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Multiplex PCR-based detection and identification of *Leuconostoc* species

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Abstract

A multiplex polymerase chain reaction (PCR) assay has been developed for rapid and reliable identification of *Leuconostoc* species, by using species-specific primers targeted to the genes encoding 16S rRNA. This assay can detect and differentiate *Leuconostoc* species from mixed populations in natural sources as well as from pure cultures, within 3 h. This assay system consists of a total of 10 primers, two primers from each target species, and comprises two multiplex PCR reactions: one reaction for *Leuconostoc carnosum*, *Leuconostoc citreum* and *Leuconostoc mesenteroides*, and another reaction for *Leuconostoc gelidum* and *Leuconostoc lactis*. This multiplex PCR assay was used to identify 31 *Leuconostoc* strains isolated from kimchi, a fermented-cabbage product, and the results showed perfect correlation with the results of a polyphasic method, including 16S rDNA sequencing and DNA–DNA hybridization. In addition, this assay enables simultaneous detection of the above-mentioned *Leuconostoc* species when chromosomal DNA from these *Leuconostoc* species was mixed. Thus, these results suggest that this multiplex PCR is a rapid and reliable method for identification of *Leuconostoc* species in pure cultures or in mixed populations.

Keywords: *Leuconostoc* species; Multiplex polymerase chain reaction; 16S rRNA gene

1. Introduction

The genus *Leuconostoc* consists of Gram-positive, facultatively anaerobic, non-sporing, catalase-negative, spherical bacteria that produce lactic acid as a main end-product of fermentation [1]. Leuconostocs have been isolated from vegetables, fermented-vegetable products and dairy products and currently consist of eight validly described species, namely, *Leuconostoc argentinum*, *Leuconostoc carnosum*, *Leuconostoc citreum*, *Leuconostoc fallax*, *Leuconostoc gelidum*, *Leuconostoc lactis*, *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*. *Leuconostoc* strains play important roles in vegetable fermentations, especially in the early phase of fermentation. Hence, evaluating the distribution of leuconostocs in food products is of major interest. These evaluations require unequivocal and reliable methods for identification of both *Leuconostoc* isolates and uncultured leuconostocs in food.

The classical phenotypic methods for the identification of *Leuconostoc* species are unreliable [2]. Some advanced molecular taxonomic techniques, developed to overcome the defect of the classical phenotypic methods, are also labor-intensive, time-consuming and sometimes unreliable. These techniques include DNA–DNA hybridization [3], sodium dodecyl sulfate–polyacrylamide gel electrophoresis of whole-cell proteins [4], and randomly amplified polymorphic DNA analysis [5]. To overcome these disadvantages, polymerase chain reaction (PCR)-based methods using species-specific primers have been considered [6].

Therefore, in this study, we established a multiplex PCR assay to identify *Leuconostoc* strains rapidly, in pure cultures or in mixed population. This assay has proven useful for rapid identification of *Leuconostoc* isolates and for evaluating the distribution of *Leuconostoc* species in mixed population.

2. Materials and methods

2.1. Bacterial strains

The *Leuconostoc* species used in this study were *L. ar-*

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gentinum DSM 8581^T, *L. carnosum* DSM 5575^T, *L. citreum* KCTC 3526^T, *L. fallax* DSM 20189^T, *L. gelidum* DSM 5578^T, *L. lactis* KCTC 3528^T, *L. mesenteroides* ssp. *mesenteroides* KCTC 3505^T, *L. pseudomesenteroides* DSM 20193^T. All *Leuconostoc* species were grown in MRS broth (Difco Laboratory) at 20°C. Non-target organisms used in this study were *Escherichia coli* K-12 W3010 (laboratory stock), *Bacillus subtilis* ATCC6051^T, *Lactobacillus brevis* DSM 20054^T, *Lactobacillus plantarum* DSM 20174^T, *Weissella confusa* KCTC 3499^T and *Weissella paramesenteroides* DSM 20288^T. For a microbial mixture from garlic or Chinese cabbage, a piece of garlic or the pieces of Chinese cabbage (from grocery) which were washed in 2.5% NaCl solution, were incubated in MRS broth at 20°C until saturation. These cultured cells in MRS broth were used to prepare a mixed chromosomal sample that was subsequently used for multiplex PCR.

