Development of PCRs for Monitoring Specific Bacteria Related to Biodegradation of Environmental Pollutants

(環境汚染物質の分解菌をモニタリングするPCR法の開発)

2000

Kazunari Sei

清　和成

Development of PCRs for Monitoring Specific Bacteria Related to Biodegradation of Environmental Pollutants

(環境汚染物質の分解菌をモニタリングするPCR法の開発)

A Thesis
Submitted to the Graduate School of Engineering
at
Osaka University

by
Kazunari Sei

in a fulfillment of the requirements for
the degree
of
Doctor of Philosophy
in
Engineering

2000

Dedicated to

my parents; my parents-in-law; and my wife Mio.

I am truly grateful for

their constant supports, cordial encouragement, and love.

Acknowledgement

First of all, I would like to express my sincerest appreciation to Professor Dr. Masanori Fujita, Department of Environmental Engineering, Graduate School of Engineering, Osaka University, for his kindest guidance and encouragement during execution of this work. He kindly provided me many opportunities to have significant experiences not only as a researcher in environmental engineering field but also as a person going out into the world.

I wish to express my sincerest gratitude to Professor Dr. Suteaki Shioya and Professor Dr. Satoshi Harashima, Department of Biotechnology, Graduate School of Engineering, Osaka University for reviewing this study and providing me valuable advises and comments.

I must express my appreciation to Professor Dr. Kenji Furukawa, Department of Civil and Environmental Engineering, Faculty of Engineering, Kumamoto University and Associate Professor Dr. Keisuke Iwahori, Institute for Environmental Sciences, The University of Shizuoka, for their helpful information and constant encouragement.

I would like to express my special gratitude to Associate Professor Dr. Michihiko Ike, Department of Environmental Engineering, Graduate School of Engineering, Osaka University, for constantly providing me valuable advises and suggestive comments, and reviewing this study. Without his patient instructions and appropriate guidance, this work would not be progressed.

I am much obliged to Professor Dr. Masao Nasu, Associate Professor Dr. Katsuji Tani, and Assistant Professor Dr. Nobuyasu Yamaguchi, Department of Pharmaceutical Science, Graduate School of Pharmacy, Osaka University, and Mr. Masahiro Suzuki, Toyobo Co. Ltd., for their helpful information and stimulating discussions. Those were much important for my basic knowledge and experimental skills on this work.

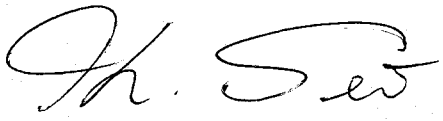
I am much grateful to Associate Professor Dr. Tetsuro Kohno and Associate Professor Dr. Kazuhiro Mori, Department of Civil and Environmental Engineering, Yamanashi University, for their helpful information and continuous encouragements. Without their sympathy, I could not complete this work.

Assistant Professor Dr. Masafumi Tateda, Technical official Ms. Yuriko Yamaoka and Ms. Tomoko Tago, Department of Environmental Engineering, Graduate School of Engineering, Osaka University, are much appreciated. Their thoughtful supports and encouragement throughout the days of study are essential for me to execute this study.

I also thank to Professor Dr. Ananda M. Chakrabarty, Department of Microbiology and Immunology, University of Illinois, Professor Dr. Kensuke Furukawa, Department of Bioscience and Biotechnology, Kyushu University, Professor Dr. Franz Lingens and Dr. Jürgen Eberspächer, Institute of Microbiology, University of Hohemheim, Professor Dr. Yu-Min Mao, Institute of Genetics, Fudan University, Professor Emeritus Dr. Teruko Nakazawa, Department of Microbiology, Yamaguchi University, Professor Dr. Toshio Ohmori, Biotechnology Research Center, University of Tokyo, Professor Emeritus Dr. Ronald H. Olsen, Department of Microbiology and Immunology, University of Michigan, Professor Dr. L. Nicholas Ornston, Department of Biology, Yale University, Professor Dr. Terumi Saito, Department of Biological Science, Kanagawa University, Professor Dr. Peter A. Williams, School of Biological Science, University of Wales, Dr. Dieter Jendrossek, Institute of Microbiology and Genetics, Georg-August-University, Dr. Ute Lechner, Institute of Microbiology, Martin Luther University, Associate Professor Dr. Maia Kivisaar, Department of Genetics, Tartu University, Associate Professor Dr. Ellen L. Neidle, Department of Microbiology, The University of Georgia, Assistant Professor Dr. Jerome J. Kukor, Department of Environmental Science, Rutgers University, Assistant Professor Dr. Chieko Nakai, Department of Biochemistry, Shiga University of Medical Science, Assistant Professor Dr. Masahiro Takeo, Department of Applied Chemistry, Himeji Institute of Technology, Dr. Atsushi Kitayama, National Institute of Basic Biology, Okazaki National Research Institutes, for kindly providing me bacterial strains and related information used in this study.

I heartily thank to Mr. Ken-ichiro Asano, Kubota Co. Ltd., Mr. Naohiro Tateishi, Ebara Co. Ltd., and Mr. Katsushi Wada and Mr. Morio Nakao, Department of Environmental Engineering, Graduate School of Engineering, Osaka University, for their continuous supports to hard and time-consuming experiments. This work could not be completed without their great cooperation.

Last but not least, I thank to Dr. Tae-Ho Lee, National Institute of Bioscience and Human Technology, Assistant Professor Dr. Satoshi Soda, Department of Global Architecture, Graduate School of Engineering, Osaka University, and JSPS PD Fellow Dr. Tomonori Ishigaki, Ms. Bandunee C. Liyanage, and all members of the Laboratory of Water Science and Environmental Biotechnology, Department of Environmental Engineering, Graduate School of Engineering, Osaka University. The days with them encouraged and improved me so much.



Kazunari SEI

TABLE OF CONTENTS

CHAPTER 1

General Introduction

Microbial monitoring Degradation of the pollutants released into environment is generally performed by specific and only a part of microorganisms in the environment. Therefore understanding the abundance and behavior of the specific bacteria responsible for degradation or removal of target pollutants is essential for evaluating the biodegradation potential in environment. Detection of specific bacteria has been traditionally performed by cultivation methods based on their phenotypes, such as detection by plate culture with selective media, enumeration by most probable number (MPN) method, and identification by investigating physiological characteristics of each isolate. However, it has come to be known that a large fraction, often up to 90-99 %, of microbial populations in environmental samples are viable but non-culturable and will, therefore, escape from the detection if cultivation-based methods are applied (Atlas and Bartha, 1987), nevertheless they must play important roles in the natural ecosystems. Furthermore, such methods require quite laborious and time-consuming processes.

To overcome these problems, molecular detection methods based on the genotypes such as DNA-DNA hybridization (Southern hybridization) (Southern, 1975), polymerase chain reaction (PCR) (Saiki *et al.*, 1985), fluorescent in situ hybridization (FISH) (DeLong *et al.*, 1989), and flow cytometry analysis of bacterial cells stained with DNA-specific fluorochromes (Amann *et al.*, 1990) have been recently developed.

PCR and FISH Among several molecular detection methods, PCR and FISH are the most frequently employed for microbial monitoring.

PCR is a technique to amplify the specific gene sequences using specific oligonucleotide primers. PCR primers targeting strain specific sequences of 16S rDNA or recombinant DNAs allowed to detect and/or monitor the selected or genetically engineered microorganisms (Steffan

and Atlas, 1988; Van Elsas *et al.*, 1991), the particular genes disseminated into indigenous microbes by gene transfer (Ravatn *et al.*, 1998), the genotypic diversity (Torsvik *et al.*, 1990), and the change of populational diversity in microbial ecosystems (Watanabe *et al.*, 1998). The advantage of this technique is its extremely high sensitivity and specificity for the detection of the target gene sequences. However, DNA extraction and recovery bias (Ogram *et al.*, 1987), amplification bias (Suzuki and Giovannoni, 1996), and poor quantitative ability inherent to amplification itself (Felske *et al.*, 1998) are the problems remaining to be solved.

FISH is a technique to hybridize the fluorescently labeled oligonucleotides to fixed microbial cells in situ. 16S rDNA sequences targeted probes allowed to detect bacterial cells (groups) responsible for metabolism of specific substrate (Amann *et al.*, 1992; Wagner *et al.*, 1995), to analyze phylogenetic structure (Amann *et al.*, 1995), and metabolic interactions between community members (Møller *et al.*, 1998). Combination with laser confocal microscopy and microelectrodes gave a three-dimensional picture of microbial associations (Møller *et al.*, 1996) and evidences of the metabolic reactions on a scale relevant for the study of stratified bacterial communities (Revsbech, 1994). The advantages of this technique are needless to extract DNA, easy to enumerate the target microbes, and easy to grasp the spatial distribution of the target microbes in the complex microbial community. However, species- and physiological condition-dependent probe permeability and detection sensitivity tightly related to the number of target molecules are the serious problems when FISH is applied to analyze environmental samples containing complex microbial community and to detect functional genes existing at a few copies in the bacterial cells (Yamaguchi and Nasu, 1997). Therefore, FISH may not be suitable for detecting and/or monitoring specific genes responsible for the degradation of the target pollutants.

This study focuses on PCR in order to achieve extremely sensitive detection and/or monitoring of specific bacteria responsible for the degradation of the target environmental pollutants. A typical schematic flow of the monitoring of specific bacterial populations in the

environment by applying PCR is illustrated as shown in Fig. 1.1. It consists of 3 main steps, (i) DNA extraction from environmental samples, (ii) design of the PCR primers and PCR amplification for the target microbes and/or genes, and (iii) enumeration of the target genes detected by PCR. The followings are literature reviews on each step.

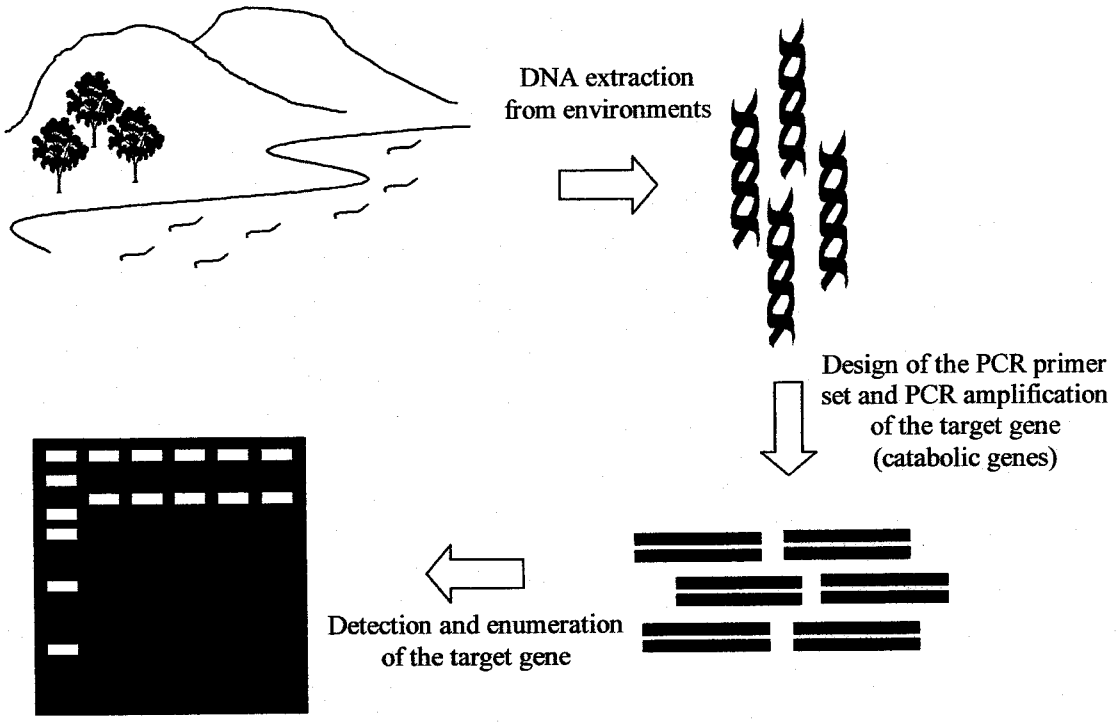


Fig. 1.1 A typical schematic flow of the PCR-based monitoring of bacterial populations responsible for biodegradation

DNA extraction from environmental samples Myriads of methods for extracting DNA from environmental samples have been already developed by many researchers up to date (Ogram *et al.*, 1987; Wilson, 1987; Steffan *et al.*, 1988; Sambrook *et al.*, 1989; Boom *et al.*, 1990; Tsai and Olson, 1991; Van Elsas *et al.*, 1991; Liesack and Stackebrandt, 1992; Picard *et al.*, 1992; Tsai and Olson, 1992; Muyzer *et al.*, 1993; Smalla *et al.*, 1993; Sparagano, 1993; Leff *et al.*, 1995; Volossiouk *et al.*, 1995; Zhou *et al.*, 1996). However, each method has been tested on a limited number of samples and/or developed for case-by-case or specialized purposes. For

example, the DNA extraction method developed by Sparagano (1993) was suitable for river water samples but not for the samples containing sediments, while the method of Volossiouk *et al.* (1995) was suitable for typical farm soils but not for soils containing large amounts of clay. Although the method of Ogram *et al.* (1987) showed a high recovery of DNA from river sediments, the DNA extracts were not suitable for PCR amplification (Leff *et al.*, 1995; Zhou *et al.*, 1996). On the other hand, the method of Zhou *et al.* (1996) extracted DNA suitable for PCR from soil samples, however, the DNA recovery was considerably lower. Further some of the methods need time-consuming, labor-full and/or complicated procedures, therefore, they are not available for routine and/or intensive uses. Consequently, the DNA extraction methods applicable to a wide variety of the environmental samples and/or purposes have not been established yet.

Design of the PCR primers for the detection of functional catabolic genes Many reports have described the monitoring of specific bacterial strains, introduced into various environment for the purpose of enhancing the bioremediation efficiency, by PCR amplification of genus-, species-, or strain-specific 16S rDNA sequences (Steffan and Atlas, 1988; Neilson *et al.*, 1994; Matheson *et al.*, 1997). However, genus-, species-, or strain-specific detection and/or monitoring results based on their 16S rDNA sequences only represent the phylogenetic classification-based information of the bacterial community. Therefore, 16S rDNA based-detection could not exhibit the specific catabolic functions of the bacterial populations, except for the group of autotrophic ammonium-oxidizing bacteria within the β-subclass of the class *Proteobacteria*, which consists of *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus*, where the detection result of this group would represent the ammonium-oxidizing potential (Voytek and Ward, 1995).

On the other hand, the detection of functional catabolic genes for evaluating the biodegradation potentials of the indigenous bacterial populations at the remediation sites or polluted environments has been reported much less often (Erb and Wagner-Döbler, 1993; Joshi

and Walia, 1996a; Chandler and Brockman, 1996; Wikström *et al.*, 1996; Braker *et al.*, 1998). Further, most of the PCR primers designed in those studies intended to cover only a limited proportion of the bacterial populations which possess the target functions, therefore, it may be said that the study on the design of PCR primers for the extensive detection of specific catabolic functions have been scarcely reported.

Enumeration of the target genes detected by PCR For the enumeration of the target genes detected by PCR, internal standard method (Wang *et al.*, 1989), competitive PCR method (Gilliland *et al.*, 1990), and MPN-PCR method (Picard *et al.*, 1992) have been developed.

Internal standard method is performed by the coamplification of the internal standard of the known amount and the sample DNA in one reaction. The amount of the target DNA is quantified by extrapolating against the standard curve generated with the internal standard.

Competitive PCR method is performed by the addition of increasing amounts of known competitor to a fixed amount of the sample DNA to be quantified, and the ratio between the final amplification products for the two is evaluated for each point. From this ratio, which precisely reflects the ratio between the initial amounts of the two templates, the amount of the target DNA can be evaluated. In these methods, since the internal standard or competitor and the target DNA are coamplified within the same tube and share the same primer recognition sites, they compete for amplification. It is considered that any variable affecting amplification has the same effect on both and, therefore, that reliable quantification can be obtained. However, differences in amplification efficiency between the primer pairs for the internal standard or competitor and the target DNA can cause the unreliable quantification result (Raeymaekers, 1995). Furthermore, the construction of internal standard or competitor is the problem which can be often a tedious and long work of mutagenesis and cloning.

MPN-PCR method is performed by the serial dilution of the sample DNA and their amplification in multiplicate. The number of the amplifiable target DNA sequences was determined according to the most-probable-number (MPN) technique. In this method, as the

sample DNA is serially diluted, the reduction of the effects of the PCR inhibitors such as humic substrates are expected. However, this method requires PCR analysis of the great number of samples.

As mentioned above, each DNA enumeration method has both merits and demerits, and therefore, the generalized method applicable for monitoring of specific bacterial populations in natural environment has not been established yet.

Objective of this study This study aims to develop the PCR-based specific microbial detection and/or monitoring method for evaluating biodegradation potentials against target pollutants in the environment. For this purpose, novel attempts were made to "extensively" detect the functional catabolic genes responsible for the degradation of the target pollutants, different from previous microbial detection and/or monitoring based on 16S rDNA or only a limited portion of the functional catabolic genes. The schematic flow of this study is shown in Fig. 1.2. In Chapter 2, DNA extraction methods from various water and soil environmental samples were investigated so as to establish the methods which can give high purity and yields of extracted DNA applicable to PCR analyses. In Chapter 3, PCR primers and gene probe for the extensive detection of poly(3-hydroxybutyrate) (PHB)-degrading bacteria were designed and applied for the enumeration of the PHB-degrading bacteria in soil samples. In Chapter 4, PCR primers and gene probes for the extensive detection of aromatic compounds-degrading bacteria were designed and applied for monitoring aromatic compounds-degrading bacteria in several kinds of water environmental microcosms. In Chapter 5, finally, applicability and limitation of the PCR-based specific microbial detection method as a tool for monitoring specific bacteria responsible for the degradation of target pollutants are discussed with the main conclusions obtained in this thesis.

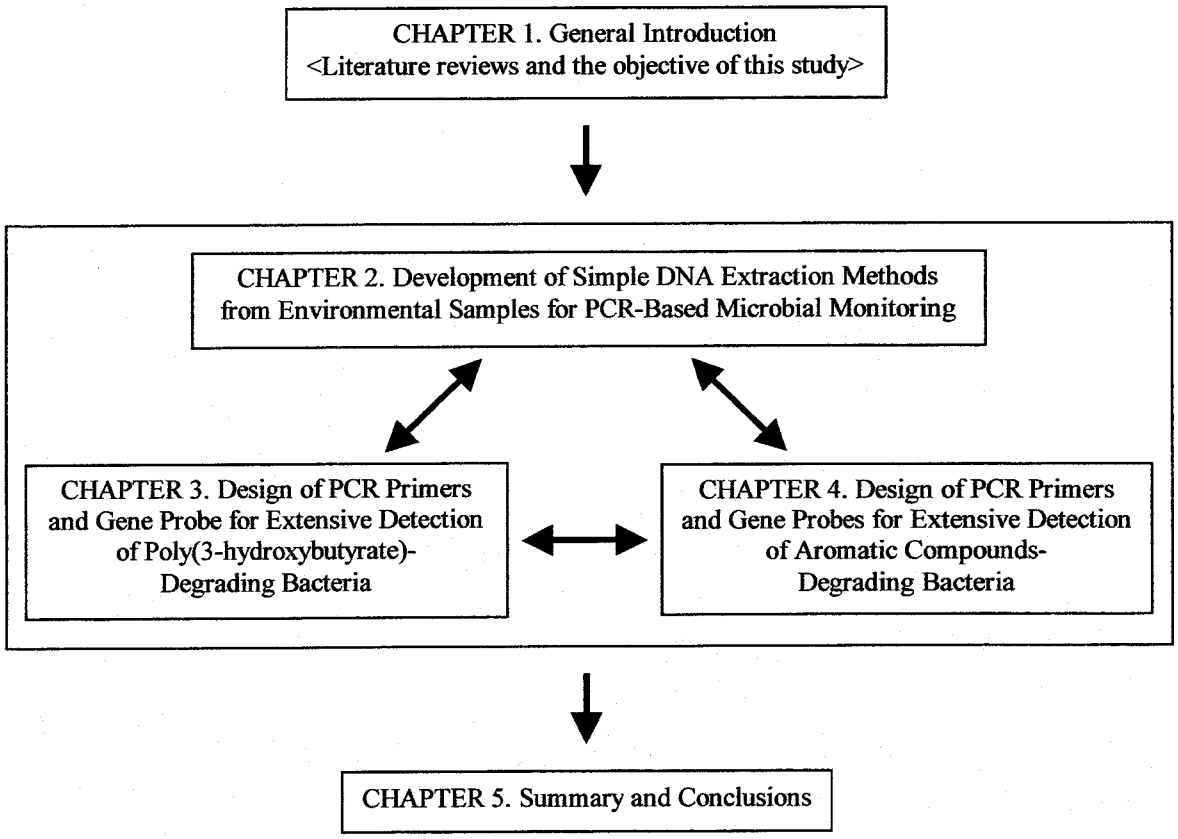


Fig. 1.2 Schematic flow of this study

CHAPTER 2

Development of Simple DNA Extraction Methods from Environmental Samples for PCR-Based Microbial Monitoring

2.1 Introduction

In this chapter, it is aimed to establish generalized DNA extraction methods applicable for intensive monitoring of microbial ecology with PCR techniques, of which requirements are (i) a whole or major portion of DNA contained in the samples should be recovered by the methods, (ii) the DNA extracts prepared by the methods should have a high purity and a least mechanical damage suitable for PCR amplification and analyses, (iii) the methods should be applicable to a wide variety of environmental samples of diverse natures, and (iv) the methods should be rapid, simple and possibly cost-effective enough for routine applications.

Previously-reported typical protocols for extraction of environmental DNA are summarized in Table 2.1. In general most of the protocols are composed of the following sequential steps; (a) cell lysis, (b) DNA extraction (and partial purification of DNA), and (c) further purification of the DNA extracts.

