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A novel nucleic lateral flow assay for screening of PHA-producing haloarchaea

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are important for biodegradable plastic production, and prokaryotes play a very important role in PHA production. PHA synthase is a key enzyme for the polymerization of PHAs. There are four classes of PHA synthase. The *phaC* gene is necessary for the production of all classes of PHA synthase, whereas the *phaE* gene is necessary for the production of class III PHA synthase. This gene is a biomarker for microorganisms that contain class III PHA synthase, such as haloarchaea. Standard techniques for screening of PHA-producing haloarchaea require time for culturing and have poor specificity and sensitivity. Thus, the *phaE* biosensor was developed to overcome these issues. PCR and DNA lateral flow biosensor techniques were combined for construction of the *phaE* biosensor. The *phaE* biosensor has a high specificity for PHA-producing haloarchaea. The lowest amount of genomic DNA of *Haloquadratum walsbyi* DSM 16854 that the *phaE* gene could be detected by the biosensor was approximately 250 fg. The *phaE* biosensor can be applied for screening of PHA-producing haloarchaea from environmental samples. The *phaE* biosensor is easy to handle and dispose. For screening PHA-producing haloarchaea, the *phaE* biosensor requires less time and costs less than the standard methods.

1. Introduction

Production of polyhydroxyalkanoates (PHAs) is increasing due to their biodegradable characteristics. The properties of PHAs are similar to petroleum-based plastics (Steinbuchel and Fuchtenbusch, 1998). PHAs are environmentally friendly because they can be produced from renewable sources. The consumption of PHAs in European countries was approximately 50,000 to 100,000 tons in 2007 and will increase to 2–4 million tons in 2020 (Crank et al., 2005). Poly(3-hydroxybutyrate) (PHB) is the most common type of PHA that is accumulated by bacteria and archaea (Steinbuchel and Fuchtenbusch, 1998). PHA-producing microorganisms are able to convert carbon sources, such as glucose and fatty acids, to intermediate substances in PHA synthesis pathways. Acetyl-CoA and acyl-CoA are important intermediate substances for PHA biosynthesis (Philip et al., 2007). PHA synthases are key enzymes in PHA biosynthesis and are classified into four groups based on their structures, substrate specificities and subunit components (Rehm, 2003). Classes I and II PHA synthases are encoded by the *phaC* gene. Class III PHA synthase is encoded by the *phaC* and *phaE* genes. Class IV PHA synthase is encoded by the *phaC* and *phaR* genes (Rehm, 2003). The accumulation of PHAs in haloarchaea has been reported in the literature; however, the number of reports on this topic is still limited (Quillaguaman et al., 2010). Haloarchaea require a high concentration of NaCl for growth. Non-halophilic microorganisms cannot grow in that condition; thus, the cost of sterilization of the equipment, media and bioreactor used for haloarchaea production is low. The salt from the cultured media can be recovered and reused in the PHA production pipeline (Hezayen et al., 2010). Isolation of PHAs from haloarchaea can be performed using hypo-osmotic shock. PHAs leak out of the haloarchaea cells when they are treated with salt-free water. This method reduces the cost of the downstream processes by approximately 40% (Choi and Lee, 1999). *Haloferax mediterranei* is a halophilic archaea that utilizes whey hydrolysate as a carbon source for PHA production. The cost of PHA production is 2.82 euro for 0.29 g^{-1} L^{-1} h^{-1} (Koller et al., 2007). This production cost is approximately 1.4 times lower than that for PHA production by recombinant *Escherichia coli* (c.a. 4.0 Euro) (Koller et al., 2007; Reddy et al., 2003).

In this study, PCR method had been applied for detection of the *phaE* gene of PHA-producing haloarchaea. DNA lateral flow biosensor was a rapid method that had been used for detection of the *phaE* gene amplicon.

2. Material and methods

2.1. Bacterial strains and culture method

Bacillus megaterium DSM 319 and *Ralstonia eutropha* DSM 428 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *Streptomyces hygroscopicus* BTCC 7028 was obtained from the National Center for Genetic Engineering and

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Biotechnology (BIOTEC). *Bacillus subtilis* ATCC 6633, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *P. fluorescens* ATCC 13525 and *Staphylococcus aureus* ATCC 13565 were obtained from the Department of Microbiology, Faculty of Pharmacy, Mahidol University. Genomic DNA of *Haloquadratum walsbyi* DSM 16854 was obtained from DSMZ. All bacterial strains were inoculated in LB broth at 37 °C with shaking at 100 rpm overnight.

2.2. Primer design for phaE gene of PHA-producing haloarchaea

The *phaE* gene sequences of PHA-producing haloarchaea were obtained from the genome database of GenBank. The length of the *phaE* genes of haloarchaea was 507 to 555 bp. The gene sequences were aligned using the iterative refinement method. The evolutionary model selection was based on Akaike information criterion (AIC) and hierarchical likelihood ratio tests (hLRTs) criteria. A phylogenetic tree was constructed using the Bayesian inference method (Yasawong et al., 2011). The conserved region of *phaE* genes was used to design the primers.