2.2. 16S rDNA-targeted species-specific primers

To design species-specific primers, which target 16S rDNA sequences, we first compared the 16S rDNA sequences of a total of eight *Leuconostoc* species using the CLUSTAL W multialignment program [7]. We then designed 12 species-specific primers, both forward and reverse primers for six *Leuconostoc* species, except *L. pseudomesenteroides* and *L. fallax* that were treated as a *L. mesenteroides* group and as a distinct group [8], respectively. The primers were selected from the most varied regions of 16S rDNA sequences of the *Leuconostoc* species. GenBank accession numbers for the 16S rDNA sequences are as follows: *L. argentinum* (AF175403), *L. carnosum* (X95977), *L. citreum* (AF111949), *L. fallax* (S63851), *L. gelidum* (AF175402), *L. lactis* (M23031), *L. mesenteroides* (M23034) and *L. pseudomesenteroides* (X95979). To avoid possibly forming self-dimers, we made a nucleotide substitution in a reverse primer for *L. lactis*.

2.3. PCR

The chromosomal DNAs were isolated by the method described previously [9]. PCR amplification of the 16S rDNA was performed in a 50-μl reaction mixture containing 0.5 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate and 1.25 U of *Taq* DNA polymerase (Perkin-Elmer) by using the following program: 1 cycle of denaturation for 5 min at 94°C; 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min; a final extension step at 72°C for 10 min. The PCR products were analyzed by electrophoresis through 1.5% (w/v) agarose gel and visualized by staining with ethidium bromide. For PCR with mixed chromosomes from different *Leuconostoc* species, each chromosomal DNA was added in the amount of 300 ng. For PCR with the chromosomal DNA from natural sources, a total of 2 μg chromosomal DNA was used. For evaluating the detection limit of PCR, PCR was performed as described above with the serial dilutions of each chromosomal DNA.

3. Results and discussion

The aim of this study was to develop a novel multiplex PCR, which uses minimum reactions and enables rapid and reliable identification of *Leuconostoc* species. For this aim, we first designed species-specific primers using the alignment of 16S rDNA sequences of *Leuconostoc* species, which were partly determined recently by Kim et al. [9]. These species-specific primers were designed in both forward and reverse direction for each *Leuconostoc* species (Table 1), because *Leuconostoc* species-specific signature regions on 16S rDNA sequences are very limited. These limitations resulted from a high homology (over 97%) between 16S rDNA sequences of seven out of eight known *Leuconostoc* species [9]. For example, the 16S rDNA se-

Table 1
Primers specific to *Leuconostoc* strains

Target	Primer	Sequence	Position[a]	Size[b] (bp)
L. argentinum	Larg-f	5′ACTGGTTAACTTGAGTCTT3′	642–660	830
(*L. citreum*[c])	Lcit-r	5′CTTAGACGACTCCCTCCCG3′	1452–1470	
L. carnosum	Lcar-f	5′CTTAGTATCGCATGATATC3′	183–195	318
	Lcar-r	5′CTGGTATGGTACCGTCAGG3′	478–496	
L. citreum	Lcit-f	5′AAAACTTAGTATCGCATGATATC3′	180–195	1298
	Lcit-r	5′CTTAGACGACTCCCTCCCG3′	1452–1470	
L. gelidum	Lgel-f	5′TCGTATCGCATGATACAAG 3′	185–197	1290
	Lgel-r	5′TAGACGGTTCCCTCCTTAC3′	1450–1468	
L. lactis	Llac-f	5′AGGCGGCTTACTGGACAAC3′	729–747	742
	Llac-r	5′CTTAGACGGCTCCTTCCAT3′	1452–1470	
L. mesenteroi-	Lmes-f	5′AACTTAGTGTCGCATGAC3′	181–192	1150
des	Lmes-r	5′AGTCGAGTTACAGACTACAA3′	1307–1326	

[a]Numbering system for *E. coli* 16S rRNA gene.
[b]Expected size of an amplified PCR fragment using species-specific primers set.
[c]This primer set also produces an 830-bp PCR product from the chromosomal DNA of *L. citreum*.