Cell lysis (a) is performed so as to release the intracellular DNA, and is performed by the treatments with enzymes (lysozyme, proteinase K, etc.), chemical agents (SDS (sodium dodecyl sulfate), CTAB (cetyltrimethyl ammonium bromide), TritonX-100, GuSCN (guanidine thiocyanate), phenol, etc.), and/or mechanical treatments (bead beating, freeze-and-thaw, ultrasonication, etc.) in most cases. Depending on sample properties and purposes different types of the cell lysis treatments have been applied in combination. The DNA extraction step (b)

Table 2.1. Operations in cell lysis, DNA extraction, and further purification steps

References	Cell lysis	DNA extraction	Further purification	Target environment
Boom *et al.* (1990)	• GuSCN and TritonX-100 [room temperature, 10 min]	• silica or diatom adsorption • GuSCN wash • 70% ethanol wash • acetone wash	–	• pure bacterial culture
Sparagano (1993)	• phenol-chloroform extraction		–	• river water
Wilson (1994)	• SDS and proteinase K [37°C, 1 hr]	• CTAB-NaCl extraction • phenol-chloroform extraction • isopropanol precipitation	–	• marine surface water
Ogram *et al.* (1987)	• SDS [70°C, 1 hr] • bead beating	• ethanol precipitation or PEG precipitation	• KAc precipitation • CsCl-EtBr gradient centrifugation • hydroxyapatite chromatography	• marine and reservoir sediments
Tsai and Olson (1991)	• lysozyme [37°C, 2 hr] • SDS and freeze-and-thaw	• Elutip-d elution • ethanol precipitation	–	• subsurface soil and settling pond sediment
Picard *et al.* (1992)	• ultrasonication • freeze-and-thaw	• Elutip-d elution • ethanol precipitation	–	• subsurface soil (silt loam)
Trevors *et al.* (1992)	• lysozyme and zymolyase [4°C, 1.5 hr] • SDS (or Brij 58, sarkosyl, deoxycholic acid) with/without PVPP and proteinase K [4°C, 16 hr]	• phenol-chloroform extraction • ethanol precipitation	–	• surface soil (loam)
Smalla *et al.* (1993)	• lysozyme [4°C or 37°C, 30 min] • SDS and bead beating	• phenol-chloroform extraction	• CsCl precipitation • KAc precipitation • glass milk or spermine purification	• loamy sand and silt loam soil
Moré *et al.* (1994)	• bead beating or SDS and freeze-and-thaw	• NH_4Ac precipitation • SpinBind DNA extraction cartridge	• agarose gel electrophoresis • SpinBind DNA extraction cartridge	• groundwater sediment
Porteous *et al.* (1994)	• ultrasonication • lysozyme and Novozym [37°C, 1 hr] • SDS and GuSCN [68°C, 1 hr]	• isopropanol precipitation	–	• surface agronomic, urban, and natural soil
Zhou *et al.* (1996)	- pretreatment for gram positive bacteria - • grinding of frozen sample • SDS and freeze-and-thaw - general procedure - • CTAB and proteinase K [37°C, 30 min] • SDS [65°C, 2 hr]	• chloroform extraction • isopropanol precipitation	• agarose gel electrophoresis • spun column	• Standard soil

is performed to separate DNA from other cell components such as polysaccharides and proteins, and environmental impurities such as soil particles and humic substances. In this step, DNA extractions with phenol or phenol-chloroform, direct DNA precipitations with PEG (polyethylene glycol), ethanol or isopropanol precipitations, and silica or glass milk adsorption of DNA were often employed. Of them phenol-chloroform extraction could serve as both cell lysis and DNA extraction steps in the study of Sparagano (1993). Further purification of DNA (c) is occasionally performed to obtain DNA of high purity enough for PCR or other molecular analyses, especially in case that the DNA extracts obtained by the previous steps can contain much impurities. Often-employed operations for this step include hydroxyapatite column chromatography, CsCl-ethidium bromide (EtBr) density gradient ultracentrifugation, and agarose gel electrophoresis followed by DNA elution (e.g., Elutip-d elution) and spun column (resin) treatments.

Generally preparation of DNA extracts from water samples was carried out through the protocols composed of the cell lysis and DNA extraction steps, while those for soil and sediment samples needed the purification step additionally. Because soil and sediment samples contain much more amounts of various organic matters and other impurities which can inhibit the recovery and PCR amplification and/or other analyses of DNA compared with water samples. Therefore, sometimes higher concentrations of chelating agents were used and/or other chemical agents such as PVPP (polyvinylpolypyrrolidone), which can reduce the effect of impurities in the environmental samples, were added to the cell lysis buffer to improve the recovery and purity of DNA for soil or sediment samples.

2.2 Experimental design

In order to develop DNA extraction methods applicable for intensive monitoring of microbial community with PCR techniques, comparative studies were performed on several types of cell lysis treatments for water and soil samples. The cell lysis (a) with enzyme

(proteinase K), chemical agents (SDS, CTAB, and PVPP), and mechanical treatments (freeze-and-thaw and ultrasonication) were investigated solely or in combinations for their DNA extracting capability.

As for the DNA extraction (b) and purification (c) steps, phenol-chloroform extraction followed by ethanol precipitation and spun column purification was used, respectively, throughout the experiments of the comparative studies, because only these methods seem to be able to satisfy all the requirements (i) to (iv) mentioned in the introduction part. The other DNA extraction and purification methods apparently possess disadvantages or problems compared with the selected methods (phenol-chloroform extraction/ethanol precipitation and spun column purification). For example, direct DNA precipitations with chemical agents tend to co-precipitate much impurities in the samples (Ogram *et al.*, 1987), and silica or glass milk adsorption causes significant damage of DNA molecules from shearing (Boom *et al.*, 1990). DNA purification with hydroxyapatite column chromatography, EtBr density gradient ultracentrifugation, and agarose gel electrophoresis with Elutip-d elution are too laborious and time-consuming to be applied routinely. Further, they require special apparatus and sometimes cause a significant loss of DNA yields (Tsai and Olson, 1991).

In total 6 and 24 different DNA extraction protocols were comparatively investigated against water and soil samples, respectively, as summarized in Table 2.2. These protocols were evaluated from the viewpoints of the recovery (yield) and purity (suitability for PCR amplification) of the DNA of the target microbe seeded to the water and soil samples.

Table 2.2. Outline of the comparative study on DNA extraction methods

Methods	Cell lysis			DNA extraction[a]	DNA purification
	Buffer	Enzymatic/chemical treatment	Mechanical treatment		
			Water samples		
W1	SB	(none)	(none)	phenol-chloroform	(none)
W2	SB	proteinase K	(none)	phenol-chloroform	(none)
W3	SB	SDS	(none)	phenol-chloroform	(none)
W4	SB	CTAB	(none)	phenol-chloroform	(none)
W5	SB	(none)	freeze-and-thaw	phenol-chloroform	(none)
W6	SB	(none)	ultrasonication	phenol-chloroform	(none)
			Soil samples		
S1-L/H	LB/HB	proteinase K	(none)	phenol-chloroform	spun column
S2-L/H	LB/HB	proteinase K + PVPP	(none)	phenol-chloroform	spun column
S3-L/H	LB/HB	proteinase K + SDS	(none)	phenol-chloroform	spun column
S4-L/H	LB/HB	proteinase K + CTAB	(none)	phenol-chloroform	spun column
S5-L/H	LB/HB	proteinase K	freeze-and-thaw	phenol-chloroform	spun column
S6-L/H	LB/HB	proteinase K + PVPP	freeze-and-thaw	phenol-chloroform	spun column
S7-L/H	LB/HB	proteinase K + SDS	freeze-and-thaw	phenol-chloroform	spun column
S8-L/H	LB/HB	proteinase K + CTAB	freeze-and-thaw	phenol-chloroform	spun column
S9-L/H	LB/HB	proteinase K	ultrasonication	phenol-chloroform	spun column
S10-L/H	LB/HB	proteinase K + PVPP	ultrasonication	phenol-chloroform	spun column
S11-L/H	LB/HB	proteinase K + SDS	ultrasonication	phenol-chloroform	spun column
S12-L/H	LB/HB	proteinase K + CTAB	ultrasonication	phenol-chloroform	spun column

[a] Coupled with ethanol precipitation for DNA recovery.

Abbreviations: SB, Standard Buffer; LB, Low Buffer; HB, High Buffer

2.3 Materials and methods

Bacterial strain *Pseudomonas putida* BH (Hashimoto and Fujita, 1987) was used as the seed microbe. The *pheB* gene encoding catechol 2,3-dioxygenase (Fujita *et al.*, 1991) carried by this strain (1 copy per cell) was chosen as the target gene for PCR amplification. For experiments, *P. putida* BH was cultivated with LB medium (Sambrook *et al.*, 1989) for about 3 hours at 30 °C at 100 rpm on a reciprocal shaker. The bacterial cells were seeded to water and soil samples to give densities of 1.0×10^5 cells/ml and 1.0×10^7 cells/g (wet weight of soil), respectively. Cell density of this strain was determined by microscopic direct counting with a counting chamber.

Water and soil samples Water samples were collected from the Yodo river, Osaka, Japan (River sample; SS 19.4 mg/*l*, pH 6.9), the Lake Biwa, Shiga, Japan (Lake sample; SS 4.6 mg/*l*, pH 7.1), and the Ise Bay, Mie, Japan (Sea sample; SS 33.2 mg/*l*, pH 7.8). Soil samples were collected from 3 sites of the agricultural fields (Tsukuba National Agricultural Experiment Station, Ibaraki, Japan). The characteristics of the soil samples are shown in Table 2.3.

DNA extraction from water samples One milliliter of the water sample seeded with *P. putida* BH was centrifuged at 8,000 × *g* for 15 min in a 1.5 ml-microcentrifuge tube and discarded the supernatant. The pellet was suspended in 500 µl of the extraction buffer (Standard Buffer; 50 mM Tris-HCl, 20 mM EDTA, 100 mM NaCl, pH 8.0) in the tube and the cell lysis

Table 2.3. Characteristics of soil samples

Soil samples	Particle distribution			Moisture content (%)	pH	Carbon content (%)	Nitrogen content (%)	CFU/g
	Sand (%)	Silt (%)	Clay (%)					
Sample A	16.0	33.8	50.2	40.2	7.0	4.5	0.39	9.3×10^8
Sample B	25.0	30.0	45.0	43.1	6.8	2.9	0.23	1.8×10^8
Sample C	40.0	30.5	29.5	50.4	6.7	1.6	0.12	8.0×10^6

treatment as follows was comparatively performed. For the proteinase K treatment, 50 μl of 2 mg/ml proteinase K solution was added to the tube and it was incubated at 37 °C for 2 hours. For the SDS or CTAB treatment, the extraction buffer was prepared with 1 % of SDS or 1 % of CTAB, respectively, and the tube was incubated in the same manner. For the freeze-and-thaw treatment, the pellet suspension in the extraction buffer was subjected to 5 cycles of freezing and thawing at -196 °C/+80 °C. For the ultrasonication treatment, the pellet suspension was sonicated with an ultrasonicator Bioruptor UCD200-T (Cosmo Bio, Tokyo, Japan) for 3 min under the condition of output 120 W and 50 % active cycle. After the cell lysis, samples were extracted with equal volumes of phenol-chloroform twice (phenol-chloroform extraction). The water phase was collected and DNA was precipitated with 0.3 M sodium acetate and 2 volumes of chilled ethanol (ethanol precipitation). The control was conducted without any enzymatic, chemical, or mechanical treatment, i.e. only phenol-chloroform extraction followed by ethanol precipitation. The recovered DNA pellet was dissolved in TE buffer (1 mM EDTA, 10 mM Tris-HCl; pH 8.0) for analyses.

DNA extraction from soil samples A 100 mg sample of fresh soil inoculated with *P. putida* BH was put into a 1.5 ml-microcentrifuge tube and suspended in 500 μl of DNA extraction buffer with a higher (High Buffer; 250 mM Tris-HCl, 50 mM EDTA, 125 mM NaCl, pH 8.0) or lower concentration of chelating agent (Low Buffer; 10 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, pH 8.0). The proteinase K treatment, which was selected as the best method for DNA extraction from water samples, was performed in combination with other chemical treatment (SDS, CTAB, or 0.5 % PVPP) and/or mechanical treatment (freeze-and-thaw or ultrasonication) in the cell lysis step. Phenol-chloroform extraction was done as the DNA extraction step as well as for water samples, followed by ethanol precipitation for DNA recovery. Further purification of the DNA extracts was carried out using a spun column, Ultrafree C3 Probind (Millipore, MA, USA), according to the supplier's instruction. The DNA pellets were suspended in TE buffer for analyses.

Determination of DNA yields The amount of *P. putida* BH-derived DNA recovered by each method was roughly measured utilizing Hoechst 33258 {bisbenzimide; 2-[2-(4-hydroxy-phenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl) benzimidazole, trihydrochloride, penta-hydrate} as previously described (Paul and Myers, 1982). The yield was determined by the following equation:

$$E(\%) = \frac{Ds - D_0}{X \times (9.0 \times 10^{-15})}$$

where E is DNA yield, D_S is weight of DNA recovered from *P. putida* BH-seeded water or soil sample, D_0 is weight of DNA recovered from the corresponding non-seeded sample, X is the number of *P. putida* BH cells seeded to the sample, and the value 9.0 x 10^{-15} represents a general DNA weight (g) per one bacterial cell (Ingraham *et al.*, 1983).

Determination of DNA purity by PCR The purity of DNA extracts prepared by each method was compared on the detection limit of PCR amplification that was targeted *pheB* gene, as previously described (Zhou *et al.*, 1996). The PCR amplification was conducted in 30 cycles with denaturation at 95 °C for 60 s, annealing at 50 °C for 30 s, and extension at 72 °C for 60 s using Program Temp Control System PC-800 (ASTEC, Fukuoka, Japan). The sequences of the primer set to amplify *pheB* gene of *P. putida* BH were 5'-ATGAAAAAAGGCGGAATTCGCC-CCG-3': PHE-1 and 5'-TCAGGTGAGCACGCTCGAGAAACGT-3': PHE-2. Aliquots (10 μl) of the PCR products were analyzed by electrophoresis on a 1.0 % agarose gel stained with 0.5 μg/ml ethidium bromide.

2.4 Results and Discussion

Comparative study on DNA extraction methods for water samples Results of the comparative study on the DNA extraction methods for water samples are summarized in Tables 2.4 and 2.5. Five frequently-employed enzymatic, chemical, or mechanical cell lysis operations

Table 2.4. Comparison of various DNA extraction methods for water samples: DNA yields (%)

Methods	Samples used for DNA extraction		
	River water	Lake water	Sea water
W1	9.3 ± 1.1	11.2 ± 2.7	7.2 ± 1.3
W2	53.8 ± 8.8	55.9 ± 4.5	23.8 ± 7.3
W3	88.3 ± 2.1	70.3 ± 5.1	63.9 ± 1.5
W4	0.2 ± 0.2	0.9 ± 0.3	0.06 ± 0.04
W5	104 ± 20.0	105 ± 10.0	21.0 ± 9.1
W6	109 ± 12.0	76.0 ± 8.0	8.9 ± 1.5

DNA yields (%) are expressed by mean value ± standard deviation in triplicated experiments. Details of each methods are given in Table 2.2.

(proteinase K, SDS, CTAB, freeze-and-thaw, and ultrasonication treatments) were tested coupled with phenol-chloroform extraction.

When phenol-chloroform extraction was applied without any other enzymatic, chemical, or mechanical treatment (control ; W1), the DNA yields were only 7.2 to 11.2 % (Table 2.4). When the samples were treated with proteinase K (W2) or SDS (W3) prior to phenol-chloroform extraction, the DNA yield was considerably increased independent of the water samples, though the use of CTAB (W4) was not effective at all. Application of the mechanical cell lysis operations, freeze-and-thaw (W5) and ultrasonication (W6), also gave very high yields of DNA for the fresh water (river and lake) samples; it seems that almost all DNA was recovered (76.0 to 109 %). On the other hand, these mechanical treatments were not so effective for enhancing the DNA yield from the sea water sample.

In order to determine whether the DNA extracts were sufficiently pure or suitable for PCR or not, amplification of the *P. putida* BH-derived sequence was tried using the primer set PHE-1

Table 2.5. Comparison of various DNA extraction methods for water samples: PCR amplification (DNA purity)

Methods	Samples used for DNA extraction		
	River water	Lake water	Sea water
W1	++−	++−	+−−
W2	+++	+++	++−
W3	−−−	−−−	−−−
W4	−−−	+−−	−−−
W5	−−−	+−−	−−−
W6	−−−	−−−	+−−

Results of the triplicated experiments are shown as positive (+) and negative (–) signals. e.g. + + + indicates that the anticipated DNA fragment was amplified (observed) in all the triplicated trials. Details of each method are given in Table 2.2.

and PHE-2 (Table 2.5). No PCR signal was observed from non-inoculated samples, indicating absence of the DNA sequence of the target microbe in the environmental samples (data not shown). The positive signal of the PCR amplification was observed from 1 to 2 of triplicated DNA extracts prepared by the phenol-chloroform extraction without additional treatment (W1), suggesting that PCR-dependent detection of a target microbe at approximately 10^5 cells in 1 ml of water samples is possible by this simplest DNA extraction procedure. The DNA extracts prepared by the proteinase K treatment showed more reproducible amplification; all the extracts except for one from the sea water showed positive signals, indicating that more sensitive detection of the target microbe was possible by this method (W2). However, the other chemical or mechanical treatments resulted in inferior PCR amplification, nevertheless some of them gave very high DNA yields. Especially no positive signal was observed from any samples when the SDS treatment was employed for extracting DNA (W3). The reason can be attributed to that SDS remaining in the DNA extracts inhibited *Taq* DNA polymerase activity in the PCR (Bej,

1995). On the other hand, the reasons for poor PCR amplification of the mechanically-treated samples (W5, W6) may be that the shearing force injured or fragmentated the extracted DNA to generate damaged or smaller-size fragments which are unsuitable for PCR analyses (Picard *et al.*, 1992; Simonet *et al.*, 1991).

Considering both DNA yield and suitability for PCR, the proteinase K treatment followed by phenol-chloroform extraction (W2) was selected as the best method from water samples among the tested methods. In order to determine the detection limit of the target microbe by PCR, water samples were inoculated with *P. putida* BH at different densities (10^0 to 10^5 cells/ml) and the selected method was applied to extract DNA from these samples. The DNA extracts from water samples which were inoculated with BH at more than 10^1 cells/ml reproducibly gave positive signals by the PCR against the background of 10^4 to 10^5 cells/ml of indigenous microbes in the samples (Fig. 2.1).

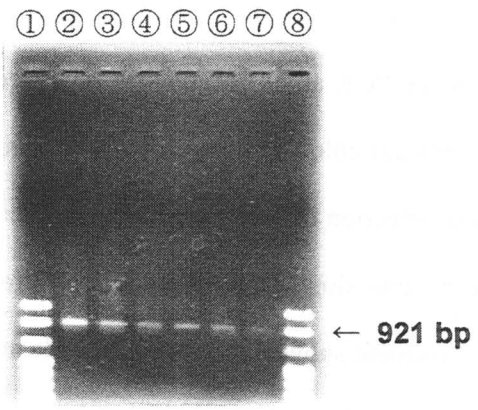


Fig. 2.1. PCR amplification of *P. putida* BH inoculated into pond water sample at various inoculum size. Lane 1, Molecular weight marker; lane 2, 10^6 cells/ml; lane 3, 10^5 cells/ml; lane 4, 10^4 cells/ml; lane 5, 10^3 cells/ml; lane 6, 10^2 cells/ml; lane 7, 10^1 cells/ml; lane 8, molecular weight marker.

Comparative study on DNA extraction methods for soil samples In a preliminary study, the DNA extraction method established for water samples was applied to soil samples, resulted in no amplification of the target DNA fragment from any samples, while the DNA yields ranged from approximately 10 to 75 %. When the DNA extracts were further purified by the spun column, loss of the DNA was trivial (less than 5 %), however, positive signals were rarely obtained by PCR (Table 2.6). This suggested that much impurities which inhibit PCR was contained in the soil samples and that they could not be sufficiently removed by the combination of proteinase K treatment, phenol-chloroform extraction, and spun column purification.

Table 2.6. Results of PCR amplification and yields of DNA extracted from soil samples with the method for water samples

		Samples used for DNA extraction														
		A					B					C				
		10^{5a}	10^{4a}	10^{3a}	10^{2a}	10^{1a}	10^{5a}	10^{4a}	10^{3a}	10^{2a}	10^{1a}	10^{5a}	10^{4a}	10^{3a}	10^{2a}	10^{1a}
Without Spun column purification	yields (%)[b]	56.0 ± 8.9					74.9 ± 6.2					9.4 ± 3.6				
	PCR	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
With Spun column purification	yields (%)[b]	52.1 ± 8.3					71.1 ± 5.9					9.0 ± 3.5				
	PCR	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] DNA extracts were diluted to the corresponding number of cells/g (wet weight). DNA was extracted from the samples inoculated *P. putida* BH to be 1.0×10^7 cells/g (wet weight).

[b] DNA yields (mean values, n = 3, ± 1 standard deviation).

+, amplification of the fragment; –, no amplification.

To overcome this problem, several modifications were added for establishing the DNA extraction methods suitable for soil samples. Comparative studies were performed on the concentration of chelating agent in the extraction buffer, and on the uses of chemical agents (SDS, CTAB, and PVPP) and mechanical treatments (freeze-and-thaw and ultrasonication) in addition to the proteinase K treatment (Table 2.2). The results are summarized in Tables 2.7 and 2.8.

As shown in the Tables 2.7 and 2.8, mechanical treatments were much effective for increasing DNA yield and purity (suitability for PCR), especially when used in the presence of higher chelating activity (High Buffer) which can protect the extracted DNA from re-absorbed onto soil-derived impurities. Though chelating agents at high concentration are known to inhibit the activity of the *Taq* DNA polymerase (Bej, 1995), no PCR inhibition was observed in this study. When comparing 2 mechanical treatments used here, the ultrasonication led to more sensitive PCR amplification than the freeze-and-thaw as a whole, while DNA yield was not so different between both treatments. As for the addition of chemical agents, SDS and PVPP generally gave higher DNA yield and purity, while CTAB was much less effective. As previously described, both SDS and mechanical treatments for water samples caused inhibitory effects on the PCR amplification in spite of high DNA yields, however, such inhibitory effects were not clearly observed for soil samples. It seems that, when applied to soil samples, SDS formed a complex with impurities and it might be removed to be lower concentrations during the DNA extraction and purification steps, resultantly it gave little or no inhibition for *Taq* DNA polymerase activity. On the other hand the reason for no PCR inhibition by the mechanical treatments for soil samples may be that the co-presence of soil particles and impurities reduced the effects of shearing force onto released DNA, caused less damage or fragmentation.

