

Phylogenetic Diversity of Lactic Acid Bacteria Associated with Paddy Rice Silage as Determined by 16S Ribosomal DNA Analysis

Said Ennahar, Yimin Cai,* and Yasuhito Fujita

National Agricultural Research Organization, National Institute of Livestock and Grassland Science,
Nishinasuno-machi, Tochigi-ken 329-2793, Japan

Received 3 June 2002/Accepted 6 October 2002

A total of 161 low-G+C-content gram-positive bacteria isolated from whole-crop paddy rice silage were classified and subjected to phenotypic and genetic analyses. Based on morphological and biochemical characters, these presumptive lactic acid bacterium (LAB) isolates were divided into 10 groups that included members of the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella*. Analysis of the 16S ribosomal DNA (rDNA) was used to confirm the presence of the predominant groups indicated by phenotypic analysis and to determine the phylogenetic affiliation of representative strains. The virtually complete 16S rRNA gene was PCR amplified and sequenced. The sequences from the various LAB isolates showed high degrees of similarity to those of the GenBank reference strains (between 98.7 and 99.8%). Phylogenetic trees based on the 16S rDNA sequence displayed high consistency, with nodes supported by high bootstrap values. With the exception of one species, the genetic data was in agreement with the phenotypic identification. The prevalent LAB, predominantly homofermentative (66%), consisted of *Lactobacillus plantarum* (24%), *Lactococcus lactis* (22%), *Leuconostoc pseudomesenteroides* (20%), *Pediococcus acidilactici* (11%), *Lactobacillus brevis* (11%), *Enterococcus faecalis* (7%), *Weissella kimchii* (3%), and *Pediococcus pentosaceus* (2%). The present study, the first to fully document rice-associated LAB, showed a very diverse community of LAB with a relatively high number of species involved in the fermentation process of paddy rice silage. The comprehensive 16S rDNA-based approach to describing LAB community structure was valuable in revealing the large diversity of bacteria inhabiting paddy rice silage and enabling the future design of appropriate inoculants aimed at improving its fermentation quality.

Using rice silage as an animal feed has proven economically viable, not only as a way of disposing of rice straw residues but also as a real alternative for feeding livestock in regions where rice is the main crop (13). As a result, in Japan and other rice-producing countries, rice is no longer grown exclusively for human consumption but increasingly as a valuable forage crop. Forage rice is in fact believed to be an ideal alternative crop, not only in helping farmers adjust grain rice production but also in preserving the soil, leading to long-term utilization of the paddy field. Yet a major drawback of forage rice is that it yields low-quality silage, due to poor digestibility of nutrients, mostly crude proteins (40). Several processes have been developed to improve the fermentation and nutritional value of whole-crop silage from forage paddy rice. Breeding programs are carried out, and newly developed rice varieties with increased yield and amount of digestible nutrients are being grown and tested. Also, harvesting, preparation, and storage techniques are constantly being improved. However, most of the processes used to date still rely on heavy chemical treatments with ammonia and sodium hydroxide (10, 20, 23, 40, 41) and were reported to reduce the palatability of silage to ruminants (23).

Of the many factors that can affect silage fermentation, the type of microorganisms that dominate the process often dictates the final quality of the silage. For instance, homolactic fermentation by lactic acid bacteria (LAB) is more desirable

than other types of fermentation because the theoretical recoveries of dry matter and energy are greatest. During this type of fermentation, LAB utilize water-soluble carbohydrates (WSC) to produce lactic acid, the primary acid responsible for decreasing the pH in silage. In contrast, other fermentations are less efficient. Natural populations of LAB on plant material are often low in number and heterofermentative. Thus, the concept of using a microbial inoculant to silage involves adding fast-growing homofermentative LAB in order to dominate the fermentation, thereby producing higher-quality silage. Some of the commonly used homofermentative LAB in silage inoculants include *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Pediococcus acidilactici*, and *Enterococcus faecium*. Commercially available microbial inoculants contain one or more of these bacteria that have been selected for their ability to dominate the fermentation.

From a microbiological point of view, to our knowledge, no information is available on the microbial ecology of paddy rice silage (PRS), especially with regard to the indigenous LAB and their effects during the fermentation process. If identified, the LAB inhabiting PRS could be evaluated for growth kinetics and ability to decrease pH during forage paddy rice fermentation. Selected strains could be examined for their competitiveness as PRS inoculants compared to commercial inoculants. Ultimately, efficacious inoculants could be identified that would lead to an optimization of the microbial action, preserve a high level of crop nutrients, allow chemical treatments to be reduced, and consequently produce a palatable PRS with a high intake potential.

The present study set out to screen, isolate, and identify the

* Corresponding author. Mailing address: National Institute of Livestock and Grassland Science, Nishinasuno, Tochigi 329-2793, Japan. Phone: 81-287-377-804. Fax: 81-287-366-629. E-mail: cai@affrc.go.jp.

LAB colonizing PRS at various stages of fermentation, taking particular interest in species that are likeliest to play an important role in the fermentation process. Isolates were identified biochemically, and selected representative strains were identified at the molecular level using 16S rDNA sequence analysis.

