Microbiology (2000), **146**, 2019–2025 Printed in Great Britain

Rapid detection of polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR

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Colony PCR and semi-nested PCR techniques were employed for screening polyhydroxyalkanoate (PHA) producers isolated from the environment. Three degenerate primers were designed based on multiple sequence alignment results and were used as PCR primers to detect PHA synthase genes. Optimized colony PCR conditions were achieved by adding 3% DMSO combined with 1 M betaine to the reaction mixture. The sensitivity limit of the colony PCR was 1×10^5 viable cells for *Ralstonia eutropha*. Nineteen PHA-positive bacteria were used to evaluate this PCR protocol; fifteen of the nineteen could be detected by colony PCR, and the other four could be detected by applying semi-nested PCR detection following colony PCR. In a preliminary screening project, 38 PHA-positive strains were isolated from environmental samples by applying the PCR protocol, and their phenotype was further confirmed by Nile blue A staining assay. By combining the colony PCR and semi-nested PCR techniques, a rapid, reliable and highly accurate detection method has been developed for detecting PHA producers. This protocol is suitable for screening large numbers of environmental isolates. The PHA accumulation ability of well-separated colonies isolated from environmental samples can be directly validated by PCR with no further culturing or chromosomal DNA extraction procedures. In addition to its application to the screening of wild-type isolates, the individual PCR-amplified product is also suitable as a specific probe for PHA operon cloning. The results suggest that the application of this PCR protocol for rapid detection of PHA producers from the environment is plausible.

Keywords: colony PCR, semi-nested PCR, polyhydroxyalkanoates, PHA synthase, degenerate primers

INTRODUCTION

Polyhydroxyalkanoates (PHAs) function as carbon and energy reserves in prokaryotic cells (Anderson & Dawes, 1990); they are accumulated by a wide range of bacteria when a carbon source is provided in excess and one essential growth nutrient is limited (Ramsay *et al.*, 1990; Steinbüchel & Schlegel, 1991). Since their physical characteristics are similar to those of petrochemical polyesters, such as polypropylene, PHAs have been studied intensively by academia and industry and are considered good candidates for biodegradable plastics and elastomers (Anderson & Dawes, 1990; Poirier *et al.*, 1995). The synthesis of PHAs requires the enzyme PHA synthase (PhaC), which uses β-hydroxyacyl-coenzyme A substrates for polymerization. The production of such substrates can occur by a variety of pathways (Madison & Huisman, 1999), including the simplest using the enzymes β-ketothiolase (encoded by *phaA*) and acetoacetyl-CoA reductase (encoded by *phaB*), β-oxidation (Page & Manchak, 1995), and a fatty acid *de novo* synthesis pathway (Rehm *et al.*, 1998). So far, more than 20 kinds of PHA synthesis operons have been cloned and analysed from a variety of bacteria; the results reveal that the proteins required for PHA biosynthesis pathways have diverged considerably (Madison & Huisman, 1999). Nevertheless, PHA synthase is a crucial enzyme in all PHA synthesis pathways (Anderson & Dawes, 1990; Madison & Huisman, 1999; Rehm & Steinbüchel, 1999). The PHA synthases can be broadly categorized into three different types based on their primary amino acid

Abbreviation: PHA, polyhydroxyalkanoate.

sequences and *in vivo* substrate specificities (Rehm & Steinbüchel, 1999). Type I PHA synthases (*Ralstonia eutropha*) are preferentially active towards coenzyme A thioesters of various short-chain-length 3-hydroxyalkanoates comprising three to five carbon atoms. Type II PHA synthases (*Pseudomonas aeruginosa*) are preferentially active towards coenzyme A thioesters of various medium-chain-length 3-hydroxyalkanoates comprising at least five carbon atoms. Type III PHA synthases (*Chromatium vinosum*) comprise enzymes consisting of two different types of subunits, the PhaC subunit and the PhaE subunit. These PHA synthases prefer coenzyme A thioesters of short-chain-length 3-hydroxyalkanoates.

