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SHORT CONTRIBUTION

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Design of PCR primers and a gene probe for extensive detection of poly(3-hydroxybutyrate) (PHB)-degrading bacteria possessing fibronectin type III linker type-PHB depolymerases

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Abstract For rapid and sensitive detection of poly(3-hydroxybutyrate) (PHB)-degrading bacteria, a PCR primer set (PHB primers) and a gene probe (PHB probe) were designed, based on the homologous regions of six fibronectin type III linker domain-encoding sequences laid on a variety of PHB depolymerase genes listed in the GenBank. PCR using PHB primers amplified DNA fragments with the expected sizes from all the tested bacterial strains used for primer design; and all of the amplified fragments gave positive signals by Southern hybridization with the PHB probe. No amplified fragments were observed from negative controls. To evaluate the availability of the PHB primers and PHB probe, they were applied to 57 wild-type, PHB-degrading bacteria newly isolated from a variety of environments. The PHB primers amplified DNA fragments with expected sizes from 50 of the 57 wild-type strains, while the PHB probe showed positive signals against the amplified fragments from 47 strains. These results suggest that the primer and probe system established in this study can detect a considerable proportion of the potential PHB-degrading bacteria and can be applied to evaluate PHB-degradation potential in a natural environment, in combination with direct DNA extraction methods.

Introduction

Poly(3-hydroxybutyrate) (PHB) is a carbon and energy reserve accumulated by several kinds of bacteria under conditions of nutrient stress, e.g. when an external carbon source is available but the concentration of nutrients such as nitrogen, phosphorus, or oxygen are limiting growth (Oeding and Schlegel 1973; Senior and Dawes 1973). Recently, PHB has attracted great interest, being one of the useful biodegradable plastics which are not derived from petroleum; and the co-polymer of PHB and poly(3-hydroxycalerate), which is more flexible than the homopolymer of PHB (Slater et al. 1999), has been commercially produced as Biopol in a large-scale fermentation process (Holmes 1985). Its agricultural and marine applications have been particularly emphasized (Holmes 1985). The level of production and consumption of environmentally friendly, biodegradable plastics, including PHB and related co-polymers, is expected to increase and, consequently, their emission into the natural environment is estimated to increase. Therefore, it is necessary to know the biodegradation potential of PHB in the natural environment to assess the effects of their discard. In order to evaluate the PHB-degradation potential, it is important to detect, enumerate, and/or monitor the behavior of PHB-degrading microbes.

This study aims to develop a molecular technique for the extensive detection and monitoring of PHB-degrading bacteria. A PCR primer set which can detect a variety of PHB depolymerase genes and a DNA probe for the specific identification of the PCR products were designed from relatively highly homologous regions on various gene sequences encoding PHB depolymerases containing the fibronectin type III (Fn3) linker domain. Their applicability was investigated by applying them to both known and newly isolated PHB-degrading bacteria.

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Materials and methods

Bacterial strains and growth conditions

Among the authentic bacterial strains known to possess well characterized PHB depolymerase genes listed in GenBank (Keller et al. 1984; Table 1), four bacteria (written in bold type in Table 1) were kindly provided to us by the researchers who isolated the strains. These authentic bacterial strains were used for experimen-

Table 1 Bacterial strains possessing known PHB depolymerase genes, with the primability and stability of the designed poly(3-hydroxybutyrate) (PHB) primers (*PHBf* and *PHBr*) and PHB probe (*PHBp*) against the corresponding genes. The *enzyme type* is the type of the PHB depolymerase. The strains and the PHB depolymerase genes used in the experimental investigation are indicated in *bold type*. – Scores could not be calculated because of the low homology with the corresponding primers and probe. *cadherin* Cadherin-like linker type, *Fn3* fibronectin type III linker type, *intracellular* intracellular PHB depolymerase, *threonine* threonine-rich linker type

