Identification of thermo tolerant lactic acid bacteria isolated from silage prepared in the hot and humid climate of Southwestern Japan

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Abstract

To develop high-quality silage starters adapted to hot and humid weather, 12 LAB isolates from silage produced in Kyushu and Okinawa, Japan were characterized based on their morphological features, growth curves and sugar utilization. In addition, the nucleotide sequences of the V2-V3 region of their 16S rRNA genes and the 16S-23S rRNA intergenic spacer (ITS) regions were determined. The isolates were also subjected to RAPD-PCR analysis, DNA-DNA hybridization, G+C content analysis and immuno-identification using species-specific monoclonal antibodies and SDS-PAGE profiling. Nearly all of the isolates exhibited high thermotolerance and rapid growth. Combining ITS sequence analysis, RAPD-PCR and immuno-identification enabled rapid and accurate identification of closely related LAB strains that other methods failed to appropriately differentiate; for example, L. plantarum was distinguished from L. pentosus, and L. casei was distinguished from L. rhamnosus. Using the aforementioned techniques, the isolated strains were identified as L. plantarum, L. rhamnosus, L. rapi, Pediococcus pentosaceus and P. loli. Our findings also showed that there is greater diversity among thermophilic LABs in silage prepared in a hot and humid environment.

Keywords: Lactic acid bacteria; Lactobacillus; Pediococcus; Silage; Hot and humid environment

Introduction

Silage is a storable fodder typically produced through a sequential microbial process involving homofermentative lactic acid bacteria (LAB) such as Lactobacillus plantarum, Lactococcus lactis, Leuconostoc pseudomesenteroides, Pediococcus acidilactici and Enterococcus faecalis (Cai et al. 1999; Ennahar et al. 2003; Giraffa et al. 2010). In recent years, the demand for dairy products has increased in many developing countries in the tropical and subtropical regions of Asia and Africa; however, production of the silage necessary for dairy farming has been hindered in these regions because the ensiling process is dependent on local environmental conditions (Namihira et al. 2010). For example, virtually all of the many available silage starters (Holzer et al. 2003; Yan et al. 2008) are hampered by high temperatures and humidity (Ohmomo et al. 2002; Mulrooney and Kung 2008). This is in part because the phage infections that occur in hot humid weather reduce the viability of LAB (Kaneshige et al. 1994). At present, therefore, there is a strong need to identify starter strains suitable for use in hot climates.

The islands of Kyushu and Okinawa in southwestern Japan have a humid, subtropical climate. Production of high-quality silage there is difficult due to unbroken periods of extremely hot days, with temperatures exceeding 30°C in summer, and to the high moisture content (over 80%) in the plant materials. Stable production of high-quality silage in these regions will require the identification and application of acid-tolerant, thermophilic LAB or homolactic acid fermented LAB as starter strains (Mulrooney and Kung 2008). Given the limits of the available technology, however, the screening, selection and construction of starter cultures for silage making remains a challenge, as classification of isolated strains is still difficult. In particular, closely related species, such
as L. plantarum, L. casei, L. rhamnosus and L. pentosus, which are the main dominant strains in silage, are difficult or impossible to distinguish on the basis of their phenotypes and genotypes (Hammes and Hertel 2006). Some PCR amplification methods for the 16S-23S rRNA, recA and dnaK genes have been used to identify L. plantarum-related species (Torriani et al. 2001; Huang et al. 2010; Ouoba et al. 2010), but there is no generally accepted or systematic approach to identification of silage-making lactobacilli. In particular, there have been few studies characterizing LAB strains grown in silage produced in tropical or subtropical regions (Namihira et al. 2010).

Here, we report the isolation and characterization of LAB from silage produced in Okinawa, Japan. We used various identification methods to assess the diversity of silage-making LAB in subtropical areas, and to identify species closely related to L. plantarum and L. pentosus and to L. casei and L. rhamnosus. Our findings represent the first use of immuno-identification for the identification of LAB from silage.

Materials and methods

Bacterial strains and culture conditions

Twelve representative LAB strains isolated from silage prepared in Okinawa prefecture, Japan were used. L. brevis JCM 1059T, L. casei JCM 1134T, L. fermentum JCM 1173T, L. kefiri JCM 5818T, L. parakefir JCM 8573T, L. paraplantarum JCM 12533T, L. plantarum JCM 1057, L. plantarum JCM 1149T, L. rapi JCM 15042T, L. rhamnosus JCM 1136T, L. pentosus JCM 1558T, Pediococcus acidilactici JCM 8797T and P. pentosaceus JCM 5890T were used as reference strains. Escherichia coli DH5α was used as a host strain for plasmid vector pTA2 (Toyobo Co., Ltd., Osaka, Japan).

Bacto™ Lactobacilli MRS Broth (Becton, Dickinson and Company, NJ, USA) and agar plates (2.0% agar) were used for transplantation and growth of Lactobacillus strains. GAM Broth (Nissui Pharmaceutical Co., Tokyo, Japan) and R-CW agar (Kojima et al. 1993) were used for growth of P. pentosaceus JCM 5890T and L. parakefir JCM 8573T, respectively. L. kefiri JCM 5818T, L. parakefir JCM 8573T, L. pentosus JCM 1558T, L. plantarum JCM 1057, L. plantarum JCM 1149T and P. acidilactici JCM 8797T were cultivated at 30°C under anaerobic conditions. Cultivation of other strains was carried out at 37°C under anaerobic conditions. A microaerobic condition (5% O2, 10% CO2, 85% N2) for cultivation of L. paraplantarum ATCC 700211T was generated using AnaeroPack Plus (Mitsubishi Gas Chemical Co., Tokyo, Japan). LB broth and agar plates (2.0%) were used for growth and transformation of E. coli. LB plates containing ampicillin (100 μg/ml), isopropyl-β-D-thiogalactopyranoside (120 Dg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (400 Dmg /ml) were used for selection of blue/white colonies.

