newby et al., 2003; Wittwer et al., 1997).

Assays for the enumeration of *Dhc* 16S rRNA biomarker gene utilize both the TaqMan and SG detection chemistries. While laboratory results demonstrate that both assays produce comparable results with template DNA from defined samples (e.g., pure culture DNA), their application to field samples has not been systematically evaluated. In this study, the direct comparison of both detection chemistries for the quantification of the *Dhc* 16S rRNA biomarker gene from laboratory and groundwater samples using a single primer set was undertaken. The effects of qPCR assay detection chemistry on *Dhc* biomarker gene abundance estimation and subsequent bioremediation decision-making are discussed.

2. Methods

2.1. Samples

DNA was obtained from *Dhc* sp. strain BAV1, the PCE-to-ethene-dechlorinating consortium KB-1, which contains two *Dhc* strains, and groundwater samples collected from chlorinated ethene-contaminated sites. For collection of biomass, 5 mL of *Dhc* sp. strain BAV1 culture was passed through a 25 mm 0.22 μm pore size Durapore hydrophilic polyvinylidene fluoride membrane (Millipore, Billerica, MA) by vacuum filtration. Dilutions of consortium KB-1 with cell titers in the range of 1.0×10^4 to 1.0×10^5 and 1.0×10^7 to 1.0×10^8 *Dhc* cells per L were obtained from Dr. E. Edwards, University of Toronto. Each polypropylene vessel contained 570 mL of KB-1 culture suspension and was shipped in a cooler on blue ice via overnight carrier. Upon receipt, the biomass was collected by vacuum filtration onto a 0.22 μm pore size, 47 mm diameter polyethersulfone (PES) membrane (MO BIO Laboratories, Carlsbad, CA). Contaminated groundwater samples were obtained from Vandenberg Air Force Base, CA (3 wells) and the Bachman Road site in Oscoda, MI (11 wells), and Sterivex cartridges were received from Naval Air Station at Cecil Field, Site 59, FL (9 wells) and Ft. Dix Army Base, Ft. Dix, NJ (9 wells). Groundwater and Sterivex cartridges were shipped by overnight carrier on blue ice. Upon receipt, the biomass was collected from groundwater samples by vacuum filtration onto a 0.22 μm pore size, 47 mm diameter PES membrane and immediately stored at −80 °C prior to DNA isolation. On site biomass collection used Sterivex-GP cartridge filters (Millipore, Billerica, MA, Catalog #SVGPL10RC) and was performed as described (Ritalahti et al., 2010a). Filters were stored at −80 °C prior to DNA isolation.

2.2. DNA isolation from membrane filters

Total DNA was isolated from the frozen MO BIO filters using the UltraClean Water DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions or directly from the frozen Durapore or Sterivex filters by addition of the filter to the PowerSoil DNA Isolation Kit bead tubes (MO BIO Laboratories, Carlsbad, CA) and proceeding according to the manufacturer's instructions (Ritalahti et al., 2010a,b). DNA from the UltraClean Water Isolation Kit was eluted into a final volume of 3 mL of elution buffer (10 mM Tris, pH 8.5) and concentrated by addition of 1/10 volume of 5 M NaCl, 2 volumes of absolute, ice cold ethanol, and incubation for 30 min at 4 °C. The DNA was collected by centrifugation (13,200 rpm, 15 min, room temperature), rinsed with 70% (v/v) ethanol, air dried and suspended in a final volume of 100 μL elution buffer. Total DNA isolated using the PowerSoil DNA isolation kit was recovered in 100 μL elution buffer. DNA concentrations were determined by absorption readings at 260 nm using a NanoDrop Spectrophotometer ND-1000 and DNA quality was inferred by the 260/280 absorbance ratios.

2.3. qPCR primers and TaqMan probe

The primer set targeting a *Dhc* 16S rRNA gene sequence was previously described and validated, and generated a 66-basepair amplicon (He et al., 2003a; Ritalahti et al., 2006). The TaqMan probe for the *Dhc* 16S rRNA gene carried the 5′ fluorescent dye 6-carboxyfluorescein (FAM) and the 3′ Black Hole-1 quencher instead of the TAMRA quencher (He et al., 2003a; Ritalahti et al., 2006). Primers and probe were purchased from Integrated DNA Technologies (San Diego, CA). All qPCR assays used a 300 nM concentration of each forward and reverse primer, with the TaqMan assay also receiving a 300 nM concentration of the linear hybridization probe.

2.4. TaqMan assays

All TaqMan assays used the TaqMan universal PCR master mix from Applied Biosystems. In a total volume of 20 μL, each reaction contained 1× PCR master mix, primers, probe and 2 μL template DNA. Three qPCR tubes received undiluted template DNA and three tubes received 1:10 diluted template DNA. This resulted in six quantifications per DNA sample and allowed for a determination of the presence of PCR inhibitors in the DNA preparations. Because PCR assays are easily contaminated, the master mix contained a mixture of dTTP/dUTP nucleotides and the AmpErase uracil N-glycosylase (UNG) (Applied Biosystems) as a safeguard against contamination by amplification products from previously run reactions. Prior to amplification, the reactions were heated for 2 min at 50 °C to activate the AmpErase UNG to hydrolyze the N-glycosidic bond between the uracil base and the deoxyribose backbone from carry-over products and prevent their use as templates in the current reaction. Following a 10 min denaturation step at 94 °C, amplification was carried out for 40 cycles of 15 s at 94 °C and 1 min at 60 °C in the presence of the TaqMan probe. The fluorescence increase due to DNA amplification at any given cycle within the exponential phase of PCR is proportional to the initial number of template copies. The number of PCR cycles (i.e., the C_T or the threshold cycle) needed for the amplification