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Detection of *bphAa* Gene Expression of *Rhodococcus* sp. Strain RHA1 in Soil Using a New Method of RNA Preparation from Soil

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To understand the response of soil bacteria to the surrounding environment, it is necessary to examine the gene expression profiles of the bacteria in the soil. For this purpose, we developed a new method of extracting RNA from soil reproducibly. Using this new method, we extracted RNA from a field soil, which was sterilized and inoculated with *Rhodococcus* sp. 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 by 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.

Key words: soil; RNA; real-time reverse transcription-polymerase chain reaction (RT-PCR); *Rhodococcus*; biphenyl

Polychlorinated biphenyl (PCB) is a toxic chemical that causes serious environmental problems because of its persistence in the environment. So far, knowledge of the biphenyl degradation pathway has been acquired from environmental bacteria which could use biphenyl as a carbon and energy source.[1–5] Gram-positive *Rhodococcus* sp. strain RHA1, an actinomycete, was originally isolated from γ-hexachlorocyclohexane-contaminated soil and was identified as a biphenyl degrader.[6] The genes involved in the early steps of biphenyl degradation in *Rhodococcus* sp. strain RHA1, *bphAaAbAcAd-bphC-bphB* (formerly *bphA1A2A3A4-bphC-bphB*), have been identified.[7] So far, it has been found that *Rhodococcus* sp. strain RHA1 metabolizes biphenyl to 2-hydroxypenta-2,4-dienoate (HPD) and benzoate, which are further metabolized through the HPD and benzoate pathways respectively.[8,9] The genes involved in these two pathways have been identified.[8,9] Recent research and a newly completed genome project indicate that *Rhodococcus* sp. strain RHA1 has one of the largest bacterial genomes sequenced to date. It contains 9.7 million base pairs arranged in a linear chromosome and three linear plasmids.[10,11] Unlike other related actinomycetes, *Rhodococcus* sp. strain RHA1 possesses highly redundant catabolic pathways and enzymes, suggesting a potential to adapt to new carbon sources.[12] This has been confirmed by recent transcriptomic analysis, which revealed several new isozymes in the biphenyl pathway and a new degradation pathway in strain RHA1.[13,14]

Many papers have reported study of *Rhodococcus* sp. strain RHA1 cultured in liquid media. However, to understand the response of soil bacteria to the surrounding environment, such as PCB-contaminated soil, it is necessary to examine the gene expression profiles of the bacteria in soil. For this purpose, RNA extraction from soil is required. For eukaryotes, it is rather easy to extract high purity mRNA even from the cells in soil, since eukaryotic mRNA possesses a poly A tail at its 3′-end, which makes it possible to use oligo(dT) beads in mRNA extraction. Thus, for gene expression analysis in eukaryotes, extraction of total RNA can be avoided.[15,16] Unlike eukaryotes, bacteria mRNA does not possess a poly A tail at its 3′-end, and hence it is not possible to extract mRNA from bacteria in soil by simple procedures like those used with eukaryotes. In the past 20 years, except for reports focusing on rRNA extraction

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Abbreviation: RT-PCR, reverse transcription-polymerase chain reaction

from soil and sediments,[17–23] there have been few reports on the technique of total RNA extraction from bacteria in soil or sediments, in which the amount of mRNA is sufficient for gene expression analysis.[24–32] In an attempt to extract RNA from bacteria in soil, we tried several methods, including the use of several commercial kits, in our preliminary experiments, but all methods handled only small amount of soil, or co-extracted humic substances together with RNA. To get sufficient quantities of high quality total RNA from soil for the study of biodegradation genes, we developed a new method, presented here, and examined the expression of a gene involved in biphenyl degradation by strain RHA1.

Materials and Methods

Soil for inoculation. A field soil sample, collected from the Ehime Agricultural Experiment Station in Ehime, Japan, was sieved 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 1.

Bacterial strain and culture conditions. Rhodococcus sp. strain 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 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 30 °C 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, pH 7.0, 1 mM EDTA, and freshly prepared 0.5% 2-mercaptoethanol)[26] 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 cleanbench 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) 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).

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

Table 1. Properties of the Field Soil Examined

pH (H_2O)	pH (KCl)	EC(1:5) dS m^{-1}	Total carbon g kg^{-1}	Total nitrogen g kg^{-1}	CEC cmol(+)kg^{-1}	Base saturation percentage %	Phosphate absorption P_2O_5mg/100 g	Available phosphate P_2O_5mg/100 g	Soil group
6.6	5.4	0.056	9.7	1.6	8	95	514	42	Brown forest soil

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′-AAGAAGCGGCG-CGTGGGCT-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* sp. strain 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.[33)]

Results and Discussion

Growth of Rhodococcus *sp. strain RHA1 in soil*

During the several days immediately after inoculation, *Rhodococcus* sp. strain RHA1 increased its population in the soil without the addition of any carbon source, and maintained its population for about one month (Fig. 1). In an attempt to determine at which growth stage of *Rhodococcus* sp. strain 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. 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. 2, among the soil cultures of bacteria in the presence of biphenyl (Fig. 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. 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.