There are many phenotypic detection methods for detecting intracellular PHA granules which are applied to the screening of PHA producers, including Sudan Black staining (Schlegel *et al*., 1970) and Nile blue A staining (Ostle & Holt, 1982), which result in dark blue or fluorescent granules. Although these methods are quite sensitive, it is rather time-consuming and labour-intensive work to screen a large number of environmental isolates. Alternative staining methods have recently been developed for directly staining colonies (Kranz *et al*., 1997) or growing bacteria on plates containing Nile blue A or Nile red (Spiekermann *et al*., 1999), resulting in fluorescent colonies that can be visualized by UV illumination. These colony-staining methods are suitable for screening large numbers of strains. However, appropriate carbon sources should be used, and a long culture time (3 d) is required for PHA granule accumulation. In addition, these methods cannot distinguish between bacteria that accumulate PHA granules and those that accumulate lipid compounds.

In this study, a genotypic detection method, which circumvents the major drawbacks inherent in phenotypic detection methods described above, was devised.

METHODS

Bacterial strains and media. Nineteen PHA-positive bacterial strains [*Ralstonia eutropha* H16 ATCC 17699 (formerly known as *Alcaligenes eutrophus*), *Alcaligenes hydrogenophilus* ATCC 33178, *A. latus* ATCC 15440, *Comamonas* (*Delftia*) *acidovorans* ATCC 15668, *Hydrogenophaga palleronii* ATCC 17724, *H. pseudoflava* ATCC 33668, *Pseudomonas alcaligenes* ATCC 14909, *P. aureofaciens* ATCC 13986, *P. citronellolis* ATCC 13674, *P. fluorescens* ATCC 12983, *P.* (*Burkholderia*) *glathei* ATCC 29195, *P. mendocina* ATCC 25411, *P. oleovorans* ATCC 8062, *P. pseudoalcaligenes* ATCC 17440, *P. putida* ATCC 12633, *Sphaerotilus natans* ATCC 15291, *Azotobacter chroococcum* ATCC 9043, *A. vinelandii* ATCC 478 and *Bacillus megaterium* CCRC 11595] and one PHA-negative bacterium, *Escherichia coli* XL-1 Blue (Bullock *et al*., 1987) were used. Mineral salt medium (MSM) pH 7·0 (Ramsay *et al*., 1990) was used for the isolation of bacterial strains from environmental samples. Carbohydrates and mineral salt solutions were sterilized separately, and combined prior to culturing.

Nucleotide sequence analysis. The nucleotide sequences of 13 PHA synthase (*phaC*) genes were collected for sequence analysis, derived from the following Gram-negative bacteria: *Acinetobacter* sp. (Schembri *et al*., 1994), *Aeromonas caviae* (Fukui & Doi, 1997), *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) (Peoples & Sinskey, 1989), *Alcaligenes* sp. (GenBank accession no. U78047), *Methylobacterium extorquens* (Valentin & Steinbüchel, 1993), *Paracoccus denitrificans* (Ueda *et al*., 1996), *Pseudomonas aeruginosa* (Timm & Steinbüchel, 1992) (containing two *phaC* genes), *P. oleovorans* (Huisman *et al*., 1991) (containing two *phaC* genes), *Rhizobium etli* (Cevallos *et al*., 1996), *Rhizobium meliloti* (Tombolini *et al*., 1995) and *Zoogloea ramigera* (GenBank accession no. U66242). Multiple sequence alignment was achieved by employing the SeqWeb software allowing a GapWeight of 10 and a GapLengthWeight of 5.

Synthetic oligonucleotide primers. Three degenerate primers (all 26-mers) were designed and synthesized according to the multiple sequence alignment results. The sequences were 5′-ATCAACAA(GGG/A)T(TT/A)CTAC(AA/G)TC(CC/T)-T(CC/G)GACCT-3′ (designated phaCF1, corresponding to nt 741–766 of *R. eutropha phaC*), 5′-GT(CCC/GG)TTC-(GGG/AA)T(GGG/CC)(AAA/GG)T(CC/G)(TT/A)(C-CC/GG)CTGGCGCAACCC-3′ (designated phaCF2, corresponding to nt 846–871 of *R. eutropha phaC*) and 5′-AGGT-AGTTGT(TT/C)GAC(CCC/GG)(AAA/CC)(AAA/CC)-(GGG/A)TAG (TTT/G)TCCA-3′ (designated phaCR4, corresponding to nt 1237–1212 of *R. eutropha phaC*).

