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Real-time quantitative PCR assay on bacterial DNA: In a model soil system and environmental samples

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Real-time quantitative PCR (RTQ-PCR) was used to quantify the bacterial target DNA extracted by three commonly used DNA extraction protocols (bead mill homogenization, grinding in presence of liquid nitrogen and hot detergent SDS based enzymatic lysis). For the purpose of our study, pure culture of *Bacillus cereus* (model organism), sterilized soil seeded with a known amount of *B. cereus* (model soil system) and samples from woodland and grassland (environmental samples) were chosen to extract DNA by three different protocols. The extracted DNA was then quantified by RTQ-PCR using 16S rDNA specific universal bacterial primers. The standard curve used for the quantification by RTQ-PCR was linear and revealed a strong linear relationship (r^2=0.9968) with a higher amplification efficiency, e=1.02. High resolution gel electrophoresis was also carried out to observe the effect of these extraction methods on diversity analysis. For the model soil system, the liquid nitrogen method showed the highest target DNA copy number (1.3×10^9 copies/μl). However, for both the environmental samples, the bead beating method was found to be suitable on the basis of the high target DNA copy numbers (5.38×10^9 and 4.01×10^8 copies/μl for woodland and grassland respectively), high yield (6.4 μg/g and 1.76 μg/g of soil for woodland and grassland respectively) and different band patterns on high resolution gel electrophoresis suggesting an overall high extraction efficiency. This difference in the extraction efficiency between the model soil system and environmental samples may be attributed to different affinity of seeded and native DNA to soil particles.

Key Words——*Bacillus cereus*; copy number; DNA extraction; model system; real-time quantitative PCR; 16S rDNA

Introduction

Quantitative estimation as well as diversity analysis of microbial population is important for better understanding of microbial processes such as biochemical, mineralization, community structure analysis etc. The development of molecular biological methods involving PCR has led to an array of new techniques that are not limited by the culturability of the microorganisms. As a result, the application of culture-independent nucleic acid techniques has greatly advanced the detection and identification of microorganisms in natural environments (Amann et al., 1995; Bornemon and Triplett, 1997; Hugenholtz et al., 1998). Thus methods using in situ cell lysis, followed by DNA extraction, quantification and diversity analyses offer new ways of studying microorganisms in the environment that make it possible to circumvent biases related to cultivation.

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Although PCR-based methods and spectroscopy have been used for the quantitative analysis of DNA, PCR-based quantification has the disadvantage of relying on end-point measurement of the amount of DNA produced, which makes it difficult to deduce the initial concentration of template DNA (Hermanson and Lindgren, 2001). On the contrary, without extensive purification, spectroscopic A_{260} determination cannot be an accurate measure for the DNA concentration. This is due to the overlap in the 260 nm absorbance range by co-extracted humic contaminants (Cullen and Hirsch, 1998). As a result, various suitable extraction and purification protocols have already been established, but they still suffer lack of precision as well as reliability for the quantification of the extracted DNA.

In order to overcome these limitations, quantification of target gene copy number has been attempted with most probable number (MPN) PCR (Hielm et al., 1996) and competitive PCR (Janse et al., 1998; Lee et al., 1996). These methods require the multiple handling of culture tubes (MPN PCR) and time-and-resource-consuming post PCR analyses (competitive PCR) (Becker et al., 2000). As an alternative, a real-time quantitative PCR (RTQ-PCR) system based on continuous monitoring of the DNA amplification having several advantages has recently been developed. i) Unlike MPN PCR, it is simple and rapid because it is a real-time assay in a closed tube without a need for any post-PCR manipulation. ii) Unlike competitive PCR, real-time PCR makes DNA quantification much more precise and reproducible, since it is based on threshold values, rather than an end-point measurement of the amount of accumulated PCR product. The RTQ-PCR has been used successfully to quantify *Porphyromonas gingivalis* and total bacteria in plaque samples (Lyons et al., 2000), ammonia-oxidizing bacteria in soil (Hermanson and Lindgren, 2001), fungal DNA in clinical samples (Loeffler et al., 2000) and so on.

Although extraction protocols have already been qualitatively compared using spectroscopy, PCR, and gel electrophoresis (Cullen and Hirsch, 1998; Zhou et al., 1996), to our knowledge quantitative comparison based on real-time PCR has not been done. Thus in the present study, RTQ-PCR has been used to compare three different extraction methods quantitatively. In addition, the length polymorphism pattern was analyzed by high resolution gel electrophoresis for diversity analysis as a tool to evaluate the extraction efficiency of these methods. A sterile soil system seeded with bacterial DNA was used as a model system and soil samples from two different environments were also used to determine the suitability of the quantification of target DNA.

