Identification of Bacillus Strains as Contaminants in Soybean Milk by RAPD (Random Amplified Polymorphic DNA) Analysis and Southern Blotting with 23S rRNA-Targeted Oligonucleotide Probes (Ribotyping)

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Even if a sporeformer is subjected to heat processing, in some cases it can survive in food. We focused on Bacillus, a sporeformer. We gathered 22 kinds of marketed soybeans, mixed them in equal amounts, heated them at 90°C for 5 min and made soybean milk for experiments in our laboratory. We isolated Bacillus cereus and Bacillus licheniformis from the milk and tried to identify the source of contamination. These bacteria were also isolated from 22 kinds of soybeans. Total DNA were extracted from the isolates and compared by RAPD analysis (Random Amplified Polymorphic DNA) and Southern blotting with 23S rRNA-targeted oligonucleotide probes, and Riboprinter® (Qualicon). It was supposed that the B. licheniformis isolated from the product was identical to the B. licheniformis from one of the raw materials. Moreover, as the B. cereus of the product differed from the B. cereus present in the original soybeans at the genomic level, it appeared that the contaminants were derived from our laboratory environment. For B. licheniformis and B. cereus, we constructed a primer that was effective in RAPD analysis. The Ribotyping reported in this paper might be effective for the polymorphic analysis of B. cereus.

Key words: RAPD / 23S rRNA / Ribotyping / Bacillus cereus / Bacillus licheniformis.

INTRODUCTION

B. cereus and B. licheniformis bacteria, which contaminate soybean milk, are aerobic sporeformers. Some of these bacteria cause food poisoning and must be controlled from the point of view of food hygiene. As bacteria which cause food poisoning in the Bacillus genus, B. cereus, found in soil, is widely distributed including in filthy water and rivers. The bacteria exist mainly as spores in crops and raw food materials such as vegetables and cereal grains. Therefore even heat treatment does not kill them. Moreover, in favorable moisture, temperature, and nutrition conditions, they begin to multiply and spoil food. B. cereus food poisoning includes "the diarrheal type" and "the emetic type". The former is seen all over the world, especially in eastern and northern Europe. The latter mostly occurs in the United Kingdom. In most cases the source of the poisoning is starchy foods, boiled rice, fried rice, and pasta. In Japan, most cases of food poisoning are of "the emetic type". B. licheniformis was recently reported to cause food poisoning more often than any other member of the Bacillus genus and is the species to which most attention should be paid in the future.

Gene analysis such as by PCR is becoming more common in bacterial inspections. It is now possible to rapidly identify pathogenic microorganisms such as Salmonella (Ashok et al., 1994 and Jorge et al.,

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and *Escherichia coli* O157 (Ito et al., 1990; Jackson et al., 1987; Takao et al., 1988 and Weinstein et al., 1988), and study the source of pathogenic microorganisms such as the dimethoxyphenyl penicillin-tolerant *Staphylococcus aureus* (De Buyser et al., 1989; Kreiswirth et al., 1993; Musser and Kapur, 1992; Wada et al., 1991 and 1993) which cause nosocomial infections. In Japan, there have been several mass outbreaks of infectious disease caused by O157 and the source of infection was made clear in part by PFGE (the pulse field gel electrophoresis) analysis (Wada et al., 1997) and RAPD analysis (Izumiya et al., 1997; Madico et al., 1995).

Our laboratory is responsible for monitoring the contamination of company products and raw materials. Generally, thermotolerant aerobic sporeformers tend to survive heat treatment. Consequently, there is a need to study the source of these sporeformers. Generally, the identification of a contaminant is not made beyond the species level. However, a comparison at the strain level is required to make the source of the contaminant clear.

The viable cell count in raw material soybeans is generally below 3,000 cfu/g and there is no regulation for spore number. In case of soybean milk where UHT sterilization is done, the viable cell count is generally below 1 cfu/ml and the number of sporeformers is below 1 cfu/ml. It is supposed that food poisoning does not break out at this degree of contamination. However, if the temperature control for soybean milk products is not properly executed, the sporeformers will proliferate and food poisoning or spoiling can occur. On the other hand, with soybean milk manually made at the tofu maker, sometimes the sterilization and production environment conditions are inadequate to suppress microbial growth. In such a case examined before, the degree of contamination was admitted to be as much as 10-100 cfu/ml in viable cell count and as much as 1-10 cfu/ml of sporeformers were also detected.

We would like to identify soybean milk contaminants at the genomic level as well as the species level in order to establish methods for elucidating their source. Therefore, using the RAPD method which has been assessed as superior to general DNA fingerprinting techniques (Akopyanz et al., 1992; Brousseau et al., 1993; Makino et al., 1994; Torriani et al., 1999; Welsh and McClelland, 1990; Williams et al., 1990) and Southern blotting (De Buyser et al., 1989 and 1992; Mangin et al., 1994; Marilena et al., 1992; Pot et al., 1993 and Tatzel et al., 1994) targeting the 23S rRNA gene (Ludwig et al., 1992) (The Ribotyping method), we attempted to identify the source of contamination. For RAPD, we referred to Izumiya et al. (1997) on screening with a primer for *E. coli* O157: H7. For Southern blotting which targeted the 23S rRNA gene, the report of Marilena et al. (1992) was used as a reference. Even if the targeted species changes, we believe that the approach reported in this paper will be effective in identifying the source of contamination.