Colony PCR. The optimized colony PCR reaction mixture contained 1 × PCR amplification buffer [20 mM $(NH_4)_2SO_4$, 72·5 mM Tris/HCl, 0·1 % Tween 20, pH 9·0], 2·5 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 2·5 μM each primer, 1·25 U Supertherm DNA polymerase (LPI) in 50 μl PCR reaction mixture. A final concentration of 100 μg ml^{-1} of acetylated BSA (New England BioLabs), 3 % dimethyl sulfoxide (DMSO) (Sigma) and 1 M betaine (Sigma) as PCR additives were also added to the reaction mixture. Colonies approximately 1 mm in diameter were picked up with a sterilized toothpick and directly transferred to the PCR tube as DNA templates. The thermal cycle programme, run on a GeneAmp PCR system 9700 (Perkin Elmer) consisted of one cycle of 94 °C for 10 min, 51 °C for 2 min, 72 °C for 2 min, and 35 cycles of 94 °C for 20 s, 57 °C for 45 s (decreased by 1 s per cycle), 72 °C for 1 min, and then incubation at 72 °C for 5 min, and a final incubation at 4 °C.

Sensitivity of colony PCR. Tenfold serial dilutions of *R. eutropha* overnight cultures were prepared in nutrient broth. Aliquots of each dilution were subjected to plate counting to determine bacterial cell concentration. Other aliquots of each dilution were centrifuged, and the cell pellet was washed once with sterilized deionized water and then used as DNA template for PCR.

Detection of PCR products. PCR-amplified DNA fragments were observed by agarose gel electrophoresis in 1·3 % agarose gels (FMC). Ten microlitres of each amplification mixture and the molecular mass marker (*Hae*III digest of ϕX174 DNA) were subjected to agarose gel electrophoresis and ethidium bromide staining. The amplified DNA fragments were visualized by UV illumination.

Southern hybridization. To confirm the amplified DNA fragment, 10 μl of colony PCR products was subjected to 1·3 % agarose gel electrophoresis and then transferred to a Hybond-N+ membrane (Amersham) by alkaline Southern transfer (Reed & Mann, 1985). An internal oligonucleotide probe, phaCF2, was labelled with [γ-^{32}P]ATP by 5′-end labelling with T4 polynucleotide kinase (New England BioLabs) as a probe (1·5 ng ml^{-1}). The membranes were air-dried and hybridized at 38 °C overnight in 1 × SSC buffer containing

1 % SDS, 1 % casein (Merck), 0·5 mg ml^{-1} of denatured herring sperm DNA (Boehringer Mannheim) and 50 % (v/v) deionized formamide (Clontech). At low stringency, the buffer and hybridization temperature were the same as those above except for the lack of formamide. After hybridization, the membranes were rinsed with 2 × SSC and 2 × SSC/0·1 % SDS at 38 °C, followed by 0·5 × SSC/0·1 % SDS at 25 °C. Then the membranes were autoradiographed with X-ray film (Fuji) and exposed at −70 °C for an appropriate length of time.

Semi-nested PCR. Semi-nested PCR was done with the primers phaCF2 and phaCR4, with a predicted product of 406 bp, to further confirm the colony PCR results. For positive products, 1 µl of 100-fold-diluted colony PCR products was subjected to semi-nested PCR. For negative products, 1 µl of undiluted colony PCR products was directly supplied as DNA templates for semi-nested PCR. The 50 µl PCR mixture contained 1 × PCR amplification buffer, 1·5 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphate, 2 µM each primer, 2 % DMSO and 0·5 U Supertherm DNA polymerase. The thermal cycle programme, run on a GeneAmp PCR system 9700 (Perkin Elmer), was 94 °C for 5 min (initial denaturation), 25 cycles of 94 °C for 15 s, 57 °C for 15 s, 72 °C for 30 s, and then incubation at 72 °C for an additional 5 min, with final incubation at 4 °C.

