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Reaction- and sample-specific inhibition affect standardization of qPCR assays of soil bacterial communities

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ABSTRACT

Quantitative PCR (qPCR) is a popular technique used to quantify target sequences from DNA isolated from soil, but PCR inhibition makes it difficult to estimate gene copy number. Here, we evaluated the extent to which inhibition associated with reaction conditions and sample-specific properties influence the linear range of amplification, and the efficiency and sensitivity of qPCR assays of three bacterial gene targets. We adopted a sample pool approach and exploited the mathematical basis of qPCR to correct for sample-specific effects on amplification. Results revealed that qPCR efficiency and sensitivity were dependent on all conditions tested. In addition, the effect of annealing temperature and SYBR green PCR kit was target-specific, suggesting that the sample pool approach is appropriate for evaluating the quality of new primers. Likewise, the efficiency and sensitivity of qPCR amplification was sample-specific and is likely a result of site and date-specific co-extractants. When relativized against calculations based on plasmid curves alone, reaction-specific and sample-specific inhibition influenced calculations of gene copy number. To account for these differences, we present a brief protocol for soil samples that will facilitate comparison of future datasets.

1. Introduction

Our understanding of soil microbial communities has greatly advanced in recent decades with the application of molecular tools and the advent of culture-independent methods. One method now widely used is quantitative polymerase chain reaction (qPCR). QPCR has become the touchstone for nucleic acid quantification for microbial ecologists because of its conceptual and practical simplicity and its relatively high sensitivity and dynamic range for quantification (Bustin, 2004; Smith and Osborn, 2009; Sieber et al., 2010). The method combines PCR and fluorescent detection of template amplification, allowing for quantification of gene copy number or gene transcript copy number of an unknown sample. When assays are relativized against a known number of gene copies (i.e. plasmid DNA containing cloned copy of a gene) or the total bacterial community (i.e. *16S rRNA* gene sequences), copy number and relative abundance of a specific gene can be obtained, respectively. In general, qPCR is a valuable tool for estimating gene copy abundance and has provided important insights into the role of microbial communities in ecosystem processes.

Despite the use and appeal of qPCR, the technique is not without problems. One of the more serious issues affecting qPCR is sub-optimal reaction performance due to inhibition. For example, thermodynamic conditions, chemical carryover from nucleic acid extraction, and interference of excessive nucleic acid concentration can affect amplification of gene targets (Gallup and Ackermann, 2006). In soil and other environmental samples (e.g. feces), sample-specific inhibition is also present. This type of inhibition is a result of substances that co-purify during nucleic acid isolation and interfere with enzyme kinetics or reaction chemistry by overwhelming and/or disrupting enzyme binding (i.e. *Taq* polymerase) (Bustin, 2004). Inhibitory substances co-extracted from soil include excess nucleic acids, phenolic compounds, humic and fluvic acids, heavy metals, and plant-derived polysaccharides and polyphenolic acids (Wilson, 1997, reviewed by). The main symptoms of qPCR inhibition are loss of efficiency and sensitivity of target amplification. Efficiency refers to the extent to which the target template is replicated each cycle and, for the basis of qPCR calculations, is assumed to be equal in all wells. In the face of inhibition, however, sample amplification can be highly variable (Huggett et al., 2008). Additionally, inhibition of template amplification may change assay sensitivity, or the detection limit, of genes found in low copy number.

While the complications associated with qPCR inhibition of soil-extracted samples are well recognized (e.g. one well-used

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commercially available DNA extraction kit touts its proprietary inhibitor removal system), there is no standard method to truly account for the effects of inhibition. It is current practice to account for inhibition by diluting samples in a relatively narrow range (e.g. 10–100×) and then standardizing gene abundance based on the characteristics of a plasmid-based standard curve. However, soil-extracted inhibitors do not affect standard curves constructed from plasmid DNA. Furthermore, target-specific kinetics may differ from the plasmid sequence and the mixed community templates, and external standard curves (i.e. standard curves generated on separate assay plate) do not reflect intra-assay differences in user or PCR amplification (Smith et al., 2006). By not accounting for this inherent variability it is difficult, if not impossible, to standardize calculations of gene copy number.

To confront the effects of inhibition, we took a sample pool approach previously shown to help identify and diagnose inhibition in mammalian tissue and cell culture (Gallup and Ackermann, 2006, 2008; Gallup et al., 2010). We aimed to determine the extent to which (i) the linear range of amplification, (ii) amplification efficiency, and (iii) qPCR sensitivity are affected by inhibition associated with reaction kinetics (annealing temperature), experiment-specific properties (e.g. potential co-extractants that may change with site or sampling date), and chemistries of different commercial SYBR green PCR kits. We chose three bacterial genes that are targeted by different types of primers used in soil microbiological research. The primers targeting the *16S rRNA* gene sequence quantify the total bacterial community and are not degenerate (Fierer et al., 2005). In contrast, primers targeting the gene encoding for nitrous oxide reductase (*nosZ*; Henry et al., 2006) and a gene encoding for nitric oxide reductase (*qnorB*; Braker and Tiedje, 2003) quantify only the denitrifiers, a functional group of nitrogen-cycling bacteria and model system for linking microbes to ecosystem process rates (Philippot and Hallin, 2005). The denitrifier-specific primer pairs differ in their degeneracy and use in the literature. The *nosZ* primers are degenerate but are well used and well characterized (Throbäck et al., 2004; Henry et al., 2006; Jones et al., 2008; Hallin et al., 2009), whereas the *qnorB* primers are relatively more degenerate and less well used (Braker and Conrad, 2011; Braker and Tiedje, 2003).

