academicJournals

Vol. 13(6), pp. 723-728, 5 February, 2014
DOI: 10.5897/AJBX2013.13563
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and its usefulness in soil microbial ecological studies - A review

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Accepted 10 January, 2014

The reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is a highly specific polymerase chain reaction (PCR) method that allows one to detect very low transcription levels of functional gene(s) in soil. RT-qPCR helps us to know the active members of the microbial community, and their activities can be linked with other ecological processes in soil. If after the extraction of RNA from soil, the mRNA is converted to cDNA which is then sequenced, one would analyze directly the active members of the microbial community.

Key words: Complementary DNA (cDNA), messenger RNA (mRNA), reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), soil microbial study, microbial community.

INTRODUCTION

If one can see or know where microbes in soil live, what roles in soil processes they play and how their abundance and activity are influenced by soil physical and chemical properties, the soil will no longer be a black box. Molecular methods are evolving newer methods that help to answer questions like "who is the active member in the soil microbial community and where are their activities located"? Through DNA/DNA hybridization experiment, studies showed that one gram of soil contained more than 4,000 different genomes of bacteria (Torsvik et al., 1990). Most of the diversity was found in the fraction that could not be isolated and cultured by standard and sophisticated plating techniques. Thus, there is lack of knowledge on the unculturable portion of the microbial population in terms of who they are and the taxanomy, their contribution to nutrient and energy flow, soil respiration, gene transfer, degradation of pollutants, disease and quorum sensing, all of whose mechanisms have yet to be better understood. Researchers have developed new methods that allow the use of RNA extractions and manipulations to study microbial gene expression in the environment. One of such method is the reverse transcriptase quantitative polymerase chain reaction **(**RT-qPCR). RT-qPCR is a combination of reverse transcription and quantitative PCR and is highly specific that allows one to detect very low transcription levels in soil (Wang and Brown, 1999). It allows for enumeration of the number of mRNA copies of a gene under defined environmental conditions. RT-qPCR involves total RNA extraction from soil or an environmental sample, followed by RNA purification and conversion to cDNA. The isolated RNA sample containing mRNA of the gene of interest is then subjected to optimized RT-qPCR conditions that allow for amplification of the gene of interest.

The presence of a gene is an indicator of potential gene

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Abbreviations: FRET, Fluorescence resonanace energy transfer; **TG,** target gene; **CG,** control gene; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **RT-qPCR,** reverse transcriptase-quantitative polymerase chain reaction.

expression, not an actual measurement of it. DNA analyses target not only active microorganisms, but also inactive microorganisms (Lindahl, 1993). Baelum et al. (2008) also demonstrated that analyzing soil microbial communities for a specific metabolic activity based on DNA sequences may be biased by detection of nonactive populations encoding homologous genes, but not actively expressing them. Measurement of mRNA (where tractable) is a far better indicator of *in situ* metabolic activity than measurement of the number of copies of the gene that are present in an environmental sample. There is an increasing need to investigate gene expression directly in soil since it can provide a more detailed understanding of the dynamics of the functional population, the activities of specific groups and the conditions required for induction of the activities in soil and devising optimal strategies for sustainable low-input farming and forestry.

Environmental studies based on functional gene transcripts are potentially complicated by many critical factors. Many processes proceed slowly in the environment due to a variety of environmental parameters and limiting factors and therefore require only low amounts of mRNA. Hence, it is important that the genes of interest are actually expressed to a detectable level, and studies of slow soil processes with correspondingly low transcription levels may always be limited. The ability to detect and quantify functional gene transcripts in a complex environmental matrix will become an important tool for microbial ecologists to link phylogeny to ecological function and should help to improve understanding of microbial processes in general. The objective of this review is to assess the importance of RT-qPCR in soil related studies and the extent RT qPCR can be used as a tool to “open” the black box of soil.