Isolation and screening of environmental strains. The screening procedure for PHA producers from the environment was modified from Gomez *et al.* (1996). Serial dilutions of active sludge were prepared in MSM. Aliquots of the dilution series were spread onto MSM plates containing 1 % (w/v) sucrose (Sigma), 1·5 % (w/v) sodium gluconate (Wako), 1 % (w/v) glycerol (Sigma) or 0·1 % (w/v) sodium octanoate (Wako) as carbon sources and were cultured at 30 °C overnight. The grown colonies were individually streaked onto MSM plates containing appropriate carbon sources to obtain well-separated single colonies. The PHA producer candidates were directly screened by colony PCR and semi-nested PCR from these well-separated colonies.

Nile blue A staining. PHA-positive strains identified by PCR were cultured in 50 ml MSM containing an appropriate carbon source at 30 °C with reciprocal shaking at 150 r.p.m. for 3 d. The bacterial cells were stained with Nile blue A and observed as described by Ostle & Holt (1982). The fluorescence microscope used was an Olympus AX 70; the excitation filter, barrier filter and dichroic mirror sets were BP450–480, BA515 and DM500, respectively. The staining results were photographed in colour on Kodak Kodacolor print film (400 ASA).

RESULTS

DNA sequence alignment

Thirteen PHA synthase gene sequences from Gram-negative bacteria (see Methods) were aligned. The PILEUP results showed that there were seven highly conserved regions among the 13 sequences. The corresponding positions of these conserved regions are depicted on the *phaC* of *R. eutropha* in Fig. 1. Based on the F1, F2 and R4 conserved region sequences, three degenerate primers, phaCF1, phaCF2 and phaCR4, were designed for PCR amplification and Southern hybridization (see Methods).

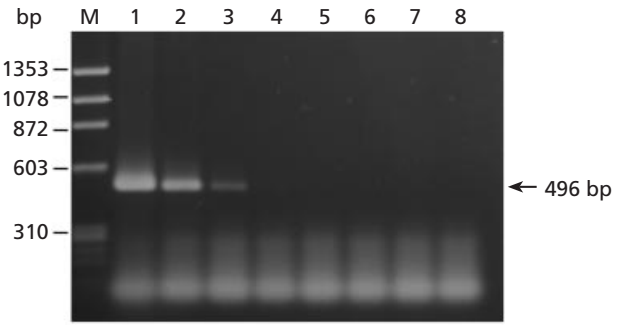

Fig. 2. Detection limit of *R. eutropha* by colony PCR with primer pair phaCF1-phaCR4 and *R. eutropha* cells as the DNA template. Lane M, molecular size marker (φX174 DNA/*Hae*III); lane 1, $1·1 \times 10^8$ c.f.u.; lane 2, $1·1 \times 10^7$ c.f.u.; lane 3, $1·1 \times 10^6$ c.f.u.; lane 4, $1·1 \times 10^5$ c.f.u.; lane 5, $1·1 \times 10^4$ c.f.u.; lane 6, $1·1 \times 10^3$ c.f.u.; lane 7, $1·1 \times 10^2$ c.f.u.; lane 8, $1·1 \times 10$ c.f.u.

Optimization of colony PCR

To establish a rapid screening protocol, a colony PCR approach with degenerate primers, phaCF1 and phaCR4, was employed. The predicted product, amplified from *R. eutropha*, was 496 bp. In each PCR experiment, 50 µl of *R. eutropha* overnight culture directly served as the DNA template following centrifuging and washing once with sterilized deionized water. In the preliminary test, no PCR product was amplified without the help of PCR additives (data not shown). However, a very specific DNA fragment, ~ 500 bp, was amplified when a final concentration of 2 % DMSO was added to the PCR mixture. Moreover, the PCR product was enriched (~ 1·9-fold) when DMSO was replaced by 1 M betaine. Finally, the optimized condition was achieved by combining 3 % DMSO and 1 M betaine in the PCR mixture to obtain an approximately 2·2-fold enrichment (data not shown) as compared to the result obtained, when only DMSO was