Physiological characterization

The shapes of all isolates were observed using an Axioskop phase-contrast microscope (Carl Zeiss, Ober kochen, Germany) and transmission electron microscopy (JEM 2000 EX; JEOL, Tokyo, Japan). Gas formation from glucose, fermentation type, lactic acid production, and peptidoglycan type were examined as described previously (Tanaka et al. 1994). In addition, the characteristics of the carbohydrate fermentation by all strains were determined using an API 50CHL kit (BioMérieux, Montalieu Vercie, France). Growth curves for the isolates were constructed using Miniophoto 518 (TAITEC Corporation, Saitama, Japan) by monitoring the optical density at 660 nm.

Total DNA extraction

Total DNA was isolated from LAB strains cultured overnight in 600 ml of liquid medium supplemented with 0.5% glucose. After washing the cells with wash buffer (50 mM Tris–HCl, 150 mM NaCl, 100 mM EDTA; pH 8.0), they were collected by centrifugation and lysed in 10 ml of lysis buffer (50 mM Tris–HCl, 10 mM EDTA, 10 mg/ml lysozyme (Seikagaku Corporation, Tokyo, Japan), 2 mg/ml N-acetylmuramidase SG (Seikagaku Corporation), 100 μg/ml RNase A; pH 8.0). The lysate was then added to 200 ml of 25% sodium dodecyl sulfate (SDS) and incubated for 2 h at 37°C, after which the DNA was extracted first in phenolchloroform-isooamyl alcohol (25:24:1) and then in chloroform-isooamyl alcohol (24:1). The extracted DNA was then precipitated in isopropanol, collected and dissolved in 100 ml of TE buffer.

PCR amplification and cloning amplified fragments

The primers used in this study are listed in Table 1. To amplify the V2-V3 regions of 16S rRNA and the 16S-23S intergenic spacer (ITS) region, primers were designed as described by Tannock et al. (1999). With all primer sets, PCR was carried out in a 50-μl reaction volume containing 5 U of KOD DNA polymerase (Toyobo), 5 μl of 10×PCR reaction buffer, 0.2 mM each primer, 0.2 mM dNTPs, 0.5 mM MgCl2 and 200 ng of purified genomic DNA. The amplified products were separated on 2.0% agarose gels containing 1×TAE electrophoresis buffer, purified using a Cyclo-Pure Gel Extraction Kit (AMRESCO Inc., Solon, OH, USA) according to the manufacturers’ instructions, and then ligated to pTA2 vector using a Takara BK2 Kit (Takara Bio Inc., Shiga, Japan). The recombinant DNAs to be transformed into E. coli DH5α competent cells were extracted and purified through alkaline lysis using QIAGEN-tip 20 (QIAGEN GmbH, Hilden, Germany).
Nucleotide sequence analysis

Nucleotide sequences were determined using a Thermo Sequenase fluorescently labeled primer cycle sequencing kit with 7-deaza-dGTP (GE Healthcare Bio-Sciences, Uppsala, Sweden) and ALF express II DNA sequencer. The sequences of the amplified DNA were scanned based on data registered in databases using GENETIX-MAC ver. 15 (GENETYX, Tokyo, Japan). Comparisons between the sequences and the databases were made using the Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments and phylogenetic analyses were accomplished using ClustalW ver. 2.1 (http://clustalw.ddbj.nig.ac.jp/) and the neighbor-joining (NJ) method. Evolutionary distance values were estimated using Kimura’s 2 parameter method.

RAPD analysis

RAPD-PCR analyses were performed as described previously (Fujino et al. 2008) using a single 10-mer random primer listed in Table 1 and 0.5 U of Gene Taq FP polymerase (Nippongene, Toyama, Japan). The PCR protocol entailed initial denaturation at 95°C for 5 min, followed by 35 cycles of 1 min denaturation at 95°C, annealing 36°C for 1 min and extension for 2 min at 72°C, and a final extension for 5 min at 72°C. RAPD-PCR products were analyzed by electrophoresis run on 2.0% agarose gels in 1X TAE for 1 h at 100 V.

SDS-PAGE profiling

SDS-PAGE was carried out using an AE-6200 electrophoresis unit (ATTO, Tokyo, Japan) to determine the size and number of whole proteins in the isolates. Electrophoresis was carried using the method of Laemmli (1970). Samples were boiled for 10 min at 100°C in SDS-PAGE buffer (125 mM Tris–HCl (pH 6.8), 10% 2-mercaptoethanol, 4% SDS, 10% glycerol, 0.004% bromophenol blue) to ensure denaturation before being loaded onto the gel. Each well was loaded with 20 μl of sample containing approximately 10 μg of protein. After electrophoresis, the gels were stained with Coomassie blue R-250, destained using 7.5% glacial acetic acid and 5% methanol, and then photographed. Numerical analysis of the protein pattern was performed using the GelCompar system (version 4.0; Applied Maths, Sint-Martens-Latem, Belgium), which normalizes fragment pattern data for band intensity, after which the relative band position is compared to molecular weight standards. The similarities between all pairs were expressed using a pair-group method with arithmetic averages (UPGMA) for construction of the dendrogram. All strains were analyzed at least three times to ensure the reproducibility of the fingerprinting pattern.

Immuno-identification

For identification using monoclonal antibodies, anti-L. casei monoclonal antibody (72-6D2) and anti-L. plantarum monoclonal antibody (55-1C3) (Asahi Food & Healthcare Co., ltd., Tokyo, Japan) were used as primary antibodies, and an affinity purified, horseradish peroxidase-conjugated rabbit anti-mouse IgG1 (MP Biomedicals, Solon, OH, USA) was used as the secondary antibody. LAB were incubated until late log growth phase and washed with TES buffer (50 mM NaCl, 30 mM Tris–HCl, 5 mM EDTA; pH 8.0), after which they were suspended in TES buffer at an optical density of 1.0 at 660 nm. Aliquots (100 ml) of the suspension were then incubated overnight at 4°C in a 96-well microtiter plate. To determine the dose response relationship, various numbers of bacteria were inoculated onto similar microtiter plates. After washing with Tween-PBS (phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4 and 1.5 mM KH2PO4) supplemented with 0.1% Tween 20), the plates were blocked by incubation for 1 h at 37°C with 120 ml of carbonate buffer containing 1% bovine serum albumin (BSA). The plates were then washed three times and treated for 2 h at 37°C with 100 ml per well of appropriate dilutions of monoclonal antibodies in Tween-PBS with 1%
BSA. The plates were then washed again and treated for 2 h at 37°C with 100 ml of a 1:1000 dilution of secondary antibody in Tween-PBS with 1% BSA. After washing, the plates were treated with 100 ml of the coloring agent (100 mM citric acid, 200 mM Na₂HPO₄, 10% DMSO containing 2% 3, 3', 5, 5', tetrathymethylbenzidine, 0.02% H₂O₂ (30% aqueous); pH 6.2) for 10 min at 37°C and finally with 50 ml per well of 2 M sulfuric acid to stop the reaction.