STUDYING OF GENE EXPRESSION IN SOIL

Total soil RNA must first be extracted from soil or from the environmental sample. Thus, a robust protocol for extraction of total RNA, (more specifically, nondegraded mRNA) is essential. However, only a small number of studies have reported successful analyses of mRNA isolated from soil or sediment (Fleming et al., 1998; Hurt et al., 2001; Mendum et al., 1998; Miskin et al., 1998; Ogram et al., 1995; Tsai et al., 1991). To obtain pure RNA from the initial total nucleic acid extracts, DNA must be selectively removed by combined use of DNase digestion and acid phenol extraction (Bornemann and Triplett, 1997). This method proved to be DNA free upon visual inspection after gel electrophoreses, however, positive amplification was observed in the control RT- qPCR not containing the reverse transcriptase, indicating that there was residual contamination with DNA (Burgmann et al., 2003). Replacing the acid phenol extraction step with heat inactivation of the added DNase resulted in extracts with the RT-qPCR procedure being frequently unsuccessful. Similarly, the use of commercially available spin columns for DNA removal from the initial extracts also resulted in unsatisfactory RNA clean up.

Later, Burgmann et al. (2003) extracted total RNA from soil based on optimized nucleic acid extraction with a highly efficient and fast bead beating method (Burgmann et al., 2001) with only small amounts of soil used (0.5 g). The extraction buffer allowed both soil DNA and total soil RNA isolation due to the nuclease (protein)-denaturing capacities of cethyltrimethyl ammoniac bromide (CTAB) and dithiothreitol (DTT) and rapid application of the phenol-chloroform extraction procedure (Cheung et al., 1994; Farrel, 1998). The extracted RNA was protected in phenol-chloroform-treated raw extracts kept at -80°C for four months. Detection of active members of natural soil microbial communities was feasible when the method of Burgmann et al. (2003) was used to extract soil RNA. Novinscak and Filion (2011) recommended that the Burgmann et al. (2003) protocol appeared to be the most robust and recommendable for extracting RNA and allowing microbial gene transcript quantification in soils varying in clay content. Later, Botero et al. (2005) increased the accessibility of prokaryotic RNA from environmental samples by modifying the purified RNA with the addition of a poly A tail. Subsequent amplification and cloning to create cDNA library followed by screening of the library revealed clones representing sequences from bacterial ribosomal RNA and mRNA.

Difficulties in extraction of RNA from soil

Some of the difficulties involved in the extraction of total RNA from soil have been the extraction of RNA at a sufficient yield and purity to allow for subsequent molecular analysis. This is challenging because soil contains substances that interfere with RNA extraction and subsequent downstream applications (England et al., 2001; England and Trevors, 2003; Sayler et al., 2001; Trevors, 1996). Clay particles and organic matter bind to nucleic acids, interfere with the extraction processes, thus reducing nucleic acid yields. Humic acids inhibit the enzymes used in RT-qPCR which are reverse transcriptase and DNA polymerase (Mendum et al., 1998). It has been suggested that humic acids consisting of polyphenolic substances are inhibitory because phenols bind to proteins by forming hydrogen bonds which could change the conformation of the enzyme (Kreader, 1996). Overcoming inhibition, challenges/limitations often requires trying different specialized soil extraction kits, modifying existing extraction procedures to optimize RNA yields and minimizing inhibitors, using small amounts of soil, trying different polymerases, using additives such as bovine serum albumin (BSA), using less template, diluting the inhibitors below levels that are not inhibitory or even devising a new or modified extraction purification method for the particular sample being researched and also using positive and negative controls to avoid the false positives.

Once extracted, the stability of RNA is affected by several factors including short half-life. mRNA has a short half life, prokaryotic mRNA half-life is an average of 1.3 min at 37°C (Arriano, 1993). Once mRNA is outside the cell, it is quickly degraded and is susceptible to RNases. Storage methods alter mRNA stability. Lypholization and storage at -20°C and storage in glycerol stocks at -80°C for soil conservation were equally effective methods of soil preservation for subsequent RNA isolation (Sessitsch et al., 2002).

