Development of a Novel Method for RNA Extraction from Soil and Its Application to the Study of Soil Microbiology

土壌から RNA を抽出する方法の開発および土壌微生物の研究への応用

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Yong WANG

Synopsis

A novel method for RNA extraction from soil was developed and improved. To remove co-extracted humic substances from soil RNA samples, the lysis conditions and purification columns were optimized, which allowed preparation of soil RNA with high purity. By using this method, real-time RT-PCR and microarray analyses of bacterial gene expression in a sterilized soil inoculated with a single bacterial strain were performed successfully. To extract RNA from Andosols (volcanic ash soils), which are the dominant agricultural soil in Japan and are well known for their strong adsorption of RNA, extraction buffer containing autoclaved casein was used, which allowed successful RNA extraction from diverse soils.

Contents

Chapter I

Introduction

Many tales about the Earth can be found in the cultures of ancient China, ancient Greece, and other nations throughout the world. Although we live on the Earth, it remains mysterious to us. Most of our food originates from soil, which forms a very thin layer on the surface of the Earth. To understand the Earth better, knowledge of soil and the microorganisms living in it should be obtained. During the last century, one of the major achievements of soil microbiologists was isolating bacterial strains from soil and surveying their population in soil environments using culture-based methods; however, the great number of bacterial species (Torsvik & Øvreås, 2002; Torsvik *et al.*, 2002; Gans *et al.*, 2005; Roesch *et al.*, 2007) in soil makes the isolation and identification of new bacterial species a never-ending task. Although much effort has been devoted to the development of new strategies to isolate new species from soil (Hattori, 1981; Suwa & Hattori, 1987; Mitsui *et al.*, 1997; Stevenson *et al.*, 2004), many bacterial species are resistant to culture. Because approximately 99% of bacteria in soil remain unidentified and/or are difficult to culture (Torsvik *et al.*, 1990), culture-based methods have limitations for the survey of bacterial populations in soil. These limitations have motivated researchers to search for breakthrough culture-independent approaches. After it was approved for use in a wide range of life science applications (Boehm, 1989; Deacon & Lah, 1989; Macintyre, 1989; Paabo *et al.*, 1989; Vosberg, 1989), the polymerase chain reaction (PCR) technique, which appeared in the mid-1980s (Mullis & Faloona, 1987), was used by soil microbiologists soon after its introduction to detect bacterial genes in soil (Chaudhry *et al.*, 1989; Henschke *et al.*, 1991; Pillai *et al.*, 1991; Selenska & Klingmüller, 1991). With the increasing use of PCR, more soil microbiological studies focused on specific

genes in soil bacteria, mainly the small subunit ribosomal RNA gene (Hahn *et al.*, 1990; Segovia *et al.*, 1991; Bruce *et al.*, 1992; Liesack & Stackebrandt, 1992). Culture-independent molecular techniques have proven that the microbial world is genetically and functionally more complex and diverse than previously hypothesized on the basis of culture-dependent studies. Culture-independent methods provide us with large amounts of information about bacterial species in soil, and this information is useful for identifying newly isolated bacterial species and surveying the bacterial community in soil environments (Janssen, 2006). Internet databases, such as the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) and Greengenes (DeSantis *et al.*, 2006), facilitate the dissemination of new information to soil researchers.

Many researchers have used bacterial genomic DNA extracted from soil as templates for PCR detection of bacterial genes in soil (Segovia *et al.*, 1991; Bruce *et al.*, 1992; Liesack & Stackebrandt, 1992). DNA only provides us with information about the existence of bacteria in soil; it cannot provide us with information about gene expression, which is important to understand bacterial activities in soil, such as bacterial growth, degradation activities of various compounds, and bacterial responses to environmental factors. For this reason, a study using reverse transcription-polymerase chain reaction (RT-PCR) to detect bacterial gene expression in soil was launched in the early 1990s (Hahn *et al.*, 1990; Tsai *et al.*, 1991; Selenska & Klingmüller, 1992). Recently, the cDNA clone library was also used to investigate active genes in soil (Botero *et al.*, 2005). Because both RT-PCR and the cDNA clone library require bacterial RNA as a template for converting RNA into cDNA, direct extraction of bacterial RNA from soil is a key procedure in both techniques and is of great interest. In the past 20 years, many methods of RNA extraction from soil have been reported (Hahn *et al.*, 1990; Tsai *et al.*, 1991; Selenska & Klingmüller, 1992; Moran *et al.*, 1993; Borneman & Triplett, 1997; Fleming *et al.*, 1998; Mendum *et al.*, 1998; Griffiths *et al.*, 2000; Hurt *et al.*,

2001; Sessitsch *et al.*, 2002; Bürgmann *et al.*, 2003; Luis *et al.*, 2005; Peršoh *et al.*, 2008); however, until now, there has been no method for RNA extraction from all types of soil, so researchers had to choose or develop soil RNA extraction methods to fit their own research purposes. The lack of a universal RNA extraction method for all soils hindered the study of bacterial gene expression in soil. Recently, the application of RNA extracted from soil has been extended to whole transcriptomic analysis (Leininger *et al.*, 2006; Urich *et al.*, 2008), which are more powerful than RT-PCR and may provide us with information about the global gene expression of soil bacteria.

The major difficulties encountered in extracting RNA from soil and corresponding strategies to overcome those difficulties, rather than each detailed procedure in the protocol, are discussed below.

Overview of RNA extraction from soil

There are two major methods of RNA extraction from soil: the direct and the indirect. In the direct method, nucleic acid is extracted from soil directly; in the indirect method, the bacteria are first isolated from the soil, and then nucleic acids are extracted from the cells collected. It has been reported that the indirect method resulted in significantly lower RNA yields than the direct extraction method (Hahn *et al.*, 1990). Also, gene expression might be affected or altered during treatment before cell lysis. Hence the direct method to extract RNA from soil is prefered by soil microbiologists.

RNA extraction from soil with a direct method can be divided into three stages: cell lysis, extraction of RNA from the soil matrix, and purification of RNA. At the cell lysis stage, bead beating has become popular over the past 10 years (Griffiths *et al.*, 2000; Sessitsch *et al.*, 2002; Bürgmann *et al.*, 2003; Luis *et al.*, 2005; Peršoh *et al.*, 2008), although several other methods, such as sonication (Hahn *et al.*, 1990), grinding after freezing in liquid nitrogen

(Hurt *et al.*, 2001), and enzymatic lysis by lysozymes (Moran *et al.*, 1993), have also been used. To protect RNA from degradation by RNase, inactivation reagents for RNase, such as guanidine thiocyanate, guanidine isothiocyanate, 2-mercaptoethanol, or dithiothreitol are normally added to the extraction buffer so that the RNase molecules can be inactivated immediately after they are released from cells. After cell lysis, RNA molecules, together with DNA and proteins, are released from cells into the soil suspension; meanwhile, humic substances are also released from soil particles; therefore, the soil suspension is a mixture of many kinds of molecules, including RNA and humic substances. At the second stage, the nucleic acids can be separated from the soil matrix, proteins, and cell debris by phenol extraction. Then, RNA precipitation by ethanol, isopropanol, or polyethylene glycol (PEG) is typically required to reduce the volume of the sample and to remove various salts. At the third stage, RNA samples are purified by spin columns, including gel filtration (size exclusion) (Moran *et al.*, 1993; Mendum *et al.*, 1998; Sessitsch *et al.*, 2002) and ion exchange (Hurt *et al.*, 2001) chromatography columns. Commercial kits for RNA extraction from soil are also available, and are summarized in Table I.1.

Difficulties in recovering bacterial RNA from soil

Contamination by humic substances

Impurities are extracted from soil along with RNA, and the majority of these impurities are humic substances, which are dark-colored, heterogeneous organic compounds in soil (Stevenson, 1994). Based on their solubility under acidic or alkaline conditions, humic substances in soils can be divided into three main groups: humic acids, which are soluble under alkaline conditions but not acidic conditions; fulvic acids, which are soluble under all pH conditions; and humin, which is the insoluble fraction (Stevenson, 1994). Because humin cannot be extracted by any water solution, the predominant humic substances co-extracted

with RNA should be humic and fulvic acids. Fulvic acids inhibit PCR amplification, but only at high concentrations (Kreader, 1996). Compared with fulvic acids, the effect of humic acids on biological experiments has been well studied because they present difficulties in various molecular biological experiments. Humic acids have been shown to interfere with enzyme reactions (restriction endonuclease, DNase, and RNase) (Tebbe & Vahjen, 1993), PCR amplification (Tsai & Olson, 1992b; Tebbe & Vahjen, 1993), DNA-DNA hybridization (Steffan *et al.*, 1988; Tebbe & Vahjen, 1993), transformation of competent cells (Tebbe & Vahjen, 1993), nucleic acid detection and measurement (Bachoon *et al.*, 2001; Zipper *et al.*, 2003), and RNA hybridization (Alm *et al.*, 2000). Thus, the removal of humic substances from soil RNA samples is critical to molecular analysis; however, complete removal is rather difficult (Harry *et al.*, 1999). As shown in Fig. I.1, only a fraction of humic and fulvic acids can be removed by phenol extraction, and both can be precipitated by ethanol, which is somewhat similar to DNA and RNA.

Adsorption of RNA by soil

As mentioned above, there have been some successful cases of RNA extraction from diverse soils; however, RNA extraction from Andosols is a challenge. Andosols (volcanic ash soils) can be found all over the world. In Japan, Andosols cover about 16.4% of land surface and 46.5% of arable upland fields (Goyal *et al.*, 2000); thus, it is necessary to establish a method for RNA extraction from Andosols to facilitate the study of bacterial gene expression. For this reason, we attempted RNA extraction from Andosols with a popular commercial kit, RNA PowerSoil Total RNA Isolation Kit (MO BIO, Carlsbad, CA, USA). Unfortunately, RNA extraction failed in all Andosol soil samples tested (Wang *et al.*, unpublished data), although this commercial kit has been proven to extract RNA from diverse soils successfully (Accinelli *et al.*, 2008; Sagova-Mareckova *et al.*, 2008). It is true that soil possesses detectable

extracellular RNase activities (Greaves & Wilson, 1970); however, recent reports suggest that RNA could survive in the presence of extracellular RNase in soil (Franchi & Gallori, 2005; Biondi *et al.*, 2007). Also, almost intact bacterial rRNA could be extracted from an Andosol with an extraction buffer amended with DNA (Hoshino & Matsumoto, 2007). Thus, the failure of RNA extraction from Andosols is possibly caused by RNA adsorption by soil but not RNA degradation by RNase. RNA adsorbs to soil very quickly. About 50–90% of the adsorbed RNA molecules were adsorbed to clay within one hour (Goring & Bartholomew, 1952), and 85% of the maximum adsorption occurred on allophane (one of the major components in Andosols) within 30 min (Taylor & Wilson, 1979). It is known that all RNA components (mononucleotides, nucleosides, bases, phosphate and ribose) and nucleotides possessing different numbers of phosphate groups can be adsorbed by soil (Goring & Bartholomew, 1952; Cortez & Schnitzer, 1981; Leytem *et al.*, 2002). Also, all of the RNA components could be adsorbed by allophane (Hashizume & Theng, 2007). Although both DNA and RNA could be adsorbed by soil (Goring & Bartholomew, 1952), it seems that RNA is more difficult to extract from soil than DNA. First, the ribose in RNA has one more hydroxyl group than the 2-deoxyribose in DNA. This hydroxyl group may result in stronger adsorption of RNA on soil than that of DNA. Second, the free extracyclic functional groups on the bases in the single-strand structure of RNA (partial base pairing may occur in some regions of RNA molecules) could form hydrogen bonds with soil surface (Robinson *et al.*, 2007), which may also result in stronger adsorption of RNA on soil than that of DNA. This is supported by a previous report in which, from the same Andosol, DNA was successfully extracted by a skim milk amended extraction buffer, whereas RNA failed to be extracted using the same buffer (Hoshino & Matsumoto, 2007). Therefore, efforts are still required to investigate the mechanism of RNA adsorption by Andosols.

Strategies to overcome these difficulties

Removal of humic substances

Many methods have been tested or used to remove humic substances from RNA extracted from soil, including chemical flocculation with $Al_2(SO_4)_3$ under alkaline conditions prior to cell disruption (Peršoh *et al.*, 2008), addition of cetyltrimethyl ammonium bromide (CTAB) to the extraction buffer (Griffiths *et al.*, 2000; Bürgmann *et al.*, 2003), precipitation of RNA by PEG (Griffiths *et al.*, 2000; Bürgmann *et al.*, 2003), adsorption by polyvinylpolypyrrolidone (PVPP) (Mendum *et al.*, 1998), co-precipitation with guanidine hydrochloride (Hahn *et al.*, 1990), and chromatography using gel filtration (Moran *et al.*, 1993; Mendum *et al.*, 1998; Sessitsch *et al.*, 2002) and ion exchange (Hurt *et al.*, 2001) columns. The half-life of bacterial mRNA is very short, ranging from no more than 30 s to more than 20 min (Ehretsmann *et al.*, 1992), and thus, unlike DNA extraction, a pre-wash step is inappropriate for RNA extraction from soil. For this reason, using an extraction buffer amended with CTAB (Griffiths *et al.*, 2000; Bürgmann *et al.*, 2003) can be more helpful than other methods of controlling the release of humic substances into the aqueous phase. Phenol extraction is a common procedure to remove proteins from the cell lysate. As shown in Fig. I.1, to some extent, phenol extraction also removes humic and fulvic acids. Precipitation of RNA is normally required before a purification procedure to reduce the volume of the RNA sample and to remove various salts and partial humic substances. Although ethanol is commonly used, isopropanol and PEG show higher recoveries of nucleic acids with low contamination of humic acids (Cullen & Hirsch, 1998). In most cases, one or multiple purification procedures are required to remove humic substances completely. Because the weight average molecular weight of humic and fulvic acids in soil is less than 20 kDa (Perminova *et al.*, 2003), which is slightly lower than typical tRNA in mass, most humic and fulvic acids possess lower molecular weights than rRNA and mRNA. Thus, an appropriate gel

filtration column could be used to remove most of the co-extracted humic substances from an RNA sample, such as Sephadex G-75 (Moran *et al.*, 1993; Mendum *et al.*, 1998) and Sepharose CL-6B (Sessitsch *et al.*, 2002). As the content of carboxyl groups in humic acids increases with a decrease in molecular weight (Shin *et al.*, 1999), humic acid molecules with a high content of carboxyl groups could be removed more efficiently than other humic acid molecules by cations of various compounds, such as the cetrimonium cation of CTAB (Griffiths *et al.*, 2000; Bürgmann *et al.*, 2003). Because the surfaces of soil humic acids are normally negatively charged (Ceppi *et al.*, 1999), the separation of RNA from humic acids can be performed successfully on an ion-exchange column, such as a silica-gel-based membrane column (Qiagen Total Nucleic Acid purification system) (Hurt *et al.*, 2001). Apparently, the column purification methods (both gel filtration and ion-exchange columns) are much easier to use and require much less operation time than chemical methods, such as co-precipitation with guanidine hydrochloride followed by phenol extraction (Hahn *et al.*, 1990); therefore, they can be expected to be a standard procedure in the protocol of RNA extraction from soil. Because there is no single purification method to remove co-extracted humic substances completely (Harry *et al.*, 1999), the appropriate combination of several methods is required to obtain high-purity RNA.

Release of RNA from soil

It is known that RNA adsorption by clays decreases with the increase of pH of soil suspensions (Goring & Bartholomew, 1952; Taylor & Wilson, 1979). Adsorption of RNA components, *e.g.*, adenine, adenosine, ribose and adenosine-5’-phosphate (5’-AMP), showed a similar tendency with RNA; in particular, the adsorption of 5’-AMP at pH 4 and pH 6 was about 60 times higher than at pH 8 (Hashizume & Theng, 2007), suggesting that an extraction buffer with a pH higher than 6 could be helpful to release RNA from Andosols. RNA

adsorption by allophane increased as the concentration of sodium chloride increased when the pH was higher than 5 (Taylor & Wilson, 1979). Divalent cations, *e.g.*, Ca^{2+} and Mg^{2+}, were much more effective at promoting RNA adsorption than mono-cations, *e,g.*, Na^{+} and K^{+} (Goring & Bartholomew, 1952; Taylor & Wilson, 1979). Thus, it is preferable for an extraction buffer to possess a pH higher than 6, without Ca^{2+} or Mg^{2+}, and with a low level of Na^{+} and K^{+}, to improve the recovery efficiency of RNA from Andosols. In successful extractions of DNA from Andosols, an appropriate additive is often required. Some additives have been tested and shown to be helpful in assisting the release of DNA from Andosols to recover DNA from soil (Volossiouk *et al.*, 1995; Takada-Hoshino & Matsumoto, 2004; Ikeda *et al.*, 2008); however, only one additive, DNA, has been shown to be helpful in recovering RNA from an Andosol (Hoshino & Matsumoto, 2007). In that case, RT-PCR amplification of rRNA was successful, but no functional gene was tested; therefore, it is unclear whether DNA can be helpful in recovering RNA from Andosols for the detection of mRNA. Since many molecules, such as ribose (Hashizume & Theng, 2007), base (Cortez & Schnitzer, 1981; Hashizume & Theng, 2007), nucleoside (Hashizume & Theng, 2007), nucleotide (Goring & Bartholomew, 1952; Leytem *et al.*, 2002), DNA (Goring & Bartholomew, 1952) and proteins (Goring & Bartholomew, 1952; Fusi *et al.*, 1989), could be adsorbed by soil, it is worth investigating which material is helpful to release RNA from Andosols.

Limitations of RNA-based techniques

Although the detection of target mRNA can provide much information about gene function and cell response to treatments or environmental conditions, there are some limitations to the use of RNA-based techniques. First, proteins are molecules that exert gene functions but not mRNA. Thus, detection of target proteins, if possible, should give us more reliable information than the detection of target mRNA. Second, the level of some proteins

might not always be consistent with that of the corresponding mRNA. This often happens in Eukarya and Archaea and may also happen in some bacteria, especially high GC Gram-positive species, because of the existence of the proteasome-dependent protein degradation mechanism (Goldberg *et al.*, 1995; Gille *et al.*, 2003). Third, enzyme proteins usually have a range of optimal conditions to exert activities. Although there is no difference at the mRNA and protein levels, the activity of enzymes may be different among samples because of differences in pH or the existence of activators or inhibitors; therefore, precautions should be taken when explaining the gene expression data obtained from soil samples. If possible, integrating multiple 'omics' analyses, including genomics, transcriptomics, proteomics, interactomics, metabolomics, and fluxomics, for soil microbiological study can be a powerful and more reliable method (Singh & Nagaraj, 2006).

The constitution of the dissertation

In the current study, as a first trial, detection of bacterial gene expresson in a sterilized soil inoculated with a *Rhodococcus jostii* RHA1 strain was conducted, which showed us the possibility to detect bacterial gene expression in soil (Chapter II). However, the problem of humic contamination in the extracted RNA samples came to us. To inspect the efficiency of humic removal during RNA extraction from soil, determination of humic substances in soil RNA samples is required. Thus, we evaluated various methods for measurement of humic acids and gave out our suggestions about how to use these methods properly (Chapter III). After that, we improved our method for RNA extraction from soil, by which humic substances were removed from RNA samples efficiently (Chapter IV). By using this new strategy, we extracted RNA from sterilized soil inoculated with *Pseudomonas putida* KT2440 for microarray analysis of genome-wide gene expression, which validated that the RNA extracted from soil with this new strategy was suitable for microarray analysis (Chapter V). Finally, we

developed a universal method for RNA extraction from various soils by addition of autoclaved casein into lysis buffer. This universal method allowed us to successfully detect the transcripts of bacterial ammonia monooxygenase subunit A gene (*amoA*) in Andosols, the most challenging soil on the Earth (Chapter VI).

Table I.1. Commercially available kits for RNA extraction from soil

Kit	Manufacturer	Soil for processing	Lysis	Purification	Principle of purification
E.Z.N.A. Soil RNA Kit	Omega Bio-Tek (Norcross, GA, USA)	2 g	Bead beating	Single spin column	Adsorption
FastRNA Pro Soil-Direct Kit	MP-Biomedicals (Q-Biogene)(Solon, OH, USA)	0.5 g	Bead beating	Binding matrix	Adsorption
ISOIL for RNA	NIPPON GENE (Tokyo, Japan)	0.5 g	Bead beating	Precipitation	Information not publicly available
IT 1-2-3 Platinum PathTM Sample Purification kit	Idaho Technology (Salt Lake City, UT, USA)	0.5 g	Bead beating	Magnetic beads	Information not publicly available
RNA PowerSoil Total RNA Isolation Kit	MO BIO (Carlsbad, CA, USA)	2 g	Bead beating	Single gravity flow column	Adsorption
Soil Total RNA Purification Kit	Norgen (Thorold, ON, Canada)	0.5 g	Bead beating	Single spin column	Adsorption
ZR Soil/Fecal RNA MicroPrep	Zymo Research (Orange, CA, USA)	0.25 g	Bead beating	Multiple spin columns	Adsorption/gel filtration

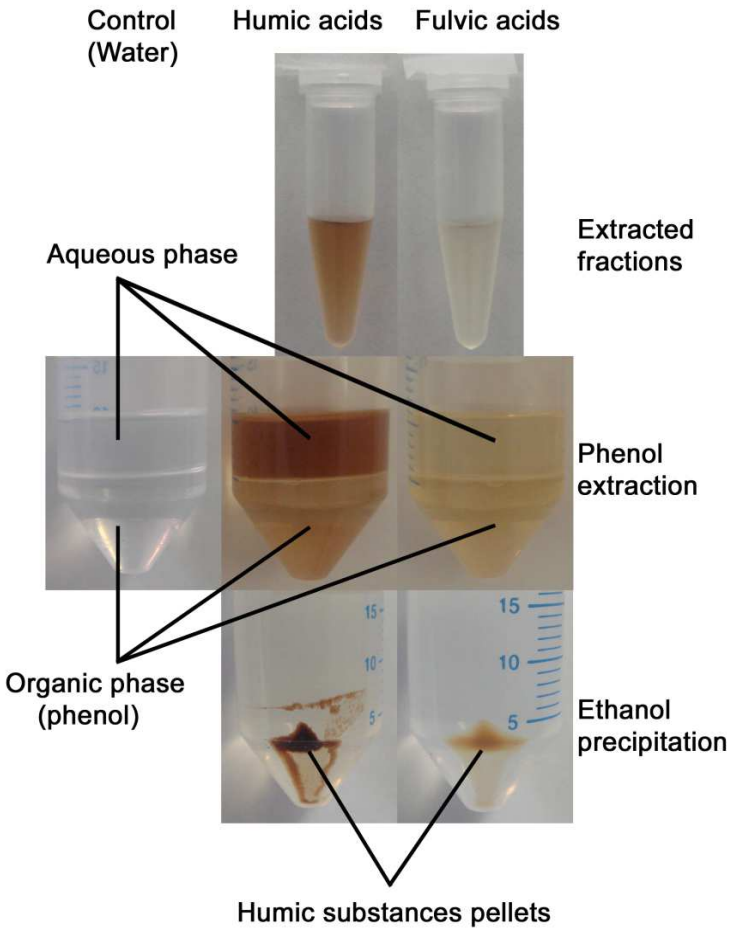

Fig. I.1. The behavior of humic and fulvic acids during phenol extraction and ethanol precipitation. Humic and fulvic acids were prepared as previously described (139). Citrate-saturated phenol at pH 4.3 was used for extraction, and water was used as a control to show the original color of the phenol reagent used. The aqueous layer was transferred to a fresh tube, followed by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol for precipitation.

Chapter II

A Trial to Detect Bacterial Gene Expression in a Sterilized Soil Inoculated with a *Rhodococcus jostii* RHA1 Strain

Summary

As a first trial, we set up a new method by combination of several commercial kits. Using this method, we extracted RNA from a sterilized brown forest soil inoculated with *Rhodococcus jostii* strain RHA1, a biphenyl degrader isolated from γ-hexachlorocyclohexane-contaminated soil. Data from agarose gel electrophoresis indicated that the extracted RNA was purified properly. This new method can be applied easily in the preparation of large amounts of RNA. Real-time reverse transcription-polymerase chain reaction (RT-PCR) experiments performed with the TaqMan method suggested that the *bphAa* gene in this strain, which is involved in the degradation of biphenyl, was induced in the biphenyl amended soil.

The ultimate purpose of my research is to develop a universal method for RNA extraction from diverse soils, which could be used for the study of soil microbiology and microbial ecology. As mentioned in the Introduction, RNA extraction from Andosols is rather difficult, thus I decided to start my research with a non-Andosol soil. To ensure the soil used for RNA extraction containing sufficient amount of RNA for extraction, an autoclaved soil inoculated with a bacterial strain could be a nice material.

In this study, the soil used was a brown forest soil, from which bacterial RNA could be extracted without any problem. The bacteria for inoculation in soil is an actinomycete, *Rhodococcus jostii* RHA1, which was originally isolated from γ-hexachlorocyclohexane-contaminated soil and was identified as a biphenyl degrader (Seto *et al.*, 1995). The genes involved in the early steps of biphenyl degradation in *Rhodococcus jostii* RHA1, *bphAaAbAcAd-bphC-bphB* (formerly *bphA1A2A3A4-bphC-bphB*), have been identified (Masai *et al.*, 1995). Because many papers have reported study of *Rhodococcus jostii* RHA1 cultured in liquid media, the condition to induce expression of *bphAa* gene in RHA1 is well established. Thus, a sterile soil inoculated with RHA1 could be used as a model system for detection of induced gene expression in soil.

As the first trial, a new method for RNA extraction from soil was set up by combination of several commercial kits. In this method, a binding spin column (Aurum Total RNA Mini Kit column) and a gel filtration column (Sephadex G-50 spin column) were included to get rid of humic substances efficiently. The principles of separation of these two columns are quite different from each other. The binding spin column is similar to an ion-exchange chromatography column, which separate molecules based on the electric charge of molecules. The gel filtration column separates molecules based on their sizes. Combination of these two types of columns can get rid of humic substances more efficiently than using a single column. Although both of these columns are not designed for RNA extraction from soil, and they have

not yet been used for RNA extraction from soil by any other researchers, the results in my preliminary experiments suggested that these two columns work well in removing humic substances from RNA samples.

Materials and Methods

Soil for inoculation

A field soil sample, collected from the Ehime Agricultural Experiment Station in Ehime, Japan, was sieved (2 mm mesh) and sterilized by autoclaving (1 h at 121°C, twice). After the sterilized soil was cooled, its water content was measured and adjusted to 60% of the maximum water-holding capacity. The properties of this field soil are shown in Table II.1.

Bacterial strain and culture conditions

Rhodococcus jostii RHA1 was grown in 1/5 LB (2 g bactotryptone, 1 g yeast extract, 1 g NaCl per liter) at 28°C. One ml of the culture was centrifuged to collect cells. After it was washed twice with 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6.8), the cell suspension was diluted by 10, 100, and 1,000 fold. The soil cultures inoculated with 10^1, 10^2, and 10^3 fold dilutions of the bacterial suspension were designated Dil 1, Dil 2, and Dil 3 respectively. At 0 day, the cell densities of Dil 1, Dil 2, and Dil 3 approximately corresponded to 10^6, 10^5, and 10^4 CFU/g soil respectively. For RNA extraction, 0.5 ml of the diluted bacteria suspension was dispensed to 4.5 g of sterilized soil in 50-ml tubes, in which 10 mg of biphenyl was added where required. For colony counting, 0.3 ml of the diluted bacteria suspension was dispensed to 2.7 g of sterilized soil in 50-ml tubes, in which 6 mg of biphenyl was added where required. The soil culture of the bacteria was incubated at 30ºC for a proper period, for example, 24 h, 48 h, or 72 h. Then RNA extraction and colony counting was done. The colony forming units of bacteria inoculated in sterilized soil were determined by the diluted plating method.

