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RESEARCH NOTE

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Characterization of a New *Leuconostoc* Species Isolated from Fresh Garlic

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Abstract Unknown bacterium isolated from garlic was characterized using phenotypic methods, phylogenetic analysis, DNA–DNA hybridization, and cultural methods. The strain was identified as typical leuconostoc; Gram-positive, non-sporeforming, heterofermentative, catalase-negative, and spherical. Although its 16S rRNA gene sequence showed high homology to *Leuconostoc argentinum* DSM 8581^T(99.8%), DNA–DNA hybridization experiments indicated it represents novel genomic species in the genus *Leuconostoc*. The garlic-specific leuconostoc was more resistant to antimicrobial activity of garlic compared to other common laboratory lactic acid bacteria, and was even stimulated by low concentrations (1-2%) of garlic extract supplemented in trypticase soy broth. Growth stimulation was concentration-dependent when tested with residual aqueous layer after solvent extraction of fresh whole garlic extract.

Keywords: garlic, leuconostocs, identification, resistance

Introduction

The genus *Leuconostoc* encompasses a phylogenetically coherent group of lactic acid bacteria and currently includes 13 species, namely *L. mesenteroides, L. lactis, L. gelidum, L. carnosum, L. citreum, L. pseudomesenteroides, L. fallax, L. argentinum, L. kimchii, L. gascomitatum, L. inhae, L. fructosum,* and *L. ficulnuem* (1-6). *L. mesenteroides* comprises three subspecies, *L. mesenteroides* subsp. *mesenteroides, L. mesenteroides* subsp. *dextranicum,* and *L. mesenteroides* subsp. *cremoris* (7). Leuconostocs are Gram-positive, facultatively anaerobic, asporogenous, catalase-negative, and spherical organisms containing DNA with relatively low G+C content (37-45 mol%); they produce lactic acid as one of the main end products of sugar fermentation and, in many cases, produce dextran from sucrose (1, 8).

The habitat of many leuconostoc species is reported to be milk and other dairy products (8) as well as plant materials (9). There have also been reports of leuconostocs occurring among the dominant population on meat products stored in vacuum packages (10-13) or under a modified atmosphere (14, 15), where they may contribute to spoilage. *L. oenos* has been isolated from wine and related habitats (8). Kyung *et al.* (16) previously reported that prepeeled commercial garlic cloves carried up to 10^8 lactic acid bacteria (LAB)/g, while freshly peeled garlic carried less than 10^3 LAB/g. They reported isolation and identification of *L. mesenteroides* that multiplied in MRS broth with 10% garlic. No other known bacteria were able to grow in the presence of 10% garlic. Kim *et al.* (17) conducted a follow-up study and reported the isolation of leuconostocs with a minimum inhibitory concentration (MIC) of 25% garlic.

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Certain groups of bacteria are better adopted to the hostile garlic environment, which is unfit for most other microorganisms (16). Damage to the skin of garlic during the peeling process (17) results in the production of antimicrobial compounds such as allicin, which inhibit or destroy the less tolerant microorganisms. Garlic isolates were identified mostly as *L. mesenteroides* subsp. *mesenteroides* using the phenotypic classification scheme (16, 17).

Because the leuconostocs isolated from garlic possess unusual characteristics, such as tolerance of high concentrations of garlic and the ability to grow at high temperatures (>40°C), further identification using more advanced technologies was performed to understand the diversity of the microbial ecology of garlic. In this study, genetic, physiological, and cultural characteristics of a new species of *Leuconostoc* isolated originally from fresh garlic were evaluated.

Materials and Methods

Bacterial strains Thirty-nine leuconostoc strains tolerant to 10% or higher concentration of garlic in MRS broth (Difco Laboratories, Detroit, MI) were selected for species-level identification. Garlic cloves were thoroughly washed, crushed, and homogenized with an equal volume of distilled water in a blender. The homogenate was filter-sterilized (0.45 μm, Gelman Sciences, Inc., Ann Arbor, MI) following centrifugation at 17600 × g (HMR-220IV, Hanil Industrial Co., Inchon, Korea) for 30 min. The sterile garlic extract was appropriately diluted with heat-sterilized culture media, which were among the 87 strains previously isolated from garlic (17) mostly identified as *L. mesenteroides* subsp. *mesenteroides* by phenotypic classification, to make garlic extracts of desired concentration. The isolates were Gram-positive, catalase-negative cocci that produced gas from glucose and dextran from sucrose. *L. citreum* ATCC49370^T, *L. argentinum*

KCTC3773^T, and *L. mesenteroides* subsp. *mesenteroides* KCTC3505^T 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 incubated at 30°C unless otherwise specified.