2.3. Polymerase chain reaction

Genomic DNA of bacterial cells was extracted using the PowerSoil® DNA isolation kit (Mo Bio, USA). *H. walsbyi* DSM 16854 is a PHA-producing haloarchaea (Burns et al., 2007). *H. walsbyi* DSM 16854 possesses PHA synthase class III and contains the *phaE* gene (Bolhuis et al., 2006). Thus, the genomic DNA of *H. walsbyi* DSM 16854 was used as a positive control for *phaE* amplification. The *phaE* primers were purchased from Bio Basic Canada Inc. The details of the *phaE* primers are shown in Table 1. The size of the PCR product was approximately 224 bp. The PCR reaction was performed in a 50 μL total volume containing 10 ng of DNA, 20 pmol of each primer, 80 μM dNTPs, 1 × reaction buffer (Vivantis, PL1202, Malaysia) and 1.25 U thermostable DNA polymerase (Vivantis, PL1202, Malaysia). The PCR began with an initial step of 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 60 °C for 10 s, and 72 °C for 10 s, and a final step of 72 °C for 1 min. PCR was performed in a T100™ Thermal Cycler (Bio-Rad, USA). Reproducibility of PCR was examined by testing three replicates of genomic DNA by each assay and repeating the experiment for five times. The amplicon was examined by 1% w/v agarose gel electrophoresis. GeneRuler 100 bp plus DNA ladder (Thermo, USA) was used as the DNA marker for the agarose gel electrophoresis.

2.4. Specificity test of the phaE biosensor

The details of the microorganisms used in this study are shown in Table 2. *H. walsbyi* DSM 16854 was used as a positive control, as it is able to produce PHA and contains the *phaE* gene (Burns et al., 2007; Bolhuis et al., 2006). Deionized water was used instead of DNA template for a no template control (NTC) reaction. Negative control group 1 (NC1) included *E. coli* ATCC 25922, *S. aureus* ATCC 13565 and *S. hygroscopicus* BTCC 7028, which are all incapable of producing PHAs and lack the *phaE* gene. Negative control group 2 (NC2) included *B. megaterium* DSM 319, *B. subtilis* ATCC 6633, *P. aeruginosa* ATCC 9027, *P. fluorescens* ATCC 13525 and *R. eutropha* DSM 428, which are all capable of producing PHAs but lack the *phaE* gene. The *phaE* biosensor procedure contained two steps, amplification and detection. First, the *phaE* gene of PHA-producing haloarchaea was amplified by PCR. Then, the *phaE* amplicon was detected on the HybriDetect DNA lateral flow biosensor (Milenia Biotec, MGHD1, Germany). The specificity of the *phaE* biosensor was dependent on the amplification step. PCR amplification was positive when the amplicon contained biotin and FITC at its flanking region. The DNA lateral flow biosensor was obtained from Milenia Biotec (Germany) and was set up according to the instructions provided with the kit (Milenia, Germany). Five microliters of PCR products were added to the DNA lateral flow biosensor. The results appeared on the biosensor within one to two minutes. One line on the biosensor (control line) meant that no amplification of the *phaE* gene was detected. The PCR product of *phaE* gene was detected when two lines appeared on the biosensor (control line and test line).

Table 2
Specific details of microorganisms used in this study.

No.	Species	PHA synthase	*phaE*	Designated
1	*B. megaterium* DSM 319	Class IV	No	Negative control group 2
2	*B. subtilis* ATCC 6633	Class IV	No	Negative control group 2
3	*E. coli* ATCC 25922	–	No	Negative control group 1
4	*H. walsbyi* DSM 16854	Class III	Yes	Positive control
5	*P. aeruginosa* ATCC 9027	Class II	No	Negative control group 2
6	*P. fluorescens* ATCC 13525	Class II	No	Negative control group 2
7	*R. eutropha* DSM 428	Class I	No	Negative control group 2
8	*S. aureus* ATCC 13565	–	No	Negative control group 1
9	*S. hygroscopicus* BTCC 7028	–	No	Negative control group 1

2.5. Sensitivity test of the phaE biosensor

The genomic DNA (gDNA) of *H. walsbyi* DSM 16854 was quantified using NanoDrop (Thermo, USA). Two-fold dilutions of the gDNA were performed from 1000 to 125 fg/μL. Each dilution of the gDNA of *H. walsbyi* DSM 16854 was used as a DNA template for the PCR reaction as previously described. The PCR products were examined by 1% w/v agarose gel electrophoresis and the DNA lateral flow biosensor (Milenia Biotec, Germany).

2.6. Testing of the phaE biosensor with environmental samples

Soil samples were collected from solar salterns and mangroves. The details of the samples are described in Table 3. Environmental DNA (eDNA) was extracted using the PowerSoil® DNA isolation kit (Mo Bio, USA). Ten nanograms of eDNA was used as the DNA template for PCR. The amplicons were tested on the HybriDetect DNA lateral flow biosensor (Milenia Biotec, Germany).

3. Results

3.1. Primer design for phaE gene of PHA-producing haloarchaea

The *phaE* phylogeny of haloarchaea was reconstructed based on the GTR + G model. The *phaE* sequence of *Allochromatium vinosum* was used as an outgroup for the analysis. The posterior probabilities were obtained by performing two separate runs with four Markov chains. Each run was conducted with four million generations and was sampled every 100 generations. The potential scale reduction factor (PSRF) for all parameters was 1.000. The average standard deviation of the split frequencies was 0.001149. A consensus tree was calculated after discarding the first 25% of the iterations as the burn-in. The *phaE* phylogeny of PHA-producing haloarchaea is shown in Fig. 1. The details of the microorganisms used for the phylogenetic analysis are shown in

Table 1
Primers for amplification of *phaE* gene of PHA-producing haloarchaea.

Primer	Sequence (5′ → 3′)	Length (bp)	5′-labeled	PCR product (bp)
PhaE3A-F	GAGTTCCGYGATATCTGGYT	20	Biotin	224
PhaE3A-R	TGCTGACGACGCTCGASYTC	20	FITC	