RNA isolation from soil

The protocol for RNA isolation from soil was as follows:

(i) Extraction. Ten grams of glass beads (diameter, 0.2 mm) (BioMedical Science, Tokyo) and one zirconia-silica ball (diameter, 15 mm) (BioMedical Science) were added into each of the 50-ml tubes containing 5 g of soil and inoculated bacteria. After 9 ml of Na_2HPO_4/NaH_2PO_4 buffer (300 mM, pH 8), 0.5 ml of 20% SDS solution, and 0.5 ml of guanidine solution (4 M guanidine isothiocyanate, 10 mM Tris-HCl, pH7.0, 1 mM EDTA, and freshly prepared 0.5% 2-mercaptoethanol) (Hurt *et al.*, 2001) were added into the tubes, the tubes were set into a ShakeMaster Auto machine (BioMedical Science) for 15-min of shaking to break the cells. Then samples were subjected to centrifugation at 10,000 rpm for 15 min at room temperature. The supernatant was extracted twice with phenol and precipitated with ethanol at room temperature. After co-precipitated oil-like humic substances were removed carefully, the nucleic acid pellet was air-dried for 10 min in a clean bench and dissolved in 100 µl of DEPC-treated water.

(ii) Purification with an Aurum Total RNA Mini Kit. The extracted nucleic acid was subjected to an Aurum Total RNA Mini Kit column (Bio-Rad Laboratories, Hercules, CA) to remove co-precipitated brownish humic substances and DNA, according to the manufacturer's instructions.

(iii) Purification with a Sephadex G-50 spin column. The RNA purified at step (ii) was applied to an RNase-free Sephadex G-50 quick spin column (Roche Applied Science, Indianapolis, IN) to remove humic substances, according to the manufacturer's instructions.

(iv) Removal of DNA with a TURBO DNA-free kit. The RNA purified at step (iii) was treated with a TURBO DNA-free kit (Ambion, Austin, TX) to remove DNA completely, according to the manufacturer's instructions.

Gel electrophoresis of RNA

Two hundred nanograms of Novagen Perfect RNA Markers (0.2-10 kb) (Merck KGaA, Darmstadt, Germany) together with 10 μl of purified RNA sample was electrophoresed in each of the lanes of 1% agarose gels, and images of the SYBR Gold (Molecular Probes, Eugene, OR) stained gels were captured with a FAS-III gel scanner (Toyobo, Osaka, Japan).

Real-time quantitative RT-PCR

One-step real-time RT-PCR was performed to examine gene expression levels using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA). For the *bphAa* (formerly *bphA1*) gene, the forward primer was 5'-GGCACGATCAGCTACGTCTACA-3', the reverse primer was 5'-TCCGGACCCATTGCGTAT-3', and the TaqMan probe was 5'-AAGAAGCGGCGCGTGGGCT -3'. For the probe, 6FAM was used as a 5'-reporter, and TAMRA (6-carboxytetramethylrhodamine) was used as a quencher. The concentration of RNA samples was adjusted to 10 ng/μl with DEPC-treated water, and 2 μl of the RNA solution was used as a template in a 50-μl volume of one-step RT-PCR reaction mixture. TaqMan quantitative RT-PCR was performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The reaction conditions were as follows: 30 min at 48°C for reverse transcription, 10 min at 95°C for activation of DNA polymerase, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Standards for the assays were prepared with PCR amplicons from *Rhodococcus jostii* RHA1 genomic DNA with the forward and reverse primer set described above. A standard curve was constructed by comparing the copy numbers of 10-fold dilutions of the standard to their respective threshold cycles.

Determination of humic acid

The level of humic acid in the extracted RNA was determined at 320 nm using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE), following a previous report (Miller, 2001).

Results and Discussion

Growth of* Rhodococcus *jostii* *RHA1 in soil

During the several days immediately after inoculation, *Rhodococcus jostii* RHA1 increased its population in the soil without the addition of any carbon source, and maintained its population for about one month (Fig. II.1). In an attempt to determine at which growth stage of *Rhodococcus jostii* RHA1 we could extract large amounts of RNA, the soil cultures inoculated with the cell suspensions (Dil 1, Dil 2, and Dil 3, as described in "Materials and Methods") were prepared in the presence and the absence of biphenyl (biphenyl(+) or biphenyl(-)) and incubated for different periods. Since the data in Fig. II.1 suggested that the exponential phase of bacteria growth in soil lasted for 1 or 2 d, we focused on the first 3 d of soil incubation in this experiment. As shown in Fig. II.2, among the soil cultures of bacteria in the presence of biphenyl (Fig. II.2A), even on the third day, all cultures with different inoculation sizes showed a tendency for the population to increase, suggesting that the samples on the third day were still in the exponential phase. On the other hand, among the soil cultures of bacteria in the absence of biphenyl (Fig. II.2B), on the second day, the cultures showed the highest bacteria population, even in culture Dil 3, which possessed the lowest inoculation size (10^4 CFU/g soil), and the population did not increase on the third day, indicating that the cultures on the first day were in the exponential phase. The reason that the highest population level reached by the three sections of biphenyl(-) were similar might be the maximum cell density of strain RHA1 that can be achieved in this soil with its original growth

substrates. This is in accordance with the fact that the highest population reached by the three sections of biphenyl(-) were lower than those of the biphenyl(+) sections.

In contrast with the biphenyl(-) cultures, the growth of the biphenyl(+) cultures was inhibited on the first day. This growth inhibition in biphenyl(+) cultures might have resulted from presence of biphenyl, although further evidence is required. On the third day, the population of biphenyl(+) cultures became higher than that of the corresponding biphenyl(-) cultures (Fig. II.2A and B). This might be explained as follows: after the genes related to the biphenyl degradation pathway were induced by biphenyl, biphenyl was degraded and the metabolite served as a carbon resource that promoted the growth of *Rhodococcus jostii* RHA1.

RNA isolation from soil

In the present study, the guanidine isothiocyanate/SDS/phosphate buffer system was used to prepare cell lysate, and then nucleic acid was separated from the protein and a portion of brownish organic substances by extraction with phenol. A high concentration of phosphate buffer (300 mM) was utilized in the lysis buffer so that the bacteria could be dissociated from the soil particles easily, and so that after cell lysis, binding between released RNA molecules and soil particles could be suppressed. To avoid co-precipitation of salt caused by the high concentration of phosphate in the lysis buffer, we performed ethanol precipitation at room temperature instead of a lower temperature.

After ethanol precipitation, the extracted nucleic acid solution showed a brown color, suggesting the presence of humic substances. The most serious problem in soil RNA extraction is the contamination of humic substances, because RNA isolation from soil results in co-extraction of humic substances. It has been reported that humic substances interfere with many enzyme reactions (Tebbe & Vahjen, 1993), nucleic acid detection and measurement

(Bachoon *et al.*, 2001; Zipper *et al.*, 2003), and RNA hybridization (Alm *et al.*, 2000). To remove humic substances, affinity/ion-exchange spin columns and gel filtration columns have been used by some researchers (Moran *et al.*, 1993; Mendum *et al.*, 1998; Hurt *et al.*, 2001; Han & Semrau, 2004; Luis *et al.*, 2005; Lakay *et al.*, 2007). In an alternative method, humic substances were removed by precipitation with 7.5 M potassium acetate from nucleic acid extract (Miskin *et al.*, 1999). Since commercially available RNase-free columns are safe and convenient, we prefer to use such columns for purification of RNA. Based on our preliminary experiments, a Bio-Rad Aurum Total RNA Mini Kit column (Bio-Rad Laboratories) and a Sephadex G-50 quick spin column (Roche Applied Science) were selected for purification of RNA. The nucleic acid extract precipitated with ethanol was subjected to the Aurum column to remove humic substances. Most of the DNA was also removed at this step by on-column DNase digestion. Since the eluted RNA solution from the Aurum column still showed a yellowish color, RNase-free Sephadex G-50 quick spin columns were used to remove the remaining humic substances. According to our real-time PCR data, such purified RNA samples contain trace amounts of DNA (data not shown). Hence we treated the RNA sample with an Ambion Turbo DNA-free kit to ensure that all DNA was removed.

The quality of the finally purified RNA samples was examined by agarose gel electrophoresis, as shown in Fig. II.3. Most samples showed three bands. Two of them might have been 23S rRNA and 16S rRNA according to their molecular sizes, and the third one at the higher position of the gel might have contained RNA molecules with special secondary structures, since RNase-free DNase digestion did not remove this band, but denaturation of the RNA sample at 70°C before it was loaded on gel did remove this band (data not shown). In all of our samples, 5S RNA was not visible due to the utilization of affinity spin columns in the Bio-Rad Aurum Total RNA Mini Kit, which was found to have low efficiency in recovering small RNA in our preliminary experiment. In all the samples, there was no smear

immediately under the 16S rRNA band, suggesting there was no detectable degradation in any of the RNA samples.

In all groups, we detected no RNA signal in the samples incubated for 0 d, that is to say, soil RNA extraction was performed immediately after inoculation. In all of the other samples, the signal intensity of RNA on the gel was consistent with the corresponding bacteria population (Figs. II2 and II3).

One of the advantages of this new method is that it can easily be applied to extract large amounts of RNA. This is especially attractive for microarray analysis. The ShakeMaster Auto device (BioMedical Science) holds up to ten 50-ml tubes for shaking at one time. Normally, we were able to finish RNA extraction and purification from the ten 50-ml tubes (5g soil/tube) within one day.

To test the large scale application of this method to gene expression analysis, we chose different soil samples where cells grew abundantly with or without substrate addition. Since the day-3 sample of biphenyl(+)-Dil 1 showed the highest bacteria population and the strongest fluorescent signal on agarose gel (Fig. II.2A and II.3A), this sample was used as the biphenyl(+) soil sample for RNA extraction. Similarly, since the day-1 sample of biphenyl(-)-Dil 1 showed the highest bacteria population and the strongest fluorescent signal on agarose gel among the three samples on day 1 (Fig. II.2B and II.3B), this sample was used as the biphenyl(-) soil sample for RNA extraction.

For Fig. II.3, from 5 g soil sample in one 50 ml tube, about 2 μg and 0.2 to 0.3 μg of RNA were extracted from the biphenyl(+) soil sample and the biphenyl(-) soil sample respectively. For large-scale application of this method, we extracted RNA within one day from four tubes containing a total of 20 g of the biphenyl(+) soil sample, or six tubes containing a total of 30 g of the biphenyl(-) soil sample. Finally, we obtained 8.1 μg RNA from the day-3 soil of biphenyl(+)-Dil 1 and 1.4 μg RNA from the day-1 soil of

biphenyl(-)-Dil 1. We detected almost the same level of *bphA* expression by RT-PCR in both the small-scale and the large-scale preparation of RNA (data not shown). This suggests that RNA extraction from soil with this method could be scale-up to fit for various demands.

***Expression of the biphenyl degradation gene,* bphAa**

We performed one-step real-time RT-PCR by the TaqMan method using all 24 RNA samples shown in Fig. II.3 to examine the expression of a biphenyl degradation gene, *bphAa*. Real-time RT-PCR was also performed to examine the expression of the 16S rRNA gene, but the expression of 16S rRNA varied during cell growth, suggesting that it cannot be used to normalize the expression of other genes. Similar results have been reported by other researchers, who reported fluctuating expression levels of several housekeeping genes, including 16S rRNA, during cell growth (Vandecasteele *et al.*, 2001). Hence normalization was done against the amount of total RNA. The real-time RT-PCR data are summarized in Fig. II.4. Only the expression data for biphenyl(+) cultures are shown, since the expression of *bphAa* in biphenyl(-) cultures was close to the background.

For all biphenyl(+) cultures, expression of *bphAa* increased during the time course. Interestingly, though the samples of all biphenyl(+) cultures 2 d after inoculation showed very similar population sizes and amounts of total RNA (Fig. II.2A and II.3A), the expression levels of *bphAa* were significantly different. That is, the day-2 sample of biphenyl(+)-Dil 1 showed a dramatic increase in the expression level of *bphAa* as compared to the day-1 sample, while the day-2 sample of biphenyl(+)-Dil 3 showed an almost undetectable expression level (Fig. II.4). This may have resulted from the different status of the nutrition consumption of cells in different soil cultures. It is apparent that the soil contained a certain amount of compounds that can be used as growth substrates of strain RHA1, considering that strain RHA1 grew even without the addition of biphenyl to the soil. Although the identity of the

substances is not known, the existence of a carbon or nitrogen source in the soil is evident in the data for total carbon and total nitrogen (Table II.1). In biphenyl(+)-Dil 1, since the initial cell density was high, the cells used up carbon and energy sources in the soil earlier than those in biphenyl(+)-Dil 3, in which the initial cell density is low (1/100 of Dil 1). The cells in biphenyl(+)-Dil 3 utilized carbon and energy sources in the soil for a longer time than those in biphenyl(+)-Dil 1 or biphenyl(+)-Dil 2 before reaching a high density. Accordingly, in the cells of biphenyl(+)-Dil 3, the biphenyl degradation pathway was switched on later than in the cells of biphenyl(+)-Dil 1 or biphenyl(+)-Dil 2. In short, although further study is required, it is possible to speculate that induction of the biphenyl degradation gene *bphAa* by biphenyl in the soil is dependent on the status of nutrition of the cells.

Comparison with commercial kits

To determine whether the new method we presented here was successful, we compared it with two commercial soil RNA extraction kits. RNA was extracted from *Rhodococcus jostii* RHA1 incubated with sterilized soil with these two commercial kits and by the new method. On agarose gel, we detected a clear RNA signal in the RNA extracted with kit B and by the new method, but there was almost no RNA signal in that extracted with kit A (Fig. II.5, upper panel). On the other hand, the co-extracted humic substances (humic acid was determined to represent the humic substances) had the highest level in the RNA extracted with kit A and a much lower level in that extracted with kit B and by the new method (Fig. II.5, lower panel). Compared with kit B, the new method extracted RNA with a lower level of humic substances (close to the background) and no detectable DNA, suggesting that the new method is a good candidate to extract RNA from soil.

Table II.1. Properties of the Field Soil Examined

Soil type	FAO soil grouping	Vegetation	Particle distribution (%)			Soil texture*	pH	Total C	Total N	Moisture content %	Moist color
			Sand	Silt	Clay			(g kg^{-1} soil)	(g kg^{-1} soil)		(hue, value/chroma)
Brown Forest Soil (BFS)	Gleyic Cambisols	No plant	67.7	14.8	17.6	Sandy clay loam	6.6	9.7	1.6	11	10YR 3/1

*Soil texture was determined according to the International Union of Soil Sciences (IUSS) classification system.

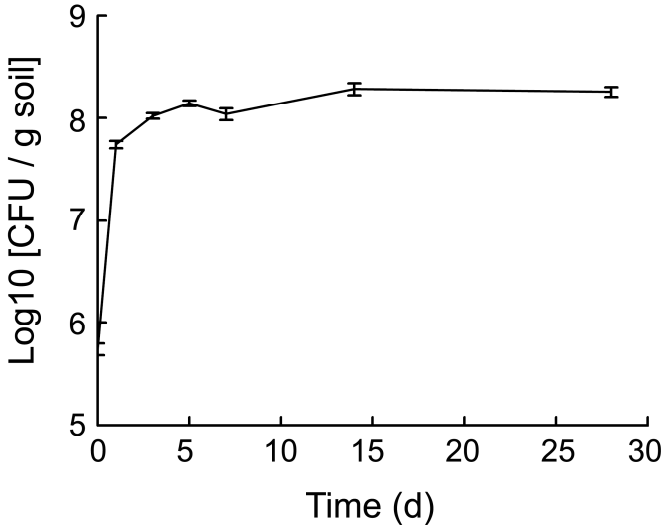

Fig. II.1. The Viability of *Rhodococcus jostii* RHA1 in Soil for a Long Period of Time.

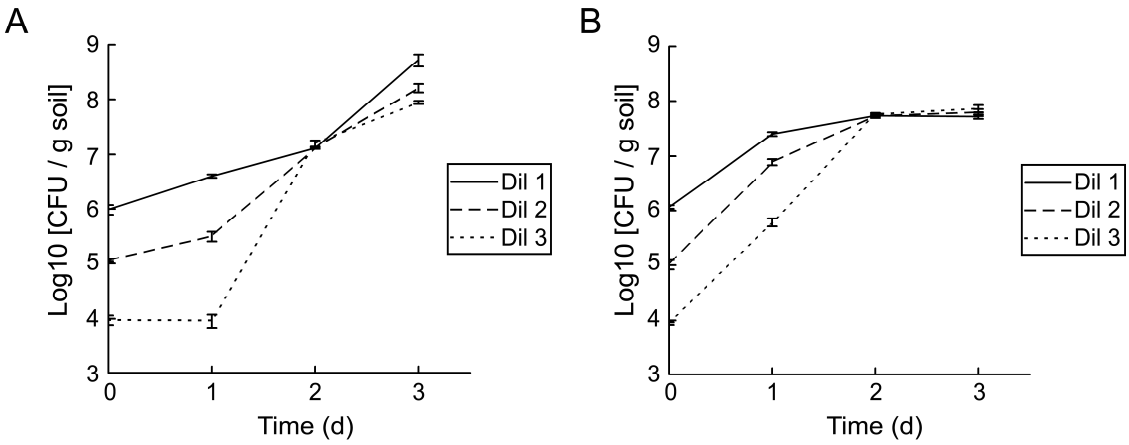

Fig. II.2. The Growth of *Rhodococcus jostii* RHA1 in Soil Amended with Biphenyl (A) or Not Amended with Biphenyl (B). CFU, colony forming unit.

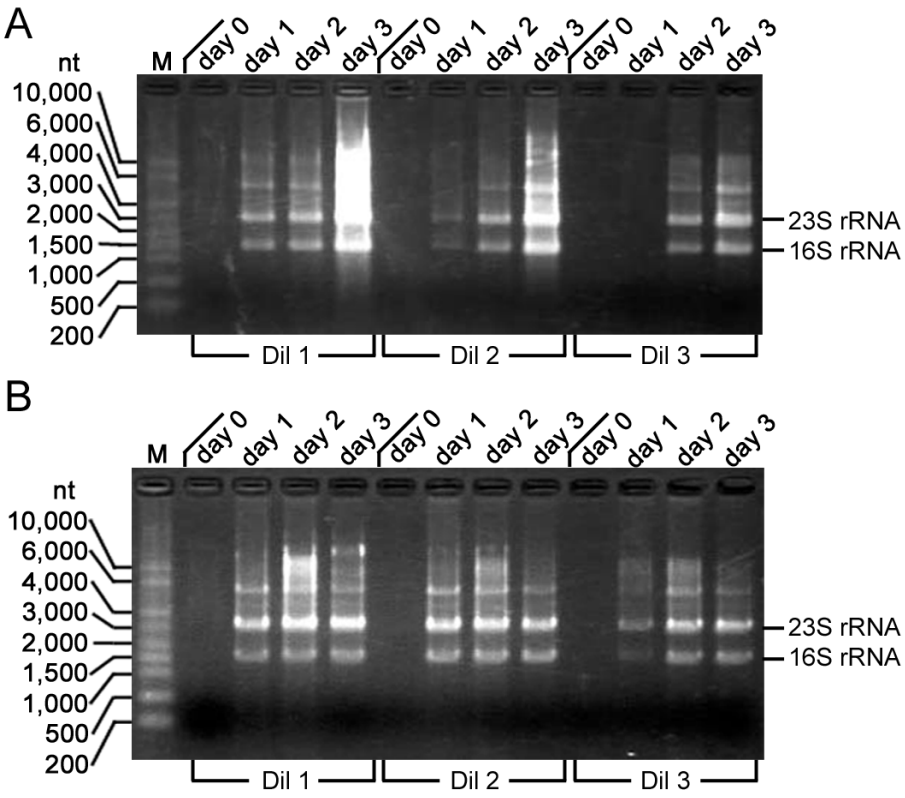

Fig. II.3. Agarose Gel Electrophoresis of RNA Samples Prepared from Soil Amended with Biphenyl (A) or Not Amended with Biphenyl (B).

M, RNA marker.

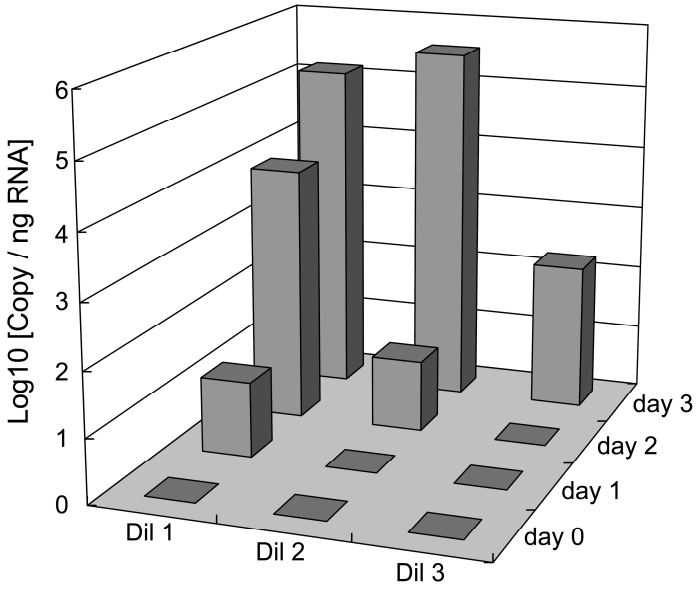

Fig. II.4. Expression of *bphAa* in *Rhodococcus jostii* RHA1 Inoculated in Biphenyl Amended Soil.

Triplicate Experiments were performed. The gene expression profiles among the experiments were quite similar to each other.

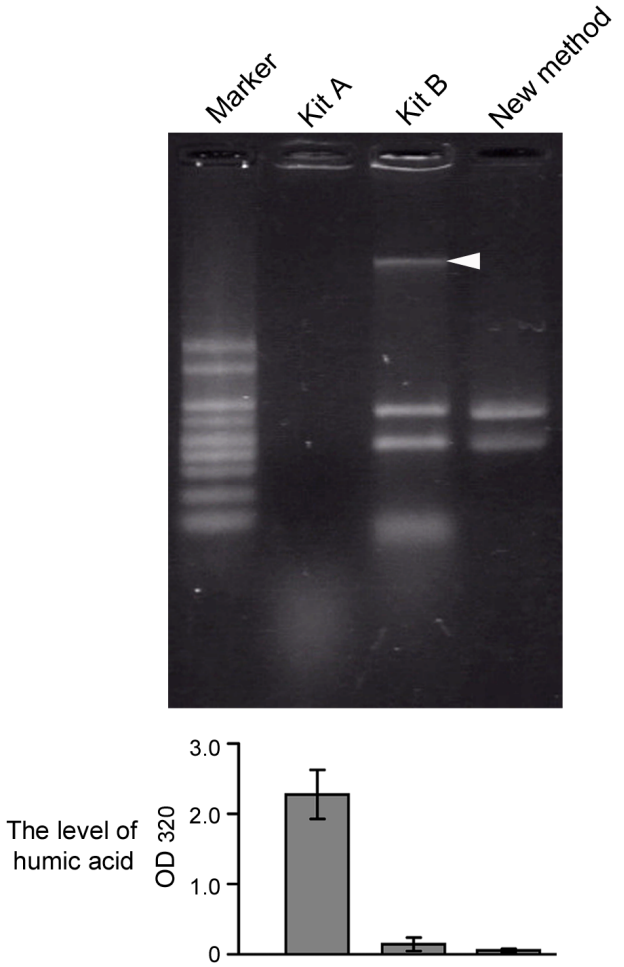

Fig. II.5. Comparison of Different Methods to Extract RNA from Soil.

RNA extracted from 0.5 g of soil was loaded in each lane. Triplicate samples were processed for each method. The white arrowhead indicates a DNA band in the RNA extracted with kit B.

Chapter III

Evaluation of Methods Determining Humic Acids in Soil RNA Samples

Summary

It has been known that even small amounts of humic substances may affect the detection of gene expression. However, we did not know how much humic substances remained in soil RNA samples. To select a proper method for measurement of humic substances, we compared the sensitivity of various methods for measurement of humic acids, and influences of DNA, RNA and proteins on the measurement. Data suggests that both ultraviolet/visible spectroscopic and fluorescence spectroscopic methods are reliable to determine the quantity of humic substances in RNA samples. Considering the results, we also give suggestions as to choice of methods for measurement of humic acids in molecular biological analyses.

We described a method to extract high quality RNA from bacteria in soil and subsequently detected functional gene expression via RT-PCR (Chapter II). However, when the extracted RNA was concentrated, a slightly yellow color appeared, indicating the presence of humic acids. It has been known that humic acids, as rich organic constituents of soil, often appear as impurities in the nucleic acids (DNA or RNA) extracted from soil. In order to evaluate the quality of the extracted DNA/RNA, several methods for determining concentrations of co-extracted humic acids were developed. These methods were divided into three types, as follows: visual colorimetry (this study), visible and ultraviolet spectroscopy (Torsvik, 1980; Miller, 2001; Howeler *et al.*, 2003; Sagova-Mareckova *et al.*, 2008), and fluorescence spectroscopy (Kuske *et al.*, 1998; Howeler *et al.*, 2003), but information on important features of most of the methods, such as detection limit, linear range, and disturbing substances, is not available. Thus, it is unclear under what conditions these methods are suitable to determine the level of co-extracted humic acids in nucleic acids extracted from soil. In this study, we compared the sensitivity of the methods to each other for measurement of humic acids using a commercial humic acid derived from soil, and the threshold concentrations of the nucleic acids and protein molecules affecting the measurement of humic acids by the various methods. Considering the results obtained, we give suggestions as to the conditions under which these methods should be used.

Materials and Methods

Measurement of humic acids

Spectrophotometric measurements of humic acids were performed using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at a wavelength of 320 nm, which has been verified as a proper wavelength for measurement of humic acids (Miller, 2001). Fluorescence measurements of humic acids were performed with

a microcell using an F-2500 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) at 25°C. The slit width for excitation and emission wavelengths was 10 nm.

A commercial humic acid (Nacalai Tesque, Kyoto), originating in soil, was dissolved in 0.1 M NaOH. After brief centrifugation to remove undissolved materials, the humic acid solution was diluted serially with Milli-Q water (Millipore, Billerica, MA). The spectroscopic characteristics of the commercial humic acid are shown in Fig. III.1A and B, and suggest that it possesses typical spectroscopic characteristics for humic acids extracted from soil. The detection limit of the visual colorimetry method was determined by comparing a set of serial diluted humic acid solutions with water (Fig. III.1C). To determine the linear range of each spectroscopic method, serially diluted humic acid solutions in triplicate (from 0.1 ng/μL to 1 μg/μL for visible and ultraviolet spectroscopy, and from 0.01 ng/μL to 20 ng/μL for fluorescence spectroscopy) were determined by each method. The linearity of the data was tested by squared correlation (R^2) on Microsoft Excel. Disturbance of DNA, RNA, and protein was measured by comparing the fluorescence intensities of DNA, RNA, BSA (bovine serum albumin) or skim milk at different concentrations with those of the humic acids. The concentration of DNA, RNA, BSA or skim milk corresponding to the signal intensity lower than the lower limit of the linear range of humic acids detection was considered to represent no effect on the determination of humic acids.