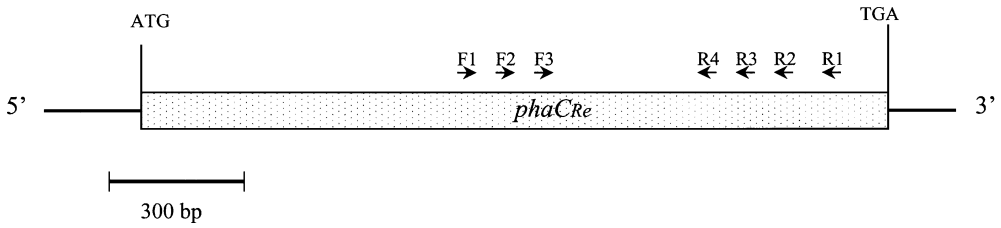

Fig. 1. Positions of primers on the *phaC* gene of *R. eutropha*. F1, phaCF1; F2, phaCF2; F3, phaCF3; R4, phaCR4; R3, phaCR3; R2, phaCR2; R1, phaCR1.

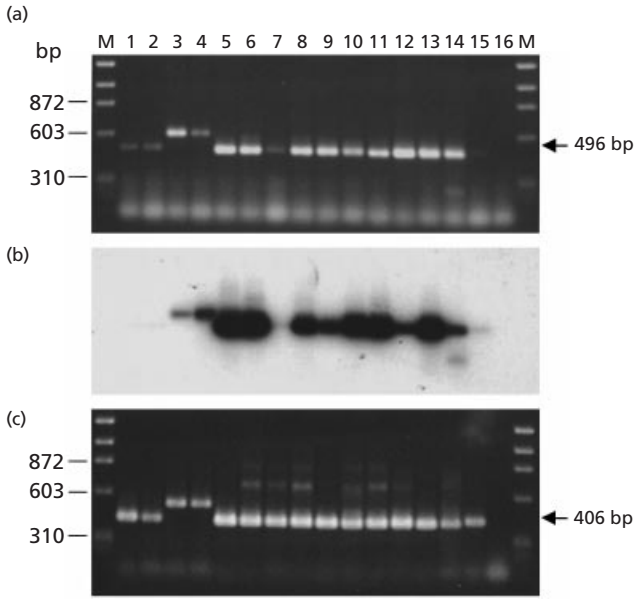

Fig. 3. Results of colony PCR amplification with primer pair phaCF1-phaCR4 among PHA-positive strains. (a) Agarose gel electrophoresis of colony PCR products. (b) Southern hybridization of the products probed with ^{32}P-labelled phaCF2. (c) Confirmation by semi-nested PCR. Lanes: M, molecular size marker (ϕX174 DNA/*Hae*III); 1, *R. eutropha*, 2, *A. hydrogenophilus*; 3, *A. latus*; 4, *C. acidovorans*; 5, *H. palleronii*; 6, *P. alcaligenes*; 7, *P. aureofaciens*; 8, *P. citronellolis*; 9, *P. fluorescens*; 10, *P. glathei*; 11, *P. mendocina*; 12, *P. oleovorans*; 13, *P. pseudalcaligenes*; 14, *P. putida*; 15, *S. natans*; 16, *E. coli* XL-1 Blue (negative control).

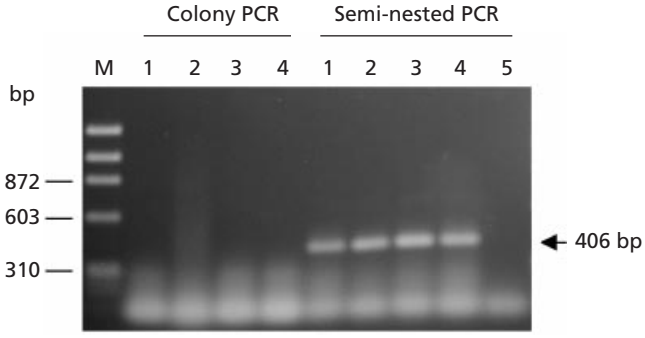

Fig. 4. Analysis of colony-PCR-negative products and their confirmation by semi-nested PCR. Primer-pair phaCF1-phaCR4 was used in colony PCR and phaCF2-phaCR4 was used in semi-nested PCR. Lanes: M, molecular size marker (ϕX174 DNA/*Hae*III); 1, *H. pseudoflava*; 2, *A. chroococcum*; 3, *A. vinelandii*; 4, *B. megaterium*; 5, *E. coli* XL-1 Blue (negative control).

added. The results clearly demonstrated that a colony-PCR-based approach with these degenerate primers could specifically amplify the DNA fragment from the PHA synthase gene without additional chromosomal DNA extraction procedures. The detailed PCR thermal program and reaction mixture composition are described in Methods.