**MATERIALS AND METHODS**

**Preparation of isolates**

Contaminants were isolated from test products (only sporeformers survived and the spore number assay was 1-2 cfu/ml) as follows. A sample from the test product (25 g) was extracted and the extract was incubated at 35°C for 24 h in 225 ml of nutrient broth. The culture was serially diluted and cultivated at 35°C for 24 h in nutrient agar. As all the colonies on the agar plates formed a simple shape, one typical colony was selected and cultivated on nutrient agar (Eikenkagaku) and NGKG agar (Nissui) at 35°C for 24 h (on NGKG agar, only one isolate gave positive reaction for prolonged cultivation time but lecithinase was not produced). After a colony on NGKG agar was checked on nutrient agar (as for the one isolate on nutrient agar) was confirmed to have spores by microscopic examination, it was once again cultivated on nutrient agar. In total, 4 isolates were prepared. Those that formed a colony on NGKG agar were named Inc1, Inc2 and Inc3, respectively, while the isolate which formed a colony very late and did not produce lecithinase on NGKG agar was named Inc4.

As shown below, the aerobic sporeformer was selectively separated from 22 kinds of soybean (viable cell count of soybeans, 100-200 cfu/g; spore number, 10-30 cfu/g; colonies of sporeformers were mainly fringe-like in these soybeans).

The soybeans (25 g) were suspended in 225 ml of saline after being smashed and heat processed at 80°C for 5 min. The suspension was then incubated in nutrient broth. A serial dilution of the culture was performed and the diluents were cultivated in nutrient agar. The colony showed a simple shape for each kind of soybean. A typical colony was picked up and cultivated on nutrient agar (Eikenkagaku) and NGKG agar (Nissui) at 35°C for 24 h. After the colony on NGKG agar was confirmed to form spores by the microscopic examination, it was once again cultivated on the nutrient agar. Twenty-two test isolates were prepared and labeled M1 to M22. On NGKG agar, in two of those isolates, the rate of colony formation was very slow and no lecithinase was produced for 48 h. For these two isolates, spores were detected on nutrient agar. They were labeled M16 and M22.
Identification of the species of isolates

For the identification at the species level of the contaminants in the test products and of bacteria from the raw material, Bergey's Manual of Systematic Bacteriology volume 2 (Sneath et al., 1986), Standard Methods of Analysis in Food Safety Regulation (the Ministry of Health and Welfare supervision (Japan), 1990), and the report of Azuma (1962) were used as references.

The identification of contaminants in the test product and raw materials was achieved as follows. First, a classification was made based on the width, whether it was above or below 1 μm, by microscopic examination. Isolates of the former group were identified based on the form of colonies on nutrient agar, the spore form, the swelling and the position (Sit), the ability to produce lecithinase (Leci), hemolysis (sheep corpuscle), and the existence of crystal toxin or not (by method staining crystal toxin). The isolates of the latter group were identified by colony shape on nutrient agar, the ability to use citrate (Cit), the deoxidation of nitrate (Nit), starch hydrolysis (Sta), the ability to generate acetyl methyl carbinol (V-P), the hydrolysis of gelatin (Gel), the generation of lecithinase (Leci), the form, the swelling (Swell) and the position (Sit) of spores, the anaerobic growth in a glucose broth (Anae. -gl), the generation of gas about the glucose semisolid medium (Gas), the maximum temperature of the growth (Temp. Max), the ability to utilize sugars (Glucose, Sucrose, Mannitol, Arabinose, Xylose and Lactose), the pH of the Voges-Proskauer (VP) preparation, Casein, NaCl tolerance (5%, 7% and 10%), Indol, and a microplate 96-well identification kit for bacterial metabolites (BioLOG).

Preparation of total DNA of isolates

After each isolate was cultivated on standard agar, microscopic examinations were done regarding the degree to which they had proliferated on the agar. Using GenomicPrep™ Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech), total DNA was extracted from the isolate (the vegetative cells) after confirmation that no spores had formed. It was confirmed that the extract of DNA had not undergone physical or chemical cleavage by the agarose gel electrophoresis and UV irradiation after the ethidium bromide staining. The concentration of Total DNA and the degree of contamination of protein (OD260/OD280) were evaluated with UV measurement receptacle GeneQuant™ II (Amersham pharmacia biotech) for DNA densitometry.