Considering both DNA yield and suitability for PCR, the best method for extracting DNA from soil samples among the tested methods seemed to be the combination of proteinase K, SDS, and ultrasonication treatments in the High Buffer followed by the phenol-chloroform

Table 2.7. Comparison of various DNA extraction methods for soil samples: DNA yield (%)

Methods	Samples used for DNA extraction		
	A	B	C
S1-L	52.1 ± 8.3	71.1 ± 5.9	9.0 ± 3.5
H	23.1 ± 6.3	2.0 ± 1.8	16.4 ± 6.7
S2-L	65.8 ± 19.7	17.6 ± 1.5	10.6 ± 8.9
H	8.6 ± 3.5	18.0 ± 3.3	34.1 ± 2.8
S3-L	65.4 ± 3.6	74.4 ± 7.9	4.6 ± 3.7
H	21.8 ± 5.3	16.6 ± 2.0	8.7 ± 1.5
S4-L	22.4 ± 3.2	48.4 ± 7.9	0.3 ± 0.2
H	26.1 ± 4.0	17.1 ± 2.5	7.6 ± 2.4
S5-L	39.7 ± 5.4	34.4 ± 10.0	14.8 ± 8.2
H	26.2 ± 4.0	34.2 ± 5.4	28.8 ± 13.9
S6-L	16.5 ± 1.9	29.5 ± 0.6	5.3 ± 1.6
H	91.0 ± 3.6	78.5 ± 2.6	64.5 ± 3.3
S7-L	76.6 ± 1.3	60.5 ± 5.6	< 0.1
H	43.8 ± 6.6	38.3 ± 12.2	68.8 ± 3.3
S8-L	62.1 ± 14.4	77.8 ± 16.3	< 0.1
H	44.8 ± 7.5	41.6 ± 3.9	43.3 ± 6.8
S9-L	70.1 ± 5.5	62.7 ± 7.1	4.8 ± 5.0
H	44.6 ± 7.2	54.3 ± 5.7	37.2 ± 5.9
S10-L	41.1 ± 4.2	14.0 ± 2.1	26.5 ± 5.9
H	89.1 ± 3.2	80.8 ± 7.8	57.5 ± 4.4
S11-L	76.5 ± 23.2	68.6 ± 1.5	8.5 ± 2.1
H	87.9 ± 2.5	80.1 ± 10.4	95.8 ± 17.3
S12-L	15.0 ± 2.0	10.6 ± 3.0	< 0.1
H	51.0 ± 5.3	80.7 ± 7.6	80.7 ± 9.4

DNA yields (%) are expressed by mean value ± standard deviation in triplicated experiments. Details of each methods are given in Table 2.2.

Table 2.8. Comparison of various DNA extraction methods for soil samples: PCR amplification (DNA purity)

Methods	Samples used for DNA extraction														
	A					B					C				
	10^{5a}	10^{4a}	10^{3a}	10^{2a}	10^{1a}	10^{5a}	10^{4a}	10^{3a}	10^{2a}	10^{1a}	10^{5a}	10^{4a}	10^{3a}	10^{2a}	10^{1a}
S1-L	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
S2-L	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
H	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
S3-L	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
S4-L	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
S5-L	+	+	–	–	–	+	+	–	–	–	+	–	–	–	–
H	+	+	+	–	–	+	+	+	–	–	+	–	–	–	–
S6-L	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–
H	+	+	+	+	–	+	+	+	+	–	+	–	–	–	–
S7-L	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
H	+	+	+	–	–	+	–	–	–	–	+	–	–	–	–
S8-L	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–
H	+	+	+	–	–	+	+	+	–	–	+	–	–	–	–
S9-L	+	+	–	–	–	+	+	–	–	–	+	–	–	–	–
H	+	+	+	–	–	+	+	+	–	–	+	–	–	–	–
S10-L	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–
H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S11-L	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–
H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S12-L	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–
H	+	–	–	–	–	+	–	–	–	–	+	–	–	–	–

[a] DNA was extracted from the soil samples inoculated with *P. putida* BH to be corresponding numbers of the cells/g-soil (wet weight). +, amplification of the fragment; –, no amplification. Details of each method are given in Table 2.2.

extraction, ethanol precipitation, and spun column purification (S11-H). By using this extraction method, very high DNA yields (> 80 %) and sensitive PCR amplification results were obtained from soil samples regardless of their soil structure and carbon contents. The combination of the proteinase K, PVPP, and ultrasonication treatments (S10-H) showed good results almost comparative to the best one, however, the DNA yield from the soil sample C was considerably lower (about 60 %).

When the selected method (S11-H) was applied, the detection limit of the inoculated microbe by PCR from 3 soil samples where 10^7 to 10^9 cells/g of indigenous microbes existed was 10^1 to 10^2 inoculated cells/g (Table 2.8). Previous studies reported similar or a little lower sensitivity of PCR-mediated detection of microbes in soil samples, though some of the employed DNA extraction methods in these studies were more complicated and/or time-consuming than the method established here; e.g. the detection limits reported were 100 *Nitrobacter* cells/g soil (Degrange and Barbin, 1995), 70 *Escherichia coli* cells or 500 copies of target DNA/g sediment (Tsai & Olsen, 1992), 100 plasmids of *Corynebacterium glutamicus*/g soil (Vahjen and Tebbe, 1994) and 800 *Desulfitobacterium frappieri* PCP-1 cells/g soil (Levesque *et al.*, 1997).

Conclusive discussion Based on the results of comparative studies performed here, the direct DNA extraction methods for water and soil samples were developed as shown in Figs. 2.2 and 2.3, respectively. The established method for water samples (Fig. 2.2) could recover more than 60 % from fresh water samples, though the yield was a little lower from a sea water samples. The PCR-mediated detection limit of the target microbe was between 10^0 and 10^1 added cells/ml, indicating no or very little inhibition of PCR reaction. The procedure was very simple and could be completed within 3 to 4 hours in a small scale (1.5-ml microcentrifuge tube).

On the other hand, the established method for soil samples (Fig. 2.3) gave the DNA yield more than 80 % and enabled sensitive detection of the inoculated microbe by PCR without

apparent inhibition (detection limit, 10^1 to 10^2 added cells/g-soil, i.e. 10^0 to 10^1 cells/micro-centrifuge tube) regardless of the soil characteristics. The procedure could be completed within 4 to 5 hours without complicated operations and required no expensive and/or specialized equipments, therefore, was routinely-applicable. Thus, the established DNA extraction methods are considered to satisfy the requirements (i) to (iv) for general protocols applicable for intensive monitoring of microbial ecology with PCR techniques.

2.5 Summary

In this chapter, studies were made so as to establish simple and rapid DNA extraction methods for PCR-based monitoring of microbial ecology in the water/soil environment.

Several kinds of cell-lysis enzyme, chemical agents, and mechanical treatments were comparatively investigated solely or in combinations for their DNA extracting capability against each 3 water and soil samples inoculated with the PCR-targeting bacterium, *P. putida* BH. For water samples, cell lysis with proteinase K allowed to detect the target bacterium at a sensitivity at 10^1 cells/ml against backgrounds of indigenous bacteria at 10^4-10^5 cfu/ml with the DNA recovery of ca. 25-55 %, when coupled with phenol-chloroform extraction and ethanol precipitation. For soil samples, ultrasonication in addition to the uses of the proteinase K and SDS in the presence of a high concentration of chelating agent was the most effective, although purification of the DNA extracts with a spun column were required in addition to the phenol-chloroform extraction and ethanol precipitation. This method enabled the PCR-mediated detection of the target bacterium at 10^1-10^2 cells/g of the soil samples where 10^7-10^9 cfu/g of indigenous bacteria existed and the DNA yield was between 80-95 %. The methods established in this chapter seem to be able to extract a most or considerable portion of the DNA from a variety of environmental samples with a sufficiently high purity for PCR amplification. These methods also seem routinely applicable, because the procedures are very simple and do not contain time-consuming and labor-full operations.

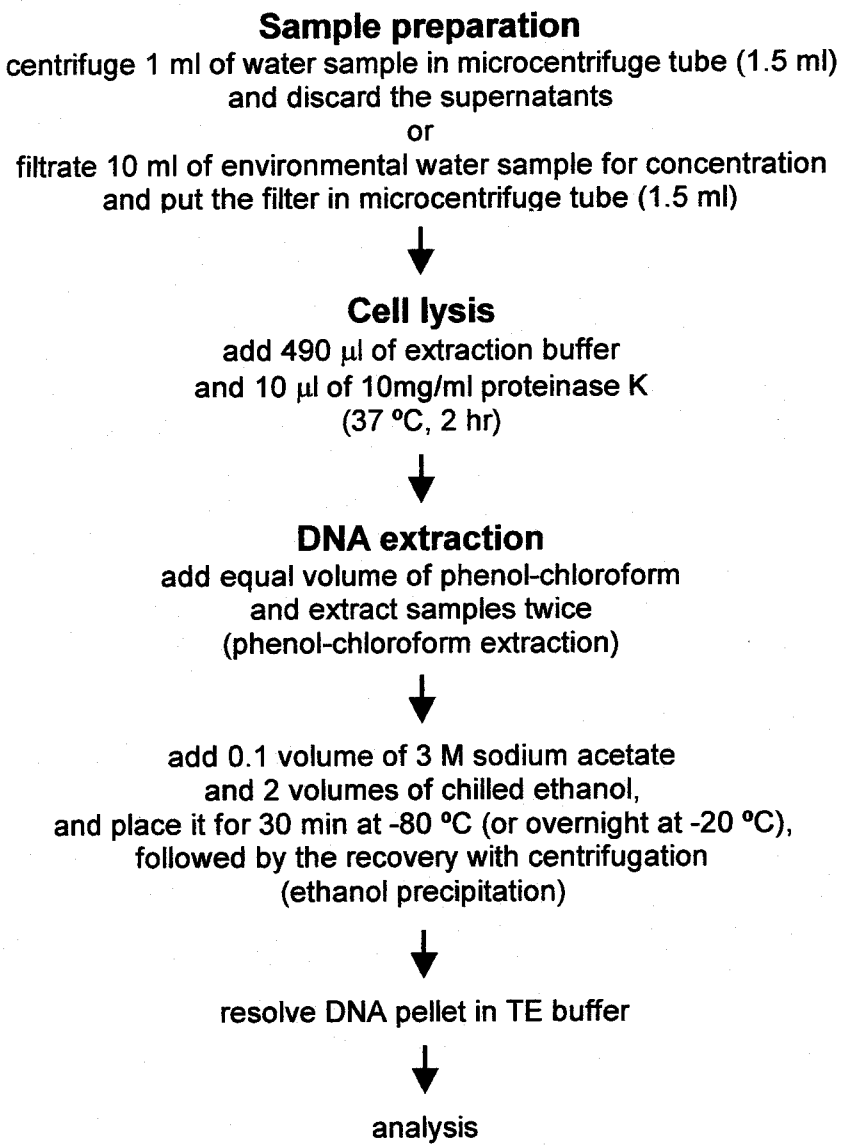


Fig. 2.2. Established DNA extraction method for water environmental samples

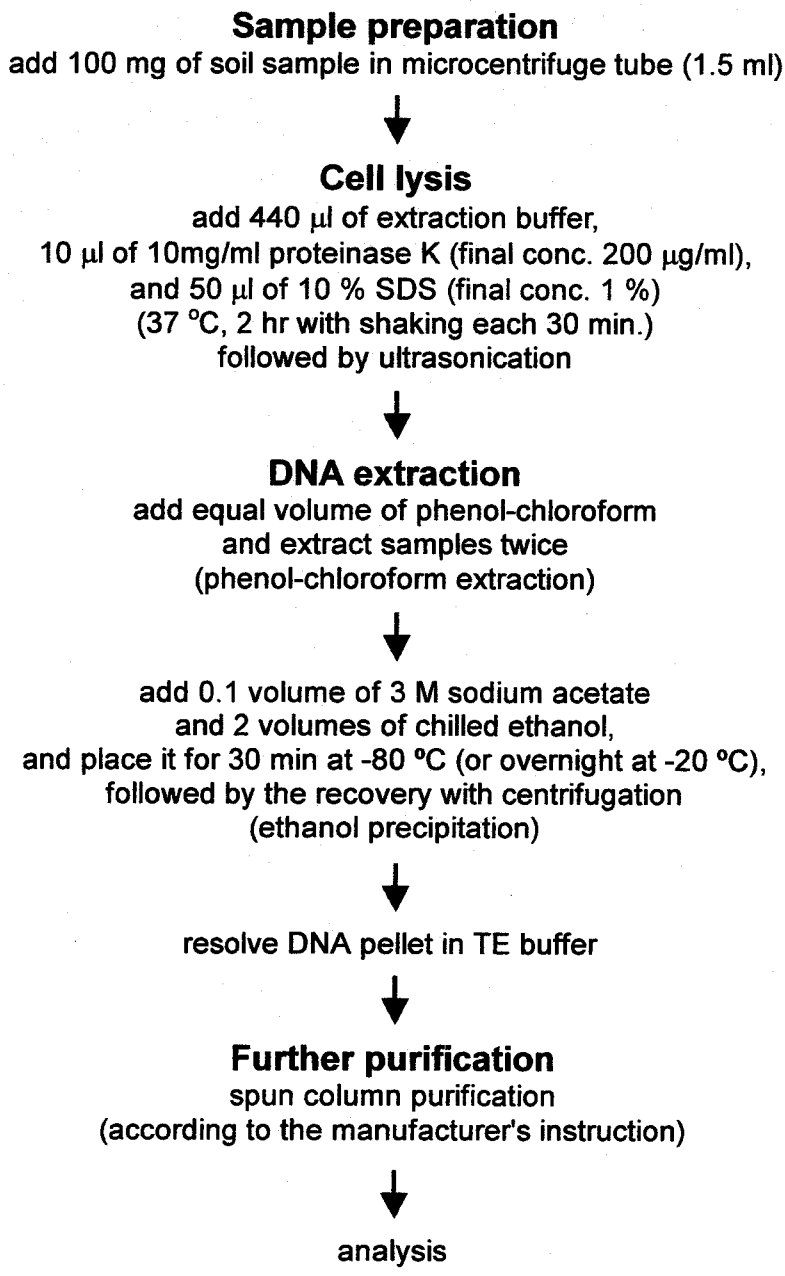


Fig. 2.3. Established DNA extraction method for soil environmental samples

CHAPTER 3

Design of PCR Primers and Gene Probe for Extensive Detection of Poly(3-hydroxybutyrate)-Degrading Bacteria

3.1 Introduction

Poly(3-hydroxybutyrate) (PHB) is a carbon and energy reserve accumulated by several kinds of bacteria under the conditions of nutrient stress, normally when an external carbon source is available but when concentrations of nutrients such as nitrogen, phosphorous or oxygen are limiting growth (Oeding and Schlegel, 1973; Senior and Dawes, 1973). Recently, PHB is attracting a great concern as one of the useful biodegradable plastics which are not derived from petroleum, and the co-polymer of PHB and poly(3-hydroxyvalerate) (PHV), which is more flexible than homopolymer of PHB (Slater *et al.*, 1999), has been commercially produced as BIOPOL in a large fermentation process (Holmes, 1985). Especially its agricultural and marine applications are emphasized (Holmes, 1985). The amount of the production and consumption of environmentally-friendly biodegradable plastics including PHB and related co-polymers is expected to increase, consequently, their emission to the natural environments 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 degrading potentials, it is important to detect, enumerate and/or monitor the behavior of PHB-degrading microbes.

This chapter aims at developing a molecular technique for the extensive detection of PHB degrading-bacteria. A PCR primer set which can detect a variety of PHB depolymerase genes

and DNA probe for specific identification of the PCR products were designed from relatively highly homologous regions lied on various PHB depolymerase genes. Their applicability was investigated by applying them to both known and newly isolated bacterial strains. Furthermore, PHB-degrading bacteria in a variety of soil environments were enumerated by both most probable number (MPN)-PCR and colony forming units (CFU) so as to investigate the applicability of MPN-PCR for monitoring PHB-degrading bacteria in natural environment.

3.2 Materials and methods

Bacterial strains and growth conditions Among the authentic bacterial strains known to possess well characterized PHB depolymerase genes listed in GenBank (Table 3.1) (Keller *et al.*, 1984), 4 bacteria (written in bold type in Table 3.1) were kindly provided us by the researchers who isolated the strains. These authentic bacterial strains were used for experimentally evaluating the specificity of the designed primer set and probe. Authentic bacterial strains not possessing PHB depolymerase genes, *Acinetobacter baumannii* ATCC19606, *Acinetobacter calcoaceticus* ATCC23055, *Acinetobacter haemolyticus* ATCC17906, *Acinetobacter johnsonii* ATCC17909, *Acinetobacter lwoffii* ATCC15309, *Bacillus megaterium* ATCC12872, *Escherichia coli* K12, *Flavobacterium breve* IFO 14943, *Moraxella bovis* ATCC10900, *Pseudomonas aeruginosa* IFO12689, *Pseudomonas fluorescens* ATCC15553, *Pseudomonas putida* IFO14164, *Vibrio campbellii* ATCC25920, and *Staphylococcus epidermidis* GTC289 were served as negative controls for the PCR amplification. Additionally, in total 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.*, 1999), and garden using a slightly modified mineral medium (Fujita and Ike, 1997) agar plate containing 0.1 % of 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 (Cowan and Steel, 1974). Furthermore, all of the gram negative bacteria except for enterobacteria

Table 3.1. Bacterial strains possessing known PHB depolymerase genes and the primability and stability of the designed PHB primers (PHBf and PHBr) and probe PHBp against the corresponding genes

Bacterial strains (gene name)	PHBf		PHBr		PHBp	Enzyme type[a]	References and Accession numbers of GenBank
	Primability (%)	Stability (%)	Primability (%)	Stability (%)	Primability (%)		
***Comamonas* sp. (*phaZ*)**	**91**	**74**	**96**	**77**	**81**	**Fn3**	**Jendrossek *et al.*, 1995b U16275**
***Ralstonia pickettii* T1 (*phaZ*)**	**92**	**78**	**99**	**74**	**94**	**Fn3**	**Saito *et al.*, 1989 J04223**
***Streptomyces exfoliatus* K10 (*phaZ*)**	**96**	**75**	**100**	**82**	**76**	**Fn3**	**Klingbeil *et al.*, 1996 U58990**
Comamonas acidovorans YM1609 (*phaZ*)	87	73	98	69	81	Fn3	Kasuya *et al.*, 1997 AB003186
Comamonas testosteroni YM1004 (*phaZ*)	91	74	96	77	81	Fn3	Shinomiya *et al.*, 1997 AB000508
Pseudomonas pickettii K1 (*phaZ*)	92	78	95	71	88	Fn3	Yukawa *et al.*, 1994 D25315
Alcaligenes faecalis AE122 (*phaZ*)	-	-	-	-	-	Fn3	U55775
***Pseudomonas lemoignei* (*phaZ4*)**	-	-	-	-	-	**Fn3**	**Jendrossek *et al.*, 1995a U12976**
Pseudomonas lemoignei (*phaZ1*)	-	-	-	-	-	threonine	Jendrossek *et al.*, 1993 Z22595
Pseudomonas lemoignei (*phaZ2*)	-	-	-	-	-	threonine	Briese *et al.*, 1994 U68039
Pseudomonas lemoignei (*phaZ3*)	-	-	-	-	-	threonine	Briese *et al.*, 1994 U68170
Pseudomonas lemoignei (*phaZ5*)	-	-	-	-	-	threonine	Jendrossek *et al.*, 1995a U12977
Pseudomonas stutzeri YM1006 (*phaZ*)	-	-	-	-	-	cadherin	Ohura *et al.*, 1999 AB012225
Ralstonia eutropha H16 (*phaZ*)	-	-	-	-	-	intracellular	AB017612

The strains and the PHB depolymerase genes used in the experimental investigation are indicated in bold type.

-, The scores could not be calculated because of the low homology with the corresponding primers and probe.

[a] The type of the PHB depolymerase. Fn3; Fn3 linker type, threonine; threonine-rich linker type, cadherin cadherin like linker type, intracellular; intracellular PHB depolymerase

were identified using API20NE (bioMerieux Japan Ltd., Japan). Their PHB degrading ability was confirmed by detecting clear zones surrounding colonies because of hydrolysis of the water-insoluble polymer by extracellular depolymerases. For the enumeration of the PHB-degrading bacteria by CFU, soil samples from agricultural fields, paddy fields, aerobic and anaerobic landfill reactors (Ishigaki *et al.*, 1999), compost reactor (Tateda *et al.*, 1998), garden, forest, pond sediment, and activated sludge were suspended in sterilized water, plated on to the PHB medium, aerobically cultivated at 30 ℃ for 1 week, and the number of the colonies surrounded by clear zones was counted. All the bacterial strains were aerobically cultivated at 30 ℃ in CGY broth (Pike *et al.*, 1972).

Design of the primer set and probe for extensive detection of PHB depolymerase genes Fourteen PHB depolymerase gene sequences were obtained from GenBank (Keller *et al.*, 1984) and the sequences were subjected to multiple alignment using CLUSTALW (Eddy, 1995). The calculations were done using the Sun SPARK station 1000 (Sun Microsystems, Inc., Tokyo) supported by the Genome Information Research Center, Osaka University. Relatively highly homologous regions of each gene group were selected to design PCR primers for their extensive detection. The designed primers were applied to the PCR simulation software, Amplify (Ver.1.2, University of Wisconsin, USA), to evaluate their primability and stability of binding to the target sequences, anticipated amplified fragment sizes and 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. Oligonucleotide probe was also designed from the relatively highly homologous regions lying within the fragments which is amplified by the PCR. It was also evaluated for its primability with the target regions.

PCR amplification DNA templates from each bacterial strain were prepared by the DNA extraction method for water samples established in Chapter 2 (i.e., proteinase K treatment followed by phenol-chloroform extraction). From soil samples, DNA templates were prepared by the method for soil samples established in Chapter 2 (i.e., proteinase K treatment in the High

buffer containing SDS followed by ultrasonication, phenol-chloroform extraction and spun column purification). PCR was conducted for 30 cycles, with denaturation at 94 ℃ for 60 s, annealing at 57 ℃ for 30 s, and extension at 72 ℃ for 30 s using the PROGRAM TEMP CONTROL SYSTEM (PC-800, 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/ml ethidium bromide. For the enumeration of the PHB-degrading bacteria by MPN-PCR (Picard *et al.*, 1992), DNA extracts from the soil environmental samples were serially diluted by 10-fold, and 3 samples of each dilution step were subjected to PCR under the condition described above. Aliquots (4 μl) of the resultant was again subjected to PCR of the same condition. The number of the targeted DNA was enumerated by the MPN technique according to the 2nd PCR amplification result.