Sensitivity of colony PCR

A dilution series of *R. eutropha* cells was prepared as DNA templates for testing the sensitivity of the colony PCR. The detection limit of the colony PCR was approximately 1×10^5 viable cells (Fig. 2). On the basis of the results, colonies approximately 1 mm in diameter, containing $\sim 1 \times 10^7$ viable cells, were picked up as DNA templates for colony PCR in the following experiments.

Evaluation of the detection efficiency of colony PCR

Nineteen PHA-positive bacteria, 18 Gram-negative and one Gram-positive, as well as one PHA-negative strain (*E. coli* XL-1 Blue) were used to evaluate the detection efficiency of the colony PCR. Among these bacterial strains, 15 were detected by colony PCR and five (*E. coli* XL-1 Blue, *H. pseudoflava*, *A. chroococcum*, *A. vinelandii* and *B. megaterium*) were not detected. The 15 detected strains belonged to six genera: *Alcaligenes*, *Comamonas*, *Hydrogenophaga*, *Pseudomonas*, *Ralstonia* and *Sphaerotilus*. Furthermore, two types of PHA synthases, type I (e.g. PHA synthase of *R. eutropha*) and type II (e.g. PHA synthase of *P. oleovorans*), were simultaneously detected by the same primer pair. When the semi-nested PCR technique was combined with colony PCR (Figs 3 and 4) all 19 PHA-positive strains were detected; the negative control *E. coli* XL-1 Blue did not produce a signal following semi-nested PCR (Fig. 4, lane 5). The detection experiments were repeated three times with the same results, confirming reproducibility. The size of the PCR products consistently matched the predicted results except for *A. latus* and *C. acidovorans*, from which larger fragments were amplified (Fig. 3, lanes 3 and 4).

Confirmation of colony PCR products by Southern hybridization and semi-nested PCR

Southern hybridization was carried out for further confirmation of the identity of the PCR products with a degenerate probe, phaCF2, targeting the internal regions (Fig. 1). Most of the colony-PCR-positive products were detected at high stringency except for four products amplified from *R. eutropha* (Fig. 3, lane 1), *A. hydrogenophilus* (Fig. 3, lane 2), *P. aureofaciens* (Fig. 3, lane 7) and *S. natans* (Fig. 3, lane 15). At low stringency (without formamide), the above four products possessed obvious signals (Fig. 5), except that *S. natans* needed a longer exposure time. No hybridization signals were observed among the colony-PCR-negative products even at low stringency (data not shown). The results suggested that the phaCF2 probe is capable of being a universal probe to confirm the identity of the colony PCR products. However, the sensitivity was not satisfactory, perhaps due to the highly degenerate probe used. Therefore, a very sensitive method, semi-nested PCR (Zhang *et al.*, 1994), employing the primer pair phaCF2-phaCR4, was developed as a fast method for

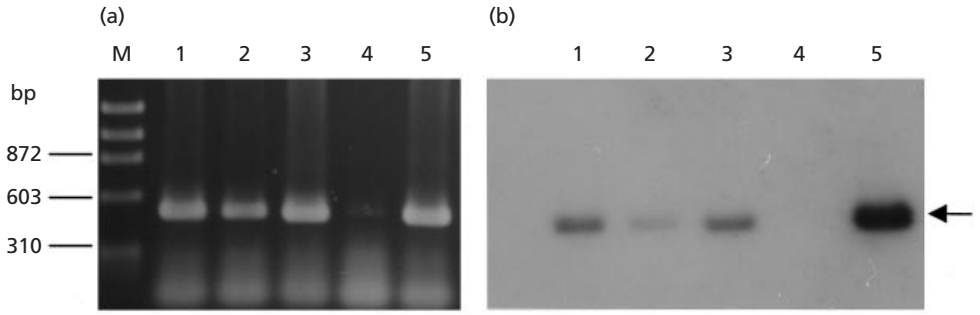

Fig. 5. Southern hybridization of phaCF1-phaCR4 colony PCR products under low-stringency hybridization conditions. (a) Agarose gel electrophoresis. (b) Southern hybridization analysis with low hybridization stringency. Lanes M, molecular size marker (ϕX174 DNA/*Hae*III); 1, *Ralstonia eutropha*; 2, *Alcaligenes hydrogenophilus*; 3, *Pseudomonas aureofaciens*; 4, *Sphaerotilus natans*; 5, *Hydrogenophaga palleronii.*