Other difficulties involving the use of RNA from soil samples includes the designing of degenerate primer sets that captures a particular gene of interest from an entire community in a soil sample. This becomes more of a challenge when diverse bacterial groups carry out the same metabolic process of interest. The presence of genomic DNA contamination in the RNA sample is troublesome because the primers used in the PCR reaction can also bind to genomic DNA and result in inaccurate quantification result. Most of the studies have been conducted on nitrogen processes including N fixation, nitrification, denitrification among others. Almost no studies have been done on the other biogeochemical cycles in soil such as carbon and sulphur cylces.

Conversion of mRNA to cDNA

Small amounts of RNA converted into cDNA by reverse-transcription reaction could be amplified with the advent of PCR. Poly -T primers bind to the polyA-tail on the mRNA. Reverse transcriptase is the enzyme that synthesizes a strand of complementary DNA. Nucleotides that can be labeled or unlabelled are incorporated into the new cDNA molecule. After the reverse transcriptase has assembled the nucleotide into a cDNA molecule, the mRNA is degraded by RNase out. Different kits have been used for the reverse transcriptase reaction such as superscript II reverse transcriptase (Invitrogen, Parsley, United Kingdom) and Omniscript reverse transcription have been used by Freitag and Prosser (2009), Treusch et al. (2005), Baelium et al. (2008) and so forth. The produced cDNA copies are used as templates in a PCR reaction along with probes designed to amplify genes of interest. The cDNA pool from sample could be used to analyze tanscripts from other genes as well.

Real time polymerase chain reaction (PCR)

The cDNA produced can be used for real-time PCR analysis. Real- time PCR has an increased capability for quantifying the number of transcript copies present in the environmental sample. Real-time PCR integrates the amplification and analysis steps by monitoring the DNA produced during each PCR cycle. The focus on real time PCR is the logarithmic phase of product accumulation rather than the end point abundance of PCR product which is a more accurate estimate of the amount of transcripts obtained, since it is less affected by the amplification efficiency of the reaction or depletion of reagent (Gruntzig et al., 2001).

A typical PCR amplification plot shows the reaction going through exponential and linear phases in which the PCR is initially not limited by enzymatic activity or substrates. Eventually, the enzyme activity and /or the substrates (for example, dNTPs) become limiting or exhausted. At this point, the reaction reaches a plateau and theoretically, all of the samples will reach the same total amount of amplified DNA. This both obscures any difference in initial cDNA abundance and is also quite variable. While end point qPCR requires that PCR products be detected and quantified by gel electrophoresis after completion of the reaction, real-time qPCR technology allows quantification of PCR products in “real time” during each PCR cycle yielding a quantitative measurement of PCR products accumulated during the course of reaction. Real-time reactions are carried out in a thermocycler that permits measurement of a fluorescent detector molecule decreasing the PCR post-processing steps and minimizes experimental error. This is most commonly achieved through the use of fluorescence-based technologies including 1) Probe sequences that fluoresce upon hydrolysis (TaqMan, Applied Biosystems, Foster City, CA,USA) or hybridization (Light Cycler, Roche, Indianapolis, IN, USA); 2) fluorescent hairpins or 3) intercalating dyes (SYBR Green). These approaches require less RNA than the end point assays.

TaqMan chemistry uses the 5’-3’exonuclease activity of Taq DNA polymerase, which degrades a nonextendable fluorescent DNA probe following hybridization and extension in PCR reaction (Heid et al., 1996). TaqMan probes are labeled with both fluorescent reporter (FAM) and a fluorescent quencher (rhodamine (TAMARA) that are bound to the 5’ and 3’ ends of the probe sequence and fluorescence resonanace energy transfer (FRET) from the reporter to the quencher. Following annealing of the forward and the reverse primers to the target sequence, the TaqMan probe is designed to anneal between these primer sites.