MATERIALS AND METHODS

Silage samples and bacterial isolates. Whole-crop rice (*Oryza sativa* var. Hamasari) grown in a local paddy field (Ohtawara, Tochigi, Japan) was harvested at ripe stage. Silage was prepared in a small-scale fermentation system using the method of Tanaka and Ohmomo (35) modified as previously described (3). A total of 40 silage samples were collected at days 2, 10, 30, and 50 of the ensiling process. The samples (10 g) were blended with 90 ml of sterilized distilled water. Serial dilutions were used for isolation of LAB by using plate count agar (Difco Laboratories, Detroit, Mich.) with bromocresol purple and GYP (glucose, yeast extract, and peptone) agar (17) and for isolation of other microorganisms using the corresponding media as previously described (4, 5). Each suspected LAB colony was purified twice by streaking on MRS agar. The pure cultures were grown on MRS agar at 30°C for 24 h, before being transferred to nutrient broth (Difco) with 10% glycerol and stored as stock cultures at -80°C for further analysis.

Morphological, physiological, and biochemical tests. Morphological characteristics and Gram staining of LAB were examined after 24 h of incubation on MRS agar. Catalase activity and gas production from glucose were determined by the methods of Kozaki et al. (17). All PRS isolates assigned to a particular LAB genus or species were identified on API 50 CH strips (bioMérieux, Tokyo, Japan) by using carbohydrate assimilation and fermentation of 49 different compounds (and one control). These were incubated at 30°C for 3 to 6 days. The organisms were identified by using the APILAB Plus software version 3.3.3 from bioMérieux and Analytab Products' computer database for comparison of assimilation and/or fermentation patterns.

DNA base composition and sequencing of the 16S ribosomal DNA (rDNA). Cells grown for 8 h in MRS broth at 30°C were used for DNA extraction and purification as described by Saito and Miura (25). DNA base composition was determined, following enzymatic digestion of DNA to deoxyribonucleosides, by the method of Tamaoka and Komagata (34) using high-performance liquid chromatography. The equimolar mixture of four deoxyribonucleotides in a Yamaoka GC Kit (Yamaoka Shoyu Co., Ltd., Choshi, Japan) was used as the quantitative standard. Amplification of the 16S rRNA gene was carried out in a ThermalCycler (GeneAmp PCR System 9700; PE Applied Biosystems, Foster City, Calif.) using the PCR method described by Suzuki et al. (33) and reagents from a Takara Taq PCR Kit (Takara Shuzo Co., Ltd., Otsu, Japan). Sequencing was performed twice on both strands by the dideoxy method of Sanger et al. (28), using a PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) in combination with an Applied Biosystems model 310A automated sequencing system.

Sequence alignments and phylogenetic inference. Sequence similarity searches were performed in the GenBank data library using the BLAST program. The sequence information was then imported into the CLUSTAL W software program (Hitachi Software Engineering Co.) for assembly and alignment. The 16S rDNA sequences of RO strains were compared to sequences from type LAB strains held in GenBank (Fig. 1 and 2). Nucleotide substitution rates were calculated (15), and phylogenetic trees were constructed by the neighbor-joining method (26). *Bacillus subtilis* NCDO 1769 was used as an outgroup organism. The topologies of trees were evaluated by bootstrap analysis of the sequence data with CLUSTAL W software based on 100 random resamplings (36).

Nucleotide sequence accession numbers. The nucleotide sequences for the 16S rDNA described in this report were deposited with GenBank under accession no. AF515219, AF515220, AF515221, AF515222, AF515223, AF515224, AF515225, AF515227, AF515228, and AF515229 for the strains RO97, RO66, RO5, RO7, RO90, RO6, RO3, RO95, RO1, and RO17, respectively.

RESULTS AND DISCUSSION

PRS is an increasingly important source of animal feed in Japan and other rice-producing countries. Yet, while LAB in several forage crops and silages have been more or less described (2, 4, 6, 19, 22, 24, 32), a similar study has yet to be

carried out on PRS. This provides a unique opportunity to study and document the composition of the microbial flora inhabiting PRS and its effects on the fermentation process of the crop. In the present investigation, 400 PRS isolates were screened, of which 161 isolates were considered to be LAB as determined by culture on MRS agar, Gram stain appearance, catalase test, and gas production from glucose. Fifty percent of the presumptive LAB were characterized further by sugar fermentation assays using API 50 CH strips. This led to the delineation of 10 groups of isolates, each one displaying a distinct carbohydrate fermentation pattern (Table 1). Groups A to E included cocci that did not produce gas from glucose, while group F was represented by heterofermentative cocci. Groups G through J included rods, with those in group G being homofermentative and the others being heterofermentative. The various groups presumably represented six different LAB genera: *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Weissella*, *Enterococcus*, and *Leuconostoc*, which accounted for various percentages of the total. This was in part confirmed by DNA base composition analysis, which showed that most strains had low G+C content within the range of the corresponding genera (Table 2): RO6 and RO3 from groups A and B with, respectively, 38.0 and 36.9 mol% (*Lactococcus* range: 38 to 40 mol%); RO95 and RO17 from groups C and D with, respectively, 39.3 and 44.4 mol% (*Pediococcus* range: 34 to 43 mol%); RO90 from group E with 39.2 mol% (*Enterococcus* range: 37 to 45 mol%); RO1 from group F with 40.8 mol% (*Leuconostoc* range: 38 to 44 mol%); RO7, RO66 and RO97 from groups G, I, and J with, respectively, 45.5, 48.0, and 46.1 mol% (*Lactobacillus* range: 32 to 53% mol%); and RO5 from group H with 48.5 mol% (*Weissella* range: 37 to 48% mol%).

