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Diversity of Leuconostocs on Garlic Surface, an Extreme Environment

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Abstract Thirty-nine strains of Leuconostocs found to be tolerant to 10% or more garlic were selected for further identification, by comparing their whole-cell protein pattern, 16S rRNA gene (first 530 bases) sequence, cellular fatty acid composition, and carbon source metabolism. Two isolates were identified as *Leuconostoc mesenteroides* and 32 others as *Leuconostoc citreum*. Five other strains belonging to a cluster could not be allocated to the existing species. 16S rRNA gene sequence and cellular fatty acid composition of the unidentified bacteria exhibited close similarity with *Leuconostoc argentinum*. The unidentified isolates were not allocated to *L. argentinum*, because they formed polysaccharide from sucrose, while *L. argentinum* strains do not. Leuconostocs tolerant to high concentration of garlic were found predominantly on garlic surface, an extreme environment which is unfit for most of other microorganisms.

Key words: Leuconostocs, garlic, identification, 16S rRNA gene, *Leuconostoc citreum*, *Leuconostoc argentinum*

Antimicrobial activity of garlic has been recognized for many years. One to two percent of garlic has been reported to inhibit microbial growth and higher concentrations are germicidal. Dababneh and Al-Delaimy [1] and Rees *et al.* [14] reported that 1% garlic inhibited *Staphylococcus aureus*, and Kyung *et al.* [12] reported that 1% garlic completely inhibited *Saccharomyces cerevisiae*, *Candida albicans*, *S. aureus*, and *Escherichia coli*. Even though lactic acid bacteria (LAB) in general are known to be least sensitive to the inhibitory activity of garlic than other microorganisms [14], *L. mesenteroides* C33 and LA183 are killed by 5% garlic in MRS broth [12]. Strains of *Pediococcus pentosaceus* are shown to be the most tolerant ones [12, 14].

Kyung *et al.* [12] previously reported that prepeeled commercial garlic cloves carried up to 10^8 LAB/g, while freshly peeled garlic carried less than 10^3 LAB/g. They isolated and identified *Leuconostoc mesenteroides* that multiplied in MRS broth with 10% garlic, however, other known bacteria were not able to grow in the presence of 10% garlic. A relatively tolerant LAB, *Pediococcus pentosaceus* ATCC 33316, was not killed but it was not able to multiply in the presence of 5–10% garlic for 48 h [12]. Shim and Kyung [19] followed-up their earlier studies and reported isolation of Leuconostocs with MIC of 25% garlic.

It was previously suggested by Kyung *et al.* [12] that certain bacteria take advantage of the hostile garlic environment which is unfit for most other microorganisms. When the skin of garlic is damaged during the peeling process [19], an antimicrobial compound like allicin is generated to inhibit or destroy less tolerant microorganisms.

The habitat of many *Leuconostoc* species has been reported to be milk and other dairy products [10] along with plant materials [2]. There have also been reports of Leuconostocs occurring among most meats which are stored in vacuum packages [3, 7, 17, 18] or under a modified atmosphere [11, 15], where they may contribute to spoilage. *L. oenos* has been isolated from wine and related habitats [10]. Garlic isolates were identified mostly as *L. mesenteroides* subsp. *mesenteroides* according to the phenotypic classification scheme [12, 19].

Since the Leuconostocs isolated from garlic possess unusual characteristics such as tolerance to high garlic concentration and ability to grow at high temperature (>40°C), we attempted to identify the bactena with more advanced technologies in order to understand the diversity of the microbial ecology of garlic, an extreme environment.

MATERIALS AND METHODS

Strains

Thirty-nine Leuconostocs which were tolerant to 10% or more garlic in MRS broth were selected for the species-

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level identification. These were from 87 strains previously isolated from garlic [19] and they were identified mostly as *L. mesenteroides* subsp. *mesenteroides* by phenotypic classification. The isolates were Gram-positive and catalase-negative cocci that produced gas from glucose and dextran from sucrose. *Leuconostoc citreum* ATCC 13146, *Leuconostoc citreum* ATCC 49370, *Leuconostoc argentinum* ATCC 51355, and *Leuconostoc mesenteroides* LA 10 were included as reference strains. The stock cultures were maintained at -66°C in MRS broth with 16% glycerol. Inocula for tests were grown in MRS broth at 30°C, unless otherwise specified.

