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Potential Application of Ribosomal Intergenic Spacer Analysis to the Microbial Community Analysis of Agronomic Products

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Ribosomal intergenic spacer analysis (RISA) has been applied to the microbial community analysis of agronomic products in combination with a simple and rapid DNA extraction method, consisting of a one-step extraction and two-step purification, for a variety of agronomic products. RISA appears to be a useful tool for the study of the community structures of food-associated microbes and their use as a unique fingerprinting signature for each agronomic product. Sequencing analyses of amplicons generated from RISA suggest that this method can detect conventional microbes. In the case of RISA of wasabi paste DNA, the sequences of the amplicons showed high similarity to the plant pathogen *Xanthomonas campestris* and the soil bacterium *Bacillus subtilis*, whereas several food-associated bacteria (*Lactococcus lactis*, *Lactococcus raffinolactis*, and *Lactococcus sakei*) were detected using this technique in sausage DNA. Unexpectedly, the sequencing analyses also revealed the presence of several microbes that possessed high similarity to human bacterial pathogens such as *Weissella confusa* and *Yersinia pestis*. The results suggest that RISA will be a useful method for routine microbial community analysis in agronomic products.

KEYWORDS: Microbial community analysis; ribosomal intergenic spacer analysis

INTRODUCTION

Molecular biological methods have now been widely applied in food science due to the increasing demands for rapid and precise clinical testing, the possible presence in foodstuffs of genetically modified organisms, quality control testing, forensic analysis, and food traceability. To address these academic and social demands, a series of techniques have now been developed for the extraction and analysis of DNA from various agronomic products (*1*−*8*). Because each agronomic product has quite distinct physical and chemical properties, most of the published DNA extraction methods for use with foodstuffs were developed for specific materials. This has led to increases in the both the costs and the time required to set up and perform DNA extractions when systematic examinations of food samples are required. Recently, several commercial kits have been developed for the rapid extraction of food DNAs, but these kits often require the use of toxic reagents, and the use of these kits has also considerably increased the running costs of routinely obtaining statistically meaningful data sets.

Polymerase Chain Reaction (PCR) based methods have also been widely applied in combination with DNA extraction protocols for a variety of analytical purposes, such as the detection of specific pathogens in foods and the protection of the variety classifications of agronomic products (*9*−*15*). Among these methods, the use of ribosomal RNA (rRNA) gene sequences is becoming one of the chief strategies for characterizing the biological content of agronomic products by taking advantage of the increasing amount of available DNA sequence information for a wide variety of organisms (*12*, *16*−*19*). The usefulness of rRNA gene sequences for the examination of biological diversity has been recognized in several fields of biology, including food science. The high levels of conservation, and thus the only moderate variability among rRNA gene sequences, have enabled species-specific design of PCR primers. However, most of the reports of the use of PCR methodologies in food science have focused on predicted target organisms, and little effort has been made to develop methods to properly analyze the DNA content of foodstuffs, including nonculturable microbes and contaminations by capricious organisms. Such methods would be useful for microbial community analysis, clinical studies, forensic examinations, and food traceability.

We and other soil microbiologists have been working on several methodologies to properly analyze microbial diversity in soil, including the extraction of DNA from the soil matrix and the visualization of the diversity of microbial community structure in soils. Recently we have newly developed a DNA extraction method for soil (DSPD method) (*20*) and proposed an efficient experimental system for DNA fingerprinting methods such as terminal restriction fragment length polymorphism (T-RFLP) and ribosomal intergenic spacer analysis (RISA) (*21*). From these experiments, we have found that the DSPD method is very reliable for extracting DNA from diverse biological materials. We have also determined that RISA is an efficient and robust DNA fingerprinting method for evaluating biological diversity in various environmental samples. Because

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soils are considered to be one of the most challenging materials for molecular biological analyses, due to the extreme complexity of their physical, chemical, and biological properties, the methodologies for molecular biological analyses employed in this field would be expected to be sufficiently robust to be applied to other diverse biological materials. In the present study, we describe the application of a DNA extraction method and a fingerprinting technique, which were originally developed for use in soil microbiology, to the molecular analyses of agronomic products. The use of these methods in this context overcomes some of the technical problems described above and provides a new strategy based on aspects of environmental microbiology for the analysis of agronomic DNA.

MATERIALS AND METHODS

Materials and Sampling. Sampling of all materials was carried out in triplicate. Seedling plants and foodstuffs were purchased locally from commercial vendors. Samples were collected from seedling plant leaves, leaves of kidney bean (*Phaseolus vulgaris*), melon (*Cucumis melo*), and watermelon (*Citrullus lanatu*) plants. Samples of boiled carrot (*Daucus carota*), boiled kidney bean, and fresh tomato fruit (*Lycopersicon esculentum*) were obtained from the same package of processed salad. Samples of shichimi togarashi (a mixture of red pepper and other spices) and wasabi paste (from the root of *Wasabia japonica*) were also obtained from supermarket packages. Samples of hamburger steak were obtained from a vacuum-packed preparation. Sausage and ice fish (*Salanx microdon*) (0.5 g of semidried) samples were also obtained from packages. To enable extensive examinations of wasabi paste and sausage materials using RISA, products from three different distributors were selected for both materials, and three packages were purchased from each company. One sample was also collected from each package as a duplicate.