Materials and Methods

Bacterial strain. *Bacillus cereus* was used in the present study to compare the DNA extraction efficiencies of various methods. This bacterium was used in this study, because it is highly diversified in soil (7–67%) (Alexander, 1977), nonpathogenic, easy to culture and is of interest in many microbial applications. Bacterial strain (*B. cereus* JCM 2152) was obtained from the Japan Collection of Microorganisms and was allowed to grow in nutrient broth for 48 h at 30°C in a shaker at 100 rpm. Cell suspension was centrifuged at 45×100 rpm for 5 min at 4°C and then used for the purpose of this study.

Samples for DNA extraction.

i) Bacteria: As described above, the bacterial cells were harvested and a known amount of bacterial cells was used for DNA extraction.

ii) Model soil system: A model soil system was obtained by autoclaving the woodland soil twice at 121°C for 60 min (Zhou et al., 1996). This sterilized soil (100 mg) was then mixed with a known amount (approximately 75 mg bacterial biomass) of bacterial cells. Seeded soil samples were kept at room temperature for 30 min prior to DNA extraction. Uninoculated sterilized soil was also used as a control.

iii) Environmental samples: Soil samples from two different environments (woodland and grassland) collected from the campus of the Yokohama National University, Yokohama, Japan were used for DNA extraction. After removing all visible roots and other materials from the soil samples, they were sieved (2-mm mesh) and then stored at −20°C. DNA was extracted from 500 mg of both woodland and grassland samples.

Extraction and purification of DNA. Three commonly used extraction methods: i) Bead mill homogenization (Cullen and Hirsch, 1998), ii) grinding in the presence of liquid N_2 (Nazar et al., 1996), and iii) hot detergent SDS based enzymatic lysis (Zhou et al., 1996) were followed to extract DNA from bacteria and soil samples. Bead mill homogenization was carried out with a high speed (50×100 rpm) mini-bead beater™ (BioSpec Products, Inc., Bartlesville, USA). Before the lysis treatment of Zhou et al. (1996), glass

beads were used to facilitate the disruption of highly resistant bacterial cell walls. In each case, the extraction procedure was carried out in triplicate.

Bead mill homogenization was performed after suspending soil samples in 3 ml sodium phosphate buffer (120 mM sodium phosphate, pH 8.0) and 1% SDS in snap-top polypropylene tubes containing glass beads of three different sizes (0.5, 0.1 and 1.0 mm diameter). Each tube was then subjected to bead beating for 30 s at 50×100 rpm. Tubes were then centrifuged at 45×100 rpm for 15 min at 10°C and the supernatant was transferred to a new tube. Immediately after adding EDTA (500 mM, pH 8.0), 1/10 vol. of potassium acetate (5 M, pH 5.5) was added. Samples were incubated on ice for 20 min and then centrifuged at 150×100 rpm for 5 min at 4°C. The DNA in the supernatant fraction was precipitated with 1 vol. of isopropanol and then recovered by centrifugation. Pellets were resuspended in sterile distilled water.

SDS-based enzymatic analysis was performed by mixing soil samples with DNA extraction buffer (1.5 M NaCl, 1% hexadecyltrimethyl ammonium bromide, 0.1 M Tris-HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 0.1 M sodium phosphate (pH 8.0), and 100 µl proteinase-K) in 2 ml tubes and then bead beating for 30 s at 50×100 rpm. After adding 300 µl of 20% SDS, the samples were incubated in a water bath at 65°C for 2 h with gentle end-over-end inversions every 20 min. The supernatants were collected after centrifugation at 50×100 rpm for 15 min. Then the soil pellets were again extracted one more time by the addition of the extraction buffer and 20% SDS. The combined supernatants were then mixed with an equal volume of chloroform and the aqueous phase was recovered by precipitation with an equal volume of isopropanol for 1 h. Following centrifugation (150×100 rpm), a DNA pellet was obtained, washed with 70% ice cold ethanol and resuspended in sterile distilled water.

For the liquid nitrogen method, soil samples were transferred to a small mortar and then ground in the presence of liquid nitrogen with the help of a pestle for 5 min until the soil had a very fine and smooth consistency. The powdered soil was then transferred to a small tube and suspended in 1 ml powdered milk solution (0.1 g powdered milk in 25 ml of H_2O) by vigorous vortexing. Following centrifugation at 45×100 rpm for 10 min, the collected supernatant was transferred to a 10 ml polypropylene tube and mixed by vortexing with 4 ml of SDS extraction buffer (0.3% SDS in 140 mM NaCl, 50 mM Na-acetate, pH 5.1) and an equal volume of phenol saturated with TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5). The upper aqueous phase was then collected by centrifugation at 150×100 rpm for 10 min and precipitated with 2.5 vol. of ethanol (95–100%) containing 0.2 M potassium acetate at −20°C overnight. The pellet was collected by centrifugation at 150×100 rpm for 10 min and rinsed twice with ethanol with centrifugation between rinses and then resuspended in sterile distilled water.