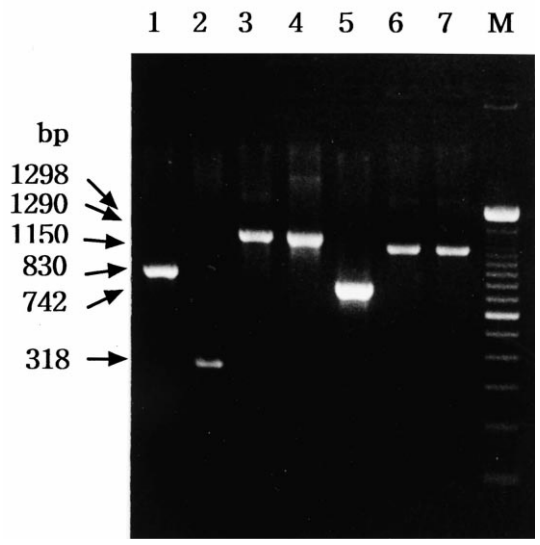

Fig. 1. PCR amplification products obtained from each species-specific primer set. Lanes: 1, *L. argentinum* DSM 8581^T (Larg-f and Larg-r); 2, *L. carnosum* DSM 5576^T (Lcar-f and Lcar-r); 3, *L. citreum* KCTC 3526^T (Lcit-f and Lcit-r); 4, *L. gelidum* DSM 5578^T (Lgel-f and Lgel-r); 5, *L. lactis* KCTC 3528^T (Llac-f and Llac-r); 6, *L. mesenteroides* ssp. *mesenteroides* KCTC 3505^T (Lmes-f and Lmes-r); 7, *L. pseudomesenteroides* DSM 20193^T (Lmes-f and Lmes-r); M, 100-bp DNA ladder.

quence of *L. mesenteroides* KCTC 3505^T is similar to that of *L. pseudomesenteroides* DSM 20193^T (99.6% identity), and the 16S rDNA sequence of *L. argentinum* DSM 8581^T is similar to both *L. lactis* KCTC 3528^T (99.6% identity) and *L. citreum* KCTC 3526^T (99.4% identity). We found that each PCR amplification reaction generated a unique DNA fragment of the expected size without producing PCR products from non-target species, proving that each primer set was species-specific (Figs. 1 and 2). For example, primer set Lcar-f and Lcar-r yielded a 318-bp PCR product for *L. carnosum*. The primer set did not react with the seven other *Leuconostoc* species, *E. coli*, *B. subtilis*, *Lb. brevis*, *Lb. plantarum*, *W. confusa*, and *W. paramesenteroides* (data not shown). PCR amplifications with other primer sets also showed specificity (Fig. 1): a 742-bp PCR band was obtained for *L. lactis* using both Llac-f and Llac-r; a 1290-bp band for *L. gelidum* (using Lgel-f and Lgel-r) and a 1298-bp band for *L. citreum* (using Lcit-f and Lcit-r). However, the primer set Larg-f and Larg-r yielded an 830-bp PCR product from both *L. argentinum*, and *L. citreum*, implying that there is no specificity for this primer set to discriminate between *L. argentinum* and *L. citreum*. Similarly, the primer set Lmes-f and Lmes-r yielded a 1150-bp PCR product for both *L. mesenteroides* and *L. pseudomesenteroides*. Our results show that species-specific primers designed in this study work well for discriminating between *L. carnosum*, *L. citreum*, *L. gelidum* and *L. lactis*.