DNA base composition The G+C content of the DNA was determined by DSMZ (Braunschweig, Germany). The G+C content was determined through HPLC as described by Mesbah *et al.* (18) using the non-methylated λ DNA (Sigma Chem. Co., St. Louis, MO, USA) as the standard.

Cellular fatty acid composition Fatty acid methyl esters were prepared from biomass that was scraped from MRS agar incubated for 24 hr at 30°C. The composition of whole cell fatty acids was determined using the MIDI system (Sherlock 6890, Hewlett-Packard Co., Avondale, PA, USA) as described elsewhere (17). The fatty acid methyl esters were analyzed using a model 6890 flame ionization gas chromatograph (Hewlett-Packard Co.) equipped with an HP Ultra 2 column at a column temperature programmed from 170 to 310°C. The 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 computer comparison of the retention times against those of authentic standards (Hewlett-Packard Co.).

Phylogenetic analysis 16S rRNA gene sequences published previously were obtained from the Ribosomal Database Project (19), EMBL, and GenBank databases. Multiple alignments of sequences, calculation of the nucleotide substitution rate (20), construction of a neighbor-joining phylogenetic tree (21), and 1000-replicate bootstrap analysis for evaluation of the phylogenetic tree topology (22) were carried out with Clustral W version 1.8 (23), in which the alignment gaps and unidentified positions were not taken into account.

DNA-DNA hybridization DNA-DNA hybridization was determined by DSMZ (Braunschweig, Germany). Spectroscopic DNA–DNA hybridization was carried out as described by De Ley *et al.* (24), with the modifications described by Huss *et al.* (25) and Escara and Hutton (26), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories Inc., Oberlin, OH, USA).

Carbon-source fermentation patterns Carbohydrate fermentation characteristics were determined using the API 50 CHL system (bioMerieux sa, Marcy-l'Etoile, France) at 30°C after 48 hr of incubation according to the manufacturer's instructions.

Results and Discussion

Differentiation of garlic leuconostoc from other known leuconostocs GL87, one of the five novel strains, showed phenotypic characteristics typical of the genus *Leuconostoc*, i.e., a Gram-positive, non-sporeforming, facultatively anaerobic, catalase-negative, coccus-shaped organism that usually occurs in pairs. The G+C content of GL87 was 41.3 mol% and was within the observed range for the genus *Leuconostoc*, i.e., 37-45 mol% (7). Identification at the genus level was confirmed by 16S rRNA gene analysis, for which an almost-complete 16S rRNA gene sequence was determined for strain GL87 (17). The unrooted tree based on the neighbor-joining method clearly placed strain GL87 in a clade corresponding to the genus *Leuconostoc* (Fig. 1). Sequence searches of the GenBank and Ribosomal Database Project libraries showed that strain GL87 had a high level of 16S rRNA gene sequence similarity to *Leuconostoc* species. The closest sequence was that of *L. argentinum* DSM8581^T, with a level of similarity of 99.8%. Levels of similarity of the 16S rRNA gene sequences between strain GL87 and other *Leuconostoc* species were lower (below 91.7%). It is likely that GL87 and *L. argentinum* are related at the species level, as indicated by 16rRNA gene sequence analysis (27). The sequences of the nearest relatives of strain GL87 were retrieved and subjected to pairwise analysis to determine its phylogenetic position. A tree depicting the phylogenetic position of strain GL87 within the *Leuconostoc* group of bacteria is shown in Fig. 1. Branching pattern of the tree showed that *L. argentinum* DSM8581^T was the closest relative of GL87 with 100% bootstrap support. The genealogical relatedness of the garlic isolate to other leuconostocs was further elucidated using DNA-DNA pairing experiments (Table 1). Strain GL87 showed relatedness of 34.5-62.2% to the type strains of the genus *Leuconostoc*. DNA–DNA hybridization data show that strain GL87 represents an independent genomic species of *Leuconostoc*.

GL87 contained large amounts of monounsaturated and straight-chain saturated fatty acids: $C_{18:1w9c}$ (57.38%), $C_{16:0}$ (20.47%), $C_{14:0}$ (9.51%), and $C_{18:1\ w7c}$ (5.05%) (Table 2). The presence of large amounts of the C_{19} cyclopane-ring acid and its precursor ($C_{18:1}$) has also been reported in other leuconostocs (13). Garlic isolates have a fatty acid profile most similar to that of *L. argentinum* (Table 1). Both GL87 and *L. argentinum* grew at 40 but not at 45°C (Table 3). GL87 produced dextran from sucrose and