Real-time PCR and real-time RT-PCR

Genomic DNA and total RNA were extracted from the *Pseudomonas putida* KT2440 strain with a Puregene DNA Purification Kit (Gentra, Minneapolis, MN) and an RNeasy Mini Kit (Qiagen, Valencia, CA) respectively. The genomic DNA or total RNA was mixed with different amounts of the commercial humic acid prior to real-time PCR or real-time RT-PCR reactions. The abundance of the genomic DNA and RNA of the 16S rRNA gene in *P. putida*

KT2440 was examined by real-time PCR or real-time RT-PCR with a TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA). The forward primer was 5'-TGGAGCATGTGGTTTAATTCGA-3', the reverse primer was 5'-CCATCTCTGGAAAGTTCTCTGCA-3' and the TaqMan probe was 5'-CAACGCGAAGAACCTTACCAGGCCTT-3', which utilized 6FAM (6-carboxyfluorescein) as a 5'-reporter and TAMRA (6-carboxytetramethylrhodamine) as a quencher. All primers and probes were designed and synthesized by Biosearch Technologies Japan (Tokyo, Japan). The final concentration of each primer or probe in the PCR reaction mixture was 200 nM. The parameters for a thermocycler were: 30 min at 48°C for reverse transcription (this step was omitted for DNA samples), 10 min at 95°C for activation of DNA polymerase, and 40 cycles of 15 s at 95°C and 1 min at 60°C.

Results and Discussion

Comparison of methods of determining humic acids

Among all of the methods examined in this study, the visual colorimetry method was the easiest to use, and was not affected by DNA, RNA, or protein, but it was less sensitive to humic acids than the others (Fig. III.1C and Table III.1). In addition, this method determined a rough quantity rather than a precise quantity of humic acids. Thus, it is useful only when high levels of humic acids must be determined roughly, for example, in evaluation of it in an early step of DNA/RNA extraction from soil.

All of the visible and ultraviolet spectroscopic methods showed similar sensitivity to humic acids and similar linear ranges of detection (Table III.1). These methods were not affected by DNA or RNA, and were affected by protein only when the concentration of protein was very high. Since such a high concentration of protein normally does not present after phenol extraction followed by spin column purification during DNA/RNA extraction,

even if an extraction buffer containing skim milk is used, as reported previously (Takada-Hoshino & Matsumoto, 2004; Hoshino & Matsumoto, 2007), disturbance of protein is negligible in the determination of humic acids using visible or ultraviolet spectroscopic methods (data not shown).

The two fluorescence spectroscopic methods showed the highest sensitivity to humic acids among all of the methods we examined (Table III.1). A previous report concluded that 10 ng/μL of DNA or 2 μg/μL of BSA did not affect the determination of humic acids at a high concentration (50 ng/μL) (Howeler *et al.*, 2003). Since a high concentration of humic acids can easily be determined by visible and ultraviolet spectroscopic methods, we tested disturbance of DNA, RNA, and protein on the determination of low-level humic acids, and found that DNA, RNA, and protein do affect the determination (Table III.1). This suggests that proper dilution of samples might be required to avoid disturbance by DNA, RNA, or protein when fluorescence spectroscopy is used.

Application of these methods to soil biological research

To determine under what conditions the aforementioned methods are suitable to measure co-extracted humic acids in the extracted nucleic acids, we collected information from papers published previously. In experiments not sensitive to humic acids, such as DNase I or RNase digestion, transformation and nucleic acid hybridization, in which humic acids at lower than 100 ng/μL do not have a strong effect on experiments (Tebbe & Vahjen, 1993; Alm *et al.*, 2000), even the visual colorimetry method is sufficient. However, in experiments sensitive to humic acids, such as restriction enzyme digestion, in which several ng per μL of humic acids inhibit enzyme activity significantly (Tebbe & Vahjen, 1993), fluorescence spectroscopy might be more helpful to measure low-level humic acids precisely if disturbance by DNA can be avoided. For PCR or real-time RT-PCR, however, there were no clear data showing the

effects of humic acids on these reactions. Hence we evaluated the effects of humic acids on them (Fig. III.2). Although we found information on the effects of humic acids on PCR (Tsai & Olson, 1992a; Tebbe & Vahjen, 1993), the lowest level of humic acids that affected PCR significantly was ambiguous because the values in the two reports were different from each other. To clarify this ambiguity, we conducted real-time PCR by the addition of different quantities of humic acids to the reaction mixtures. As shown in Fig. III.2A, determination of abundance of genomic DNA was significantly affected by humic acids at a level of ≥ 10 ng/μL, which was consistent with one of the reports (Tsai & Olson, 1992a), suggesting that this value is reliable. Since we did not find any information on the effects of humic acids on RT-PCR, one of the popular techniques in molecular biology laboratories, we conducted real-time RT-PCR. As shown in Fig. III.2B, determination of abundance of RNA was affected by humic acids at a level of ≥ 5 ng/μL. This suggests that RT-PCR is more sensitive to humic acids than PCR, probably because disturbance of humic acids occurred in two reactions, both the reverse transcription and the PCR. Since the methods of A_{465}, A_{320}, $A_{340,}$ and A_{350} detected humic acids at levels as low as 5 ng/μL, all of the visual and ultraviolet spectroscopic methods were sufficient to evaluate the quality of soil DNA and RNA for routine PCR or RT-PCR analysis.

Table III.1. Comparison of the methods for determining humic acids

Methods	Detection limit (ng/μl)	Linear range (ng/μl)	Linearity (R^2)	Disturbance[a]			References
				RNA (ng/μl)	DNA (ng/μl)	Protein[b] (ng/μl)	
Visual colorimetry	25	—	—	No effect	No effect	No effect	This study
A465	—	5 − 500	0.9996	No effect	No effect	> 1 000	Sagova-Mareckova, et al., 2008
A320	—	5 − 200	0.9993	No effect	No effect	> 500	Miller, 2001
A340	—	5 − 500	0.9998	No effect	No effect	> 1 000	Howeler, et al., 2003
A350	—	5 − 500	0.9997	No effect	No effect	> 1 000	Torsvik, 1980
$\lambda_{excitation}/\lambda_{emission}$							
276/445	—	0.05 − 5	0.9997	> 50	> 20	> 5	This study
471/529	—	0.05 − 20	0.9995	> 100	> 10	> 50	Kuske, et al., 1998; Howeler, et al., 2003

[a]The disturbance molecules affect determination of humic acids only when their concentrations are higher than the thresholds presented here.

[b]The disturbance of protein was examined using BSA (bovine serum albumin) or skimmed milk.

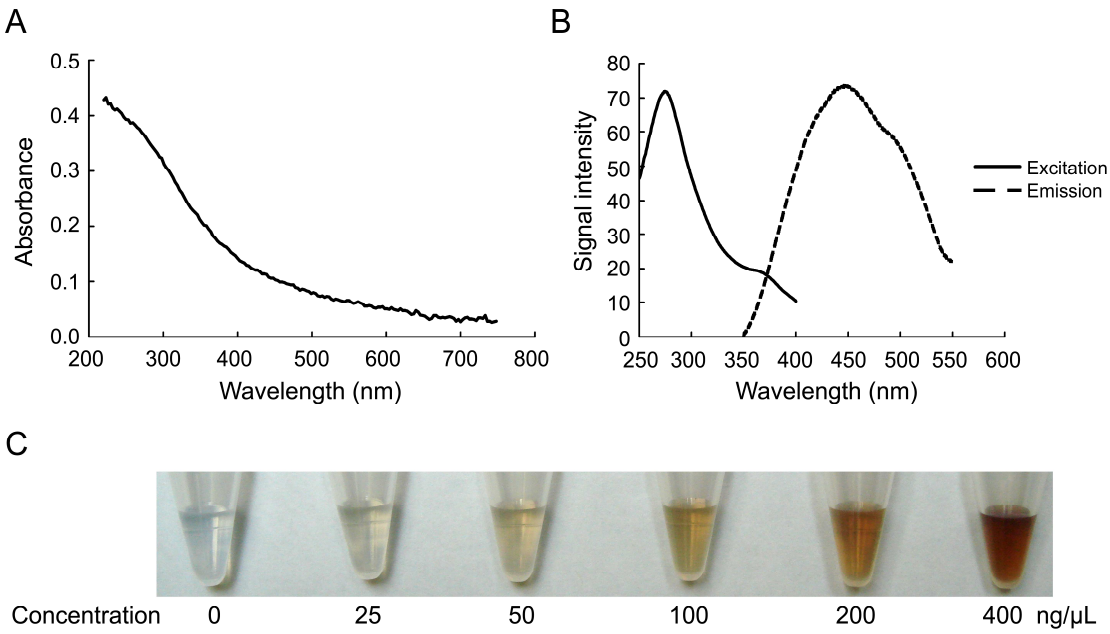

Fig. III.1. Ultraviolet-Visible Absorption Spectrum (A), Fluorescence Spectrum (B), and a Set of Serial Diluted Solution (C) of a Commercial Humic Acid.

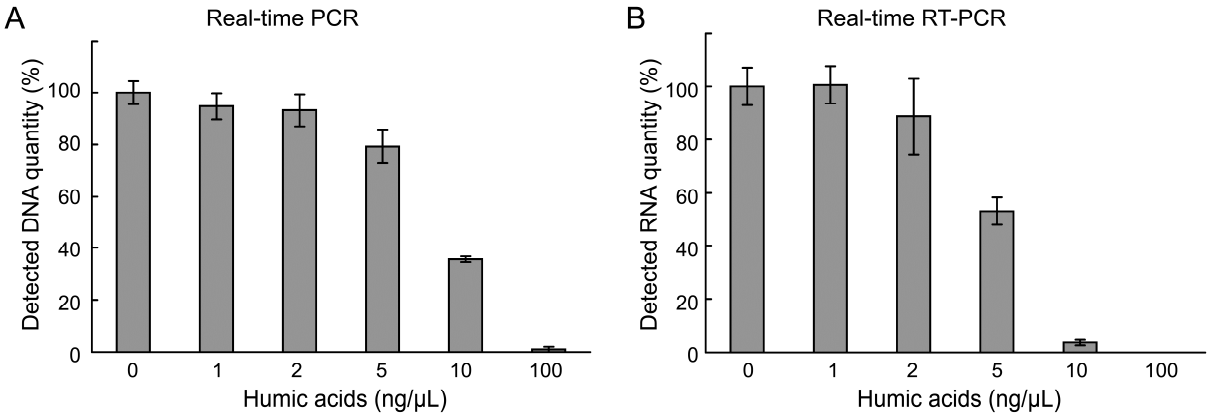

Fig. III.2. Inhibitory Effects of Humic Acids on Real-Time PCR (A) and Real-Time RT-PCR (B). Two μL of DNA or RNA was used in each 50-μL reaction mixture, and triplicate samples were examined. Error bars indicate standard derivations.

Chapter IV

Optimization of the Conditions to Remove Humic Substances from Soil RNA Samples

Summary

In an attempt to remove humic substances from RNA extracted from soil, our previous method for soil RNA extraction was improved by optimization of lysis conditions and purification columns. Fluorescence spectroscopy confirmed efficient removal of both humic and fulvic acids by the improved method. The sensitivity of detection by real-time RT-PCR increased 10-fold compared with that using the previous method. Using this method, we extracted RNA from a sterilized field soil, which was inoculated with *Pseudomonas putida* KT2440 transformed with a chloroaromatic degrading plasmid, in the presence or absence of 3-chlorobenzoate (3CB). Real-time RT-PCR performed using the extracted RNA as a template confirmed the induction of chloroaromatic degrading genes in 3CB-amended soil. Thus, this improved method is suitable for the extraction of RNA to detect gene expression in soil.

As described in the Chapter III, even small amounts of humic acids may affect PCR and RT-PCR reactions. To acquire reliable information of bacterial gene expression in soil, we optimized the previous method so that humic acids could be removed more efficiently.

Using the improved method, RNA was extracted from a soil sample which was sterilized and then inoculated with a *Pseudomonas putida* KT2440 strain containing a plasmid (pSL1) (Liu *et al.*, 2001) encoding chloroaromatic degradative enzymes. *P. putida* KT2440 is a frequently studied bacterium isolated from soil with catabolism of various aromatic compounds (Jiménez *et al.*, 2002; Reva *et al.*, 2006; Yuste *et al.*, 2006; del Castillo & Ramos, 2007). It is the first Gram-negative soil bacterium to be certified as a biologically safe strain by the Recombinant DNA Advisory Committee (Nelson *et al.*, 2002). The strain has been used as a host in the development and utilization of genetic tools for studying the functions of a variety of bacteria (Bagdasarian *et al.*, 1981; Panke *et al.*, 1998; Henning *et al.*, 2006; Miyakoshi *et al.*, 2007), and also has been used for behavioral studies of microorganisms in the environment (Wang *et al.*, 2004; Ude *et al.*, 2006; Dechesne *et al.*, 2008; Shintani *et al.*, 2008). This strain is a useful model for developing methods to extract RNA from soil. The genome of the strain has been sequenced (Nelson *et al.*, 2002), allowing detection of individual genes. In this study, improvement of the RNA extraction method was verified by the determination of remaining humic substances and by the detection of gene expression by real-time RT-PCR using catabolic genes located on the chromosome and on the plasmid pSL1.

Materials and methods

Soil for RNA extraction

A field soil sample, a brown forest soil, collected at the Ehime Agricultural Experiment Station in Japan, was sieved (2 mm mesh) and sterilized by autoclaving (1 h at 121°C, twice). After the sterilized soil had cooled to ambient temperature, its water content was measured

and adjusted to 60% of the maximum water-holding capacity. The properties of this field soil have been described in the Chapter II. A subsample of this soil without sterilization by autoclaving was used for RNA extraction from raw soil.

Bacteria culture conditions

Pseudomonas putida KT2440 (Bagdasarian *et al.*, 1981) carrying the plasmid pSL1 conferring the ability to degrade chlorocatechols grows on 3-chlorobenzoate (Liu *et al.*, 2001). The strain was grown overnight in LB medium with kanamycin (50 µg ml^{-1}) at 28°C. One ml of this liquid culture (OD_{600} = 1.2) was centrifuged to collect cells. After washing twice with buffer (10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.8)), cells were resuspended and diluted 10-fold in the same buffer. For RNA extraction, the diluted bacteria suspension (0.5 ml) was mixed with 4.5 g sterilized soil in 50-ml tubes, to which 3-chlorobenzoate (3CB) was added at a final concentration of 250 ppm in the case of 3CB+ samples. To ensure a homogeneous distribution of 3CB in the soil, 3CB was first mixed with Celite® powder (Wako, Osaka, Japan) as previously described (Morimoto *et al.*, 2005). The soil culture of bacteria was incubated at 30ºC for 24 h (3CB− samples) or 48 h (3CB+ samples) prior to RNA extraction and colony counting. The number of bacteria inoculated in sterilized soil was determined by the dilution plating method, as described in the Chapter II. In all experiments, duplicate inoculations were prepared as a minimum.

RNA isolation from soil

The protocol for RNA isolation from soil is based on that described in the Chapter II with the following modifications.

(i) Extraction: RNA extraction from soil was done as described in the Chapter II. To optimize the pH of the lysis buffer, lysis buffers with different pH values (pH 6.6, pH 7, pH

7.6 and pH 8) were used. The metal chamber in the ShakeMaster Auto machine (BioMedical Science, Tokyo, Japan) which held the tubes for shaking at the default speed, was incubated at 14°C or 28°C overnight.

(ii) Purification with Aurum Total RNA Mini Kit. The extracted nucleic acid was purified with an Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA) as described in the Chapter II.

(iii) Purification with gel filtration columns. A Sephadex G-50 spin column (Roche, Indianapolis, IN) and a MicroSpin S-400 HR spin column (GE Healthcare, Little Chalfont, UK) were used to remove humic substances from the RNA fractions acquired at step (ii) according to the manufacturer's instructions.

(iv) Removal of DNA with the TURBO DNA-free kit. Co-extracted DNA was digested with TURBO DNA-free DNase (Ambion, Austin, TX).

(v) Desalting and Concentration: The RNA purified at step (iv) was applied to an RNA Clean-Up Kit-5 (Zymo Research, Orange, CA) or an RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions.

RNA extraction from the raw brown forest soil was performed under the optimized conditions.

Extraction methods for comparison

RNA extraction from the raw brown forest soil with the PowerSoil Total RNA Isolation Kit (MO BIO, Carlsbad, CA, USA) and a recently reported method (Peršoh *et al.*, 2008) were performed according to the manufacturer's instructions or following the original report (Peršoh *et al.*, 2008). In the method of Peršoh et al. (2008), the optimal volume of 0.2 M $Al_2(SO_4)_3$ was 300 µl so that this volume was applied in the current study.

Gel electrophoresis of RNA

The Novagen Perfect RNA Markers (0.2-10 kb) (Merck KGaA, Darmstadt, Germany) together with the purified RNA were resolved on 1% agarose gels, and images of gels stained with SYBR Gold (Molecular Probes, Eugene, OR) were captured with an FAS-III gel scanner (Toyobo, Osaka, Japan). The size of the bands in the RNA marker was as follows: 200, 500, 1000, 1500, 2000, 3000, 4000, 6000, 10000 nucleotides.

UV spectroscopy for humic acids determination

To determine the absorbance of humic acids, an aliquot (2 μl) of RNA extracted from the soil was loaded on a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at a wavelength of 320 nm.

Extraction of humic substances

Sterilized field soil (2 g) was processed to prepare humic acids and fulvic acids fractions as previously described (Hiradate *et al.*, 2006).

Fluorescence spectroscopy for humic substance determination

Fluorescence measurements were performed with a microcell using an F-2500 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) at 25°C. The slit width for excitation and emission wavelengths was 10 nm.

(i) Method 1 (Using a mixture of humic and fulvic acids as a standard): The humic acid and fulvic acid fractions prepared from the field soil were adjusted to equal volumes. Then, equal volumes of each fraction were combined to yield a mixture of humic substances, which was diluted serially to serve as a standard. Samples of the extracted humic acids, fulvic acids, and lysate were diluted with DEPC-treated water; all diluted samples had a pH of 6. Based on

our preliminary experiments, the samples were excited at a wavelength of 332 nm, and the fluorescence intensity was measured at 445 nm.

(ii) Method 2 (Using a commercial humic acid as a standard): The commercial humic acid (nacalai tesque, Kyoto, Japan) was dissolved in 0.1 M NaOH. After a brief centrifugation to remove undissolved materials, the humic acid solution was diluted serially with Milli Q water to serve as a standard. The samples were excited at a wavelength of 276 nm, and the fluorescence intensity was measured at 445 nm.

Real-time quantitative RT-PCR

One-step real-time RT-PCR was performed to examine gene expression levels using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA). For the *benA* gene (PP_3161), the forward primer was 5’-GAAGAAGTCTTCGTACTGGCGAATA-3’, the reverse primer was 5’-GTGAACAAGACCGAAATCACCAT-3’ and the TaqMan probe was 5’-ACGAGCATCGGCGCTCTCGC-3’. For the *tfdC* gene (Liu *et al.*, 2001), the forward primer was 5’-AACTCAGGGTCGGTCGTGAT-3’, the reverse primer was 5’-ATCGTTGGGAATCTGATATGCA-3’ and the TaqMan probe was 5’-CAGTTTAGCGTGCAAACGACGATGCC-3’. For the 16S rRNA gene (PP_16SA), the forward primer was 5’-TGGAGCATGTGGTTTAATTCGA-3’, the reverse primer was 5’-CCATCTCTGGAAAGTTCTCTGCA-3’ and the TaqMan probe was 5’-CAACGCGAAGAACCTTACCAGGCCTT-3’. For all probes, 6FAM (6-carboxyfluorescein) was used as a 5’-reporter and TAMRA (6-carboxytetramethylrhodamine) was used as a quencher. All primers and probes were designed and synthesized by Biosearch Technologies Japan (Tokyo, Japan). The final concentration of each primer or probe in the PCR reaction mixture was 200 nM. The

concentration of RNA samples was adjusted to 10 ng μl^{-1} with DEPC-treated water, and 2 µl of the RNA solution was used as a template in a 50-µl volume of the one-step RT-PCR reaction mixture. Real-time quantitative RT-PCR was performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The reaction conditions were: 30 min at 48°C for reverse transcription, 10 min at 95°C for activation of DNA polymerase, and 40 cycles of 15 s at 95°C and 1 min at 60°C. All reactions were performed in triplicate, and the data were normalized using the real-time RT-PCR signal for the 16S rRNA as follows: $Target_{3CB-} = Target_{3CB+} \times (16S\ rRNA_{3CB-} / 16S\ rRNA_{3CB+})$. Standards for the assays were prepared with PCR amplicons from total DNA of *Pseudomonas putida* KT2440 with the forward and reverse primer sets described above. A standard curve was constructed by comparing the copy numbers of 10-fold dilutions of the standard to their respective threshold cycles. The slope of the standard curve was used to calculate the PCR efficiency with the equation $E = 10^{-1/slope} - 1$. The PCR efficiencies of 16S rRNA, *benA* and *tfdC* are 92.6%, 91.4% and 95.0%, respectively.

Results

Optimization of RNA extraction from soil

To optimize the procedures, we focused initially on the extraction step, and examined different combinations of lysis temperature and pH of the lysis buffer. As shown in Fig. IV.1A, under the same temperature, the color of cell lysate varied from bright to dark as the pH value increased. On the other hand, under the same pH condition, the color of cell lysate prepared at higher temperature (28°C) was darker than that prepared at 14°C. These findings indicated that at lower temperature and pH, the RNA solution extracted from the soil contained lower levels of humic substances. At temperatures below 14°C, the SDS in the lysis buffer precipitated. We also examined RNA recovery under different pH conditions. RNA recovery

was similar at pH 7, pH 7.6, and pH 8 but lower at pH 6.6 (Fig. IV.1B). There was no detectable difference in RNA recovery between lysis temperatures of 14°C and 28°C (data not shown). We concluded that RNA extraction was performed best at 14°C with a lysis buffer of pH 7.

Next, we optimized the purification step. Although major portions of the contaminants including humic substances in the sample prepared at the extraction step could be removed at the step using an Aurum Total RNA Mini Kit, it became necessary to remove the remaining low levels of humic substances which inhibited enzyme-catalyzed reactions such as RT-PCR as shown in the Chapter III. We compared the efficiency of two commercially available RNase-free columns, the Sephadex G-50 spin column (Roche) and the MicroSpin S-400 HR spin column (GE Healthcare), in removing humic substances. Phenol-extracted RNA that had not been purified first with the Aurum Total RNA Mini Kit was used in this experiment. RNA samples purified with any of the two RNase-free columns exhibited similar RNA recovery (Fig. IV.2, upper panel). However, the RNA samples purified with a Sephadex G-50 column had a darker color than those purified with a MicroSpin S-400 HR column (Fig. IV.2, lower panel), suggesting that the latter removed humic substances more efficiently.

The ability of the improved method to remove humic acids was compared to that of the one described in the Chapter II, by parallel analyses using the same inoculation method of KT2440/pSL1. The improved method reduced the level of humic acids to about 5 % of that detected in samples prepared by the older protocol (Fig. IV.3A). To examine whether the interference of humic substances with real-time RT-PCR was reduced, the expression of 16S rRNA was examined. The expression signal detected in RNA fractions prepared by the improved method was 10-fold higher than that obtained from the same amount of RNA prepared by the older method (Fig. IV.3B). We concluded that the undesired effects of humic substances had been reduced significantly by the modifications of the RNA purification

procedure.

The efficiency of humic substances removal of the improved method

To quantify the efficiency of the improved method in removing humic substances, the quantities of the total humic substances in the lysate obtained at step (i) as well as in the final purified samples were determined by fluorescence spectroscopy using method 1, described in Materials and Methods. As shown in Table IV.1, 99.998% of humic substances had been removed after purification. The humic substances extracted together with RNA might include humic acids and fulvic acids (see Discussion). To confirm that the improved method removed both of them efficiently, we extracted humic acids and fulvic acids from the field soil and subjected them to the RNA extraction procedure independently. The quantities of humic substances in the start material at step (i) and the final samples obtained at step (v) were determined by fluorescence spectroscopy. After purification, more than 99.9% of humic acids and fulvic acids were removed (Table IV.1). The percentage of fulvic acids removed was higher than that of humic acids (Table IV.1), suggesting that fulvic acids can be removed more efficiently than humic acids by the improved method.

In an attempt to compare the efficiency of this improved method with other available methods, RNA extraction from the raw brown forest soil was performed with this method, the PowerSoil Total RNA Isolation Kit (MO BIO, Carlsbad, CA, USA) and a recently reported method (Peršoh *et al.*, 2008). Because the improved method removed fulvic acids more efficiently than humic acids (Table IV.1) and the fluorescence spectra of the purified RNA was similar to that of humic acids but different from that of fulvic acids (data not shown), humic acids could be the major component of the humic substances remained in the purified soil RNA solution. The quantities of humic acids in the purified RNA were determined by fluorescence spectroscopy using the method 2 described in the Materials and Method. As

shown in Fig. IV.4, the improved method showed the lowest level of co-extracted humic acids in the extracted RNA among all methods. The co-extracted humic acids in the RNA extracted with the PowerSoil Kit and the method of Peršoh et al. (2008) were reduced to a low level after further purification with a MicroSpin S-400 HR column (Fig. IV.4).

Expression of 3-chlorobenzoate degrading genes

We chose the *benA* and *tfdC* genes that are encoded by genomic DNA and plasmid DNA, respectively, as representatives of the 3CB degrading genes, to examine gene expression induced by 3CB that was added to the soil. Real-time quantitative RT-PCR was performed using the RNA extracted by the improved method as a template (Fig. IV.5). The expression levels of *benA* and *tfdC* were strongly increased in the 3CB-amended soil (3CB+) as compared to the control (3CB−), indicating that the expression of 3CB degrading genes was induced by 3CB in the soil. The bacterial numbers in the two inoculated soils were very similar ($1.02 \times 10^8 \pm 1.04 \times 10^7$ CFU g^{-1} soil in 3CB− soil and $1.01 \times 10^8 \pm 1.18 \times 10^7$ CFU g^{-1} soil in 3CB+ soil), so the difference in expression was due to gene expression in the soil.

Discussion

Optimization of the method for RNA extraction from soil

Although our previous method could handle large amounts of soil and was successful in detection of gene expression in soil, significant levels of humic substances remain in the extracted RNA. In the present study, we optimized several steps to remove humic substances from the RNA preparation more efficiently.

We optimized the lysis conditions in the extraction step because removal of as much of the humic substances as possible at this early step facilitated further purification. The amount of co-extracted humic substances varied with the ambient temperature. The cell lysate

prepared under high ambient temperature had a darker color than that prepared under low ambient temperature. Therefore, we speculated that the temperature might affect the level of co-extracted humic substances in the lysate. Humic substances can be classified into three fractions: fulvic acids that are soluble in alkali and acid, humic acids that are precipitated in alkaline extracts by acidification, and humin that cannot be extracted by alkali or acid (Zipper *et al.*, 2003). Humin was unlikely to be extracted by our lysis buffer so that the co-extracted humic substances likely consisted of fulvic acids and humic acids. Since fulvic acids are soluble in alkali and acid, it is not possible to reduce their levels by optimizing the pH. On the other hand, humic acids are extracted by alkali but not acid, hence it is possible to reduce their level in the lysate by lowering the pH of the lysis buffer. Based on these considerations, we optimized the pH and temperature conditions.

In the subsequent step, the Bio-Rad Aurum Total RNA Mini Kit was used, which has been shown to work well as an ion exchange column-based technique. In the previous study, the Sephadex G-50 quick spin column was used as a gel filtration column. The Sephadex G-50 column has an exclusion limit around 10 kDa so that low molecular weight humic substances will be trapped in the column. While the molecular weights of humic substances are conventionally assumed to range from a few hundred to millions of Dalton, it was reported recently that the weight average molecular weight of soil fulvic and humic acids were 9-14 kDa and 15-20 kDa, respectively (Perminova *et al.*, 2003). Based on this information, we concluded that the Sephadex G-50 column, which allows humic substances of high molecular weight (MW > 10 kDa) pass together with RNA molecules, was not the most appropriate choice. An alternative commercially available RNase-free gel filtration column, the MicroSpin S-400 HR column, separates larger molecules than the Sephadex G-50 column, suggesting that a greater proportion of high molecular weight fulvic and humic acids might be retained in the column while RNA molecules were eluted. This was confirmed by

the results (Fig. IV.2).