DNA Extraction. DNA isolates were extracted from 0.5 g of the various food products described above using the extraction method (DSPD method) previously reported by Ikeda et al. (*20*) with slight modifications. Briefly, all extractions were performed without the addition of skim milk to the extraction buffer, and each DNA sample from plant leaves, vegetables, meats, and fishes was homogenized and mixed with the extraction buffer using a mortar and pestle. After homogenization of these materials, the samples were transferred into 2 mL screw-capped tubes. In the case of shichimi togarashi and wasabi paste, samples were directly collected into 2 mL screw-capped tubes. After the addition of 0.5 g of glass beads (0.1 mm diameter, B. Braun Biotech International, Melsungen, Germany), the tubes were processed in a bead beater (Micro-Dismembrator S, B. Braun Biotech International) for 1 min at 2600 rpm. The supernatants were transferred into fresh 1.5 mL microtubes and mixed by inversion with 0.2 volume of 8 M potassium acetate. Following incubation for 5 min at room temperature, the microtubes were centrifuged for 5 min at 16000*g* at room temperature. The supernatants were collected, transferred into fresh 2 mL microtubes, and mixed by inversion with 0.6 volume of 2-propanol. After incubation for 5 min at room temperature, the tubes were centrifuged for 5 min at 16000*g*, also at room temperature. The pellets were then washed with 70% ethanol and resuspended in 100 μL of TE buffer (pH 7.6). For the final purification step, DEAE-cellulose column treatment was carried out. The eluted DNA preparations were precipitated by adding an equal volume of 2-propanol and $^1/_{10}$ volume of 3 M sodium acetate (pH 5.2). The pellets were then washed with 70% ethanol. After air-drying, the pellets were resuspended in 50 μL of sterilized water.

Qualitative and Quantitative Evaluation of DNA Extracts. DNA purity was assessed by calculating A_{260}/A_{230} and A_{260}/A_{280} ratios using an ND-1000 spectrophotometer (Nano Drop Technologies, Inc., Rockland, DE). Any possible fragmentation of the DNA samples was evaluated by running an extract (5 μL) on a 1% agarose gel (0.5× TBE) for 45 min. A fluorescence-based assay reported by Kuske et al. (*22*) was employed for DNA quantification to avoid interference by contaminating substances such as RNA and other organic compounds. For quantification of the DNA, samples were diluted in 0.1× TAE to an absorbance maximum in the 200−300 nm range of <0.05. An equal volume of a 200-fold-diluted PicoGreen dye (Molecular Probes, Eugene, OR) was then added. Samples were incubated at room temperature in the dark for 20 min, and the fluorescence intensities of the intercalated PicoGreen dye were determined using a Molecular Imager FX (Bio-Rad Laboratories, Inc., Hercules, CA), using band path filters for Sybr Green I and II. DNA concentrations were then determined relative to a lambda DNA standard curve.

The quality of the extracted DNA was also examined by PCR amplification. For PCR amplification, Ex *Taq* DNA polymerase (Takara, Kyoto, Japan) was used according to the manufacturer's protocol, except that BSA was added to each reaction to a final concentration of 0.2 μg/μL. Universal primers for the 18S rRNA gene, EukA and EukB, were used in the amplification reactions (*23*), and 0.5 μL of each final DNA extract was used as a template in a 50 μL reaction volume. The thermal cycler program was as follows: initial denaturation for 2 min at 94 °C; then 25 cycles consisting of 30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C; and a final extension for 7 min at 72 °C. PCR product (5 μL) was analyzed by 1% agarose gel electrophoresis in 0.5× TBE.

Ribosomal Intergenic Spacer Analysis. RISA was carried out using modifications previously reported by Ikeda et al. (*21*). Briefly, PCR primers for amplification of ribosomal intergenic spacers of bacteria were 1406F (5′-TGY ACA CAC CGC CCG T-3′) and 23SR (5′-GGG TTB CAT TCR G-3′) (*24*). For fluorescence detection, the 5′ end of the forward primer was labeled with 6-carboxyrhodamine (Sigma Genosys Japan, Hokkaido, Japan). The PCR reaction mixture (total 50 μL) contained 5 μL of 10× buffer, 10 μg of BSA, 0.5 μM of primers, 200 μM of each dNTP, 4 units of Ex *Taq* DNA polymerase (Takara), and 0.5 μL of DNA extract. The PCR amplification program was as follows: 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C, and a final extension of 7 min at 72 °C. PCR products (2.5 μL) were then mixed with 2.5 μL of loading dye (95% formamide, 10 mM EDTA, pH 8.0, 0.1% bromophenol blue). After incubation at 80 °C for 2 min, samples were chilled on ice and directly loaded on 5% polyacrylamide gels (19:1 ratio of acrylamide to bisacrylamide, 0.4 mm thick, 40 cm long) containing 7.7 M urea and 0.5× TBE and were electrophoresed at a constant 2000 V in 1× TBE for 2.5 h. Following gel electrophoresis, a digital fingerprinting image was obtained using a fluorescent scanner (Molecular Imager FX, Bio-Rad Laboratories, Inc.). Bands of interest were then excised from the gel for cloning and sequencing analysis.

