J. Microbiol. Biotechnol. (2002), **12**(1), 166–171

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Reevaluation of the Change of *Leuconostoc* Species and *Lactobacillus plantarum* by PCR During Kimchi Fermentation

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Received: September 5, 2001
Accepted: December 26, 2001

Abstract The genus *Leuconostoc* is generally recognized as a favorable microorganism associated with a good taste of Kimchi and *Lactobacillus plantarum* is responsible for the overripening and acidification of Kimchi. A rapid and reliable PCR-based method to monitor the change of these lactic acid bacterial populations during Kimchi fermentation was attempted. A *Leuconostoc*-specific primer set was chosen from the conserved sequences of 16S rRNA genes among *Leuconostoc* species. The *Lb. plantarum*-specific primer set was the internal segments of a *Lb. plantarum*-specific probe which was isolated after randomly amplified polymorphic DNA (RAPD) analysis and tested for identification. The specificity of this protocol was examined in DNA samples isolated from a single strain. In agarose gel, as little as 10 pg of template DNA could be used to visualize the PCR products, and quantitative determination was possible at the levels of 10 pg to 100 ng template DNA. For the semi-quantitative determination of microbial changes during Kimchi fermentation, total DNAs from the 2 h-cultured microflora of Kimchi were extracted for 16 days and equal amounts of DNA templates were used for PCR. The intensities of DNA bands obtained from PCR using *Leuconostoc*-specific and *Lb. plantarum*-specific primer sets marked a dramatic contrast at the 1 ng and 100 ng template DNA levels during Kimchi fermentation, respectively. As the fermentation proceeded, the intensity of the band for *Leuconostoc* species increased sharply until the 5th day and the levels was maintained until the 11th day. The sharp increase for *Lb. plantarum* occurred after 11 days with the decrease of *Leuconostoc* species. The results of this study indicate that *Leuconostoc* species were the major microorganisms at the beginning of Kimchi fermentation and reach their highest population during the optimum ripening period of Kimchi.

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Key words: *Leuconostoc*, *Lactobacillus plantarum*, Kimchi, 16S rRNA, PCR

Kimchi is a group of traditional fermented vegetable foods in Korea and known to be the product of a natural mixed-fermentation process carried out principally by lactic acid bacteria (LAB) [7]. The flavor of Kimchi is dependent on the ingredients and fermentation conditions. The fermentation of Kimchi usually results from the growth and interaction between various LAB from a naturally occurring microflora of ingredients. More than 30 species belonging to different genera of LAB have been reported in Kimchi. In particular, the genera *Leuconostoc*, *Lactobacillus*, and *Pediococcus* are known to play an important role in the fermentation of Kimchi [8, 10, 12, 16, 17, 19]. In the previous microbial studies on the LAB from Kimchi, *Leuconostoc* species have been shown to be the major LAB found in the initial stage of Kimchi fermentation. After the maximum growth of *Leuconostoc* species, the number of *Leuconostoc* species decreased as pH decreased, and that of lactobacilli with a strong pH tolerance continued to increase until the last stage of fermentation [8, 10, 12, 19]. For these reasons, *Leuconostoc* species are generally recognized as the favorable microorganisms associated with a good taste of Kimchi and *Lactobacillus* species are considered to be responsible for the overripening and acidification of Kimchi. In particular, *Lactobacillus plantarum* appeared at the time when the *Leuconostoc* species were decreasing and became dominant, thus being considered as the main acidifying organism [8, 12]. Recently, in order to suppress acidification by *Lb. planturum*, researches on bacteriocins as candidates for prolonging the preservation period of Kimchi have became of a great interest in Korea [3, 9].

It is widely recognized that identification of LAB based on conventional methods is very ambiguous and complicated

Table 1. Properties of the 16S rDNA-targeted PCR primers used in this study.

16S rDNA-targeted primer		Nucleotide (5'→3')	Product size (bps)	Reference
Type	Name			
LAB-detectable primer set				
forward	LabF	TGATGCATAGCCGAGTTGAGAGACTG	782	this study
reverse	LabR	GACCATGCACCACCTGTCACTTTGTC		
***Leuconostoc*-specific primer set**				
forward	LabF	TGATGCATAGCCGAGTTGAGAGACTG	745	this study
reverse	LeuR	CACTTCTATCTCTAAAAGCTTCA		
***Lb. plantarum*-specific primer set**				
forward	LbP11	AATTGAGGCAGCTGGCCA	253	[14]
reverse	LbP12	GATTACGGGAGTCCAAGC		

because of their similar nutritional and growth requirements. Also, these methods are time-consuming and labor-intensive [5]. Recently, the rRNA sequence data and polymerase chain reaction (PCR) have been increasingly used in microbial ecology studies to detect not only qualitatively, but also quantitatively foodborne pathogens and unculturable organisms [2, 5, 6, 11, 14, 18, 20].