In the improved RNA extraction method, a desalting/concentration step was included to facilitate downstream experiments such as real-time RT-PCR. The RNA Clean-Up Kit-5 (Zymo Research) and the RNeasy Mini Kit (QIAGEN) both worked well in our experiments. The maximal RNA binding capacity of the spin column in the RNA Clean-Up Kit-5 (Zymo Research) is 5 µg (RNA Clean-Up Kit-5 Handbook); the RNeasy Mini Kit (QIAGEN), which has a maximal RNA binding capacity of 100 µg (RNeasy Mini Handbook), should be used if greater amounts of RNA are to be handled.

Compared with the previous method for RNA extraction from soil (described in Chapter II), this improved method was modified in three steps. The first is the extraction step, which requires low temperature and an extraction buffer at pH 7 for beads beating. The second is the purification step, at which the Sephadex G-50 column was replaced by a MicroSpin S-400 HR column. Finally, a desalting/concentration step was added, at which a binding spin column was used to ensure high quality RNA.

The efficiency of humic removal

Fulvic acids were removed more efficiently than humic acids in the optimized procedure. First, the phenol extraction (step (i)) removed higher proportions of fulvic acids than humic acids (data not shown). Second, the molecular weights of fulvic acids are smaller than those of humic acids, which made fulvic acids more easily removable by gel chromatography than humic acids.

Although the absorption at 320 nm in principle can be used to determine the levels of humic acids (Miller, 2001), it is unclear whether the method enables the measurement of fulvic acid contents. On the other hand, fluorescence spectroscopy is more sensitive than UV spectroscopy and has a higher specificity since it works with two specific wavelengths for a

given material, the excitation and emission wavelengths. For this reason, fluorescence spectroscopy has been used to detect and characterize humic substances for more than twenty years (Ghosh & Schnitzer, 1980; Senesi, 1990; Belin *et al.*, 1993; De Souza Sierra *et al.*, 2000; Schepetkin *et al.*, 2003; Rezácová & Gryndler, 2006). Since humic substances from different sources have different excitation and emission wavelengths (Belin *et al.*, 1993; De Souza Sierra *et al.*, 2000), it is not possible to determine the absolute quantity of humic substances extracted from various samples accurately using commercial humic substances as standards. In addition, the excitation and emission wavelengths of humic acids and fulvic acids are close to each other (Schepetkin *et al.*, 2003) and hence it is impossible to discriminate one from the other in a mixture. Therefore, we relied on a set of serial dilutions of a mixture of humic and fulvic acids which were extracted from the field soil we used in the present study, as standards for the determination of relative levels of humic substances.

To evaluate the efficiency of the improved method, the raw brown forest soil was processed with the improved method, the PowerSoil Total RNA Isolation Kit (MO BIO, Carlsbad, CA, USA) and a recently reported method (Peršoh *et al.*, 2008), in which $Al_2(SO_4)_3$ was used to trap humic acids prior to RNA extraction. The improved method was more efficient in humic acid removal than these two methods (Fig. IV.4). Since the method of Peršoh et al. (2008) has been already evaluated by its authors as superior to the FastDNA Spin Kit for Soil (Qbiogene, Irvine, CA, USA), the PVPP-purification method (Mendum *et al.*, 1998) and two previously reported methods (Griffiths *et al.*, 2000; Hurt *et al.*, 2001), the improved method may be more efficient in removal of humic acids than those methods.

Although we focused on optimization of our soil RNA extraction method to reduce the contamination of humic substances, the optimized conditions (or strategy for optimization) in this study could be applied for some other soil RNA extraction methods, especially those with purpose requiring highly purified RNA, such as detection of gene expression. Firstly, the

composition of the lysis buffer in a given soil RNA extraction method will not be changed if we only adjust the pH of lysis buffer to neutral and perform cell lysis under lower temperature. Secondly, the MicroSpin S-400 HR spin column could be an optional module in any soil RNA extraction method. In the case that heavy contamination of humic acids occurs, the RNA sample prepared with a given method could be further purified with this column to get rid of humic acids efficiently.

Detection of gene transcripts in soil

The purpose behind our attempts to improve the soil RNA extraction method was the acquisition of high quality RNA for gene expression analyses. Therefore, we examined the expression of two 3CB degrading genes, *benA* and *tfdC*, in the soil using the well-studied strain *Pseudomonas putida* KT2440 as a model microorganism. The real-time RT-PCR data clearly demonstrated 3CB-dependent gene induction in the soil. The genes *benA* and *tfdC* encode benzoate 1,2-dioxygenase and chlorocatechol 1,2-dioxygenase, respectively, both of which are key enzymes in the degradation of chlorobenzoate and chlorocatechols (Cowles *et al.*, 2000; Liu *et al.*, 2001; Ogawa *et al.*, 2003). *benA* is located in the genomic DNA while *tfdC* is located on the plasmid pSL1 (Liu *et al.*, 2001; Nelson *et al.*, 2002). Both of them are located at the first position in the respective gene cluster responsible for the successive enzymatic steps (Ogawa *et al.*, 2003), and thus appeared to be good targets to analyze the expression of the two clusters. Our improved method reliably detected the change of expression levels of the two genes that occurred in the soil due to induction.

Table IV.1 Efficiency of the improved method in removing humic substances

Sample type	Humic substances in the start materials (%)[a]	Humic substances in the final samples (%)[a]
Lysate	100 ± 1.07	0.0021 ± 0.00130
Fulvic acids	100 ± 1.74	0.0007 ± 0.00003
Humic acids	100 ± 2.89	0.0149 ± 0.00175

[a] Triplicate samples were examined.

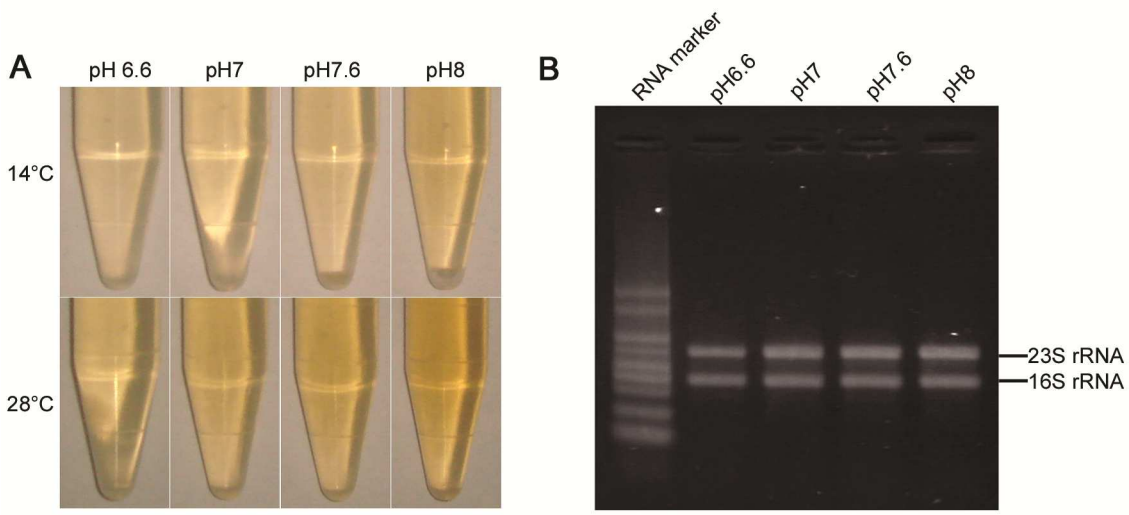

Fig. IV.1 Optimization of cell lysis conditions. (A) The effect of the lysis buffer pH and of lysis temperatures on the levels of co-extracted humic substances. (B) Agarose gel electrophoresis showing the efficiency of RNA recovery under different pH conditions.

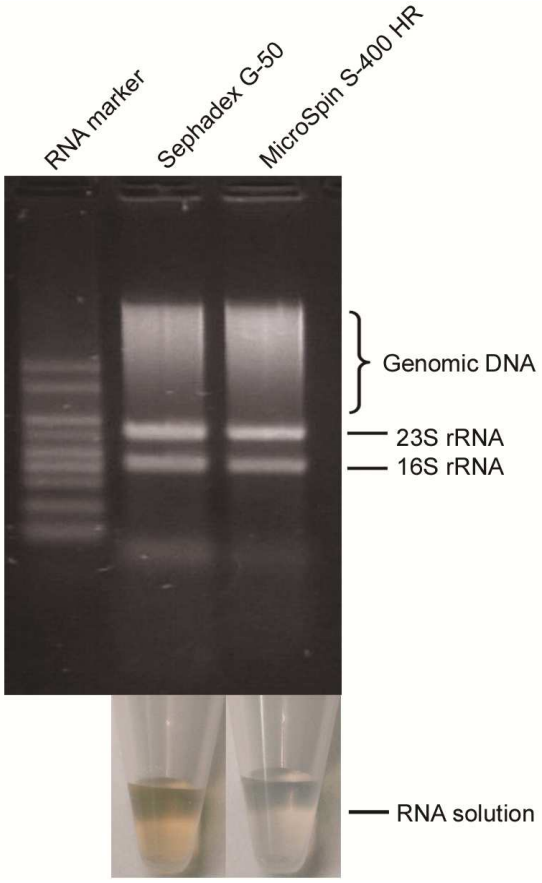

Fig. IV.2 Selection of the gel filtration column for purification. RNA solutions purified by different columns are shown together with the agarose electrophoresis gel. The yellowish tone of the RNA solutions is caused by co-extracted humic substances.

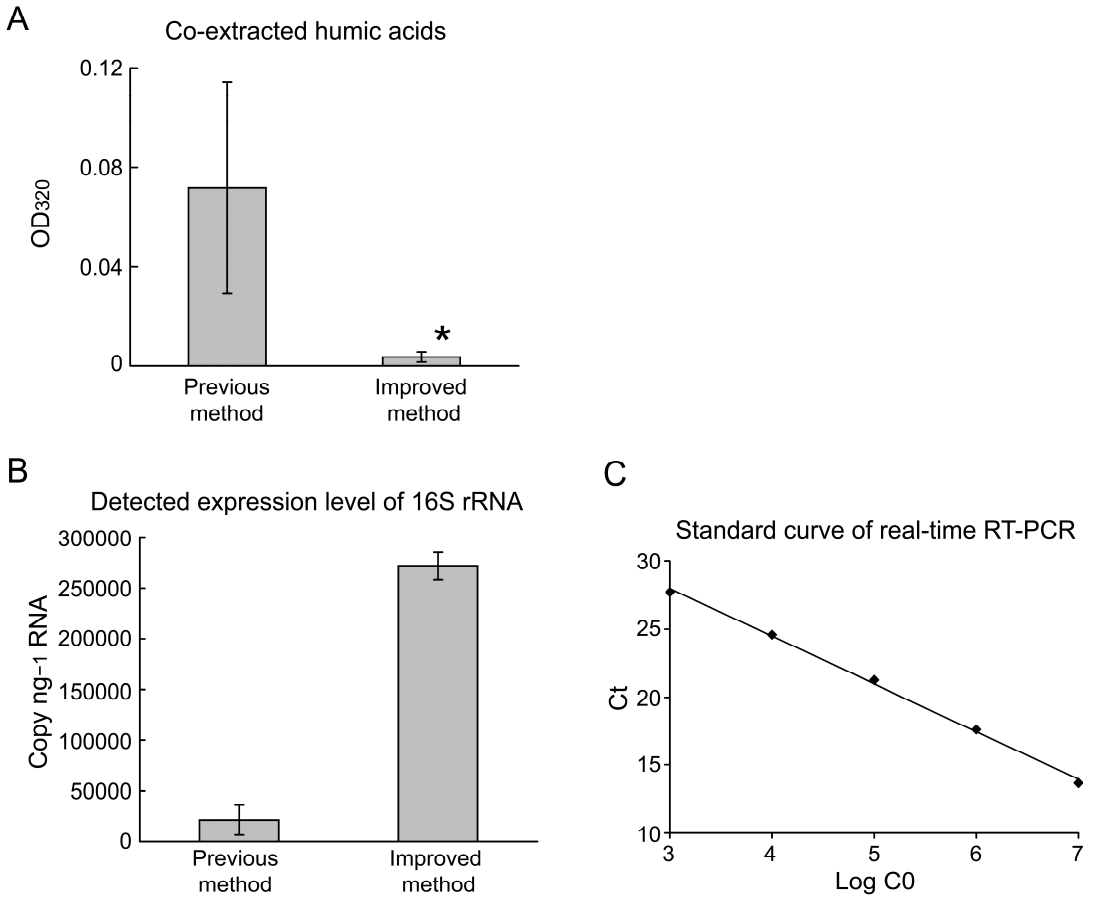

Fig. IV.3 Improvement of the soil RNA extraction method. (A) UV spectrophotometric measurement of humic acids in the RNA extracted from soil. *This was calculated from measurement of 10-folds concentrated RNA solutions since the signals of the original solutions were under the detectable level (The value of OD_{320} lower than 0.02 could be considered as undetectable for humic acid measurement). (B) Expression of 16S rRNA as detected by real-time RT-PCR. (C) The standard curve of real-time RT-PCR for the 16S rRNA gene. Linear regression coefficient R^2 = 0.99 ($y = -3.51x + 38.54$). Samples from triplicate soil incubations were examined in (A) and (B).

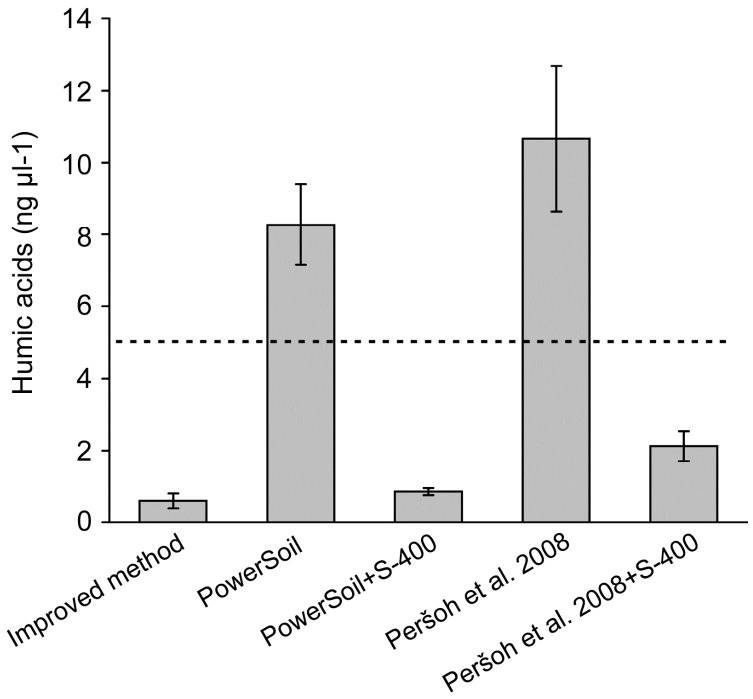

Fig. IV.4 Fluorescence spectroscopic measurement of humic acids in the RNA extracted from soil. For each method, samples from triplicate extractions were examined. The signal intensities of all RNA solutions for measurement were at detectable levels. The broken line indicates the threshold value, lower than which the humic acids in an RNA sample did not inhibit RT-PCR reaction significantly (Chapter III).

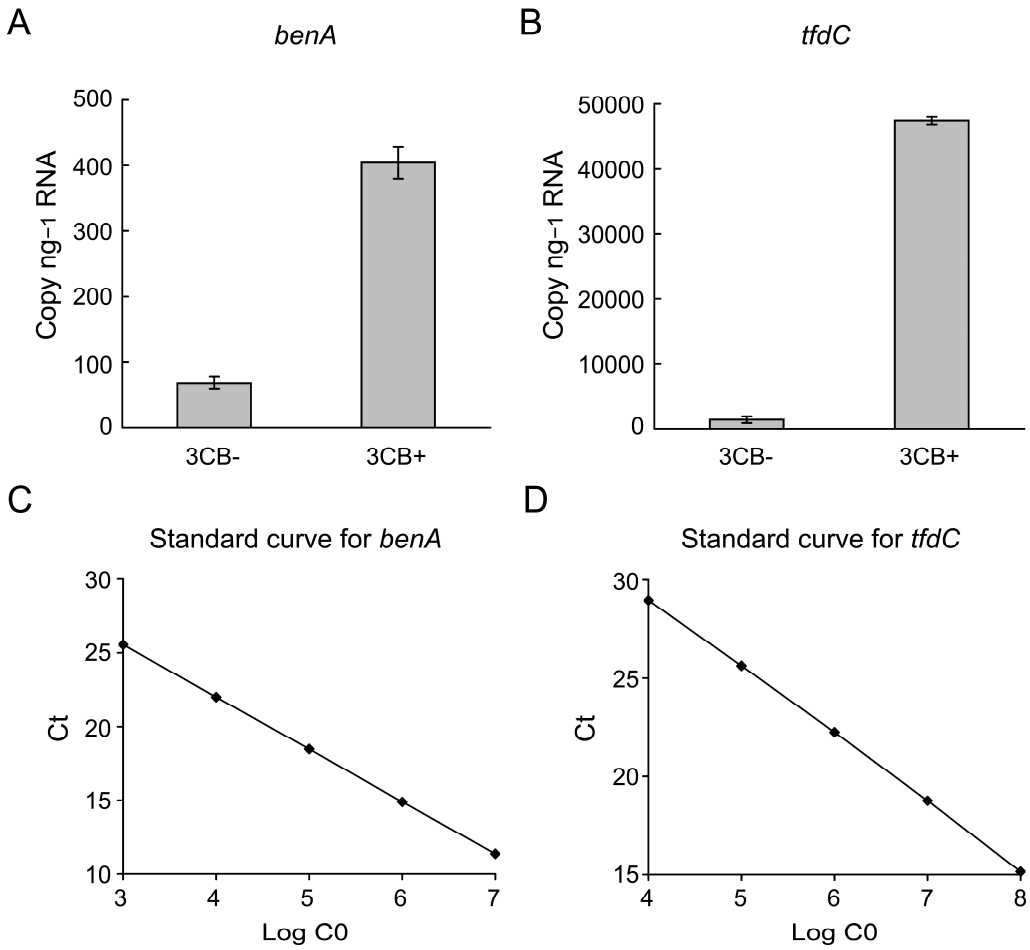

Fig. IV.5 Expression of the *benA* (A) and *tfdC* (B) genes in *Pseudomonas putida* KT2440/pSL1 inoculated in sterilized soil. (C) The standard curve of real-time RT-PCR for the *benA* gene. Linear regression coefficient R^2 = 0.99 (y = −3.55x + 41.20). (D) The standard curve of real-time RT-PCR for the *tfdC* gene. Linear regression coefficient R^2 = 0.99 (y = −3.45x + 42.83).

Chapter V

Microarray Analysis of Global Gene Expression in *Pseudomonas putida* KT2440 Growing in a Sterilized Soil

Summary

To examine whether the RNA extracted from soil with this improved method was suitable for microarray analysis, genome-wide scanning of gene expression by microarray techniques was performed on RNA extracted from sterilized soil inoculated with *Pseudomonas putida* KT2440/pSL1. The genes showing significant changes in their expression in both the triplicate-microarray analysis using amplified RNA and the single-microarray analysis using unamplified RNA were investigated. Pathway analysis revealed that the benzoate degradation pathway underwent the most significant changes following treatment with 3CB. Analysis based on categorization of differentially expressed genes against 3CB revealed new findings about the cellular responses of the bacteria to 3CB. The genes encoding a K^+/H^+ antiporter complex, a universal stress protein, two cytochrome P450 proteins and an efflux transporter were upregulated. The downregulated expression of several genes involved in carbon metabolism and the genes belonging to a prophage in the presence of 3CB was observed. This study demonstrated the applicability of the method of soil RNA extraction for microarray analysis of gene expression in bacteria growing in sterilized soil.

Microarray techniques are powerful tools to monitor gene expression genome-wide, and have been extensively applied to many aspects of biological studies (Watson *et al.*, 1998; Epstein & Butow, 2000). Soon after this methodology became available, soil microbiologists speculated on its usefulness for the detection of bacterial gene expression in soils (Insam, 2001). However, because of the difficulties involved in RNA extraction from soil, these expectations have not yet been realized. One of the major problems in soil RNA extraction is contamination with humic acids, which affect the accurate measurement of nucleic acids (Bachoon *et al.*, 2001; Zipper *et al.*, 2003), suppress enzyme activity (Tebbe & Vahjen, 1993), and inhibit hybridization (Alm *et al.*, 2000). Recently, we developed a new strategy to extract bacterial RNA from soils, enabling us to acquire high quality RNA with very low levels of humic acid contamination, allowing for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to obtain reliable gene expression data (Chapter IV). While our ultimate target is to detect bacterial gene expression in raw soil, we reasoned that a useful first step would be the successful microarray analysis of a sterilized soil inoculated with a bacterial strain whose genome had been fully sequenced. In this study, we used *Pseudomonas putida* KT2440 as a target strain, whose genome sequence (Nelson *et al.*, 2002) and catabolic potential against a wide range of natural aromatic compounds (Jiménez *et al.*, 2002) has been determined. We extracted RNA from sterilized soil inoculated with a *Pseudomonas* strain (*Pseudomonas putida* KT2440/pSL1) containing a plasmid that carried genes for the degradation of chloroaromatic compounds (Liu *et al.*, 2001), in the presence or absence of 3-chlorobenzoic acid (3CB). Microarray analysis and subsequent validation by qRT-PCR provided us with new knowledge about the cellular responses of bacteria to 3CB, including induction of several genes involved in transport and stress response, and downregulation of the genes belonging to a prophage and several genes involved in carbon metabolism.

Materials and methods

Bacterial culture conditions

Pseudomonas putida KT2440/pSL1 (Liu *et al.*, 2001) was transferred from a glycerol stock to an LB agar plate (Sambrook & Russell, 2001) with kanamycin (50 μg mL^{-1}), and incubated at 30 °C overnight. The strain was then subcultured overnight in LB medium (Sambrook & Russell, 2001) with kanamycin (50 μg mL^{-1}) at 28 °C. A 1-mL volume of the liquid culture (OD_{600} = 1.0) was centrifuged to collect cells. After washing twice with 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6.8), cells were resuspended and diluted 10-fold with the same buffer. To prepare bacterial soil cultures, 0.2 mL of the diluted bacterial suspension was mixed with 1.8 g sterilized soil in 15 mL tubes [about 5×10^6 CFU $(g\ soil)^{-1}$], to which 3CB had been already added at a final concentration of 250 μg $(g\ soil)^{-1}$ (the 3CB+ group). Brown forest soil (FAO classification: Gleyic Cambisols) was sampled from the surface of a field at the Ehime Agricultural Experimental Station in Ehime, Japan (33° 50' 24" N and 132° 46' 12" E). Preparation of the sterilized soil samples and the properties of the soil were same as described in the Chapter II. To ensure homogeneous distribution of 3CB in the soil, 3CB was first mixed with Celite® powder (Wako, Osaka, Japan) as previously described (Morimoto *et al.*, 2005). Equal amounts of Celite powder were added to control soil samples (3CB- group). The soil cultures were incubated at 30 °C for the appropriate periods. To determine the concentration of 3CB in soil, soil samples were subjected to HPLC analysis with a reversed phase C18 column as previously described (Morimoto *et al.*, 2008).

RNA extraction from soil

Total RNA was extracted from 2 g of soil culture using an RNA PowerSoil Total RNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's instructions, with some modifications. Centrifugation (2,500 × *g*, 10 min, room temperature) was

conducted between the cell lysis and the phenol extraction steps to separate the cell lysate from the soil. Purification using a MicroSpin S-400 HR spin column (GE Healthcare, Little Chalfont, UK), DNase digestion using a TURBO DNA-free DNase (Ambion, Austin, TX, USA), and concentration using an RNeasy Mini Kit column (Qiagen, Valencia, CA, USA) were conducted as described in the Chapter IV. The concentration of RNA was determined using a Nanodrop system (Nanodrop, Wilmington, DE, USA). The integrity and purity of extracted soil RNA was assessed by agarose gel electrophoresis, ultraviolet spectrometry, and 23S/16S ratio on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Microarray analysis – Single array without RNA amplification

In this analysis, the pooled RNA extracted from 32 (3CB-) and 48 (3CB+) soil cultures was directly applied to microarray analysis. A single microarray was used for each treatment (3CB+ or 3CB-), therefore two NimbleGen Custom Prokaryotic Gene Expression 385K Arrays were used in this analysis. Each array contained five sets of fourteen sequence-specific 60-mer probes per gene corresponding to 5,341 genes from the *Pseudomonas putida* KT2440 genome and five genes (*tfdT*, *tfdC*, *tfdD*, *tfdE* and *tfdF*) from the pSL1 plasmid.
Five micrograms of total RNA was processed and labeled according to the standard protocols from Roche NimbleGen. Briefly, double-stranded cDNA was synthesized using a SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), then labeled with Cy3-random nonamers using a NimbleGen One-Color DNA Labeling Kit (Roche NimbleGen) and hybridized to the microarrays for 16 h at 42 °C on a NimbleGen Hybridization System. The arrays were washed, dried, and scanned at 5 µm resolutions using a GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA). NimbleScan v2.5 (Roche NimbleGen) was used to extract data from scanned images, and to perform quantile normalization (Bolstad *et al.*, 2003) and robust multi-array average (RMA)

analysis (Irizarry *et al.*, 2003) across arrays to generate gene expression values. Statistical analysis and fold change calculations were performed using NANDEMO Analysis v1.0.2 (Roche NimbleGen). The Student's *t*-test with Bonferroni correction for multiple testing [a total of 5,346 open reading frames (ORFs) on arrays] was applied to evaluate genes with significantly altered signal intensity.

Microarray analysis – Triplicate arrays with RNA amplification

In this analysis, the total RNA extracted from three replicate soil cultures of each treatment (3CB+ or 3CB-) was amplified by a MessageAmp™ II-Bacteria Kit for Prokaryotic RNA Amplification according to the manufacturer's instructions (ABI Ambion, Tokyo, Japan). For each soil culture, 100 ng total RNA was used as start material in the RNA amplification reaction. One array was used for each of the triplicate RNA samples in each treatment, therefore six NimbleGen Custom Prokaryotic Gene Expression 4x72K Arrays were used in this analysis. Each array contained two sets of six sequence-specific 60-mer probes per gene corresponding to 5,341 genes from the *Pseudomonas putida* KT2440 genome. Both the NimbleGen 385K and the 4x72K arrays used in this study were designed and manufactured by Roche NimbleGen.

Amplified RNA (10 μg) was processed and labeled according to the standard protocols from Roche NimbleGen as described above. Data were extracted from scanned images using NimbleScan v2.5 (Roche NimbleGen). The ArrayStar v4.0 software (DNASTAR, Madison, WI, USA) was used to perform quantile normalization (Bolstad *et al.*, 2003) and RMA analysis (Irizarry *et al.*, 2003) across arrays to generate gene expression values, fold change calculation and statistical analysis. The Student's *t*-test with false discovery rate (FDR) correction for multiple testing [a total of 5,341 open reading frames (ORFs) on arrays] was applied to evaluate genes with significantly altered signal intensity.