Cloning and Sequencing. Excised gel pieces containing bands of interest were soaked in 50 μL of sterilized water and boiled for 15 min. The supernatant was transferred to a new microtube and mixed with 5 μL of 3 M sodium acetate, 2.5 μL of glycogen (10 mg/mL), and 225 μL of ethanol. After incubation for 30 min at −80 °C, the tube was centrifuged at 4 °C for 10 min at 15000 rpm. The supernatant was then discarded, and the pellet was rinsed with 100 μL of 85% ethanol, air-dried, and suspended in 10 μL of sterilized water. For reamplification of the excised DNA, PCR was performed under the same conditions described above for RISA, except that 1 μL of extract was used as the template DNA. Reamplified PCR products were purified using Wizard PCR purification columns (Promega, Madison, WI), and each was eluted in a final volume of 50 μL. Purified DNA fragments were then ligated to the pCR2.1-TOPO plasmid vector and subsequently transformed into *Escherichia coli* using a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA). After overnight incubation on LB medium at 37 °C, three colonies from each transformation were selected at random and grown at 37 °C in 4.5 mL cultures of LB medium containing 50 μg/mL kanamycin overnight. Plasmid DNA was extracted using a plasmid DNA extraction kit (Promega). To select clones corresponding to each RISA fingerprinting band for wasabi paste and sausage, the RISA procedure was carried out as described above using 1 μL of plasmid extract as the template DNA.

Sequencing reactions were prepared for selected plasmid clones using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems, Foster, CA) according to the manufacturer's instructions. Using a Perkin-Elmer 9600 thermal cycler and an ABI 377 Prism DNA Sequencer (Perkin-Elmer Applied Biosystems), both ends of each PCR clone were sequenced with M13

Table 1. Comparison of DNA Yields and Purities from Agronomic Products

sample	A_{260}/A_{230}	A_{260}/A_{280}	yield (μg/g of material)
kidney bean[a]	2.29 ± 0.01[b]	2.07 ± 0.01	46.6 ± 1.8
melon[a]	2.35 ± 0.02	2.06 ± 0.00	40.2 ± 1.5
tomato[a]	2.20 ± 0.05	2.05 ± 0.01	43.1 ± 1.8
watermelon	2.28 ± 0.01	2.06 ± 0.01	39.8 ±3.2
carrot	1.89 ± 0.06	1.98 ± 0.01	2.3 ± 0.3
kidney bean	2.19 ± 0.05	1.97 ± 0.03	7.4 ± 5.5
tomato	1.93 ± 0.10	1.95 ± 0.03	4.7 ± 2.1
shichimi togarashi	1.63 ± 0.09	1.78 ± 0.03	4.3 ± 1.4
wasabi	0.81 ± 0.06	1.64 ± 0.05	2.0 ± 0.1
hamburger steak	2.15 ± 0.01	1.95 ± 0.01	27.7 ± 0.9
sausage	2.07 ± 0.03	1.83 ± 0.01	20.9 ± 2.1
ice fish	1.96 ± 0.08	1.95 ± 0.01	15.4 ± 0.4

[a] Plant leaf. [b] Mean ± SD of three extractions.

forward and reverse primers. Sequences were manually edited to remove vector backbone, primer regions, and ambiguous sequences, and contiguous sequences were generated using overlapping regions of sequence data from both ends of the fragments. These data were compared to public database entries using the BLASTN subroutine (*25*), and sequence matches were considered to be significant when the score was >50 (*26*).

RESULTS

In our present study, the DSPD method originally developed for soil DNA extraction (*20*) was successfully applied to the extraction of DNA of good quality and quantity from most of the agronomic products that were examined (**Table 1**). In the case of the leaf samples that were tested (kidney bean, melon, and watermelon), the DNA extracts were deemed to be of sufficient quality following A_{260}/A_{230} and A_{260}/A_{230} spectrophotometric analyses, and the corresponding DNA yields were ∼40 μg/(g material) or greater for all three plant species. For processed salad vegetables (carrot, kidney bean, and tomato) and semiprocessed vegetables (shichimi togarasahi and wasabi paste), the quality of the DNA samples was also high following A_{260}/A_{230} and A_{260}/A_{230} measurements, with the exception of wasabi paste, and the DNA yields in these cases ranged from 2 to 7 μg/g of material. For the meat and fish products under examination (hamburger steak, sausage, and ice fish), high-quality DNA was again obtained, and a good amount of DNA (16−27 μg/g of material) was stably recovered from all three products.