Southern hybridization The designed probe was labeled by digoxigenin-11 dUTP using the Dig Oligonucleotide 3'-End Labeling Kit (Boehringer Mannheim, Germany) in accordance with the procedure described by the manufacturer, and used for hybridization. Transfer was performed using a Model 785 Vacuum Blotter (BIO-RAD, USA) as per the manufacturer's instructions. Detection of the digoxigenin-labeled probe was performed using the Dig DNA Labeling and Detection Kit (Boehringer Mannheim, Germany) in accordance with the instructions of the manufacturer, except that the hybridization temperature was set at 37 ℃.

3.3 Results

Design of the primer set for extensive detection of PHB depolymerase genes Multiple alignment was performed against 14 PHB depolymerase gene sequences, and they were confirmed to be considerably divergent (Fig. 3.1). However, 3 relatively highly homologous regions were found among 6 gene sequences encoding PHB depolymerase containing the fibronectin type III (Fn3) linker domain (Fn3 linker type-PHB depolymerase), and 2 regions which seemed to be suitable for designing primer set were chosen to design the primer set for

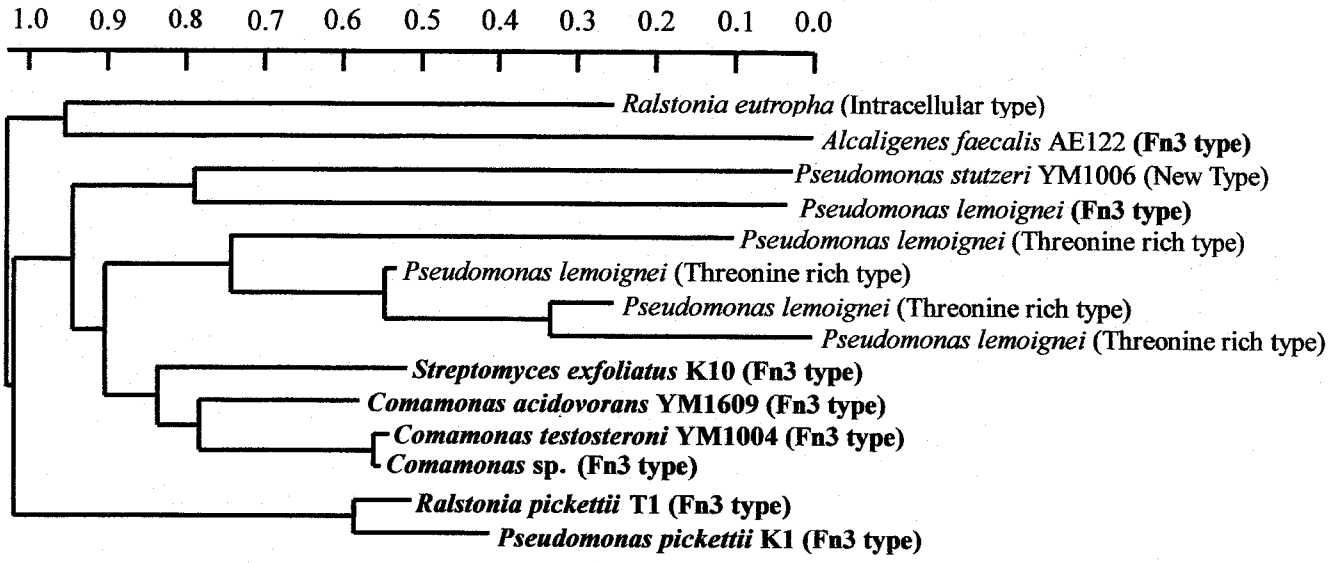


Fig. 3.1. Phylogenetic tree based on PHB depolymerase gene sequences. Calculation was performed by NJ method. Scale bar shows the phylogenetic distance calculated as replacement bases/total bases. Types of PHB depolymerases are given in parentheses after the bacterial names.

the extensive detection of the Fn3 linker type-PHB depolymerase genes (Fig. 3.2). 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. 3.3), although the dominant base at the 6th position from 3' is A, C was introduced instead for improving 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 the simulation of the PCR reaction by Amplify to maximize the possibility of detection of all the gene sequences used for the primer design. Reverse primer (PHBr) was also designed based on the same principle. The sequences of the designed primers are shown in Table 3.2.

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

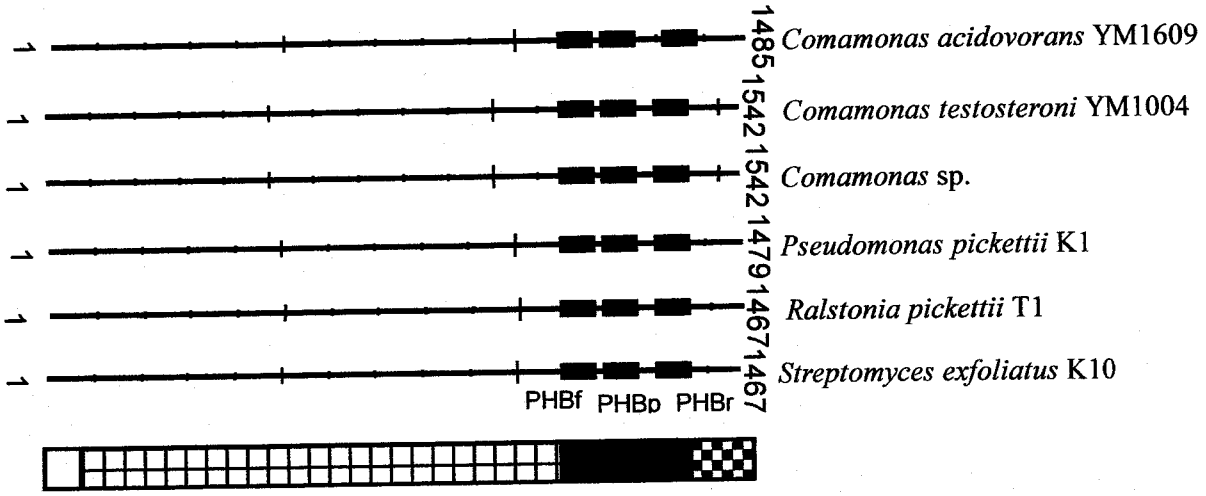


Fig. 3.2. Relatively highly homologous regions of the Fn3 linker type-PHB depolymerase genes. The homologous regions among 6 of all the Fn3 linker type-PHB depolymerase genes are expressed by the squared boxes. Names below the homologous regions show the priming region of each primer and probe. The nucleotide sequences are numbered from the initiation codon to termination codon of each PHB depolymerase gene. □··· signal peptide, ▣··· catalytic domain, ■··· linker domain, ⊠·· substrate binding domain.

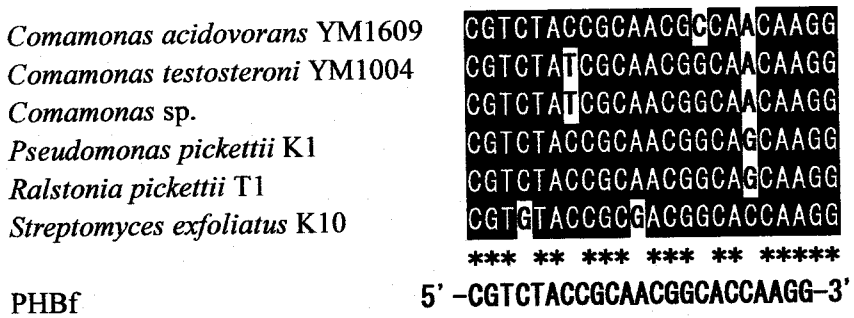


Fig. 3.3. Sequence alignment of the Fn3 linker type-PHB depolymerase genes and design of the forward primer for the Fn3 linker type-PHB depolymerase genes (PHBf). Inverted bases match against those of the primer. *, 100 % match.

Table 3.2. Sequences of the designed PHB primers and probe 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

the target PHB depolymerase gene sequences are summarized in Table 3.1. The anticipated PCR product sizes were 218 bp from *C. acidovorans* YM1609, 215 bp from *Comamonas* sp. and *C. testosteroni* YM1004, and 203 bp from other 3 strains.

Based on homologous region on all the 6 anticipated amplified fragments, DNA probe for the Fn3 linker type-PHB depolymerase genes (PHB probe : PHBp) was also designed (Fig. 3.2 and Table 3.2) on the same principle as for the design of PHBf, except for employing dITP and multiple bases at several positions of divergent bases. The primability of PHBp for the target gene sequences is also shown in Table 3.1.

The results of the FASTA analyses revealed that PHBf showed a homology to chitinaseA gene of *Streptomyces lividas* (GenBank accession No. D13775), proteinase gene of *Streptomyces albogriseolus* (No. D83672), cosmid of *Saccaromyces pombe* (No. AL034433), and 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 depolymerase genes, PCR and Southern hybridization were conducted on 4 strains 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. 3.4A). Additional larger fragment (710 bp) was also amplified from *S. exfoliatus* K10. No fragments were amplified from the strains that served as negative controls.

Among the 3 amplified fragments of the expected sizes, all the 3 showed the positive signals by Southern hybridization with the PHBp (Fig. 3.4B). Furthermore, the nonspecific amplified fragment of larger size from *S. exfoliatus* K10 also showed weakly positive signal by hybridization with PHBp.

Application of PHB primers and PHBp to wild-type PHB-degrading bacteria Fifty-seven PHB-degrading bacteria surrounded by clear zones on the PHB medium were isolated from various environments, and taxonomically characterized. They were considered to belong to *Bacillus*, *Kurthia*, *Rothia*, *Streptococcus*, *Ralstonia pickettii*, *Orchrobactrum anthropi*, *Burkholderia cepacia*, *Sphingomonas paucimobilis*, *Acinetobacter lwoffii*, *Chrysemonas luteola*, *Pseudomonas fluorescens*, *Enterobacter*, and unknown categories (Table 3.3). 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 the 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. No significant interspecific detection bias was observed (Table 3.3).

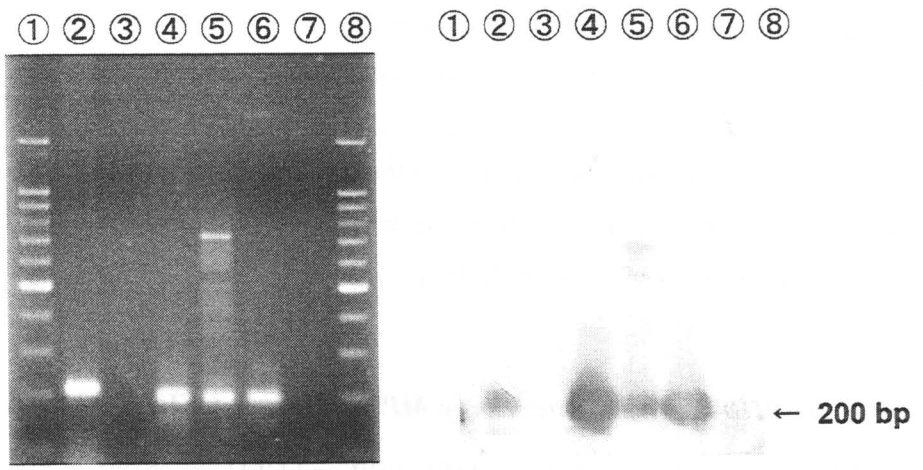


Fig. 3.4. (A) PCR amplification of the PHB depolymerase genes from the authentic strains with PHB primers, and (B) corresponding Southern hybridization with PHBp for identification of the amplified fragments. Lane 1, molecular weight marker; lane 2, *Comamonas* sp.; lane 3, *P. lemoignei*; lane 4, *R. pickettii* T1; lane 5, *S. 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 observed in lane 5 shows false-positive signal.

Table 3.3. 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

Taxonomical groups		PCR[a]	PCR + Hybridization[b]
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%)

[a] Number of strains which showed amplified fragments of expected size.

[b] Number of strains which showed amplified fragments of expected size by PCR, and also positive signal by hybridization.

Enumeration of the PHB-degrading bacteria by MPN-PCR and CFU The enumeration results of the PHB-degrading bacteria by both MPN-PCR and CFU are shown in Fig. 3.5. The extremely sensitive detection results were obtained by MPN-PCR, which gave 100 fold or higher sensitivity than CFU in most of the samples, although CFU was higher than MPN-PCR in one sample from compost reactor. No obvious correlation was observed between the MPN-PCR and CFU, although the samples of higher CFU generally gave higher counts by MPN-PCR.

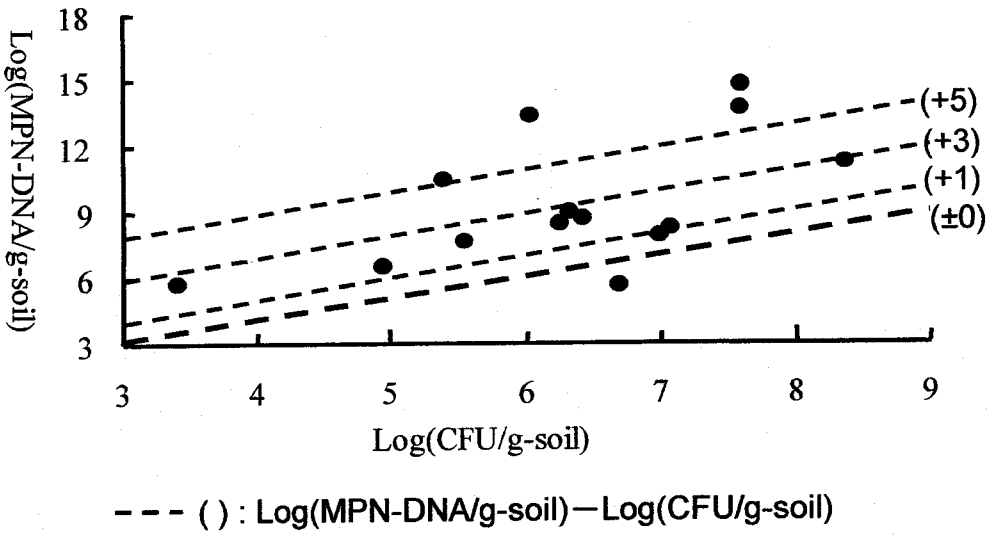


Fig. 3.5. Correlation between MPN-PCR and CFU for enumerating PHB degrading genes and bacteria in a variety of soil environmental samples.

3.4 Discussion

In this chapter, an attempt was made to design a set of PCR primer and DNA probe which would be able to detect a wide range of PHB-degrading bacteria, i.e., PHB depolymerases. There has been no report so far concerning primers and probes for the detection of PHB-degrading bacteria.

The 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, 5 of them were the isozymes possessed by *P. lemoignei*, and therefore 10 kinds of well characterized PHB-degrading bacteria were reported. Among them, 8 possess Fn3 linker type-PHB depolymerases. Since relatively-highly homologous regions were found among 6 of 8 Fn3 linker type-PHB depolymerase gene sequences, the PHB primers were designed based on the sequences of those regions. On the other hand, all the 4 of 4 threonine-rich linker type-PHB depolymerases were only found in *P. lemoignei*. Cadherin-like linker type- and intracellular

type-PHB depolymerases were only found in *P. stutzeri* YM1006 and *Ralstonia eutropha* H16, respectively. These PHB depolymerases possess 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 designed primers and probe, which were designed to be able to detect 6 of 8 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, which exhibit very low primability and stability with PHB primers on the Amplify simulation (Table 3.1), yielded PCR products of the expected sizes. These results imply the primer set can detect all the PHB-degrading bacteria used for the primer design (see Table 3.1). On the other hand, 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, therefore, it was regarded as a negative control strain, suggested that the designed primers are specific enough to detect most of Fn3 linker type-PHB depolymerase genes. However, additional PCR products with larger sizes were amplified from *S. exfoliatus* K10 by the PHB primers, which implies the amplification due to nonspecific binding of the primers. Therefore, Southern hybridization with PHBp was carried out for further specifically identifying the amplified fragments, 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 sequences of *S. exfoliatus* K10.

In total, 57 wild-type PHB-degrading bacteria were isolated from a variety of environment 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 exist a variety of unknown gram positive PHB-degrading bacteria in natural environment. On the other hand, among 33 gram negative PHB-degrading bacteria, 24 strains were identified as pseudomonads. All the 57 isolates were classified into at least 12 taxonomical group (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 can 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 the PHBp. 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 identified by the hybridization was slightly smaller than that by the PCR reaction. This is probably attributable to the lower homology between the probe and target regions, since the amplified fragments obtained by the PCR reaction were of the anticipated sizes. In 46 isolates, additional fragments larger or smaller than the anticipated sizes were amplified, though all of them were excluded by the Southern hybridization with PHBp. Thus, the designed probe seems available for specifying the amplified fragments. The reason for the amplification of the nonspecific fragments from such a greater part of the isolates might be attributed to that the PHB primers were designed from the gene sequences encoding Fn3 linker domain. As the Fn3 type linkers were widely distributed among various bacterial enzymes (Little *et al.*, 1994), possibly these nonspecific fragments seem to be derived from genes encoding other enzymes possessing Fn3 linker domain. On the other hand, no PCR products

were generated using the PHB primers from 7 strains (12.3 %). The possible reasons for the failure of detection are considered to be as follows; (i) these isolates possess other types of PHB depolymerase (i.e. threonine rich linker type- and cadherin-like linker type-PHB depolymerases), (ii) although these isolates possess Fn3 linker type-PHB depolymerase genes, these had lower homology with the designed PHB primers.

For the enumeration of the PHB depolymerase genes in soil samples, MPN-PCR was employed. This study aims at developing the method for the detection and enumeration of PHB depolymerase genes with considerable sequence divergence in a variety of environmental samples, therefore, quantitative PCR methods such as internal standard method and competitive PCR method were considered to be unsuitable. Because (i) the sequence divergence of the target genes might cause the difference of the amplification efficiency, and (ii) PCR inhibitors such as humic substrates might cause severe interference with amplification. On the contrary, MPN-PCR seemed suitable because it contains the dilution step of the DNA extracts, which might reduce the effect of the PCR inhibitors. No obvious correlation was observed between MPN-PCR and CFU of the PHB-degrading bacteria, however, the samples with higher counts by CFU also gave higher counts by MPN-PCR as a whole. MPN-PCR gave extremely higher counts than CFU in 13 of 14 soil samples. Therefore, the MPN-PCR using the designed primer and probe system seems to be applicable for sensitive counting of the PHB degrading bacteria, i.e. PHB degradation potential.

It may be concluded that the primer and probe system established in this chapter is useful for detecting and/or monitoring a majority of PHB-degrading bacteria existing in the natural environment.

3.5 Summary

In this chapter, for rapid and sensitive detection of poly(3-hydroxybutyrate) (PHB) degrading-bacteria, a PCR primer set and a gene probe were designed to be able to amplify

specific fragments from a wide variety of PHB depolymerase genes. The primer set (PHB primers) was designed based on the homologous regions of 6 fibronectin type III linker domain-encoding sequences laid on a variety of PHB depolymerase genes listed in the GenBank. The oligonucleotide probe (PHB probe) was also designed from the internal, homologous regions to specify the amplified fragments. The specificity of the primer set and the probe was confirmed using authentic bacterial strains used for the primer and probe design. Various authentic bacterial strains not carrying PHB depolymerase gene were used as negative controls. PCR using PHB primers amplified DNA fragments with the expected sizes from all the tested 3 PHB depolymerase-carrying bacterial strains used for primer design, and all of the amplified fragments gave positive signals by Southern hybridization with PHB probe. No amplified fragments were observed from negative controls. To evaluate the availability of the designed primers and probe, they were applied to 57 wild-type PHB-degrading bacteria newly isolated from a variety of environments. The designed primer set amplified DNA fragments with expected sizes from 50 of 57 wild-type strains, while the designed probe showed positive signals against the amplified fragments from 47 strains. No obvious correlation was observed between MPN-PCR and CFU of the PHB-degrading bacteria, however, the samples of higher CFU gave higher MPN-PCR result as a whole, and MPN-PCR gave extremely higher counts than CFU in 13 of 14 soil samples. These results suggest that the primer and probe system can detect a considerable proportion of PHB-degrading bacteria, and can be applied to analysis of PHB degradation potential in natural environment.

CHAPTER 4

Design of PCR Primers and Gene Probes for Extensive Detection of Aromatic Compounds-Degrading Bacteria

4.1 Introduction

Aromatic compound-degrading bacteria are of the greatest concern, because aromatic compounds, including chlorinated ones, are frequently a major category of pollutants at sites requiring remediation. For example, a considerable portion of petroleum hydrocarbons which are the most prevalent soil and groundwater contaminants is comprised by aromatic compounds, including BTEX (benzene, toluene, ethylbenzene, xylene) (Mikesell *et al.*, 1993). The second most prevalent hazardous waste, creosote, also consists mainly of aromatic compounds, 85 % polyaromatic hydrocarbons and 12 % phenolic compounds (Cookson, 1995). Further, it is well known that several types of aromatic compound-degrading bacteria possess the ability to co-metabolically degrade trichloroethylene and dichloroethylenes, the main constituents of solvent wastes which are the third most prevalent contaminants (Fujita and Ike, 1997). Thus, the necessity of extensive detection of indigenous aromatic compound-degrading bacteria in most bioremediation activities has been emphasized.

This chapter aims at developing a molecular technique for the extensive detection of aromatic compound-degrading bacteria. Two PCR primer sets and gene probes which can, respectively, detect a variety of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) genes were designed and their applicability was investigated by applying them to both known and newly isolated bacterial strains. Furthermore, as a case study, the behavior of the

aromatic compound-degrading bacteria in aquatic microcosms were monitored during phenol degradation processes using designed PCR primer sets and gene probes. Most of the aromatic compounds, including polyaromatics, are known to be metabolized to a common intermediate, catechol, which is further oxidized through the two ring-cleavage pathways, *ortho* and *meta* cleavage pathways, catalyzed by C12O and C23O, respectively (Fig. 4.1). There are two additional common intermediates, protocatechuic acid and gentisic acid, found in the metabolic pathways of aromatic compounds. However, most of the gene sequences encoding aromatic ring cleavage pathways listed in GenBank are those for catechols. Therefore, C12O and C23O genes seem to be good markers for the detection of a whole range of aromatic compound-degrading bacteria.

