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Short Communication

Isolation and characterization of lactic acid bacteria from soils in vineyards

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Lactic acid bacteria (LAB) have played a long and important role in food technology. LAB include rod, short-rod and coccus in cell types and physiological and biochemical characteristics. Isolates of LAB have been frequently reported from a variety of environments, including milk products, fermented foods and plants. However, studies on the isolation of LAB from soil remain scarce, even though it is well known that spore-forming LAB exist in soil (Nakayama and Yanoshi, 1967a, b; Yanagida et al., 1997).

Rich nutrition, such as from carbohydrates, minerals, nitrogen compounds and other substances, is necessary for the growth of LAB. However, it has not been determined whether the nutritional condition of soil is suitable for the growth of LAB. Since grapes contain a large quantity of sugar, we considered that the necessary nutrition for the growth of LAB in the soil of vineyards may be richer than that of other soil, due to fruit fall. This study aimed to isolate and identify LAB from soil, primarily from vineyards but also from other sites.

Eighty-seven soil samples were collected from Yamanashi and Nagano prefectures in Japan, mainly in the rhizospheres of vines (Table 1). Two methods, viz. direct spreading and accumulation with incubation, were used for the isolation of acid-producing bacteria. Each collected sample was incubated in 5 ml of two media containing GYP (2% glucose, 1% yeast extract, 1% peptone, 1% sodium acetate and 0.5% vol/vol salts solution, adjusted to pH 7.0) and BM (2% polypeptone, 0.5% tryptose, 2% Bacto-liver extract, 0.5% yeast extract, 0.001% Tween 80, 1% glucose, 0.5% fructose, 0.1% DL-malic acid, 0.008% $MnCl_2 \cdot 4H_2O$, 25% filtered tomato juice and 75% distilled water, adjusted to pH 5.4) under anaerobic conditions (BBL™ GasPak™, H_2+CO_2) at 30°C for 2–3 days. After incubation, acid-producing bacteria were isolated. These bacteria were tested with a Gram stain, and only Gram-positive bacteria were kept.

The isolates were examined for their phenotypic features, including physiological and biochemical characteristics. Examinations were conducted to determine the cell shape, lactic acid isomer, fermentation type, growth temperature, ability to ferment carbohydrates, type of cell wall and GC content (Kozaki et al., 1992). The API50CHL fermentation test was performed in some isolates.

Isolates were classified and identified using 16S rDNA RFLP (Restriction Fragment Length Polymorphisms) (Gurtler et al., 1991; Jang et al., 2003; Johansson et al., 1995; Ramos and Harlander, 1990; Sato et al., 2000) and 16S rDNA sequences (Sato et al., 2001). The DNA of each isolate was extracted, and PCR reactions were performed using a Gene Amp

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PCR System 2400 (PerkinElmer Corp., USA) under the following conditions: 95°C for 3 min; 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 90 s; a final extension of 72°C for 10 min; completion at 4°C. For assays of 16S rDNA RFLP, amplified DNA fragments (10 µl, about 500 ng) were mixed with 1.22-µl 10× M buffer and 4 units of the restriction enzyme *Acc*II (CG/CG) or *Hae*III (GG/CC), and incubated at 37°C for at least 3 h. After reactions, DNA fragments (5 µl) were mixed with a 0.5 µl loading buffer and electrophoresed through a 3% agarose gel in 1× TAE buffer for about 42 min. For the 16S rDNA sequence analysis, a Thermo Sequenase Cy™5 Dye Terminator Kit (Amersham Pharmacia Biotech, Inc., USA) was used and DNA sequencing was performed on an ALFexpress™ DNA Sequencer (Pharmacia Biotech). Partial sequencing, about 500 bp from the head, was done and three primers, 27FC: 5′-AGTTTGATCCTGGCTCAG-3′, 350R: 5′-CTGCTGCCTCCCGTAG-3′ and 520R: 5′-ACCGCGGCTGCTGGC-3′, were used in this study. The 16S rDNA sequences determined in this study were compared to previously published sequences of reference strains obtained from the database of the DNA Data Bank of Japan (DDBJ: http://www.ddbj.nig.ac.jp/) and software of Genetyx (Ver. 5.1).

In this study, high sequence homologies were observed among *Enterococcus* species when identifying these strains from 16S rDNA sequences. Other studies have shown the same results and found it difficult to identify these strains to species level based on the sequencing results alone (Devriese et al., 1993). Therefore, isolated *Enterococcus* strains were identified by their ability to ferment carbohydrates using an API50CHL kit (data not shown).