Electrophoretic analysis of the agronomic DNAs showed that most contained high molecular weight fragments and had undergone little degradation except for the wasabi paste sample, which showed DNA smearing, indicating fragmentation (**Figure 1A**). PCR testing of each DNA extract using universal primers for the 18S rRNA gene further showed successful amplification of the 18S product (**Figure 1B**). RISA was then employed as a means of visualizing the complexity of the DNA content from the food samples. **Figure 2** shows RISA fingerprinting patterns obtained from duplicate samples from each agronomic product, clearly indicating, with high reproducibility in most cases, that a unique fingerprinting pattern is obtained for each sample. As expected, the DNA fingerprinting profiles of the leaf samples and vegetables are relatively simple, which contrasted with the highly complicated patterns that were obtained for semiprocessed products (shichimi togarashi and wasabi paste) and processed products (hamburger steak and sausage). The results of our extended RISA examinations of wasabi pastes and sausage are shown in **Figure 3**. The fingerprinting patterns were again highly reproducible among triplicate samples from each individual supplier of both wasabi paste and sausage with a notably higher complexity for sausage than for wasabi pastes. Length polymorphisms were observed, however, between samples from different producers of both these foods.

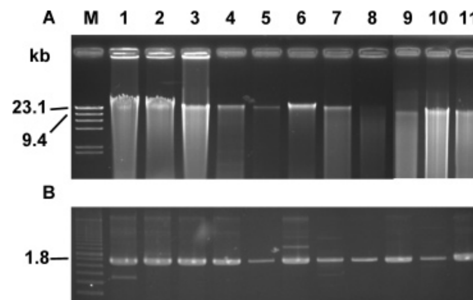

Figure 1. Total DNA samples from agronomic products, extracted using the DSPD method, and their corresponding PCR products amplified with an 18S rRNA primer set. (**A**) Agarose gel electrophoresis of total DNA extracts of agronomic products: (lane M) *Hin*dIII-cut lambda bacteriophage molecular size markers; (lane 1) kidney bean leaf; (lane 2) melon leaf; (lane 3) watermelon leaf; (lane 4) boiled carrot; (lane 5) boiled kidney bean; (lane 6) tomato fruit; (lane 7) shichimi togarashi; (lane 8) wasabi paste; (lane 9) hamburger steak; (lane 10) sausage; (lane 11) ice fish. (**B**) Amplification of 18S rRNA gene sequences: (lane M) 200 bp ladder molecular size markers; (lanes 1–11) amplicons from corresponding DNA samples in panel **A**.

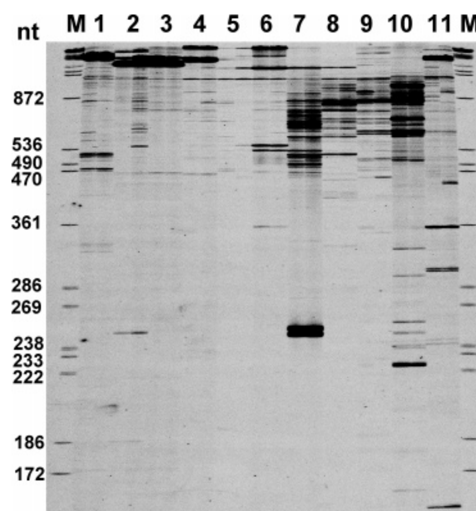

Figure 2. Complexity of the DNA content of agronomic products revealed by RISA: (lane M) GeneScan-2500 ROX molecular size markers (Applied Biosystems); (lane 1) kidney bean leaf; (lane 2) melon leaf; (lane 3) watermelon leaf; (lane 4) boiled carrot; (lane 5) boiled kidney bean; (lane 6) tomato fruit; (lane 7) shichimi togarashi; (lane 8) wasabi paste; (lane 9) hamburger steak; (lane 10) sausage; (lane 11) ice fish. Duplicate results for each sample are shown. Numbers indicate marker fragment lengths.

To reveal the identity of the amplified bands from the RISA of the agronomic DNAs examined, we attempted to clone each of the bands that had been stably detected among replicate samples of wasabi paste and sausage, and the results of the subsequent sequencing analyses of these amplicons are shown in **Table 2**. Following RISA of wasabi paste DNA, 15 amplified bands were excised from the gel, and 11 of these were successfully subcloned using the TA system. Subsequent sequencing analysis revealed that 2 of these wasabi subclones were generated by amplification with only the forward primer, and these clones were excluded from further analysis. The remaining clones were confirmed for the presence of both