This study was undertaken to reevaluate the change of *Leuconostoc* species and *Lb. plantarum* during the Kimchi ripening process by means of PCR amplification.

Specificity and Sensitivity of PCR

Oligonucleotide primers for the detection of *Leuconostoc* species and *Lb. plantarum* were synthesized by Takara (Japan), and their sequences are presented in Table 1. For the designing of primers, comparative analysis of 16S rRNA sequences from *Leuconostoc mesenteroides* (M23035), *Leu. lactis* (ABO23968), *Leu. paramesenteroides* (M23033), *Leu. gelidum* (AF175402), *Leu. citreum* (AF173986), *Leu. gasicomitatum* (AF231132), *Leu. carnosum* (ABO22925), *Leu. argentinum* (AF175403), *Leu. pseudomesenteroides* (ABO23237), *Pediococcus pentosaceus* (M58834), *Lb. plantarum* (AJ271852), *Lb. hilgardii* (M58821), *Lb. amylophilus* (M58806), and *Lb. confusus* (X52567) was performed. All sequences are available in GenBank and aligned by CLUSTAL W software. LabF and LabR primers were chosen from the conserved sequences of 16S rRNA genes among *Leuconostoc* strains and various LAB. The LeuR primer was selected from the specific region among *Leuconostoc* strains except *Leu. paramesenteroides*. *Lb. plantarum*-specific primers, LbP11 and LbP12, determined by Quere *et al.* [14], were used for the detection of *Lb. plantarum*.

The specificity of the primers was tested by performing PCR with the various strains of LAB listed in Table 2. Amplification of 16S rDNA fragments of total DNAs from bacterial strains was carried out in an UNOII Thermocycler (Biometra Inc., Germany). The PCR solution included template DNA, 0.5 μM of each primer, 1 U of *Taq* polymerase (Takara, Japan), 2.5 mM $MgCl_2$, 100 mM dNTPs, and 1× PCR buffer supplied by the manufacturer. The total DNAs were extracted from LAB with a DNeasy Tissue Kit (Qiagen, Germany). Samples were preheated for 5 min at 95°C, and amplified for 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C. PCR products were separated by electrophoresis in 1.2% agarose gel and stained with ethidium bromide.

Amplification using the *Leuconostoc*-specific primer set produced a PCR product of approximately 745 bp from the chromosomal DNAs of *Leuconostoc* strains except *Leu. paramesenteroides* (Fig. 1B). The LeuR primer was selected from the specific region among *Leuconostoc* species, except

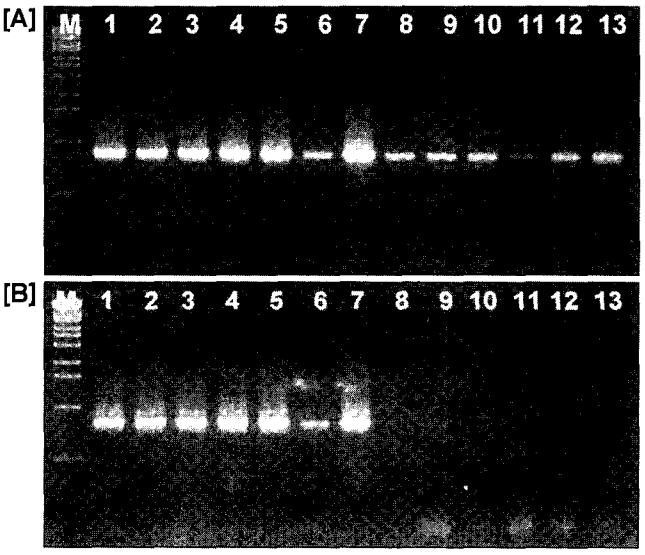

Fig. 1. PCR products obtained from amplification of chromosomal DNA with LAB-detectable (A) and *Leuconostoc*-specific (B) primer sets.