Quantitative RT-PCR

Expression of genes selected from the microarray screening was validated by two-step qRT-PCR; the genes for validation and corresponding primers are listed in Table S1. Total RNA (700 ng) extracted from 3CB+ or 3CB- soil cultures (three independent pools of RNA were used and each pool contained RNA recovered from two soil cultures) in either the logarithmic or transition phases was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions for random hexamer primed reactions. Quantitative RT-PCR was performed using SYBR Premix DimerEraser (Perfect Real Time) (Takara, Shiga, Japan) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 2 μL sample of 4-fold diluted cDNA was used as a template in a 20 μL reaction mixture. The final concentration of each primer in the PCR mixture was 300 nM. The reaction conditions were: 30 s at 95 °C for activation of DNA polymerase, 40 cycles of 5 s at 95 °C and 1 min at the temperature indicated in Table S1, followed by a melting curve stage, which generated curves with continuous fluorescence acquisition from 60–95 °C at a rate of 0.3 °C s^{-1}. Standards for the assays were prepared with PCR amplicons from genomic DNA or cDNA of *Pseudomonas putida* KT2440, or the plasmid pSL1 with the same primers used in the qRT-PCR. A standard curve was constructed by comparing the copy numbers of 10-fold dilutions of the standard to their respective threshold cycles. The amplification efficiencies of all standard curves, which were calculated with StepOne Software (Version 2.1;Applied Biosystems, Foster City, CA, USA), were higher than 90%. The negative controls without template and RT- controls (RNA without reverse transcriptase treatment) for all examined genes showed Cq values at least five cycles higher than those of samples. A recent report found that actually no gene is universally stable enough to serve as a general reference gene to normalize qRT-PCR data, and a subset of stable genes that has smaller variance than commonly used reference genes exist in each biological context

(Hruz *et al.*, 2011). Thus, we searched for proper reference genes using the current gold standard method, which combines the evaluation of a set of reference genes together with a method for selecting reference genes with the most stable expression (Huggett *et al.*, 2005; Nolan *et al.*, 2006). The candidate reference genes for normalization of qRT-PCR data were selected according the criteria as followed: in both the triplicate-array and the single-array analyses, fold changes were smaller than ±1.20 and signal intensities were 20–60% of the maximum signal on arrays to avoid signal saturation or undetectable signals. Six genes fitted these criteria: flagellar protein FliS (PP_4375); translation initiation factor IF-3 (PP_2466); 50S ribosomal protein L1 (PP_0444); ornithine carbamoyltransferase (PP_1000); flagellar cap protein FliD (PP_4376); and tRNA (guanine-N(1)-)-methyltransferase (PP_1464). The best reference gene was selected by evaluation of the qRT-PCR data for these genes as well as for the 16S rRNA gene using BestKeeper (Pfaffl *et al.*, 2004). This analysis suggested that the gene encoding the flagellar protein FliS (PP_4375) was the more stable gene. Thus, the expression level of each gene was normalized using the qRT-PCR signal for PP_4375. Student's *t*-test (two-sided) was applied to identify genes with significantly altered signal intensity. A *p*-value less than 0.05 was considered statistically significant.

Analysis of biochemical pathways and cellular responses

To perform automatic biochemical pathway analysis, the Entrez Gene ID numbers of genes to be analyzed were submitted to the DAVID Bioinformatics Resources server (Dennis *et al.*, 2003; Huang *et al.*, 2009), followed by running the Functional Annotation Chart with default settings. Categorization of differentially expressed genes against 3CB was conducted by manual searches on the website of the Comprehensive Microbial Resource (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi). The latest information for each differentially expressed gene was confirmed, or updated when necessary, by referring to the

Entrez Gene database (http://www.ncbi.nlm.nih.gov/gene). Annotation of hypothetical proteins was done by performing a BLASTP search against the NCBI non-redundant protein sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and domain search against the NCBI CDD (Conserved Domain Database, http://www.ncbi.nlm.nih.gov/cdd) and the Pfam (http://pfam.sanger.ac.uk/) databases. The hypothetical proteins with assigned function by homology search were re-categorized accordingly. Manual analysis of biochemical pathways was conducted, where required, by locating differentially expressed genes on the corresponding pathway maps which were downloaded from the KEGG website (http://www.genome.jp/kegg/).

Microarray data accession number

The microarray data discussed in this publication have been deposited in the NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002; Barrett *et al.*, 2011) and are accessible through GEO Series accession number GSE19516 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19516) and GSE28215 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28215).

Results

Bacterial growth and 3CB degradation in soil

In preliminary experiments, we observed that a 10-fold dilution of the *P. putida* KT2440/pSL1 liquid culture (OD_{600} = 1.0) for soil inoculation was appropriate to generate a growth curve that reached a maximum level within several days (Fig. V.1A). Thus, we applied this dilution step to prepare all soil cultures used in this study. The 3CB is degraded completely by the *P. putida* KT2440/pSL1 strain as it possesses genomic genes for the breakdown of 3CB to 3-chlorocatechol, and an introduced plasmid, which contains

3-chlorocatechol degradation genes (Liu *et al.*, 2001). When soil was treated with 250 μg (g soil)$^{-1}$ 3CB, this strain degraded 3CB almost completely within 3 days of incubation (Fig. V.1B). To detect expression of 3CB degradation genes in populations at comparable growth stages by microarray analysis, bacterial cells were harvested in the transition phase, 2 days and 1 day after incubation for 3CB+ and 3CB- groups, respectively. For qRT-PCR analysis to validate the data obtained by microarray analysis, bacterial cells were harvested after 1 day (log phase of 3CB+ treatment) or 2 days (transition phase of 3CB+ treatment) and half a day (log phase of 3CB− treatment) or 1 day (transition phase of 3CB− treatment) after incubation.

Quality of RNA extracted from soil

For microarray analysis, the extracted soil RNA possessed the typical ultraviolet absorption spectrum of pure RNA, in which the ratios of OD_{260}/OD_{280} were higher than 2.00 and OD_{260}/OD_{230} were higher than 1.95, suggesting a successful removal of impurities such as humic acids during RNA purification. The rRNA ratio (23S/16S) examined with an Agilent 2100 Bioanalyzer was 1.0 for all samples, indicating that the integrity of the extracted RNA was appropriate for microarray analysis. The suggested 23S/16S ratio should be greater than or equal to 1.0 as suggested by Roche.

Overview of microarray analysis and qRT-PCR validation

In the triplicate array analysis, 197 genes possessed fold changes higher than 3 and the maximum FDR *p*-value among these genes was lower than 0.005. In the single array analysis, 217 genes possessed fold changes higher than 3 and low Bonferroni ($p < 0.001$). These data suggested that there was no false positive data among these genes in both analyses. Because false positive genes could be selected if the threshold of fold change was lower than 3, we used a 3-fold change as a common criterion to select significantly altered genes from both

analyses. The genes showing significant changes in their expression levels in both analyses were collected for further analysis, covering 51 upregulated genes and 59 downregulated genes. Among them, the genes involved in 3CB degradation and transport, and major cellular responses were validated by qRT-PCR (Tables V.1 and V.2).

Expression of genes involved in 3CB degradation and transport

To identify biologically significant cellular responses, we analyzed the 51 upregulated genes and 59 downregulated genes using the DAVID Bioinformatics Resources server. This analysis suggested that benzoate degradation *via* the hydroxylation pathway was the pathway most significantly affected by 3CB treatment in multiple tests (Bonferroni, $p < 0.001$).
For the benzoate degradation-related genes located in the chromosome (*benABCD* operon), the upregulated expression on microarrays was validated by qRT-PCR (Table V.1). It was also confirmed by qRT-PCR analysis that the 3CB degradation genes located in the plasmid (*tfdC, tfdD, tfdE and tfdF*) exhibited strong responses to 3CB treatment in both the log and transition phases (Table V.1).

For small compound transport in Gram-negative bacteria, a porin protein in the outer membrane and transporter proteins in the inner cell membrane are required (Schirmer, 1998; Klebba, 2005). In the *P. putida* KT2440 genome, there were seven candidate genes involved in benzoate transport according to the KEGG gene annotation (http://www.genome.jp/kegg/), including three porin genes (PP_1383, PP_2517, *benF*) and four transporter genes (PP_1820, *benK*, *benE*-1, *benE*-2). However, according to our microarray and qRT-PCR data (Table V.1), only two transporter genes (*benE*-2 and *benK*) and two porin genes (*benF* and PP_1383) responded to 3CB treatment.

Survey of cellular responses to 3CB

To characterize the cellular responses of *P. putida* KT2440/pSL1 to 3CB, 110 differentially expressed genes (51 upregulated and 59 downregulated genes), which had been used for pathway analysis, were categorized according to their cellular roles as annotated by the Comprehensive Microbial Resource database (Fig. V.2). The categories “energy metabolism” and “transport and binding proteins” included 47% of the upregulated genes, and the categories “prophage” and “energy metabolism” included 64% of the downregulated genes. These suggested that major cellular responses to 3CB occurred in these categories. In terms of the ratio of the number of differentially expressed genes to the total gene number in each category, “central intermediary metabolism” (2.5%) and “energy metabolism” (2.7%) were the categories affected most by 3CB exposure, suggesting that important cellular responses occurred also in “central intermediary metabolism”.

Transport and binding proteins

Among the 3CB responsive genes, 12 were related to transport (11 upregulated and one downregulated; Figs. V.2 and V.3). The upregulated genes included transporter genes for benzoate and its analogues and for other compounds (Tables V.1 and V.2). The genes coding for predicted subunits of a K^+/H^+ antiporter complex (PP_2225–PP_2228) were upregulated in both the log and transition phases. As a response to 3CB, the expression of a drug resistance efflux transporter gene (PP_1271) increased in both the log and transition phases. This suggested that this gene was involved in the extrusion of excess 3CB molecules or its toxic metabolites from *P. putida* KT2440/pSL1 cells, although further study is required to confirm this.

Cytochrome P450 and stress response proteins

The two upregulated genes in the “central intermediary metabolism” category, PP_1950

and PP_1955, encode cytochrome P450 family proteins, which are involved in the oxidative degradation of various compounds, especially environmental toxins and mutagens (Werck-Reichhart & Feyereisen, 2000). PP_1950 showed a dramatic fold change by the 3CB treatment (72- and 46-fold in the single array and triplicate array analyses, respectively), suggesting a high response to the treatment. In the "cellular processes" category, PP_1269 encodes a UspA-like universal stress protein. The UspA protein is a small cytoplasmic protein whose expression is enhanced when the cell is exposed to stress agents (Nystrom & Neidhardt, 1994). It is likely the universal stress protein in strain KT2440 was responsive to the 3CB treatment to protect cells from 3CB molecules or the intermediate product molecules generated during 3CB degradation (Fig. V.3).

Carbon metabolism

Genes involved in carbon metabolism were mostly found in the "energy metabolism" category, and included the benzoate degradation genes. As shown in Table V.2, the genes encoding 2,3-butanediol dehydrogenase and the components of the acetoin-cleaving system (PP_0552 to PP_0557), which are required for the conversion of 2,3-butanediol to central metabolites (Huang *et al.*, 1994), were downregulated following 3CB treatment in both the log and transition phases (Fig. V.3), suggesting a reduced consumption of carbon sources other than 3CB in the soil. Utilization of carbon sources was switched from acetoin-related compounds to 3CB in the cells exposed to 3CB in a soil environment.

Prophage genes

There are four prophages in the *P. putida* KT2440 genome (Canchaya *et al.*, 2003). Among 53 genes (from PP_1532 to PP_1584) belonging to phage 04, 29 genes were downregulated in the cells exposed to 3CB (Fig. V.3). The qRT-PCR data of the genes

encoding a capsid protein (PP_1567) and a major tail protein (PP_1573) confirmed the microarray data (Table V.2). In the 3CB- samples, the expression levels of these two prophage genes were increased at the transition phase (1.8 ± 0.1-fold for PP_1567 and 2.3 ± 0.3-fold for PP_1573, $p < 0.05$, Student's *t* test) compared with the log phase, but such a tendency was not observed in the 3CB+ samples.

Discussion

Optimization of the microarray technique for soil RNA samples: RNA preparation, RNA amplification and data analysis

For successful microarray analysis, a large quantity of high quality RNA is required. It is easy to obtain sufficient quantities of pure RNA (tens of micrograms) from a liquid culture. Conversely, the RNA yield from soil is low, ranging from tens to hundreds of nanograms per gram of soil. For this reason, large amounts of source soil and concentration of the extracted RNA are required prior to microarray analysis. Furthermore, humic acids co-extracted with nucleic acids from the soil interfere with microarray analysis. Because humic acids can be concentrated together with nucleic acids during ethanol precipitation (Torsvik, 1980), the co-extracted humic acids need to be reduced to an extremely low level before the final concentration procedure. These problems make it difficult to analyze gene expression of microorganisms living in soils by microarray methods. Using the method developed by us, approximately 99.9% of the humic acids could be removed. To render the method more feasible for routine laboratory use so that the microarray data can be compared with those obtained in future studies, we used a combination of commercial kits (PowerSoil Total RNA Isolation Kit plus MicroSpin S-400 HR spin column) to purify soil RNA, which proved to be highly successful in removing humic acids from soil RNA. To establish the reliability of the method, we conducted microarray analyses using RNA extracted from sterilized soil

inoculated with a single bacterial strain.

In the microarray analysis using a single array per treatment, we collected 30 µg RNA from 48 independent soil cultures treated with 3CB and an equal quantity of RNA from 32 soil cultures untreated with 3CB. Since it is difficult to prepare such large amounts of RNA for triplicate array analysis, we adopted an RNA amplification strategy to obtain a sufficient amount of RNA for triplicate microarray analysis, in which around 100 µg amplified RNA was generated from as little as 100 ng total RNA. This made microarray analysis using a small amount of soil (e.g. 2 grams of soil) become available so that it saved much effort in RNA extraction from soil. High reproducibility of microarray data using RNA amplified by the MessageAmp II-Bacteria Kit has been verified by the manufacturer (http://www.ambion.com/) and other researchers (Frias-Lopez *et al.*, 2008). This technique has been previously used by researchers successfully (Frias-Lopez *et al.*, 2008; Shi *et al.*, 2009; Stewart *et al.*, 2010). However, the correlation between gene expression data acquired using amplified RNA and those acquired using unamplified RNA (r^2 = 0.85–0.92) was a little lower than that for biological replicates (r^2 = 0.94–0.99) (Frias-Lopez *et al.*, 2008), suggesting that a small bias was generated during RNA amplification. Although this small bias might be ignored in some cases, to obtain reliable microarray data, we selected the significantly differentially expressed genes in both the single array analysis using unamplified RNA and the triplicate array analysis using amplified RNA. Because there was no replicate sample and the unit of observation in the statistical test was a probe (70 probes/gene), Student's *t*-test with Bonferroni correction was applied to select differentially expressed genes in the single array analysis. In the triplicate array analysis, there were three replicate samples per treatment and the unit of observation in the statistical test was a sample. In such cases, Bonferroni correction would be too stringent to select significantly differentially expressed genes and FDR correction was more appropriate as discussed previously (Leung & Cavalieri, 2003; Reimers,

2010). Thus, Student's *t*-test with FDR correction was applied to this analysis.

Changes of the expression level of the genes involved in 3CB degradation and transport

P. putida KT2440 can metabolize many xenobiotic compounds such as benzoate, but cannot convert 2-, 3- and 4-chlorobenzoate to the central intermediate compounds, which can then enter the citrate cycle (Jiménez *et al.*, 2002; Ogawa *et al.*, 2003). To confer *P. putida* KT2440 the ability to degrade 3CB completely, a plasmid containing the *tfd* operon was introduced into *P. putida* KT2440 (Liu *et al.*, 2001). In the single array analysis, the 3CB degradation genes located in the plasmid (*tfd* operon) showed almost no significant change of expression in the microarray (Table V.1). The signal intensities of these genes on the microarray were very high, indicating that the expressions of these genes were saturated even in 3CB- samples, possibly because of multiple copy numbers. The qRT-PCR analysis confirmed that four enzyme genes (*tfdC*, *tfdD*, *tfdE* and *tfdF*) in the plasmid exhibited strong responses to 3CB (Table V.1). For the benzoate degradation-related genes located in the chromosome (*benABCD* operon), the upregulated expression on the microarray was validated by qRT-PCR (Table V.1). The gene expression data of the *tfd* operon together with the *benABCD* operon are consistent with the HPLC data, which indicated complete degradation of 3CB after a 3 day incubation (Fig. V.1B).

In biodegradation studies, most efforts have been directed towards identifying or characterizing enzymes that can degrade a certain compound. Therefore, other cellular responses to the treatment of the compound have been rarely analyzed. Although it has been known that *benK* and *benF* are involved in transport of benzoate and its analogous molecules (Cowles *et al.*, 2000), there are five other genes in the *P. putida* KT2440 genome putatively involved in this process. In this study, besides *benK* and *benF*, we confirmed that a BenF-like porin (PP_1383) and a transporter gene (*benE*-2) were also induced by 3CB treatment in both

the log and transition phases, suggesting that these genes are involved in 3CB transport. Recent experimental evidence suggests that *benE*-1, *benE*-2 and *benK* are benzoate transporter genes in the KT2440 strain (Nishikawa *et al.*, 2008). However, *benE*-1 and two other genes (PP_1820 and PP_2517) were not induced by 3CB, an analogue of benzoate, suggesting that these three genes might be expressed in response to exposure to substrates more specific than *benK*, *benE-2* and *benF*.

Other cellular responses to 3CB treatment

In this study, we detected induction of an efflux transporter protein, a universal stress protein and a K^+/H^+ antiporter gene operon under existence of 3CB in soil by microarray analysis, these results were subsequently validated by qRT-PCR (Table V.2). All of these genes were first reported to be closely related to 3CB degradation. The universal stress protein and the multidrug efflux transporter probably represented two strategies adopted by the bacteria to deal with excess 3CB molecules. The multidrug efflux transporter pumped out excess 3CB molecules; meanwhile, the universal stress protein helped the cells to survive in the presence of excess 3CB. Although it seemed that these genes might be involved in resistance against 3CB, we still cannot exclude the possibility that it might be also involved in resistance against the intermediate products during 3CB degradation. On the other hand, the K^+/H^+ antiporter complex shuttles K^+ into cells while extruding H^+ to maintain the intracellular pH at an appropriate level when bacterial cells are exposed to a low pH environment (Moat *et al.*, 2002). The pH of both 3CB+ and 3CB- soil samples slightly increased during incubation (from 6.04 to 6.26 in 3CB+ samples and from 6.13 to 6.37 in 3CB- samples), and at each time point, the pH of 3CB+ samples was lower than that of 3CB- samples. The difference of pH between 3CB+ and 3CB- samples at the transition phase was 0.08. Because addition of 3CB acidifies the soil, the upregulation of the K^+/H^+ antiporter

genes could be helpful in maintaining cytoplasmic pH in bacteria taking up 3CB, therefore these genes were induced in both the log and the transition phases. It remains to be elucidated whether there is any other gene affected by change of pH in KT2440 containing pSL1.

It has been known that several members of the cytochrome P450 protein family are involved in oxidation of benzoic acid (Matsuzaki & Wariishi, 2005) and its derivatives, such as 3-chloroperoxybenzoic acid (Spolitak *et al.*, 2005), 4-methoxybenzoic acid and 4-ethylbenzoic acid (Bell *et al.*, 2008). In a recent study, it was found that 3-chlorobenzoic acid induced the expression of a fungus P450 protein, which was demonstrated to be involved in degradation of benzoic acid (Ning *et al.*, 2010). Thus, we speculate that the two P450 proteins (PP_1950 and PP_1955) in the *P. putida* KT2440 strain might play a role in 3CB degradation or its conversion to nontoxic compounds; however, further investigation is required.

A cluster of genes highly responsive to 3CB treatment

Because the P450 proteins mainly catalyze the oxidation reaction in compound degradation, there should be some other related genes encoding corresponding enzymes either to convert a compound to the substrate of P450 proteins or to convert the product generated by the P450 proteins to a downstream product. When we survey the list of differentially expressed genes in the microarray analysis, we noticed that a large gene cluster showed high fold changes in both microarray analyses, and contained the genes encoding the two cytochrome P450 proteins mentioned previously. This gene cluster possesses 15 genes ranging from PP_1943 to PP_1957 with the same orientation of transcription, suggesting that co-expression may occur among these genes. More than half of the genes in this cluster showed several tens to more than 100-fold changes in both microarray analyses and expression of two genes, PP_1943 and PP_1950, were validated by qRT-PCR (Table V.2).

This suggested that these genes are highly responsive to 3CB treatment.

In this gene cluster, three genes, PP_1943 to PP_1945, are involved in one carbon pool (the pathway of conversion of different types of tetrahydrofolate), suggesting that transfer of the methyl group may become more active in the cells exposed to 3CB. If some enzymes catalyzed demethylation reactions, it is reasonable to observe this phenomenon. Two genes coding for demethylase were found in the list of upregulated genes, PP_1957 and PP_3736. Both of them are vanillate demethylase. We did not add vanillate into the soil for bacteria culture, therefore it is unclear through what mechanism 3CB induced expression of these genes. There are five genes encoding oxidoreductases in the gene cluster (PP_1946, PP_1949, PP_1951, PP_1953 and PP_1957). Except for PP_1957 (a vanillate demethylase), all of them have unknown function(s). It is possible that these oxidoreductases play a role in 3CB conversion or degradation as they seem to be co-expressed with the two cytochrome P450 genes.

In a BLASTP search against the NCBI database using the amino acid sequence of each gene in this gene cluster, we found that the genes from PP_1943 to PP_1955 had high similarity to the genes belonging to bacteria that were not pseudomonads. A comparative genomic analysis of 19 genomes in the *Pseudomonadaceae* family using the RECOG server (http://mbgd.nibb.ac.jp/RECOG/) revealed that the organization of this gene cluster is only present in *P. putida* KT2440 (Fig. S1). The integrated microbial genomes (IMG) database (Markowitz *et al.*, 2010) predicted these genes to be putative horizontally transferred genes (Table S2). Among them, most genes (PP_1943, PP_1944, PP_1945, PP_1948, PP_1951 and PP_1952) were possibly transferred from alphaproteobacteria, and others were possibly transferred from betaproteobacteria (PP_1949 and PP_1956), actinobacteria (PP_1950, PP_1953, PP_1954 and PP_1955), chloroflexi (PP_1946) and firmicutes (PP_1947). It is likely that *P. putida* KT2440 acquired these genes from different donors so that a complete set

of genes for conversion or degradation of a certain compound (probably 3CB-like molecules) was organized as the current status. Although the functions of these genes have not yet been experimentally confirmed till now, the current study firstly reported under what condition these genes were expressed, which is critical for the study of gene functions.

Prophage genes—the major downregulated genes

Among the downregulated genes, the genes belonging to the phage 04 outnumbered the genes in all other categories, suggesting that the response of this prophage was one of the major responses to 3CB (Fig. V.3). The phage 04 is a putative lysogenic bacteriophage with high similarity to the bacteriophage D3 (Canchaya *et al.*, 2003). The bacteriophage D3 belongs to the unclassified *Siphoviridae* of double-stranded DNA viruses. Organization of the phage 04 genome (Fig. S2) is similar to that of the lambda phage. However, the mechanism that 3CB or its intermediate products affected the behavior of this prophage during degradation remains unclear. Since the gene expression profile changes dramatically at the transition phase (Clark *et al.*, 2006), even small difference in the incubation time could result in big difference in the gene expression profile. Thus, we cannot exclude the possibility that the downregulated expression of prophage genes was caused by the difference in the incubation time, if there was, between the 3CB+ and 3CB− soils although both were at the transition phase. We also found that expression of prophage capsid and tail genes in the transition phase was higher than that at the log phase in the 3CB- samples. This suggested that the lytic activity of the prophage was more active at the transition phase than at the log phase, which was consistent with previous reports (Webb *et al.*, 2003; Clark *et al.*, 2006). The mechanism under this phenomenon, as suggested by recent studies, is involved in quorum-sensing signaling, which is RecA-independent and does not involve an SOS response (Ghosh *et al.*, 2009; Oinuma & Greenberg, 2011). It has been known that about 30% of the

cultivable soil bacteria may contain inducible prophages (Williamson *et al.*, 2008). Probably, the phage 04 in *P. putida* KT2440, which was downregulated by 3CB treatment in sterilized soil, could be used as a model system to investigate the transition of prophage between the lytic and lysogenic life cycles in a soil-like environment.

One of the major contributions of this work is that it is the first successful genome-wide microarray analysis using RNA extracted from a bacterial strain growing in sterilized soil. The new knowledge obtained from this analysis regarding genes involved in transport allows us to obtain a more precise understanding regarding the 3CB degradation process, which might also be helpful in understanding the degradation process of other compounds.

Table V.1. Validation of microarray data by qRT-PCR for genes involved in 3CB degradation and transport

Locus tag	Gene	Definition	Microarray (transition phase)				Fold changes in qRT-PCR[b] (3CB+/3CB−)	
			Single-array analysis		Triplicate-array analysis			
			Fold change[a]	*p*-values in a *t*-test with Bonferroni correction	Fold change[a]	*p*-values in a *t*-test with FDR correction	Log phase	Transition phase
3CB degradation								
PP_3161	*benA*	benzoate dioxygenase, alpha subunit	6.8	1.7×10^{-8}	18.8	6.1×10^{-5}	36.7 ± 32.6	14.2 ± 2.9
PP_3162	*benB*	benzoate dioxygenase, beta subunit	5.9	1.7×10^{-8}	13.4	2.2×10^{-4}	30.6 ± 27.0	14.0 ± 4.5
PP_3163	*benC*	benzoate 1,2-dioxygenase reductase	7.8	1.7×10^{-8}	32.3	4.0×10^{-5}	29.2 ± 27.9	19.0 ± 5.1
PP_3164	*benD*	cis-diol dehydrogenase	6.7	1.7×10^{-8}	23.2	2.4×10^{-4}	26.1 ± 16.7	19.8 ± 4.5
Plasmid	*tfdC*	chlorocatechol 1,2-dioxygenase	1.4[c]	2.3×10^{-8}	–	–	71.4 ± 12.6	37.1 ± 6.4
Plasmid	*tfdD*	chloromuconate cycloisomerase	2.9[c]	1.7×10^{-8}	–	–	15.9 ± 4.0	14.6 ± 2.9
Plasmid	*tfdE*	dienelactone hydrolase	1.1[c]	2.4×10^{-3}	–	–	7.0 ± 1.8	8.8 ± 1.8
Plasmid	*tfdF*	maleylacetate reductase	1.2[c]	4.3×10^{-6}	–	–	6.1 ± 1.4	10.8 ± 4.1

3CB transport								
PP_1383		BenF-like porin	1.9	1.7×10^{-8}	2.8	1.4×10^{-4}	6.3 ± 4.6	17.4 ± 2.9
PP_1820		benzoate transport protein	1.0	1.0	-1.1	0.05	NC	NC
PP_2035	*benE*-1	benzoate transport protein	1.0	1.0	1.0	0.72	NC	NC
PP_2517		BenF-like porin	1.1	2.3×10^{-3}	1.1	0.48	NC	NC
PP_3165	*benK*	benzoate MFS transporter BenK	8.3	1.7×10^{-8}	22.7	6.6×10^{-5}	28.4 ± 25.4	23.5 ± 8.0
PP_3167	*benE*-2	benzoate transport protein	4.1	1.7×10^{-8}	23.3	4.1×10^{-5}	14.0 ± 11.4	25.4 ± 7.7
PP_3168	*benF*	benzoate-specific porin	6.4	1.7×10^{-8}	19.8	9.8×10^{-5}	11.0 ± 9.1	19.2 ± 4.5

NC, no change (fold changes with $p > 0.05$ in a *t*-test was considered as no change).

[a] A fold-change of 1.0 is the same as no change.

[b] qRT-PCR was conducted using different pooled samples of RNA.

[c] The fluorescent signals of genes *tfdC*, *tfdD*, *tfdE* and *tfdF* were saturated on the microarray.

Table V.2. Validation of microarray data by qRT-PCR for genes involved in major cellular responses.