Bacterial strain (gene name)	PHBf		PHBr		PHBp	Enzyme type	Accession number in GenBank
	Primability (%)	Stability (%)	Primability (%)	Stability (%)	Primability (%)		
Comamonas sp. (*phaZ*)	91	74	96	77	81	Fn3	**U16275**
Ralstonia pickettii T1 (*phaZ*)	92	78	99	74	94	Fn3	**J04223**
Streptomyces exfoliatus K10 (*phaZ*)	96	75	100	82	76	Fn3	**U58990**
Comamonas acidovorans YM1609 (*phaZ*)	87	73	98	69	81	Fn3	AB003186
Comamonas testosteroni YM1004 (*phaZ*)	91	74	96	77	81	Fn3	AB000508
Pseudomonas pickettii K1 (*phaZ*)	92	78	95	71	88	Fn3	D25315
Alcaligenes faecalis AE122 (*phaZ*)	–	–	–	–	–	Fn3	U55775
Pseudomonas lemoignei (*phaZ4*)	–	–	–	–	–	Fn3	**U12976**
Pseudomonas lemoignei (*phaZ1*)	–	–	–	–	–	Threonine	Z22595
Pseudomonas lemoignei (*phaZ2*)	–	–	–	–	–	Threonine	U68039
Pseudomonas lemoignei (*phaZ3*)	–	–	–	–	–	Threonine	U68170
Pseudomonas lemoignei (*phaZ5*)	–	–	–	–	–	Threonine	U12977
Pseudomonas stutzeri YM1006 (*phaZ*)	–	–	–	–	–	Cadherin	AB012225
Ralstonia eutropha H16 (*phaZ*)	–	–	–	–	–	Intracellular	AB017612

tally evaluating the specificity of the designed primer set and probe. Authentic bacterial strains not possessing PHB depolymerase genes, *Acinetobacter baumannii* ATCC19606, *A. calcoaceticus* ATCC23055, *A. haemolyticus* ATCC17906, *A. johnsonii* ATCC17909, *A. lwoffii* ATCC15309, *Bacillus megaterium* ATCC12872, *Escherichia coli* K12, *Flavobacterium breve* IFO 14943, *Moraxella bovis* ATCC10900, *Pseudomonas aeruginosa* IFO12689, *P. fluorescens* ATCC15553, *P. putida* IFO14164, *Staphylococcus epidermidis* GTC289, and *Vibrio campbellii* ATCC25920 served as negative controls for the PCR amplification. Additionally, a total of 57 wild-type, PHB-degrading bacteria were newly isolated from a variety of environmental samples from agricultural fields, aerobic and anaerobic landfill reactors (Ishigaki et al. 2000), and gardens, using a slightly modified mineral medium agar plate (Fujita and Ike 1997) containing 0.1% PHB as the sole carbon source (PHB medium). They were morphologically and physiologically characterized, classified, and tentatively identified, according to the diagnostic tables of bacteria proposed by Cowan and Steel (1974). Furthermore, all of the gram-negative bacteria, except for enterobacteria, were identified using API20NE (BioMerieux Japan). Their PHB-degrading ability was confirmed by detecting the clear zones around colonies due to hydrolysis of the water-insoluble polymer by extracellular depolymerases.

Design of the primer set and probe for extensive detection of PHB depolymerase genes

A total of 14 PHB depolymerase gene sequences were obtained from GenBank (Keller et al. 1984) and the sequences were subjected to multiple alignment, using CLUSTAL W (Eddy 1995). These calculations were done using the Sun Spark Station 1000 (Sun Microsystems, Tokyo) supported by Genome Information Research Center, Osaka University. Relatively highly homologous regions were selected to design a PCR primer set for extensive detection. The designed primers were applied to the PCR simulation software, Amplify (ver. 1.2, University of Wisconsin), to evaluate their primability and stability of binding to the target sequences, the anticipated amplified fragment sizes and the strength of amplification. A homology search of the database was performed using FASTA (Pearson and Lipman 1988) to investigate the specificity of the designed primers. An oligonucleotide probe was also designed from the relatively highly homologous regions lying within the fragments of expected sizes amplified by PCR. Primability with the target regions was also evaluated.

PCR amplification

A DNA template for each bacterial strain (grown overnight) was prepared by the methods previously described (Sei et al. 2000). PCR was conducted for 30 cycles, with denaturation at 94 °C for 60 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s, using the PC-800 temperature control system (Astec, Fukuoka, Japan). Aliquots (10 µl) of the PCR products were analyzed by electrophoresis on a 1.0% agarose gel stained with 0.5 µg ethidium bromide/ml.

Southern hybridization

The designed probe was labeled by digoxygenin-11 dUTP using the Dig oligonucleotide 3′-end-labeling kit (Boehringer Mannheim, Germany), according to the procedure described by the manufacturer, and was used for hybridization. Transfer was performed using a model 785 vacuum blotter (Bio-Rad, USA), as per the manufacturer's instructions. Detection of the digoxygenin-labeled probe was performed using the Dig DNA labeling and detection kit (Boehringer Mannheim, Germany), according to the instructions of manufacturer, except that the hybridization temperature was set at 37 °C.

