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Real Time PCR Assay for Polyphosphate Kinase Genes in Activated Sludge

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Abstract: Polyphosphate kinase gene (*ppk*) encodes the enzyme polyphosphate kinase (PPK) that is thought to be responsible for the synthesis of polyphosphate (poly-P) in phosphorus accumulating organisms (PAOs). Methods to detect and quantify *ppk* gene simultaneously may be useful to detect microbial communities containing PPK. The objective of this study was to develop a real time polymerase chain reaction (RLT-PCR) assay for the detection and quantification of *ppk* gene in activated sludge. RLT-PCR conditions were optimized to amplify a 1.2 kb fragment of known *ppk* gene sequences in a plasmid and in *Pseudomonas aeruginosa* using degenerate primers. The protocol was then modified to use with the ABI Prism 7700 sequence detection system using SYBR® Green I for real time quantification of the amplicons. Four activated sludge microbial communities expected to contain varying levels of PAOs were analyzed to quantify the abundance of *ppk*. The results indicated that communities enriched for PAOs contained 10- to 100-fold higher levels of *ppk* (10^3-10^5 copies of *ppk* gene per 100 ng of total DNA) than the non-enriched communities (10^2 or less copies per 100 ng of total DNA). RLT-PCR is indeed a promising method to monitor environmental samples of complex nature and high similarity.

Key words: Polyphosphate kinase, *ppk*, real-time PCR, activated sludge

INTRODUCTION

Polyphosphate kinase (PPK) encoded by *ppk* gene has been found in many organisms (Kulaev *et al*., 1999). PPK mediates the transfer of the terminal phosphate from adenosine triphosphate to inorganic polyphosphate chain (poly-P), a biopolymer containing many phosphate residues linked together by phosphoanhydride bonds. Sequence analysis has revealed the high degree of conservation among various PPK homologs (Kornberg *et al*., 1999) in many bacteria (e.g., *Pseudomonas aeruginosa*, *Mycobacter tuberculosis*, *Neisseria meningitidis*, *Helicobacter pylori*, *Vibrio cholerae*, *Salmonella typhimurium* etc.,) including pathogenic bacteria as well as phosphorus accumulating organisms (PAOs) (McMahon *et al*., 2002). This has prompted the retrieval of their *ppk* gene to determine the accumulation of poly-P and its biological role in virulence or enhanced biological phosphorus removal (EBPR) in municipal wastewater treatment plants.

Although, poly-P is found in most organisms (Kulaev *et al*., 1999), some PAOs may accumulate it to more than 10% of their cellular dry weight when subjected to alternating anaerobic and aerobic conditions (Park *et al*., 1997; Keasling *et al*., 2000). Accumulation of poly-P through this approach followed by cell wasting forms the basis of biological phosphorus removal in municipal wastewater treatment plants (Crocetti *et al*., 2000).

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PPK containing microorganism have generally been accomplished using phylogenetic probes targeting 16S ribosomal RNA (rRNA) (Amann *et al*., 1998; Dabert *et al*., 2002) and by Polyphosphate staining (Lee *et al*., 1999; Crocetti *et al*., 2000; Liu *et al*., 2001; Keller *et al*., 2002). However, it still remains a poorly characterized group. Detection approaches based on the 16S rRNA are excellent tools for phylogenetically anchoring the microorganisms but they may not be capable of detecting all the microorganisms involved in poly-P accumulation. For example, recent studies (Liu *et al*., 2001; Zilles *et al*., 2002) identified *Rhodocyclus*-related organisms in the EBPR using the combination of 16S rRNA with fluorescence *in situ* hybridization (FISH) and poly-P staining techniques. Still this hampered the identification of multiple microorganisms that might be present or involved in EBPR processes. Up till now, there is no general consensus about putative PAOs. The reason for this would be the unavailability of an easy method to identify the microbial community using phosphorus removal phenotype/genotype.