16S rRNA Gene Sequencing

The sequencing of 16S rRNA gene (first 530 bases) of garlic isolates was performed by MIDI Labs, Inc. (Newark, DE, U.S.A.). The 16S rRNA gene was PCR amplified from genomic DNA isolated from bacterial colonies. Primers used for the amplification corresponded to *E. coli* positions 005 and 531. Amplification products were purified from excess primers and dNTPs by using Microcon 100 (Amicon) molecular weight cut-off membranes, and checked for quality and quantity by running a portion of the products on an agarose gel. Cycle sequencing of the 16S rRNA amplification products were carried out by using both AmpliTaq FS DNA polymerase and dRhodamine dye terminators. Excess dye-labeled terminators were removed from the sequncing reactions by using a Sephadex G-50 spin column. The products were collected by centrifugation, dried under a vacuum, and frozen at -20°C until they were ready to load. Samples were resuspended in a solution of foramide/blue dextran/EDTA and denatured prior to loading. The samples were electrophoresed on a ABI Prism 377 DNA Sequenser, and data were analyzed by using PE/Applied Biosystems DNA editing and assembly software.

Total Soluble Cell Protein Patterns

Bacteria were incubated for 24 h at 30°C in the MRS broth and centrifuged at 12,000 ×g for 3 min at 4°C. Bacterial pellet was resuspended in an equal volume of the sample buffer. The bacterial suspension was vortexed for 5 min after adding 0.05 g of glass beads to the tube. The suspension was heated for 10 min at 95°C and centrifuged at 12,000 ×g for 1 min at 4°C before the samples were analyzed on a gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; [6]). Gels were stained for 2 h with Coomassie brilliant staining solution and destained with 5% acetic acid-16.5% methanol.

Cellular Fatty Acid Composition

Fatty acid methyl esters were prepared from biomass that was scraped off from the MRS agar incubated for 24 h at 30°C. The composition of whole cell fatty acids was determined by using the MIDI system (Sherlock 6890, Hewlett Packard Co., PA, U.S.A.). Fifty mg of the cells were transferred to a screw-cap tube and saponified with 1 ml of 15% (wt/vol) NaOH in 50% aqueous methanol for 30 min at 100°C. After cooling, the released fatty acids were converted to methyl esters by adding 2 ml of 54% (vol/vol) 6 N-HCl in methanol followed by heating at 80°C for 10 min and then rapid cooling in cold running water. The methyl esters were extracted into 1.25 ml of hexane-ether (1:1) by gentle mixing on a specimen tumbler for 10 min. The phases were allowed to separate, and the lower (aqueous) phase was discarded. The sample was then washed with 3 ml of 1.2% (wt/vol) NaOH by mixing on a specimen tumbler for 5 min. Then, 2 drops of saturated NaCl solution were added to assist in separating the phases. The upper phase was transferred to a gas chromatography autosampler vial and sealed with a clamp cap. The fatty acid methyl esters were analyzed by using a model 6890 flame ionization gas chromatography (Hewlett-Packard Co., Avondale, PA, U.S.A.) equipped with HP ultra 2 column at a column temperature programmed from 170 to 310°C. Flow rate of the carrier gas (H_2 99.999%) was 30 ml/min. Data were recorded with an electronic integrator, and fatty acid methyl esters were identified by using computer comparison of retention times with retention times of authentic standards (Hewlett-Packard Co.).

Carbon-Source Utilization Patterns

The test strains were subcultured onto BUG (Biolog universal growth; Biolog, Inc., Hayward, CA, U.S.A.) agar and incubated for 48 h at 30°C. The procedure that followed was in the manual of the Biolog system (Biolog, Inc.). The utilization patterns of carbon sources were analyzed by using the Micrologrelease 4.0 software (Biolog, Inc.).

Carbohydrate fermentation characteristics were determined by using the API 50 CHL system (bioMerieux sa, Marcy-l'Etoile, France) at 30°C after 48 h. The test was carried out according to the instructions of the API 50 CHL system.