Results

Design of the primer set for extensive detection of PHB depolymerase genes

Multiple alignment was performed against 14 PHB depolymerase gene sequences; and the sequences were confirmed to be considerably divergent (data not shown). However, three relatively highly homologous regions were found among six gene sequences encoding PHB depolymerase containing the Fn3 linker domain (Fn3 linker type-PHB depolymerase); and two regions which seemed to be suitable for designing a primer set were chosen to design the primer set for the extensive detection of the Fn3 linker type-PHB depolymerase genes. In principle, the dominant bases among the aligned, relatively highly homologous sequences were selected for the primer design, allowing mismatches against certain genes at positions of sequence divergence. However, nondominant bases were occasionally introduced instead of dominant ones. For example, in designing the forward primer (PHBf; Fig. 1), the dominant base at the sixth position from 3′ is A, but C was introduced instead, to improve the primability and stability of the primer for the PHB depolymerase gene sequence of *S. exfoliatus* K10. Such selection of a nondominant base was determined based on a simulation of the PCR reaction by Amplify to maximize the possibility of detecting all the gene sequences used for the primer design. The reverse primer (PHBr) was also designed on the same principle as for PHBf. The sequences of the designed primers are shown in Table 2.

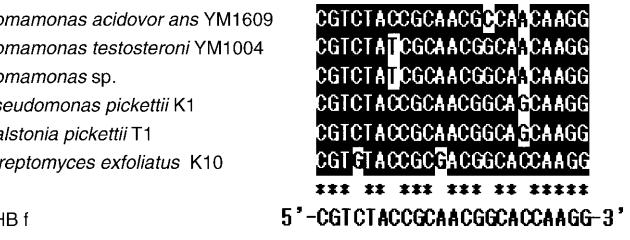

Fig. 1 Sequence alignment of the fibronectin type III (Fn3) linker type-poly(3-hydroxybutyrate) (PHB) depolymerase genes and design of the forward primer for the Fn3 linker type-PHB depolymerase genes (PHBf). *Inverted bases* match those of the primer. * 100% match

Simulation results of Amplify revealed that the primer set for the Fn3 linker type-PHB depolymerase genes (PHB primers) could amplify specific DNA fragments from all the PHB depolymerase genes used for the design. The primability and stability of the PHB primers for the target PHB depolymerase gene sequences are summarized in Table 1. The anticipated PCR product sizes were 218 bp from *Comamonas acidovorans* YM1609, 215 bp from *Comamonas* sp. and *C. testosteroni* YM1004, and 203 bp from other three strains.

Based on the homologous region in all six of the anticipated amplified fragments, a DNA probe for the Fn3 linker type-PHB depolymerase genes (PHB probe: PHBp) was also designed (Table 2) on the same principle as for the design of PHBf, except for employing dITP and multiple bases at the positions of several divergent bases. The primability of PHBp for the target gene sequences is also shown in Table 1.

The results of the FASTA analyses revealed that PHBf showed a homology to the chitinaseA gene of *Streptomyces lividas* (GenBank accession No. D13775), the proteinase gene of *S. albogriseolus* (No. D83672), the cosmid of *Saccaromyces pombe* (No. AL034433), and the cosmid of *Mycobacterium leprae* (No. Z99263), in addition to the genes related to the Fn3 linker type-PHB depolymerases, while PHBr and PHBp only showed homology with genes related to the Fn3 linker type-PHB depolymerase (data not shown).

Evaluation of the PHB primers and PHBp using authentic strains

To experimentally confirm the possibility of extensive and specific detection of the Fn3 linker type-PHB de-

Table 2 Sequences of the designed PHB primers and PHBp

Primers and probe	Sequences	Length (bp)	GC contents (%)
PHBf	5′-CGTCTACCGCAACGGCACCAAGG-3′	23	65.2
PHBr	5′-TGGGCGTAGTTGCTGGCCGT-3′	20	65.0
PHBp	5′-CTGATCICTG(G/C)CCACACCTACAGCTACACGGT-3′	32	56.3