Locus tag	Gene	Definition	Microarray (transition phase)				Fold changes in qRT-PCR (3CB+/3CB−)	
			Single-array analysis		Triplicate-array analysis			
			Fold change	*p*-values in a *t*-test with Bonferroni correction	Fold change	*p*-values in a *t*-test with FDR correction	Log phase	Transition phase
Transporter								
PP_2225		putative monovalent cation/H+ antiporter subunit G	5.6	1.7×10^{-8}	5.3	1.1×10^{-4}	7.9 ± 1.8	9.3 ± 2.4
PP_2226 (PP_2225 operon)		putative monovalent cation/H+ antiporter subunit F	4.9	1.7×10^{-8}	5.1	1.2×10^{-4}	–	–
PP_2227 (PP_2225 operon)		putative monovalent cation/H+ antiporter subunit E	4.4	1.7×10^{-8}	5.4	1.4×10^{-4}	–	–
PP_2228 (PP_2225 operon)		putative monovalent cation/H+ antiporter subunit D	3.9	1.7×10^{-8}	4.1	1.8×10^{-4}	–	–
PP_1271		multidrug efflux MFS transporter, putative	3.1	1.7×10^{-8}	4.7	2.8×10^{-4}	11.1 ± 2.0	9.0 ± 2.9

Carbon metabolism									
PP_0552		*adh*	2,3-butanediol dehydrogenase	−8.4	1.7×10^{-8}	−5.9	1.4×10^{-4}	−3.4 ± 1.1	−1.7 ± 0.8
PP_0553	(adh operon)	*acoC*	dihydrolipoamide acetyltransferase	−7.4	1.7×10^{-8}	−6.1	6.0×10^{-5}	–	–
PP_0554	(adh operon)	*acoB*	acetoin dehydrogenase, beta subunit	−12.5	1.7×10^{-8}	−6.8	6.2×10^{-5}	–	–
PP_0555	(adh operon)	*acoA*	acetoin dehydrogenase, alpha subunit	−8.4	1.7×10^{-8}	−6.2	1.4×10^{-4}	–	–
PP_0557	(adh operon)	*acoR*	acetoin catabolism regulatory protein	−6.8	1.7×10^{-8}	−4.7	1.4×10^{-4}	–	–
Prophage									
PP_1567			phage major capsid protein, HK97 family	−8.1	1.7×10^{-8}	−5.2	1.1×10^{-4}	NC	−4.1 ± 0.8
PP_1573			major tail protein, putative	−11.7	1.7×10^{-8}	−7.8	8.9×10^{-5}	NC	−4.0 ± 0.9
Others									
PP_1269			universal stress protein family	10.4	1.7×10^{-8}	6.6	1.4×10^{-4}	15.4 ± 4.1	11.6 ± 2.7
PP_1950			cytochrome P450 CYP199	72.6	1.7×10^{-8}	46.0	9.7×10^{-5}	53.1 ± 23.6	84.5 ± 26.7
PP_1943		*purU*	formyltetrahydrofolate deformylase	310	1.7×10^{-8}	66.1	6.7×10^{-5}	430 ± 99	662 ± 159

NC: no change (fold-changes with $p > 0.05$ in a *t*-test was considered as no change).

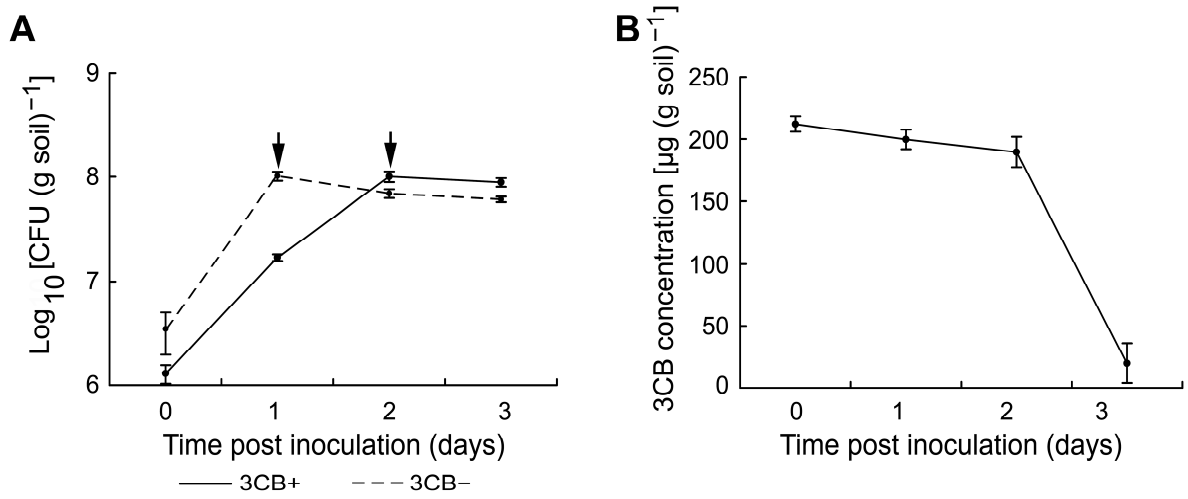

Fig. V.1. Time-courses of bacterial growth (A) and 3CB degradation (B) of *P. putida* KT2440/pSL1 in soil cultures. Arrows indicate sampling time for microarray analysis.

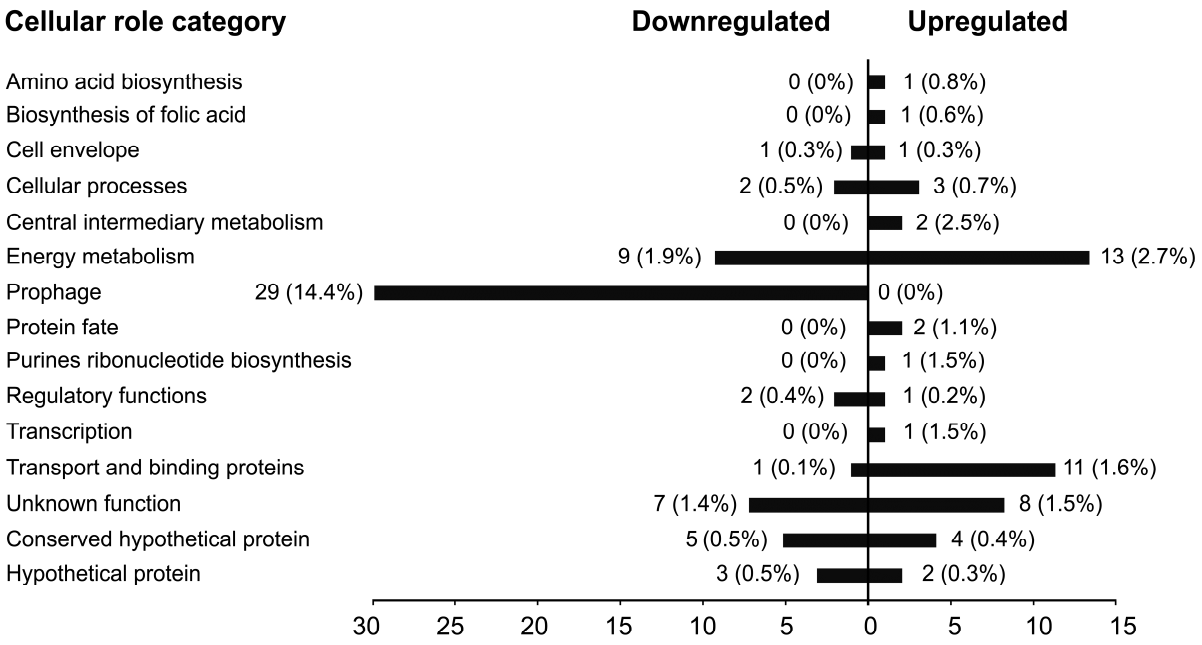

Fig. V.2. Number of differentially expressed genes in different functional categories. Percentages in parentheses indicate the ratio of the number of differentially expressed genes to the total number of genes in each category.

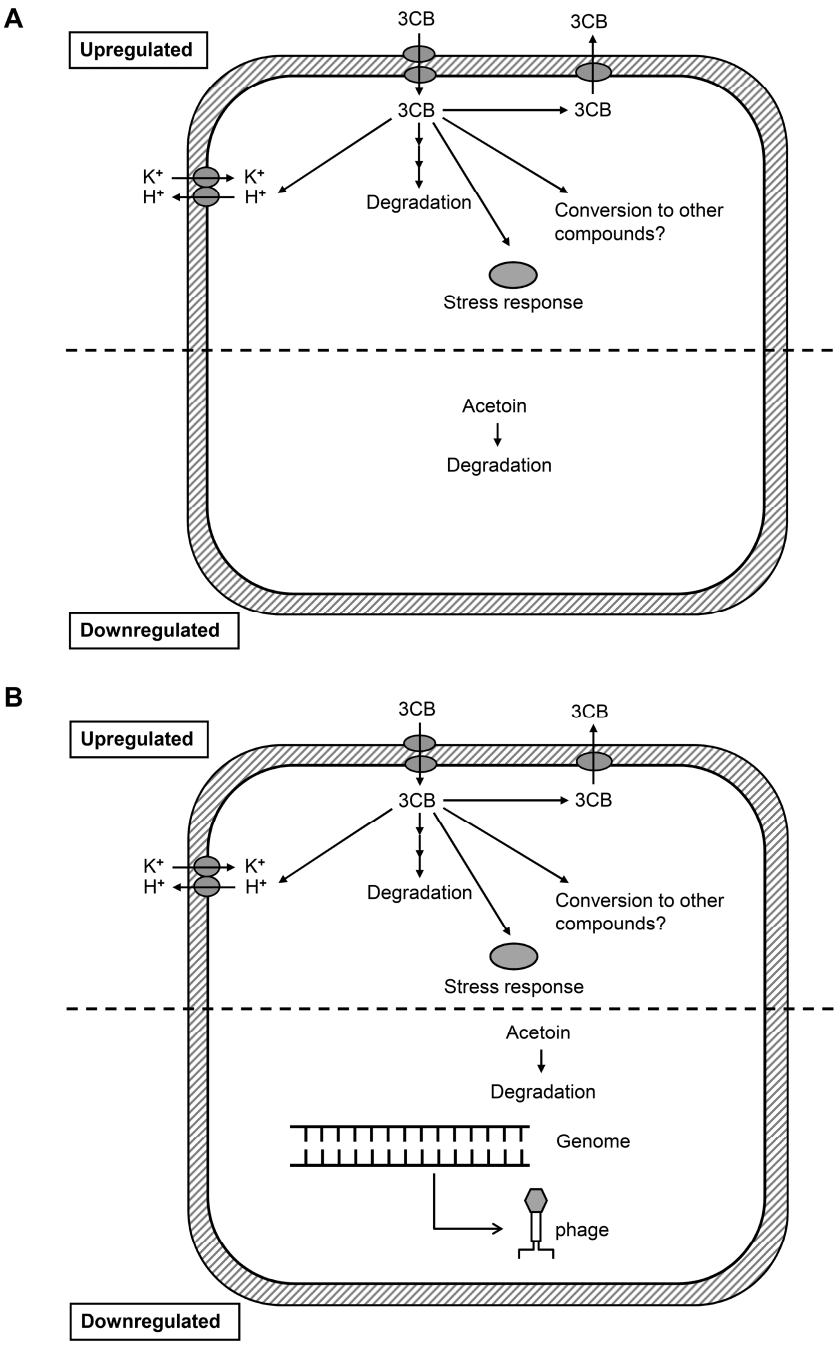

Fig. V.3. Summary of biochemical pathways and major cellular responses in *P. putida* KT2440/pSL1 cells exposed to 3CB in the log phase (A) and the transition phase (B). The horizontal broken line serves as a boundary between the upregulated (upper part) and downregulated (lower part) biochemical pathways and cellular responses.

Supplementary Data Containing Detailed Information

Table S1. Oligonucleotides[a] used in qRT-PCR to evaluate the expression of genes selected according to the microarray analyses

Locus tag	Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplicon size (bp)	Annealing (°C)
PP_0124	PP_0124	GCAAGTCAAGAACCCCATC	AAGCCACCTCGTAACCCT	104	60
PP_0444	*rplA*	GCAATCGCCGAGAAAAT	GGGTCAACACCGAGGTTA	128	60
PP_0552	*adh*	CCCAGCGAGTTCAACTTCTTC	CAGCCGACCATCAGCAATG	114	60
PP_1000	*argI*	GGCGTTCAACATTCACAA	GTTGCCCTTCAGGTGCT	139	60
PP_1269	PP_1269	CTACACCGCCGAGGAACT	TTGGTCACAATCGCTTCA	159	60
PP_1271	PP_1271	TTTCTGCCGCCAGGTATCA	CCATTGCCAACCGACGTATT	120	60
PP_1383	PP_1383	AAGGCACCACCAACACTCACC	GGCATCCATTCACCGACTTTC	130	60
PP_1464	*trmD*	TTGCTTCAGGTGACTTGCT	CTTCCAGAGGCTTGATTTTC	118	60
PP_1567	PP_1567	CAGTCCGACCTGTCCTTT	GCAGTTGCTCTTCTTCCTT	160	60
PP_1573	PP_1573	GGGCATTAAACCCACAGT	TCGAACCCAACCACCTT	165	60
PP_1820	PP_1820	GCTGGACTTTAGCGGCTTCC	CCACCACGAACAGCGGAAT	107	60
PP_1943	*purU*	TAGGTGAGTTGGATATGGAAGT	CAGGTGTAACAGGCAGATAGTG	111	60
PP_1950	PP_1950	AGCACATTCCCAATATCCTT	TCAGAAAAGCCATCCACAT	178	60
PP_2035	*benE*-1	CGCAACGATGGATTCAAGACC	CCCCGACGAGGATGTAACAGA	205	60

PP_2225	PP_2225	TCATCTACTTTTCGTGGCTCAAG	CAGCAACAGCGTGGTTATCG	98	60
PP_2466	PP_2466	GAAGAAGCAGGCTAACGAA	ATCTCACGACCACGGAAT	174	60
PP_2517	PP_2517	TTCACCTCGGACCGTTTCAA	AGCGGTGCGGTTGTTCAGTT	228	60
PP_3161	*benA*	GAAGAAGTCTTCGTACTGGCGAATA	GTGAACAAGACCGAAATCACCAT	96	60
PP_3162	*benB*	CACCTCGCACAACATCAGCAA	CGTTCTTCAGCACCACCTTCTT	191	60
PP_3163	*benC*	GCTATGTCACCCAGCACA	AGCCGCAAACTTCTCGTA	155	60
PP_3164	*benD*	CAAGTGGTGGCGGTAGAC	ATGGACGAGACATTGACGA	317	60
PP_3165	*benK*	GCCATCTGTTTCGCCTTGTT	GCTTGGGTGCGTATTCGTTC	154	60
PP_3167	*benE*-2	AAACAAGAGCCCGCCACAAA	CCCAGACCCAGGATGACAACT	199	60
PP_3168	*benF*	CTGGAGGACATCTACCAACAAGG	GCAGTGAACAGCGAGAAGAACG	161	60
PP_4375	*fliS*	GATTTCTGAAGCCACTCCG	CTTGCCCAGCATTACACC	127	60
—	*tfdC*[b]	AACTCAGGGTCGGTCGTGAT	ATCGTTGGGAATCTGATATGCA	74	60
—	*tfdD*[b]	CGTGACGGCTACGCAGAAAGT	CGCAAGGACAAAGGGACCAA	169	60
—	*tfdE*[b]	GGCGTGTTGGGGTATTGTCTAG	CTCCTCCGTCACATAATGGTCC	180	60
—	*tfdF*[b]	CGGGAAGTCTATCTGCGTTGC	TGCCCCATATCGTCGTCATCT	339	60

[a]All primers used in this study were solely designed for detection of genes when the single strain *Pseudomonas putida* KT2440 was used but not for cases in which multiple species were used.

[b]The accession number for the genes on the pSL1 plasmid is AB050198.

Table S2. Summary of the putative horizontally transferred genes

Locus	IMG Gene Object ID	Product Name	Transferred from Gene (IMG Gene Object ID)	Transferred from Product	Transferred from Genome	Taxonomy
PP_1943	637145348	formyltetrahydrofolate deformylase	648120599	formyltetrahydrofolate deformylase	*Brevundimonas subvibrioides* ATCC 15264	Alpha proteobacteria
PP_1944	637145349	aminomethyltransferase, putative	648279007	aminomethyltransferase, putative	*Maritimibacter alkaliphilus* HTCC2654	Alpha proteobacteria
PP_1945	637145350	5,10-methylene-tetrahydrofolate dehydrogenase/cyclohydrolase	637623544	Methylene-THF dehydrogenase	*Gluconobacter oxydans* 621H	Alpha proteobacteria
PP_1948	637145353	benzaldehyde dehydrogenase	641560929	Aldehyde Dehydrogenase_	*Caulobacter* sp. K31	Alpha proteobacteria
PP_1951	637145356	oxidoreductase, short chain dehydrogenase/reductase family	640444617	short-chain dehydrogenase/reductase SDR	*Novosphingobium aromaticivorans* DSM 12444	Alpha proteobacteria
PP_1952	637145357	metallo-beta-lactamase family protein	640880607	beta-lactamase domain protein	*Xanthobacter autotrophicus* Py2	Alpha proteobacteria

PP_1949	637145354	oxidoreductase, GMC family	642598481	glucose-methanol-choline oxidoreductase	*Burkholderia phymatum* STM815	Beta proteobacteria
PP_1956	637145361	hypothetical protein	637760849	protein involved in meta-pathway of phenol degradation	*Burkholderia cepacia* 383	Beta proteobacteria
PP_1950	637145355	cytochrome P450 CYP199	638089533	cytochrome P450 CYP199	*Rhodococcus jostii* RHA1	Actinobacteria
PP_1953	637145358	oxidoreductase, short chain dehydrogenase/reductase family	645207689	short chain dehydrogenase/reductase family oxidoreductase	*Streptomyces ghanaensis* ATCC 14672	Actinobacteria
PP_1954	637145359	beta-lactamase	645207690	hypothetical protein	*Streptomyces ghanaensis* ATCC 14672	Actinobacteria
PP_1955	637145360	cytochrome P450 family protein	645415942	cytochrome P450 family protein	*Streptomyces griseoflavus* Tu4000	Actinobacteria
PP_1947	637145352	leucyl aminopeptidase	637830213	hypothetical protein	*Moorella thermoacetica* ATCC 39073	Firmicutes
PP_1946	637145351	oxidoreductase, short chain dehydrogenase/reductase family	643566460	short-chain dehydrogenase/reductase SDR	*Chloroflexus aggregans* DSM 9485	Chloroflexi

Fig. S1. Comparative genomic analysis of the *Pseudomonadaceae* family using the RECOG (Research Environment for Comparative Genomics) server. The abbreviations of species' names are shown at the top line (*Pseudomonas putida* KT2440 was in yellow), the gene names are shown at the left column and the genes labeled with either locus number or the number of homologous genes (in the case that there are more than one homologues in the same genome) are in dark green (*P. putida* KT2440) or green (other species). The species used for analysis are as followed: avn, *Azotobacter vinelandii* DJ; cja, *Cellvibrio japonicus* Ueda107; pag, *Pseudomonas aeruginosa* LESB58; pap, *Pseudomonas aeruginosa* PA7; pae, *Pseudomonas aeruginosa* PAO1; pau,

Pseudomonas aeruginosa UCBPP-PA14; pmy, *Pseudomonas mendocina* ymp; pen, *Pseudomonas entomophila* L48; pfl, *Pseudomonas fluorescens* Pf-5; pfo, *Pseudomonas fluorescens* PfO-1; pfs, *Pseudomonas fluorescens* SBW25; ppf, *Pseudomonas putida* F1; ppg, *Pseudomonas putida* GB-1; ppu, *Pseudomonas putida* KT2440; ppw, *Pseudomonas putida* W619; psa, *Pseudomonas stutzeri* A1501; psb, *Pseudomonas syringae* pv. *syringae* B728a; psp, *Pseudomonas syringae* pv. *phaseolicola* 1448A; pst, *Pseudomonas syringae* DC3000.

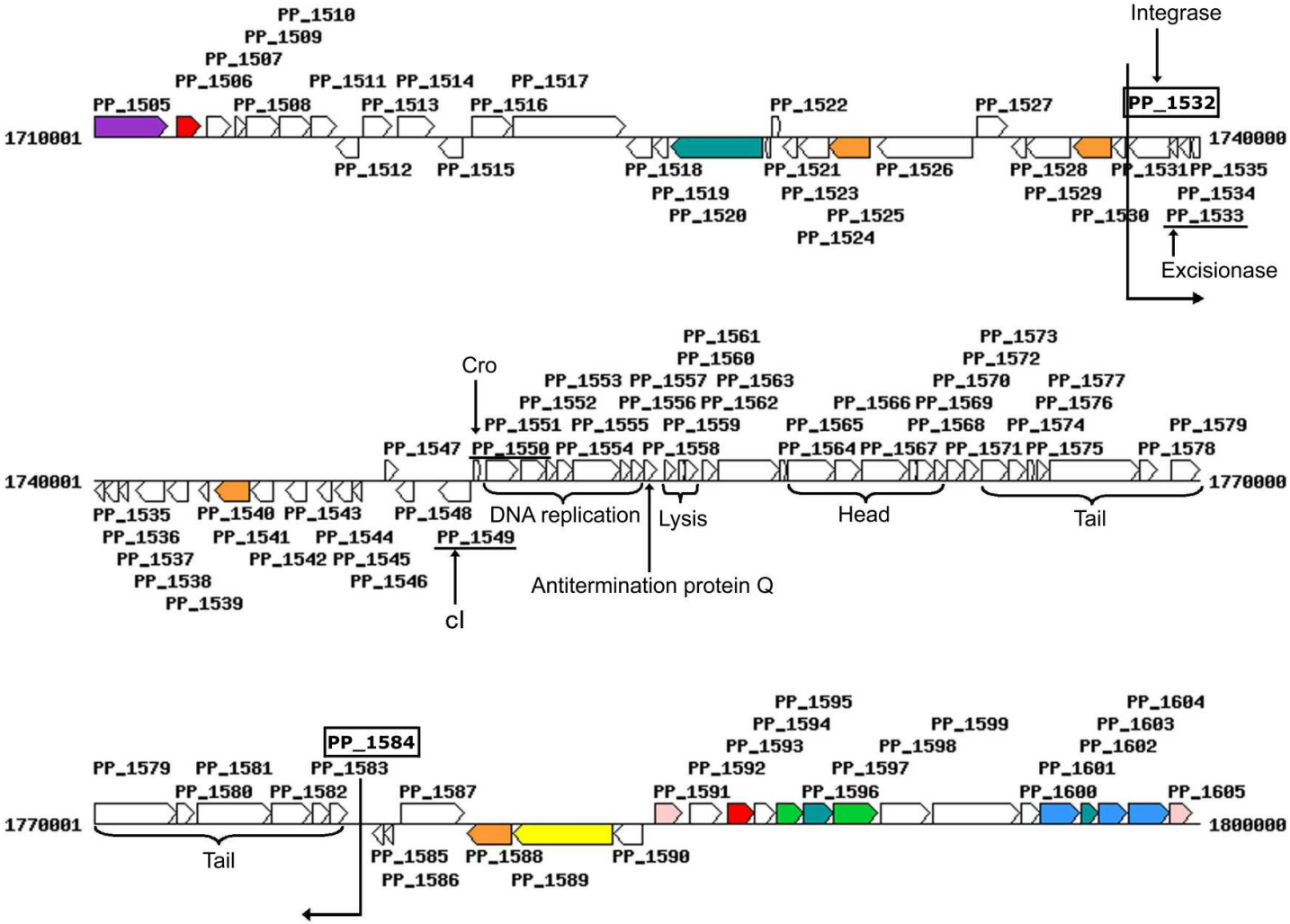

Fig. S2. The organization of the phage 04 genome in *Pseudomonas putida* KT2440. The drawing of gene organization was downloaded from the KEGG database, and the annotation was done by referring to the NCBI Gene database.

Chapter VI

Development of a Universal Method for RNA Extraction from Diverse Soils and Its Application to *amoA* Gene Expression Study in Andosols

Summary

In an attempt to extract RNA from Andosols (volcanic ash soils), which are the dominant agricultural soils in Japan and are well known for their strong adsorption of RNA, extraction buffer containing autoclaved casein was used. Using this buffer, high-quality RNA was successfully extracted from eight types of agricultural soils that were significantly different in their physicochemical characteristics. To detect bacterial ammonia monooxygenase subunit A gene (*amoA*) transcripts, bacterial genomic DNA and messenger RNA were co-extracted from two different types of Andosols during incubation with ammonium sulfate. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and reverse transcription polymerase chain reaction-denaturing gradient gel electrophoresis (RT-PCR-DGGE) analyses of *amoA* in soil microcosms revealed that only few *amoA* genes, which had the highest similarities to those in *Nitrosospira multiformis*, were expressed in these soils after treatment with ammonium sulfate, in spite of the fact that multiple *amoA* genes were present in the soil microcosms examined. This study demonstrated that the casein method could be considered as a universal method for bacterial RNA extraction from soil.

Since the contamination of humic substances in RNA samples, which is one of the two major difficulties in soil RNA extraction, has already been solved (Chapter IV), another difficulty, adsorption of RNA by soil, became our research target.

Skim milk has been successfully used to extract DNA from Andosols (Ikeda *et al.*, 2004; Hoshino & Matsumoto, 2005). However, it was not useful in extracting RNA from an Andosol for unknown reasons (Hoshino & Matsumoto, 2007). Because skim milk is a mixture of proteins and other materials, we speculated that a pure protein may be better than skim milk. In this study, we developed a new method to extract RNA from soil using casein as a competitor.

The nitrogen cycle, an important biological process for producing human foods, involves the consumption and conversion of fertilizers that are added to arable land. The nitrogen cycle includes nitrogen fixation, nitrification, and denitrification reactions. Each of these reactions requires the contribution of numerous microorganisms found in natural soil environments (Hayatsu *et al.*, 2008; Klotz & Stein, 2008). Thus, in order to determine the mechanisms involved in the nitrogen cycle in agricultural soils, it is necessary to garner information on soil microorganisms. Apparently, the genes involved in the nitrogen cycle are good targets for the study of microbial ecology. So, in this study, expression of a gene involved in the nitrification process was selected as our research target to test whether our RNA extraction method could be applied to the study of microbial ecology.

Materials and methods

Soil sampling

Soil was collected from five sites in Japan from 2006 to 2009: (1) the Tsukuba campus at the National Agricultural Research Center (Ibaraki, Japan, 36°2'N, 140°6'E) from where Yellow Soil (YS), Gray Lowland Soil (GLS), Cumulic Andosols (CA), and Low-humic

Andosols (LHS) were collected; (2) a field at the Ehime Agricultural Experiment Station (Ehime, Japan, 33°50'N, 132°46'E) from where Brown Forest Soil (BFS) was collected; (3) an agricultural field in Kyoto, Japan (35°18'N, 135°15'E) from where Brown Lowland Soil (BLS) was collected; (4) a rice paddy in Aomori, Japan (40°41'N, 140°35'E) from where Wet Andosols (WA) were collected; and (5) a rice paddy in Toyama, Japan (36°44'N, 137°10'E) from where Gley Soil (GS) was collected. The properties of these field soils are shown in Table VI.1.

At each site, top soil (5−10 cm for WA soil and 0−15 cm for the other soil types) was collected from five locations, mixed, and passed through a 2-mm mesh sieve before storage at −20°C (WA) or at 4°C (other soils). The soil samples stored at 4°C were incubated at 25°C for several days before RNA extraction, whereas the WA samples were directly subjected to RNA extraction.

Soil analyses

The soil texture was determined using a pipette method (Gee & Bauder, 1986). Total carbon, total nitrogen and pH of the soil samples were determined as previously described (Suzuki *et al.*, 2009). The moist color of soil samples was evaluated by visual examination under outdoor sunlight with Munsell color plates, in which a lower value or chroma indicated a darker color.