In an effort to identify PRS LAB isolates at the species level, molecular phylogeny analysis was conducted and phylogenetic trees were constructed based on the 16S rDNA sequences from evolutionary distances by the neighbor-joining method. Following phylogenetic analysis, strains RO95 and RO17, respectively, representative of groups C and D, were placed in the cluster making up the genus *Pediococcus*, which, with the exception of *Pediococcus urinaeequi*, was recovered in 100% of bootstrap analyses (Fig. 1). They both formed a distinct cluster together with *P. acidilactici* and *Pediococcus pentosaceus*, supported with a bootstrap value of 100%. While RO95 was monophyletic with *P. pentosaceus* with a bootstrap of 100%, RO17 grouped with *P. acidilactici* in a 99% bootstrap cluster. The group E strain RO90 was placed in the enterococcal cluster on the phylogenetic tree, with *E. faecalis* being the most closely related species in 100% of bootstrap analyses (Fig. 1). RO1, the representative strain of group F, was unambiguously enclosed in the *Leuconostoc* phylogenetic group, which is very well delimited with a bootstrap of 100%. The strain distinctly clustered with the species *Leuconostoc pseudomesenteroides*, as indicated by the high bootstrap value (96%). The representative strains of groups A (RO6) and B (RO3) were clearly identified as *Lactococcus lactis* strains by forming a very well-defined cluster (100% bootstrap) with this species (Fig. 1). Furthermore, both strains were ascribed to the subspecies *lactis* on the phylogenetic tree, with a bootstrap of 99% supporting monophyly. Nevertheless, as reported by Stackebrandt and Goebel (31), the 16S rDNA sequence analysis method, while very good at identifying the organisms by genus and species,

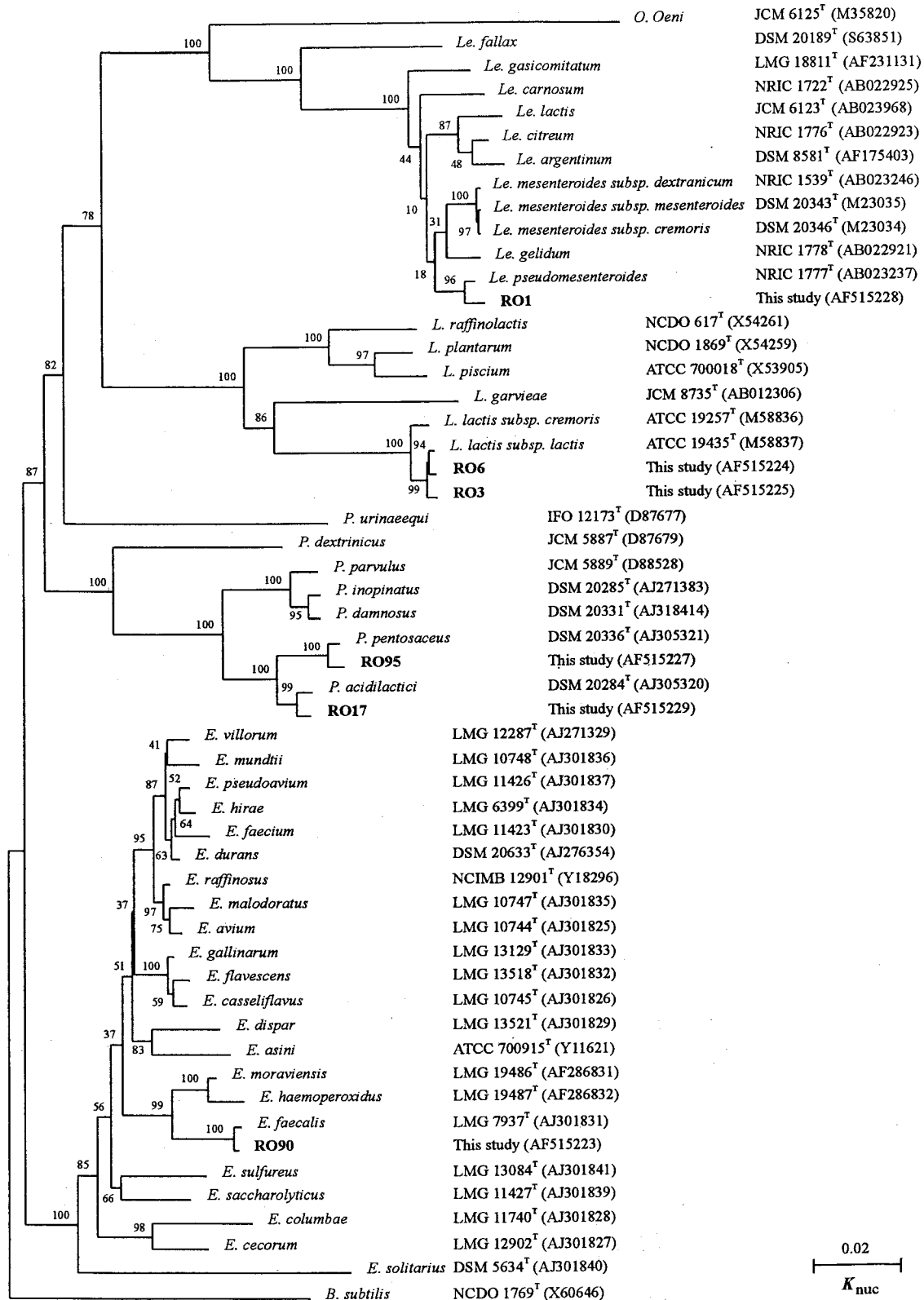


FIG. 1. Phylogenetic tree showing the relative positions of PRS isolates RO1, RO3, RO6, RO17, RO90, and RO95 as inferred by the neighbor-joining method of complete 16S rDNA sequences. Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. References of the type strains used for comparison are given, as well as the accession numbers for all 16S rDNA sequences (between brackets). *B. subtilis* is used as an outgroup. The bar indicates 2% sequence divergence. *E.*, *Enterococcus*; *L.*, *Lactococcus*; *Le.*, *Leuconostoc*; *O.*, *Oenococcus*; and *P.*, *Pediococcus*. K_{nuc} , nucleotide substitution rates.