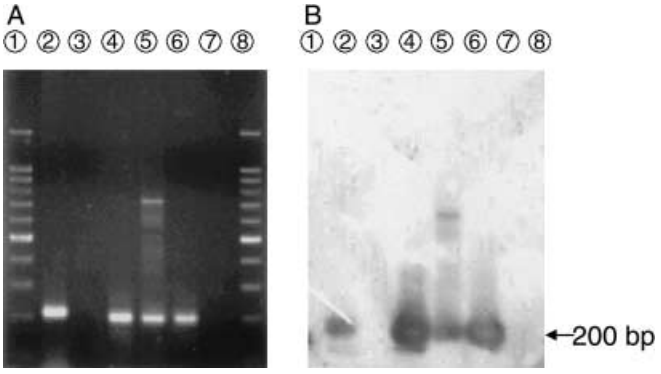

Fig. 2 **A** PCR amplification of the Fn3 linker type-PHB depolymerase genes from authentic strains with PHB primers; and **B** corresponding Southern hybridization with PHB probe for identification of the amplified fragments. *Lane 1* Molecular weight marker, *lane 2 Comamonas* sp., *lane 3 Pseudomonas lemoignei*, *lane 4 Ralstonia pickettii* T1, *lane 5 Streptomyces exfoliatus* K10, *lane 6* positive control (*R. pickettii* K1), *lane 7* negative control (no DNA template), *lane 8* molecular weight marker. Amplified fragments and positive signals of Southern hybridization corresponding to the calculated sizes were observed around 200 bp. Amplified fragment with larger size (*lane 5*) shows false-positive signal.

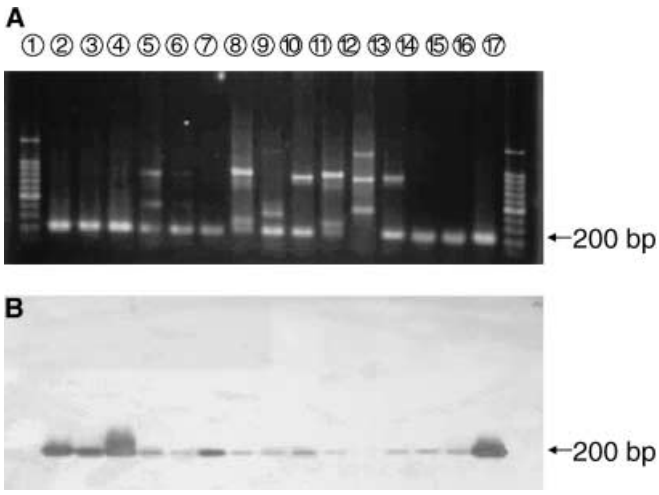

Fig. 3 **A** PCR amplification of the Fn3 linker type-PHB depolymerase genes from newly isolated strains with PHB primers; and **B** corresponding Southern hybridization with PHB probe for identification of the amplified fragments. *Lane 1* Molecular weight marker, *lanes 2–5* newly isolated strains from aerobic landfill reactor, *lanes 6–9* newly isolated strains from anaerobic landfill reactor, *lanes 10–12* newly isolated strains from agricultural field, *lanes 13–15* newly isolated strains from garden, *lane 16* positive control (*R. pickettii* K1), *lane 17* molecular weight marker. Amplified fragments and positive signals for Southern hybridization corresponding to the calculated sizes were observed around 200 bp. All amplified fragments with larger or smaller sizes (*lanes 4, 5, 7, 8–14*) were excluded by Southern hybridization with PHB probe

polymerase genes, PCR and Southern hybridization were conducted on four strains (*Comamonas* sp., *Pseudomonas lemoignei*, *Ralstonia pickettii* T1, and *Streptomyces exfoliatus* K10) possessing the Fn3 linker type-PHB depolymerase genes listed in GenBank. Fragments of the expected sizes were amplified from all the tested strains, except for *P. lemoignei*, which was not used for the primer and probe design (Fig. 2A). An additional larger fragment (710 bp) was also amplified from *S. exfoliatus* K10. No fragments were amplified from the strains that served as negative controls (data not shown).

Among the three amplified fragments of the expected sizes, all three showed positive signals by Southern hybridization with PHBp (Fig. 2B). The nonspecific, amplified fragment of larger size from *S. exfoliatus* K10 also showed a weakly positive signal by hybridization with PHBp.