G+C content and DNA-DNA hybridization
To determine the genomic G+C content, extracted DNA was purified by ultracentrifugation (Optima T1X Ultracentrifuge, Beckman Coulter Inc.) for 24 h at 80,000 g in cesium chloride (1 g/ml) and ethidium bromide (0.8 mg/ml). The DNA was then dissolved in ultrapure distilled water, and the ethidium bromide was removed with isopropanol, after which the G+C content of the purified DNA was determined using DNA-GC kits (Yamasa-Shoyu, Chiba, Japan) according to the manufacturer’s instructions. The nucleoside mixture obtained was then separated by HPLC (model 600E, Waters Corporation, Massachusetts, MA, USA) using a YMC pack AQ-312 column (YMC Co., Ltd., Kyoto, Japan). The mobile phase consisted of 10 mM H₃PO₄ and 10 mM KH₂PO₄ (pH3.5). As a control, genomic DNA from phage λ was analyzed along with the sample.

DNA-DNA hybridization was carried out as described previously (Ezaki et al. 1989; Adnan et al. 1993). Samples of purified DNA from each of the 12 isolated and 6 type strains were suspended in TE buffer (100 μg/ml) and denatured at 100°C for 5 min. Ice-cold PBS (8 mM Na₂HPO₄, 1.5 mM KH₂PO₄ (pH 7.2), 137 mM NaCl, 2.7 mM KCl) containing 10% DMFO was then added to the DNA solution to a final concentration of 10 μg/ml, and aliquots of the resultant single-stranded DNA solution (100 μl/well) were dispensed into a FluoroNunc Plate (Thermo Fisher Scientific Inc., Waltham, MA, USA). The plate was then sealed and incubated for 3 h at 37°C, after which the mixture was discarded and the plate was dried overnight at 48°C. Photobiotin-labeled DNA was then prepared using a PHOTOPROBE (VECTOR lab., Burlingame, CA, USA) according to the manufacturer’s instructions. About 25 ng of labeled DNA were distributed into each well of a microplate, and hybridization was carried out at for 3 h at 40°C in the hybridization mixture (2xSSC, 5xDenhardt, 3% dextran sulfate, 50% formamide and 200 μg of denatured salmon sperm DNA). Streptavidin-conjugated horseradish peroxidase (Thermo Fisher Scientific Inc.) was diluted 2000-fold in PBS containing 0.5% bovine serum albumin and 0.1% Triton X-100, and 100 μl of the diluted enzyme solution were added to each well. The plate was then incubated for 30 min at 37°C and washed three times in 300 μl of 2xSSC. After washing, 100 μl of chromogenic substrate solution (0.1 M citric acid-0.2 M Na₂HPO₄ (pH 6.2)-10% DMFO containing of 2% 3, 3’, 5, 5’, tetrathymethylbenzidine in DMFO and 2% H₂O₂ aqueous (0.3%)) were added to each well. After incubating the samples and standards for 10 min, the absorbance at 655 nm was read on a Multiskan FC microplate reader (Thermo Fisher Scientific Inc.).

Results
Phenotypic characterization of the isolates
All of the isolates grew in MRS broth at 30-43°C, and strains NGRI 0001, 0110 and 0130 grew at 45°C (data not shown). When cultivated at 37°C, strains NGRI 0101 and 0529 reached the stationary phase within 8 h after inoculation (Figure 1). With the exception of strains NGRI 0130 and 0305, the isolated strains were homofermentative and did not produce gas from glucose. The peptidoglycans of strains NGRI 0110, 0130, 0304 and 0305 were the non-diamino pimericacid type, while those of the other strains were diamino pimelicacid. None of the strains was able to assimilate soluble starch, and the sugar utilization properties of strains NGRI 0001, 0315 and 0524 were identical. Based on their properties, strain NGRI 0110 was coarsely categorized as a L. casei group, and strains NGRI 0001, 0101, 0225, 0315, 0404, 0524 and 0529 were categorized as a L. plantarum group. Strains NGRI 0304, 0305 and 0510 were grouped together as Pediococcus species. That said, the properties of the isolates were not all identical to those of the reference strains.

Analysis of the V2-V3 variable region of 16S rDNA and the 16S-23S rDNA ITS region
An approximately 200-bp band amplified using primers HDA1 and HDA2 was detected in all LAB isolates tested (data not shown). The nucleotide sequences from the isolated strains NGRI 0225, 0304, 0305, 0315, 0404, 0524 and 0529 showed 100% identity to those of L. plantarum, L. paraplantarum and L. pentosus. The highest sequence homology shown by strain NGRI 0110 was 99% to L. casei, L. paracasei subsp. tolerans and L. rhamnosus, while strain NGRI 0510 showed 99%, 98% and 92% identity to P. acidilactici, P. pentosaceus and P. damnosus, respectively. The nucleotide sequence in the V2-V3 region of strain NGRI 0001 perfectly matched that of L. kefri. The sequences from strain NGRI 0130 showed significant identity to the V2-V3 regions of L. rapi and L. parabuchneri. Using these partial 16S rRNA sequences, we constructed a phylogenetic tree (Figure 2(A)). Four clusters were formed at a similarity level of 97%, with the L. plantarum cluster containing the largest number of isolates (8) and the L. casei cluster containing the fewest (1). Strains NGRI 0001 and 0130 were categorized in the L. kefri cluster.
Figure 1 Growth curves for isolated silage-making LABs. Cells were grown at 37°C for 15 h in MRS media and monitored based on absorbance at 660 nm. Relative cell densities are an average of four independent cultures.