RNA recovery from soil in the presence of casein

Total RNA from *Pseudomonas putida* KT2440 (a Gram-negative bacterium), *Rhodococcus jostii* RHA1 (a Gram-positive bacterium) and BFS soil, a commercial product of casein (Nacalai Tesque, Kyoto, Japan), and CA soil were used to test the RNA recovery from Andosols. To test the ability of casein to block RNA binding sites on soil particles, 0.2 g of

soil was mixed with 300 µl of a casein solution (20 mg ml^{-1} in 300 mM sodium phosphate, pH 7) by vortexing and then mixed with 100 µl of RNA (2 µg). To test the ability of casein to release adsorbed RNA from soil, the soil was mixed with RNA prior to mixing with the casein solution. RNA mixed with soil was used as a control. After centrifugation, supernatants were subjected to phenol extraction and isopropanol precipitation. RNA pellets were dissolved in 20 µl of RNase-free water. Five microliters of each sample was separated by agarose gel electrophoresis.

Quality control analysis of commercial casein product

RNase activity was evaluated by incubating casein solution (20 mg ml^{-1} in 300 mM sodium phosphate, pH 7) with 1 µg of total RNA extracted from *Pseudomonas putida* KT2440 cells for 2 h at 37°C, followed by agarose gel electrophoresis. KT2440 total RNA dissolved in sodium phosphate buffer was used as a negative control, and mixtures of total RNA and RNase A (Sigma-Aldrich, St. Louis, MO, USA) were used as positive controls.

To examine RNA contamination in the commercial casein, a casein solution (2 mg ml^{-1} in 300 mM sodium phosphate, pH 7) was subjected to phenol extraction and isopropanol precipitation, followed by RNase-free DNase treatment and purification with a RNA Clean & Concentrator-5 kit (Zymo Research, Orange, CA, USA). Reverse transcription was performed using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions for random hexamer primed reactions. Water was used as a negative control and the total RNA extracted from GS soil was used as a positive control.

A bacterial 16S rRNA fragment and an eukaryotic 18S rRNA fragment were amplified by PCR using the primers F984 (5′- AACGCGAAGAACCTTAC -3′) and R1378 (5′- CGGTGTGTACAAGGCCCGGGAACG -3′) (Costa et al. 2006) and the primers NS1 (5′-

GTAGTCATATGCTTGTCTC -3′) and Fung (5′- ATTCCCCGTTACCCGTTG -3′) (May *et al.*, 2001), respectively. The PCR products (approximately 470 bp for 16S and approximately 390 bp for 18S rRNA) were separated on 2% agarose gels, and the images of ethidium bromide (0.5 µg ml^{-1}) stained gels were acquired with a FAS-III gel imaging device (Toyobo, Osaka, Japan).

Nucleic acid extraction from soil

An RNA extraction buffer was prepared by dissolving casein in a sodium phosphate buffer (300 mM, pH 7), followed by autoclaving at 121°C for 20 min. The optimum concentration of casein for efficient RNA recovery was determined for each soil type. RNA was extracted from 2 g of soil sample as follows:

(i) After adding 2 g of glass beads (diameter = 0.2 mm; BioMedical Science, Tokyo, Japan) and 4 ml of the RNA extraction buffer (pre-cooled on ice) to 15-ml plastic tubes containing soil samples, beads beating was performed by vortexing at the maximum speed on a vortex adaptor (Mo Bio, Carlsbad, CA, USA) for 2 min to disrupt microorganism cells, followed by centrifugation at 10, 000 ×*g* for 5 min. The supernatant was subjected to phenol extraction, phenol/chloroform/isoamyl alcohol extraction, and precipitation at room temperature with a 0.1 volume of sodium acetate (3 M, pH 5.2) and a 0.7 volume of isopropanol.

(ii) Subsequent purification with a column from a PowerSoil Total RNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) and a MicroSpin S-400 HR spin column (GE Healthcare, Little Chalfont, UK), DNase digestion with a TURBO DNA-free DNase (Ambion, Austin, TX, USA), and concentration with a Zymo Research RNA Clean & Concentrator-5 kit were performed according to the manufacturers' instructions. Absorbances of RNA samples at 320 nm, 340 nm, 350 nm, and 465 nm, which were determined to be appropriate methods for

humic acid measurements (Chapter III), were measured using UV-Vis module in a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

To co-isolate DNA and RNA from the same soil sample, after eluting RNA from the PowerSoil column, DNA elution from the same column was performed using an RNA PowerSoil DNA Elution Accessory Kit (MO BIO), followed by purification with a MicroSpin S-400 HR column and a DNA Clean & Concentrator-5 column (Zymo Research, Orange, CA, USA).

Measurement of nitrogen in incubated soil microcosms

To detect ammonia-oxidizing bacterial ammonia monooxygenase subunit A gene (AOB *amoA*) transcripts in agricultural soils, a model system was established by incubating soil microcosms amended with ammonium sulfate. CA and LHA soils were incubated independently. Four hundred grams of soil was pre-incubated at 30°C for a week. At the end of this pre-incubation period, 300 g of soil was amended with an ammonium sulfate solution (0.4 mg NH_4-N g^{-1} dry soil and 60% of the maximum water-holding capacity). Fifteen grams of amended soil was transferred to a glass flask and soil microcosms were incubated at 30°C for 8 days. Every two days, distilled water was added to the incubated soils to compensate for evaporation. Soil was sampled after incubation for 0, 2, 4, and 8 days. NH_4-N and NO_x-N (NO_3-N plus NO_2-N) in 5 g of soil were extracted with 20 ml of 2 M KCl for 1 h and their concentrations were determined with an Autoanalyzer QuAAtro 2HR (BLTEC, Osaka, Japan).

***PCR-DGGE and RT-PCR-DGGE analysis of the AOB* amoA**

Total RNA (approximately 300 ng) extracted from 2 g of incubated soil was reverse transcribed as described above, followed by cDNA purification with a DNA Clean & Concentrator-5 column. The AOB *amoA* was amplified by PCR using the primers

AmoA-1F-GC Clamp

(5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGGGGTTTCTACTG GTGGT -3’) and AmoA-2R-GG (5’-CCCCTCGGGAAAGCCTTCTTC -3′). A 50-µl PCR reaction mixture comprised 0.4 µM of each primer, 200 µM of each deoxynucleotide triphosphate, 5 µl of 10× *Ex Taq* buffer (20 mM Mg^{2+} plus), 1 µl of bovine serum albumin (20 mg ml^{-1}), 2.5 U of *Ex Taq* polymerase (Takara Bio, Otsu, Japan), 3 µl of soil DNA (1/10 the total volume of the DNA extracted from 2 g of soil) or 10 µl of purified cDNA (converted from ½ the total volume of the RNA extracted from 2 g of soil), and sterile water. The PCR reaction conditions were as follows: 5 min at 94°C, and 30 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, followed by 5 min at 72°C. Denaturing gradient gel electrophoresis (DGGE) analysis was performed as previously described (Chu *et al.*, 2007). The RT-PCR-DGGE bands detected and the corresponding PCR-DGGE bands were excised and suspended in 20 µl TE buffer. After overnight incubation at 4℃, 1µl of the supernatant was used as the template DNA. PCR was performed as described above except that bovine serum albumin was not added. PCR products were applied to DGGE again to verify its migration. After purification with QIAquick PCR Purification Kit (Qiagen), DGGE band sequencing was performed by Hokkaido System Science Co., Ltd. (Sapporo, Japan).

Phylogenetic analysis

The sequences obtained from reverse transcription-polymerase chain reaction-denaturing gradient gel electrophoresis (RT-PCR-DGGE) analysis along with the nucleotide sequences of *amoA* retrieved from the NCBI database were aligned on the MAFFT server (http://mafft.cbrc.jp/alignment/server/index.html) and trimmed with BioEdit version 7. The processed sequences containing 138 amino acid residues per sequence were used to construct a neighbor-joining tree with the Jones-Taylor-Thornton amino acid substitution model in

MEGA version 5 (Tamura *et al.*, 2011).

Nucleotide sequence accession numbers

The nucleotide sequences of *amoA* determined in this study were deposited in the GenBank/EMBL/DDBJ databases under accession numbers AB719960-AB719971.

Results

RNA recovery from soil in the presence of casein

Skim milk has been successfully used to extract DNA from Andosols (Ikeda *et al.*, 2004; Hoshino & Matsumoto, 2005). Casein, the major protein in skim milk, can also be used to extract DNA from Andosols (Ikeda *et al.*, 2008). Thus, we investigated if casein could also be used to extract RNA from Andosols. The RNA that was added to an Andosol sample pre-incubated with casein could be recovered in spite of its origin (Fig. VI.1A, B and C). This suggested that casein was a good competitor for successful RNA extraction from Andosols. However, once adsorbed by Andosols, it was difficult to release RNA from soil particles with casein (Fig. VI.1A, B and C). These results suggested that the binding between an Andosol and RNA is much stronger than that between an Andosol and casein. Thus, during RNA extraction from Andosols, casein can prevent RNA adsorption by an Andosol only before RNA is released from cells. An extra rRNA band (indicated by an arrow in Fig. VI.1B) could be seen in the RHA1 RNA sample because RNA was not heat-denatured prior to be loaded on gel, which is a phenomenon reported in the Chapter II. In the RNA samples recovered from the soil pre-incubated with casein, a band with high molecular weight (indicated by arrowheads in Fig. VI.1A B and C) could be seen on gels. Such a band was also observed in the sample of soil pre-incubated with casein at the absence of foreign RNA (indicated by an arrowhead in Fig. VI.1D), and disappeared after incubation with DNase (data not shown).

These suggest that it could be extracellular DNA (free DNA) in soil.

Quality control of casein for RNA extraction from soil

Because the commercial casein used was not guaranteed to be RNase-free and the existence of RNase activity in the soil RNA extraction buffer could disrupt RNA integrity, we examined if this commercial casein had RNase activity. As shown in Fig. VI.2A, strong RNase activity was detected in the casein solution, which is almost similar to RNase A activity at a concentration of 1 μg ml^{-1}. However, after autoclaving, RNase activity was undetectable in the casein solution (Fig. VI.2B). We noted that the 23S rRNA band of the RNA mixed with autoclaved casein ran more slowly than that of controls (Fig. VI.2B). This suggested that binding between RNA and casein had occurred during incubation. After phenol extraction, the RNA samples showed normal band patterns (Fig. VI.2B), indicating the complete separation of RNA from casein. During phenol extraction, the loss of RNA due to presence of casein was negligible (p = 0.51; paired t-test, n = 3).

Next, we investigated if this commercial casein had RNA contamination using RT-PCR analyses with the casein extract as a template. As shown in Fig. VI.3A and B, 16S and 18S rRNA were not detected in the casein extract, although both were detected in the positive controls.

RNA extraction from diverse soils

The optimum concentration of casein for each soil type was investigated by extracting RNA from each of the eight soil types using RNA extraction buffers containing different concentrations of casein. As shown in Table VI.2, high quality RNA was successfully extracted from BFS and GS soils in the absence of casein, whereas the other soils required different amounts of casein in the extraction buffers. In particular, in the absence of casein,

RNA extraction failed for the four soil types: GLS, YS, LHA, and CA. Thus, these were challenging soils for successful RNA extraction.

Using the optimum concentrations of casein in the extraction buffers, the RNA extracted from soils showed the highest RNA yields with low levels of humic acids. The OD_{320}, OD_{340}, OD_{350}, and OD_{465} readings were lower than 0.01 for all soil RNA samples (final volume = 10 µl per sample), indicating high purity for the RNA samples. The integrity of the RNA extracted from all eight soil types using the optimum casein concentrations was examined using a 2% agarose gel (Fig. VI.4A). All RNA samples showed clear rRNA bands.

The RNA extracted from GS soil showed one thick band corresponding to the large subunit rRNA and two bands corresponding to the small subunit rRNA. Based on the sizes of these bands, eukaryotic 18S rRNA and 28S rRNA had been co-extracted with prokaryotic RNA from this soil. Using these RNA samples as templates, a fragment of bacterial 16S rRNA was successfully amplified in all RT-PCR reactions (Fig. VI.4B), suggesting that the quality of these RNA samples was sufficiently high for downstream molecular biological analysis.

Identification of the AOB amoA in two Andosols

To examine induced bacterial gene expression using RNA extracted from Andosols, the most challenging of these soils, we incubated two groups of soil microcosms (CA and LHA) amended with ammonium sulfate and performed PCR-DGGE and RT-PCR-DGGE analyses that targeted *amoA*, the gene encoding the ammonia monooxygenase subunit A in AOB. In both soil microcosms, with the consumption of ammonium sulfate, the levels of nitrate/nitrite gradually increased (Fig. VI.5A), which indicated that nitrification had progressed in these soil microcosms.

Because the first step in nitrification, the conversion from ammonia to hydroxylamine, is catalyzed by ammonia monooxygenase, the increased level of nitrification could have resulted

from the induced expression of the gene encoding this enzyme. As shown in Fig. VI.5B, *amoA* transcripts were detected in both soil microcosms after four days of incubation. By PCR-DGGE analysis, multiple bands were detected during eight days of incubation, but only one (from the CA soil microcosms) or two (from the LHA soil microcosms) bands were detected by RT-PCR-DGGE analysis (Fig. VI.5B). These results suggested that multiple AOB *amoA* genes were present in both soil microcosms, but only one (from the CA soil microcosms) or two (from the LHA soil microcosms) genes showed induced expression in response to treatment with ammonium sulfate.

The upper bands (bands 1–8 in Fig. VI.5B) were identical to each other in terms of their sequences (431 bp in length), as were the lower bands (bands 9–12 in Fig. VI.5B). Both sequences were grouped into cluster 3a of beta-proteobacterial AOB (Fig. VI.6). BLASTN searches against the KEGG GENES database (http://www.genome.jp/tools/blast/) and the NCBI Genomic BLAST database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) indicated that these two sequences had the highest similarities (94% for the upper band and 95% for the lower band) to three *amoA* genes in *Nitrosospira multiformis* ATCC 25196.

Discussion

Utilization of autoclaved casein during RNA extraction from soil

In this study, we found that casein could be used as a competitor of RNA to aid in RNA recovery from Andosols. Casein possibly blocked the RNA binding sites on soil particles, but was not involved in the release of adsorbed RNA from soil particles (Fig. VI.1). Casein, which is abundant in cow milk (Van Slyke & Bosworth, 1915), is a family of phosphoproteins, including α_{s1}-, α_{s2}-, β- and κ-casein, with molecular masses of approximately 30 kDa (Strange *et al.*, 1992) and isoelectric points (pI) of approximately 4.6 (Michaelis and Pechstein 1912). The degradation temperature for casein is 180°C (Maréchal, 2007). β-casein, the major

component of casein, behaves as a non-compact and largely flexible structure at approximately 100°C (Holt & Sawyer, 1988). Thus, the temperature routinely used for autoclaving (121°C) has no effect on casein stability, whereas RNase can be inactivated under these conditions. The autoclaved commercial casein also had undetectable levels of RNase activity (Fig. VI.2). In addition, casein can be obtained at large scale and low cost because it is currently the cheapest pure protein on the market; it is only a small fraction of the cost of other commercial proteins. Thus, autoclaved casein could be an ideal competitor for RNA extraction from soil.

To completely dissolve casein, the pH of RNA extraction buffers should be far from the pI of casein. It is known that RNA adsorption by clays decreases with increase in pH of soil suspensions (Goring & Bartholomew, 1952; Taylor & Wilson, 1979); thus, a high pH is preferable for increasing the RNA yield. However, a high pH RNA extraction buffer results in greater release of humic acids from soil than a low pH as shown in the Chapter IV; thus, a low pH is preferable to control humic acids contamination in RNA samples (Mettel *et al.*, 2010). As a balance for these concerns, a neutral pH (pH 7), which also allows casein to be dissolved completely, could be the optimum condition.

Because different soil types have different physicochemical characteristics, the adsorption characteristics of soils are also expected to be different. Here, we investigated the optimum concentrations of casein required for successful RNA extraction from diverse, challenging soils. As shown in Table VI.2, based on the amount of casein required for successful RNA extraction, half of the soils examined were challenging soils. Among these, two Andosols (CA and LHA) were considered to be the most challenging. To our knowledge, this is the first trial in the world to quantitatively characterize challenging soils for successful RNA extraction. Thus, these results could be used as references for the study of other soil types or the same soil types in other countries.

The mechanism behind RNA adsorption by soil

RNA adsorption by soil is caused by both chemical and physical characteristics of soil. It has been known that high soil pH was helpful for RNA recovery from soil, whereas presence of divalent cations (Ca^{2+} and Mg^{2+}) resulted in low recovery of RNA(Goring & Bartholomew, 1952; Taylor & Wilson, 1979). A recent study revealed that humic acids strongly adsorbed DNA (Saeki et al. 2011), thus it is likely that humic acids also adsorb RNA as concerned previously (Peršoh *et al.*, 2008). Although a recent report suggested that the clay content was one of the causes for RNA adsorption by soil (Novinscak & Filion, 2011), the composition of clay may also contribute to RNA adsorption. It has been reported that different clay fractions have different adsorption capacities for RNA, and the order of adsorption capacity was bentonite > illite > kaolinite (Goring & Bartholomew, 1952). Taken together, the adsorption capacity of a certain soil should be an integrated result caused by all of the chemical and physical characteristics of the soil. The effect of a single factor could be seen only in the case that all of the other factors of the soils for comparison contribute almost equally. Because we used diverse soils with quite different physicochemical characteristics in the current study, it is reasonable that different soils required different amount of casein for RNA extraction (Table VI.2), and that Table VI.2 did not show a clear relationship between soil characteristics (e.g., soil clay content) and RNA recovery.

Importance of detection of bacterial mRNA in soil

In our RT-PCR-DGGE analysis of the AOB *amoA*, two sequences similar to those of *amoA* in beta-proteobacterial AOB (*Nitrosospira multiformis* ATCC 25196) were identified to be responsive to treatment with ammonium sulfate. In BLASTN searches against the NCBI Non-Redundant Nucleotide Collection database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), we

found that each of these had one identical sequence (Identities = 100%).

The sequence (Accession number: EF207192) identical to that of the upper bands detected in two Andosols in Japan (Fig. VI.5B) was obtained from a red soil (FAO: Agri-Udic Ferrosols) in China (He *et al.*, 2007). The sequence (Accession number: AB621412) identical to that of the lower bands detected in one Andosol in Japan (Fig. VI.5B) was also obtained from an Andosol in Japan, although the field used for soil sampling was different (Shimomura *et al.*, 2012). It might be interesting to investigate geographic distribution of these two AOB *amoA* genes.

Although our PCR-DGGE analysis detected the presence of multiple *amoA* genes in these soil microcosms, it was difficult to determine which was responsive to the treatment with ammonium sulfate. Nevertheless, our RT-PCR-DGGE analysis clearly detected responsive genes. Thus, to obtain information regarding which bacterial gene is important in the nitrogen cycle in soil, it will be necessary to survey the bacterial genes that are responsive to changes in a nitrogen source using soil RNA techniques.

In this study, we successfully detected bacterial mRNA in two Andosols using a new method for RNA extraction from soil. The difference between the new method and our previous method described in Chapter IV is that the extraction buffer in the current method contained casein and no guanidine or SDS. The reason that guanidine and SDS were not used in the new method is that they may interact with casein so that the effect of casein might be reduced. This method was applicable for both Andosol and non-Andosol soils. To our knowledge, we are the first to report the detection of mRNA in Andosols. The eight soil types that were tested for RNA extraction in this study were classified into major soil groups that cover 90% of the agricultural lands and forest lands in Japan, and 40% agricultural land on the earth. Therefore, we believe this method has great potential for application to a broad

spectrum of studies as a universal method. Moreover, the successful application of this method for detecting the AOB *amoA* transcripts in two Andosols is a good example for showing the importance of detecting bacterial RNA in soil.

Table VI.1 Properties of the examined field soils

Soil type	FAO soil grouping	Vegetation	Particle distribution (%)			Soil texture*	pH	Total C	Total N	Moisture content %	Moist color	References
			Sand	Silt	Clay			($g\ kg^{-1}$ soil)	($g\ kg^{-1}$ soil)		(hue, value/chroma)	
Yellow Soil (YS)	Gleyic, Haplic Alisols	Corn	55.1	24.5	20.4	Clay loam	5.4	11.4	1	20	10YR 6/8	(Hoshino et al. 2011; Suzuki et al. 2009)
Gray Lowland Soil (GLS)	Entric Fluvisols	Corn	65.2	20.7	14.1	Loam	5.7	11.8	1.1	38	10YR 3/1	(Hoshino et al. 2011; Suzuki et al. 2009)
Cumulic Andosol (CA)	Gleyic, Mollic, Umbric Andosols	Corn	67.9	21.0	11.1	Sandy loam	5.5	50.5	3.7	51	10YR 2/1	(Hoshino et al. 2011; Suzuki et al. 2009)
Low-humic Andosol (LHA)	Gleyic, Haplic Andosols	Corn	58.3	29.0	12.7	Loam	6.3	32.8	2.7	55	10YR 3/2	(Hoshino et al. 2011; Suzuki et al. 2009)
Brown Forest Soil (BFS)	Gleyic Cambisols	No plant	67.7	14.8	17.6	Sandy clay loam	6.6	9.7	1.6	11	10YR 3/1	This study
Brown Lowland Soil (BLS)	Fluvisols	Spinach	57.7	21.2	21.1	Clay loam	6.6	18.3	1.2	14	10YR 3/2	This study
Wet Andosol (WA)	Andic Gleysols	Rice	60.5	18.9	20.6	Sandy clay loam	5.2	41.5	3.2	42	10YR 2/1	This study
Gley Soil (GS)	Dystric Gleysols	Rice	67.1	17.6	15.3	Sandy clay loam	5.0	15.6	1.4	20	10YR 3/1	(Makino et al. 2006)

*Soil texture was determined according to the International Union of Soil Sciences (IUSS) classification system.

Table VI.2 Optimum casein concentrations in RNA extraction buffers

Soils	Casein concentration (mg g^{-1} wet soil)				
	0	10	20	40	60
Brown Forest Soil (BFS)	O	+	+	+	NA
Gley Soil (GS)	O	+	+	−	NA
Brown Lowland Soil (BLS)	+	O	+	+	NA
Wet Andosol (WA)	+	O	+	−	−
Gray Lowland Soil (GLS)	−	−	O	+	NA
Yellow Soil (YS)	−	−	O	+	NA
Low-Humic Andosol (LHA)	−	−	−	O	+
Cumulic Andosol (CA)	−	−	−	O	+

−, no detectable RNA; + low RNA yield; O, highest RNA yield; NA, data not available.

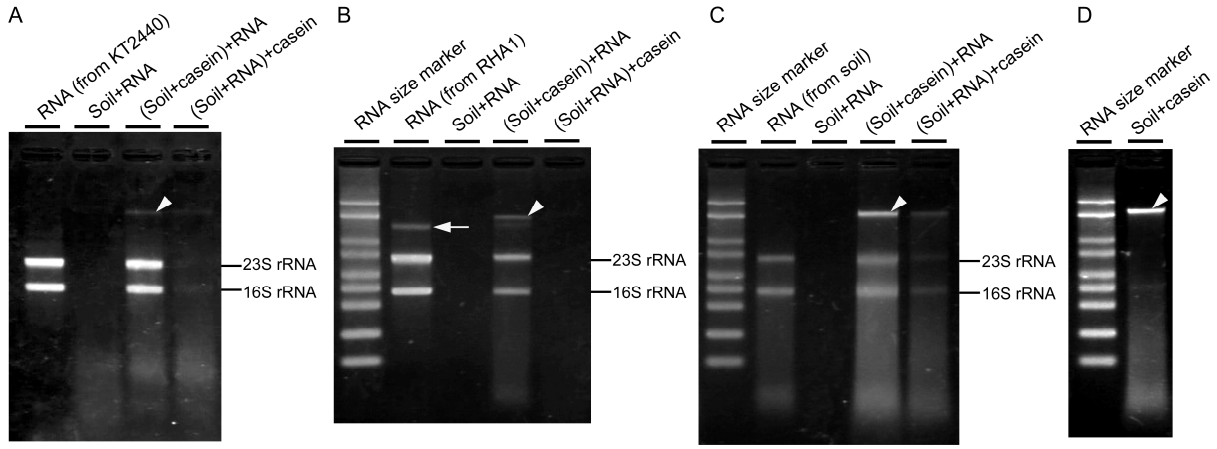

Fig. VI.1 Nucleic acids recovery from an Andosol pre-incubated with casein at the presence of total RNA from a Gram-negative bacterium (A), a Gram-positive bacterium (B) and BFS soil (C), or at the absence of foreign RNA (D). RNA: RNA not mixed with soil or casein; Soil + RNA: soil was mixed with RNA; (Soil + casein) + RNA: soil was mixed with casein before mixing with RNA; (Soil + RNA) + casein: soil was mixed with RNA before mixing with casein. An arrow indicates an extra rRNA band in an RHA1 RNA sample, and arrowheads indicate the bands of extracellular DNA in soil.

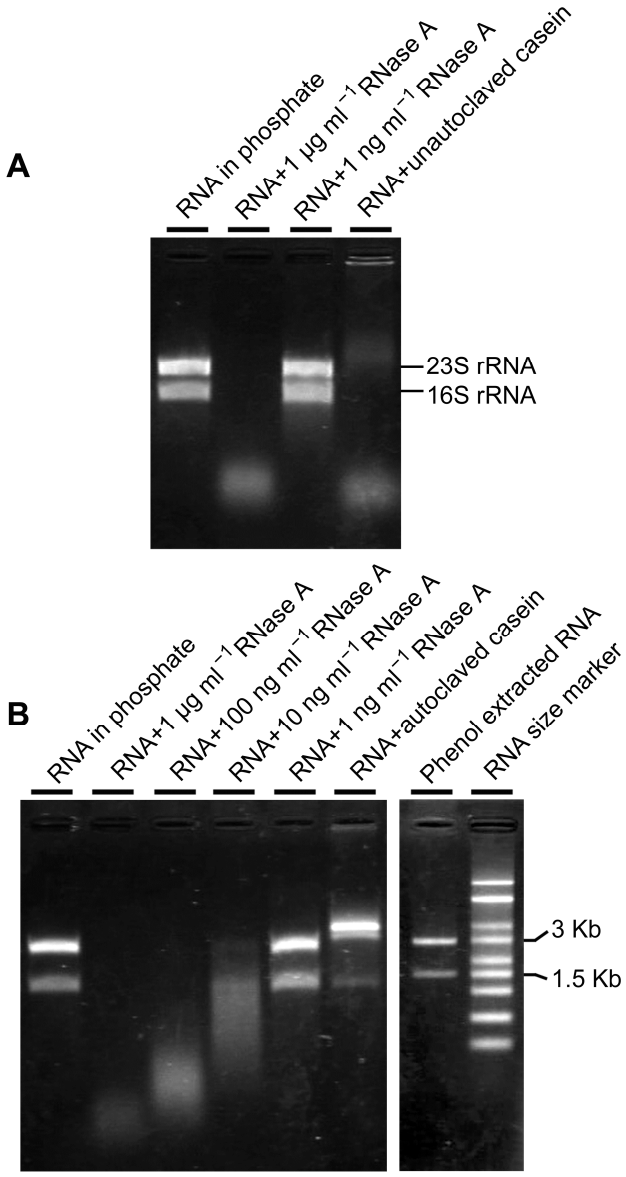

Fig. VI.2 RNase activities of (a) non-autoclaved casein and (b) autoclaved casein. “Phenol-extracted RNA” indicates the RNA that was extracted with phenol from a mixture of RNA and autoclaved casein.