Results and Discussion

16S rRNA Gene Sequence

Two strains from the 39 garlic isolates were identified as *L. mesenteroides* (Table 1). Subspecies level differentiation, however, was not possible by using the 16S rRNA gene sequence analysis. In fact, they should belong to either *L. mesenteroides* subsp. *mesenteroides* or *L. mesenteroides* subsp. *dextranicum*, since they produced dextran from sucrose. Furthermore, the 16S rRNA gene sequence was identical to strain GL 40 with the exception of one bp and perfectly identical with strain GL 50.

Table 1. Cellular fatty acid composition of Leuconostocs.

Cluster	Strains	Fatty acid composition (%)						
		C14:0	C16:0	C18:1(9)	C18:0	C16:1(9)/15iso 2OH	C18:1(11)	C19:0cyclo
I	GL09	23.31	48.21	11.75	2.17	9.85	-	-
	GL24	23.15	47.89	11.50	2.44	11.78	-	-
	GL67	22.88	49.08	11.95	1.83	9.06	-	-
	GL86	22.41	47.48	13.60	2.60	8.74	-	-
	GL87	23.24	47.65	11.41	2.08	9.67	-	-
II	GL25	4.99	30.94	4.98	1.42	4.80	25.31	24.64
	GL28	10.94	32.89	13.60	0.82	6.92	27.30	4.94
	GL32	5.77	31.62	4.87	1.06	6.45	21.42	25.13
	GL35	7.81	30.60	14.90	1.92	5.20	26.30	9.42
	GL43	6.50	31.95	16.32	1.02	6.03	33.13	0.98
	GL44	4.54	30.58	8.77	0.75	4.80	30.22	17.48
	GL56	9.09	28.66	3.57	0.82	8.52	25.63	22.61
	GL59	6.52	36.13	5.21	0.84	6.97	18.98	21.82
	GL61	5.24	35.13	3.89	1.66	4.52	20.67	25.70
	GL62	6.64	29.41	14.94	0.95	5.50	37.71	1.85
	GL71	4.84	32.30	13.29	0.84	5.66	23.60	17.47
	GL78	11.13	25.12	4.50	0.40	9.65	34.20	14.21
III	GL40	2.49	21.04	35.69	-	6.95	25.60	8.22
	GL50	4.82	25.01	27.80	0.43	10.50	26.26	2.47
Reference strains								
L. argentinum ATCC 51355		27.94	43.20	7.23	2.15	11.43	-	-
L. citreum ATCC 13146		3.43	33.83	4.63	0.95	5.24	13.51	34.60
L. citreum ATCC 49370		5.59	33.72	12.53	0.49	7.72	23.56	13.72
L. mesenteroides LA 10		4.75	21.70	19.84	0.53	11.43	32.49	4.90

Beside the two *L. mesenteroides* strains among the 39 isolates, 37 strains were temporarily identified as *Leuconostoc citreum* through the 16S rRNA gene sequence analysis. The rRNA gene sequences were identical, with the exception of 1, 2, 5 and 11 bp in 28, 1, 3 and 5 strains, respectively (Fig. 1). The percent gene differences (GD) from *L. citreum* were 0.19, 0.28, 0.93, and 2.06%, respectively. Those isolates with GD 0.93% or less showed protein pattern and cellular fatty acid composition identical with *L. citreum*, and were naturally allocated to *L. citreum*. The isolates with GD 2.06% had totally different protein pattern and cellular fatty acid composition, compared with those of *L. citreum*. 16S rRNA gene sequences of five isolates in the cluster I were homologous, having high degree of similarity (99%) to the GenBank reference strain of *Leuconostoc argentinum*.

Fatty Acid Composition

Those strains in the clusters I, II, and III showed fatty acid profiles similar to that of *L. argentinum, L. citreum,* and *L. mesenteroides*, respectively (Table 1). The cluster I had fewer types of cellular fatty acids, lacking C18:1 w9c, C19:0cyclo w10c/19w6, and C19:0cyclo w8c, and the fatty acid composition profiles were similar to that of *L. argentinum* ATCC 51355 (Table 1). Over 70% of the cellular fatty acids of the cluster I was relatively short-chain saturated fatty acids including C14:0 (23%) and C16:0 (48%).