Figure 2 Phylogenetic trees constructed using the neighbor-joining method with the sequences of the V2-V3 region of the 16S rRNA gene (A) and the ITS region (B) from the isolated LAB strains and their taxonomic neighbors. The V2-V3 gene sequence from E. coli DSM 30083T (HQ012019) was used as the outgroup in (A). The ITS sequence from E. coli K-12 (X12420) was used as the outgroup in (B). Bootstrap values based upon 1000 replicates are included at the major branch points. The bars show the number of nucleotide substitutions per site.
Amplification of the ITS region using primers 16A-1 and 23B-1 yielded an approximately 540-bp band with all samples tested (data not shown). The ITSs of strains NGRI 0225, 0315, 0404 and 0524, showed 99%, 100% and 100% identity to those of *L. plantarum*, *L. pentosus* and *L. paraplantarum*, respectively, while the ITSs of strains NGRI 0304 and 0305 showed 99%, 95% and 92% identity to the those of *P. pentosaceus*, *P. acidilactici* and *P. damnosus*, respectively. Using the ITS sequences, we constructed a phylogenetic tree, which included four clusters: an *L. plantarum* group, *L. casei* group, *L. buchneri* group and *Pediococcus* group (Figure 2(B)).

The dendrograms for both the V2-V3 and ITS regions did not separate the positions of strains closely related on the evolutionary tree, such as among *L. plantarum*, *L. paraplantarum* and *L. pentosus*, or among *L. casei*, *L. paracasei* and *L. rhamnosus*. The sequences of the 16S rRNA V2-V3 regions of strains NGRI 0101, 0315, 0404, 0524 and 0529 were identical to those of *L. paraplantarum*, *L. pentosus* and *L. plantarum*. However, the isolated strains were able to utilize raffinose, which is not fermented by *L. paraplantarum* (Curk et al. 1996) (Table 2). Therefore, these strains should be *L. pentosus* or *L. plantarum*. The V2-V3 and ITS sequences of the isolates have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB090373 to AB090383 and AB083113 to AB083123, respectively.

Genotyping using RAPD-PCR profiles

RAPD-PCR analyses were performed using single oligonucleotide primers (10 mer). No amplified fragment was generated with the AP-A-6 or AP-A-8 primer (data not shown). On the other hand, the genotypes obtained with the AP-A-2 (Figure 3(A)), AP-A-19 (data not shown) and AP-A-23 (Figure 3(B)) primers were completely consistent with the phenotypes of the isolates. The amplified patterns were detectable in all isolates and reference strains, and RAPD-PCR profiles recognized some clusters. In Figure 3(A), a 0.5-kb fragment (arrowhead a) was amplified in the *L. plantarum* strains JCM 1057 and JCM 1149T (lanes 13

### Table 2 Characteristics of isolated strains

| Strain | NGRI 0001 | NGRI 0101 | NGRI 0110 | NGRI 0130 | NGRI 0225 | NGRI 0304 | NGRI 0305 | NGRI 0315 | NGRI 0404 | NGRI 0510* | NGRI 0524 | NGRI 0529 |
|--------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Shape  | Rods      | Rods      | Rods      | Rods      | Rods      | Cocci     | Coci      | Rods      | Rods      | Rods      | Rods      | Rods      |
| Gas from glucose | - | - | - | + | - | - | + | - | - | - | - | - |
| Growth at 15°C | + | + | + | + | + | + | + | + | + | + | + | + |
| Growth at 45°C | + | - | + | + | + | + | + | + | + | + | + | + |
| Fermentation type | Homo | Homo | Homo | Hetero | Homo | Homo | Hetero | Homo | Homo | Homo | Homo | Homo |
| Lactate formed | DL | DL | L | DL | DL | L | DL | DL | DL | DL | DL | DL |
| Peptidoglycan type | A2pm | A2pm | Non- A2pm | Non- A2pm | A2pm | Non- A2pm | Non- A2pm | A2pm | A2pm | A2pm | A2pm | A2pm |
| Acid from: | | | | | | | | | | | | |
| Na-Gluonate | + | + | + | + | + | + | + | + | + | + | + | + |
| L- Arabinose | + | - | + | + | - | + | + | + | + | + | + | + |
| Lactose | + | + | + | + | - | + | - | + | + | - | + | + |
| Galactose | + | + | + | + | - | + | + | + | + | + | + | + |
| Sucrose | + | + | + | + | - | + | + | + | + | + | + | + |
| Mannose | + | + | + | + | - | + | + | + | + | + | + | + |
| Fructose | + | + | + | + | - | + | + | + | + | + | + | + |
| Soluble starch | - | - | - | - | - | - | - | - | - | - | - | - |
| D-Xylose | + | - | + | - | - | + | + | + | + | + | + | + |
| D-Ribose | + | + | + | + | + | + | + | + | + | + | + | + |
| Mannitol | + | + | + | + | - | + | + | + | + | + | + | + |
| Sorbitol | + | + | + | + | - | + | + | + | + | + | + | + |
| Rhamnose | + | - | + | - | - | + | - | - | - | + | + | + |
| Trehalose | + | + | + | + | - | + | + | + | + | - | + | + |
| Raffinose | + | + | + | + | - | + | + | + | + | - | + | + |
| DNA G+C content (%) | 45.1 | 45.3 | 47.3 | 46.2 | 45.1 | 39.7 | 39.0 | 44.4 | 45.1 | 41.0 | 46.1 | 44.0 |

*Data was obtained from Doi et al (2009).*
and 16) using primer AP-A-2, and equal-sized bands were observed in lanes 2, 3, 5, 8, 9, 11, 12 and 13, suggesting the amplified fragments are common to all *L. plantarum* strains. Although amplification of a 0.3-kb fragment (arrowhead b) was also observed in lanes 6, 7, 8, 9, 11, 12 and 17, no other amplified fragments were identical to *L. pentosus* JCM 1558T (lane 17). Moreover, the profiles of strains NGRI 0304 and 0305 (lanes 6 and 7) were virtually identical to one another, but did not resemble the profiles of any other strains. In Figure 3(B), two major bands, at 1.4 kb (arrowhead c) and 0.4 kb (arrowhead d), appeared in the *L. plantarum* JCM 1057 (lane 13) and *L. plantarum* JCM 1149T (lane 16) profiles obtained using primer AP-A-23. Similar patterns were observed with strains NGRI 0101, 0225, 0315, 0404, 0524 and 0529 (lanes 2, 5, 8, 9, 11 and 12), suggesting these major amplified bands are indicative of the *L. plantarum* group.