Application of PHB primers and PHBp to wild-type, PHB-degrading bacteria

A total of 57 PHB-degrading bacteria surrounded by clear zones on the PHB medium were isolated from various environments and were taxonomically characterized. They were considered to belong to *Acinetobacter lwoffii*, *Burkholderia cepacia*, *Chrysemonas luteola*, *Orchrobactrum anthropi*, *Pseudomonas fluorescens*, *Ralstonia pickettii*, *Sphingomonas paucimobilis*, *Bacillus*, *Enterobacter*, *Kurthia*, *Rothia*, *Streptococcus*, and unknown

Table 3 Number of strains detected by PCR and Southern hybridization applied to the taxonomically identified PHB-degrading bacteria newly isolated from a variety of soil environments. The *PCR* column gives the number of strains which showed amplified fragments of expected size. The *PCR + hybridization* column gives the number of strains which showed amplified fragments of expected size by PCR and a positive signal by hybridization

Taxonomic group	Total	PCR	PCR + hybridization
Gram-positive bacteria	24	21 (87.5%)	18 (75.0%)
Bacillus	13	11 (84.6%)	10 (76.9%)
Kurthia	3	3 (100.0%)	3 (100.0%)
Rothia	1	1 (100.0%)	1 (100.0%)
Streptococcus	1	0 (0.0%)	0 (0.0%)
Unknown	6	6 (100.0%)	4 (66.7%)
Gram-negative bacteria	33	29 (87.9%)	29 (87.9%)
Ralstonia pickettii	10	9 (90.0%)	9 (90.0%)
Orchrobactrum anthropi	7	5 (71.4%)	5 (71.4%)
Acinetobacter lwoffii	1	1 (100.0%)	1 (100.0%)
Burkholderia cepacia	2	2 (100.0%)	2 (100.0%)
Enterobacter	1	1 (100.0%)	1 (100.0%)
Chrysemonas luteola	1	1 (100.0%)	1 (100.0%)
Pseudomonas fluorescens	1	1 (100.0%)	1 (100.0%)
Sphingomonas paucimobilis	1	1 (100.0%)	1 (100.0%)
Unknown	8	7 (87.5%)	7 (87.5%)
Total	57	50 (87.7%)	47 (82.5%)

categories (Table 3). The designed primer set and probe were applied to all the wild-type isolates. Among 57 PHB-degrading bacteria, fragments of the anticipated sizes were amplified from 50 strains (87.7%), using PHB primers. When PHBp was used for hybridization against the corresponding amplified fragments, 47 strains (82.5%) showed positive signals. Fragments larger or smaller than the anticipated sizes were amplified from 46 strains, although none of those fragments showed positive signals by hybridization with PHBp (Fig. 3). No significant interspecific detection bias was observed (Table 3).

Discussion

In this study, we attempted to design a set of PCR primers and a DNA probe which would be able to detect a wide range of PHB-degrading bacteria possessing Fn3 linker type-PHB depolymerases. Within the range of our reference search, there has been no report so far concerning primers and probes for the detection of PHB-degrading bacteria.

Our concept for the primer design was to detect as many kinds of PHB-degrading bacteria as possible. Therefore, all of the known PHB depolymerase genes listed in the GenBank were multiple-aligned. Though 14 PHB depolymerase gene sequences were listed in the GenBank as of June 1999, five of them were the isozymes possessed by *Pseudomonas lemoignei*; and therefore ten kinds of well characterized PHB-degrading bacteria were reported. Among them, eight possessed Fn3 linker type-PHB depolymerases. Since relatively highly homologous regions were found among six of the eight Fn3 linker type-PHB depolymerase gene sequences, the PHB primers were designed from the sequences of those regions. In contrast, all four of the threonine-rich linker type-PHB depolymerases were found only in *P. lemoignei*. Cadherin-like linker type and intracellular type-PHB depolymerases were found only in *P. stutzeri* YM1006 and *Ralstonia eutropha* H16, respectively. These PHB depolymerases possessed no obvious homology with Fn3 linker type-PHB depolymerase. Since a previous report suggested that most of the PHB-degrading bacteria in the natural environment possess Fn3 linker type-PHB depolymerases (Jendrossek 1998), the PHB primers and PHB probe, which were designed to be able to detect six of the eight previously reported Fn3 linker type-PHB depolymerase genes, seemed to have a possibility to detect a majority of PHB-degrading bacteria existing in the natural environment.