Lanes: M, DNA size marker (DNA digested with *Eco*T14I); 1, *Leu. mesenteroides* KFRI817; 2, *Leu. mesenteroides* ssp. *mesenteroides* KCTC3100; 3, *Leu. mesenteroides* ssp. *mesenteroides* KCTC3505; 4, *Leu. mesenteroides* ssp. *cremoris* KCTC3529; 5, *Leu. mesenteroides* ssp. *dextranicum* KCTC3530; 6, *Leu. mesenteroides* C7; 7, *Leu. lactis* KFRI232; 8, *Leu. paramesenteroides* B4; 9, *Lb. confusus* KFRI227; 10, *Lb. hilgardii* KFRI229; 11, *Lb. plantarum* KCTC3108; 12, *Lb. amylophilus* KFRI238; 13, *Ped. pentosaceus* KFRI833.

Table 2. LAB strains and their reactions with primer sets.

Bacterial strain	PCR product		
	LAB-detectable primer set	*Leuconostoc*-specific primer set	*Lb. plantarum*-specific primer set
Leuconostoc			
Leu. mesenteroides			
KFRI817	+	+	-
ssp. *mesenteroides*			
KCTC3100	+	+	-
KCTC3505	+	+	-
ssp. *dextranicum*			
KCTC3530	+	+	-
ssp. *cremoris*			
KCTC3529	+	+	-
Leu. mesenteroides C7	+	+	-
Leu. lactis KFRI232	+	+	-
Leu. paramesenteroides B4	+	-	-
Lactobacillus			
plantarum KCTC3104	+	-	+
plantarum KCTC3108	+	-	+
amylophilus KFRI238	+	-	-
hilgardii KFRI229	+	-	-
confusus KFRI227	+	-	-
Pediococcus			
pentosaceus KFRI833	+	-	-

The strains used in this study were grown facultative-anaerobically at 30°C in MRS broth. Strains were purchased from Korea Food Research Institute (KFRI) and Korean Collections for Type Cultures (KCTC). *Leu. mesenteroides* C7 and *Leu. paramesenteroides* B4 isolated from Kimchi were kindly donated by Prof. Jeong Hwan Kim of Gyeongsang National University (Chinju, Korea).

Leu. paramesenteroides, in order to test the sensitivity of amplification. Meanwhile, amplification using LAB-detectable primer sets (LabF and LabR) produced PCR products of approximately 782 bp from the chromosomal DNAs of all tested strains (Fig. 1A). The PCR results are summarized in Table 2. Therefore, the primer combination of LabF and LeuR can be used successfully to detect the existence of *Leuconostoc* species except *Leu. paramesenteroides*. *Lb. plantarum*-specific primers, LbP11 and LbP12, were introduced to amplify the internal segments of the *Lb. plantarum*-specific probe, which was isolated after randomly amplified polymorphic DNA (RAPD) analysis and also tested for identifying *Lb. plantarum* [14]. Contrary to the other LAB species tested, a 253-bp fragment was amplified from the tested *Lb. plantarum* strains (Fig. 2). The nucleotide sequence of the fragment was determined and it was in agreement with that of Quere *et al.* (data not shown) [14].

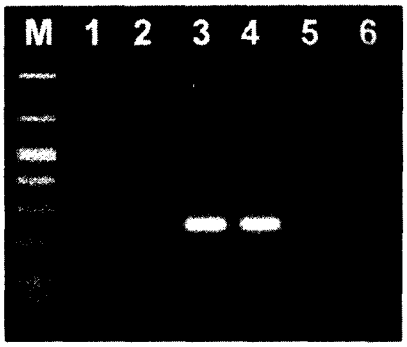

Fig. 2. PCR products obtained from amplification of chromosomal DNA with a *Lb. plantarum*-specific primer set.
Lanes: M, 50–1,000 bp DNA size marker; 1, *Lb. confusus* KFRI227; 2, *Lb. amylophilus* KFRI238; 3, *Lb. plantarum* KCTC3104; 4, *Lb. plantarum* KCTC3108; 5, *Ped. pentosaceus* KFRI833; 6, *Leu. mesenteroides* ssp. *mesenteroides* KCTC3100.

In order to assess the sensitivity of the PCR amplification with primer sets, different amounts of template DNAs ranging from 100 ng to 1 pg were used for PCR. As shown in Fig. 3, the amount of PCR product was dependent on the amount of template DNA in an amplification reaction. In agarose gel, as little as 10 pg of template DNA could be used to visualize the PCR products. The results suggest the possible quantitative determination of template DNA amount at the levels of 10 pg to 100 ng.

Therefore, the primer sets and PCR method mentioned above can be applied to monitor rapidly and semi-quantitatively the change of *Leuconostoc* species and *Lb. plantarum*.