**Immuno-identification using anti-lactobacilli antibodies**

The antigenicity of the LAB isolates to anti-*L. plantarum* and anti-*L. casei* antibodies was assessed using an enzyme-linked immunosorbent assay (ELISA). Six samples from silage (NGRI 0101, 0225, 0315, 0404, 0524 and 0529) and three reference samples (*L. plantarum* strains JCM 1057 and JCM 1149T, and *L. pentosus* JCM 1558T) reacted positively with the anti-*L. plantarum* antibody (Figure 4(A)). In addition, one reference sample (*L. casei* JCM 1134T) reacted with the anti-*L. casei* antibody. *L. rhamnosus* JCM 1136T, which is a *L. casei*-related strain, showed a negative response to the antibody (Figure 4(B)). Among the LAB isolates tested, only NGRI 0110 cells showed faint positivity for the anti-*L. casei* antibody. These results suggest that the anti-*L. casei* antibody tested could differentiate between *L. casei* and *L. rhamnosus*, and that there was no strain which could be identified as *L. casei* among the 12 LAB isolates.

**Whole-cell protein fingerprinting**

Whole-cell protein fingerprinting of the 12 isolates and 5 reference strains yielded distinctly different band patterns (Figure 5), with an intragel reproducibility of approximately 90%. Numerical analysis of the SDS-PAGE patterns obtained with the isolated strains resolved 5 clusters (A-E), emerging at a similarity level of 60%, and confirmed
the phenotypic characterization of the isolates. Cluster A includes *L. plantarum* and *L. pentosus*, while cluster C includes *L. casei* and *L. rhamnosus*.

### Analysis of G+C content and DNA-DNA relatedness

The G+C content of the isolates ranged from 39.0% to 47.3% (Table 2). Particularly noteworthy was the finding that the G+C contents of NGRI 0101, 0315, 0404, 0524 and 0529 were all around 45 mol%, which is consistent with those of *L. plantarum*, whereas strains NGRI 0304 and 0305 showed lower G+C contents (less than 40%).

DNA relatedness was analyzed using the isolated strains along with the 6 type strains, *L. casei* JCM 1134T, *L. plantarum* JCM 1149T, *L. rhamnosus* JCM 1136T, *L. pentosus* JCM 1558T, *P. acidilactici* JCM 8797T and *P. pentosaceus* JCM 5890T (Table 3). Levels of DNA relatedness among strains NGRI 0001, 0130 and 0510 and the 5 type strains were lower than 57%. This clearly indicates that these isolates do not belong to the *L. casei* group, *L. plantarum* group or *Pediococcus* species, as these values are well below the 70% threshold for definition of bacterial species (Wayne *et al.* 1987). Strains NGRI 0101, 0315, 0404, 0524 and 0529 all showed high levels of DNA relatedness (> 70%) with *L. plantarum* JCM 1149T, while strains NGRI 0110 and 0225 showed more than 80% DNA relatedness to *L. rhamnosus* JCM 1136T and *L. pentosus* JCM 1558T.

The experimental results summarized above confirmed *L. plantarum* NGRI 0101, *L. rhamnosus* NGRI 0110, *P. pentosus* NGRI 0225, *P. pentosaceus* NGRI 0304, *P. acidilactici* NGRI 0305, *L. plantarum* NGRI 0315, *L. plantarum* NGRI 0404, *L. plantarum* NGRI 0524 and *L. plantarum* NGRI 0529. However, because strains NGRI 0001, NGRI 0130 and NGRI 0510 showed significant similarities to the reference strains used, it seemed best to analyze the complete nucleotide sequences of their 16S rRNA gene.

### Phylogenetic analysis of the complete 16S rRNA gene sequence

The complete nucleotide sequences of the 16S rRNA coding genes were determined for two isolated strains,
NGRI 0001 and NGRI 0130, which were not identified in the experiments summarized above. The full 16S rDNA sequences of the isolates have been deposited in the DDBJ under accession numbers AB362983 and AB362987. Blast analysis revealed that the 16S rRNA gene sequence of strain NGRI 0001 showed similarity with those of *L. buchneri* CD034 (CP003043) (98.64%), *L. buchneri* NRRL B-30929 (CP002652) (98.57%) and *L. buchneri* JCM 1115T (NR_041293) (98.54%), while the sequence of strain NGRI 0130 showed similarity to those of *L. rapi* YIT 11204T (NR_041659) (98.9%), *L. rapi* YIT 11688 (AB366399) (98.9%) and *L. buchneri* CD034 (97.5%) (Figure 6). From these results, strains NGRI 0001 and NGRI 0130 were identified as *L. buchneri* and *L. rapi*, respectively.

**Discussion**

In tropical and subtropical regions, the ensiling process often yields only poor quality silage. Two of the reasons are butyric fermentation by butyric acid bacteria and consumption of the nutrients in the silage by coliform bacteria (Tanaka et al. 1994). Butyric acid is usually produced at high temperatures (30-50°C) by butyric acid bacteria, which have the ability to grow under those conditions. Therefore, to prevent proliferation of butyric acid bacteria and coliform bacteria, silage-making LAB must be capable of rapid growth and high production of lactic acid at high temperatures. Much effort has gone into improving the quality of silage (Weinberg et al. 2007; Lindsey and Kung Jr. 2010; Parvin et al. 2010). In those studies, LABs grown at room temperature showed great ability to improve the quality of silage. However, it was unclear whether strains showing high lactic acid production could grow ahead of butyric acid bacteria or coliform bacteria at higher temperatures (>37°C), which will be necessary for potentially useful candidates to adapt to tropical and subtropical regions. To prepare potential inoculants for ensilage at high temperature, we isolated LABs from silage prepared in southwestern Japan, where the weather can be hot and humid.