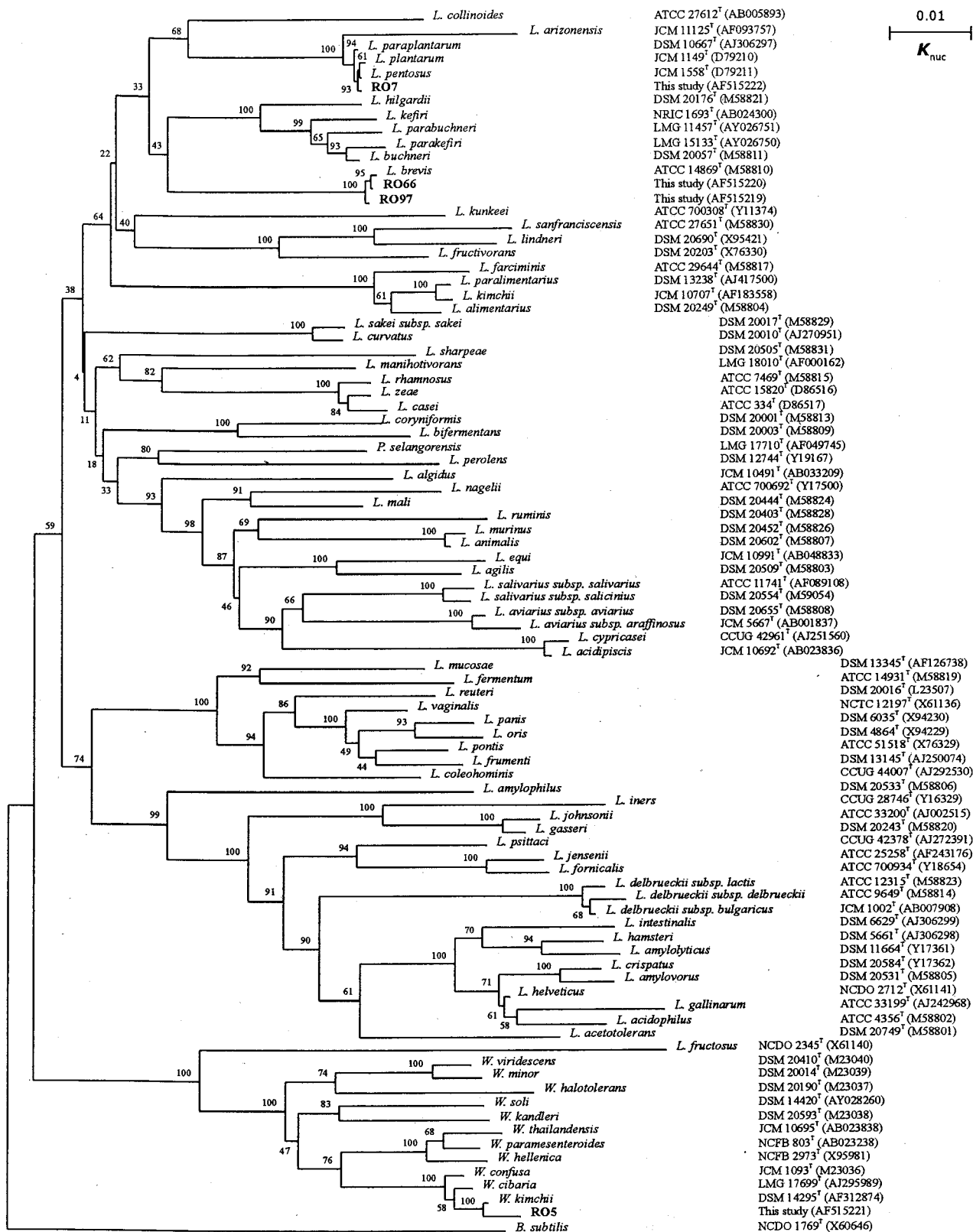


FIG. 2. Phylogenetic tree showing the relative positions of PRS isolates RO5, RO7, RO66, and RO97 as inferred by the neighbor-joining method of complete 16S rDNA sequences. Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. References of the type strains used for comparison are given, as well as the accession numbers for all 16S rDNA sequences (between brackets). *B. subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *L.*, *Lactobacillus*; *P.*, *Paralactobacillus*; and *W.*, *Weissella*. K_{nuc} , nucleotide substitution rates.