4.2 Materials and methods

Bacterial strains and growth conditions Among the authentic bacterial strains known to possess well characterized C12O or C23O genes listed in GenBank (Tables 4.1 and 4.2) (Keller *et al.*, 1984), 6 C12O- and 12 C23O-carrying bacteria (written in bold type in Tables 4.1 and 4.2, respectively) were kindly provided to us by the researchers who isolated the strains. These authentic bacterial strains were used for experimentally evaluating the specificity of the designed primers and probes. Authentic bacterial strains possessing neither C12O nor C23O genes, *Acinetobacter baumannii* ATCC19606, *Acinetobacter calcoaceticus* ATCC23055, *Acinetobacter haemolyticus* ATCC17906, *Acinetobacter johnsonii* ATCC17909, *Acinetobacter lwoffii* ATCC15309, *Bacillus megaterium* ATCC12872, *Escherichia coli* K12, *Flavobacterium breve* IFO14943, *Moraxella bovis* ATCC10900, *Pseudomonas aeruginosa* IFO12689, *Pseudomonas fluorescens* ATCC15553, *Pseudomonas putida* IFO14164, *Vibrio campbellii* ATCC25920 and *Staphylococcus epidermidis* GTC289 served as negative controls for the PCR amplification. Additionally, a total of 106 wild-type aromatic compound-degrading bacteria were newly isolated from a variety of environmental samples, including soils from a garden,

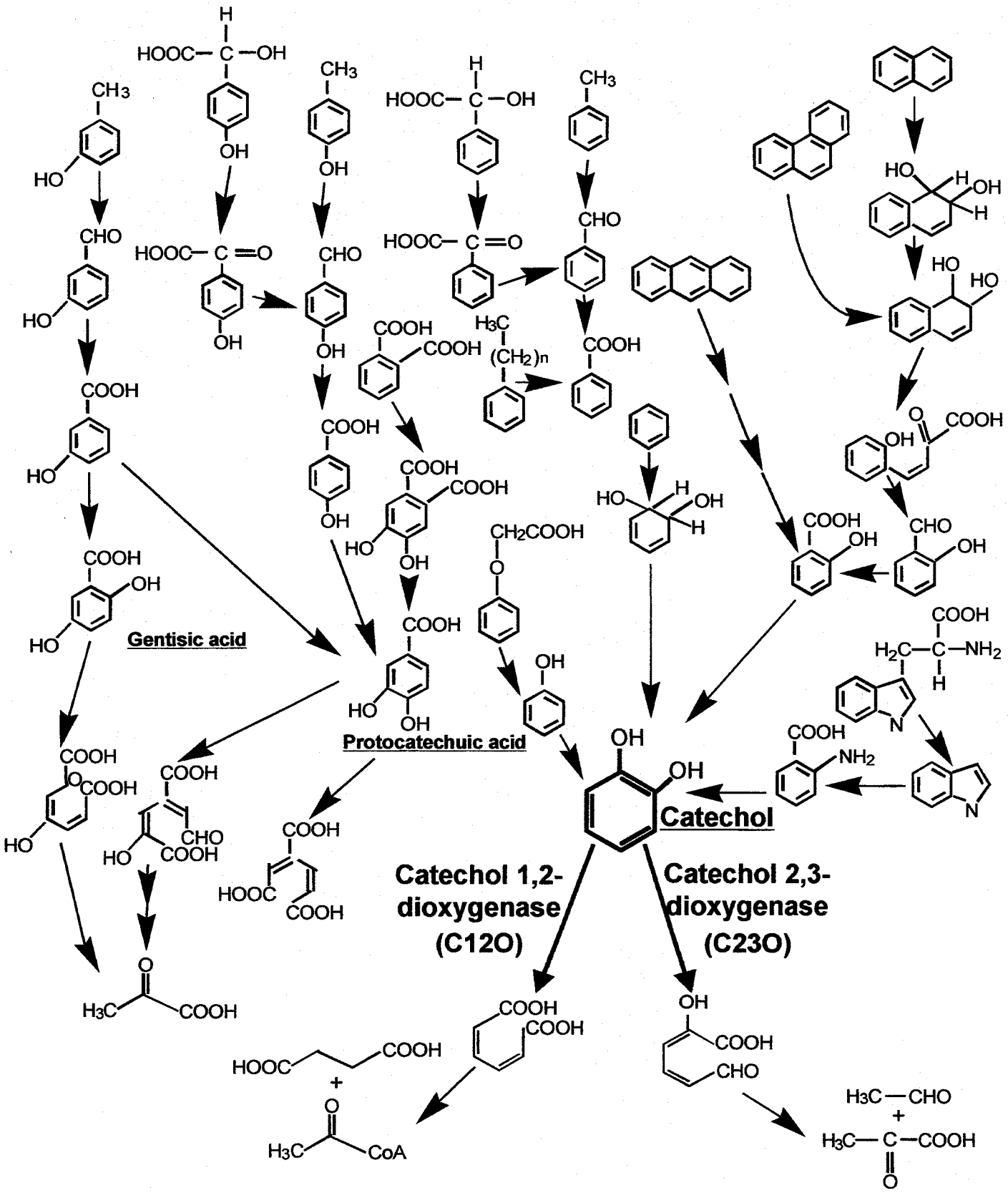


Fig. 4.1 Degradation pathways of aromatic compounds

forests and agricultural fields, ditch sediments, pond waters and sea water. These strains were isolated using a slightly modified mineral medium (Fujita and Ike, 1997) containing 5 mM phenol or benzoate as the sole carbon source. They were morphologically and physiologically characterized, classified, and tentatively identified according to the diagnostic tables of bacteria (Cowan and Steel, 1974). All the bacterial strains were aerobically cultivated at 30 ℃ in Luria Bertani broth (Sambrook *et al.*, 1989), except for *Bacillus stearothermophilus* FDTP3 at 55 ℃.

Design of the primer sets and probes for the extensive detection of the C12O and C23O genes Eleven C12O and 20 C23O gene sequences were obtained from GenBank (Keller *et al.*, 1984). Analyses of the obtained PHB depolymerase gene sequence data, design of the primer set and probe, and evaluation of the designed primers and probe by computer simulation were performed as described in the previous chapter using CLUSTALW (Eddy, 1995), Amplify (Ver.1.2, University of Wisconsin), and FASTA (Pearson and Lipman, 1988).

PCR amplification DNA templates from each bacterial strain were prepared by the DNA extraction method for water samples established in chapter 2 (i.e. proteinase K treatment followed by phenol-chloroform extraction). PCR was conducted for 40 cycles, with denaturation at 94 ℃ for 60 s, annealing for 30 s, and extension at 72 ℃ for 30 s using the Program Temp Control System (PC-800, ASTEC, Fukuoka, Japan). Here, considering that each C12O or C23O target sequence has a different homology score with the designed primers, a step down method was employed (Hecker and Roux, 1996). For the C12O genes, the annealing temperature was 61 ℃ in the first 10 cycles followed by a step down to 59 ℃ in the next 15 cycles, and 57 ℃ in the last 15 cycles, while for the C23O genes, the annealing temperature was 59 ℃ in the first 10 cycles, 57 ℃ in the next 15 cycles, and 55 ℃ in the last 15 cycles. Aliquots (10 μl) of the PCR products were analyzed by electrophoresis on a 1.0 % agarose gel stained with 0.5 μg/ml ethidium bromide.

Southern hybridization Labeling of the designed probes, Southern transfer, hybridization, and the detection of the labeled probes were performed in the same way as described in the

previous chapter.

Table 4.1. Bacterial strains possessing known C12O genes and the primability and stability of the designed C12O primers (C12Of and C12Or) and probe C12Op against the corresponding genes

Bacterial Strains (C12O gene)	C12Of		C12Or		C12Op	References and Accession Numbers of GenBank
	Primability (%)	Stability (%)	Primability (%)	Stability (%)	Primability (%)	
***Acinetobacter calcoaceticus* ADP1 (*catA*)**	**84**	**65**	**78**	**50**	**86**	Neidle *et al.*, 1988 M76991
***Pseudomonas arvilla* C1 (*catA*β)**	**85**	**73**	**99**	**81**	**85**	Nakai *et al.*, 1995 D37783
***Pseudomonas cepacia* CSV90 (pMAB1) (*tfdC*)**	**86**	**48**	**87**	**65**	**74**	Bhat *et al.*, 1994 D16356
***Pseudomonas putida* mt2 (*catA*)**	**85**	**73**	**98**	**78**	**85**	Nakai *et al.*, 1995 D37782
***Pseudomonas putida* (pAC27) (*clcA*)**	**87**	**62**	**82**	**61**	**84**	Frantz and Chakrabarty, 1987 M16964
***Pseudomonas* sp. EST1001 (pEST1226) (*pheB*)**	**76**	**59**	**89**	**65**	**78**	Kivisaar *et al.*, 1991 M57500
Acinetobacter calcoaceticus NCIB8250 (*catA*)	83	57	77	53	80	Ehrt *et al.*, 1995 Z36909
Alcaligenes eutrophus JMP134 (pJP4) (*tfdC*)	86	48	87	65	74	Perkins *et al.*, 1990 X07754
Arthrobacter sp. mA3 (*catA*)	85	60	81	66	75	Eck *et al.*, 1993 M94318
Pseudomonas putida PRS1 (*catA*)	93	80	97	70	79	Stanier *et al.*, 1966 U12557
Pseudomonas sp. P51 (pP51) (*tcbC*)	87	66	88	71	85	van der Meer *et al.*, 1991 M57629

Strains or C12O genes used in the experimental investigation are written in bold type.

Table 4.2. Bacterial strains possessing known C23O genes and the primability and stability of the designed C23O primers (C23Of and C23Or) and probe C23Op against the corresponding genes

Bacterial Strains and Plasmids	C23Of		C23Or		C23Op	References and Accession Numbers of GenBank
	Primability (%)	Stability (%)	Primability (%)	Stability (%)	Primability (%)	
Acinetobacter sp. YAA (_atdB_)	**66**	**43**	**85**	**70**	**82**	Fujii _et al._, 1997 AB008831
Alcaligenes sp. KF711	**73**	**44**	**88**	**67**	**88**	Moon _et al._, 1995 S77084
Bacillus stearothermophilus FDTP3 (_pheB_)	**83**	**49**	**79**	**52**	**50**	Dong _et al._, 1992 X67860
Burkholderia pickettii PKO1 (_tbuE_)	**76**	**50**	**96**	**68**	**67**	Kukor and Olsen, 1996 U20258
Pseudomonas aeruginosa JI104 (_xylE_)	**84**	**60**	**84**	**65**	**89**	Kitayama _et al._, 1996 X60740
Pseudomonas fluorescens IP01 (_cumC_)	-	-	-	-	-	Habe _et al._, 1996 D37828
Pseudomonas putida BH (_pheB_)	**75**	**53**	**97**	**76**	**84**	Takeo _et al._, 1995
Pseudomonas putida MT15 (_cdo_)	**73**	**49**	**66**	**52**	**65**	Keil _et al._, 1985 U01826
Pseudomonas putida mt2 (pWW0) (_xylE_)	**75**	**53**	**87**	**64**	**95**	Nakai _et al._, 1983 M64747
Pseudomonas putida PpG7 (NAH7) (_nahH_)	**77**	**56**	**88**	**68**	**97**	Ghosal _et al._, 1987 M17159
Pseudomonas sp. IC (_bphE_)	**84**	**60**	**84**	**65**	**89**	Carrington _et al._, 1994 U01825
Rhodococcus rhodochrous CTM (pTC1) (_dma_)	**69**	**41**	**74**	**50**	**81**	Candidus _et al._, 1994 X69504
Bacillus subtilis (_xylE_)	75	53	87	64	95	A14852
Beijerinckia yanoikyae B1 (_xylE_)	87	55	90	62	85	Kim and Zylstra, 1995 U23375
Pseudomonas putida H (pPGH1) (_phlH_)	76	56	97	76	84	Herrmann _et al._, 1995 X80765
Pseudomonas putida HS1 (pDK1) (_xylE_)	74	48	87	64	96	Benjamin _et al._, 1991 M65205
Pseudomonas putida KF715	-	-	-	-	-	Lee _et al._, 1995 S78585
Pseudomonas putida P35X (_phhB_)	75	52	97	76	84	Ng _et al._, 1994 X77856
Rhodococcus rhodochrous (_catA_)	-	-	-	-	-	L77225
Sphingomonas sp. HV3 (_catE_)	79	54	93	58	82	Yrjälä _et al._, 1994 L10655

Strains or C23O genes used in the experimental investigation are written in bold type.

-, The scores could not be calculated because of the low homology against the corresponding primers and probe.

Studies using aquatic microcosms Seawater sample was collected from Taisho port, Osaka, Japan, and phenol was added to 250 ml of the samples to be final concentration of 0, 20, and 100 mg/*l* in 500 ml flask. The microcosms were incubated at 30 ℃ on a rotary shaker with 160 rpm for 10 days, and the behavior of aromatic compound-degrading bacteria was monitored every other day by enumerating 16SrDNA, C12O, and C23O genes by MPN-PCR. Phenol concentration and total organic carbon (TOC) were also monitored. For MPN-PCR, DNA templates from aquatic microcosms were prepared by the DNA extraction method for water environmental samples established in Chapter 2 with little modification that 10 ml of samples were filtrated (Isopore polycarbonate membrane filter, pore size 0.2 μm, diameter 47 mm, Millipore, MA, USA) for concentration of the samples (see Fig. 2.2). Extracted DNA was serially diluted by 10-fold, and 3 samples of each diluted step were subjected to PCR under the conditions described above. The number of the targeted DNA was enumerated by the MPN technique. Phenol concentration was analyzed by HPLC (CCPE, Tosoh, Tokyo, Japan) connected with UV detector (UV-8000, Tosoh) at 270 nm. A reverse phase column (TSKgel ODS-80T_M; 4.6 mm × 150 mm, Tosoh) was utilized with a 50 % diluted acetonitrile as eluting solution (1 ml/min.). TOC was analyzed by TOC analyzer (TOC-5000A, Shimadzu, Tokyo, Japan) according to the instructions of manufacturer.

4.3 Results

Design of the primer sets for the extensive detection of the C12O and C23O genes Multiple alignment was performed against 11 C12O and 20 C23O gene sequences, and the C12O and C23O gene sequences were confirmed to be considerably divergent (Figs 4.2 and 4.3). However, 3 and 11 relatively highly homologous regions were found among 11 C12O and 17 C23O gene sequences, respectively, and 2 regions of each which seemed to be suitable for designing primers for the C12O and C23O genes were chosen to design the primers for their extensive detection (Figs. 4.4 and 4.5). Because the concept of the primer design was same as

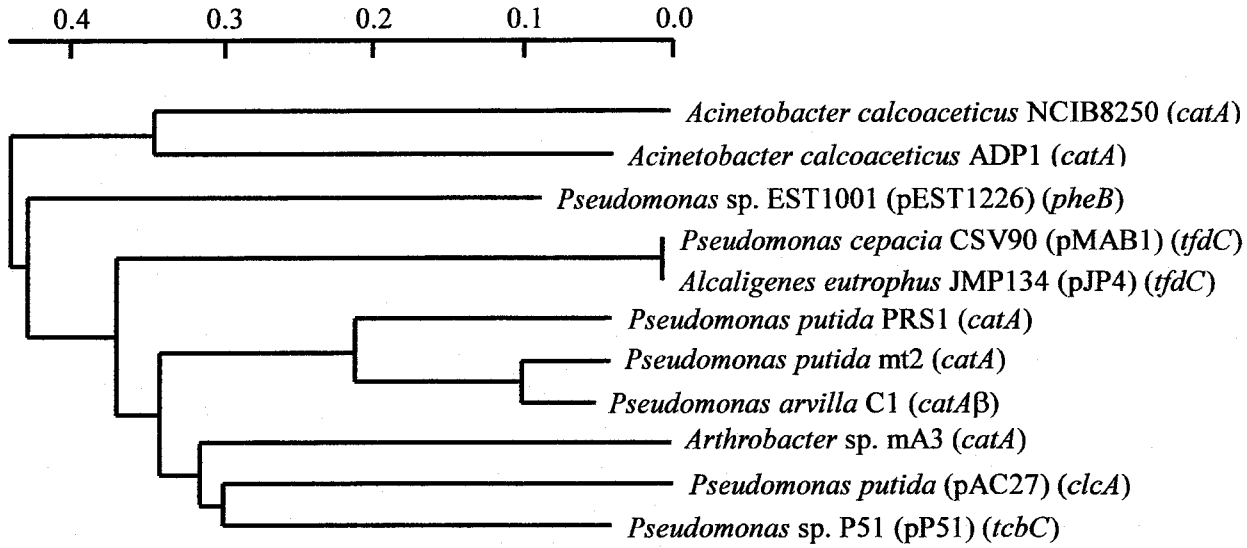


Fig. 4.2. Phylogenetic tree based on the nucleotide sequence of C12O genes. Calculation was performed by NJ method. Scale bar shows the phylogenetic distance calculated as replacement bases/total bases.

the previous chapter, nondominant bases were occasionally introduced instead of dominant ones as shown in Fig. 4.6, where in designing the forward primer for the C12O genes, A was introduced instead of C at the 5th position from 5' for improving the primability and stability of the primer for the C12O gene sequence of *Arthrobacter* sp. mA3. Other primers for the C12O and C23O genes (C12Or, C23Of and C23Or) were also designed based on the same principle. The sequences of the designed primers are shown in Table 4.3.

Simulation results of Amplify revealed that the primer set for the C12O genes (C12O primers) could amplify specific DNA fragments from all the C12O genes used for the design. The primability and stability of the C12O primers for the target C12O gene sequences are summarized in Table 4.1. The anticipated PCR product sizes were 288 bp from *A. calcoaceticus* ADP1 and 282 bp from the other 10 strains. Similarly, the primer set for the C23O genes (C23O primers) was revealed to be capable of amplifying specific DNA fragments from all the C23O genes used for the design (Table 4.2). The anticipated amplified fragment sizes were 377 bp from *Alcaligenes* sp. KF711, 383 bp from *Bacillus stearothermophilus* FDTP3, *Beijerinckia yanoikyae* B1, *Rhodococcus rhodochrous* CTM (pTC1) and *Sphingomonas* sp. HV3, 407 bp

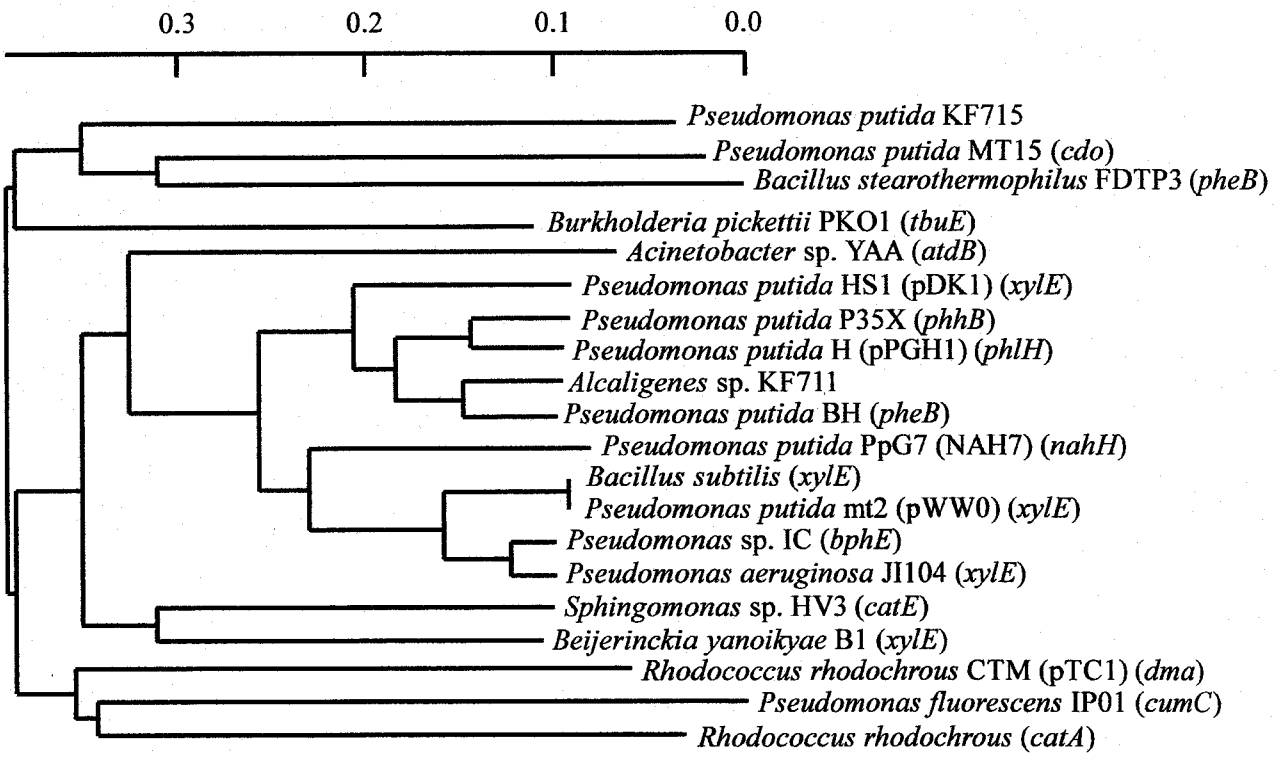


Fig. 4.3. Phylogenetic tree based on the nucleotide sequence of C23O genes. Calculation was performed by NJ method. Scale bar shows the phylogenetic distance calculated as replacement bases/total bases.

from *Burkholderia pickettii* PKO1 and *P. putida* MT15, and 380 bp from the other 10 strains.

Based on homologous regions of the anticipated amplified fragments, DNA probes for the C12O genes (C12O probe : C12Op) and C23O genes (C23O probe : C23Op) were also designed (Figs. 4.4 and 4.5, and Table 4.3) based on the same principle as for the design of C12Of. The primability of C12Op and C23Op for the target gene sequences is also shown in Tables 4.1 and 4.2, respectively. The results of the FASTA analyses revealed that the designed primer sets only showed homology with genes related to the C12O and/or C23O genes, except for C23Of, which showed a homology to *S*-adenosylmethionine-dependent methylase gene of *Rhodococcus erythropolis* (GenBank accession number L21196).