If a product is present, the probe binds to the product and is degraded, hence the reporter remains unquenched. Probe hydrolysis results in desuppression of the reporter and a subsequent cumu-lative increase in fluorescence proportional to the amount of transcriptome present. This oligonucleotide primer/probe approach increases accuracy and specificity of PCR product detection due to the requirement for precise, gene-specific matching of three independent nucleotide sequences (Wang and Brown, 1999).

SYBR Green is an example of an intercalating dye that fluoresces upon binding to double-stranded DNA. During PCR, multiple molecules of SYBR Green bind to the product and emit a strong fluorescent signal that is easily detected. Inercalating dyes are inexpensive and simple to

use compared to sequence probes and because they are not sequence specific, they can be used for any reaction. Limitations of real-time PCR are similar to microarrays. A specific probe used in the amplification reactions may fail to capture the sequence diversity that is present in environmental populations. Humic acid, clay material and enzymatic inhibitors that co-extract with the RNA may interfere with the PCR reaction. Finally, the RNA extract from environmental samples is often not of sufficient yield to be representative of the soil microbial population.

Data analysis of real-time PCR reactions

Data can be analyzed by the use of the absolute and the relative methods. For absolute quantization (or the absolute method), an RNA standard curve of the gene of interest is required in order to calculate the number of copies. In this case, a serial dilution of a known amount (number of copies) of pure RNA is diluted and subjected to amplification. The unknown signal is compared with the curve so as to extrapolate the starting concentration. The major limitation of the absolute method is its inability to account for any procedure that may introduce inter - or intra sample variability (Bustin et al., 2005).

The relative quantification method ($2^{-\Delta\Delta CT}$ method) is commonly used to analyze data obtained from real time PCR reactions (Livak and Schmittgen, 2001). This method relies on two assumptions (a) the PCR reaction is occurring with 100% efficiency, in other words, with each cycle of PCR, the amount of product doubles. This assumption is also one of the reasons for using a low cycle number when the reaction is still in the exponential phase and substrates are not limiting and there is no degradation of products. This requires setting the crossing threshold (C_T) at the earliest cycle possible. The C_T is the number of cycles that it takes each reaction to reach an arbitrary amount of fluorescence. The second assumption of the $2^{-\Delta\Delta CT}$ method is that there is a gene /genes that are expressed at a constant level between the samples. This endogenous control will be used to correct for any difference in sample loading. The choice of the endogenous control is important. When C_T is known for a reaction, it can be used to generate the relative expression level.

Assuming that there are two samples which are the control and treated samples, hence we have the gene expression level of target sample (target gene- TG) and ii) endogenous gene expression level of control gene (control gene -CG). C_T value can be used to generate the relative gene expression level; C_T for the gene of interest (TG) is measured and C_T for control gene (CG) is also measured, and the difference between the C_Ts for the target gene and control gene is found,

$C_T(TG) - C_T(CG) = \Delta C_T$

$-\Delta\Delta CT$ = Control condition ΔC_T – treated condition ΔC_T

Or

$2^{-\Delta\Delta CT}$ = Control condition ΔC_T – treated condition ΔC_T

The negative value of this subtraction, the $-\Delta\Delta CT$ is used as the exponent of 2 in the equation and represents the difference in 'corrected' number of cycles to threshold.

Choice of endogenous controls

Intersample variation between biological and technical replicates can interfere with data analysis and therefore must be normalized to one or more endogenous control genes. Properly selected, the control genes will normalize differences in the amount and quality of starting material as well as in reaction efficiency. Normalization uses endogenous housekeeping or reference genes with the assumption that their expression is i) similar between all samples in a given study ii) is resistant to experimental controls iii) undergoes all steps of the PCR with the same kinetics as the target gene (Bustin, 2000; Thellin et al., 1999). Housekeeping genes such as β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin, or tubulin are commonly used since they are ubiquitously expressed in cells and tissues. Quantification of mRNA from functional genes might be normalized to transcripts of housekeeping gene that is constitutively and evenly transcribed during all growth phases and states of metabolic activity (Eleaume and Jabbouri, 2004; Johnson et al., 2005). However, no such gene has been suggested across all of prokaryotic diversity and such normalization would require extensive *a* prior knowledge of the suites of genes present in natural soil microorganisms which is currently not available (Baelium et al., 2008).