TABLE 1. Phenotypic characteristics of LAB isolated from PRS^a

| Characteristic or sugar assayed | Group | | | | | | | | | |
|---------------------------------|-------|-------|-------|-------|-------|--------|------|--------|--------|--------|
| | A | B | C | D | E | F | G | H | I | J |
| No. of isolates | 14 | 21 | 4 | 18 | 11 | 32 | 39 | 4 | 11 | 7 |
| Representative strain | RO6 | RO3 | RO95 | RO17 | RO90 | RO1 | RO7 | RO5 | RO66 | RO97 |
| Shape | Cocci | Cocci | Cocci | Cocci | Cocci | Cocci | Rods | Rods | Rods | Rods |
| Gram stain | + | + | + | + | + | + | + | + | + | + |
| Fermentation type | Homo | Homo | Homo | Homo | Homo | Hetero | Homo | Hetero | Hetero | Hetero |
| Catalase | - | - | + | - | - | - | - | - | - | - |
| Glycerol | - | - | - | - | + | - | - | - | - | - |
| Erythritol | - | - | - | - | - | - | - | - | - | - |
| D-Arabinose | - | - | - | - | - | - | - | - | - | - |
| L-Arabinose | + | - | + | + | - | + | + | + | + | + |
| Ribose | + | + | + | + | + | + | + | + | + | + |
| D-Xylose | + | + | + | - | - | + | - | + | + | + |
| L-Xylose | - | - | - | - | - | - | - | - | - | - |
| Adonitol | - | - | - | - | - | - | - | - | - | - |
| β-Methyl-xyloside | - | - | - | - | - | - | - | - | + | - |
| Galactose | + | + | + | + | + | + | + | - | + | + |
| D-Glucose | + | + | + | + | + | + | + | + | + | + |
| D-Fructose | + | + | + | + | + | + | + | + | + | + |
| D-Mannose | + | + | + | + | + | + | + | + | - | - |
| L-Sorbose | - | - | - | - | - | - | - | - | - | - |
| Rhamnose | - | - | - | - | w | - | - | - | - | - |
| Dulcitol | - | - | - | - | - | - | - | - | - | - |
| Inositol | - | - | - | - | w | - | - | - | - | - |
| Mannitol | + | + | - | - | + | - | + | - | - | - |
| Sorbitol | - | - | - | - | + | - | + | - | - | - |
| α-Methyl-D-mannoside | - | - | - | - | - | - | + | - | - | - |
| α-Methyl-D-glucoside | - | - | - | - | - | + | - | - | + | + |
| N-Acetyl glucosamine | + | + | + | + | + | + | + | + | - | + |
| Amygdalin | + | + | + | + | + | - | + | + | - | - |
| Arbutin | + | + | + | + | + | + | + | + | - | - |
| Esculin | + | + | + | + | + | + | + | + | - | + |
| Salicin | + | + | + | + | + | + | + | + | - | - |
| Cellobiose | + | + | + | + | + | + | + | + | - | - |
| Maltose | + | + | + | - | + | + | + | + | + | + |
| Lactose | + | + | - | - | + | - | + | - | - | - |
| Melibiose | - | - | - | - | - | + | + | - | + | - |
| Saccharose | + | + | - | - | + | + | + | + | - | - |
| Trehalose | + | + | + | - | + | + | + | - | - | - |
| Insulin | - | - | - | - | - | - | - | - | - | - |
| Melezitose | - | - | - | - | + | - | + | - | - | - |
| D-Raffinose | - | - | - | - | - | + | + | - | - | - |
| Starch | w | w | - | - | w | - | - | - | - | - |
| Glycogen | - | - | - | - | - | - | - | - | - | - |
| Xylitol | - | - | - | - | - | - | - | - | - | - |
| β-Gentiobiose | + | + | + | w | + | + | + | - | - | - |
| D-Turanose | - | - | - | - | - | + | + | - | - | - |
| D-Lyxose | - | - | - | - | - | - | - | - | - | - |
| D-Tagatose | - | - | + | + | + | - | - | - | - | - |
| D-Fucose | - | - | - | - | - | - | - | - | - | - |
| L-Fucose | - | - | - | - | - | - | - | - | - | - |
| D-Arabitol | - | - | - | - | - | - | w | - | - | - |
| L-Arabitol | - | - | - | - | - | - | - | - | - | - |
| Gluconate | w | w | w | - | + | + | + | w | w | w |
| 2-Ceto-gluconate | - | - | w | - | - | w | - | - | - | - |
| 5-Ceto-gluconate | - | - | - | - | - | + | - | - | - | w |

^a +, positive; -, negative; w, weakly positive; Homo, homofermentative; Hetero, heterofermentative.

cannot differentiate strains at the subspecies level and is therefore not the appropriate method to measure intraspecies relationships. Despite clustering together on the phylogenetic tree, RO3 and RO6 consistently showed discrepancy with regard to L-arabinose utilization (Table 1) and their 16S rDNA sequences were different even though by only 7 bp. In the same way, strains RO66 and RO97 from groups I and J, which were

both phylogenetically identified (100% bootstrap) as *L. brevis* (Fig. 2), were shown to display marked differences in four fermentation reactions (Table 1). The group G strain RO7 was clearly assigned to the genus *Lactobacillus*, since it grouped on the phylogenetic tree together with *L. pentosus*, *L. plantarum*, *L. paraplantarum*, and *L. arizonensis* in a 100% bootstrap cluster (Fig. 2). Furthermore, RO7 appeared to be equally linked

TABLE 2. G+C content of LAB isolated from PRS

| Group | Representative strain | G+C (%) |
|-------|-----------------------|---------|
| A | RO6 | 38.0 |
| B | RO3 | 36.9 |
| C | RO95 | 39.3 |
| D | RO17 | 44.4 |
| E | RO90 | 39.2 |
| F | RO1 | 40.8 |
| G | RO7 | 45.5 |
| H | RO5 | 48.5 |
| I | RO66 | 48.0 |
| J | RO97 | 46.1 |