Functional gene-based approaches, such as reported by McMahon *et al*. (2002) using the conserved regions of the many *ppk* gene sequences may serve as additional complementary tools to study *ppk* gene containing microorganisms and ultimately, PAOs. As a first step towards this approach, here, we describe the development of a quantitative real time (RLT)-PCR technique to detect and measure the abundance of *ppk* gene in activated sludge using the degenerate primers reported earlier (McMahon *et al*., 2002) and SYBR® Green I, a fluorescent dye specific to double stranded DNA (Witter *et al*., 1997). The method is capable of detecting 100 copies of *ppk* gene and has high dynamic range and reproducibility, typical of RLT-PCR methods (Ririe *et al*., 1997; Harms *et al*., 2003). We also present data related to *ppk* gene abundance in several activated sludge communities expected to contain varying levels of PAOs. This method can be extended to measure the abundance of the corresponding messenger RNA after reverse transcribing the mRNA into complementary DNA.

MATERIALS AND METHODS

Samples

A plasmid containing a 1.2 kb fragment of *Candidatus accumulibacter phosphatis ppk* gene (pPPK1) cloned in *Escherichia coli* DH10B using pCR4 vector (Invitrogen Corp., CA, USA), was provided by Dr. Katherine McMahon (insert sequence Genbank accession number AF502189) (McMahon *et al*., 2002). *Pseudomonas aeruginosa* (ATCC 15692) as a positive strain for *ppk* gene was obtained from the culture collection at the Center for Microbial Ecology, Michigan State University, Michigan. Activated sludge samples enriched for PAOs were collected from the anaerobic and aerobic phases of two municipal wastewater treatment plants carrying out biological phosphorus removal, one at Ann Arbor and the other at Ypsilanti, both located in Michigan, USA. Samples expected to contain non-enriched levels of PAOs were collected from a 1L aerobic sequencing batch reactor maintained on phenol for more than 7 years and from the start-up phase of a 5 L anaerobic-aerobic sequencing batch reactor treating liquid manure.

DNA Extraction

E. coli clone containing pPPK1 and *P. aeruginosa* were grown overnight at 37°C using Luria-Bertani (LB) medium (Sambrook *et al*., 1989) with and without Kanamycin (50 mg L^{-1}), respectively. Plasmid DNA from the *E. coli* clone was isolated using Qiagen mini plasmid DNA extraction kit (Qiagen, Inc., CA, USA) and genomic DNA from *P. aeruginosa* was isolated using QIAamp DNA mini kit (Qiagen) as per manufacturer's instructions. Genomic DNA from the activated sludge samples was isolated from 10 g of sludge (wet pellet weight) using Ultra Clean Soil DNA Mega Kit (MoBio Lab. Inc., CA, USA) as per manufacturer's instructions. DNA was quantified by

absorbance at 260 nm and visualized by gel electrophoresis using standard protocols (Sambrook *et al*., 1989) as well as using PicoGreen quantitation method as described (Ahn *et al*., 1996), to ensure the amount of DNA in each sample.

Conventional PCR Amplification

Conventional PCR amplification was carried out in 0.2 mL PCR tubes using Advantage-GC cDNA kit (CloneTech Lab. Inc.) with GC-melt as per manufacture's instructions. GC-melt is a proprietary buffer containing DMSO designed to help amplify GC-rich sequences. The 25 μL reaction mixture contained 100 to 200 ng of total genomic DNA, 300 nM each of the degenerate primers NLDE-0199F and TGNY-1435R (Rose *et al*., 1998; McMahon *et al*., 2002), 2.5 mM $MgCl_2$, 1 M GC-melt and 200 μM each dNTPs using a gradient thermal cycler (Master Cycler; Eppendorf AG, Hamburg, Germany). Only 10 ng DNA was used to amplify *ppk* gene from pPPK1. The PCR program consisted of the following four stages: one 12 min denaturation step at 94°C; 10 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 45 sec and extension at 72°C for 2 min with the annealing temperature decreasing by 0.5°C per cycle; 25 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 45 sec and extension at 72°C for 2 min and one 12 min extension step at 72°C (McMahon *et al*., 2002).