In total, 34 acid-producing bacteria were isolated from 87 soil samples. Among these acid-producing bacteria, 25 were isolated from soil in the rhizospheres of vines (Table 1). The physiological and biochemical characteristics of these isolates are shown in Table 2. The 34 isolates were divided into 11 groups (A to K) based on their 16S rDNA RFLPs (Table 2; Fig. 1). Further, based on their partial sequences (approximately 500 bp ahead), the evolutionary distance (the K_{nuc} value) and similarity values of representative strains of each group were generated using the software CLUSTAL W (http://www.ddbj.nig.ac.jp/search/clustalw-e.html), and a phylogenetic tree was constructed by the neighbor-joining method with the program TreeView (version 1.66) (Fig. 2). Based on previous studies from our laboratory (Chen et al., 2005), we identified 10 isolated cocci as *Enterococcus* species from 16S rDNA RFLPs. However, it was difficult to identify the isolates to species level with physiological tests or patterns of 16S rDNA RFLP. Thus, all 10 isolated cocci of Group A were identified with 16S rDNA and API50CHL kits. In Group A, strain L28-1 was identified as *Enterococcus durans*, L98-43-1 and L2000-15-2 as *Enterococcus avium*, L2000-6-1 and L2000-6-2 as *Enterococcus mundtii*, L2000-14-1 as *Enterococcus faecium*, L2000-16-1 as *Enterococcus raffinosus* and L2000-18-1, L2000-27-1 and L2000-29-1 as *Enterococcus hirae*. In the other Groups, representative strains were identified with 16S rDNA and their physiological characteristics. The results were as follows: L98-8-1 of Group B as *Lactobacillus plantarum*; L98-1-2 of Group C as *Lactobacillus brevis*; L98-3-1 of Group D as *Weissella confuse* (Björkroth et al., 2002); L2000-27-2 of Group E as *Leuconostoc mesenteroides* subsp. *mesenteroides*; L2000. 10-1 of Group F as *Streptococcus thermophilus*; L2000-29-2 of Group G as *Lactococcus lactis* subsp. lactis; L98-32-1 of Group H as *Lactobacillus casei* subsp. *casei*; L2000-16-2 of Group I as *Sporolactobacillus kofuensis* (Yanagida et

Table 1. Sampling sites and summary of isolations.

Place	Source	Number of soil samples	Strain number
Yamanashi prefecture	soil in rhizospheres of vines	48	L98-1-1, L98-1-2, L98-3-1, L98-4-2, L98-6-1, L98-8-1, L98-9-1, L98-18-1, L98-18-2, L98-20-1, L98-22-1, L98-32-1, L98-34-1, L2000-6-1, L200-6-2, L2000-14-1, L2000-15-1, L2000-15-2, L2000-16-1, L2000-16-2, L2000-18-1
	soil in rhizospheres of other trees	34	L28-1, L98-43-1, L98-51-1, L98-51-2, L98-52-1, L98-55-1, L98-55-2, L2000-10-1, L2000-10-2
Nagano prefecture	soil in rhizospheres of vines	5	L2000-27-1, L2000-27-2, L2000-29-1, L2000-29-2

Table 2. Grouping, physiological and biochemical characteristics of isolates.