(93% bootstrap) to both *L. plantarum* and *L. pentosus* and its 16S rDNA sequence showed a similarity of 99.7% to *L. plantarum* and 99.8% to *L. pentosus*. This is not surprising, given that the two species themselves have very similar 16S rDNA sequences that differ only by 2 bp. In fact, it is widely acknowledged that *L. plantarum* and *L. pentosus* belong to the same 16S rRNA phylogenetic group and could only be distinguished using phylogenetic analysis of sequences of the 16S-23S large spacer region (12) or partial sequences of the *recA* gene (37). In the present study, we referred to carbohydrate fermentation patterns that showed unambiguously that the pattern of strain RO7 was an exact match of that of the *L. plantarum* type strain and different from that of the *L. pentosus* type strain. In fact, it appeared that, while the *L. plantarum* type strain produced acid from melezitose, D-raffinose, and α -methyl-D-mannoside, the *L. pentosus* type strain did not. On the other hand, as opposed to *L. plantarum*, *L. pentosus* could grow using glycerol or D-xylose as a carbon source (data not shown). All other isolates from group G were similarly identified as *L. plantarum*. Strain RO5, which is representative of group H, was placed within the *Weissella* phylogenetic group, which is supported by a 100% bootstrap value (Fig. 2). The two newly described species *Weissella cibaria* (1) and *Weissella kimchii* (7), were the nearest phylogenetic relatives to RO5, with similarities exceeding 99%. On the phylogenetic tree RO5 formed, however, a well-defined cluster with *W. kimchii*, while *W. cibaria* appeared to be a less related species.

Phylogenetic trees based on the 16S rDNA sequence displayed high consistency regarding the relationships between the organisms included. All the nodes leading to RO strain clusters are supported by high bootstrap values and should be considered significant. Also, RO strains were designated to the correct species with close homology. In fact, BLAST search analyses using the whole 16S rDNA sequence resulted in identifications above the 99% level, although in two instances (RO1 and RO95) it was slightly lower. All homologies displayed were, however, well above 97%, which is considered to be the cutoff value indicating species identity (31). On the other hand, genetic characterization of PRS isolates correlated well with their biochemical classification, which was obtained at percentages of identification of more than 99% using the APILAB Plus computer program. Only strain RO1 (*Leuconostoc pseudomesenteroides*) was biochemically misidentified as *Leuconostoc mesenteroides*, and strain RO5 (*W. kimchii*) could not be identified biochemically to the species level.

Following biochemical and phylogenetic analyses, PRS iso-

lates fell within well-recognized groups of LAB and, for the majority of them, were clearly related to particular species. Most of the LAB characterized belonged to the genera *Lactobacillus*, *Lactococcus*, and *Leuconostoc*, with *L. plantarum* (24%), *L. lactis* (22%), and *L. pseudomesenteroides* (20%) totaling two-thirds of the entire LAB flora. Yet a great species diversity was observed since five other species were also identified: *P. acidilactici* (11%), *L. brevis* (11%), *E. faecalis* (7%), *W. kimchii* (3%), and *P. pentosaceus* (2%). Furthermore, homofermentative species accounted for two-thirds (66%) of the total LAB microflora.

LAB species identified in PRS are common inhabitants of a variety of forage crops and silages. This is in agreement with the results of our previous investigations (2, 4, 6), as well as those of other authors (19, 22, 24, 32), which showed that the natural fermentation processes in crop silages, including corn, sorghum, alfalfa, Italian ryegrass, alfalfa, and guinea grass silages, are dominated by species of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Lactococcus*. Particularly, the prevalent species in PRS, *L. plantarum*, is reportedly the most dominant during the fermentation of crop silage (6). Other LAB, such as leuconostocs and pediococci, have often been found at low frequencies together with *Weissella* strains on plant material and in various silages (4, 16, 19). They are also widely used as starter cultures or control barriers for food pathogens in vegetables (38). The presence of *E. faecalis* suggests a fecal origin of some PRS microorganisms. The natural habitat of lactococci is milk, but *L. lactis* subsp. *lactis* has been isolated previously from plants, vegetables, and cereals (9, 27).

Silage fermentation begins with aerobic respiration immediately upon harvest of the plant. During this phase, WSC are converted to CO₂, heat, and water by both plant cells and aerobic microbes (4, 19). This process will continue until the oxygen is depleted. Once anaerobic conditions are established, the anaerobic fermentation begins with the growth of heterofermentative LAB. More nutrients are retained in the silage when this phase is rapidly completed. As the pH of the ensiled mass falls below 5, the growth of heterofermentative bacteria is inhibited and there is a shift towards increasing populations of homofermentative LAB (4, 19). Rapid growth and acidification by these bacteria are the basic aim of the silage fermentation so that sugars are efficiently converted to lactic acid, leading to suppression of nonbeneficial microbes (by both direct competition and lowering of the pH). This ultimately leads to improvements in preservation of plant proteins in a digestible form, in aerobic stability of the silage during the feedout phase, and in nutritional quality in relation to intake levels and livestock productivity (8). However, the rate and extent of final pH in the ensiled forage depend largely on the type and moisture of the forage being ensiled. As opposed to commonly ensiled crops, such as corn, with paddy rice, low WSC levels (1 to 4%) and a high buffering capacity are less conducive to rapid acidification by epiphytic LAB populations and hence to adequate ensilage in the absence of additives (10, 13, 23, 40). Moreover, under farm conditions, the populations of epiphytic LAB are not always large enough or do not have a composition suitable for promoting efficient homolactic fermentation (11). Thus, efforts to obtain PRS that has higher nutritional value and good storage properties have to be pursued with the aim of

developing additives that stimulate and direct the fermentation process.