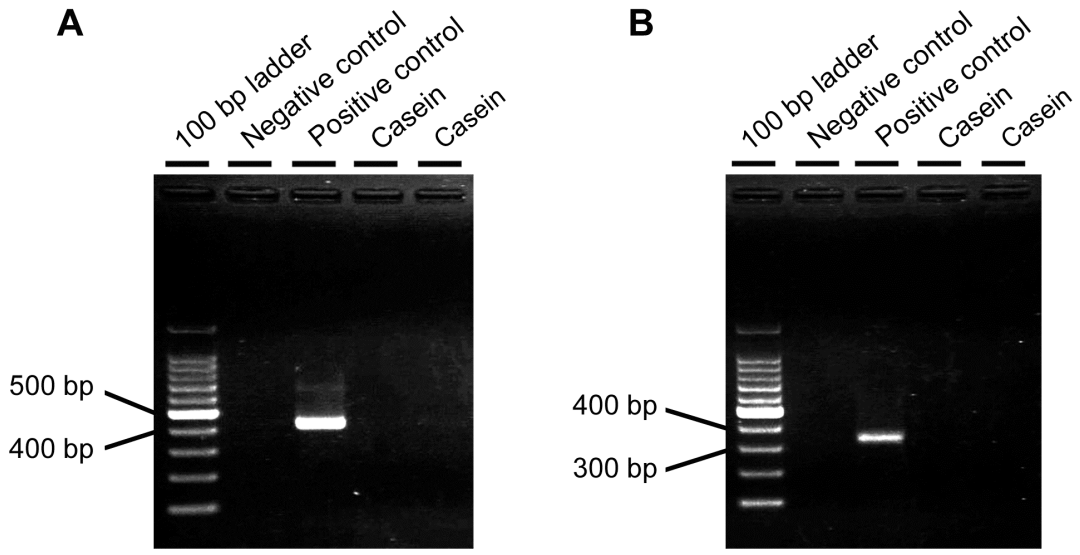

Fig. VI.3 Evaluation of RNA contamination in casein. (A) RT-PCR detection of 16S rRNA in casein. (B) RT-PCR detection of 18S rRNA in casein. Duplicate casein extracts were examined in both experiments.

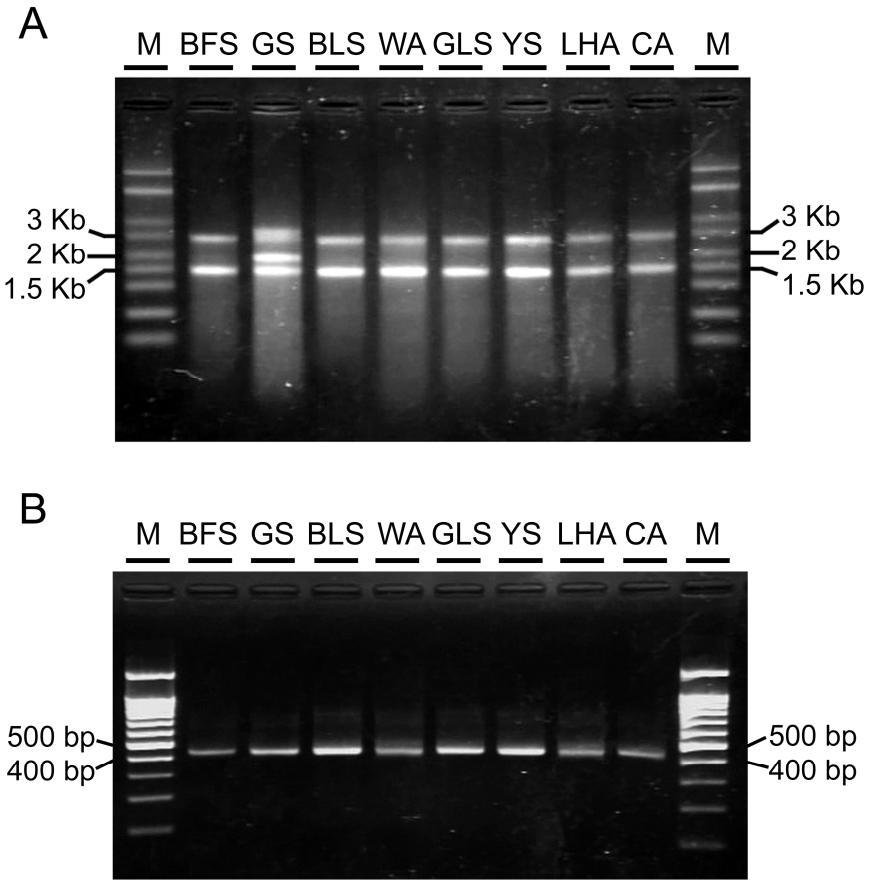

Fig. VI.4 (A) Agarose gel electrophoresis of the RNA samples prepared from diverse soils. M: RNA size marker. (B) Agarose gel electrophoresis of the RT-PCR products amplified from 16S rRNA transcripts.

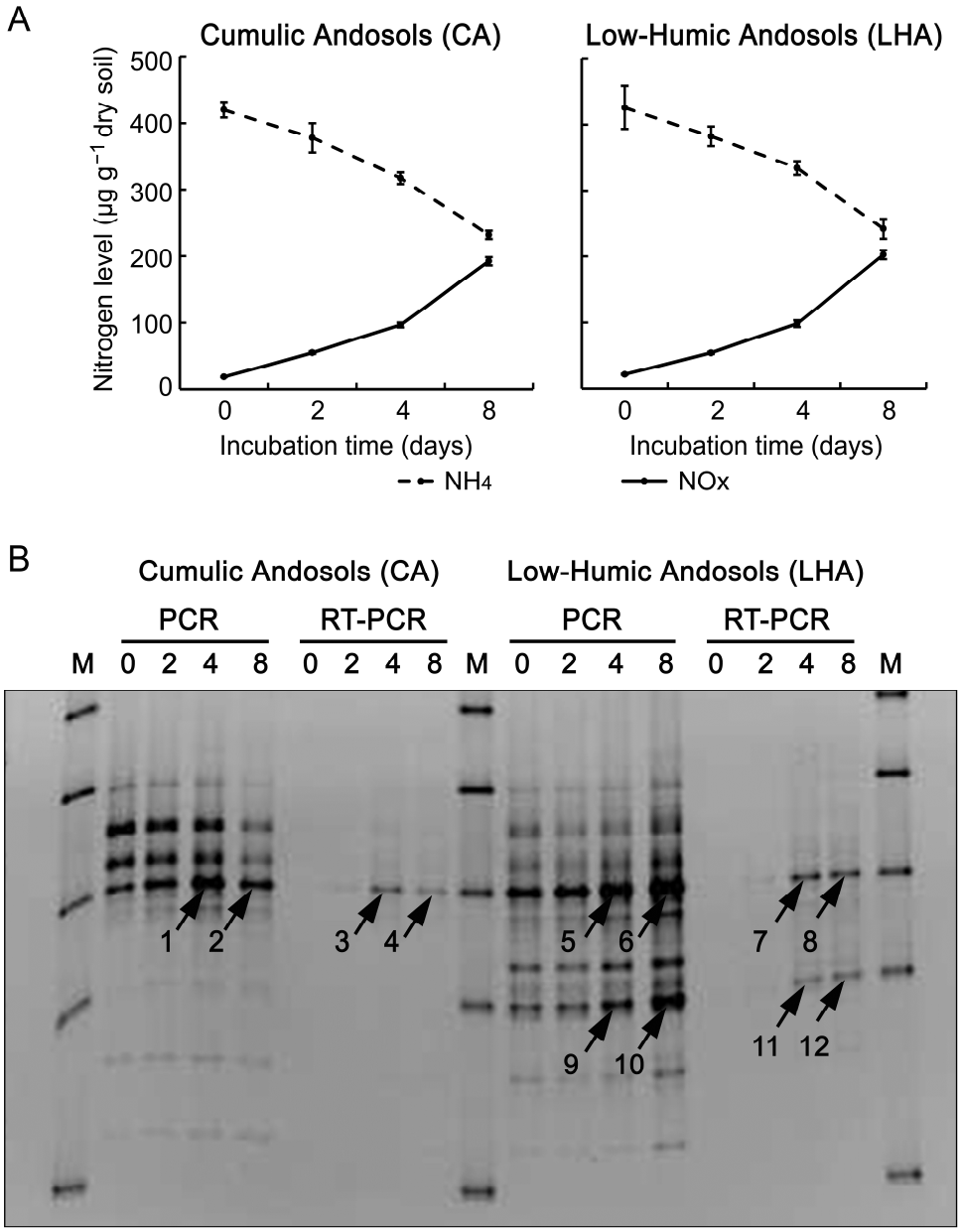

Fig. VI.5 (A) Nitrogen levels of ammonia and nitrate/nitrite in the CA and LHA soils during incubation. (B) PCR-DGGE and RT-PCR-DGGE analyses for the AOB *amoA* in the incubated soil microcosms.

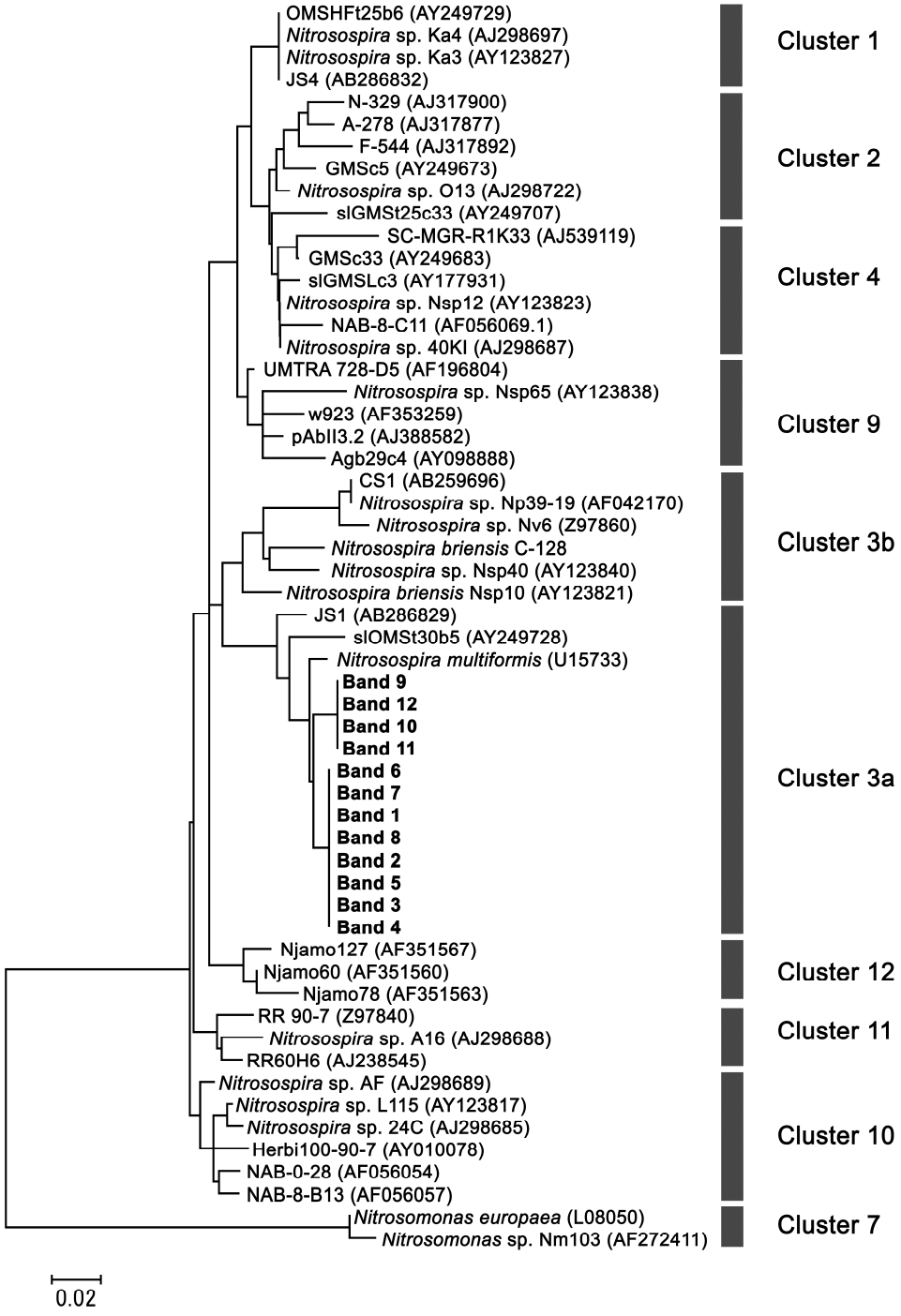

Fig. VI.6 Phylogenetic tree of *amoA* based on their partial sequences (138 amino acid residues). The scale bar indicates the number of substitutions per amino acid position.

Chapter VII

Conclusions and perspectives

Until now, no method of RNA extraction from soil could be used to fit all research purposes. Thus, significant efforts are still required to develop a universal method, which is expected to facilitate researchers in generating comparable data worldwide.

During the past six years, we developed a new method to extract high quality and high purity bacterial RNA from diverse soils. The soil RNA extracted with this method has been successfully applied to different technical platforms for analyzing bacterial gene expression, which included real-time RT-PCR, genome-wide microarray, and RT-PCR-DGGE analyses. Because the soils we tested covered the major soil groups in Japan and about 40% of the agricultural lands on the Earth, we believe this method has great potential to be used worldwide.

However, we still think there is a room for developing this technique. As stated in the motto of the Zymo Research Corporation (Irvine, CA, USA), “The beauty of science is to make things simple.” Thus, we believe that the “new version” of a universal method should be as simple as possible so that it can be mastered by regular researchers without much experience with RNA experiments. To reach this goal, the removal of humic substances should be as simple as possible without loss of purification power. Because biologists have already spent two decades improving the methodology of RNA extraction from soil, it is apparently difficult to simplify the purification procedures based on the current technologies. This may require contributions from chemists or physicists to develop new technologies.

Although we have already tested diverse soils for RNA extraction, much more soil groups should be tested in the future to generate a more powerful technique. For this purpose,

RNA extraction from diverse soil groups collected worldwide is required. Because it is difficult to conduct such a systematic test for technical, economic, and political reasons, the "final version" of a universal method for RNA extraction from soil seems to be far from us.

Gene expression, as an important tool in the study of soil microbial ecology and physiology, can be expected to be more popular (because of the ease of use than before) and more important (because more new information could be obtained) with the development of methods of bacterial RNA extraction from soil. It could be expected that this technique will be applied to the detection of bacterial responses in field soils to various treatments (e.g., fertilization, waterlogging, etc.) and various changes of the environment (e,g., heavy rain, drought, etc.). In the current method, 2-5 g of soil is required for processing. If the amount of soil for processing could be reduced, e.g., less than 1 g, this technique could be used to investigate the bacterial information at a higher resolution along the soil depth, or even in the soil aggregates, which will open a new door for us to learn the bacterial behavior in soil.

List of Abbreviations

3-CB: 3-chlorobenzoate;

5’-AMP: Adenosine-5’-phosphate;

6FAM: 6-carboxyfluorescein;

BSA: Bovine serum albumin

CTAB: cetyltrimethyl ammonium bromide

DEPC: Diethylpyrocarbonate;

DGGE: Denaturing gradient gel electrophoresis;

FDR: False discovery rate;

HPD: 2-hydroxypenta-2,4-dienoate;

ORFs: Open reading frames

PCR: Polymerase chain reaction;

PVPP: Polyvinylpolypyrrolidone;

qRT-PCR: Quantitative reverse transcription-polymerase chain reaction;

RT-PCR: Reverse transcription-polymerase chain reaction;

TAMRA: 6-carboxytetramethylrhodamine.

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Summary

Detection of bacterial gene expression in soil emerged in the early 1990s and provided information on bacterial responses in their original soil environments. As a key procedure in the detection, extraction of bacterial RNA from soil has attracted much interest, and many methods have been reported in the past 20 years. In addition to various RT-PCR-based technologies, new technologies for gene expression analysis, such as microarrays and high-throughput sequencing technologies, have recently been applied to examine bacterial gene expression in soil. These technologies are driving improvements in RNA extraction protocols. However, until now, no commercial kit or method could be considered as a "universal" method, by which RNA could be extracted from diverse soil.

1) A trial to detect bacterial gene expression in a sterilized soil inoculated with a *Rhodococcus jostii* RHA1 strain

Under such a situation, we tried to develop a universal method for extraction of bacterial RNA from soil. As a first trial, we set up a new method by combination of several commercial kits. Using this method, we extracted RNA from a sterilized soil inoculated with *Rhodococcus jostii* RHA1, a biphenyl degrader isolated from γ-hexachlorocyclohexane-contaminated soil. Data from agarose gel electrophoresis indicated that the extracted RNA was purified properly. This new method can be applied easily in the preparation of large amounts of RNA. Real-time reverse transcription-polymerase chain reaction (RT-PCR) experiments performed with the TaqMan method suggested that the *bphAa* gene in this strain, which is involved in the degradation of biphenyl, was induced in the biphenyl amended soil.

2) Evaluation of methods determining humic acids in soil RNA samples

It has been known that even small amounts of humic substances may affect the detection of gene expression. However, we did not know how much humic substances remained in soil RNA samples. To select a proper method for measurement of humic substances, we compared

the sensitivity of various methods for measurement of humic acids, and influences of DNA, RNA and proteins on the measurement. Data suggests that both ultraviolet/visible spectroscopic and fluorescence spectroscopic methods are reliable to determine the quantity of humic substances in RNA samples. Considering the results, we also give suggestions as to choice of methods for measurement of humic acids in molecular biological analyses.

3) Optimization of the conditions to remove humic substances from soil RNA samples

Then, the soil RNA extraction method was improved by optimization of lysis conditions and purification columns, to efficiently remove humic substances that may hinder enzymatic reactions of extracted RNA. Fluorescence spectroscopy confirmed efficient removal of both humic and fulvic acids by the improved method. The sensitivity of detection by real-time RT-PCR increased 10-fold compared with that using the previous method. Using this method, we extracted RNA from a sterilized field soil, which was inoculated with *Pseudomonas putida* KT2440 transformed with a chloroaromatic degrading plasmid, in the presence or absence of 3-chlorobenzoate (3CB). Real-time RT-PCR performed using the extracted RNA as a template confirmed the induction of chloroaromatic degrading genes in 3CB-amended soil. Thus, this improved method is suitable for the extraction of RNA to detect gene expression in soil.

4) Microarray analysis of global gene expression in *Pseudomonas putida* KT2440 growing in a sterilized soil

Next, we examined whether the RNA extracted from soil with this improved method was suitable for microarray analysis. For this purpose, genome-wide scanning of gene expression by microarray techniques was performed on RNA extracted from sterilized soil inoculated with *Pseudomonas putida* KT2440/pSL1, which contains a chloroaromatic degrading plasmid, in the presence or absence of 3-chlorobenzoic acid (3CB). The genes showing significant changes in their expression in both the triplicate-microarray analysis using amplified RNA and the single-microarray analysis using unamplified RNA were investigated. Pathway

analysis revealed that the benzoate degradation pathway underwent the most significant changes following treatment with 3CB. Analysis based on categorization of differentially expressed genes against 3CB revealed new findings about the cellular responses of the bacteria to 3CB. The genes encoding a K^+/H^+ antiporter complex, a universal stress protein, two cytochrome P450 proteins and an efflux transporter were upregulated. The downregulated expression of several genes involved in carbon metabolism and the genes belonging to a prophage in the presence of 3CB was observed. This study demonstrated the applicability of the method of soil RNA extraction for microarray analysis of gene expression in bacteria growing in sterilized soil.

5) Development of a universal method for RNA extraction from diverse soils and its application to *amoA* gene expression study in Andosols

In an attempt to extract RNA from Andosols (volcanic ash soils), which are the dominant agricultural soils in Japan and are well known for their strong adsorption of RNA, extraction buffer containing autoclaved casein was used. Using this buffer, high-quality RNA was successfully extracted from eight types of agricultural soils that were significantly different in their physicochemical characteristics. To detect bacterial ammonia monooxygenase subunit A gene (*amoA*) transcripts, bacterial genomic DNA and messenger RNA were co-extracted from two different types of Andosols during incubation with ammonium sulfate. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and reverse transcription polymerase chain reaction-denaturing gradient gel electrophoresis (RT-PCR-DGGE) analyses of *amoA* in soil microcosms revealed that only few *amoA* genes, which had the highest similarities to those in *Nitrosospira multiformis*, were expressed in these soils after treatment with ammonium sulfate, in spite of the fact that multiple *amoA* genes were present in the soil microcosms examined. This study demonstrated that the casein method could be considered as a universal method for bacterial RNA extraction from soil.

After six years effort, I have successfully developed a universal method for RNA extraction from diverse soils including the most challenging soil, Andosols, by using autoclaved casein and getting rid of humic substances efficiently. The soil RNA extracted with this method has high quality and high purity, and has been successfully applied to different technical platforms including qRT-PCR, genome-wide microarray, and RT-PCR-DGGE analyses. It could be expected that this technique will be applied to a wide range of research area in the soil microbiology and microbial ecology.

土壌から RNA を抽出する方法の開発および土壌微生物の研究への応用

王　勇

要　　旨

土壌中の細菌遺伝子発現の検出は、1990 年代初頭に登場し、土壌中に生きている細菌の応答に関する情報を提供してきた。検出において重要な手順として、土壌から細菌 RNA を抽出する方法が多くの関心を集めている。更に、過去 20 年間、様々な土壌 RNA の抽出方法が報告されてきた。RT-PCR に基づく様々な技術に加えて、マイクロアレイや次世代シーケンシング技術など遺伝子発現解析の新しい技術は、土壌中の細菌の遺伝子発現を検出するために使用されている。しかし、今まで、多様な土壌から RNA を抽出する方法は開発されていない。

1) *Rhodococcus jostii* RHA1株を接種した滅菌土壌中の遺伝子発現の検出

このような状況下で、我々は多様な土壌から細菌RNAを抽出するための方法の開発を試みた。最初に、我々はいくつかの市販のキットを組み合わせて、新しい方法を開発した。この方法を用いて、我々はγ-ヘキサクロロシクロヘキサンで汚染された土壌から分離されたビフェニル分解菌*Rhodococcus jostii* RHA1株を接種した滅菌土壌から細菌RNAを抽出した。抽出されたRNAはアガロースゲル電気泳動によって、分析に十分な純度に精製されたことが示された。この新しい方法は、多量のRNAの調製に簡単に利用することができる。またTaqMan法を用いたリアルタイムRT-PCR実験により、ビフェニルの分解に関与している*bphAa*遺伝子の発現が、ビフェニルを添加した土壌に誘導されたことが示唆された。

2) 土壌RNA試料中のフミン酸を測定する方法の評価

一方、少量の腐植物質でも、遺伝子発現の検出に影響を与える可能性があることが知られている。しかし、これまで土壌RNAサンプル中に残っている腐植物質を評価する方法はなかった。そこで腐植物質を測定する方法を選択するために、様々なフミン酸の測定方法の感度とDNA、RNAおよびタンパク質の影響を調べた。その結果、紫外/可視分光法と蛍光分光法は、RNAサンプル中の腐植物質の量を測定するために信頼性が高いことを示した。以上から、この方法を分子生物学実験に使用するサンプル中のフミン酸測定に使用することを提案した。

3) 土壌RNA試料から腐植物質を除去するための条件の最適化

次に、土壌から抽出した RNA サンプル中の腐植物質を除去するために、土壌中の微生物細胞破砕条件と精製カラムを最適化した。この改良した方法によってフミン酸とフルボ酸を効率的に除去できることを蛍光分光法で確認した。リアルタイム RT-PCR 法による検出感度は、以前の抽出方法を使用した場合と比較して 10 倍に増加した。この方法を用いて、3-クロロ安息香酸（3CB）の存在下または非存在下で、3CB 分解プラスミドで形質転換された *Pseudomonas putida* KT2440 株を接種した滅菌畑土壌から RNA を抽出した。抽出した RNA を用いてリアルタイム RT-PCR を行い、3CB を添加した土壌中の 3CB 分解遺伝子の発現が確認された。したがって、この改良された RNA 調製方法は、土壌中の遺伝子発現を検出するための高純度の RNA の調製に適している。

4) 滅菌土壌における *Pseudomonas putida* KT2440 の遺伝子発現マイクロアレイ解析

さらに、この改良された方法で土壌から調製した RNA が、マイクロアレイ解析に利用することが可能かどうかを検討した。そこで、3CB の存在下または非存在下で *Pseudomonas putida* KT2440/pSL1 を接種した滅菌土壌から抽出した RNA を用い

て、マイクロアレイ技術による遺伝子発現のゲノムワイドスキャンを行った。増幅されていない RNA を用いたシングルマイクロアレイ解析と増幅した RNA を用いた三重マイクロアレイ解析の両方で、発現に有意な変化を示す遺伝子を調べた。パスウェイ解析は、安息香酸分解経路が 3CB で処理した後の最も大きく変化したことを示した。3CB によって発現した遺伝子の解析結果から、土壌中における細菌の 3-CB に対する細胞応答に関する新たな知見を得ることができた。具体的には、K^+/H^+アンチポーター複合体、ストレスタンパク質、二つのシトクロム P450 タンパク質および排出系トランスポーターをコードする遺伝子がアップレギュレートされていた。いくつかの炭素代謝に関与する遺伝子とプロファージ遺伝子の発現が 3CB 存在下にダウンレギュレートされていた。以上の結果から、我々が開発した土壌 RNA 抽出の方法は、土壌中の細菌の遺伝子発現のマイクロアレイ解析に適用できることが実証できた。

5) 多様な土壌から RNA を抽出する方法の開発及び黒ボク土における *amoA* 遺伝子発現研究への応用

黒ボク土（火山灰土壌）は日本の農耕地における主要な土壌であり、RNA を強く吸着する特性を持っており、これまで RNA を抽出する好適な方法はなっかた。そこで黒ボク土から RNA を抽出する方法を検討した。滅菌済みカゼインを含有する抽出バッファーを用いることにより、高品質の RNA が 8 種類の農耕地土壌から抽出できることを示した。この開発した方法で細菌のアンモニア酸化酵素遺伝子（*amoA*）転写産物を検出するために、硫酸アンモニウムで処理した 2 つの黒ボク土から細菌の土壌 DNA と土壌 mRNA を抽出した。PCR-DGGE 分析よって、多様な *amoA* 遺伝子が土壌中に存在することを示された。しかし、*amoA* 遺伝子の mRNA の RT-PCR-DGGE 分析よって、*Nitrosospira multiformis* の *amoA* 遺伝子と高い相同性を

持つ少数の *amoA* 遺伝子だけが発現していることを明らかにした。以上から、カゼイン利用した RNA 抽出法は黒ボク土壌だけでなく多様な土壌から細菌 RNA を抽出するの有効な方法であることを実証した。

六年間の努力を通じて、滅菌済みカゼインを使用し、効率的に腐植物質を取り除くことで黒ボク土を含む多様な土壌から細菌 RNA を抽出する有効な方法を開発した。この方法で抽出した RNA は高品質、高純度を有しており、定量 RT-PCR、マイクロアレイ、および RT-PCR-DGGE 解析を含む様々な技術に適用されている。なお、この技術は土壌微生物学と微生物生態学の広い範囲の研究領域に適用されることが期待できる。

Publication list (2008 – 2012)**:** (All research presented here has been published in the followed papers)

Research papers:

Wang Y, Shimodaira J, Miyasaka T, Morimoto S, Oomori T, Ogawa N, Fukuda M, Fujii T. (2008). Detection of bphAa gene expression of *Rhodococcus* sp. strain RHA1 in soil using a new method of RNA preparation from soil. *Biosci. Biotechnol. Biochem.* 72, 694-701.

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Review article:

Wang Y, Hayatsu M, Fujii T. (2012) Extraction of bacterial RNA from soil: challenges and solutions. Microbes Environ. 27, 111-121.

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