The 12 strains of silage-making LABs tested showed distinct phenotypes that were irreconcilable with the reference strains, which included the type strains of *L. plantarum*, *L. pentosus*, *L. casei* and *L. rhamonsus*. 5 strains frequently used in silage preparation and frequently isolated from silage (Filya et al. 2000; Nishino et al. 2004; Okine et al. 2005; Klocke et al. 2006). It was impossible to distinguish among closely related LAB strains (Doi et al. 2003), we were unable to do a complete identification. We therefore attempted to identify the isolated LABs by comparing the sequences of the 16S-23S rRNA ITS and the 16S rRNA V2-V3 region. Both approaches enabled identification of several clusters, including the *L. plantarum* and *L. casei* groups, among others (Figure 2). Although strain NGRI 0110 was distributed to the *L. casei* group based on their ITS sequences, it was impossible to distinguish among *L. casei*, *L. paracasei* and *L. rhamonsus*. Strains NGRI 0304 and 0305, which were grouped with *P. pentosaceus* based on their ITS sequences, were categorized to the *L. plantarum* group based on their V2-V3 region sequences. These results indicate that although we were able to classify the isolated LABs at the genus level, we could not resolve them at the species level.

RAPD-PCR profiles obtained using primers AP-A-2 and AP-A-23 enabled us to distinguish among closely related silage-making LABs when *L. casei*, *L. rhamonsus*, *L. plantarum* and *L. pentosus* were used as references. The RAPD-PCR patterns of *L. plantarum* (lanes 13 and 15 in Figure 3) obtained with primer AP-A-23 were

| Strain         | NGRI 0001 | NGRI 0110 | NGRI 0130 | NGRI 0225 | NGRI 0304 | NGRI 0305 | NGRI 0315 | NGRI 0404 | NGRI 0510 | NGRI 0524 | NGRI 0529 | JCM 1134T | JCM 1136T | JCM 1149T | JCM 1558T | JCM 5885T | JCM 5890T |
|----------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| *L. casei* JCM 1134T | 30.2      | 34.1      | 45.3      | 40.1      | 20.3      | 21.9      | 19.8      | 25.1      | 18.2      | 10.9      | 25.2      | 36.2      | 100       | -         | -         | -         | -         | -         |
| *L. rhamonsus* JCM 1136T | 28.6      | 26.4      | 89.3      | 36.2      | 21.3      | 14.2      | 26.7      | 12.4      | 21.5      | 56.3      | 42.3      | 31.2      | -         | 100       | -         | -         | -         | -         |
| *L. plantarum* JCM 1149T | 32.3      | 83.9      | 10.2      | 15.3      | 25.6      | 18.9      | 21.5      | 90.1      | 82.3      | 12.9      | 79.2      | 84.3      | -         | -         | 100       | -         | -         | -         |
| *L. pentosus* JCM 1558T | 28.3      | 30.2      | 11.2      | 19.3      | 82.9      | 34.2      | 45.6      | 42.3      | 35.2      | 26.3      | 39.1      | 36.8      | -         | -         | 100       | -         | -         | -         |
| *P. pentosaceus* JCM 5890T | 10.8      | 12.8      | 17.6      | 20.3      | 21.6      | 83.1      | 87.1      | 18.1      | 16.2      | 17.3      | 15.9      | 14.3      | -         | -         | -         | -         | 100       | -         |
| *P. acidilactici* JCM 8797T | 15.6      | 14.9      | 17.1      | 20.5      | 12.3      | 16.5      | 23.1      | 13.7      | 18.7      | 19.3      | 18.1      | 23.8      | -         | -         | -         | -         | -         | 100       |

- Not tested.

Identities over 70% were indicated by boldface.
identical, and the patterns in lanes 2, 8, 9, 11 and 12 were identified as L. plantarum. These primers thus show a potential for L. plantarum-specific typing, along with those reported previously (Johansson et al. 1995; Quere et al. 1997). With the 25 primers used in this study, however, we were unable to resolve profiles specific for L. rhamnosus or L. pentosus.

There have been few studies involving the immuno-identification of bacteria using monoclonal antibodies, and those that have been done focused mainly on pathogenic bacteria (Hearty et al. 2006; Klesius et al. 2006). With respect to LABs, only immuno-identification of L. casei strain Shirota in the gastrointestinal tract has been reported (Yuki et al. 1999). In the present study, an anti-L. plantarum monoclonal antibody reacted positively with species belonging to L. plantarum (NGRI 0101, 0315, 0404, 0524, 0529 and JCM 1149T) and L. pentosus (NGRI 0225 and JCM 1558T) (Figure 4(A)). According to the manufacturer, this antibody reacts positively with only L. plantarum among 25 strains of LAB, 2 strains of yeast and 5 other microorganisms; however, its reactivity with L. pentosus has never been tested. From the above results, we suggest this antibody could be used to identify L. plantarum and L. pentosus, and that it is impossible to differentiate between those two closely related species. The cell surfaces of L. plantarum and L. pentosus, which both contain meso-diaminopimelic acid in their peptidoglycan, likely have similar structures and antigens. On the other hand, the anti-L. casei monoclonal antibody tested could distinguish between L. casei JCM 1134T and L. rhamnosus JCM 1136T. Strain NGRI 0110, which should be identified as L. rhamnosus, reacted slightly to this antibody. Differences in the immuno-reactions among the isolates and reference strains were also observed in clustering constructed based on the cell protein profiles (Figure 5).

Figure 6 Phylogenetic trees constructed using the neighbor-joining method with the full-length 16S rRNA gene sequences from the isolated LAB strains and their taxonomic neighbors. The gene sequence of 16S rRNA from E. coli ATCC 11775 (X80725) was used as the outgroup. Bootstrap values based upon 1000 replicates are included at the major branch points. The bars show the number of nucleotide substitutions per site.
could be caused by cell surface reactions similar to those mediating adhesion to intestinal mucus membranes (Ouwehand et al. 1999).