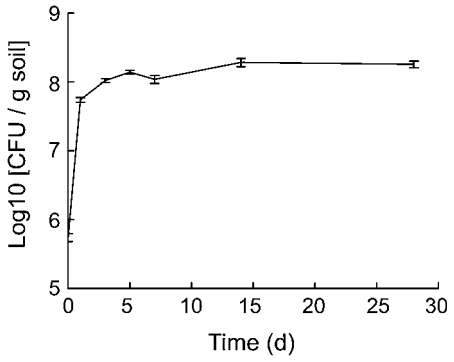

Fig. 1. The Viability of *Rhodococcus* sp. Strain RHA1 in Soil for a Long Period of Time.

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. 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* sp. strain RHA1.

RNA isolation from soil

There are two major methods of nucleic acid 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 acid is extracted from the cells collected. It has been reported that the indirect method resulted in significantly lower RNA yields than the direct extraction method.[18)] Also, we were afraid that gene expression might be affected or altered during treatment before cell lysis. Hence we adopted the direct method to extract RNA from soil.

In most RNA isolation methods, the protocol can be divided into four steps: (i) cell lysis, (ii) inactivation of nucleases, (iii) extraction of RNA from the environmental matrix, and (iv) purification.[25,34)] Of these steps, the most important is cell lysis. Many lysis techniques have been tried, including bead beating,[27,28,30,32,35)] solubilization of cell membranes by detergent,[24,25,36,37)] enzymatic degradation of cell wall and cell mem-

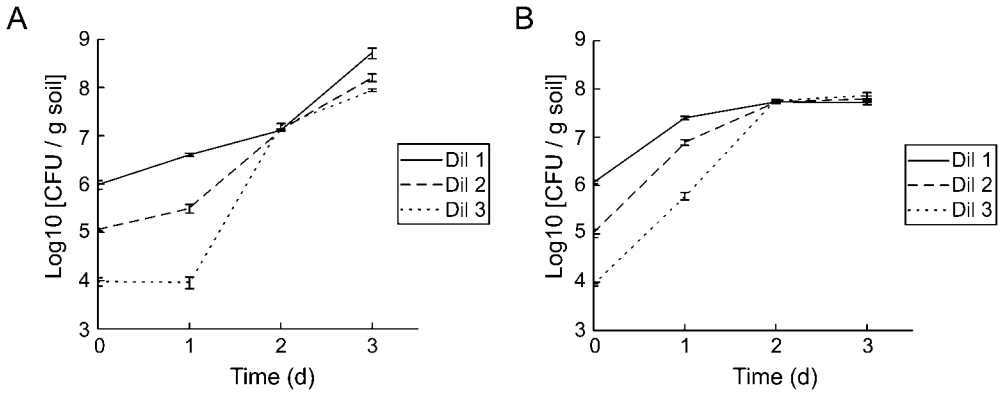

Fig. 2. The Growth of *Rhodococcus* sp. Strain RHA1 in Soil Amended with Biphenyl (A) or Not Amended with Biphenyl (B). CFU, colony forming unit.

brane,[17] liquid nitrogen grinding,[26,31] and microwave thermal shock.[23] A recent report on soil DNA extraction pointed out that the bead beating method might be the best choice, since it gave significantly higher DNA yields than the microwave-based and liquid nitrogen grinding extraction methods,[35] which implies that the efficiency of cell lysis in the bead beating method is higher than in the two other methods. Hence we adopted the bead beating method combined with the detergent-based method in the present study. Also, we combined the first two steps into one by adding guanidine solution into lysis buffer, since nuclease is released in the process of cell lysis, so that separation between cell lysis and inactivation of nucleases results in unexpected RNA degradation.[24,26] However, a combination of the first three steps, *i.e.*, adding phenol into lysis buffer to perform cell lysis and phenol extraction at the same time, causes contamination of large amounts of humic substances from the soil (data not shown). In our 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.

In our present study, 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,[38] nucleic acid detection and measurement,[39,40] and RNA hybridization.[41] To remove humic substances, affinity/ion-exchange spin columns and gel filtration columns have been used by some researchers.[17,26,29–31,35] In an alternative method, humic substances were removed by precipitation with 7.5 M potassium acetate from nucleic acid extract.[32] 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 Bio-Rad 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. 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.