Many lab-scale fermentation studies have been conducted to evaluate the effect that inoculants have on the fermentation of various crops. In general, favorable improvements in the rate of pH decline and increased lactic acid levels have been noted with legumes, grasses, and cereal silages. In previous investigations (3, 4, 6), we showed that the inoculation of various forages with homofermentative *Lactobacillus* spp. (predominantly *L. plantarum*), in combination with *Pediococcus*, *Enterococcus*, or *Lactococcus* spp., had beneficial effects, which is in agreement with other authors' studies (14, 29, 30, 39). In these studies, selective inoculants have been shown to (i) hasten the fermentation process and reduce nutrient loss, (ii) improve fiber digestibility, and (iii) reduce protein degradation, all of which yield silage with higher nutritional value. However, a recent literature review found that only a few of the published trials reported positive effect from silage inoculation, with only 28% of studies showing an increase in silage intake and only 47% showing an increase in milk production (18). Given this scientific evaluation of a 1-in-4 chance of intake improvement and a 50:50 chance of improved animal performance, it is not surprising that many producers remain unconvinced that silage inoculants can offer any real economic benefit.

The effectiveness of silage inoculants depends, among other things, upon the quality (growth rate and environmental adaptability) and quantity of the microorganisms used. Research trials have in fact shown that individual strains differ in their crop preference, ability to ferment various substrates, growth potential in various moistures and temperatures, and ability to enhance fiber digestibility (19, 21, 22). However, since most research on silage fermentation is of a strategic or applied nature, little information is available on the complex microbial and biochemical processes involved. As far as PRS is concerned, it is safe to assume that homofermentative LAB isolated in this study are adapted to the particular ecosystem of rice fermentation and could therefore be suitable as inoculants for silage making from paddy rice. For instance, the greater frequencies of recovery of *L. plantarum* and *P. acidilactici* strains from PRS samples than from paddy rice forage samples (data not shown) suggested that these species participated actively in the fermentation process. Trials are on the way in our laboratory in order to develop suitable crop-specific additive combinations by screening potentially useful PRS homofermentative LAB strains for desirable characteristics in laboratory media and then performing ensilage experiments. In the presence of an adequate supply of available nutrients at an early stage, the selected competitive LAB strains should be able to compete with the epiphytic PRS microflora and ensure a highly cost-effective means of improving the quality of silage.