Group	Strain number	Shape	Isomer of lactic acid	Fermentation type	Cell wall type	Fermentation of carbohydrates																		Species	Sequence homology	Source
						L-Arabinose	Sorbitol	D-Ribose	D-Xylose	Glucose	Melibiose	Fructose	D-Raffinose	Galactose	Mannitol	Mannose	Rhamnose	Cellobiose	Lactose	Maltose	Sucrose	Salicin	Trehalose	Starch			
A	L28-1	C	L	Homo	non-DAP	+	−	+	−	+	w	+	−	+	+	+	+	+	+	+	+	+	+	+	*Ent. durans*	99.8	other tree-yard
	L98-43-1	C	L	Homo	non-DAP	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	w	*Ent. avium*	99.0	other tree-yard
	L2000-6-1	C	L	Homo	non-DAP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Ent. mundtii*	98.8	vineyard
	L2000-6-2	C	L	Homo	non-DAP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Ent. mundtii*	99.2	vineyard
	L2000-14-1	C	L	Homo	non-DAP	w	−	+	−	+	w	+	−	+	−	+	−	+	+	+	+	+	+	w	*Ent. faecium*	99.1	vineyard
	L2000-15-2	C	L	Homo	non-DAP	+	+	+	w	+	w	+	−	+	w	+	w	+	w	+	+	+	+	−	*Ent. avium*	99.3	vineyard
	L2000-16-1	C	L	Homo	non-DAP	+	+	+	−	+	+	+	+	+	+	+	w	+	+	+	+	+	+	+	*Ent. raffinosus*	99.3	vineyard
	L2000-18-1	C	L	Homo	non-DAP	−	−	+	−	+	w	+	−	+	−	+	−	+	+	+	+	+	+	+	*Ent. hiare*	98.9	vineyard
	L2000-27-1	C	L	Homo	non-DAP	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	*Ent. hiare*	99.2	vineyard
	L2000-29-1	C	NT	NT	non-DAP	−	+	+	+	+	+	+	+	+	+	+	w	+	+	+	+	+	+	−	*Ent. hiare*	99.0	vineyard
B	L98-1-1	SR	DL	Homo	meso-DAP	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Lact. plantarum*		vineyard
	L98-4-2	SR	DL	Homo	meso-DAP	−	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	*Lact. plantarum*		vineyard
	L98-8-1	SR	DL	Homo	meso-DAP	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	*Lact. plantarum*	99.5	vineyard
	L98-18-1	SR	DL	Homo	meso-DAP	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Lact. plantarum*	99.1	vineyard
	L98-18-2	SR	DL	Homo	meso-DAP	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Lact. plantarum*		vineyard
	L98-34-1	SR	DL	Homo	NT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	*Lact. plantarum*	98.8	vineyard
C	L98-1-2	R	DL	Hetero	NT	+	−	+	+	+	+	+	−	+	−	−	−	−	−	+	N	−	−	−	*Lact. brevis*	99.3	vineyard
	L98-55-1	R	NT	NT	NT	+	−	+	+	+	+	+	−	+	−	−	−	−	−	+	N	−	−	−	*Lact. brevis*	99.4	other tree-yard
	L98-55-2	R	DL	Hetero	NT	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	N	+	+	−	*Lact. brevis*	98.7	other tree-yard
D	L98-3-1	R	DL	Hetero	non-DAP	+	−	+	+	+	w	w	−	+	−	+	−	−	−	−	−	−	+	−	*Weissella confusa*	98.9	vineyard
	L98-6-1	R	DL	NT	non-DAP	+	+	+	N	+	N	+	N	+	N	+	−	+	+	+	+	+	+	−	*Weissella confusa*		vineyard
	L98-9-1	R	DL	Hetero	non-DAP	+	+	+	N	+	N	+	N	+	N	+	−	+	+	+	+	+	+	+	*Weissella confusa*		vineyard
	L98-20-1	R	DL	Hetero	non-DAP	+	−	+	+	+	w	w	−	+	−	+	−	w	w	−	−	−	+	−	*Weissella confusa*	99.0	vineyard
E	L98-51-1	C	D	Hetero	non-DAP	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	*Leuc. mes.* ssp. *mes.*	99.2	other tree-yard
	L98-51-2	C	D	Hetero	non-DAP	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	*Leuc. mes.* ssp. *mes.*		other tree-yard
	L98-52-1	C	D	Hetero	non-DAP	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Leuc. mes.* ssp. *mes.*		other tree-yard
	L2000-27-2	C	D	Hetero	non-DAP	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	*Leuc. mes.* ssp. *mes.*	99.6	vineyard
F	L2000-10-1	C	L	Homo	non-DAP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Strep. thermophilus*	99.5	other tree-yard
G	L2000-29-2	C	L	Homo	non-DAP	−	−	+	−	+	+	+	+	+	−	+	−	+	+	+	+	+	+	w	*L. lactis* ssp. *lactis*	99.8	vineyard
H	L98-32-1	R	L	Homo	non-DAP	+	−	+	+	+	+	+	w	+	−	+	+	+	+	−	+	+	+	−	*Lact. casei* ssp. *casei*	99.2	vineyard
I	L2000-16-2	R	DL	Homo	meso-DAP	+	−	−	+	+	−	+	+	w	+	+	+	−	−	+	+	−	+	w	*Sporolact. kofuensis*	99.5	vineyard
J	L2000-10-2	R	D	Homo	meso-DAP	−	−	−	−	+	−	+	+	w	+	+	−	−	−	+	+	−	+	+	*B. laevolacticus*	98.9	other tree-yard
	L2000-15-1	R	D	Homo	meso-DAP	−	−	w	−	+	−	+	+	−	+	+	−	−	−	+	+	−	+	−	*B. laevolacticus*	99.2	vineyard
K	L98-22-1	C	DL	Homo	non-DAP	+	−	+	+	+	w	+	−	+	−	+	+	+	+	+	−	+	+	w	*Ped. pentosaceus*	99.7	vineyard

R: rod, SR: short rod, C: cocci, +: positive, −: negative, w: weak, NT and N: not tested.