DeCoste et al. (2011) first used the human GAPDH gene transcripts as exogenous spike-in RNA in their experimental system. The choice and logic of using the GAPDH mRNA centered on the fact that it was highly characterized (Schmittegen and Zakrajsek, 2000), available in copious amounts from commercial sources and being of human origin, assumed to exhibit negligible cross-reactivity to coextracted soil components. However, the qRT-PCR results with the GAPDH mRNA exogenous spike-in were inconsistent and very difficult to interpret, possibly due to higher cross-reactivity than that expected of GAPDH primers/probes to coextracted nontarget sequences and other unexplainable factors. De Coste et al. (2011) later extracted total soil RNA and spiked in a defined quantity of *in vitro*-synthesized myIC RNA (synthetic RNA internal amplification control) for relative quantification and generated specific and reproducible results in their controlled expremental set up.

Baelium et al. (2008) normalized their data to quantify gene expression *in situ* by utilizing the 'DNA equivalents' which is described as mRNA:DNA. They believed that this general approach represented the most reliable norma-

lization protocol available to quantify gene expression *in situ* of environmental samples.

THE USE OF THE RT-qPCR AS A TOOL IN SOIL RELATED STUDIES

Devers et al. (2004) investigated the expression of atrazine degrading genes (*atz*) using real-time RT-PCR in *Pseudomonas* sp. strain ADP and *Chelatobacter heintzii*. Their results showed that all the atrazine degrading genes were expressed in *Pseudomonas* sp. while only *atzA* was basally expressed in *C. heintzii*. When atrazine was added to the test medium, *atz gene* expression increased in *Pseudomonas* sp., while in *C. heintaii*, only *atzA* and *atzB* were up regulated in response. The atrazine degradation rate was also two-fold lower for *C. heintzii*. This study indicates that the host microorganism may be a factor in determining the degree of gene expression under basal and stimulated conditions and this is an important consideration in bioremediation.

Based on functional gene DNA sequences, studies showed that in a soil dominated by bacteria carrying class I *tfdA* genes, only bacteria harboring class III *tfdA* genes were able to proliferate during the degradation of MCPA. However, in this DNA based study, it was not possible to tell whether the class I *tfdA* gene was actually expressed in the standing population of bacteria harboring this gene in the presence of MCPA or if these bacteria were inactive for MCPA degradation. Hence, methodologies to detect and quantify actual gene expression related to specific microbial function in soil are needed (Baelum et al., 2006).

RT-qPCR helps to link gene expression with a specific measurable microbial activity. Studies have been conducted to link gene expression with activity, but most of these were conducted on bacterial strains isolated from soil which necessitates that the strain be culturable (Tao et al., 1999; Devers et al., 2004). To better understand ecosystem processes, there is the need to identify and characterize environmental factors affecting gene expression and ultimately the activity of soil microorganisms. Also, linking gene expression with methods that detect the presence and activity of corresponding protein either by antibody-based techniques or biochemical tests would further enhance our understanding on ecosystem processes in soil.

CONCLUSION

Major strides to date have been made with the use of RT-qPCR in soil related studies. With the use of RT-qPCR, one will know the active members of the microbial community as far as functional genes are concerned. More insight will be obtained if after the extraction of total RNA from soil, the mRNA is converted to cDNA and then sequenced. The active microbial members, diversity index, evenness, abundance and species richness in the microbial community will be known. Thus, apart from knowing the abundance of the relative genes expressed, the biodiversity changes and the microbial community structure of the active population would be known. The use of mRNA provides information about the metabolic state at the moment of testing and if combined with analysis of rDNA, very detailed information may be obtained about the involvement of certain populations in a particular metabolic activity (Gottschal et al., 1997).

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