However, for all the methods tested, the purification procedure was carried out using a spin column packed with PVPP (polyvinylpolypyrollidone) and Sepharose-4B to remove humic acids (Cullen and Hirsch, 1998; Steffen et al., 1988) and low molecular weight RNA (Jackson et al., 1997) from the extracted DNA. For the purification process, the crude DNA pellet suspended in sterile water was passed through a spin column packed with 0.15 g PVPP by centrifuging at 45×100 rpm for 10 min. The partially purified DNA solution was then passed through another spin column packed with 3 ml Sepharose-4B by centrifuging at 39×100 rpm for 5 min (Kang et al., 2001).

Qualitative determination of the extracted DNA. The quality of the extracted DNA (absence of degradation) was estimated based on the size of the DNA fragments or the relative position of the DNA smears after electrophoresis of an aliquot of the DNA solution on 0.8% agarose gel.

Quantitative determination of the extracted DNA.

Real-time quantification: Bacterial target DNA copy numbers in extracted DNA were quantified with a LightCycler™ PCR and detection system (Roche Diagnostic, Mannheim, Germany) as described below.

Preparation of external standard for LightCycler™ and amplification efficiency. DNA extracted from *B. cereus* was used for the preparation of the standard. The bacterial primers used for the amplification were 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519r (5′-GWATTACCGCGGCKGCTG-3′), as reported by Lane et al. (1991). This primer pair amplifies a 538-bp region of the *B. cereus* small subunit rDNA molecule and contains the variable regions V1 to V3. The extracted bacterial DNA was subjected to conventional PCR (PERKIN ELMER GeneAmp PCR system 2400, Perkin Elmer, Wellesley, MA, USA) in a volume of 50 µl containing 45 µl of PCR SUPERMIX® (22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 µM dGTP, 220 µM dATP, 220 µM dTTP, 220 µM dCTP and 22U Taq

DNA polymerase/ml), 2 µl of each primer (0.5 µM) and 1 µl of DNA template. The following thermocycling program was used: 2 min at 95°C (1 cycle); 1 min at 95°C, 2 min at 59°C, and 1.5 min at 72°C (30 cycles); and 5 min at 72°C (1 cycle).

After amplification, PCR products were analyzed by NUSieve® GTG® agarose gel electrophoresis (2%) with ethidium bromide staining. Expected DNA bands (538 base pairs) were excised from the gel and the DNA was extracted with the aid of Qiaquick® Gel Extraction Kit (Qiagen AG, Basel, Switzerland) according to the manufacturer's instruction. The extracted bands were accurately quantified by spectroscopy at 260 nm and the number of copies was calculated based on the molecular weight of the base pairs. Serial dilutions were done (8.8×10^5 to 8.8×10^9 copies/µl) and used as an external standard for the quantification with a LightCycler™.

For the determination of PCR amplification efficiencies for both the standard DNA and soil DNA, slopes of the standard curve lines were constructed from several dilutions of each DNA. From these slopes, the amplification efficiencies (e) were estimated by the formula $e=10^{-1/s}-1$, where 's' is the slope.

LightCycler™ based PCR assay and the specificity of the primers. The LightCycler™ PCR and detection system (Roche Diagnostic) was used for the amplification and online quantification. Amplification and detection were carried out in glass capillaries in a total volume of 20 µl containing 2 µl LightCycler DNA Master SYBR® Green I, 2.4 µl (4 mM) $MgCl_2$, 1 µl (0.6 µM) of each primer, 11.6 µl H_2O (sterile PCR grade) and 2 µl DNA template. Standards (8.8×10^5 to 8.8×10^9 copies/µl) were also quantified along with the sample and a negative control (sterile PCR-grade water). In order to confirm the specificity of the bacterial primers, DNA extracted from *Aspergillus fumigatus* (filamentous fungi) was also used in the real-time PCR assay. All reactions were run in duplicate by performing 40 cycles of repeated denaturation (0 s at 95°C), annealing (5 s at 59°C) and enzymatic chain extension (8 s at 72°C).

Quantification was performed by online monitoring for the identification of the exact time point at which the logarithmic linear phase could be distinguished from the background (crossing point). The cycle numbers of the logarithmic linear phase were plotted against the logarithm of the initial copy number of standard DNA. The copy number of the target bacterial DNA in pure culture, sterilized soil seeded with bacteria and environmental samples were calculated by comparing the cycle number of the logarithmic linear phase of the samples with the cycle number of the external standard using LightCycler™ Data Analysis (LCDA) software (version 3).

All reactions were analyzed by agarose gel electrophoresis with TBE (Tris—Boric acid—EDTA) and 2% agarose gel followed by DNA staining with ethidium bromide to provide an independent validation check of the presence of an amplicon.

