qPCR detection of *Rhodobacter sphaeroides* Phage in the CoQ_{10} Production

Kejun Li[1]*, Haina Wang[2] and Ruiping Zhang[1]

[1] Lab of Space Microbiology, Center of Space Biology Research,
China Academy of Space Technology, Peking, China.
[2]Institute of Microbiology, Chinese Academy of Science, Peking, China.

(Received: 26 April 2015; accepted: 05 June 2015)

Coenzyme Q_{10} is industrially produced by fermentation and extraction from *Rhodobacter sphaeroides*. However, phage pollution is a fatal phenomenon in the fermentation industry. The infection of *R. sphaeroides* by phase was reported for the first time in this study. Based on the obtained genetic sequence from the phage, a quantitative polymerase chain reaction (qPCR) phage detection profile was developed for early detection during the pilot culture to prevent phage pollution in the production lines.

Key words: *Rhodobacter sphaeroides*; phage pollution; phage detection; qPCR.

Coenzyme Q (CoQ) is found in many types of organisms and is confirmed to be an essential component of the electron transport chain for aerobic respiration (Lenaz *et al*., 2007). In humans, the administration of CoQ_{10} presents promising uses in the prevention and treatment of several diseases (Sharma *et al*., 2004; Somayajulu *et al*., 2005). CoQ_{10} is industrially mass produced for the raw materials of many medicines and healthcare products. Among all industrial production technologies, the fermentation and extraction from *Rhodobacter sphaeroides* is considered the most economical and efficient method for obtaining the pure substance of CoQ_{10}.

However, in the fermentation industry, phage pollution is a fatal phenomenon that can lead to the reduction or even the extinction of output. When phage pollution occurs, the production is usually stopped to clean the fermentation equipment thoroughly. An efficient way to avoid phage attacks is the early detection of phages during the pilot culture. Tests can be performed by using standard microbiological methods, such as plaque assays (Everson, 1991), but such assays are time consuming. When the results are obtained, the culture is usually inoculated for enlargement. Therefore, the rapid detection of phages is important for their earlier management. Molecular methods for the rapid detection of phages are much better candidates for this task as they produce results within a shorter period. Some techniques that are based on quantitative polymerase chain reaction (qPCR) have been developed for detecting phages, mainly in food fermentation (del Rio*et al*., 2008; Martin *et al*., 2008). To our knowledge, the qPCR detection of *R. sphaeroides* phage has never been reported in previous studies.

During the summer of 2012, severe phage pollution led to a halt in production for several months in a factory that produced around 250 tons of CoQ_{10} per year by fermenting *R. sphaeroides*. To recover and ensure normal production, the phage was isolated and one section of its genomic sequence was obtained. Based on this sequence, a standard operation profile for rapid phage detection was established based on qPCR techniques. This study is the first to report the detection of *R. sphaeroides* phage in CoQ_{10} production.

* To whom all correspondence should be addressed.
E-mail: kejunli@pku.edu.cn

MATERIALS AND METHODS

The phage lysate was obtained from the production fermentors (130 m^3 in volume and 100 m^3 in fermentation) of the factory. The lysate was centrifuged for 10000 rpm for 5 min and the supernatant was collected for electron microscopic observation (Institute of Microbiology, Chinese Academy of Science, Peking, China) and phage isolation. The genetic material was purified directly from the lysate using the TaKaRa MiniBEST Plasmid Purification Kit (Ver. 4.0, TaKaRa, Japan). The purified genetic material was digested using nucleases DNaseI, S1, and RNaseA to judge the genetic material type. Some DNA sequences were obtained using random primers amplification, cloning, and sequencing (Sangon, Peking, China). *Rhodobacter sphaeroides* chromosomal DNA was used as the control in PCR reactions. Moreover, the *R. sphaeroides* phage was isolated from the lysate supernatant. The resuspended individual plaques were prepared based on the instructions for coliphage λ isolation in a laboratory manual (Molecular Cloning, version 3), boiled for 10 min and used as templates in the PCR reactions by using several primer pairs that were designed from the above-mentioned DNA sequences. The chromosomal DNA from *R. sphaeroides* and isolated sundry bacteria were used as the control. The amplified DNA fragments were recovered from the agarose gel by using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (Ver.4.0, TaKaRa), cloned into T-vector by using the pGEM-T easy Vector System (Promega, USA), and sequenced (Sangon). Based on the obtained sequence, a primer pair was selected to establish the qPCR phage-detecting method by using the SYBR® Premix Ex Taq™ II kit (TaKaRa) and the Applied Biosystems 7500 Real-Time PCR System (ABI, USA). In the qPCR exercises, the program and mixture were prepared based on the recommendations from the product manuals of the qPCR system and kit, and the PCR amplicons were examined by agarose gel electrophoresis and sequencing to test the validity of the primers and qPCR. The standard samples for the standard curve were prepared following the methods of Li *et al.* [2007 and 2011]. Using the qPCR determination technique established in this study, the efficiency of the genetic material purification technique using the Plasmid Purification Kit (TaKaRa) was compared with the coliphage λ DNA purification methods (precipitating the phage particles with PEG8000 and extracting DNA with phenol and chloroform) prescribed by the above-mentioned manual.