Strains in the cluster II and strains of *L. citreum* had most kinds of fatty acids and their fatty acid profiles were similar to each other (Table 1). Dykes *et al.* [7] reported almost similar pattern of fatty acid composition of *L. citreum* with the exception of differences in C18:1(11) and C19:0cyclo. They found that *L. citreum* DSM 20188 contained less amount of C18:1(11) (0.37%) and more of C19:0cyclo (41.48%), whereas in this study, ATCC 13146 and 49370 strains of *L. citreum* contained large amount of C18:1(11) (18.98–37.71%) and small amount of C19:0cyclo (0.98–25.7%).

The relative percentages of individual fatty acid for a given strain are often reported to be different [7]. Medium composition and culture conditions may affect the cellular fatty acid composition of lactic acid bacteria [3, 16]. When Schmitt *et al.* [16] and Dykes *et al.* [7] analyzed the same reference strain (*L. mesenteroides* DSM 20343) independently, Schmitt *et al.* found a lower amount of C18:1(9) (0.05%) and higher amounts of C19:0cyclo (9.79%) and C14:0 (1.5%), while Dykes *et al.* reported a higher amount of

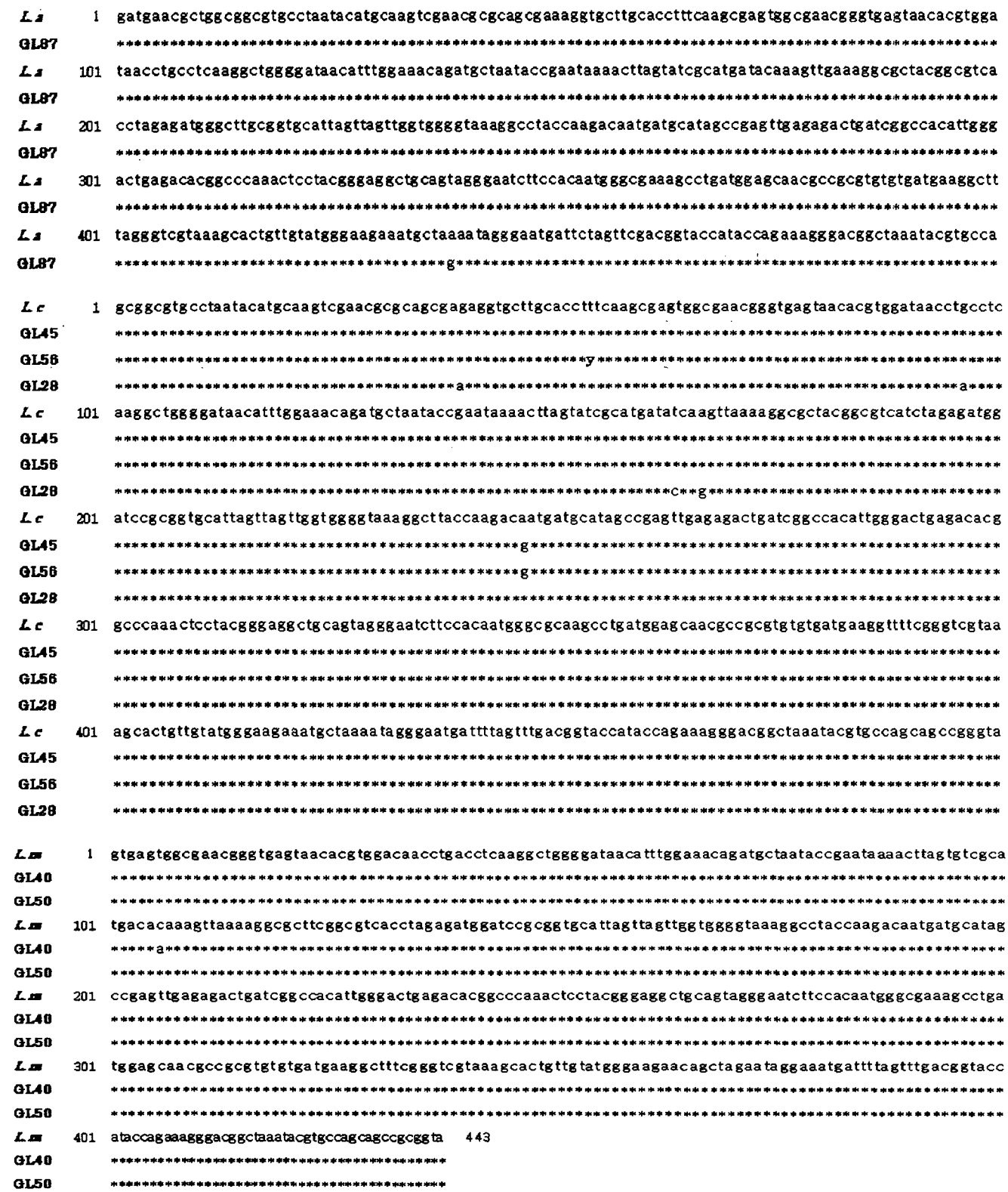

Fig. 1. Comparison of 16S rRNA gene sequence of GL87 in the cluster I with *L. argentinum*, GL45, 56, 28 in the cluster II with *L. citreum*, and GL40 and GL50 in the cluster III with *L. mesenteroides*.

L.a; *L. argentinum* AF175403, *L.c*; *L. citreum* AB022923, *L.m*; *L. mesenteroides* AF375902. GD (%) of GL87 (0.19) is from *L. argentinum*. GD (%) of GL45 (0.19), GL56 (0.28), and GL28 (0.93) are from *L. citreum*. GD (%) of GL40 (0.19) and GL50 (0.00) are from *L. mesenteroides*. Star mark indicates identical base.