Evaluation of the C12O primers and C12Op using authentic strains To experimentally confirm the possibility of extensive and specific detection of the C12O genes, PCR and

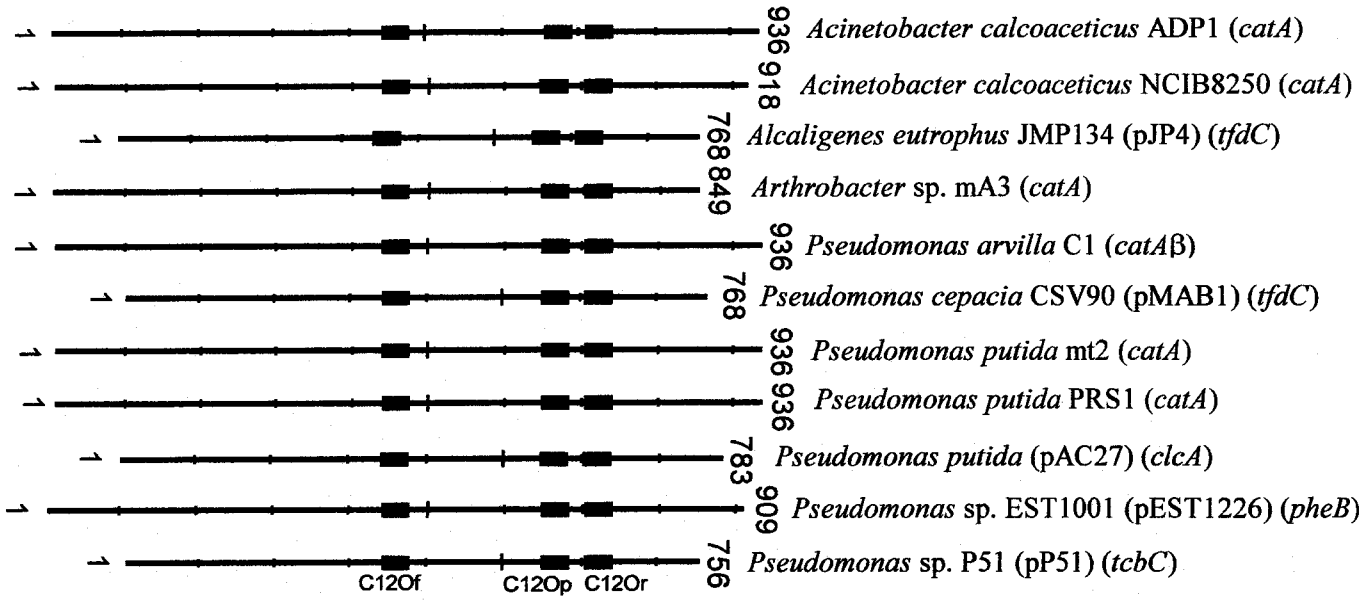


Fig. 4.4. Relatively highly homologous regions of the C12O genes. The homologous regions among 11 of all the C12O genes are indicated by squared boxes. Names below the homologous regions show the priming region of each primer and probe. The nucleotide sequences are numbered from the initiation to the termination codon of each C12O gene.

Southern hybridization were conducted on 6 strains possessing the C12O gene listed in the GenBank. Among the 6 strains, 5 (*Pseudomonas arvilla* C1, *Pseudomonas cepacia* CSV90 (pMAB1), *P. putida* DC15 (pDC15), *P. putida* mt2, and *P. putida* KT2442 (pAT1141-CatR)) showed the amplified fragments with expected sizes (Fig. 4.7A). *P. cepacia* CSV90 (pMAB1) showed additional larger fragments (576 bp and 407 bp) and *A. calcoaceticus* ADP1 only showed a larger fragment (407 bp). On the other hand, no amplified fragments were observed from the strains that served as the negative controls (data not shown).

Among the 5 amplified fragments of the expected size, 4 showed positive signals by Southern hybridization with the C12Op, although that from *P. cepacia* CSV90 (pMAB1) did not (Fig. 4.7B). Amplified fragments of larger sizes did not hybridize to the C12Op.

Evaluation of the C23O primers and C23Op using authentic strains To experimentally confirm the possibility of extensive and specific detection of the C23O genes, PCR and Southern hybridization were conducted on 12 strains possessing the C23O gene listed in the

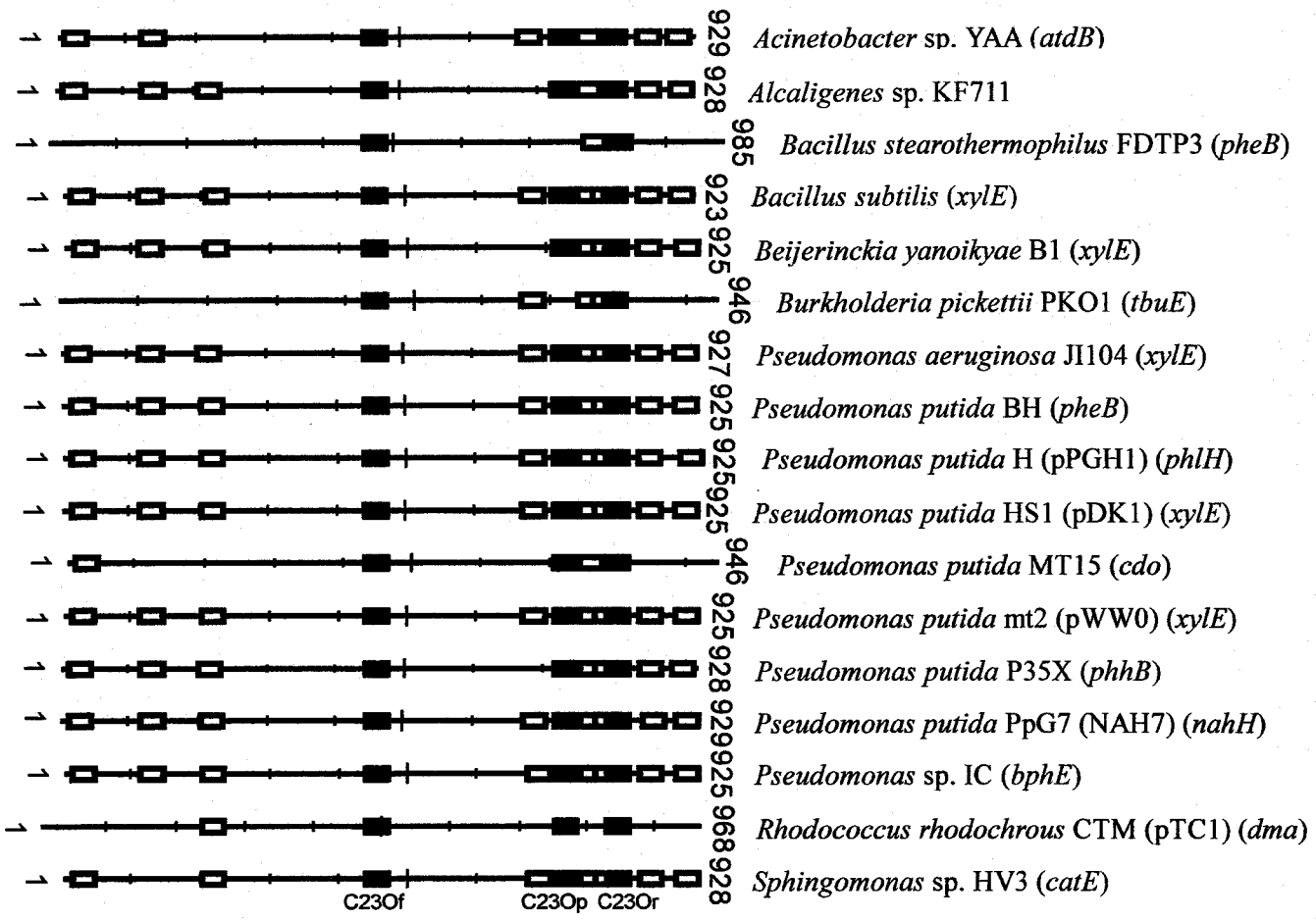


Fig. 4.5. Relatively highly homologous regions of the C23O genes. The homologous regions among most of the C23O genes are indicated by squared boxes. Names below the homologous region show the priming regions of each primer and probe. The nucleotide sequences are numbered from the initiation to the termination codon of each C23O gene.

Table 4.3. Sequences of the designed C12O and C23O primers, and probes C12Op and C23Op

Primers and probes	Sequences	Length (bp)	GC contents (%)
C12Of	5'-GCCAACGTCGACGTCTGGCA-3'	20	65
C12Or	5'-CGCCTTCAAAGTTGATCTGCGTGGT-3'	25	52
C12Op	5'-GGCGGTCATGGCTAGCGCCCCGCGCACGTTCA-3'	32	72
C23Of	5'-AAGAGGCATGGGGGCGCACCGGTTCGATCA-3'	30	63
C23Or	5'-CCAGCAAACACCTCGTTGCGGTTGCC-3'	26	62
C23Op	5'-CGTGAGTAATGCCGTGACTGGTCGGGCCGATATCGA-3'	36	58

GenBank. Fragments of the expected sizes were amplified from all the tested strains except for *P. fluorescens* IP01, which was not used for the designing of the primer and the probe (Fig. 4.8A). In 4 strains, additional larger fragments were also amplified (2110 bp from *B. pickettii* PKO1, 982 bp from *P. putida* BH, 415 bp from *P. putida* MT15, and 1301 bp from *P. putida* mt2 (pWW0)). No fragments were amplified from the strains that served as the negative controls.

Among the 11 amplified fragments of the expected sizes, 9 showed positive signals by Southern hybridization with the C23Op (Fig. 4.8B) ; those from *B. pickettii* PKO1 and *R. rhodochrous* CTM (pTC1) were not hybridized. Furthermore, the nonspecific amplified fragments of larger sizes were also not detected by hybridization except for that from *P. putida* BH (982 bp).

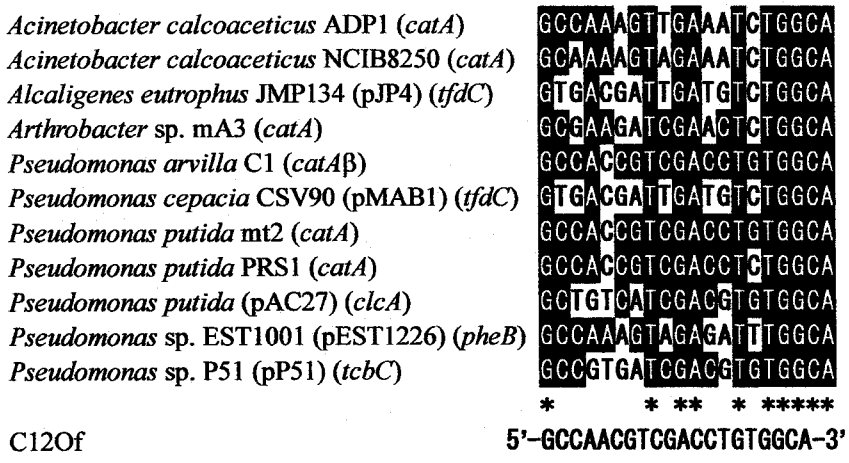


Fig. 4.6. Sequence alignment of C12O genes and design of the forward primer for the C12O genes (C12Of).
The inverted bases match with those of the primer. ✳, 100% match.

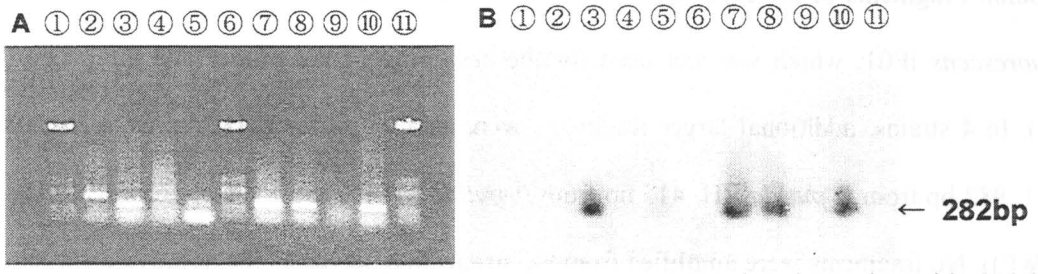


Fig. 4.7. (A) PCR amplification of the C12O genes from authentic strains with C12O primers, and (B) corresponding Southern hybridization with C12Op for identification of the amplified fragments. Lane 1, Molecular weight marker; lane 2, *A. calcoaceticus* ADP1; lane 3, *P. arvilla* C1; lane 4, *P. cepacia* CSV90 (pMAB1); lane 5, *P. putida* DC15 (pDC15) carrying C12O gene derived from *P. putida* (pAC27); lane 6, molecular weight marker; lane 7, *P. putida* mt2; lane 8, *P. putida* KT2442 (pAT1141-CatR) carrying C12O gene derived from *Pseudomonas* sp. EST1001 (pEST1226); lane 9, negative control (no DNA templates); lane 10, positive control (*P. arvilla* C1); lane 11, molecular weight marker. Amplified fragments and positive signals of Southern hybridization corresponding to the calculated sizes are observed around 282 bp. The amplified fragments of larger sizes observed in lanes 2 and 6 were excluded after Southern hybridization with C12Op.

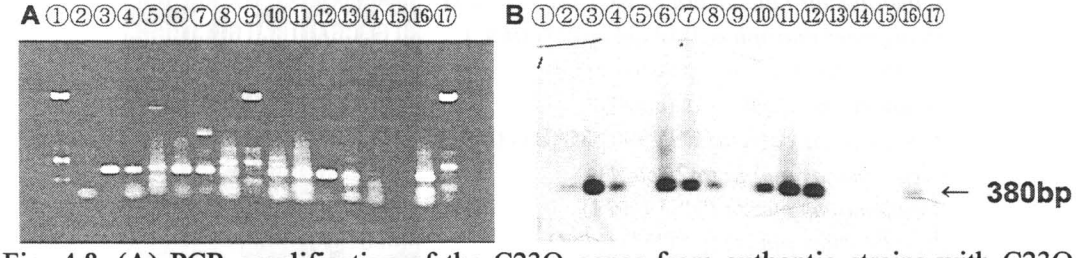


Fig. 4.8. (A) PCR amplification of the C23O genes from authentic strains with C23O primers, and (B) corresponding Southern hybridization with C23Op for identification of the amplified fragments. Lane 1, Molecular weight marker; lane 2, *Acinetobacter* sp. YAA; lane 3, *Alcaligenes* sp. KF711; lane 4, *B. stearothermophilus* FDTP3; lane 5, *B. pickettii* PKO1; lane 6, *P. aeruginosa* JI104; lane 7, *P. putida* BH; lane 8, *P. putida* MT15; lane 9, molecular weight marker; lane 10, *P. putida* mt2 (pWW0); lane 11, *P. putida* PpG1064 (NAH) carrying C23O gene derived from *P. putida* PpG7 (NAH7); lane 12, *Pseudomonas* sp. IC; lane 13, *R. rhodochrous* CTM (pTC1); lane 14, *P. fluorescens* IP01; lane 15, negative control (no DNA template); lane 16, positive control (*Pseudomonas* sp. IC); lane 17, molecular weight marker. Amplified fragments and positive signals of Southern hybridization corresponding to the calculated sizes were observed around 380 bp. The amplified fragments of larger sizes observed in lanes 5, 7, 8 and 10 were excluded by Southern hybridization with C23Op, though a false-positive signal is observed in lane 7.

Application of C12O and/or C23O primers and probes to wild-type aromatic compound-degrading bacteria Seventy phenol- and 36 benzoate-degrading bacteria were isolated from various environmental samples, and taxonomically characterized. They were considered to belong to *Pseudomonas*, *Flavobacterium*, *Acinetobacter*, *Micrococcus*, *Bacillus*, *Cardiobacterium*, *Streptococcus*, *Haemophilus*, *Kurthia*, *Neisseria*, *Rothia*, *Branhamella*, *Corynebacterium*, *Lactobacillus*, *Listeria*, *Mycobacterium* and unknown categories. The designed primers and probes were applied to all the wild-type isolates (Tables 4.4 and 4.5). Among 70 phenol-degrading bacteria, fragments of the anticipated sizes were amplified from 41 strains (58.6 %) using C12O and/or C23O primers. When the C12Op or C23Op was used for hybridization against the corresponding amplified fragments, 35 strains (50.0 %) showed positive signals. On the other hand, among 36 benzoate-degrading bacteria, fragments of the anticipated sizes were amplified from 29 strains (80.6 %) using C12O and/or C23O primers. When the C12Op or C23Op was used for hybridization against the corresponding amplified fragments, 28 strains (77.8 %) showed positive signals. In total, amplified fragments of the expected sizes were obtained from 70 aromatic compound-degrading bacteria (66.0 %) of the 106 isolates using the C12O and/or C23O primers, and positive hybridization signals with the C12Op and/or C23Op were obtained from 63 strains (59.4 %). Fragments larger or smaller than the anticipated sizes were amplified from 6 strains, however these did not show positive signals with the C12Op and/or C23Op. No significant interspecific detection bias was observed (Table 4.4).

Monitoring the behavior of the aromatic compound-degrading bacteria in aquatic microcosms Results of the monitoring the behavior of the aromatic compound-degrading bacteria in aquatic microcosms by MPN-PCR with the C12O and C23O primer sets are shown in Fig. 4.9. When the initial phenol concentration was low (20 mg/*l*) (Fig. 4.9(B)), C12O genes were increased at the early stage and phenol was completely degraded during this period. On the contrary, C23O genes were kept constant throughout the experimental period. When the initial

Table 4.4. Results of the taxonomical classification of 106 phenol- and benzoate-degrading bacteria, and number of the strains detected by PCR and Southern hybridization

Genera (Number of isolates)	C12O (PCR / Hybridization)	C23O (PCR / Hybridization)	Both C12O+C23O (PCR / Hybridization)	Total detected (PCR / Hybridization)
Phenol-degrading bacteria				
Micrococcus (14)	6 (42.9 %) / 5 (35.7 %)	8 (57.1 %) / 8 (57.1 %)	4 (28.6 %) / 3 (21.4 %)	10 (71.4 %) / 10 (71.4 %)
Bacillus (9)	7 (77.8 %) / 4 (44.4 %)	3 (33.3 %) / 3 (33.3 %)	3 (33.3 %) / 2 (22.2 %)	7 (77.8 %) / 5 (55.6 %)
Pseudomonas (6)	1 (16.7 %) / 0 (0 %)	4 (40.0 %) / 4 (40.0 %)	3 (30.0 %) / 3 (30.0 %)	5 (50.0 %) / 4 (40.0 %)
Cardiobacterium (5)	4 (80.0 %) / 4 (80.0 %)	1 (20.0 %) / 1 (20.0 %)	1 (20.0 %) / 1 (20.0 %)	4 (80.0 %) / 4 (80.0 %)
Streptococcus (5)	3 (60.0 %) / 3 (60.0 %)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)	3 (60.0 %) / 3 (60.0 %)
Flavobacterium (3)	1 (33.3 %) / 1 (33.3 %)	1 (33.3 %) / 1 (33.3 %)	1 (33.3 %) / 1 (33.3 %)	1 (33.3 %) / 1 (33.3 %)
Haemophilus (3)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)
Kurthia (3)	2 (66.7 %) / 1 (33.3 %)	1 (33.3 %) / 1 (33.3 %)	2 (66.7 %) / 1 (33.3 %)	1 (33.3 %) / 1 (33.3 %)
Rothia (3)	1 (33.3 %) / 1 (33.3 %)	2 (66.7 %) / 2 (66.7 %)	1 (33.3 %) / 1 (33.3 %)	2 (66.7 %) / 2 (66.7 %)
Acinetobacter (2)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)
Branhamella (2)	0 (0 %) / 0 (0 %)	2 (100 %) / 2 (100 %)	0 (0 %) / 0 (0 %)	2 (100 %) / 2 (100 %)
Corynebacterium (1)	1 (100 %) / 1 (100 %)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)	1 (100 %) / 1 (100 %)
unknown (11)	4 (36.4 %) / 1 (9.1 %)	5 (45.5 %) / 4 (36.4 %)	3 (27.3 %) / 1 (9.1%)	6 (54.5 %) / 4 (36.4 %)
Total phenol-degrading bacteria (70)	31 (44.3 %) / 22 (31.4 %)	25 (35.7 %) / 24 (34.3 %)	15 (21.4 %) / 11 (15.7 %)	41 (58.6 %) / 35 (50.0 %)
Benzoate-degrading bacteria				
Bacillus (12)	4 (33.3 %) / 3 (25.0 %)	9 (75.0 %) / 8 (66.7 %)	4 (33.3 %) / 3 (25.0 %)	9 (75.0 %) / 8 (66.7 %)
Corynebacterium (6)	3 (50.0 %) / 1 (16.7 %)	4 (66.7 %) / 4 (66.7 %)	2 (33.3 %) / 0 (0 %)	5 (83.3 %) / 5 (83.3 %)
Pseudomonas (4)	3 (75.0 %) / 3 (75.0 %)	4 (100 %) / 4 (100 %)	3 (75.0 %) / 3 (75.0 %)	4 (100 %) / 4 (100 %)
Mycobacterium (3)	1 (33.3 %) / 1 (33.3 %)	1 (33.3 %) / 1 (33.3 %)	1 (33.3 %) / 1 (33.3 %)	1 (33.3 %) / 1 (33.3 %)
Rothia (2)	0 (0 %) / 0 (0 %)	1 (50.0 %) / 1 (50.0 %)	0 (0 %) / 0 (0 %)	1 (50.0 %) / 1 (50.0 %)
Acinetobacter (1)	1 (100 %) / 1 (100 %)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)	1 (100 %) / 1 (100 %)
Flavobacterium (1)	0 (0 %) / 0 (0 %)	1 (100 %) / 1 (100 %)	0 (0 %) / 0 (0 %)	1 (100 %) / 1 (100 %)
Haemophilus (1)	1 (100 %) / 1 (100 %)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)	1 (100 %) / 1 (100 %)
Kurthia (1)	1 (100 %) / 1 (100 %)	1 (100 %) / 1 (100 %)	1 (100 %) / 1 (100 %)	1 (100 %) / 1 (100 %)
Lactobacillus (1)	0 (0 %) / 0 (0 %)	1 (100 %) / 1 (100 %)	0 (0 %) / 0 (0 %)	1 (100 %) / 1 (100 %)
Listeria (1)	1 (100 %) / 1 (100 %)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)	1 (100 %) / 1 (100 %)
Streptococcus (1)	1 (100 %) / 1 (100 %)	0 (0 %) / 0 (0 %)	0 (0 %) / 0 (0 %)	1 (100 %) / 1 (100 %)
unknown (2)	2 (100 %) / 2 (100 %)	2 (100 %) / 2 (100 %)	2 (100 %) / 2 (100 %)	2 (100 %) / 2 (100 %)
Total benzoate-degrading bacteria(36)	18 (50.0 %) / 15 (41.7%)	24 (66.7 %) / 23 (63.9 %)	13 (36.1 %) / 10 (27.8%)	29 (80.6 %) / 28 (77.8 %)
Total isolates(106)	49 (46.2 %) / 37 (34.9 %)	49 (46.2 %) / 47 (44.3 %)	28 (26.4 %) / 21 (19.8 %)	70 (66.0 %) / 63 (59.4 %)

Table 4.5. Summary of the results of PCR and Southern hybridization against phenol- and benzoate- degrading bacteria

Character	PCR[a]	PCR+Hybridization[b]
Phenol-degrading bacteria (70 strains)		
C12O+	30 (42.9 %)	22 (31.4 %)
C23O+	25 (35.7 %)	24 (34.3 %)
Both C12O+ and C23O+	15 (21.4 %)	11 (15.7 %)
Total detected strains	40 (57.1 %)	35 (50.0 %)
Benzoate-degrading bacteria (36 strains)		
C12O+	18 (50.0 %)	15 (41.7 %)
C23O+	24 (66.7 %)	23 (63.9 %)
Both C12O+ and C23O+	13 (36.1 %)	10 (27.8 %)
Total detected strains	29 (80.6 %)	28 (77.8 %)
Total (106 strains)		
C12O+	48 (45.3 %)	37 (34.9 %)
C23O+	49 (46.2 %)	47 (44.3 %)
Both C12O+ and C23O+	28 (26.4 %)	21 (19.8 %)
Total detected strains	69 (65.1 %)	63 (59.4 %)

[a] Number of the strains which showed the amplified fragment with expected size of corresponding genes.