2. Materials and methods

2.1. Study sites

Soil used in this study was sampled from three sites located in two counties in central Iowa, U.S.A. The first two sites were located in Pocahontas county (42°34′14.10″N, 94°33′45.88″W) and include the Kalsow Prairie, a 160-acre tall grass prairie state preserve, and an adjacent cultivated field in corn-soybean rotation. Both of these sites have gently sloping prairie pothole topography and four soil series, Clarion (Typic Hapludoll) on the summit, Webster and Canisteo (Typic Endoaquolls) on the side-slope and foot-slope, respectively, and Okoboji (Cumulic Vertic Endoaquoll) in the closed depression. The third sampling site was at the Uthe Research and Demonstration Farm in Boone county (41°55′52.44″N, 93°45′42.63″W). Soil from this site was sampled under fallow areas from Coland (Cumulic Endoaquolls) and Clarion soil types. Soils types from all three sites are common over a wide geographical area.

2.2. Soil collection and DNA extraction

At the Pocahontas county sites, three transects encompassing the four soil types were established in each of the prairie and cultivated fields, for a total of six transects. At each transect seven soil cores (2.2 cm in diameter and 10 cm in depth) from each soil type were collected in May, August, and October 2009, and consolidated for a total of 24 sampling locations per date. At the Uthe site, five soil cores (10 cm in diameter and 10 cm in depth) from each soil type were collected in October 2009, for a total of two samples. Cores from each soil type and site were composited and homogenized, and a subsample for nucleic acid analysis was stored at −80 °C. Soil was freeze-dried the day prior to grinding (Dandie et al., 2007; Miller et al., 2008). DNA was extracted from ground soil using the MoBio PowerSoil DNA kit (MoBio Laboratories, Carlsbad, CA) and quantified by absorption using a Nanodrop spectrophotometer. DNA samples were combined as described in Section 2.3.

2.3. Sample preparation

To address qPCR inhibition resulting from substances that co-purify with nucleic acids during isolation, we used a dilution series generated from a pool of DNA samples, referred to as Stock I. The Stock I method was proposed and developed by Gallup and Ackermann (2006) as a way to optimize reverse transcription qPCR (RT-qPCR) reactions using a representation of the potentially inhibitory co-extractants found in samples. Studies comparing various DNA extraction and purification methods have shown that DNA yield and purity vary depending on methodology and soil type (Lloyd-Jones and Hunter, 2001; Roh et al., 2006; Whitehouse and Hottel, 2007; Frostegård et al., 1999; Krsek and Wellington, 1999). However, there is almost certainly carryover of some concentration of inhibitors from all extraction and purification methods (Gallup and Ackermann, 2008). Furthermore, different extraction and purification methods can introduce bias such as a change in diversity or the loss of rare species in a sample (Martin-Laurent et al., 2001; Miller et al., 1999; Robe et al., 2003; Sagova-Mareckova et al., 2008). Therefore, we chose to couple a widely used commercial DNA extraction kit with a downstream mathematical correction to account for inhibition of soil-extracted DNA samples. The sample pool approach we employed is a practical option to correct for general characteristics of samples in an experiment, rather than running dilutions of every sample, which is cost prohibitive, or picking one or a few samples, which introduces bias.

To construct the dilution series, each DNA sample was pre-diluted separately using one part DNA and two parts nuclease-free water. Sub-aliquots (15 μL each) of diluted samples were combined by sampling site and sampling date to yield four distinct Stock I pools: all DNA samples collected from prairie and cultivated fields in Pocahontas (P) county were combined by sampling date to produce three Stock I pools (P-May, P-August, and P-October), and DNA samples collected at the Boone county (B) site were combined to produce a fourth Stock I pool (B-October). After these most concentrated Stock I solutions were made, each pool was serially diluted 10 times by combining 32.1 μL of previously diluted Stock I and 50 μL nuclease-free water in a nuclease-free microcentrifuge tube (Table 1; Gallup and Ackermann, 2006). For all four pools, the genomic DNA concentration of the most concentrated Stock I solution was determined with a Nanodrop spectrophotometer.

2.4. Experimental design

To address our objectives, we conducted three experiments. Experiment 1 tested the sensitivity and efficiency of *nosZ* amplification in response to four commercially available SYBR green PCR kits that are readily available in the U.S.A. and made by different manufacturers (Kit 1, Brilliant™SYBR® Green; Kit 2, KAPA SYBR® FAST; Kit 3, Quanta BioSciences PerfeCTa® SYBR® Green SuperMix; Kit 4, QuantiFast SYBR Green). For each SYBR green PCR kit, 10-point