In order to experimentally confirm the specific detection of Fn3 linker type-PHB depolymerase genes, the designed primer set and probe were applied on authentic bacterial strains known to carry Fn3 linker type-PHB depolymerase genes. The PHB primers generated PCR products of the expected sizes from all the tested strains. It should be emphasized that even *Comamonas* sp. and *P. pickettii* K1, the PHB depolymerase genes of which exhibited very low primability and stability with PHB primers in the Amplify simulation (Table 1), yielded PCR products of the expected sizes. These results imply the primer set could detect all the PHB-degrading bacteria used for the primer design (see Table 1). In contrast, no PCR product was obtained from negative control strains. Although *P. lemoignei* has been known to possess a Fn3 linker type-PHB depolymerase gene (Jendrossek et al. 1995), its sequence was not taken into consideration for the primer and probe design; and therefore, it was regarded as a negative control strain, suggesting that the designed primers were specific enough to detect most of Fn3 linker type-PHB depolymerase genes. However, additional PCR products with larger sizes were amplified from *Streptomyces exfoliatus* K10 by the PHB primers, which implies that amplification was due to nonspecific binding of the primers. Therefore, Southern hybridization with PHBp was carried out to further specifically identify the amplified fragments; but this, however, failed to exclude the unexpected larger fragments from *S. exfoliatus* K10. A possible reason for this false-positive hybridization may be that there are additional homologous regions in the whole gene sequence of *S. exfoliatus* K10.

In total, 57 wild-type, PHB-degrading bacteria were isolated from a variety of environments for evaluating the designed primers and probe. Among 57 isolates, 24 strains were gram-positive bacteria including *Bacillus*, *Kurthia*, *Rothia*, and *Streptococcus* species, although most of the previously reported PHB-degrading bacteria were gram-negative. This indicates that there exists a variety of unknown gram-positive, PHB-degrading bacteria in the natural environment. Among 33 gram-negative, PHB-degrading bacteria, 24 strains were identified as pseudomonads. All 57 isolates were classified into at least 12 taxonomic groups (genera), indicating that the library included various types of PHB-degrading bacteria and that it was suitable for evaluating how extensively the designed primers and probe could detect PHB-degrading bacteria. PCR products of expected sizes were amplified from 50 isolates (87.7%) with the PHB primers; and the amplified fragments from 47 isolates (82.5%) were hybridized with PHBp. The designed primers and probe could detect over 80% of the wild-type, PHB-degrading bacteria randomly isolated from various environments. This indicates that most of the PHB-degrading bacteria possess Fn3 linker type-PHB depolymerases and that other enzymatic types (i.e., threonine-rich linker type and cadherin-like linker type) were the minority. The number of positive strains detected by hybridization was slightly smaller than that detected by PCR. This can probably be attributed to the lower homology between the probe and target regions, since the amplified fragments obtained by PCR were of the anticipated sizes. In 46 isolates, additional fragments larger or smaller than the anticipated sizes were amplified, although all of them were excluded by the Southern hybridization with PHBp. Thus, the designed probe seems consistently accurate for specifying the amplified

fragments. The reason for the amplification of nonspecific fragments from such a great number of the isolates might be attributed to the fact that the PHB primers were designed from gene sequences encoding the Fn3 linker domain. As the Fn3-type linkers were widely distributed among various bacterial enzymes (Little et al. 1994), possibly these nonspecific fragments could be derived from genes encoding other enzymes possessing the Fn3 linker domain. In contrast, no PCR products were generated using the PHB primers from seven strains (12.3%). The possible reasons for the failure of detection are considered to be as follows: (1) these isolates possess other types of PHB depolymerase (i.e., threonine rich linker type and cadherin-like linker type-PHB depolymerases), or (2) although these isolates possess Fn3 linker type-PHB depolymerase genes, these had lower homology with the designed PHB primers.

Although the detection of PHB depolymerase genes only showed the potential for PHB-degrading ability, it may be concluded that the primer and probe system established here is useful for detecting and/or monitoring the majority of PHB-degrading bacteria existing in the natural environment, in combination with direct DNA extraction methods from a variety of environmental samples (Sei et al. 2000). Application of the designed primers and probe can provide quantitative information not only by most-probable-number PCR but also by in situ PCR (Degrange and Bardin 1995; Hodson et al. 1995), or fluorescent in situ hybridization (DeLong et al. 1989). PCR-denaturing gradient gel electrophoresis (Muyzer et al. 1993; Iwamoto et al. 2000) can then give qualitative information, such as the dominant species among the detected PHB-degrading bacteria. Accurate evaluation of the PHB-degrading activity of environmental samples may be also possible using reverse transcriptase-PCR (Bogan et al. 1996) techniques with the designed primers and probe.

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