[b] Number of the strains which showed the amplified fragment with expected size by PCR and also showed positive signal by hybridization of corresponding genes.

phenol concentration was high (100 mg/*l*) (Fig. 4.9(C)), in addition to the increase of the C12O genes at the early stage, C23O genes were also increased from the middle period to the latter period. In this experiment, phenol remained at a certain concentration even after 10days though gradually decreased. All the parameters in the control experiment (i.e., 0 mg/*l*-phenol : Fig. 4.9(A)) were almost unchanged throughout the experimental period.

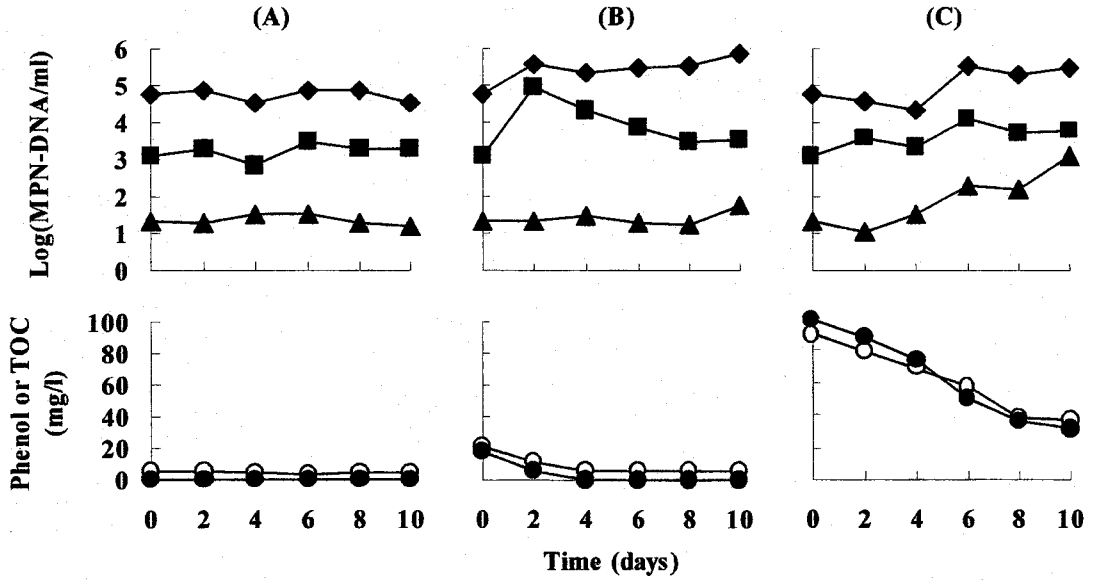


Fig. 4.9. Monitoring the phenol degradation processes in aquatic microcosms constructed using seawater from Taisho port . Initial phenol concentrations were (A) 0 mg/*l*, (B) 20 mg/*l*, and (C) 100 mg/*l*. Symbols; ◆: 16SrDNA, ■: C12O DNA, ▲: C23O DNA, ●: phenol, ○: TOC

4.4 Discussion

In this chapter, an attempt was made to design a set of PCR primers and DNA probes which would be able to detect a whole range of aromatic compound-degrading bacteria. The target functional genes for the PCR amplification and DNA hybridization were those encoding C12O and C23O, which are the key enzymes in a common pathway of aromatic compound degradation, namely, the catechol cleavage pathway.

There has been no report until now of primers and probes for the detection of C12O genes, although primers and probe specific for the chlorocatechol 1,2-dioxygenase genes in the β-subdivision of proteobacteria have been reported recently (Kleinsteuber *et al.*, 1998). On the other hand, there have been some reports on the application of PCR amplification targeting the C23O genes for evaluating the aromatic degradation potentials of the indigenous microbes in petroleum-contaminated groundwater (Joshi and Walia, 1996a), a jet fuel JP-5-contaminated

remediation site (Chandler and Brockman, 1996), and a polyaromatic-contaminated sediment undergoing *ex situ* composting (Wikström *et al.*, 1996). Although the researchers designed the primers and/or probes using dITP and multiple bases to compensate for the divergent bases, the primer sets designed in these studies intended to cover only a limited proportion of C23O-carrying bacteria. For example, Chandler and Brockman designed a primer set targeting the C23O genes only in *Pseudomonas* strains (Chandler and Brockman, 1996). Based on a theoretical calculation using Amplify, this primer set could amplify specific DNA fragments from 13 among the 20 known C23O gene sequences (calculation data not shown), which had so far been the most extensive detection of C23O genes by PCR amplification.

The concept for the primer design in this study was to detect as many kinds of C12O and C23O gene sequences as possible. Therefore, all of the known C12O and C23O genes listed in the GenBank were multiple-aligned, and their relatively highly homologous regions were selected. As a result, all the 11 of 11 C12O and 17 of 20 C23O gene sequences were taken into consideration for the design of the C12O and C23O primers. Based on the calculation by Amplify, the primer sets could theoretically generate specific DNA fragments from all the corresponding gene sequences referred for their design. DNA probes were also designed from the internal, homologous regions to further specifically identify the amplified fragments. The aforementioned theoretical calculation suggests the possibility of the detection of all the 11 of the 11 C12O-derived fragments and 15 of the 17 C23O-derived fragments with the designed C12O and C23O probes, respectively.

In order to experimentally confirm the specific detection of C12O and C23O genes, the designed primers and probes were applied on authentic bacterial strains known to carry the C12O and/or C23O genes. The C12O and C23O primers generated PCR products of the expected sizes from all the tested authentic C12O and/or C23O-carrying strains, with the exception, *A. calcoaceticus* ADP1. It should be emphasized that *P. putida* KT2442 (pAT1141-CatR), *P. cepacia* CSV90 (pMAB1) and *P. putida* DC15(pDC15), the C12O genes of

which exhibit very low primability and stability with C12O primers (Table 4.1), and *Acinetobacter* sp. YAA, *R. rhodochrous* CTM (pTC1) and *P. putida* MT15, the C23O genes of which exhibit very low primability and stability with C23O primers (Table 4.2), yielded PCR products of the expected sizes. These results imply the possibility of the detection of strains not used in experimental investigation, because the primability and stability of the primers for most of these are higher than that for the strains described above (Tables 4.1 and 4.2). On the other hand, no PCR product was obtained from negative control strains. Although *P. fluorescens* IP01 has been known to possess a C23O gene, its sequence was not taken into consideration for the primer design, therefore, it was regarded as a negative control strain. These results experimentally confirmed that the designed primers are sufficiently specific enough to detect most of the C12O and C23O genes used for their design. However, the C12O primers did not generate DNA fragments of the anticipated sizes, but a larger fragment from *A. calcoaceticus* ADP1. Further, additional PCR products with larger sizes were amplified from *P. cepacia* CSV90 (pMAB1) by the C12O primers and from *P. pickettii* PKO1, *P. putida* BH, *P. putida* MT15 and *P. putida* mt2 (pWW0) by the C23O primers. Observation of such fragments implies amplification due to nonspecific binding of the primers. Therefore, Southern hybridization with C12O or C23O probes were carried out for further specifically identifying the amplified fragments. The designed probes could exclude all the unexpected larger fragments as negative signals except for that from *P. putida* BH, a larger fragment from which showed a weak positive signal. A possible reason for this false-positive hybridization may be that C23Of, which has a lower primability than C23Or, bound to an upper site of the target region, and consequently that the larger fragment including the target region was amplified. On the other hand, 13 of the 16 PCR products of the expected sizes gave positive signals with the C12Op or C23Op; those from *P. cepacia* CSV90 (pMAB1), *B. pickettii* PKO1 and *R. rhodochrous* CTM however did not due to the absent or lower primability between those fragments and the corresponding probes. Thus, the designed probes seem available for specifying amplified fragments derived from a

considerably diverse number of C12O and C23O genes, but not all.

In total, 106 wild-type phenol- and benzoate-degrading bacteria were isolated and used for evaluating how extensively the designed primers and probes can detect aromatic compound-degrading bacteria. The 106 isolates were classified into at least 16 taxonomical groups (genera), indicating that the library included various types of aromatic compound-degrading bacteria and that it was suitable for evaluating the universality of the primers and probes. PCR products of the expected sizes were amplified from 70 isolates (66.0 %) with the C12O or C23O primers, and the amplified fragments from 63 isolates (59.4 %) were hybridized with the corresponding probes. The number of positive strains identified by the hybridization was slightly smaller than that by the PCR reaction. This is probably attributable to the lower homology between the probes and the target regions, since the amplified fragments obtained by the PCR reaction were of the anticipated sizes. On the other hand, no PCR products of the anticipated sizes were generated using the C12O and/or C23O primers from 37 isolates (34.9 %). The possible reasons for this failure of detection are considered to be as follows; (i) although these isolates possess C12O and/or C23O genes, these exhibit lower homology with the C12O and/or C23O primers, (ii) these isolates do not have catechol catabolic pathways and, instead, use other pathways such as the protocatechuate and/or gentisate pathways for aromatic compound degradation, (iii) these isolates possess neither C12O nor C23O genes, but have genes encoding a novel catechol catabolic enzyme(s) (Nordlund *et al.*, 1993). Considering that the screened library should include diverse bacterial species, and no significant interspecific detection bias was observed, the designed primers and probes are useful for detecting a major proportion of the aromatic compound-degrading bacteria existing in natural environments.

As a case study, aromatic compound-degrading bacteria were monitored during phenol degradation processes in aquatic microcosms using designed primer sets. It seems that against the relatively low phenol load C12O-carrying bacterial strains were mainly responsible for the

phenol degradation, while against the relatively high phenol load, C23O-carrying bacterial strains gradually increased and contributed to phenol degradation. It was reported that *Alcaligenes eutrophus* JMP134 (pJP4), which possess both catechol *ortho* and *meta* cleavage pathways (i.e., both C12O and C23O genes), exclusively expressed C12O activity at low phenol load, while C23O activity as well as C12O activity was strongly expressed at high phenol load. Though the experiment of the previous report was performed by single strain, and therefore, the result can not be simply compared with the present study, there might be a possibility that the similar phenomenon occurred in the complex microbial community. As it is laborious to monitor the behavior of C12O- and C23O-carrying bacterial strains separately by phenotypic methods, it can be concluded that the primer and probe system established in this chapter is available for the detailed clarification of the behavior of aromatic compound-degrading bacteria.

4.5 Summary

In this chapter, for the extensive detection of bacterial populations capable of degrading aromatic compounds, two PCR primer sets were designed which can, respectively, amplify specific fragments from a wide variety of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) genes. The C12O-targeting primer set (C12O primers) was designed based on the homologous regions of 11 C12O genes listed in the GenBank, while the C23O-targeting one (C23O primers) was designed based on those of 17 known C23O genes. Oligonucleotide probes (C12Op and C23Op) were also designed from the internal homologous regions to identify the amplified fragments. The specificity of the primer sets and probes was confirmed using authentic bacterial strains known to carry the C12O and/or C23O genes used for the primer and probe design. Various authentic bacterial strains carrying neither C12O nor C23O genes were used as negative controls. PCR with the C12O primers amplified DNA fragments of the expected sizes from 5 of the 6 known C12O-carrying bacterial strains tested, and positive signals were obtained from 4 of the 5 amplified fragments on Southern

hybridization with the C12Op. The C23O primers amplified DNA fragments of the expected size from all the 11 tested C23O-carrying bacterial strains used for their design, while the C23Op detected positive signals in the amplified fragments from 9 strains. On the other hand, no DNA fragments were amplified from the negative controls. To evaluate the applicability of the designed primers and probes for the extensive detection of aromatic compound-degrading bacteria, they were applied to wild-type phenol- and/or benzoate-degrading bacteria newly isolated from a variety of environments. The C12O and/or C23O primers amplified DNA fragments of the expected sizes from 69 of the 106 wild-type strains tested, while the C12Op and/or C23Op detected positive signals in the amplified fragments from 63 strains. The result of the monitoring aromatic compound-degrading bacteria in aquatic microcosms clarified the behaviors of the C12O- and C23O-carrying bacterial strains separately. These results suggest that the primer and probe system established in this chapter can detect a considerable portion of bacteria which can degrade aromatic compounds *via* catechol cleavage pathways and is applicable for monitoring the behavior of them in the natural environment.

CHAPTER 5

Summary and Conclusions

Microbial monitoring is essential for understanding the behavior of the bacterial populations responsible for degradation of target pollutants and evaluating the biodegradation potential. In this study, for the development of microbial monitoring methods based on molecular detection techniques, especially detecting and enumerating corresponding functional catabolic genes by PCR, investigations were performed on DNA extraction from a variety of environmental samples, design of PCR primers and gene probes for the detection of the functional catabolic genes, and their application for enumerating and monitoring target bacterial populations responsible for the biodegradation.

In Chapter 2, for the establishment of simple and rapid DNA extraction methods for PCR-based monitoring of microbial community in the water/soil environment, several kinds of methods were comparatively investigated for their DNA extracting capability. For water samples, cell lysis with proteinase K followed by phenol-chloroform extraction and ethanol precipitation allowed to detect the target bacterium at a sensitivity at 10^1 cells/ml against backgrounds of indigenous bacteria at 10^4-10^5 cfu/ml with the DNA recovery of ca. 25-55 %. For soil samples, cell lysis with ultrasonication in addition to the use of the proteinase K and SDS in the presence of a high concentration of chelating agent followed by phenol-chloroform extraction, ethanol precipitation, and spun column purification was the most effective, which allowed to detect the target bacterium at a sensitivity at 10^1-10^2 cells/g-soil against backgrounds of indigenous bacteria at 10^7-10^9 cfu/g-soil with the DNA recovery of ca. 80-95 %.

In Chapter 3, for rapid and sensitive detection of PHB degrading bacteria, a PCR primer set and a gene probe were designed based on the homologous region of 6 Fn3 linker domain-encoded sequences laid on a variety of PHB depolymerase genes listed in GenBank to

be able to amplify specific fragments from a wide variety of PHB depolymerase genes. Their specificity was confirmed using authentic bacterial strains and availability was confirmed by detecting over 80 % of wild-type PHB-degrading bacteria. The applicability of MPN-PCR was also confirmed as the samples of higher CFU gave higher MPN-PCR results and MPN-PCR gave extremely sensitive results than CFU, however, no obvious correlation was observed.

In Chapter 4, for the extensive detection of bacterial populations capable of degrading aromatic compounds, two PCR primer sets and probes, which can detect a wide variety of C12O and C23O genes, were designed. The C12O primer set and probe were designed based on the homologous region of 11 C12O genes and the C23O-targeting ones were of 17 C23O genes listed in GenBank. Their specificity was confirmed using authentic bacterial strains and availability was confirmed by detecting ca. 60 % of wild-type phenol- and benzoate-degrading bacteria. In a case study, the different behavior of C12O- and/or C23O-carrying bacterial strains in aquatic microcosms could be successfully monitored.

These results indicate that the molecular based detection system developed in this study can achieve simple, rapid, sensitive, specific, and extensive detection of bacterial populations which possess particular biodegradation function from complex microbial community of diverse nature, and are applicable for routine and/or intensive microbial monitoring in natural environment.

On the other hand, DNA recovery of ca. 25-55 % by the use of DNA extraction method for water samples established in Chapter 2 implies the need for the improvement of the method, though quite sensitive PCR detection could be achieved. DNA extraction bias depends on the species and physiological conditions of each bacterial cell, and removal of the DNA extraction- and PCR-inhibitive impurity from the samples must be further examined in DNA extraction. In designing PCR primers and PCR amplification, problems on difference of primability and stability of the primers and probes against target genes with sequence diversity, and amplification bias which can be caused by the above-mentioned "performance" of the primers

and probes should be further investigated. As these problems finally brought about unreliable quantification of the target genes, additional improvements in all of them are necessary for the accurate microbial monitoring by PCR.

However, throughout this study, it can be concluded that microbial monitoring methods based on PCR, especially by detecting genes encoding specific biodegradative functions, are revealed to be useful tool for monitoring the behavior of the microbial populations related to particular biodegradation. Application of the established microbial monitoring methods can give quantitative information not only by MPN-PCR but also by in situ PCR (Hodson *et al.*, 1995) or FISH (DeLong *et al.*, 1989). PCR-DGGE (Muyzer *et al.*, 1993; Iwamoto *et al.*, 2000) can give the qualitative information of the dominant species among the detected bacteria responsible for the degradation of the target pollutants. Evaluation of biodegrading activity of the environmental samples may be also possible using RT-PCR (Bogan *et al.*, 1996) techniques. Further investigation is expected to make the method more polished and developmental tool.

References

Amann, R. I., Stromley, J., Devereux, R., Key, R., and Stahl, D. A. (1992) Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Appl. Environ. Microbiol.*, **58,** 614-623.

Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.*, **59,** 143-169.

Atlas, R. M. and Bartha, R. (1987) Microbial ecology, 2nd ed., The Benjamin Cummings Publishing Co., Inc., Reading, Mass., Redwood City, CA.

Bej, A. K. (1995) PCR amplification of DNA recovered from the aquatic environment. *In* Trevors, J. T. and van Elsas, J. D. (ed.), Nucleic acids in the environment: methods and applications., pp. 179-218., Springer-Verlag, Berlin.

Benjamin, R. C., Voss, J. A., and Kunz, D. A. (1991) Nucleotide sequence of *xylE* from the TOL pDK1 plasmid and structural comparison with isofunctional catechol 2,3-dioxygenase genes from TOL pWW0 and NAH7. *J. Bacteriol.*, **173,** 2724-2728.

Bhat, M. A., Tsuda, M., Horiike, K., Nozaki, M., Vaidyanathan, C. S., and Nakazawa, T. (1994) Identification and characterization of a new plasmid carrying genes for degradation of 2,4-dichlorophenoxyacetate from *Pseudomonas cepacia* CSV90. *Appl. Environ. Microbiol.*, **60,** 307-312.

Bogan, B. W., Schoenike, B., Lamar, R. T., and Cullen, D. (1996) Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic hydrocarbon-contaminated soil with *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.*, **62,** 2381-2386.

Boom, R., Sol, C. J. A., Salimans, M. M. M., Jansen, C. L., Wertheim-van Dillen, P. M. E., and van der Noornaa, J. (1990) Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.*, **28,** 495-503.

Braker, G., Fesefeldt, A., and Witzel, K.-P. (1998) Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Appl. Environ. Microbiol.*, **64,** 3769-3775.

Candidus, S., Pée, K.-H., and Lingens, F. (1994) The catechol 2,3-dioxygenase gene of *Rhodococcus rhodochrous* CTM: nucleotide sequence, comparison with isofunctional dioxygenases and evidence for an active-site histidine. *Microbiology*, **140,** 321-330.

Carrington, B., Lowe, A., Shaw, L. E., and Williams, P. A. (1994) The lower pathway operon for benzoate catabolism in biphenyl-utilizing *Pseudomonas* sp. strain IC and the nucleotide sequence of the *bphE* gene for catechol 2,3-dioxygenase. *Microbiology*, **140,** 499-508.

Chandler, D. P. and Brockman, F. J. (1996) Estimating biodegradative gene numbers at a JP-5 contaminated site using PCR. *Appl. Biochem. Biotechnol.*, **57/58,** 971-982.

Cookson, J. T., Jr. (1995) Bioremediation engineering, design and application., McGraw-Hill Inc., New York.

Cowan, S. T. and Steel, K. J. (1974) Manual for the identification of medical bacteria, 2nd ed., Cambridge Univ. Press, Cambridge.

Degrange, V. and Bardin, R. (1995) Detection and counting of *Nitrobacter* populations in soil by PCR. *Appl. Environ. Microbiol.*, **61,** 2093-2098.

DeLong, E. F., Wickham, G. S., and Pace, N. R. (1989) Phylogenetic strains: ribosomal RNA-based probes for the identification of single cells. *Science*, **243,** 1360-1363.

Dong, F.-M., Wang, L.-L., Wang, C.-M., Cheng, J.-P., He, Z.-Q., Sheng, Z.-J., and Shen, R.-Q. (1992) Molecular cloning and mapping of phenol degradation genes from *Bacillus stearothermophilus* FDTP-3 and their expression in *Escherichia coli*. *Appl. Environ. Microbiol.*, **58,** 2531-2535.

Eck, R. and Belter, J. (1993) Cloning and characterization of a gene coding for the catechol 1,2-dioxygenase of *Arthrobacter* sp. mA3. *Gene*, **123,** 87-92.

Eddy, S. R. (1995) Multiple alignment using hidden Markov models. *Ismb.*, **3,** 114-120.

Ehrt, S., Schirmer, F., and Hillen, W. (1995) Genetic organization, nucleotide sequence and regulation of expression of genes encoding phenol hydroxylase and catechol 1,2-dioxygenase in *Acinetobacter calcoaceticus* NCBI8250. *Mol. Microbiol.*, **18,** 13-20.