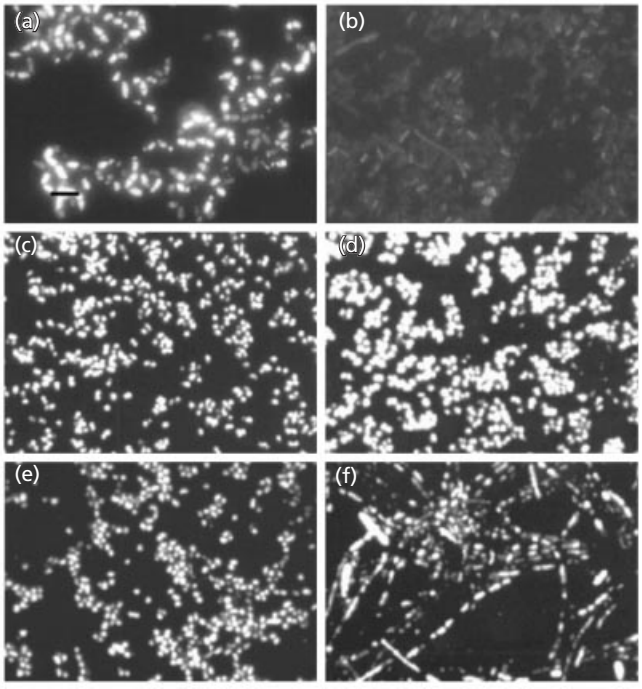

Fig. 6. Fluorescence microscopy of Nile-blue-A-stained cells grown in nitrogen-limited medium for 3 d. (a) Positive control, *Hydrogenophaga palleronii*. (b) Negative control, *E. coli* XL-1 Blue. (c), (d), (e) and (f) are environmental isolates CS5, GS28, GG45 and GG47, respectively. Bar, 5 μm.

further confirmation of the PCR products. From the results in Fig. 3, DNA fragments of the predicted size (406 bp) could all be amplified from colony PCR products by semi-nested PCR, even from the four PHA-positive strains that did not produce a colony PCR product (Fig. 4). This result suggested that colony-PCR-negative products still contain trace amounts of specifically amplified DNA fragments, which could not be detected by ethidium bromide staining. By semi-nested PCR, the unobserved signal was amplified and detected again. Moreover, the Gram-positive and PHA-positive *B. megaterium* strain could also be detected by semi-nested PCR following colony PCR (Fig. 4, lane 4). This suggested that the primers derived from *phaC* genes of Gram-negative bacteria may also be efficient for the detection of Gram-positive PHA producers. In the 19 semi-nested PCR products, two larger-than-predicted PCR products were amplified from *A. latus* and *C. acidovorans* (Fig. 3, lanes 3 and 4). This phenomenon is the same as that seen with colony PCR.

Screening of PHA producers from the environment by colony PCR and semi-nested PCR

A total of 38 PHA-positive strains was isolated from environmental samples by colony PCR and semi-nested PCR. Thirty of these isolates were Gram-negative, five were Gram-positive and three were Gram-variable. The results suggest that the degenerate primers were not only specific for *phaC* genes from Gram-negative but also for those from Gram-positive bacteria. The phenotypes of PHA-genotype-positive isolates were further confirmed by Nile blue A staining (Fig. 6). The staining assay revealed that 33 of the 38 genotype-positive isolates possessed significant PHA accumulation ability; the remaining five strains were not confirmed by Nile blue A staining.