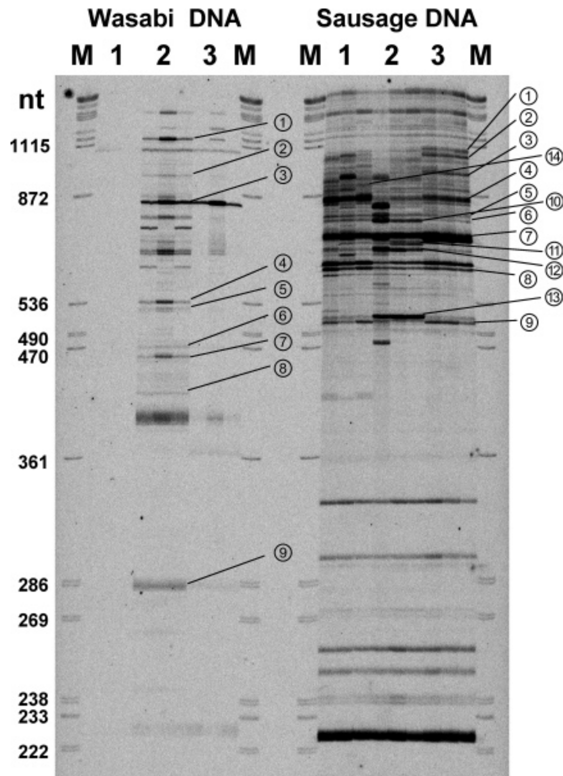

Figure 3. Comparison of DNA fingerprinting profiles of sausage and wasabi paste generated by RISA: (lane M) GeneScan-2500 ROX molecular size markers (Applied Biosystems); (lanes 1–3) results of triplicate samples from different producers of each product. The leftmost numbers indicate marker fragment lengths, and circled numbers indicate the corresponding clone numbers shown in **Table 2**.

forward and reverse primers and were subjected to BLAST searches with the public DNA database. All of these clones showed high similarities to rRNA operon regions in several species of bacteria; for example, clone AB198400 showed high similarity to the rRNA operon region of *Xanthomonas campestris* pv. *campestris*, which is a known bacterial pathogen in several plant species but had not been identified previously in wasabi. Clone AB198405 also showed high homology to the rRNA operon region of *Bacillus subtilis*, which is a known common soil bacterium. Unexpectedly, two clones (AB198402 and AB198406) showed high sequence similarity to the rRNA operon region of human bacterial pathogens. In the case of clone AB198406, this sequence similarity was limited to the region of the 16S rRNA gene for *Porphyromonas macacae*, with a homology of 96%. In contrast, in the case of clone AB198402, the entire sequence showed a homology of 97.8% to the ribosomal operon of *Weissella confusa*.

For sequencing analysis of sausage RISA amplicons, 29 bands were excised from the gel, and 25 of these were subcloned using TA cloning. Following sequencing, 11 of these subclones were shown to be amplified by the forward primer alone, and these fragments were again excluded from subsequent analyses. The remaining 14 clones were again subjected to BLAST searching, and all clones showed some degree of sequence similarity with rRNA operon regions from several species of bacteria. As expected, several clones showed homology to rRNA operons from food-associated bacteria. The entire sequences of clones AB198415, AB198419, and AB198420 showed a homology of 99% to *Lactobacillus lactis*, *Lactobacillus raffinolactis*, and *Lactobacillus sakei*, respectively, which are very commonly detected in meat products generated by both cultural-dependent and cultural-independent methods (*27*, *28*). The full sequence of clone AB198416 was also found to have a homology of 98% to a food-associated bacterium, *Carnobacterium divergens*. The sequences of several sausage clones also showed some homology to known bacterial pathogens. Clone AB198417 was shown to have only partial similarity to 16S rRNA from *Chryseobacterium meningosepticum*, which was considered to be insufficient for phyllogenetic assignment using the current DNA database. In the case of clone AB198421, >90% sequence similarity was found to the coding sequence regions of the ribosomal operon and corresponding regions of *Edwardsiella tarda*, whereas the similarity between clone AB198421 and the entire *E. tarda* sequence was 68%, suggesting that clone AB198421 may not in fact be closely related to this human pathogen. In contrast to these clones, we found that clone AB198412 and AB198413 showed high similarity to *Yersinia pestis,* which can cause the serious epidemic disease, plague. The homology between these two clones was 94.8%, and slight differences were found in intergenic spacer regions. In comparisons with the coding regions of the *Y. pestis* ribosomal operon, AB198413 showed 100% similarity with 16S rRNA and tRNA-Glu and 96% homology to 23S rRNA with an overall similarity of 88% (**Figure 4**).

DISCUSSION

The DSPD method was originally developed for the rapid extraction of DNA from soils, which are generally considered to be challenging environmental samples for the purposes of nucleic acid sampling and associated molecular biological analyses due to the extreme complexity of their physical, chemical, and biological properties. Consequently, due to the necessary robustness of DSPD, this protocol was predicted to be a suitable and rapid DNA extraction method for a wide range of biological materials. The results of DNA extraction from various agronomic products examined in the present study confirmed this prediction. The DSPD method is also a cost-effective and simple procedure and is free of hazardous reagents, making it a highly useful technique that is also environmentally friendly. These advantages of DSPD also make it very suitable for routine use in food traceability analysis, and it is noteworthy that this method not only yields eukaryotic DNAs but can also effectively extract microbial DNA from foods and agronomic products. Use of this technique therefore has the potential to provide information regarding the status and/or history of environmental microbiological conditions during crop harvesting and also the transportation and processing of agricultural products.