Preparation of RAPD reaction solution, primers and thermal profile (basic profile)

In Ready-To-Go RAPD Analysis Beads (Amersham Pharmacia Biotech), each primer (25 pmol) and total DNA (10 ng) were poured, and the volume was adjusted to 25 μl by adding sterile extra pure water. The primers used this time were AP1 (5'-GGTCTGGGAA-3'), AP2 (5'-GTTGCTGGCTCC-3'), AP3 (5'-GTAGACCGCT-3'), AP4 (5'-AAGAGCCCGCT-3'), AP5 (5'-AACGCGCAAC-3') and AP6 (5'-CCCGTCAGCA-3'). A GeneAmp PCR System 9700 (Perkin-Elmer) was used as a thermalcycler. The cycle profile was 1 cycle of 95°C for 5 min, 45 cycles of 95°C for 1 min, 36°C for 1 min and 72°C for 2 min, 1 cycle of 72°C for 7 min, and then continuously at 4°C.

Polyacrylamide gel electrophoresis and the silver staining method (the RAPD product), and agarose gel electrophoresis and ethidium bromide staining method (the RAPD product)

GeneGel Excel 12.5/24 Kit (Amersham Pharmacia Biotech) was used as the polyacrylamide gel and the electrophoresis buffer. GenePhor and Electrophoresis Power Supply EPS 600 (Amersham Pharmacia Biotech) were used as the apparatus for electrophoresis. The conditions for the electrophoresis were 600 V, 25 mA, 15 W and 80 min. Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech) was used for DNA silver staining (hereafter referred to as the silver staining method).

A 2% Seakem DTG agarose gel (w/v), 0.5 × TBE Buffer, and Mupid (Aavance) were used as the gel, electrophoresis buffer, the electrophoresis apparatus and the Power-Supply, respectively. Electrophoresis was performed at 100 V. The agarose gel was stained with the ethidium bromide and after UV irradiation, a Polaroid photograph was taken (hereafter, we refer to this detection system as the ethidium bromide staining method).

Comparison of the isolate derived from the test product with the isolate derived from the raw material by RAPD

Comparison of Inc4 with M22 was made with using three primers (AP1, AP3 and AP4). First, Inc4 and M22 DNA were analyzed by RAPD with AP1 and AP4. The basic profile of thermal cycling was adopted. The analysis was performed in duplicate. That is, DNA was independently extracted twice, and 10 ng of the extract was used for the RAPD analysis. The amount of RAPD product applied to the well was 1 μl (including 6 × loading Dye). Silver stain was for detection. A similar experiment was performed with AP3.

Moreover, comparison of Inc4 with M22 was made by using two primers (AP5 and AP6). To restrain
nonspecific reactions involving AP5 and AP6 at 36°C (annealing temp.), and to improve the repeatability of the RAPD analysis, we conducted the following experiment. Primers AP5 and AP6 were used for Inc4 or M22 DNA, with the annealing temperature changed to 45°C and 55°C (Izumiya et al., 1997), but all other conditions were the same as for the basic profile. The specificity and repeatability of the analysis were examined under these conditions. The examination was implemented four times for each DNA sample. The contents were as follows. The DNA extraction was done twice and the RAPD analysis of each extract was performed twice. The amount of the RAPD product applied to each well was 0.6 μl (the amount recommended by the manufacturer). Silver stain was used for detection.

For the RAPD analysis at 55°C (Izumiya et al., 1997), ethidium bromide stain was also used to stain 2%(w/v) agarose gels. The amount of the RAPD product applied to each well was 6 μl (the amount recommended by the manufacturer).

Next, comparison of isolates derived from test products that were identified as B. cereus (Inc1, Inc2 and Inc3) with isolates from the raw material (M1 to M15 and M17 to M21) by the RAPD method was made as follows. First of all, the screening of a primer effective for comparison of Inc1, Inc2 and Inc3 with M1 was done. M1, Inc 1, Inc2 and Inc3 were analyzed by RAPD analysis with AP1, AP2, AP3, AP4, AP5 and AP6. The annealing temperature of the thermal cycle profile was set at 55°C (Izumiya et al., 1997) but all other conditions were as they were for the basic profile. The examination was performed once for each DNA sample. The amount of RAPD product applied to each well of agarose gel was 6 μl. Ethidium bromide was used for staining.

The comparison of Inc1, Inc2 and Inc3 with M1-M15 and M17-M21 by RAPD analysis with AP2 was made. Inc1, Inc2, Inc3 and M1-M21 (containing M16) were analyzed by RAPD analysis with AP2, using the same thermal cycle profile employed for the screening. The analysis was performed in duplicate. That is, DNA was extracted twice from the same isolate, and then each DNA extract (10ng) was subject to RAPD analysis.

**Comparison of Inc4 (B. licheniformis from the test product) with M22 (B. licheniformis from soybeans) by Southern blotting targeting the 23S rRNA gene**