DISCUSSION

G+C content analysis of 13 PHA synthase (*phaC*) genes, all derived from Gram-negative bacteria, revealed that these genes possess relatively high G+C contents, at approximately 61–68 mol%, except for *phaC* from *Acinetobacter* sp. (38·8 %). Part of the PCR amplicon amplified from *R. eutropha* even possesses up to 89 mol% G+C (50 GC bases in a 56-base length). DNA templates with a high G+C content usually hamper PCR amplification; the reagents formamide, glycerol, DMSO and betaine often serve as PCR additives to overcome these problems (Dieffenbach & Dveksler, 1995; Henke *et al.*, 1997). In this study, different concentrations of the above PCR additives were tried; however, only DMSO and betaine were helpful (data

not shown) in amplifying a correct-sized DNA fragment from the *phaC* gene. Six thermostable DNA polymerases, Taq (Promega), TaKaRa Taq (TaKaRa), KlenTaq1 (Ab Peptides), DyNAzyme II DNA polymerase (Finnzymes), $Vent_R$ DNA polymerase (New England BioLabs) and Supertherm DNA polymerase (LPI), were also evaluated for their ability to amplify the high-G+C template in the process of colony PCR detection. The Supertherm DNA polymerase showed the best detection results. Regarding the detection limits, the sensitivity of colony PCR is low, which might be due to the high degeneracy of the primers as well as to the low purity of chromosomal DNA. However, by omitting the tedious chromosomal DNA extraction procedures, a large number of samples can rapidly be screened at the same time. Another merit of PCR detection is that the PHA producers can be detected when grown on nutrient medium and no PHA granules have accumulated.

The sizes of products obtained by colony PCR and semi-nested PCR were consistent with the predicted results except those of *A. latus* and *C. acidovorans*, for which sizes were larger than predicted (Fig. 3, lanes 3 and 4). Recently, the *phaC* genes of *A. latus* (Choi *et al*., 1998) and *C. acidovorans* (Sudesh *et al*., 1998) have been cloned and sequenced. Aligning the primer positions, the corresponding products, 593 and 518 bp, would be amplified from *A. latus* by primer pairs phaCF1-phaR4 and phaCF2-phaCR4, respectively. In *C. acidovorans*, the corresponding products were 621 and 555 bp by the above primer pairs. The predictive products were larger than the former; however, they obviously matched our agarose gel analysis results.

Thirty-eight PHA-positive strains isolated from the environment by PCR were further confirmed by Nile blue A staining assay. Among these isolates, 87% (33/38) possessed significant PHA accumulation ability, which strongly supports the accuracy of the genotype screening results. However, there are still five isolates (13%) for which no PHA granules were detected by Nile blue A staining. This outcome may be due to an inappropriate carbon source used in the culture medium or to a low yield of PHA granule accumulation resulting in negative detection by Nile blue A staining. These results also reflected the possibility that there may be some bacterial isolates that harbour a non-functional PHA synthase gene. The PHA-positive strains identified included five Gram-positive and five Gram-variable strains. This suggested that the colony PCR protocol is also applicable for detecting Gram-positive PHA producers.

It is often difficult to carry out gene cloning of PHA operons due to the lack of a universal probe. The degenerate primers used in this study were capable of amplifying a partial DNA fragment of *phaC* from all 19 reference strains, including strains from which *pha* operons have not yet been cloned (e.g. *A. vinelandii* and *A. chroococcum*). In addition, these individual PCR products are very suitable for use as specific probes for genomic library screening. The PCR protocol can also be regarded as a universal method to prepare a PHA-synthase-specific probe from individual strains for gene cloning. Alternatively, genomic library screening can also be rapidly achieved by using colony PCR directly. The colony PCR technique proposed in this study will significantly accelerate the discovery of new PHA operons.

ACKNOWLEDGEMENTS

This study was partially supported by grant 88-EC-2-A-17-0235 from the Development Center for Biotechnology, Taipei, Taiwan. We wish to thank Associate Professor I-Ceng Tseng (Department of Biology, National Cheng Kung University, Tainan, Taiwan) for kindly providing active-sludge samples and Professor Liang-Ping Lin (Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan) for providing strains of *Azotobacter vinelandii* and *Azotobacter chroococcum*.

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Received 3 February 2000; revised 24 April 2000; accepted 12 May 2000.