Table 2. Comparisons of 16S rRNA gene analysis (GD%), cellular fatty acid composition, soluble protein pattern, carbon source utilization and fermentation responses of *Leuconostoc* isolates.

Cluster	Strains	GD (%)	Fatty acid composition type	SDS-PAGE pattern	Utilization of			Acid from						I D
					Raf	Mel	Gal	Ara	Rib	Xyl	Lac	Raf	Mel	
I	GL09	2.06	A	a	–	+	+	+	–	+	+	+	+	Novel species
	GL24	2.06	A	a	+	+	+	+	–	+	+	+	+	
	GL67	2.06	A	a	+	+	+	–	–	+	–	+	+	
	GL86	2.06	A	a	+	+	+	–	–	+	+	+	+	
	GL87	2.06	A	a	+	+	+	–	–	+	+	+	+	
II	GL25	0.19	B	b	–	–	–	+	–	–	–	+	–	*L. citreum*
	GL28	0.93	B	b	–	–	+	+	–	–	–	+	+	
	GL32	0.19	B	b	–	–	–	+	–	–	–	+	+	
	GL35	0.93	B	b	–	–	–	+	–	–	–	+	+	
	GL43	0.93	B	b	–	–	+	+	–	–	–	+	+	
	GL44	0.19	B	b	–	–	–	+	–	–	–	+	+	
	GL56	0.28	B	b	–	–	–	–	–	–	–	–	+	
	GL59	0.19	B	b	–	–	–	+	–	–	–	+	+	
	GL61	0.19	B	b	–	–	–	+	–	–	–	+	+	
	GL62	0.19	B	b	–	–	–	+	–	–	–	+	+	
	GL71	0.19	B	b	–	–	–	+	–	–	–	+	+	
	GL78	0.19	B	b	–	–	–	+	–	+	–	+	+	
III	GL40	0.19	C	c	+	+	–	+	+	+	+	+	+	*L. mesenteroides*
	GL50	0.00	C	c	–	–	+	+	+	+	+	+	+	
Reference strains														
L. argentinum ATCC 51355			A	a	–	+	+	–	–	–	+	+	+	
L. citreum ATCC 13146			B	b	–	–	–	+	–	–	–	–	+	
L. citreum ATCC 49370			B	b	–	–	–	+	–	–	–	+	+	
L. mesenteroides LA 10			C	c	+	+	–	+	+	+	+	+	+	

Carbon source utilization of Ara, Xyl, Rib; all negative. Acid from Gal, Man, Mal; all positive. GD (%) of cluster I and II is from *L. citreum* and that of cluster III is from *L. mesenteroides*. A, B, C and a, b, c are different patterns of fatty acid composition and SDS-PAGE, respectively.