High resolution gel electrophoresis: To obtain a high resolution (1–2 bp) in the chain length analysis with minimum labor, pre-cast Spreadex® EL 1200 Mini Gel (Elchrom Scientific, Switzerland) having an optimal separation range of 250–800 bp was used. The TAE buffer (30 mM) was used as the electrophoresis buffer and M3 marker was used as a size marker. Electrophoresis was carried out in Elchrom Scientific Submerged Gel Electrophoresis Apparatus P/N 2000 at 100 V for 5 h at 55°C. The gel was then stained with SYBR® Green I (Molecular Probes, USA) fluorescent dye for 1 h. The resultant bands were observed and recorded under UV illumination and then analyzed by densitometry (ATTO Co., Ltd., Tokyo).

Results and Discussion

Qualitative analysis of different extraction methods

The quality of extracted bacterial DNA obtained from the three extraction methods were compared (Fig. 1, A and B). The absence of DNA bands (lanes 4, 7, 10 in Fig. 1A) for the sterilized soil samples (control) by three different methods revealed that the unseeded sterilized soil was either free from in situ bacterial DNA or the amount of DNA was below the detection limit. From these results, it could be suggested that there was no possible contribution of in situ bacterial population to the extracted DNA from the sterilized seeded soil. In the case of both pure culture and sterilized seeded soil (Fig. 1A), both the bead beating (lanes 2, 3) and liquid nitrogen (lanes 5, 6) methods generated a sharp and distinct band, which was 23 kb in size. The band intensity was much higher in the case of liquid nitrogen than that observed for bead beating or enzymatic lysis. DNA extracted by the hot detergent SDS-based enzymatic lysis method resulted in shearing of DNA (lanes 8 and 9). However, for environmental samples neither of these methods could generate

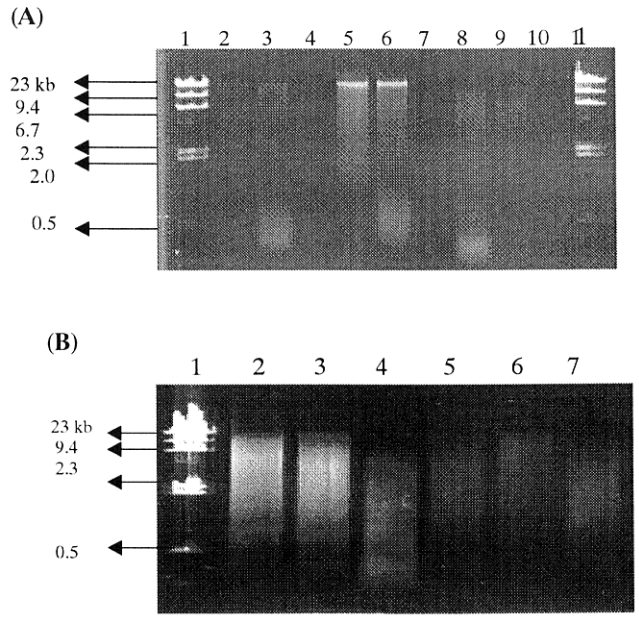

Fig. 1. Comparison of total DNA fragment size and yield obtained from pure culture and model soil system (A) and environmental samples (B) by agarose gel (0.8%) electrophoresis.

(A) Lanes: 1 and 11, λDNA size marker; 4,7 and 10, sterile unseeded soil extracted by bead beating, liquid nitrogen and hot detergent SDS based enzymatic lysis methods, respectively; 2, 5 and 8, extraction of bacterial DNA from pure culture by bead beating, liquid nitrogen and hot detergent SDS based enzymatic lysis methods, respectively; 3, 6 and 9, extraction of bacterial DNA from sterile seeded soil by bead beating, liquid nitrogen and hot detergent SDS based enzymatic lysis methods, respectively. (B) Lanes: 1, λDNA size marker; 2, 3 and 4, total soil DNA extracted from woodland samples by bead beating, liquid nitrogen and enzymatic lysis methods, respectively; 5, 6 and 7, total soil DNA extracted from grassland sample by bead beating, liquid nitrogen and enzymatic lysis methods, respectively.

high molecular weight DNA, suggesting that the lysis pattern of indigenous bacteria is different from that of seeded bacteria. The better part of the lysed DNA might be the DNA liberated from easily lysed microorganisms which have undergone ballistic degradation following bead beating using a mini bead beater or homogenization using a mortar and pestle. The band intensity was higher for the woodland (lanes 2 to 4 in Fig. 1B) than the grassland sample (lanes 5 to 7) irrespective of the method followed for the extraction of DNA.