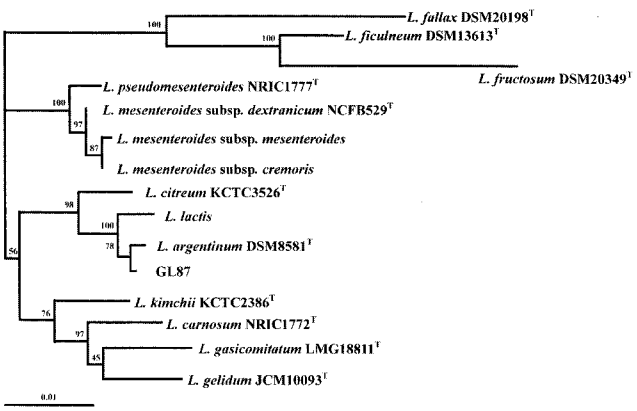

Fig. 1. Unrooted neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain GL87 and members of the genus *Leuconostoc*. Numbers at the nodes indicate levels of bootstrap support-based on neighbour-joining analysis of 1000 resampled datasets. The scale bar indicates 0.1 nucleotide substitutions per nucleotide position. *L.*, *Leuconostoc*.

Table 1. Levels of DNA relatedness (%) among strain GL87 and *Leuconostoc* species

Strains	% DNA homology with GL87
Leuconostoc argentinum DSM8581^T	52.2
Leuconostoc citreum DSM5577^T	58.4
Leuconostoc mesenteroides subsp. *mesenteroides* DSM20343^T	34.5
Leuconostoc mesenteroides subsp. *cremoris* DSM20346^T	62.2
Leuconostoc mesenteroides subsp. *dextranicum* DSM20484^T	57.7
Leuconostoc gelidum DSM5578^T	47.7
Garlic strain GL87	100

Percentage reassociation is shown.

Table 2. Cellular fatty acid profiles of strain GL87 and reference strains

Strains	Fatty acid composition (%)						
	C14:0	C16:0	C18:0	C18:1 ω7c	C18:1 ω9c	C16:1/C15:0 iso2OH	C19:0ω cyclo 8-9/ω cyclo 10-11
GL87	9.51	20.47	0.92	5.05	57.38	4.14	-
KCTC3773$^{T1)}$	13.49	18.61	0.79	3.08	57.37	3.70	-
ATCC49370$^{T2)}$	5.59	33.72	0.49	12.53	23.56	7.72	13.72
KCTC3505$^{T3)}$	4.75	21.70	0.53	19.84	32.49	11.43	4.90

[1)]KCTC3773^T *Leuconostoc argentinum* KCTC3773^T.
[2)]ATCC49370^T *Leuconostoc citreum* ATCC49370^T.
[3)]KCTC3505^T *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC3505^T.

Table 3. Differential characteristics of strain GL87 and related leuconostocs

Characteristic	GL87	KCTC3773$^{T1)}$	ATCC49370$^{T2)}$	KCTC3505$^{T3)}$
Acid produced from:				
Arabinose	-	-	+	+
Ribose	-	-	-	+
Xylose	+	-	-	+
Lactose	+	+	-	+
Raffinose	+	+	+	+
Melibiose	+	+	+	+
Dextran formation	+	-	+	+
Growth at 40°C	+	+	-	-

+, more than 90% of strains positive; -more than 90% of strains negative.
[1)]KCTC3773^T *Leuconostoc argentinum* KCTC3773^T.
[2)]ATCC49370^T *Leuconostoc citreum* ATCC49370^T.
[3)]KCTC3505^T *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC3505^T.

utilized xylose, while *L. argentinum* did not (Table 3). Sugar fermentation, however, is believed to be of limited value in the classification of non-acidophilic leuconostocs (2), because sugar fermentation patterns vary among strains that belong to the same species, and some strains belonging to different species also show identical patterns (8).

Resistance of garlic leuconostoc to the antimicrobial activity of garlic The resistance of garlic leuconostocs to antimicrobial activity of garlic was more apparent at low temperatures (<25°C; Fig. 2), with no difference in growth pattern observed at 20 (data not shown) and 25°C. At 5% whole garlic concentration, garlic leuconostoc could multiply more than 10 times at low growth temperatures, but was rapidly destroyed at 30°C. Although the reason for this phenomenon has not yet been elucidated, careful care should be given in choosing experimental temperatures when antimicrobial activity of garlic is tested.

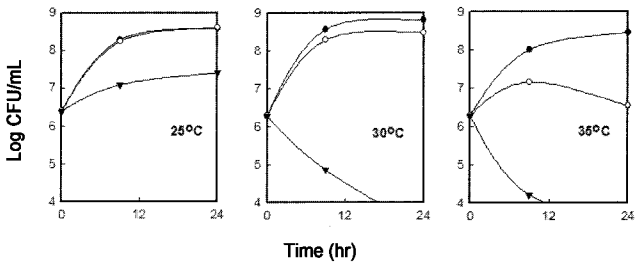

Fig. 2. The effects of growth temperature of GL87 on the resistance to whole garlic extract supplemented in TSB with 1% glucose. ●, 0%; ○, 3%; ▼, 5%

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