Design of a DNA probe that targets the 23S rRNA gene was made. *Escherichia coli* strain JCM1649 was cultivated on standard agar plates. An amount corresponding to 1 platinum loop was extracted from the colony and suspended in 1ml of TE buffer. A 10-fold dilution of the suspension was made with TE Buffer and boiled at 95°C for 5 min. The boiled diluent was centrifuged at 12000 ×g and 4°C for 10 min (TOMY MRX-150) and 2.5 μl of the supernatant was then used as template DNA. In Ready-To-Go RAPD Analysis Beads (Amersham pharmacia biotech), Primers, 23S rRNAF (5'-CAGTCAGAGGCGATGAAGGACGTGC-3') (25 pmol) and 23 S rRNA primer (5'-CCGGTTAGCTCAACCCATCGCTGCG-3') 25 pmol and the above template DNA, 2.5 μl of the supernatant, were poured. A volume of 25 μl was made by adding sterile extra pure water. The PCR profile was 1 cycle of 95°C for 5 min., 30 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 90 s, and 4 °C continuously. PCR products (10 μl) were electrophoresed on 0.8% seakem GTG agarose gels, and the target product, selected by taking the 37th cytosine (C) from the 5' end of the *E. coli* JCM1649 23S rRNA as the amplification starting point and the 28th guanine (G) from the 3' end as the end point, was separated. The target PCR product was cut out from the agarose gel and refined with a DNA Gel Band and Purification Kit (Amersham pharmacia biotech). It was examined by an ALF expressDNA auto sequencer (Amersham pharmacia biotech) and by evaluating the size of DNA fragments after digestion with the restriction enzymes, *EcoR* I, *BssH* II, *Sac* I and *Nco* I as to whether the PCR amplification product had the base sequence (Brosius et al., 1981) of the *E. coli* 23S rRNA gene or not. The purified PCR amplification product was labeled with AlkPhos Direct Genelimages system Kit (Amersham Pharmacia Biotech) according to the manufacturer’s recommendation.

**DNA digestion with the restriction endonuclease and the separation of the DNA fragments**

Inc4, M22 or M14-M20 (5-10 μg) in the buffer recommended by the manufacturer (Takara) was digested by *Hind* III and *EcoR* I (40-80 units / μg DNA) overnight. These were refined by ethanol precipitate after PCI processing and dissolved in 10 μl of TE buffer. They were then electrophoresed on 0.8% Seakem GTG agarose gel (W/V) for 2 h at 50V (using 1× TBE buffer). λ-*EcoT*14 I digest (Takara) or Lambda-pUC Mix Marker, 4 (MBI Fermentas) was used as a size marker. The gel was stained with ethidium bromide. The result was visualized with an UV-Trans illuminator at 254-nm of UV light and recorded on Polaroid type 57 film.

**Conditions for Southern blotting targeting the 23S rRNA gene**

Design of a DNA probe that targets the 23S rRNA gene was made. *Escherichia coli* strain JCM1649 was cultivated on standard agar plates. An amount corresponding to 1 platinum loop was extracted from the
DNA fragments, after having been electrophoresed, were denatured and transferred to Hybond-N+ membranes (Amersham Pharmacia Biotech). The capillary transfer method was used. Prehybridization, hybridization, the first wash and a second wash were done at 55°C and in part at 42°C (Marilena et al., 1992) according to the manual of the AlkPhos Direct Genelimages system (Amersham Pharmacia Biotech).

Autoradiograms on Hyper film ECL (Amersham Pharmacia Biotech) were exposed with intensifying screens at room temperature.

Identification by the RiboPrinter(R) Microbial Characterization System (EcoR I) (Qualicon)

Using colonies of the isolate derived from the test product which was identified as *B. licheniformis* (Inc4), the isolate derived from the raw material which was identified as *B. licheniformis* (M22), and isolates derived from the raw material which were identified as *B. cereus* (M12 and M15), Ribotyping was performed with the RiboPrinter(R) Microbial Characterization System (Qualicon).

### RESULTS

#### Identification of the species of tested isolates

All results of examinations on tested isolates are shown in Tables 1 and 2. Inc1-Inc3, M1-M15 and M17-21 were identified in the following ways. It was suggested that they could be *B. cereus* because all colonies formed on nutrient agar were fringed and had a bacterial width of more than 1 μm. To identify them, the following point was also considered (Azuma, 1962; Ministry of Health and Welfare Environmental Health Bureau supervision, 1990; Sneath et al., 1986). Their identity was narrowed down to 2 species: *B. cereus* or *B. thuringiensis*, from the colony form, bacterial width, the form of the spores, Swell and Sit, positivity in the Lecithinase test and negativity in the hemolysis (the sheep corpuscle) reaction. However, as no crystal toxin was found by staining (Ministry of Health and Welfare Environmental Health Bureau supervision, 1990) in the cytoplasm, the isolates were identified as *B. cereus*. The results are shown in Table 1. Inc4, M16 and M22 were identified in the following manner.