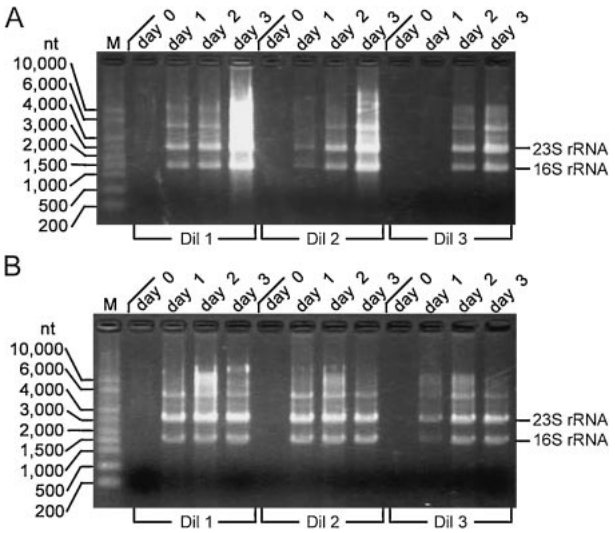

Fig. 3. Agarose Gel Electrophoresis of RNA Samples Prepared from Soil Amended with Biphenyl (A) or Not Amended with Biphenyl (B).

M, RNA marker.

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. 2A, B and 3A, B).

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 (5 g 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 (Figs. 2A and 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 (Figs. 2B and 3B), this sample was used as the biphenyl(−) soil sample for RNA extraction.

For Fig. 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). Although the yield of RNA from biphenyl(−)-Dil 1 sample was not enough for microarray analysis which normally requires about 5 to 6 μg RNA for one particular bacterium, the amounts of RNA extracted from the different soil samples were in proportion to the numbers of tubes used, indicating that this new method can be applied easily to the preparation of large amounts of RNA.

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. 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.[42] Hence normalization was done against the amount of total RNA. The real-time RT-PCR data are summarized in Fig. 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 (Figs. 2A and 3A), the expression levels of *bphAa* were significantly different. That is, the

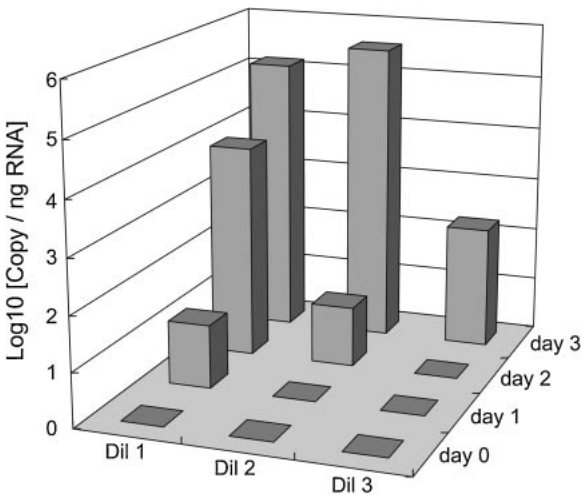

Fig. 4. Expression of *bphAa* in *Rhodococcus* sp. Strain RHA1 Inoculated in Biphenyl Amended Soil.

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

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. 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 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* sp. strain 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. 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. 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.

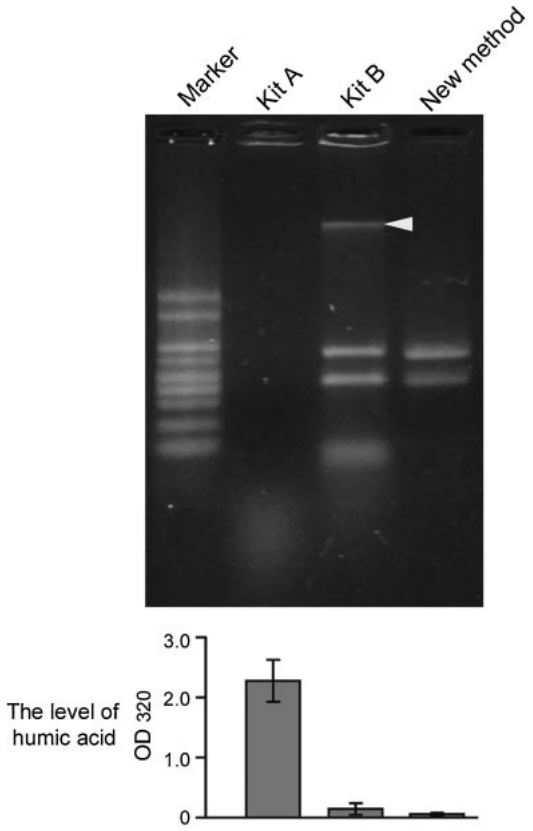

Fig. 5. Comparison of Different Methods to Extract RNA from Soil. RNA extracted from 0.5 g of soil was loaded in each lane. Triplicate samples were processed for each method. The white arrowhead indicates a DNA band in the RNA extracted with kit B.

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