Standard curve and the amplification efficiency

The standard curve (Fig. 2A) generated using standard DNA revealed a strong linear relationship

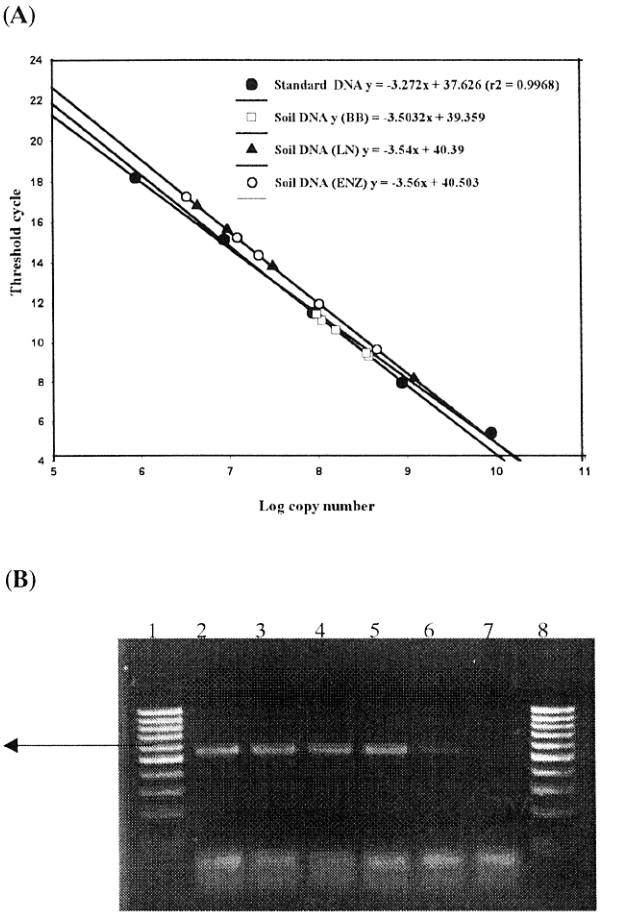

Fig. 2. (A) LightCycler™ based standard curves for several dilutions of *B. cereus* (8.8×10^5 to 8.8×10^9 copies/μl) and diluted soil DNA extracted by different methods. (B) Agarose gel electrophoresis of LightCycler™ amplified standards (*B. cereus*) showing a single, specific band at 538 bp. Lanes: 1 and 8, 100 bp ladder; 2, 8.8×10^9 copies/μl; 3, 8.8×10^8 copies/μl; 4, 8.8×10^7 copies/μl; 5, 8.8×10^6 copies/μl; 6, 8.8×10^5 copies/μl; 7, negative control (PCR grade water). Arrow indicates the 538 bp amplification product.

(r^2=0.9968) between the log of the starting copy number and the threshold cycle and a higher amplification efficiency of 1.02 (calculated from the slope [−3.272] of the standard curve using the formula described in MATERIALS AND METHODS) was obtained. However, when the soil DNA extracted by bead beating, liquid nitrogen and enzymatic lysis was used to construct standard curves, the slopes of these curves were shifted to −3.5032, −3.54 and −3.56, respectively (Fig. 2A). As a result, the amplification efficiency also changed to 0.93, 0.92 and 0.91 for soil DNA extracted by bead beating, liquid nitrogen and enzymatic lysis methods, respectively. The shifting of slope as well as the change of amplification efficiency can be attributed to

interference of coexisting substances such as humic acid.

After the amplification, agarose gel (2%) electrophoresis was carried out to confirm the presence of an amplicon (Fig. 2B). A single, specific band at 538 bp was clearly observed for the standards and no band was visible for the negative control (lane 7 in Fig. 2B) except the band for primer-dimers. The T_m of the 538 bp amplicon was 88°C. Non specific primer-dimers showed lower T_m around 78°C (data not shown). Thus melting curve analysis showed discrimination between primer-dimers and 538 bp specific PCR product.

Precision and reliability of the LightCycler™ technique to quantify bacterial target DNA copy number

There was a significant agreement between the input and output values for standard bacterial DNA copy number suggesting high precision as well as reliability of the LightCycler™ (Fig. 3). The coefficients of variation (8% for 8.8×10^9 copies, 14% for 8.8×10^8 copies, 6% for 8.8×10^7 copies, 0.07% for 8.8×10^6 copies and 12% for 8.8×10^5 copies) were also low.

Quantification of extracted bacterial DNA based on LightCycler™ (DNA copy number)

Mean values of the target DNA copy numbers for pure bacterial DNA extracted by three different methods were within a range of 10^9 copies/µl, indicating that all three of these methods are more or less equally effective for extracting DNA from pure bacterial culture (Fig. 4). However, the samples of model soil (sterilized soil with bacteria) showed vast differences in target DNA copy numbers among different methods, especially for hot detergent SDS-based enzymatic lysis method. The target DNA copy number of seeded bacterial DNA in sterilized soil was found to be 1.08×10^9, 1.32×10^9 and 4.70×10^7 copies/µl for bead beating, liquid nitrogen and enzymatic lysis method, respectively. Simultaneous involvement of mechanical and enzymatic lysis in the hot detergent SDS-based enzymatic lysis method might release nuclease during the earlier steps or might reinforce the interaction between DNA and soil particles. Similar trends were also observed when soil DNA was extracted from both woodland and grassland samples using the hot detergent SDS-based enzymatic lysis method. For the woodland sample, both the bead beating and the liquid nitrogen method showed comparable values of target DNA copy number and these values were found to be