REFERENCES

- Bjorkroth, K. J., U. Schillinger, R. Geisen, N. Weiss, B. Hoste, W. H. Holzapfel, H. J. Korkeala, and P. Vandamme. 2002. Taxonomic study of *Weissella confusa* and description of *Weissella cibaria* sp. nov., detected in food and clinical samples. *Int. J. Syst. Evol. Microbiol.* **52**:141–148.
- Cai, Y., S. Ohmomo, and S. Kumai. 1994. Distribution and lactate fermentation characteristics of lactic acid bacteria on forage crops and grasses. *J. Jpn. Grassl. Sci.* **39**:420–428.
- Cai, Y., S. Ohmomo, M. Ogawa, and S. Kumai. 1997. Effect of NaCl-tolerant lactic acid bacteria and NaCl on the fermentation characteristics and aerobic stability of silage. *J. Appl. Microbiol.* **83**:307–313.
- Cai, Y., Y. Benno, M. Ogawa, S. Ohmomo, S. Kumai, and T. Nakase. 1998. Influence of *Lactobacillus* spp. from an inoculant and of *Weissella* and *Leuconostoc* spp. from forage crops on silage fermentation. *Appl. Environ. Microbiol.* **64**:2982–2987.
- Cai, Y., S. Kumai, M. Ogawa, Y. Benno, and T. Nakase. 1999. Characterization and identification of *Pediococcus* species isolated from forage crops and their application for silage preparation. *Appl. Environ. Microbiol.* **65**:2901–2906.
- Cai, Y., S. Ohmomo, M. Ogawa, and S. Kumai. 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.
- Choi, H. J., C. I. Cheigh, S. B. Kim, J. C. Lee, D. W. Lee, S. W. Choi, J. M. Park, and Y. R. Pyun. 2002. *Weissella kimchii* sp. nov., a novel lactic acid bacterium from kimchi. *Int. J. Syst. Evol. Microbiol.* **52**:507–511.
- Davies, Z. S., R. J. Gilbert, R. J. Merry, D. B. Kell, M. K. Theodorou, and G. W. Griffith. 2000. Efficient improvement of silage additives by using genetic algorithms. *Appl. Environ. Microbiol.* **66**:1435–1443.
- De Vuyst, L., and E. J. Vandamme. 1994. Antimicrobial potential of lactic acid bacteria, p. 91–141. *In* L. De Vuyst and E. J. Vandamme (ed.), *Bacteriocins of lactic acid bacteria*. Blackie Academic and Professional, New York, N.Y.
- Enishi, O., and K. Shijimaya. 1998. Changes of chemical composition of plant parts and nutritive value of silage in male sterile rice plant (*Oryza sativa* L.). *Grassl. Sci.* **44**:260–265.
- Fenlon, D. R., A. R. Henderson, and J. A. Rooke. 1995. The fermentative preservation of grasses and forage crops. *J. Appl. Bacteriol.* **79**:118–131.
- Hammes, W. P., and R. F. Vogel. 1995. The genus *Lactobacillus*, p. 19–54. *In* B. J. Wood and W. H. Holzapfel (ed.), *The lactic acid bacteria*, vol. 2. The genera of lactic acid bacteria. Blackie Academic and Professional, London, United Kingdom.
- Han, Y. W., and A. W. Anderson. 1974. The problem of rice straw waste. A possible feed through fermentation. *Econ. Bot.* **28**:338–344.
- Hellings, P., G. Bertin, and M. Vanbelle. 1985. Effect of lactic acid bacteria on silage fermentation, p. 932–933. *In* Proceedings of the 15th International Grassland Congress. Kyotosyppan, Kyoto, Japan.
- Kimura, M., and T. Ohta. 1972. On the stochastic model for estimation of mutation distance between homologous proteins. *J. Mol. Evol.* **2**:87–90.
- Kingamkono, R., E. Sjögren, U. Svanberg, and B. Kaijser. 1995. Inhibition of different strains of enteropathogens in a lactic-fermenting cereal gruel. *World J. Microbiol. Biotechnol.* **11**:299–303.
- Kozaki, M., T. Uchimura, and S. Okada. 1992. Experimental manual of lactic acid bacteria, p. 34–37. Asakurasyoten, Tokyo, Japan.
- Kung, L., Jr., and R. E. Muck. 1997. Animal response to silage additives, p. 200. *In* Silage: field to feedbunk. Proceedings from the Silage: Field to Feedbunk North American Conference, Hershey, Pennsylvania, February 11–13, 1997. Northeast Regional Agricultural Engineering Service, Ithaca, N.Y.
- Lin, C., K. K. Bolsen, B. E. Brent, and D. Y. C. Fung. 1992. Epiphytic lactic acid bacteria succession during the pre-ensiling and ensiling periods of alfalfa and maize. *J. Appl. Bacteriol.* **73**:375–387.
- Maeda, Y., M. Okamoto, and N. Yoshida. 1988. Heat damage in hay-making of big round bale. *J. Jpn. Grassl. Sci.* **34**:193–201.
- Muck, R. E. 1988. Factors influencing silage quality and their implications for management. *J. Dairy Sci.* **71**:2992–3002.
- Muck, R. E. 1989. Initial bacterial numbers on lucerne prior to ensiling. *Grass Forage Sci.* **44**:19–25.
- Nakui, T., S. Masaki, T. Aihara, N. Yahara, and S. Takai. 1988. The making of rice whole crop silage and an evaluation of its value as forage for ruminants. *Bull. Tohoku Natl. Agric. Exp. Stn.* **78**:173–180.
- Ruser, B. 1989. Erfassung und Identifizierung des epiphytischen Milchsäurebakterienbesatzes auf Gras und Mais in Abhängigkeit von Standort, Sorte, Entwicklungsstadium sowie Ernte- und Klimaeinflüssen. Ph.D. dissertation. Institute of Grassland and Forage Research, Braunschweig, Germany.
- Saitou, H., and K. Miura. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. Biophys. Acta* **72**:619–629.
- Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
- Salama, M. S., T. Musafija-Jeknic, W. E. Sandine, and S. J. Giovannoni. 1995. An ecological study of lactic acid bacteria: isolation of new strains of *Lactococcus* including *Lactococcus lactis* subspecies *cremoris*. *J. Dairy Sci.* **78**:1004–1017.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
- Sebastian, S., L. E. Phillip, V. Fellner, and E. S. Idziak. 1996. Comparative assessment of bacterial inoculated corn and sorghum silages. *J. Anim. Sci.* **71**:505–514.
- Sharp, R., P. G. Hooper, and D. G. Armstrong. 1994. The digestion of grass silages produced using inoculants of lactic acid bacteria. *Grass Forage Sci.* **49**:42–53.
- Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**:846–849.
- Stirling, A. R., and R. Whittenbury. 1963. Sources of lactic acid bacteria occurring in silage. *J. Appl. Bacteriol.* **26**:86–92.

33. Suzuki, K., J. Sasaki, M. Uramoto, T. Nakase, and K. Komagata. 1996. *Agromyces mediolanus* sp. nov., nom. rev., comb. nov., a species for "*Corynebacterium mediolanum*" Mamoli 1939 and for some aniline-assimilating bacteria which contain 2,4-diaminobutyric acid in the cell wall peptidoglycan. *Int. J. Syst. Bacteriol.* **46**:88–93.
34. Tamaoka, J., and K. Komagata. 1984. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol. Lett.* **124**:11–16.
35. Tanaka, O., and S. Ohmomo. 1994. A repeatable model system for silage fermentation in culture tubes. *Biosci. Biotechnol. Biochem.* **58**:1407–1411.
36. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
37. Torriani, S., G. E. Felis, and F. Dellaglio. 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.
38. Vescovo, M., S. Torriani, C. Orsi, F. Macchiarolo, and G. Scolari. 1996. Application of antimicrobial-producing lactic acid bacteria to control pathogens in ready-to-use vegetables. *J. Appl. Bacteriol.* **81**:113–119.
39. Weinberg, Z. G., G. Ashbell, Y. Hen, and A. Azrieli. 1993. The effect of applying lactic acid bacteria at ensiling on the aerobic stabilizing of silages. *J. Appl. Bacteriol.* **75**:512–518.
40. Yahara, N., S. Takai, and T. Numakawa. 1981. Studies on utilization of rice plant as whole crop silage. *Bull. Tohoku Natl. Agric. Exp. Stn.* **63**:159–163.
41. Yoshida, N., M. Tomita, Y. Takemasa, and T. Takahashi. 1987. Forage paddy rice for whole crop utilization. *J. Jpn. Grassl. Sci.* **33**:109–115.