For Inc4 and M22, the colony form on nutrient agar

<p>| TABLE 1. Biochemical characteristics of Inc4 and M22 identified as <em>B. licheniformis</em>. |
|---------------------------------|---------------|---------------|</p>
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Inc4</th>
<th>M22</th>
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<tbody>
<tr>
<td>Utilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Temp. (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>55</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth and metabolite</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaerobic-glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gas</td>
<td>Weakly positive</td>
<td>Weakly positive</td>
</tr>
<tr>
<td>Generation of acid</td>
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<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pH of Voges-Proskauer</td>
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<tr>
<td>Casein</td>
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<td>NaCl tolerance (%):</td>
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</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
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<td>10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>Size (width) (μm)</td>
<td>Colony form on nutrient agar</td>
</tr>
<tr>
<td>--------</td>
<td>------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Inc1</td>
<td>&gt;1</td>
<td>Fringe</td>
</tr>
<tr>
<td>Inc2</td>
<td>&gt;1</td>
<td>Fringe</td>
</tr>
<tr>
<td>Inc3</td>
<td>&gt;1</td>
<td>Fringe</td>
</tr>
<tr>
<td>Inc4</td>
<td>0.8</td>
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<tr>
<td>M14</td>
<td>&gt;1</td>
<td>Fringe</td>
</tr>
<tr>
<td>M15</td>
<td>&gt;1</td>
<td>Fringe</td>
</tr>
<tr>
<td>M16</td>
<td>0.8</td>
<td>Likely fringe</td>
</tr>
<tr>
<td>M17</td>
<td>&gt;1</td>
<td>Fringe</td>
</tr>
<tr>
<td>M18</td>
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<td>Fringe</td>
</tr>
<tr>
<td>M19</td>
<td>&gt;1</td>
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</tr>
<tr>
<td>M20</td>
<td>&gt;1</td>
<td>Fringe</td>
</tr>
<tr>
<td>M21</td>
<td>&gt;1</td>
<td>Fringe</td>
</tr>
<tr>
<td>M22</td>
<td>0.8</td>
<td>Hairy root</td>
</tr>
</tbody>
</table>
identification of Bacillus strains

Soybean milk

\[ \text{FIG. 1. Isolates from soybean milk and the isolates from 22 kinds of soybeans.} \]

was protean, the growth was hair-like and cells were strongly attached to the agar. For M16, the colony form on the agar was fringe-like, similar to *B. cereus*, cells did not adhere strongly to the agar, and the growth was not hair-like. The bacterial width was less than 1 \( \mu \text{m} \) in 3 isolates. Identification tests of isolates whose width was less than 1 \( \mu \text{m} \) were carried out with Inc4 and M22 (Azuma, 1962; Ministry of Health and Welfare Environmental Health Bureau supervision, 1990; Sneath et al., 1986). For example, several characteristics of *Bacillus* were examined, namely Cit, Nit, Leci and the utilization of various sugars, and the results are shown in Table 2. Properties of *B. licheniformis* were shown. Moreover, their isolates were identified as *B. licheniformis* by the Microplate 96-well test.

The results are shown below. Also, their relations are shown in Fig. 1.

For the product contaminants, three isolates, Inc1, Inc2 and Inc3, were identified as *B. cereus*. One isolate, Inc4, was identified as *B. licheniformis*.

For soybean contaminants, twenty isolates, M1-M15 and M17-M21, were identified as *B. cereus*. One isolate, M22, was identified as *B. licheniformis*. One isolate, M16, could not be identified.

**Comparison of Inc4 (the *B. licheniformis* from the test product) with M22 (the *B. licheniformis* from the soybeans) by RAPD analysis**

The RAPD patterns obtained with AP1 and AP4 at 36°C (annealing temperature) and 1 \( \mu \text{l} \) of sample are shown in Fig. 2. The analysis was performed from the DNA extraction step in duplicate for each isolate and the repeatability of RAPD patterns was good in each case.

RAPD patterns of Inc4 and M22 were the same. A similar experiment using AP3 was carried out as well and again the repeatability of patterns was good. RAPD patterns of Inc4 and M22 were the same (data not shown). On the other hand, when AP5 and AP6 were used at the general annealing temperature of 36°C, nonspecific reactions occurred, and the repeatability of results was poor (data not shown). Then, it was necessary to improve the specificity as nonspecific reactions occurred at the annealing temperature of 36°C and the repeatability of results was poor (Izumiya et al., 1997). As for the RAPD pattern...
obtained at 45°C and 55°C with 0.6 µl of a sample, an almost identical thick band which has a high amplification efficiency was detected at each temperature, but a nonspecific reaction was seen at 45°C. RAPD patterns at 55°C are shown in Fig. 3.

For each primer, a more repeatable reaction was achieved by setting an annealing temperature higher than 36°C. As the result, the number of thick bands increased, in both the low molecular weight area below 500bp and from the low molecular weight to 1500bp area.

The patterns for the RAPD product at 55°C detected by ethidium bromide were almost equal to the pattern obtained by silver staining in Fig. 3.

Comparison of isolates derived from test products that were identified as B. cereus (Inc1, Inc2 and Inc3) with isolates from soybeans (M1 to M15 and M17 to M21) by the RAPD analysis

RAPD patterns obtained at an annealing temperature of 55°C with AP1, AP2 and AP5 are shown in Fig. 4. There were few differences in the patterns for M1 and Inc with AP1 or AP5 (as mentioned below, M1 was not equal to Inc by the RAPD analysis with AP2) and the number of bands was too high, in spite of the fact that the annealing temperature was high and nonspecific reactions were restrained. Those primers were not suitable for screening the B. cereus strain equaling the Inc1-Inc3 at the genomic level from soybeans.