Next we tested a multiplex PCR assay, in which the species-specific forward and reverse primers were mixed in a single reaction tube for discriminating members of the genus *Leuconostoc*. However, the multiple primers in a single reaction revealed problems: first, chromosomal DNA of *L. citreum* made two PCR products, namely 742- and 1298-bp fragments, which elicited confusion with the 742-bp-long PCR product from *L. lactis*. Second, two PCR products from *L. citreum* (1298 bp) and *L. gelidum* (1290 bp) were not separated well by electrophoretic analysis, due to their similar size. Therefore, we separated this multiplex PCR reaction into two tubes containing different sets of PCR primers: Set-A mixture consists of primers for *L. carnosum*, *L. citreum* and *L. mesenteroides*, and Set-B mixture for *L. gelidum* and *L. lactis*. In this system, the primers for *L. argentinum* were removed, because these primers were not species-specific as mentioned earlier, while the primers for *L. mesenteroides* were added despite their nonspecificity toward *L. pseudomesenteroides*, because these primers were likely still useful in detecting widespread strains of *L. mesenteroides*. We found that multiplex PCR in two reactions can specifically identify *Leuconostoc* species (Fig. 2). Set-A primer set produced specific PCR bands for *L. carnosum* DSM 5576^T (318 bp), *L. citreum* KCTC 3526^T (1298 bp) and *L. mesenteroides* ssp. *mesenteroides* KCTC 3505^T (1150 bp) (Fig. 2, lanes 1–5), while Set-B primer set produced specific bands for *L. gelidum* DSM 5578^T (1290 bp) and *L. lactis* KCTC 3528^T (742 bp) (Fig. 2, lanes 6–10). The detection limit of the multiplex PCR (100–500 pg of the chromosomal DNA) was higher than that of species-specific PCR using two primers (5 pg of DNA). We further questioned whether multiplex PCR assay could identify *Leuconostoc* strains isolated from food products. To test this question, we carried out multiplex PCR using chromosomal DNA from a total of 31 *Leuconostoc* strains, isolated from kim-

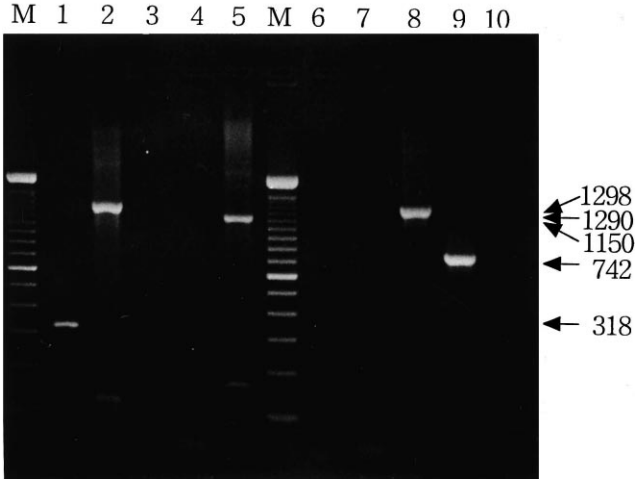

Fig. 2. PCR amplification products obtained by multiplex PCR from type strains of the genus *Leuconostoc*. Lanes: 1 and 6, *L. carnosum* DSM 5576^T; 2 and 7, *L. citreum* KCTC 3526^T; 3 and 8, *L. gelidum* DSM 5578^T; 4 and 9, *L. lactis* KCTC 3528^T; 5 and 10, *L. mesenteroides* ssp. *mesenteroides* KCTC 3505^T; M, 100-bp DNA ladder (BRL). Set-A mixture covers lanes 1 through 5 and contains the following primers: Lcar-f, Lcar-r, Lcit-f, Lcit-r, Lmes-f and Lmes-r. Set-B mixture covers lanes 6 through 10 and contains Lgel-f, Lgel-r, Llac-f and Llac-r.

chi, a fermented-cabbage product. We found that multiplex PCR successfully enabled us to identify a total of 31 strains, which were previously identified by a polyphasic classification method including 16S rDNA sequencing (unpublished results), as follows: 10 strains of *L. citreum*, 10 strains of *L. gelidum*, nine strains of *L. mesenteroides* and two strains of *L. lactis*. These results show that multiplex PCR developed in this study, is a reliable identification method for *Leuconostoc* species.