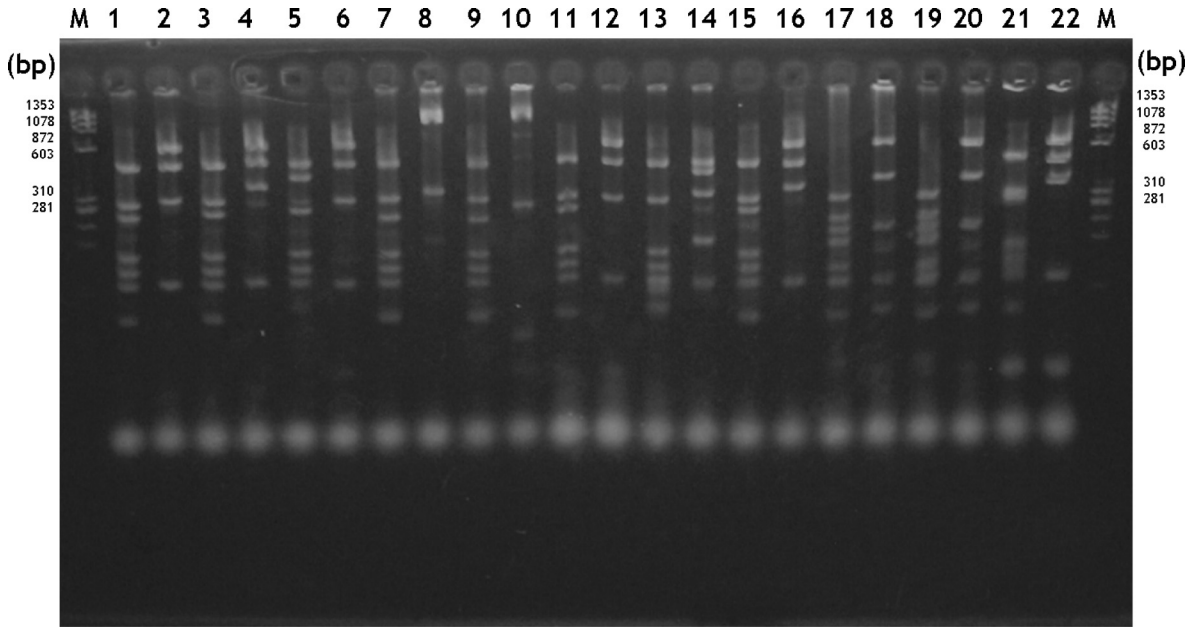

Fig. 1. 16S rDNA RFLP patterns of *Acc*II and *Hae*III digests, from Group A to Group K.

M: Size marker (ϕX174/*Hae*III). Lane 1: *Ent. durans* L28-1/*Acc*II, Lane 2: *Ent. durans* L28-1/*Hae*III, Lane 3: *Lact. plantarum* L98-1-1/*Acc*II, Lane 4: *Lact. plantarum* L98-1-1/*Hae*III, Lane 5: *Lact. brevis* L98-1-2/*Acc*II, Lane 6: *Lact. brevis* L98-1-2/*Hae*III, Lane 7: *W. confusa* L98-3-1/*Acc*II, Lane 8: *W. confusa* L98-3-1/*Hae*III, Lane 9: *Leuc. mes.* ssp. *mes.* L98-51-1/*Acc*II, Lane 10: *Leuc. mes.* ssp. *mes.* L98-51-1/*Hae*III, Lane 11: *Strep. thermophilus* L2000-10-1/*Acc*II, Lane 12: *Strep. thermophilus* L2000-10-1/*Hae*III, Lane 13: *L. lactis* ssp. *lactis* L2000-29-2/*Acc*II, Lane 14: *L. lactis* ssp. *lactis* L2000-29-2/*Hae*III, Lane 15: *Lact. casei* ssp. *casei* L98-32-1/*Acc*II, Lane 16: *Lact. casei* ssp. *casei* L98-32-1/*Hae*III, Lane 17: *Sporolactobacillus kofuensis* L2000-16-2/*Acc*II, Lane 18: *Sporolactobacillus kofuensis* L2000-16-2/*Hae*III, Lane 19: *B. laevolacticus* L2000-10-2/*Acc*II, Lane 20: *B. laevolacticus* L2000-10-2/*Hae*III, Lane 21: *Ped. pentosaceus* L98-22-1/*Acc*II, Lane 22: *Ped. pentosaceus* L98-22-1/*Hae*III.