Also, in the case of AP3, AP4 and AP6, there were few bands specific to each isolate (data not shown) and the primers could be not suitable for the screening at an annealing temperature below 55°C (if the

![FIG.4](image-url)  
**FIG.4.** Screening of primers able to discriminate between B. cereus strains. Three primers, AP1, AP2, and AP5, were used. In the first, second, third, and fourth lanes the primer is M1, Inc1, Inc2, and Inc3, respectively.

![FIG.5](image-url)  
**FIG.5.** Comparison of the RAPD patterns of each isolate. The most effective primer, AP2, was used. M, 100bp DNA ladder. The first and second lanes for each isolate contain profiles for independent DNA extracts.
temperature had been set lower, the primers might have been effective.

On the other hand, when using AP2, although there were a few bands that are supposed to be specific to M1 and Inc, the primer produced a decisive difference between M1 and Inc.

The primer, AP2, could be one of the effective primers for screening the *B. cereus* strain that is equal to Inc at the genomic level from soybeans.

**Comparison of Inc1, Inc2 and Inc3 from test products with M1-M15 and M17-M21 from soybeans using AP2**

RAPD patterns obtained at an annealing temperature of 55°C with AP2 are shown in Fig. 5. A, 5. B, 5. C and 5. D. Patterns of *B. cereus* found in Inc1-Inc3 were not found in the isolates from the soybeans. However, using AP2, there was comparative repeatability for each isolate of Inc1-Inc3 and M1-M21 (M16 was an exception because it was not *B. cereus*) and, moreover, the RAPD patterns differed among isolates.

**Comparison of Inc4 (*B. licheniformis* from the test product) with M22 (*B. licheniformis* from soybeans) by Southern blotting which targeted the 23S rRNA gene**

A PCR fragment supposed to be 2841bp long between 2690bp and 3281bp of the Lambda-pUC Mix Marker 4 (MBI Fermentas) was identified by the 23S rRNAF primer and the 23S rRNAAR primer (data not shown). Its DNA base sequence was the same as the *E. coli* 23S base sequence in the report of Brosius et al. (1981) and GenBank (accession number: V00331). Moreover the amplification product was digested by restriction endonucleases EcoRI, BssHII, SacI and NcoI and the restriction fragments cut in the expected places. It was confirmed that the amplification product was derived from the 23S rRNA gene by these results.

Then, after Hind III digests of Total DNA from Inc4, M22 and M14-M20 were electrophoresed and the gel was stained by ethidium bromide, the result was visualized with a UV-trans illuminator at 254nm (UV light) and was recorded on Polaroid type 57 film. The results are shown in Fig. 6. A. The Southern blots of the gel are shown in Fig. 6. B. Incidentally, all processes from pre-hybridization and hybridization to the first and the second wash were implemented at 42°C and 55°C (Marilena et al., 1992). After the Southern blotting at 42°C (data not shown), the probe was detached from the membrane as recommended by the manufacturer, and Southern blotting was performed again at 55°C. When those Southern blots were compared, no significant differences were seen, but the one obtained at 55°C was clearer. The Southern blots of Inc4 and M22 were the same. They were different from M16, however, and were rather different from the isolates identified as *B. cereus* (M14-M15 and M17-M20).

The Southern blots differed little among the isolates identified as *B. cereus*, but two patterns emerged, namely whether there was a DNA fragment with a mo-

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**FIG.6.** Agarose gel electrophoresis (A) of Hind III-digests of DNA extracted from each isolate shown below and each corresponding Southern blot (B). Lanes: M, Lambda-pUC Mix Marker 4; (A) 1, M14; 2, M15; 3, M17; 4, M18; 5, M19; 6, M20; 7, M16; 8, M22; 9, Inc4; (B) Each number corresponds to that in (A).
FIG. 7. The RiboPrint(R) Pattern(C) of EcoR I of each isolate shown below. (C) 1, M12; 2, M15; 3, M22; 4, Inc4.

Molecular weight of between 7.74 kb and 19.33 kb or not. Four sub-patterns appeared to exist.

Southern blotting of the same DNA was implemented at a hybridization temperature of 55°C with the restriction endonuclease EcoR I. The blots of Inc4 and M22 were the same. Their isolates were different from M16, too, and were clearly different from isolates identified as B. cereus (as overlapping identification results of the RiboPrint(R) Pattern System, data not shown).

Moreover, RiboPrint(R) patterns of the isolate from the test product which was identified as B. licheniformis (Inc4), the isolate from soybeans which was identified as B. licheniformis (M22) and isolates from soybeans which were identified as B. cereus (M12 and M15) are shown in FIG. 7.

RiboPrint(R) patterns of Inc4 and M22 appeared to be the same, but different from those of M12 and M15.

DISCUSSION

We consider all identifications of B. cereus in this paper to meet the standards of the Ministry of Health and Welfare in our country. According to TABLE 1. A and 1. B, the possibility that M16 was the same species as the test product contaminant Inc1-Inc4 was low. Based on the study of the source of pollution, we judged that our goal had been achieved at this level. Therefore, neither an identification nor a genetic examination of M16 was performed.