PCR amplification was also performed using AmpliTaq Gold™ polymerase, (PE Applied Biosystem, Foster city, CA), Advantage genomic PCR kit and Advantage-GC genomic PCR kit (Clontech Lab. Inc.) and a simpler temperature program (one 2 min denaturation at 94°C followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 45 sec and extension at 72°C for 1 min).

Real Time PCR Amplification and Data Analysis

RLT-PCR amplification was performed with the Advantage-GC cDNA PCR kit, as described above, in the presence of 40,000-fold dilution of SYBR® Green I (Molecular Probes, Inc. Eugene, OR) using the ABI Prism 7700 sequence detection system (Applied Biosystem, Foster City, CA). The PCR program was modified as: one 2 min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 45 sec and extension at 72°C for 1 min. The final extension step and touch down protocol were not necessary for the ABI Prism 7700 sequence detection system.

During RLT-PCR cycling, accumulation of PCR product is detected as fluorescence caused by SYBR® Green I intercalating into the amplified (and added) double stranded DNA. This fluorescence is first normalized relative to an arbitrarily established threshold. We used 10 times the standard deviation of the mean base line emission between the 3rd and 10th cycles as the threshold. The cycle at which the amplification curve crosses the threshold is defined as Ct (threshold cycle). Ct signifies the cycle number at which amplification of the target gene is positively observed. Knowing the copy number of the target gene (added) and the corresponding Ct value, a standard curve can be prepared. Following the above procedure, a standard curve for *ppk* gene was generated using 0, 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 copies of pPPK1. Since this curve was always linear over the 8-log using pPPK1, often one or two dilutions in the middle were not used. The absolute abundance of *ppk* gene in an unknown sample was determined using standard curves obtained concurrently on the ABI Prism 7700 sequence detection system as described (SYBR Green PCR Core Reagents, Applied Biosystems, P/N 4304886; Morrison *et al*., 1998). All analysis was carried out in triplicate.

After amplification, a melting curve for the amplified product(s) was also obtained using the ABI Prism 7700 sequence detection system by heating the reaction mixture at 95°C for 20 sec followed by rapid cooling to 52°C for 20 sec and then heating at the rate of 0.035°C sec^{-1} to 95°C and collecting

fluorescence data at 0.2°C intervals. Presence of two peaks in a melting curve is a definite indication of the presence of multiple amplicons if the variation is more than 2°C (Ririe *et al*., 1997; Morrison *et al*., 1998).

RESULTS AND DISCUSSION

Conventional PCR Amplification

In this study, we developed a quantitative real time PCR assay specific for *ppk* gene in activated sludge samples using RLT-PCR. Development and optimization of a RLT-PCR technique was carried out to measure the absolute abundance of *ppk* gene in activated sludge. First, a conventional PCR protocol was optimized to amplify a 1.2 kb sequence of the *ppk* known to be present in pPPK1 and *P. aeruginosa* and expected to be present in varying concentrations in four environmental samples. The protocol and the reagent kit were then supplemented with SYBR® Green I and used in ABI 7700 sequence detection system for real time quantification of the amplified products. Amplification of the cloned *ppk* gene from the pPPK1 was successful using several other simplified protocols and kits. For instance, Fig. 1a, shows the amplification of a 1.2 kb fragment of pPPK1 using AmpliTaq Gold™ polymerase (PE Applied Biosystem, Foster city, CA). However, this enzyme was not able to amplify *ppk* gene from *P. aeruginosa* (Fig. 1a, Lane 1). Similarly, other tested commercial kits such as Advantage genomic PCR kit and Advantage-GC genomic PCR kit (Clontech Lab. Inc.) were also not able to amplify *ppk* gene from *P. aeruginosa*, except the Advantage-GC cDNA kit (Clontech Lab. Inc.). The amplification of the *ppk* gene from *P. aeruginosa* was successful only by using Advantage-GC cDNA kit (Fig. 1a, Lane 2 and 3). *P. aeruginosa* has a relatively large genome of more than 6.3 million base pairs and 5,570 predicted open reading frames (ORFs) with GC content 55-64 mol% (Stover *et al*., 2000). The Advantage™ GC Genomic PCR Kit containing DMSO, hot start and GC-Melt reagent in addition to other standard PCR reagents improves amplification by disrupting base pairing and reducing the formation of secondary structure caused by GC-rich regions.