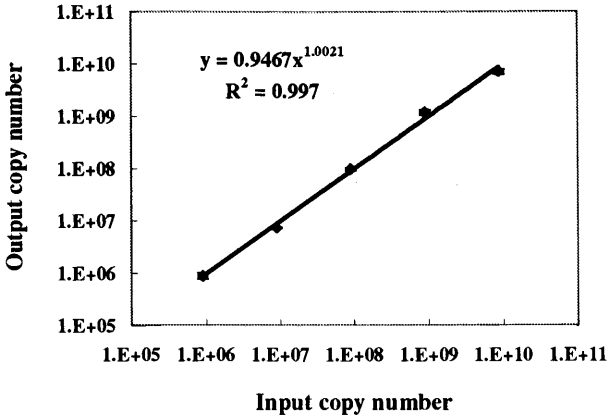

Fig. 3. Reliability of quantification of *B. cereus* DNA.
Known copy numbers of template molecules (based on A_{260}) were amplified and quantified in LightCycler™.

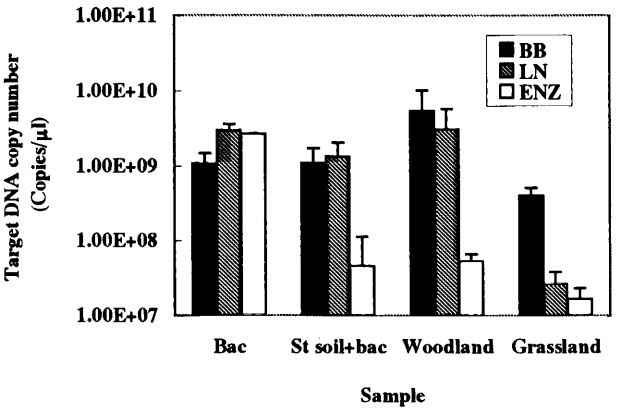

Fig. 4. Quantitative estimation of target DNA copy number in DNA extracted by bead beating (BB), liquid nitrogen (LN) and enzymatic lysis methods (ENZ).

higher than the values obtained for the model soil system. This reveals that the amount of biomass added to the sterilized soil was not high but the amount is comparable to in situ DNA. In the case of the grassland sample, bead beating was the only method that provided the highest DNA copy number (4.01×10^8 copies/µl) (Fig. 4). Results of the agarose gel electrophoresis confirmed the amplification of target part in samples as shown in Fig. 5, A and B. There was no amplification when the fungal DNA was used as a template, suggesting that there was no contribution of fungal DNA in the overall quantification of bacterial DNA from the environmental samples (lane 9 in Fig. 5B). The negative control also remained negative (lane 10 in Fig. 5B).

Recovery of added bacterial biomass

The recovery efficiency was calculated from the tar-

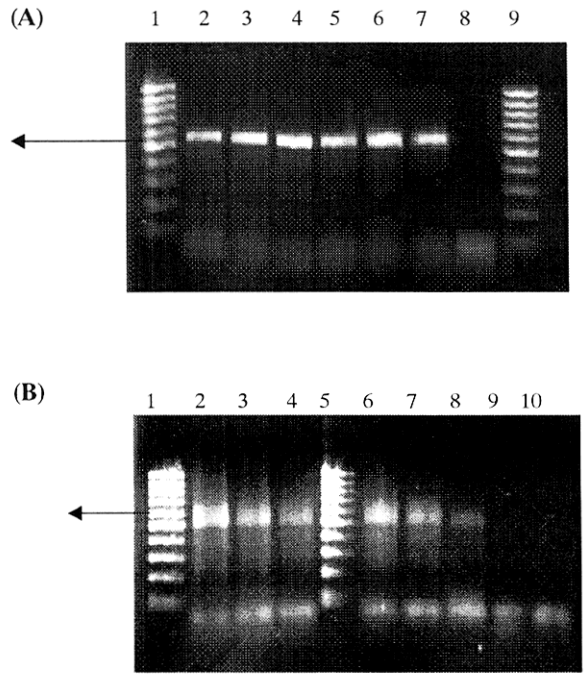

Fig. 5. Agarose gel electrophoresis (2%) of the amplified products obtained from LightCycler™ for pure culture of *B. cereus* and sterilized seeded soil DNA (A) and soil DNA (B).