Dendrograms obtained using SDS-PAGE with whole-cell protein extracts from LAB isolates and reference strains have been applied for rapid differentiation of species from fermented sausages, bread, yoghurt, cheese, plants and human faecal samples (Dimitrov et al. 2005; Ricciardi et al. 2005; Benito et al. 2008). When we applied this method to differentiation of silage-making LAB, we found good agreement between the SDS-PAGE profiles and the physiological and genetic characteristics of the LAB tested. There appeared to be obvious differentiation among the L. plantarum group (cluster A), L. casei group (cluster C) and P. pentosaceus group (cluster D), and strains NGRI 0001, 0130 and 0510 were separated into the known groups. Moreover, since strain NGRI 0510 was identified as P. lolií, a novel Pediococcus species (Doi et al. 2009; Doi et al. 2013), its phylogenetic position may be specific.

From the results of phylogenetic analysis using the complete 16S rRNA sequences, strains NGRI 0001 and 0130 were identified as L. buchneri and L. rapi, respectively (Figure 6). Because L. buchneri, L. kefiri, L. parabuchneri and L. rapi are phylogenetically the most closely related species (Watanabe et al. 2009), this result is consistent with the finding that the partial sequence of the 16S rRNA from strain NGRI 0001 was similar with that from L. buchneri and L. kefiri (Figure 2A).

In conclusion, our results show that representative strains isolated from silage produced in hot and humid weather contain six strains belonging to L. plantarum (NGRI 0101, 0315, 0404, 0524 and 0529), L. pentosus (NGRI 0225), L. rhamnosus (NGRI 0110), L. buchneri (NGRI 0001), L. rapi (NGRI 0130) P. pentosaceus (NGRI 0304 and 0305) and P. lolií (NGRI 0510). Although strain NGRI 0315 was identified as L. plantarum, its growth rate was slower than those of L. plantarum strains (Figure 1). Such slow growing strains exhibit greater acid tolerance than rapidly growing strains (data not shown). It therefore seems likely that rapidly growing LABs would produce lactic acid to reduce pH in silage, after which large numbers of cells from acid-tolerant strains living at low pH could competitively inhibit the growth of spoilage microbes, thereby contributing to silage stability. We will report on the application and evaluation of these strains as silage starters adapted to hot and humid weather.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
The work presented here was carried out in collaboration between all authors. KD, SO (Sadahiro Ohmomoto) and SO (Seiya Ogata) defined the research theme. KD, VN and HK designed methods and experiments, carried out the laboratory experiments, analyzed the data, interpreted the results and wrote the paper. KM co-designed the SDS-PAGE profiling experiments, and co-worked on associated data collection and their interpretation. YF co-designed experiments, discussed analyses, interpretation, and presentation. All authors have contributed to, seen and approved the manuscript.