Both PCR programs amplified *ppk* gene of *P. aeruginosa* using Advantage-GC cDNA kit, however, nonspecific amplification was observed using touchdown PCR program (Fig. 1a, Lane 2). This nonspecific amplification was reduced by shortening the PCR cycle, diluting the target DNA amount (Fig. 1a, Lane 3) and reducing the primers concentration from 400 nM to 300 nM in a PCR reaction. PCR samples were analyzed on 1% agarose gel (Fig. 1b). The initial problems in amplifying the *ppk* gene from *P. aeruginosa* suggests that care must be taken to ensure that failure to detect the amplification in microbial samples is solely due to the absence of the target gene and not a problem with amplification protocol. PCR amplification can be inhibited due to the complex nature of the target sequence as well as due to the presence of other substances (mostly happens when extracted DNA from the environmental samples) with the target. This modified protocol was then tested to detect and quantify *ppk* gene in the environmental samples collected from Ann Arbor and Ypsilanti WWTPs. Our results showed the presence of a 1.2 kb amplified product in the analyzed samples (Fig. 1b).

This revealed that Advantage-GC cDNA kit can be successfully used to amplify target DNA from complex environmental samples.

Real Time PCR Amplification

After optimization of primers and PCR conditions, we performed experiments with confirmed known amounts of diluted DNA from 10-10^8 copies of pPPK1 plasmid to generate a standard curve (Fig. 2). The Ct values ranged between 11 to 40 cycles. The calculated R^2 value was 0.9933 and 0.9945 (Fig. 2a and b). There was a linear correlation between the amount of DNA used as template in the beginning of PCR reaction and the Ct values for the *ppk* product generated at the end of the PCR reaction (Fig. 2a and b). However, determination of this correlation by only visualizing PCR products on agarose gel was not possible (Fig. 2c).