To date, several DNA fingerprinting methods have been employed in food science and, in particular, PCR-based techniques have become major tools in the molecular analysis of food and agronomic product DNA in daily examinations for clinical, forensic, and other purposes. Most of these methods have focused on the analysis or detection of specific organisms that are established as predictable targets for PCR detection, but less effort has been made in investigating the complexity of DNAs in food products such as the presence of nonculturable microorganisms or contamination by unknown microbes, which may not be labeled ingredients on such items. The results of the analyses undertaken in our present study clearly show, however, that RISA has the potential to be an efficient tool for profiling DNA extracts from food products. In our experiments, we employed universal primers for targeting ribosomal RNA gene sequences that are conserved in most known bacterial species. This approach allowed us to amplify DNA fragments from a

Table 2. Sequence Similarities between RISA PCR Products Amplified from either Wasabi Paste or Sausage Products and Known Bacterial Sequences

clone ID[a]	length (bp)	score[b]	expect[c]	DB no.[d]	nacterial species	description
wasabi clones						
AB198399 (1)	1020	242	1e-60	AF484065	uncultured bacterium	
AB198400 (2)	734	1415	0	AE012540	*Xanthomonas campestris*	plant pathogen
AB198401 (3)	751	266	7e-68	Y08853	*Terrebacter* sp.	
AB198402 (4)	498	525	e-146	AY342323	*Weissella confusa*	human pathogen
AB198403 (5)	486	252	7e-64	AY755378	uncultured actinobacterium	
AB198404 (6)	428	262	6e-67	AY593480	*Collimonas fungivorans*	soil bacterium
AB198405 (7)	420	825	0	Z82044	*Bacillus subtilis*	soil bacterium
AB198406 (8)	381	145	9e-32	AY546488	*Porphyromonas macacae*	human pathogen
AB198407 (9)	236	240	1e-60	AY485405	rice phyllosphere bacterium	
sausage clones						
AB198408 (1)	1033	256	9e-65	AB035150	*Haloanella gallinarum*	
AB198409 (2)	1027	454	e-124	CR543861	*Acinetobacter* sp.	
AB198410 (3)	928	220	6e-78	AY484737	*Rhizosphere bacterium*	
AB198411 (4)	765	278	2e-71	AY593480	*Collimonas fungivorans*	soil bacterium
AB198412 (5)	716	626	e-176	AE017139	*Yersinia pestis*	human pathogen
AB198413 (6)	704	630	e-177	AE017139	*Yersinia pestis*	human pathogen
AB198414 (7)	638	270	4e-69	AF095537	uncultured bacterium	
AB198415 (8)	553	1072	0	X64887	*Lactococcus lactis*	food-associated bacterium
AB198416 (9)	468	617	e-173	AF374292	*Carnobacterium divergens*	food-associated bacterium
AB198417 (10)	694	228	1e-56	AY683476	*Chryseobacterium meningosepticum*	human pathogen
AB198418 (11)	625	307	2e-80	AB103093	*Micrococcus luteus*	meat-associated bacterium
AB198419 (12)	609	646	0	AF284220	*Lactococcus raffinolactis*	food-associated bacterium
AB198420 (13)	477	930	0	AF401673	*Lactobacillus sakei*	meat-associated bacterium
AB198421 (14)	821	392	e-106	AY706748	*Edwardsiella tarda*	human pathogen

[a] DDBJ accession number of PCR clones reported in this study. The number in parentheses indicates the circled numbers in **Figure 3**. [b] BLAST search score. [c] BLAST expect score. [d] Accession number of the sequence of the closest relative found in the current databases.

wide range of bacteria, including unknown or irregular bacterial species that may be harbored in different foodstuffs or agronomic products. The presence of such species, revealed by RISA, can potentially provide new information on the status of an agronomic product from an environmental and microbiological viewpoint. Furthermore, the high reproducibility and uniqueness of the RISA fingerprinting patterns found in our experiments indicate that RISA could also be a useful tool for the traceability of agronomic products as a type of bar code, in addition to its usefulness in clinical and forensic examinations. The robustness of our RISA data also showed that our sampling volumes for DNA extraction were sufficient to enable profiling of the entire DNA content for each of the products examined in this study. Moreover, the high efficiency of RISA for detecting DNA polymorphisms among closely related microbes would also be useful for differentiating certain types of local strains, which in different foods could assist in the examination of biogeographic aspects of "Protected Designation of Origin" foods such as cheese, as pointed out by Giraffa and Neviani (*29*).