The Change of *Leuconostoc* Species and *Lb. plantarum* During Kimchi Fermentation

In order to reevaluate the change of *Leuconostoc* species and *Lb. plantarum* during Kimchi fermentation, the juice of Kimchi (Table 3) was filtered by sterilized gauze and

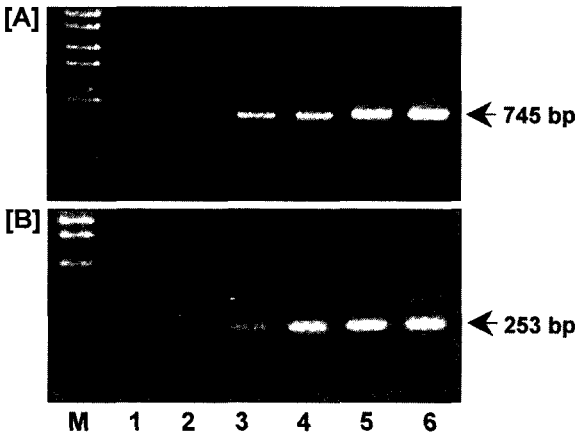

Fig. 3. Correlation between the concentration of PCR product and the amount of template DNA. Total DNA from *Leu. mesenteroides* KFRI817 (A) and *Lb. plantarum* KCTC3108 (B) was used as the template, and *Leuconostoc*-specific (A) and *Lb. plantarum*-specific (B) primer sets were used for each amplification.
Lane M, DNA size marker (DNA digested with *Eco*T14I). The amounts of template DNA used: lane 1, 1 pg; lane 2, 10 pg; lane 3, 100 pg; lane 4, 1 ng; lane 5, 10 ng; lane 6, 100 ng.

the pH of the filtrate was determined using a pH meter (Orion 410A, U.S.A.) at room temperature. The juice of Kimchi was inoculated in MRS broth and grown facultative-anaerobically for 2 h at 30°C. Total DNAs from the cultured cells were isolated with a DNeasy Tissue Kit (Qiagen, Germany). The extracted DNAs were suspended in TE buffer (pH 8.0) and determined quantitatively by spectrophotometric method. Total DNAs from the 2 h-cultured microflora of Kimchi were extracted for 16 days and the equal amounts of DNA templates were used for PCR. The culture in MRS broth can influence the proportion of microbial population of Kimchi, but can minimize the effect from dead cells.

For the semi-quantitative determination of microbial change during Kimchi fermentation, several different amounts of DNA samples were tested for PCR amplification. Different intensities of DNA bands obtained from PCR using a *Leuconostoc*-specific and LAB-detectable primer sets were observed at the concentration of 1 ng template DNA during Kimchi fermentation. Furthermore, when the same DNA samples were also used for PCR using a *Lb. plantarum*-specific primer set, the different intensities of bands appeared at the concentration of 100 ng during the fermentation (Fig. 4).

Table 3. The ratio (w/w) of ingredients used for Baechu (Chinese cabbage) Kimchi preparation.

Ingredients	Ratio (%)
Brined Chinese cabbage	71.3
Seasoning Mixture	
Sliced Chinese radish	13.0
Sliced green onion	2.0
Chopped garlic	4.5
Chopped ginger	0.5
Red pepper powder	3.0
Salt	2.0
Starch powder	3.0
Sugar	0.5
MSG (Monosodium glutamate)	0.2

The ingredient ratios and preparation methods of Baechu Kimchi, a general type of Kimchi, were employed as described by KFRI (http://kimchi.kfri.re.kr) and Park *et al.* [13]. Chinese cabbage was cut lengthwise into halves and quarters, and immersed in 12% (w/w) salt solution for 19 h until softened. It was washed three times with running water and then drained gravitationally for 1 h. The brined cabbage sections were mixed with prepared seasoning mixture. The prepared Kimchi was stored for 16 days at 15°C.