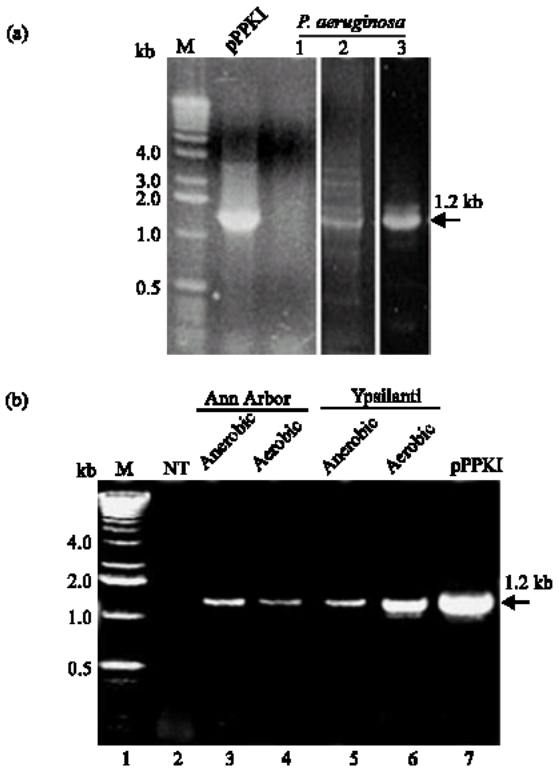

Fig. 1: (a) PCR amplification of *ppk* gene in pPPK1 and *P. aeruginosa*. Lanes: 1, using AmpliTaq Gold™ polymerase; 2, PCR amplification of *ppk* gene in *P. aeruginosa* using Advantage-GC cDNA kit; 3, Amplification of *ppk* gene in *P. aeruginosa* after certain modifications in the PCR conditions. (b) PCR amplification of environmental samples using 100 ng DNA. Lanes 3-6: activated sludge samples from Anaerobic and Aerobic phases collected from Ann Arbor and Ypsilanti wastewater treatment plants. M: 1 kb DNA ladder; NT: No template control

In RLT-PCR using SYBR® Green I absolute quantitation of genomic DNA can be performed using a standard curve (SYBR Green PCR Core Reagents, Applied Biosystems, P/N 4304886; Morrison *et al*., 1998). This quantitation is the method of choice, especially, when working with samples whose contents or communities, such as environmental samples, are not known, or when there is no internal standard or reference available for relative quantitation of a target.

There was no difference observed in the abundance of *ppk* gene between anaerobic and aerobic sludge from Ann Arbor and Ypsilanti WWTPs (Table 1). Approximately 10^4 to 10^5 copies of *ppk* per 100 ng of DNA were detected in all samples. There was less amount of *ppk* gene detected in laboratory scale reactor samples (between 10^2-10^3 copies of *ppk* gene per 200 ng of DNA). Since laboratory scale reactors were either maintained on phenol or treated with liquid manure, might contained very less number of *ppk* gene containing microorganisms. Consistent with the results of other study in our laboratory in which, these reactors did not show significant removal of phosphorus from the tested samples (data not shown).

The specificity of the amplified products using SYBR® Green I in RLT-PCR was monitored by the corresponding dissociation curve. A dissociation curve with a single peak suggests amplification of a specific product (Fig. 3) (Ririe *et al*., 1997). The T_m in °C for the four samples as calculated from the dissociation curve was found to be 85.5, 86.2, 86.2 and 86.6 for aerobic and anaerobic samples

Table 1: Absolute abundance of *ppk* gene in samples from two municipal wastewater treatment plants and two laboratory scale reactors

Sample	Phase	Ct	*ppk* abundance*
Ann Arbor WWTP	Anaerobic	28.94±0.07	9.1×10^{3}
	Aerobic	29.16±0.01	8.0×10^{3}
Ypsilanti WWTP	Anaerobic	27.97±0.02	1.6×10^{4}
	Aerobic	25.72±0.03	5.8×10^{4}
SBR treating phenol	Aerobic	34.25±1.90	2.1×10^{2}
SBR treating liquid manure	Anaerobic-Aerobic	30.43±0.80	1.9×10^{3}