The technical simplicity and ease of use of the RISA method make rapid sequencing analysis possible, as shown in the present study. We were thus able to identify several bacterial sequences, derived from both wasabi pastes and sausage. As expected, some wasabi clones had high homology to soil- and plant-associated bacteria such as *B. subtilis*, *Collimonas fungivorans*, and *X. campestris*. These sequences may be derived from bacteria from arable lands in the case of soil bacteria or from plants that are natural habitats for phyllospheric bacteria. Significantly, these bacterial species are common environmental microbes and pose no threat to human health. However, we also found that one of the wasabi clone sequences had significant similarity to *W. confusa*. This bacterium has been reported to be present in several foods as an environmental microbe (*30*). However, recent studies suggest that this bacterium may be a clinically important pathogen because of its resistance to vancomycin (*31*−*33*). When one takes into consideration the quite significant differences in the complexity of the RISA fingerprinting patterns between the same kinds of products from different producers, the results of our wasabi DNA analysis may well reflect differing microbiological and clinical states during the culturing or harvesting of plant materials or when these products are processed in factories.

Our RISA experiments with sausage revealed that the sequences of several clones were most likely derived from food- or meat-associated bacteria. In addition, as shown for the clones characterized from wasabi pastes, we also found homology between the clones from sausage to known human bacterial pathogens. Surprisingly, one of the sausage clones showed significant similarity to *Y. pestis*, and the degree of the similarity is likely to be sufficient for the phylogenetic assignment of this clone at the level of genus, species, or subspecies. Significantly, although antibiotic treatments are effective against the plague, this microbe is still considered to be one of the most devastating known infectious agents, due to its extreme pathogenicity and high mortality rates (*34*).

In the environmental microbiology field, it is well accepted that most species of microbes are generally nonculturable using currently available technologies. Hence, culture-independent methodologies have become essential tools for the better understanding of microbial activity in different environments. The activity of nonculturable microbes may also have a significant impact upon food quality, particularly in the case of processed foods. Several recent papers have now focused on the status of nonculturable microbes in food and revealed the relatively complex structures of microbial communities in various agronomic products (*18*, *29*, *35*). More recently, denaturing gradient gel electrophoresis (DGGE) has become a major tool in the visualization of microbial community complexity within foodstuffs (*32*, *36*, *37*).

In our present study, we employed RISA instead of DGGE to analyze the diversity of the microbial communities that were associated with agronomic products for a number of reasons. First, a simple gel electrophoresis system is sufficient to perform