RESULTS AND DISCUSSIONS

The electron micrographs demonstrated abundant phage particles in the lysate supernatant (Fig. 1). Therefore, phage pollution was the cause of bacteria lyses. The genetic material that was directly purified from the lysate was analyzed using three nucleases. The analysis results revealed that such material could be digested by using DNaseI and S1, but not by using RNaseA. In addition, the genetic material and phage M13 DNA (single-chain DNA) could be digested using nuclease S1 at 0.1 U/reaction, but not at concentrations of 0.01 and 0.001 U/reaction. The linearized plasmid pUC18

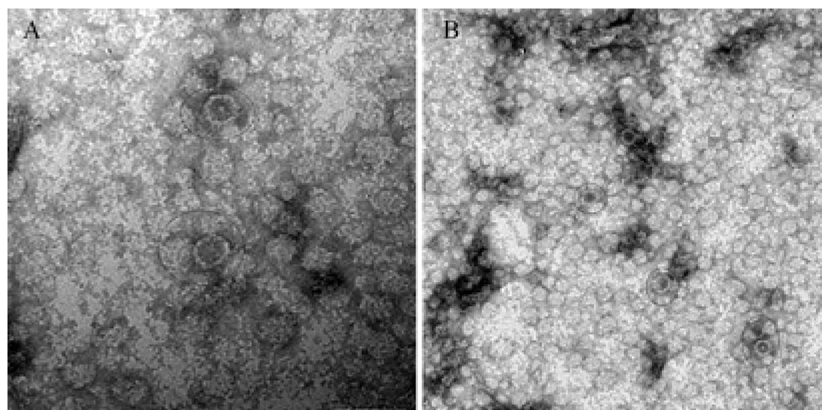

Fig. 1. Electron micrographs of phages infecting Rhodobacter sphaeroides which is fermented and extracted for Coenzyme Q_{10}. Bars represent 50 nm (A) or 100 nm (B)

(double-chain DNA) could not be digested under all three concentrations. Through enzyme digestion analysis, the genetic material type was estimated to be single-chain DNA.

Several sequences were also obtained from the isolated genetic material using random primer amplification. As a fingerprint technique, random primer amplification was often considered unacceptable over the recent years because of its low repeatability. However, in this study, random primer amplification was used to obtain some fragments from template DNA regardless of its repeatability and primers.

Given that the sequences were obtained directly from the lysate in the factory fermentors, confirming whether these sequences were from the phage genome was necessary to avoid the possibility of the sundry microbe origin. The above-mentioned sequences can be used to design primer pairs to amplify the isolated phage strain genomic DNA and to obtain amplicons. Therefore, 11 pure phage strains were isolated from the lysate supernatant and 7 resuspended plaques were successfully amplified with the primer phageZH12F (5'-TTTCGTCGTATCCGTCTCGGT-3') and phageZH12R (5'-GATGTCTACCTCTTGATATAC-3'). The DNA sequence obtained from the amplicons was the same as that obtained from the lysate, thereby confirming the phage genome source. The sequence has been submitted to GenBank under accession number KP996514. No highly similar neighbor was found in the GenBank database using the NCBI BLAST software. In this study, not all 11 strains were successfully amplified. Four unsuccessful amplifications were not repeated because of the low phage concentration in the suspended plaques used as templates and of a similar sequence obtained from seven successful amplifications.

Several pairs of primers were designed from the obtained sequence and compared in the qPCR determinations. The optimal primer pairs were phage-PF2 (5'-CTCGTCGCCATTCCAATCGG-3') and phage-PR2 (5'-CGGGGCCACAAACTTAGGTA-3'). Using this pair of primers, the qPCR phage detection had a low limit of approximately 26 DNA templates in each reaction (R^2> 0.99 for standard curves), which was considered sufficient for magnitude determination in the factory (Fig. 2 and 3). Using this technique, two previously-mentioned phage DNA purification methods were also compared using the same samples. The efficiency of the plasmid purification kit was higher than the λ DNA purification profile in this study, thereby indicating that more phage DNA existed in the bacterial cells than in the phage particles in the lysate and in the dust samples from the factory. Several high-concentration samples were also found from the sewer conduits. Therefore, adjustment and alteration were included in the disinfection operations of the factory.

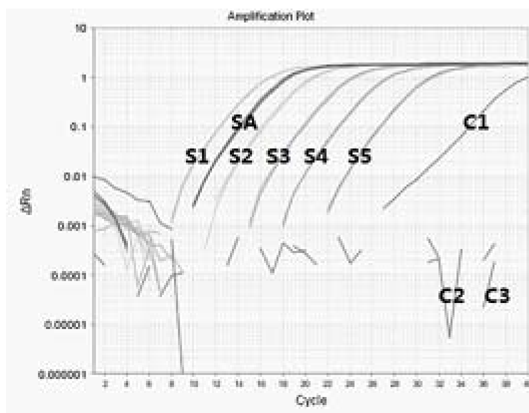

Fig. 2. Standard curve showing the linear relationship between the threshold cycles (Ct) and input copy number of standard samples. Triplicate determinations for each sample