*Detected number of *ppk* copies per 100 ng of the sludge DNA samples

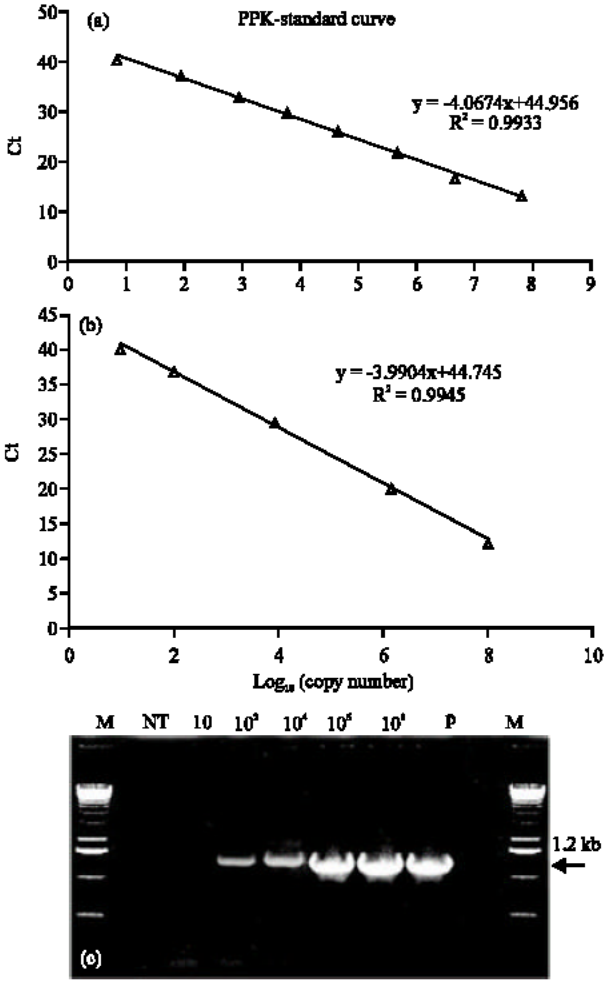

Fig. 2: Standard curve generated by Real Time PCR amplifying known amounts of *ppk* (10-10^{8} copies) obtained from pPPK1 (a) with 10 fold dilutions (b) with 100 fold dilutions © PCR amplification of pPPK1. M: 1 kb DNA ladder; 10-10^{8}: copies of pPPK1; P: pPPK1 10 ng DNA.

from Ypsilanti and aerobic and anaerobic samples from Ann Arbor, respectively. This might represent the amplification of three different types of *ppk* containing microbial communities. The identification of which, can be done using other molecular biological techniques such as sequencing, dot-blot analysis and fluorescent *in situ* hybridization etc.

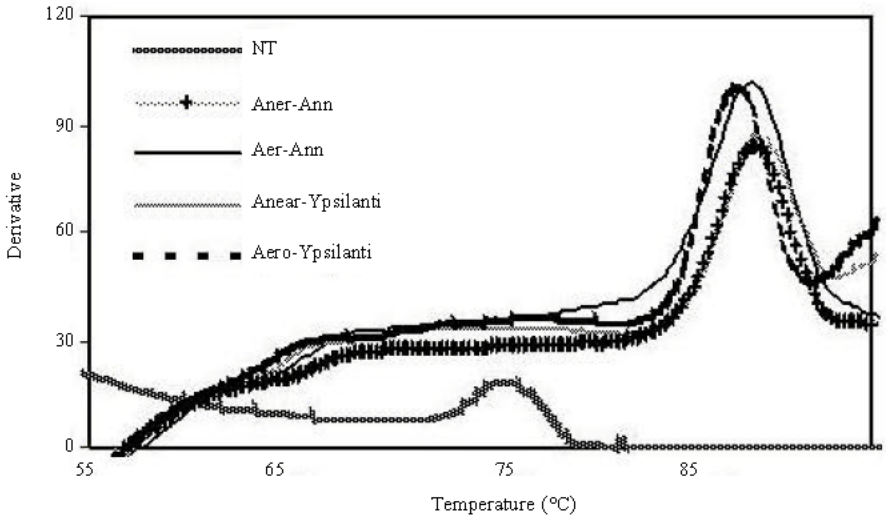

Fig. 3: The dissociation curves for activated sludge samples from Ann Arbor and Ypsilanti following RLT-PCR. NT: No template control

CONCLUSIONS

We optimized the PCR program to amplify the sequences of high similarity and complexity in activated sludge samples. Compared to other conventional PCR methods, RLT-PCR offers a reliable and high throughput analysis with a much wider detection range. Further, this modified RLT-PCR requires less cost and efforts to analyze multiple samples as compared to other methods such as FISH and poly-P staining techniques. However, it is possible that environmental samples may vary in their organic and inorganic composition that may require further optimization. Additional efforts to design more robust protocols or universal degenerate primers able to amplify various environments related significant genes, individually, from different kind of templates will be of great significance for the environmental research. Further developments using these primers may focus on validation at the mRNA level and intensive characterization of the detected populations.

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