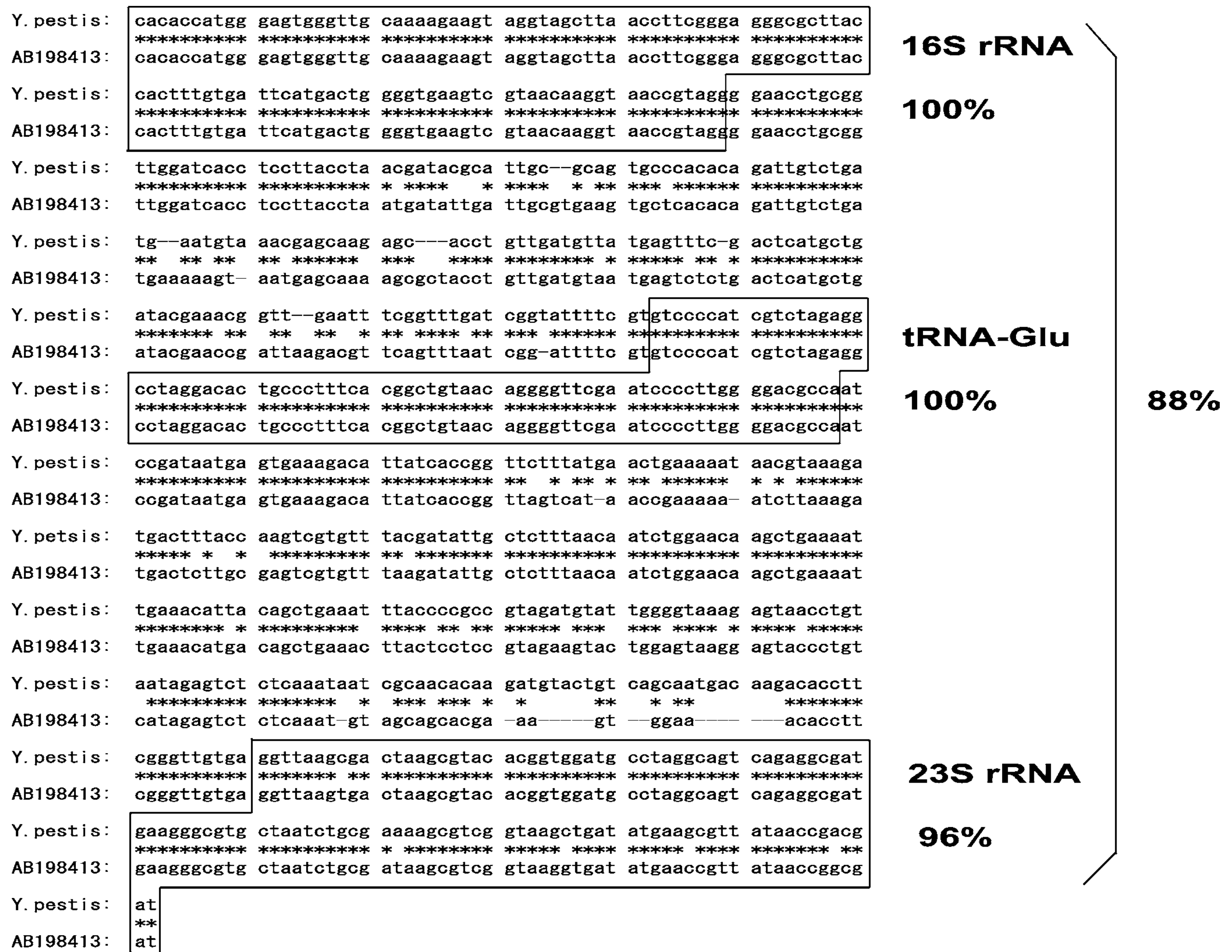

Figure 4. Sequence comparisons of the ribosomal operon regions between clone AB198413 and *Y. pestis*. Asterisks indicate identical nucleotide matches. Coding sequences are indicated by boxed regions: 16S rRNA (upper), tRNA-Glu (middle), and 23S rRNA (lower). Levels of similarity are indicated on the right.

RISA, whereas DGGE methods require expensive and complex experimental systems. Second, RISA is more suitable for processing multiple samples as it uses a fixed gel concentration for resolving DNA fingerprinting patterns and it is well-known that analysis of complex fingerprinting patterns using DGGE may be difficult when gel to gel comparisons are needed. Third, the use of sequencing gels with a one base pair resolution capability provides the highest degree of sensitivity for the detection of fragment length polymorphisms. Fourth, the RISA procedure is simpler and more rapid when compared to DGGE and other fingerprinting techniques. Fifth, the simplicity of the RISA method makes it a potentially good high-throughput analytical procedure for the large-scale screening of specific microbes and DNA libraries, particularly in the statistically meaningful monitoring and analysis of microbial communities. The high reproducibility, simplicity, and rapidity of RISA therefore make this method highly suitable for the analysis of microbial communities in foods.

There are, however, weaknesses in the RISA technique for the examination of community structure that must be taken into consideration. One such drawback is that some nonclonable amplicons were found to be present in our current study, which seems mostly to be higher molecular weight fragments. These species may prove to be clonable under PCR conditions that use longer extension times, as 2 min may not be sufficient for the cloning of large amplicons. Another concern with the use of RISA fingerprinting is the specificity of the PCR products, as shown in our sequencing analysis of the amplicons generated in our experiments. Our results showed that the reliability of the PCR product specificity was significantly different between the DNA extracted from wasabi paste and that extracted from sausage. In the case of the clonable amplicons from sausage, nearly half of all these clones were generated by amplification with one forward primer. This situation may be addressed by modifying the primer sequences, and a new primer set for RISA was in fact reported recently by Cardinale et al. (*38*) during the preparation of this paper. Last, the current DNA database does not hold adequate information for ribosomal intergenic spacer regions to cover all bacterial genetic variations in nature at either the genus or species level, although there are rapid and continuing increases in new sequence data from a variety of studies worldwide. Another concern in relation to the current level of information in the DNA database is that new microbial sequence data may have a large bias toward clinically important organisms, to the detriment of data concerning diverse microbes that occur in nature. Hence, some caution should be taken when one is assessing sequence data from environmental samples, as shown in this study.

Recently, Cocolin et al. (*27*) reported the characterization of microbial communities in sausage meat produced using both

cultural-dependent and cultural-independent methods. In their study, they employed DGGE to analyze culture-independent products and detected only a few bands at most from each set of fingerprinting data. Furthermore, most of the DGGE amplicons in this case were identified as lactic acid bacteria, which are commonly found in a wide variety of foods, and no similarities with known human pathogens were obtained from their sequencing analyses. In our analysis, we found more sequence variation in clones derived from sausage compared to previous reports, in terms of both band number and sequence diversity. These differences between our results and the findings of other studies may be due to the difference in the status of the material under study, because our materials are end products available from a supermarket. However, it is now evident from a number of studies in environmental microbiology that the genetic diversity of the regions targeted by the RISA technique is much broader than that of the 16S ribosomal rRNA gene targeted by other methods such as DGGE. Our finding of a wide variety of bacterial sequences in commercial sausage products indicates both the usefulness and the effectiveness of RISA for microbial community analysis in foods. Because the genetic variation in the ribosomal intergenic spacer regions of eukaryotes is also expected to be high enough for use as a molecular marker, a similar application of RISA to these regions may be effective for detecting eukaryotic microbes such as fungal species or for examining the true origins of diverse agronomic products and detecting false claims about their identity, which is currently a serious social issue in food production in Japan.

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