For AP1, AP3 and AP4, because the repeatability of RAPD analysis for each isolate in spite of a low annealing temperature (36°C) was good, we concluded that all three primers were suitable for Inc4 and M22. It was suggested that Inc4 could be similar to M22 at the genomic level. AP1, AP3 and AP4 could be effective primers in the polymorphic analysis of the B. licheniformis strains.

As for AP5 and AP6, because at an annealing temperature of 36°C the nonspecific reactions broke out with both isolates and the repeatability of the result was poor, an attempt to improve the specificity was made (Izumiya et al., 1997). The annealing temperature was raised to 55°C. In terms of the existence of bands with a high amplification efficiency, Inc4 and M22 showed similar patterns. On the other hand, bands with a low amplification efficiency that do not have repeatability still existed in the genome, but we considered that this meant a limit to the compatibility between the genome and the primer. Therefore, we did not make any more improvements.

Using the five kinds of primers, the RAPD pattern of Inc4 was found to be the same as that of M22, and the two isolates to be similar at the genomic level. We recognized that the repeatability was improved by raising the annealing temperature. Then, in case that there are very few primers which have repeatability even at a general annealing temperature of 36°C, we think that this is an effective technique. Of course, if it is possible to identify the bacteria and RAPD primers showing the difference among strains of the same species are found, we consider it wise to study the source of pollution by this method. For instance, if contaminants and the bacteria from the raw material are identified as B. thuringiensis, we will refer to the report of Brousseau et al. (1993). If we use the primers mentioned in that report, an effective analysis of the source of pollution can be accomplished.

We will explain why the RAPD analysis was implemented two or four times per isolate. Because non-specific amplification was expected and the repeatability of the results was poor (AP5 and AP6 are equivalent to this case in this examination), the analysis was done four times per isolate at most. Generally speaking, because repeatability was an issue with the RAPD analysis, the analysis should be implemented repeatedly at least. Almost no variance in the RAPD patterns must be grasped. After achieving this, we should finally compare the genomes in terms of the existence or non-existence of bands that have repeatability with high amplification efficiency.

Although we compared the isolates derived from the test product which were identified as B. cereus (Inc1-Inc3) with the isolates from the raw material (M1-M15 and M17-M21) using AP2 that had been suggested to be effective in the screening stage, at the genomic level, no strains that were equal to isolates derived from the test product, Inc1-Inc3, were found among isolates from raw soybeans. However, it is expected that there has been contamination by B. cereus in the past and that the necessity to compare B. cereus strains at the genomic level will increase as
sources of contamination are studied in the future. Generally, for the *B. cereus* species, for which it is difficult to identify polymorphism by Ribotyping, we think that AP2 (with 55°C as the annealing temperature) is one of effective RAPD primers.

Incidentally, M16 was found to be different from *B. cereus* by regular bacterial inspection.

As the 23S rRNA probe, we chose the PCR fragment of 2841bp by making the 37th cytosine (C) from the 5' end of the 23S rRNA gene the starting point and the 28th guanine (G) from the 3' end of the 23S rRNA gene the end point for the following reason. When we analyzed the polymorphic patternings of various species by Southern blotting, we wanted to detect the variation in parts which neighbor the 5' end of the 23S rRNA gene and the 3' side of it. Therefore, we made all parts of the 23S rRNA gene a probe. It is supposed that the restriction enzyme had more than one recognition site in 23 S and part of the 3' side of 23S was made a probe, we considered that variation in the base sequence of the part which neighbors the 5' end could not be detected and that the precision to detect differences among the genomes would decline.

In cases in which we must analyze the genome of a novel species or species whose strict identification is difficult from the study of the source of pollution, we gathered information on the 23S rRNA gene and the spacer region from numerous species. We used the report of Wada et al. (1993) and the report of Brosius et al. (1981) about the rRNA operon of *E. coli* as references. It was reported that six 23S rRNA genes of *S. aureus* strain NCTC8325 exist on the chromosome DNA and that there is considerable difference in the base sequence between the parts which neighbor the 3' end even among those copies of 23S according to the report of Wada et al. (1993). Moreover, it was stated that if *S. aureus* differs at the strain level, it was expected that parts neighboring the 23 S would be different among strains. Also, if the bacteria differ at the species level, it is possible to expect the base sequences of 23S and the neighboring region to be even more different. Therefore, it should be possible to make a mutual comparison among the strains focusing on the region neighboring the 3' end of 23S only. On the other hand, when focusing on 16S rRNA-23S rRNA spacer region among *rmb*, *rmd*, *rnx* and the *rrnE* operon of *E. coli*, Brosius et al. (1981) reported that differences in the base sequence could be seen. Therefore, we considered that there was a possibility that the precision could be generally improved by aiming at the 5'side of 23S.

Also, we made the 23S rRNA gene of the *E. coli* JCMI649 a probe, and we intend to apply this probe to the study of the source of pollution first.