C18:1(9) (36.98%) and lower amounts of C19:0cyclo (9.79%) and C14:0 (1.5%).

Pattern of Soluble Cell Protein

The overall protein profiles of the strains of a cluster were identical with each other. The patterns of the strains in clusters II and III were identical with those of *L. citreum* and *L. mesenteroides*, respectively. The protein pattern of the strains in the cluster I was similar but not quite identical with that of *L. argentinum* ATCC 51355 (Table 2, Fig. 2). Differences in protein bands between *L. argentinum* ATCC 51355 and GL24 belonging to the cluster I were observed in the region of 64 and 39 kDa (Fig. 2).

Biochemical and Physiological Test

Dicks *et al.* [4] stated that nonacidophilic Leuconostocs are not easily distinguished from each other because they share many phenotypic characteristics [10]. Sugar fermentation has been considered to be not very useful in classifying the non-acidophilic Leuconostocs [4], since sugar fermentation patterns may vary among strains which belong to the same species and may be identical for strains belonging to

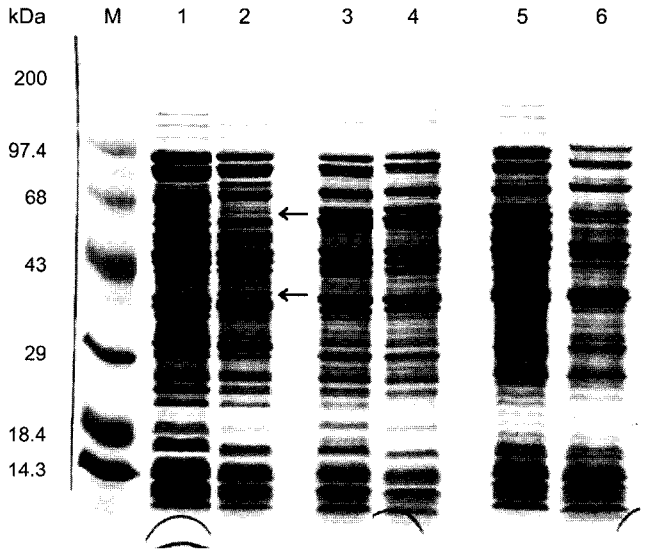

Fig. 2. SDS-PAGE of whole cell proteins of Leuconostocs isolated from garlic.
M, molecular mass standards (in kDa); lane 1: *L. argentinum* ATCC 51355, lane 2: GL24, lane 3: *L. citreum* ATCC 49370, lane 4: GL56, lane 5: *L. mesenteroides* LA10, lane 6: GL50.

different species [9, 10]. The API 50CHL system has been reported to be of little value as an identification method, as shown previously for many other lactic acid bacteria [4, 5, 8, 9, 13, 20].

Strains in clusters II and III grew at 37°C but not at 40°C, similar to the reference strains of *L. citreum* and *L. mesenteroides*. Strains in the cluster I grew at 40°C, similar to the reference strain of *L. argentinum*. All the garlic isolates produced dextran from sucrose, since they were selected on the basis of dextran formation from sucrose. Reference strains of *L. argentinum* did not produce polysaccharide in the present study, in agreement with other reports [4]. It was not possible to allocate these five cluster I strains to either *L. citreum* or *L. argentinum*.

CONCLUSION

Garlic surface, an extreme environment for microorganisms, favored those that are tolerant to garlic antimicrobial activity. Heterofermentative dextran-forming lactic acid bacteria in the genus *Leuconostoc* were among the predominant microbial groups that are tolerant to 10% or more garlic. *L. citreum* was the dominant species, followed by species presently not well characterized and *L. mesenteroides*. Identification process of the unknown is in progress, however, it was temporarily designated as *Leuconostoc garlicum* sp. nov.

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