Our third question was whether multiplex PCR enabled us to identify different *Leuconostoc* species from mixed chromosomal DNA. We carried out a multiplex PCR using a chromosomal DNA mixture from the above-mentioned five *Leuconostoc* species (300 ng chromosomal DNA per strain). Set-A multiplex PCR yielded three specific bands for *L. carnosum*, *L. citreum* and *L. mesenteroides* (Fig. 3, lane 1) and Set-B PCR yielded two specific bands for *L. gelidum* and *L. lactis* (Fig. 3, lane 2). These results indicate that multiplex PCR is useful for species-specific detection from the mixture of chromosomal DNA. However, we also found that the ratio of a chromosomal DNA to other DNA in DNA mixture is important. When chromosomal DNA of *L. mesenteroides* was reduced to one sixth the amount (50 ng) of our standard amount (300 ng) in a chromosomal mixture, our multiplex DNA assay did not produce a 1150-bp PCR product, while the assay produced all other four bands clearly (data not shown). On the other hand, species-specific primers (Lmes-f and Lmes-r) for *L. mesenteroides* successfully produced a 1150-bp band with the same DNA mixture. These results suggest that our multiplex PCR can detect several *Leuconostoc* species simultaneously that exist together in mixed population even though it appears that this assay has some limitation when the ratio of a chromosomal DNA to other DNA in the DNA mixture falls below 1:6.

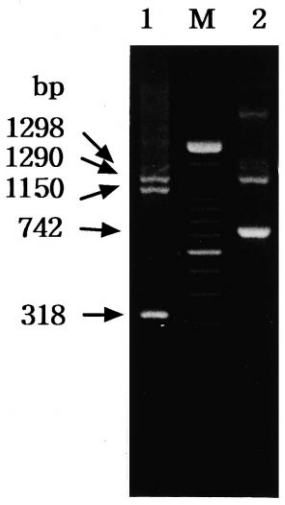

Fig. 3. Multiplex PCR-based identification of *Leuconostoc* species in mixed chromosomes. A chromosomal mixture containing an equal amount of chromosomal DNA (300 ng each) from *L. carnosum*, *L. citreum*, *L. gelidum*, *L. lactis* and *L. mesenteroides*, was used for multiplex PCR. Lanes: 1, multiplex PCR carried out by using Set-A mixture (Lcar-f, Lcar-r, Lcit-f, Lcit-r, Lmes-f, Lmes-r); 2, Set-B mixture (Lgel-f, Lgel-r, Llac-f, Llac-r); M, 100-bp DNA ladder.

Our final question was whether this multiplex PCR assay could be applied to a chromosomal mixture prepared from a microbial population derived from natural sources such as garlic or cabbage. We next cultured inborn *Leuconostoc* species from garlic or Chinese cabbage in MRS media and used them to prepare chromosomal DNA. Then these mixtures was subjected to multiplex PCR of the chromosomal DNA. We found the presence of several