(A) Lanes: 1 and 9, 100 bp size marker; 2, 4 and 6, amplified products of *B. cereus* DNA extracted by bead beating, liquid nitrogen and hot detergent SDS based enzymatic lysis methods respectively; 3, 5 and 7, amplification products of sterile seeded soil DNA extracted by bead beating, liquid nitrogen and hot detergent SDS-based enzymatic lysis methods respectively; 8, negative control (PCR grade water). Arrow indicates 538 bp amplification product. (B) Lanes: 1 and 5, 100 bp size marker; 2, 3 and 4, amplified products of soil DNA extracted from woodland soil by bead beating, liquid nitrogen and enzymatic lysis method respectively; 6, 7 and 8, amplified products of soil DNA extracted from grassland soil by bead beating, liquid nitrogen and enzymatic lysis methods, respectively; 9, fungal DNA using bacterial primers; 10, negative control (PCR grade water). Arrow indicates target DNA amplification product.

get DNA copy numbers obtained from the sterilized seeded soil and from the pure bacterial culture samples. The DNA recovery efficiencies on the basis of DNA copy number for bead beating, liquid nitrogen and enzymatic lysis methods were 119.2%, 44.4%, and 1.8%, respectively (Table 1). One possible explanation of extraction efficiencies slightly greater than 100% for the bead beating method might be the presence of extracellular DNA as the sterilized uninoculated soil sample extracted by the bead beating method showed a faint band near 538 bp (lane 2 in Fig. 6), suggesting a positive amplification. However, other sterilized uninoculated soil remained negative (lanes 3 and 4 in Fig. 6).

DNA yield. The DNA yields (0.42 to 6.4 μg/g) showed significant variations between the environmental soil samples and also among the extraction methods used (Table 1). For both the woodland and the grassland samples, bead beating method yielded high DNA that was significantly higher than those obtained with the liquid nitrogen and enzymatic lysis methods,

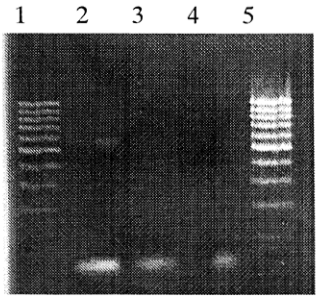

Fig. 6. Agarose gel electrophoresis of LightCycler™ amplified sterilized unseeded soil extracted by different methods.

Lanes: 1 and 5, 100 bp size marker; 2, 3 and 4, sterile unseeded soil extracted by bead beating, liquid nitrogen and enzymatic lysis methods, respectively.

Table 1. Recovery of DNA by three extraction protocols and DNA yield from the environmental samples.

Method	DNA recovery[a] (%)	DNA yield (μg/g of soil)	
		Woodland	Grassland
Bead beating	119.2	6.4±0.83	1.76±0.11
Liquid nitrogen	44.4	4.6±0.25	0.48±0.08
Enzymatic lysis	1.8	1.4±0.12	0.42±0.04

[a] DNA recovery was calculated from the target DNA copy numbers obtained from the sterilized seeded soil and from the pure bacterial cultures.

indicating an overall high extraction efficiency for the bead beating method. Nevertheless, sample variability and the possible contribution of extracellular bacterial DNA and eucaryotic DNA may also be factors. However, the high range of the copy number of target bacterial DNA for bead beating method revealed a significant contribution of soil bacterial DNA in DNA yield.

Comparison on the basis of soil diversity. To know whether these three extraction procedures have any effect on bacterial diversity analysis, amplification of extracted soil DNA was done in a conventional PCR with the same primer pairs as described previously and then subjected to high resolution gel electrophoresis. Overall the length polymorphism pattern (LPM) of amplified 16S rDNA genes indicated that amplified 16S rDNA genes were quite similar whatever extraction method was used or soil samples tested (Fig. 7, A and B). However, some differences were observed between the woodland and the grassland samples with different extraction methods. For woodland soil, all the methods showed bands around 540, 533, 524, 504 and 478 bp (Fig. 7A). A band just above 478 bp was observed in the case of bead beating (lane 2) and liquid nitrogen (lane 3) with a slightly higher intensity in the case of bead beating (Fig. 7A). For grassland soil, bead beating showed a strong band at 540 bp (lane 2 in Fig. 7B) while it was not possible to distinguish this band in DNA samples extracted by the two other extraction methods. Such variation in the brightness of the bands obtained for the grassland soil using different DNA extraction methods suggests a preferential DNA recovery or preferential DNA amplification for the bead beating method.