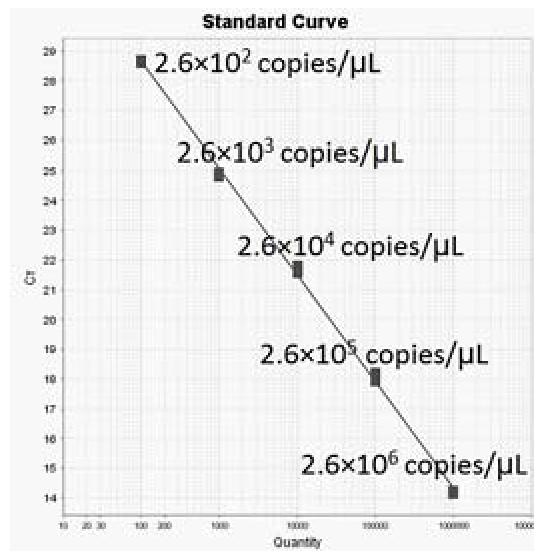

Fig. 3. Standard curve showing the linear relationship between the threshold cycles (Ct) and input copy number of standard samples. Triplicate determinations for each sample.

Based on the established qPCR techniques, one standard operation profile for phage detection was confirmed and used in the factory. The factory resumed its production in March 2013 when the phage concentration in the environmental samples was lowered after a long-time disinfection. The bacterial culture was also gradually magnified from a bacterial clone to 100-m^3 fermentation. Therefore, the phage pollution must be determined during the amplification before these phages are inoculated in bulky fermentors, particularly in 130-m^3 production fermentors. After adopting the standard operation profile, phage pollution was not observed in the fermentors since production recovery.

qPCR-based methods allow highly sensitive, rapid, and real-time monitoring of specific phages during the fermentation process (Marco *et al*., 2012; Samson *et al*., 2013). In this study, the assay process lasts no longer than 3 h after the sampling. However, when the plaque assay was used, the culture process lasted almost 3 d because of the slow bacterial growth rate. Given that qPCR detects DNA fragments, the absence of amplicons from contaminating bacteria must be ensured. To guarantee that only phage DNA was amplified and to avoid overestimation, the primer sequences were designed and compared with those in the GenBank database, using the NCBI Primer-BLAST software. Some contaminating bacteria were isolated from the lysate and used as controls. Theoretically, the contaminating bacterial strains cannot be entirely isolated. The phage magnitude in this study was usually large. The determination results require more evidence while the very low signal found in qPCR. These molecular methods do not discriminate between active and non-active phage particles and are also not very convenient for factory operators. To overcome these limitations, qPCR-based methods and classical microbiological assays should be used simultaneously to obtain more data from the factory.

REFERENCES

1. del Rio, B., Martin, M.C., Martinez, N., Magadan, A.H. Alvarez, M.A. Multiplex Fast Real-Time Polymerase Chain Reaction for quantitative detection and identification of cos and pac *Streptococcus thermophilus* bacteriophages. *Appl. Environ. Microb.*, 2008; **74**: 4779-4781.
2. Everson, T.C. Control of phage in the dairy plant. *Bull. Int. Dairy. Fed.*, 1991; **263**: 24-28.
3. Lenaz, G., Fato, R., Formiggini, G,, Genova, M.L. The role of Coenzyme Q in mitochondrial electron transport. *Mitochondrion,* 2007; **7**: S8-S33.
4. Li, K., Guan, W., Wei, G., Liu, B., Xu, J., Zhao, L., Zhang, Y. Phylogenetic analysis of intestinal bacteria in the Chinese mitten crab (Eriocheir sinensis). *J. Appl. Microbiol.*, 2007;**103**: 675-682.
5. Li, K. Molecular comparison of the sampling efficiency of four types of airborne bacterial samplers. *Sci. Total. Environ.*, 2011;**409**: 5493-5498.
6. Marco, M.B., Moineau, S., Quiberoni, A. Bacteriophages and dairy fermentations. *Bacteriophage*, 2012; **2**: 1-10.
7. Martin, M.C., del Rio, B., Martinez, N., Magadan, A.H., Alvarez, M.A. Fast real-time polymerase chain reaction for quantitative detection of *Lactobacillus delbrueckii* bacteriophages in milk. *Food Microbiol,* 2008; **25**: 978-982.
8. Samson, J.E., Moineau, S. Bacteriophages in Food Fermentations: New Frontiers in a Continuous Arms Race. *Annu. Rev. Food. Sci. T.*, 2013; **4**: 347-68.
9. Sharma, S., Kheradpezhou, M., Shavali, S., El Refaey, H., Eken. J., Hagen, C., Ebadi, M. Neuroprotective actions of coenzyme Q10 in Parkinson's disease. *Method. Enzymol.*, 2004; **382**: 488-509.
10. Somayajulu, M., McCarthy, S., Hung, M., Sikorska, M. Borowy-Borowski, H., Pandeya, S. Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by Coenzyme Q10. *Neurobiol. Dis.*, 2005; **18**: 618-627.