Erb, R. W. and Wagner-Döbler, I. (1993) Detection of polychlorinated biphenyl degradation genes in polluted sediments by direct DNA extraction and polymerase chain reaction. *Appl. Environ. Microbiol.*, **59,** 4065-4073.

Felske, A., Akkermans, A. D. L., and de Vos, W. M. (1998) Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription-PCR in temperature gradient gel electrophoresis fingerprints. *Appl. Environ. Microbiol.*, **64,** 4581-4587.

Frantz, B. and Chakrabarty, A. M. (1987) Organization and nucleotide sequence determination of a gene cluster involved in 3-chlorocatechol degradation. *Proc. Natl. Acad. Sci. USA*, **84,** 4460-4464.

Fujii, T., Takeo, M., and Maeda, Y. (1997) Plasmid-encoded genes specifying aniline oxidation from *Acinetobacter* sp. strain YAA. *Microbiology*, **143,** 93-99.

Fujita, M., Kamiya, T., Ike, M., Kawagoshi, Y., and Shinohara, N. (1991) Catechol 2,3-dioxygenase production by genetically engineered *Escherichia coli* and its application to catechol determination. *World J. Microbiol. Biotechnol.* **7,** 407-414.

Fujita, M. and Ike, M. (1994) Extension of catabolic range. *In* Waste water treatment using genetically engineered microorganisms., pp. 93-108., Technomic Publishing Company Inc., Lancaster.

Fujita, M. and Ike, M. (1997) Trichloroethylene degradation by phenol-degrading bacteria. *Recent Res. Devel. Microbiol.*, **1,** 333-343.

Ghosal, D., You, I.-S., and Gunsalus, I. C. (1987) Nucleotide sequence and expression of gene *nahH* and homology with gene *xylE* of TOL pWW0. *Gene*, **55,** 19-28.

Gilliland, G., Perrin, S., Blanchard, K., and Bunn, H. F. (1990) Analysis of cytokine mRNA

and DNA: detection and quantification by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci. USA*, **87,** 2725-2729.

Habe, H., Kasuga, K., Nojiri, H., Yamane, H., and Omori, T. (1996) Analysis of cumene (isopropylbenzene) degradation genes from *Pseudomonas fluorescens* IP01. *Appl. Environ. Microbiol.*, **62,** 4471-4477.

Hashimoto, S. and Fujita, M. (1987) Identification of three phenol-degrading microorganisms isolated from activated sludge and their characteristics. *J. Japan Sew. Wks.*, **9,** 655-660. (in Japanese)

Hecker, K. H. and Roux, K. H. (1996) High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. *BioTechniques*, **20,** 478-485.

Herrmann, H., Müller, C., Schmidt, I., Mahnke, J., Petruschka, L., and Hahnke, K. (1995) Localization and organization of phenol degradation genes of *Pseudomonas putida* strain H. *Mol. Gen. Genet.*, **247,** 240-246.

Hodson, R. E., Dustman, W. A., Garg, R. P., and Moran, M. A. (1995) In situ PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. *Appl. Environ. Microbiol.*, **61,** 4074-4082.

Holmes, P. A. (1985) Applications of PHB-A microbially produced biodegradable thermoplastic. *Phys. Technol.*, **16,** 32-37.

Ingraham, J. L., Maaloe, O., and Neidhardt, F. C. (1983) Growth of the bacterial cell., pp.1-48., Sinauer Associates, Sunderland, Mass.

Ishigaki, T., Sugano, W., Nakanishi, A., Ike, M., and Fujita, M. (2000) Enhancement of the stabilization of waste landfill with *in situ* bioventing. *Proceedings of The International Symposium of the COE Project on Establishment and Evaluation of Advanced Water Treatment Technology Systems Using Functions of Complex Microbial Community.* 349-350.

Iwamoto, T., Tani, K., Nakamura, K., Suzuki, Y., Kitagawa, M., Eguchi, M., and Nasu, M. (2000) Monitoring impact of in situ biostimulation treatment on groundwater bacterial

community by DGGE. *FEMS Microbiol. Ecol.*, **32,** 129-141.

Jendrossek, D., Frisse, A., Behrends, A., Andermann, M., Kratzin, H. D., Stanislawski, T., and Schlegel, H. G. (1995a) Biochemical and molecular characterization of the *Pseudomonas lemoignei* polyhydroxyalkanoate depolymerase system. *J. Bacteriol.*, **177,** 596-607.

Jendrossek, D., Backhaus, M., and Andermann, M. (1995b) Characterization of the extracellular poly(3-hydroxybutyrate) depolymerase of *Comamonas* sp. and of its structural gene. *Can. J. Microbiol.*, **41,** 160-169.

Jendrossek, D. (1998) Microbial degradation of polyesters: a review on extracellular poly(hydroxyalkanoic acid) depolymerases. *Polym. Degrad. Stab.*, **59,** 317-325.

Joshi, B. and Walia, S. (1996a) PCR amplification of catechol 2,3-dioxygenase gene sequences from naturally occurring hydrocarbon degrading bacteria isolated from petroleum hydrocarbon contaminated groundwater. *FEMS Microbiol. Ecol.*, **19,** 5-15.

Joshi, B. and Walia, S. K. (1996b) Detection of metapyrocatechase homologous genes in petroleum hydrocarbon contaminated groundwater by polymerase chain reaction. *J. Microbiol. Methods*, **27,** 121-128.

Keil, H., Lebens, R. M., and Williams, P. A. (1985) TOL plasmid pWW15 contains two nonhomologous, independently regulated catechol 2,3-dioxygenase genes. *J. Bacteriol.*, **163,** 248-255.

Keller, C., Corcoran, M., and Roberts, R. J. (1984) Computer programs for handling nucleic acid sequences. *Nucl. Acids Res.*, **12,** 379-386.

Kim, E.-B. and Zylstra, G. J. (1995) Molecular and biochemical characterization of two *meta*-cleavage dioxygenases involved in biphenyl and *m*-xylene degradation by *Beijerinckia* sp. B1. *J. Bacteriol.*, **177,** 3095-3103.

Kitayama, A., Achioku, T., Yanagawa, T., Kanou, K., Kikuchi, M., Ueda, H., Suzuki, E., Nishimura, H., Nagamune, T., and Kawakami, Y. (1996) Cloning and characterization of

extradiol aromatic ring-cleavage dioxygenases of *Pseudomonas aeruginosa* JI104. *J. Ferment. Bioeng.*, **82,** 217-223.

Kivisaar, M., Kasak, L., and Nurk, A. (1991) Sequence of the plasmid-encoded catechol 1,2-dioxygenase-expressing gene, *pheB*, of phenol-degrading *Pseudomonas* sp. strain EST1001. *Gene*, **98,** 15-20.

Kleinsteuber, S., Hoffmann, D., Müller, R. H., and Babel, W. (1998) Detection of chlorocatechol 1,2-dioxygenase genes in proteobacteria by PCR and gene probe. *Acta Biotechnol.*, **18,** 231-240.

Kukor, J. J. and Olsen, R. H. (1996) Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments. *Appl. Environ. Microbiol.*, **62,** 1728-1740.

Lee, J.-R., Min, K.-R., Kim, Y.-C., Kim, C.-K., Lim, J.-Y., Yoon, H.-J., Min, K.-H., Lee, K.-S., and Kim, Y.-S. (1995) Cloning of salicylate hydroxylase gene and catechol 2,3-dioxygenase gene and sequencing of an intergenic sequence between the two genes of *Pseudomonas putida* KF715. *Biochem. Biophys. Res. Commun.*, **211,** 382-388.

Leff, L. G., Dana, J. R., McArther, J. V., and Shimkets, L. J. (1995) Comparison of methods of DNA extraction from stream sediments. *Appl. Environ. Microbiol.* **61,** 1141-1143.

Levesque, M.-J., La Boissiere, S., Thomas, J.-C., Beaudet, R., and Villemur, R. (1997) Rapid method for detecting *Desulfitobacterium frappieri* strain PCP-1 in soil by the polymerase chain reaction. *Appl. Microbiol. Biotechnol.* **47,** 719-725.

Liesack, W. and Stackebrandt, E. (1992) Occurrence of novel groups of the domain bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *J. Bacteriol.*, **174,** 5072-5078.

Little, E., Bork, P., and Doolittle, R. F. (1994) Tracing the spread of fibronectin type III domains in bacterial glycohydrolases. *J. Mol. Evol.*, **39,** 631-643.

Matheson, V. G., Munakata-Marr, J., Hopkins, G. D., McCarty, P. L., Tiedje, J. M., and Forney, L. J. (1997) A novel means to develop strain-specific DNA probes for detecting

bacteria in the environment. *Appl. Environ. Microbiol.*, **63,** 2863-2869.

Mikesell, M. D., Kukor, J. J., and Olsen, R. H. (1993) Metabolic diversity of aromatic hydrocarbon-degrading bacteria from a petroleum-contaminated aquifer. *Biodegradation*, **4,** 249-259.

Møller, S., Pedersen, A. R., Poulsen, L. K., Arvin, E., and Molin, S. (1996) Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative in situ hybridization and scanning confocal laser microscopy. *Appl. Environ. Microbiol.*, **62,** 4632-4640.

Møller, S., Sternberg, C., Andersen, J. B., Christensen, B. B., Ramos, J. L., Givskov, M., and Molin, S. (1998) In situ gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. *Appl. Environ. Microbiol.*, **64,** 721-732.

Moon, J., Chang, H., Min, K.-R., and Kim, Y.-S. (1995) Cloning and sequencing of the catechol 2,3-dioxygenase gene of *Alcaligenes* sp. KF711. *Biochem. Biophys. Res. Commun.*, **208,** 943-949.

Moré, M. L., Herrick, J. B., Silva, M. C., Ghiorse, W. C., and Madsen, E. L. (1994) Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Appl. Environ. Microbiol.*, **60,** 1572-1580.

Muyzer, G., de Waal, E. C., and Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.*, **59,** 695-700.

Nakai, C., Kagamiyama, H., Nozaki, M., Nakazawa, T., Inouye, S., Ebina, Y., and Nakazawa, A. (1983) Complete nucleotide sequence of the metapyrocatechase gene on the TOL plasmid of *Pseudomonas putida* mt-2. *J. Biol. Chem.*, **258,** 2923-2928.

Nakai, C., Uyeyama, H., Kagamiyama, H., Nakazawa, T., Inouye, S., Kishi, F., Nakazawa, A., and Nozaki, M. (1995) Cloning, DNA sequencing, and amino acid sequencing of

catechol 1,2-dioxygenases (pyrocatechase) from *Pseudomonas putida* mt-2 and *Pseudomonas arvilla* C-1. *Arch. Biochem. Biophys.*, **321,** 353-362.

Neidle, E. L., Hartnett, C., Bonitz, S., and Ornston, L. N. (1988) DNA sequence of the *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase I structural gene *catA*: evidence for evolutionary divergence of intradiol dioxygenases by acquisition of DNA sequence repetitions. *J. Bacteriol.*, **170,** 4874-4880.

Neilson, J. W., Josephson, K. L., Pepper, I. L., Arnold, R. B., Di Giovanni, G. D., and Sinclair, N. A. (1994) Frequency of horizontal gene transfer of a large catabolic plasmid (pJP4) in soil. *Appl. Environ. Microbiol.*, **60,** 4053-4058.

Ng, L. C., Shingler, V., Sze, C. C., and Poh, C. L. (1994) Cloning and sequences of the first eight genes of the chromosomally encoded (methyl) phenol degradation pathway from *Pseudomonas putida* P35X. *Gene*, **151,** 29-36.

Nicoletti, A. and Sassy-Prigent, C. (1996) An alternative quantitative polymerase chain reaction method. *Anal. Biochem.*, **236,** 229-241.

Nordlund, I., Powlowski, J., Hagström, Å., and Shingler, V. (1993) Conservation of regulatory and structural genes for a multi-component phenol hydroxylase within phenol-catabolizing bacteria that utilize a meta-cleavage pathway. *J. Gen. Microbiol.*, **139,** 2695-2703.

Oeding, V. and Schlegel, H. G. (1973) Beta-ketothiolase from *Hydrogenomonas eutropha* H16 and its significance in the regulation of poly-beta-hydroxybutyrate metabolism. *Biochem. J.*, **134,** 239-248.

Ogram, A., Sayler, G. S., and Barkay, T. (1987) The extraction and purification of microbial DNA from sediments. *J. Microbiol. Methods*, **7,** 57-66.

Paul, J. H. and Myers, B. (1982) Fluoremetric determination of DNA in aquatic microorganisms by use of Hoechst 33258. *Appl. Environ. Microbiol.*, **43,** 1393-1399.

Pearson, W. R. and Lipman, D. J. (1988) Improved tools for biological sequence comparison.

Proc. Natl. Acad. Sci. USA, **85,** 2444-2448.

Perkins, E. J., Gordon, M. P., Caceres, O., and Lurquin, P. F. (1990) Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. *J. Bacteriol.*, **172,** 2351-2359.

Picard, C., Ponsonnet, C., Paget, E., Nesme, X., and Simonet, P. (1992) Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl. Environ. Microbiol.*, **58,** 2717-2722.

Pike, E. B., Carrington, E. G., and Ashburner, P. A. (1972) An evaluation of procedures for enumerating bacteria in activated sludge. *J. Appl. Bacteriol.*, **35,** 309-321.

Porteous, L. A., Armstrong, J. L., Seidler, R. J., and Watrud, L. S. (1994) An effective method to extract DNA from environmental samples for polymerase chain reaction amplification and DNA fingerprint analysis. *Curr. Microbiol.*, **29,** 301-307.

Raeymaekers, L. (1995) A commentary on the practical applications of competitive PCR. *PCR Methods Applic.*, **5,** 91-94.

Ravatn, R., Zehnder, A. J. B., and van der Meer, J. R. (1998) Low-frequency horizontal transfer of an element containing the chlorocatechol degradation genes from *Pseudomonas* sp. strain B13 to *Pseudomonas putida* F1 and to indigenous bacteria in laboratory-scale activated-sludge microcosms. *Appl. Environ. Microbiol.*, **64,** 2126-2132.

Revsbech, N. P. (1994) Analysis of microbial mats by use of electrochemical microsensors: recent advances. *In* Stal, L. J. and Caumette, P. (ed.), Microbial mats: structure, development, and environmental significance., pp. 149-163., Springer, New York.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, **230,** 1350-1354.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor.

Senior, P. J. and Dawes, E. A. (1973) The regulation of poly-beta-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. *Biochem. J.*, **134,** 225-238.

Simonet, P., Groshean, M.-C., Misra, A. K., Nazaret, S., Cournoyer, B., and Normand, P. (1991) *Franckia* genus-specific characterization by polymerase chain reaction. *Appl. Environ. Microbiol.*, **57,** 3278-3286.

Slater, S., Mitsky, T. A., Houmiel, K. L., Hao, M., Reiser, S. E., Taylor, N. B., Tran, M., Valentin, H. E., Rodriguez, D. J., Stone, D. A., Padgette, S. R., Kishore, G., and Gruys, K. J. (1999) Metabolic engineering of *Arabidopsis* and *Brassica* for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer production. *Nat. Biotechnol.*, **17,** 1011-1016.

Smalla, K., Cresswell, N., Mendonea-Hagler, L. C., Wolters, A., and van Elsas, J. D. (1993) Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *J. Appl. Bacteriol.*, **74,** 78-85.

Sparagano, O. (1993) Detection of *Naegleria fowleri* cysts in environmental samples by using a DNA probe. *FEMS Microbiol. Lett.*, **112,** 349-352.

Stanier, R. Y., Palleroni, N. J., and Doudoroff, M. (1966) The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.*, **43,** 159-271.

Steffan, R. J. and Atlas, R. M. (1988) DNA amplification to enhance detection of genetically engineered bacteria in environmental sample. *Appl. Environ. Microbiol.*, **54,** 2185-2191.

Suzuki, M. T. and Giovannoni, S. J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.*, **62,** 625-630.

Takeo, M., Maeda, Y., Okada, H., Miyama, K., Mori, K., Ike, M., and Fujita, M. (1995) Molecular cloning and sequencing of the phenol hydroxylase gene from *Pseudomonas putida* BH. *J. Ferment. Bioeng.*, **79,** 485-488.

Tateda, M., Kisaki, O., Trung, L. D., and Fujita, M. (1998) A case study of the determination

of the optimal temperature monitoring point and correlation between inflow air rate and temperature distribution in a high rate composting system for sewage sludge., Proc. Jap. Wast. Manag. Experts, **Vol. I,** 241-243 (1998). (in Japanese)

Torsvik, V., Goksoyr, J., and Daae, F. L. (1990) High diversity in DNA of soil bacteria. *Appl. Environ. Microbiol.*, **56,** 782-787.

Trevors, J. T., Lee, H., and Cook, S. (1992) Direct extraction of DNA from soil. *Microb. Releases*, **1,** 111-115.

Tsai, Y.-L. and Olson, B. H. (1991) Rapid method for direct extraction of DNA from soil and sediments. *Appl. Environ. Microbiol.*, **57,** 1070-1074.

Tsai, Y.-L. and Olson, B. H. (1992) Rapid method for separation of bacterial DNA for humic substances in sediments for polymerase chain reaction. *Appl. Environ. Microbiol.*, **58,** 2292-2295.

Vahjen, W. and Tebbe, C. C. (1994) Enhanced detection of genetically engineered *Corynebacterium glutamicum* pUN1 in directly extracted DNA from soil, using T4 gene 32 protein in the polymerase chain reaction. *Eur. J. Soil Biol.* **30,** 93-98.

Van der Meer, J. R., Eggen, R. I. L., Zehnder, A. J. B., and deVos, W. M. (1991) Sequence analysis of the *Pseudomonas* sp. strain P51 *tcb* gene cluster, which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates. *J. Bacteriol.*, **173,** 2425-2434.

Van Elsas, J. D., van Overbeek, L. S., and Fouchier, R. (1991) A specific marker, *pat*, for studying the fate of introduced bacteria and their DNA in soil using a combination of detection techniques. *Plant Soil*, **138,** 49-60.

Volossiouk, T., Robb, E. J., and Nazar, R. N. (1995) Direct DNA extraction for PCR-mediated assays of soil organisms. *Appl. Environ. Microbiol.* **61,** 3972-3976.

Voytek, M. A. and Ward, B. B. (1995) Detection of ammonium-oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with the PCR. *Appl. Environ.*

Microbiol., **61,** 1444-1450.

Wagner, M., Rath, G., Amann, R., Koops, H.-P., and Schleifer, K.-H. (1995) In situ identification of ammonia-oxidizing bacteria. *Syst. Appl. Microbiol.*, **18,** 251-264.

Wang, A. M., Doyle, M. V., and Mark, D. F. (1989) Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA*, **86,** 9717-9721.

Watanabe, K., Yamamoto, S., Hino, S., and Harayama, S. (1998) Population dynamics of phenol-degrading bacteria in activated sludge determined by *gyrB*-targeted quantitative PCR. *Appl. Environ. Microbiol.*, **64,** 1203-1209.

Watanabe, K., Teramoto, M., Futamata, H., and Harayama, S. (1998) Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. *Appl. Environ. Microbiol.*, **64,** 4396-4402.

Wikström, P., Wiklund, A., Andersson, A.-C., and Forsman, M. (1996) DNA recovery and PCR quantification of catechol 2,3-dioxygenase genes from different soil types. *J. Biotechnol.*, **52,** 107-120.

Wilson, K. (1994) Preparation of genomic DNA from bacteria. *In* Ausbel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. (ed.), Current Protocols in Molecular Biology., Unit 2.4.1., Wiley, New York.

Yamaguchi, N. and Nasu, M. (1997) Rapid *in situ* enumeration of microbes in natural aquatic environment using fluorescent gene probes. *J. Wat. Waste*, **39,** 308-315. (in Japanese)

Yrjälä, K., Paulin, L., Kilpi, S., and Romantschuk, M. (1994) Cloning of *cmpE*, a plasmid-borne catechol 2,3-dioxygenase-encoding gene from the aromatic- and chloroaromatic-degrading *Pseudomonas* sp. HV3. *Gene*, **138,** 119-121.

Zhou, J., Bruns, M. A., and Tiedje, J. M. (1996) DNA recovery from soils of diverse composition. *Appl. Environ. Microbiol.*, **62,** 316-322.

List of Publications Related to This Study

<Research Papers>

Sei, K., Asano, K., Tateishi, N., Mori, K., Ike, M., and Fujita, M. (1999) Design of PCR primers and gene probes for the general detection of bacterial strains capable of degrading aromatic compounds via catechol cleavage pathways. *J. Biosci. Bioeng.*, **88,** 542-550.

Sei, K., Asano, K., Mori, K., Ike, M., Kohno, T., and Fujita, M. (2000) Development of simple methods of DNA extraction from environmental samples for monitoring microbial community based on PCR. *Japanese J. Wat. Treat. Biol.*, **36** (Accepted)

Sei, K., Nakao, M., Mori, K., Ike, M., Kohno, T., and Fujita, M. (2000) Design of PCR primers and gene probe for the general detection of bacterial populations capable of degrading poly(3-hydroxybutyrate). *Appl. Microbiol. Biotechnol.* (Submitted)

Sei, K., Wada, K., Tateishi, N., Mori, K., Ike, M., Kohno, T., and Fujita, M. PCR-based monitoring of the behavior of aromatic compound-degrading bacteria in activated sludge during phenol acclimation process. (In preparation)

<Proceedings of the International Conferences>

Sei, K, Asano, K., Ike, M., and Fujita, M. (2000) Design of PCR primers and gene probe for extensive detection of bacterial populations capable of degrading poly(3-hydroxybutyrate). *Proceedings of the International Symposium of the COE, Center of Excellence, Project on Establishment and Evaluation of Advanced Water Treatment Technology Systems Using Functions of Complex Microbial Community.*, pp. 89-96.

Fujita, M., Ike, M., Mori, K., and Sei, K. (2000) Design of primers and probes for extensive detection of aromatic compound-degrading bacteria. *In* Wickramanayake, G. B., Gavaskar, A. R., Kelley, M. E., and Nehring K. W. (eds) Risk, Regulatory, and Monitoring Considerations: Remediation of Chlorinated and Recalcitrant Compounds (C2-1). *Proceedings of the Second International Conference on Remediation of Chlorinated and Recalcitrant Compounds.* pp.183-190, Battelle Press, Ohio, USA.

<Articles>

Fujita, M., Ike, M., and Sei, K. (1997) Introduction to microbial ecology using DNA techniques. *J. Wat. Waste*, **39,** 301-307. (in Japanese)