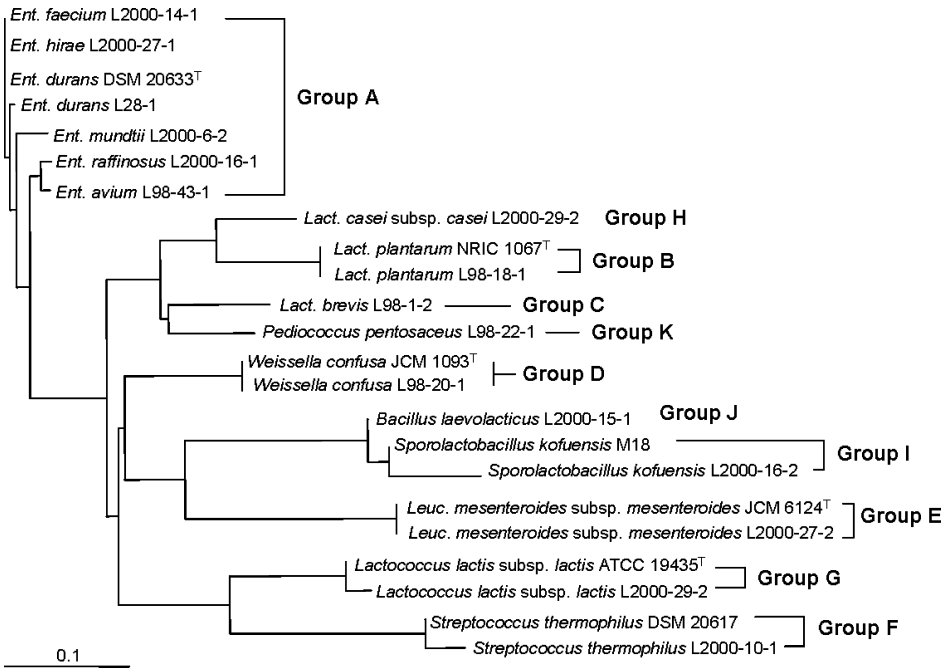

Fig. 2. Phylogenetic relationships of representative strains from each group.
The tree was constructed using the neighbor-joining method.

al., 1997); L2000-10-2 of Group J as *Bacillus laevolacticus* and L98-22-1 of Group K as *Pediococcus pentosaceus*.

From the results obtained above, it is considered that various lactic acid bacteria (LAB) can be isolated from soil. A diversity of LAB genera were found that are different from those of traditionally isolated sources. Rod, short-rod or cocci shapes were isolated and identified from soil for the six genera *Enterococcus*, *Lactobacillus* (Rodas et al., 2005), *Weissella* (Björkroth et al., 2002; Collins et al., 1993), *Leuconostoc, Lactococcus*, *Streptococcus* and *Pediococcus*. Although *Sporolactobacillus kofuensis* and *Bacillus laevolacticus* were also isolated from the soil samples, due to the common definition of LAB (Kozaki et al., 1992), we did not count these strains into the number of LAB. From the vineyard soils, 23 isolates were identified as LAB. Further, *Enterococcus* species were the most abundant of the vineyard-isolated LAB. *Lactobacillus* species were the secondarily abundant. On the other hand, *Leuconostoc mesenteroides* subsp. *mesenteroides* were the most abundant of the non-vineyard isolates. The results indicate a tendency for a greater diversity of LAB to be isolated from soil samples of vineyards than it from other soil samples. However, more evidence is necessary to confirm this, such as by large-scale sampling and isolation.

In conclusion, various LAB do exist in the soil of vineyards. In this study, it was found that the rhizospheres of vines may be good sources from which to isolate LAB. As for the methods of isolation, accumulation under anaerobic conditions may be the better method to isolate LAB from soil. Furthermore, 16S rDNA RFLP offers an effective and rapid method to distinguish the LAB coccus from their genera, such as *Enterococcus* genus, *Lactococcus* genus and *Leuconostoc* genus. In identification of *Enterococcus* species, 16S rDNA sequencing and using an API50CHL kit may offer a more correct result. In the previous studies, LAB could be isolated from many kinds of sources, such as milk products, fermented food, animal intestines or plants, but studies on the isolation of LAB from soils in vineyards remain scarce. The present study revealed that soil can be a new and different source to the isolation of LAB. Future studies in our laboratory will identify the bacteriocin activity of these isolates.

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