Marilena et al. (1992) cloned the 16S *rrnB* rRNA operon of *E. coli* for genome analysis of *B. sphaericus*, and used it as a probe for Southern blotting analysis. Therefore, we cloned the 23S rRNA gene of *E. coli*, and performed Southern blotting of isolates which were identified as the *B. cereus* and isolates which were identified as *B. licheniformis* using the cloned 23S rRNA gene as probe. However, because the complementation between 23S of the tested isolates and this probe appeared not to be completely good, the Hind III restriction fragments, touched by Materials and Methods, were tested for hybridization at 42°C at first. Although we also examined hybridization at 55°C and tried to compare hybridization at 42°C with that at 55°C, a significant change was not seen in the Southern blots. Although we failed to identify the source of pollution for isolates which were identified as *B. cereus*, we assume that the frequency of the study of the source of pollution of *B. cereus*, with relation to our company, will increase in future, and this time we will make the hybridization temperature not 42°C but 55°C.

Bourque et al. (1995) reported that there were no significant differences in the intergenic spacer region (ISR) between 16 S and 23 S with *B. cereus*, *B. thuringiensis* and *B. anthracis*. Moreover, it has been said that there are few differences among the strains in the base sequences of the area neighboring the 23S rRNA of the *B. cereus* strains, and the present examination supported this. However, it was suggested that the Southern blot patterns of tested isolates identified as *B. cereus* could be classified into 2 patterns based on the existence of an approximate 19kb band in the blots with Hind III, which would be effective for polymorphic analysis among the *B. cereus* strains and the study of the source of contamination.

The main purpose of the Southern blotting was the genomic analysis of isolates that were identified as *B. licheniformis*, but we should explain why the analysis was implemented with isolates identified as *B. cereus*.

The reason is that the frequency of the source of contamination by *B. cereus* can increase in the case of our laboratory as described above and we would like to evaluate whether Southern blots show polymorphisms of *B. cereus*. Also, we assumed at the worst case that the Southern blots might be the same for most strains which belong to *B. licheniformis*. Indeed the method must be applied to other representative strains of the species, but the application of more than two restriction endonucleases was suitable for the substitution method which improves the results with polymorphism. Therefore, we used two restriction endonucleases.

According to the results of the RAPD analysis and
Southern blots including those of RiboPrint(R) Pattern, we judged that the isolate identified as _B. licheniformis_, Inc4 which contaminated the test product, could be derived from raw material M22. In the first instance, the product contaminants Inc1-Inc3 were estimated to be derived from the laboratory environment, as they differed from the bacteria derived from the raw material at the genomic level. In the second instance, there was a small possibility that in each raw material soybean some kinds of _B. cereus_ strain including product contaminants existed but colonies of contaminants were not picked up for RAPD analysis (as in appearance colonies of _B. cereus_ strains were same).

Prepotency evaluations (Arias et al., 1998; Bjorkroth et al., 1996; Hoi et al., 1997) by RAPD analysis and Ribotyping including by PFGE analysis are frequently used. We used RAPD analysis and Southern blotting which targeted the 23S rRNA gene in order to improve the accuracy of the study of the source of contamination. Future approaches to the study of the sources are described, when contamination of the product occurs. As contamination of a sterile product is likely to cause problems and the studies of contamination by mainly the _Bacillus_ genus, _B. cereus_, will probably increase in our laboratory, the following approach may be used. If we confirm the product contaminant to be a sporeformer, we should identify the species by bacterial inspection (from experience, the contaminant is most likely to be _B. cereus_). Bacteria of the same species (the _B. cereus_) must be separated from the raw materials and the manufacturing environment. The strain supposed to have contaminated the product is screened from the raw materials and the environment by our RAPD method (AP2 and 55°C as the annealing temperature). We would also like to use Southern blotting with strains screened by the RAPD analysis.

For the other species in the _Bacillus_ genus, we would like to examine at least twenty primers including the six primers reported in this paper and try the introduction of the RAPD technique to make the annealing temperature rise step-by-step when there are few repeatable and effective primers. Moreover, we should reevaluate the results of RAPD analysis by Southern blotting.

As far as it is possible, in the Southern blotting we will also use another restriction enzyme.

In the future, for the RAPD analysis, we would like to increase the number of primers, which are effective for polymorphic analysis of _B. cereus_. Concretely, first of all it must be examined whether AP3, AP4 and AP6 can be effective, if the annealing temperature is set lower. Moreover, for AP2, it must be assessed whether the primer may be more effective with lower annealing temperatures.

As for the Southern blotting reported in this paper, we consider it a weak point that there are too many bands and few differences in the Southern blot patterns of the _B. cereus_ strains (Bourque et al., 1995). Therefore, we would like to find more effective restriction enzymes. Moreover, because _B. licheniformis_ has emerged recently as a food poisoning bacteria, we would like to investigate a lot of _B. licheniformis_ strains. For the RAPD analysis, we would like to further evaluate the effectiveness of AP1, AP3 and AP4 examined in this paper and, moreover, we want to search for a more effective primer. As for the Southern blotting, we would like to evaluate how the method examined in this paper is effective in distinguishing between _B. licheniformis_ strains. Under such conditions, we want to identify more effective restriction enzymes (Salkinoja-Salonen et al., 1999).

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