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References
Adnan S, Li N, Miura H, Hashimoto Y, Yamamoto H, Ezaki T (1993) Covalently immobilized DNA plate for luminometric DNA-DNA hybridization to identify viridans streptococci in under 2 hours. FEMS Microbiol Lett 106:139–142
Benito MJ, Serradilla MJ, Ruiz-Moyano S, Martin A, Pérez-Nevado F, Córdoba MG (2008) Rapid differentiation of lactic acid bacteria from autochthonous fermentation of Iberian dry-fermented sausages. Meat Sci 80:656–661
Cai Y, Benno Y, Ogawa M, Kumai S (1999) Effect of applying lactic acid bacteria isolated from forage crops on fermentation characteristics and aerobic deterioration of silage. J Dairy Sci 82:520–526
Curt MC, Hubert JC, Bringel F (1996) Lactobacillus paraparatrum sp. nov., a new species related to Lactobacillus plantarum. Int J Syst Bacteriol 46:595–598
Dimitrov Z, Michaylova M, Mincova S (2005) Characterization of Lactobacillus helveticus strains isolated from Bulgarian yoghurt, cheese, plants and human faecal samples by sodium dodecylsulfate polyacrylamide gel electrophoresis of cell-wall proteins, ribotyping and pulsed field gel fingerprinting. Int Dairy J 15:998–1005
Doi K, Zhang Y, Nishizaki Y, Umeda A, Ohmomo S, Ogata S, Mino K, Otsuka Y (2009) Rapid differentiation of lactic acid bacteria isolated from autochthonous fermentation of Iberian dry-fermented sausages. Meat Sci 80:656–661
Ezaki T, Hashimoto Y, Yabuchi E (1989) Fluorometric deoxyribonucleic acid immobilized DNA plate for luminometric DNA-DNA hybridization to identify viridans streptococci in under 2 hours. FEMS Microbiol Lett 106:139–142
Filya I, Ashbell G, Hen Y, Weinberg ZG (2000) The effect of bacterial inoculants on the fermentation and aerobic stability of whole crop wheat silage. Anim Feed Sci Tech 88:39
Fujino Y, Kawatsu R, Inagaki F, Umeda A, Yokoyama T, Okaue Y, Iwai S, Ogata S, Filya I, Ashbell G, Hen Y, Weinberg ZG (2000) The effect of bacterial inoculants on the fermentation and aerobic stability of whole crop wheat silage. Anim Feed Sci Tech 88:39–46
Giraffa G, Chanishvili N, Widyastuti Y (2010) Importance of lactobacilli in food and feed biotechnology. Res Microbiol 161(6):480–487
Hammes WP, Hertel C (2006) The genera Lactobacillus and Carnobacterium. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (ed) The Prokaryotes, 3rd edition. Springer-Verlag, New-York, pp 320–403
Hearty S, Leonard P, Quinn J, O’Kennedy R (2006) Production, characterisation and potential application of a novel monoclonal antibody for rapid identification of virulent Listeria monocytogenes. J Microbiol Meth 66:294–312
Holzer M, Mayrhuber E, Danner H, Braun R (2003) The role of Lactobacillus buchneri in forage preservation. Trends Biotechnol 21:282–287
Huang CH, Lee FL, Liou JS (2010) Rapid discrimination and classification of the Lactobacillus plantarum group based on a partial dnaA sequence and DNA fingerprinting techniques. Antonie Van Leeuwenhoek 97:289–296
Johansson ML, Quednau M, Molin G, Arnedt S (1995) Randomly amplified polymorphic DNA (RAPD) for rapid typing of Lactobacillus plantarum. Lett Appl Microbiol 21:155–159
Kaneshige N, Zong Y, Yishiyama K, Doi K, Tanaka O, Ohmomo S, Ogata S (1994) Relationship between the quality of Italian ryegrass silage and the presence of phages for silage-making lactobacilli. Nippon Nogeik Kaishi (in Japanese) 68:1219–1221
Klesus P, Evans J, Shoemaker C, Yeh H, Goodwin AE, Adams A, Thompson K (2004) Rapid detection and identification of Streptococcus iniae using a monoclonal antibody-based indirect fluorescent antibody technique. Aquaculture 258:180–189
Klocke M, Mundt K, Ider C, McEnery J, O’Keely P, Barth S (2006) Monitoring Lactobacillus plantarum in grass silages with the aid of 165DNA-based quantitative real-time PCR assays. Synt Appl Microbiol 29:49–58
Kojima S, Takizawa S, Tamura S, Fujinaga S, Benno Y, Nakase T, (1993) A new medium for the isolation of lactobacilli from kefir grains. Biosci Biotech Bioch 57:119–120
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
Lindsey JR, Kung L, Jr (2010) Effects of combining Lactobacillus buchneri 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. Anim Feed Sci Technol 159:105–109
Mulrooney CN, Kung L, Jr (2008) The effect of water temperature on the viability of silage inoculants. J Dairy Sci 91:236–240
Namihira T, Shinzato N, Akamine H, Maekawa H, Matsui T (2010) Influence of nitrogen fertilization on tropical-grass silage assessed by ensiling process monitoring using chemical and microbial community analyses. J Appl Microbiol 108:1954–1965
Nishino N, Wada H, Yoshida M, Shiota H (2004) Microbial counts, fermentation products, and aerobic stability of whole crop corn and a total mixed ration ensiled with and without inoculation of Lactobacillus casei or Lactobacillus buchneri. J Dairy Sci 87:2563–2570
Ohmomo S, Tanaka O, Kitamoto HK, Cai Y (2002) Silage and microbial performance, old story but new problems. JARQ 36:59–71
Ohkawara A, Hanada M, Aibobrba Y, Okamoto M (2005) Ensiling of potato pulp with or without bacterial inoculants and its effect on fermentation quality, nutrient composition and nutritive value. Anim Feed Sci Technol 121:329–343
Ouboi LB, Nyanga-Koumou CA, Parkouda C, Sawadogo H, Kobawila SC, Idler C, McEniry J, Olliver MA, Torriani S, Felis GE, Dellaglio F (2001) Differentiation of Parvin S, Wang C, Li Y, Nishino N (2010) Effects of inoculation with lactic acid bacteria isolated from African traditional alkaline-fermented foods. J Appl Microbiol 98:159–166
Ouiba LI, Parkouda C, Sawadogo H, Kobawila SC, Idler C, McEniry J, Olliver MA, Torriani S, Felis GE, Dellaglio F (2001) Differentiation of Parvin S, Wang C, Li Y, Nishino N (2010) Effects of inoculation with lactic acid bacteria isolated from African traditional alkaline-fermented foods. J Appl Microbiol 98:159–166
Parvin S, Wang C, Li Y, Nishino N (2010) Effects of inoculation with lactic acid bacteria on the bacterial communities of Italian ryegrass, whole crop maize, guinea grass and Rhodes grass silages. Anim Feed Sci Technol 160:160–166
Quere E, Deschamps A, Urdaci MC (1997) DNA probe and PCR-specific reaction for Lactobacillus plantarum. J Appl Microbiol 82:783–790
Riccardi A, Parente E, Pirano P, Paraggio M, Romano P (2005) Phenotypic characterization of lactic acid bacteria from sourdoughs for Altamura bread produced in Apulia (Southern Italy). Int J Food Microbiol 98:63–72
Tanaka O, Kimura H, Takahashi E, Ogata S, Ohmomo S (1994) Screening of lactic acid bacteria for silage inoculants by using a model system of silage fermentation. Biosci Biotech Bioch 58:1412–1415
Tannock GW, Tikala-Timisjarvi A, Rodtrong S, NG J, Munro K, Akatsbasa T (1999) Identification of Lactobacillus isolates from the gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons. Appl Environ Microbiol 65:4264–4267
Tomi S, Felis GE, Dellaglio F (2001) Differentiation of Lactobacillus plantarum, L. pentosus, and L. paraplantarum by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. Appl Environ Microbiol 67:3450–3454
Watanabe K, Fujimoto J, Tomi Y, Sasamoto M, Makino H, Kudo Y, Okada S (2009) Lactobacillus kisonensis sp. nov., Lactobacillus otakensis sp. nov., Lactobacillus rapi sp. nov. and Lactobacillus sunki sp. nov., heterofermentative species isolated from sunki, a traditional Japanese pickle. Int J Syst Evol Microbiol 59:754–760
Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Truper HG (1987) International committee on systematics bacteriology. Report of the ad hoc committee on reorganization of approaches to bacterial systematics. Int J Syst Microbiol 37:463–464
Weinberg ZG, Shatz O, Chen Y, Yosef E, Nikbahat M, Men-Ghedalia D, Miron J (2007) Effect of lactic acid bacteria inoculants on in vitro digestibility of wheat and corn silages. J Dairy Sci 90:4754–4762
Yan PM, Xuea WT, Tan SS, Zhang H, Chang XH (2008) Effect of inoculating lactic acid bacteria starter cultures on the nitrite concentration of fermenting Chinese paocai. Food Control 19:50–55
Yuki N, Watanabe K, Mike A, Taigami Y, Tanaka R, Ohwaki M, Morotomi M (1999) Survival of a probiotic, Lactobacillus casei strain Shirotia, in the gastrointestinal tract: selective isolation from faeces and identification using monoclonal antibodies. Int J Food Microbiol 48:51–57