To develop an efficient protocol for the extraction of bacterial DNA from environmental samples, sterilized soil seeded with *B. cereus* cells was used as a model soil system in this study. This study was then carried out with two different environmental samples to evaluate the suitability of the method that provided high recovery of DNA for the model soil system. Our study il-

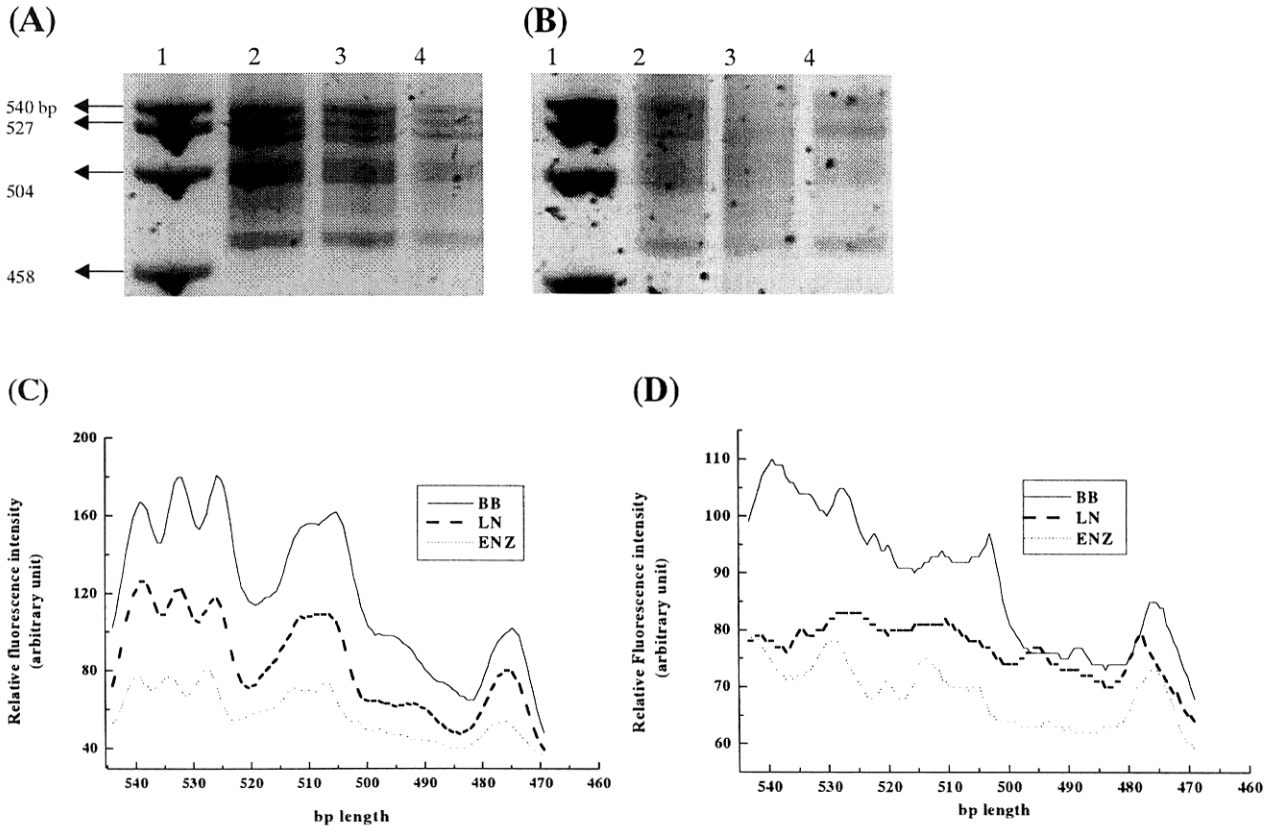

Fig. 7. Comparison of the different direct DNA extraction methods from woodland (A) and grassland (B) samples by means of high resolution gel electrophoresis.

Lanes: 1, M3 markers; 2, 3 and 4, soil DNA extracted by bead beating (BB), liquid nitrogen (LN) and enzymatic lysis (ENZ) methods, respectively. Densitometric analyses of soil DNA extracted from woodland (C) and grassland (D) samples respectively.

lustrated the application of RTQ-PCR and the LPM pattern for the comparative study of different extraction methods. Although in the case of the model soil system, the liquid nitrogen method showed a sharp, distinct band on agarose gel electrophoresis and comparatively high copy number, however, both for the woodland and the grassland samples, it was observed that the bead beating method extracted DNA with a higher extraction efficiency, high copy number and different band patterns on high resolution gel electrophoresis. Our results suggest that the interaction of seeded DNA with the model soil system is different than the interaction of indigenous DNA to soil particles. On the basis of the high values obtained by LightCycler™, it can be suggested that the bead beating method may be suitable for routine analysis. It is more attractive because it is a faster method and a large number of samples can be handled within a short period of time. In addition, it requires neither lysis enzymes (proteinase-K or lysozyme) nor organic solvent (phenol/chloroform) for protein precipitation; rather, it involves EDTA and potassium acetate. It is important to recognize that no single method of cell lysis will be appropriate for all soils and experimental goals. However, on the basis of RTQ-PCR results and LPM patterns in the present study, the bead beating method in the present study is expected to be appropriate for different types of environmental samples having a wide variety of microbial biomass.

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