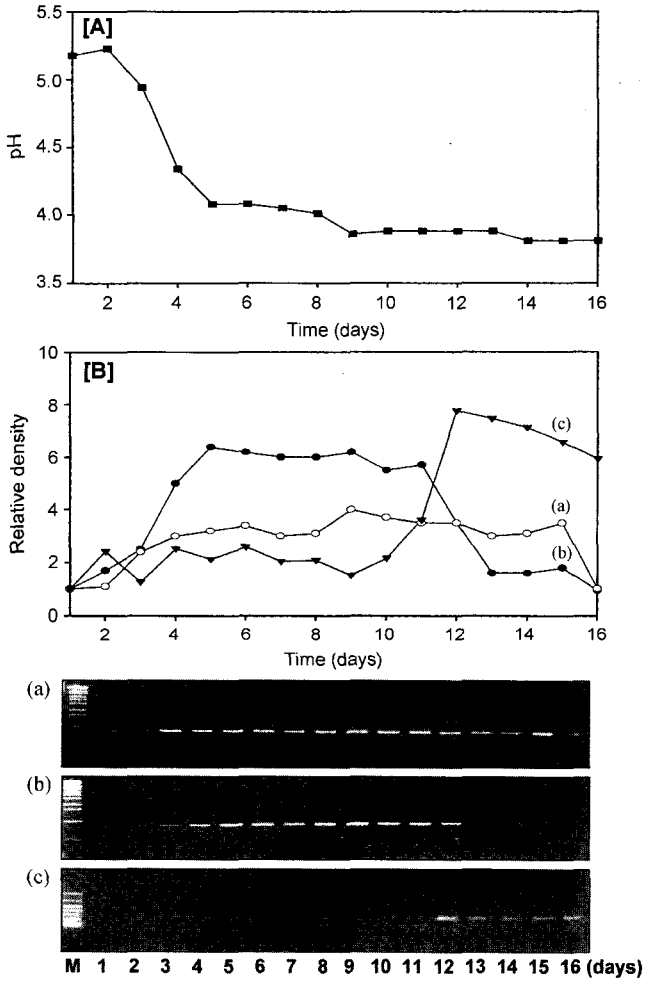

Fig. 4. The change of pH and PCR band intensity of *Leuconostoc* species and *Lb. plantarum* during Kimchi fermentation.
A, pH change; B, Intensity of PCR product. Primer sets used were LAB-detectable (a), *Leuconostoc*-specific (b), and *Lb. plantarum*-specific (c). The intensity of the band from the first day (preparation day) sample was defined as 1 for the determination of relative intensity by a densitometer (Image Master VDA, APBiotech).

As the fermentation proceeded, the intensity of the band for the *Leuconostoc* species increased sharply until the 5th day and the level was maintained until the 11th day. The sharp increase of *Lb. plantarum* occurred after 11 days with the decrease of the *Leuconostoc* species. During 16 days, the change of band intensity for LAB was insignificant compared with those for *Leuconostoc* species and *Lb. plantarum*. The weak intensity of bands at the initial day seemed to reflect the low proportion of LAB in the total microflora of Kimchi. The band intensity for *Lb. plantarum* reached a maximum at the 12th day and decreased gradually. Decreasing band intensities were observed in all cases of PCR amplification on the 16th day. These results might have been due to the increase in the population of other acid-tolerant microorganisms such as yeast. According to the results on the population of microorganisms during Kimchi fermentation, the viable total cell number of LAB reached a maximum after 6 days at 14°C, but the number of yeast increased exponentially after 6 days at the same temperature [4, 12].

Kimchi is known to have its best flavor, taste, and texture when optimally fermented at pH around 4.2 [12]. The initial pH of Kimchi was 5.2, and the value decreased to 3.8 during 16 days of fermentation. At around pH 4.2, the band intensity of *Leuconstoc* species was maximum and decreased with the decrease of pH. As the pH changed, the population change of *Leuconostoc* species and *Lb. plantarum* also changed, and this observation was consistent with the results obtained by earlier researchers. The results of this study confirmed that *Leuconostoc* species were the major microorganisms at the beginning of fermentation and reached their highest population during the optimum ripening period of Kimchi. However, further studies are in need to define *Lb. plantarum* as the main acidifying organism during Kimchi fermentation, because the detection level of *Lb. plantarum* was only 1% of *Leuconostoc* species in this PCR.

The results of Mheen and Kwon [12] showed that *Lb. plantarum* appeared at 30, 20, and 14°C, but could not be detected at lower temperature (5°C), and similar results were also reported by Lim *et al.* [10] and Lee *et al.* [8]. The difficulty of discrimination between *Lb. plantarum* and *Lb. pentosus* was recognized in other LAB studies [1, 15]. In addition, Lim *et al.* [10] also pointed out the difficulty of discrimination between *Lb. plantarum* and *Lb. brevis* by biochemical methods. Therefore, the suggestion that *Lb. plantarum* was responsible for overripening of Kimchi has to be further tested by careful studies at several temperatures.

Acknowledgment

This work was supported by a research grant from Kyonggi University (2001).

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