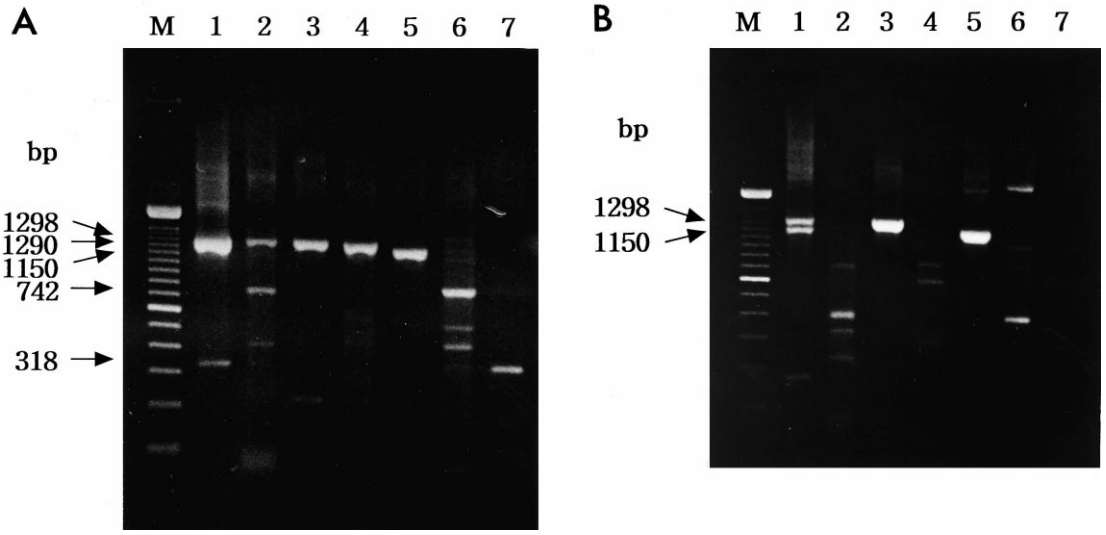

Fig. 4. Multiplex PCR-based identification of *Leuconostoc* species from a mixed culture that was cultured using garlic (A) or Chinese cabbage (B). Lanes: 1, multiplex PCR with Set-A primer set; 2, multiplex PCR with Set-B primer set. PCR amplification with species-specific primer set (lanes 3–7): lane 3, Lcit-f and Lcit-r (1298 bp); 4, Lgel-f and Lgel-r (1290 bp); 5, Lmes-f and Lmes-r (1150 bp); 6, Llac-f and Llac-r (742 bp); 7, Lcar-f and Lcar-r (318 bp).

Leuconostoc species together in garlic (Fig. 4A) or Chinese cabbage (Fig. 4B). Three distinct PCR products from the Set-A PCR reaction represent *L. carnosum* (318 bp), *L. mesenteroides*/*L. pseudomesenteroides* (1150 bp) and *L. citreum* (1298 bp) (Fig. 4A, lane 1). DNA sequencing confirmed that the 1150-bp PCR band represented *L. mesenteroides* (data not shown). Two PCR bands from the Set-B reaction indicate *L. lactis* (742 bp) and *L. gelidum* (1290 bp) (Fig. 4A, lane 2). PCR amplification using each species-specific primer sets confirmed that these results from multiplex PCR are specific (Fig. 4A, lanes 3–7). In addition, we found that our multiplex PCR assay simultaneously detects *L. citreum* (1298 bp) and *L. mesenteroides* (1150 bp) from a chromosomal mixture of Chinese cabbage (Fig. 4B, lane 1). On the other hand, other *Leuconosotoc* species were not detected by the multiplex PCR (Fig. 4B, lane 2) or by each species-specific primer set (Fig. 4B, lanes 3–7). Some non-specific background PCR products due to non-specific annealing appear weak and especially differ in product size from those of the target PCR products, resulting in no confusion in identification of *Leuconostoc* species (Fig. 4). These results show that this multiplex PCR assay can be applied to chromosomal mixtures prepared from microbial populations derived from natural sources. These results also support the previous findings that leuconostocs have their ecological niche on plants and vegetables [10].

In conclusion, these results show that this multiplex PCR assay is a rapid and reliable method for identification of *Leuconostoc* species in pure cultures or in mixed populations. Therefore, this multiplex PCR assay may be useful for routine detection of *Leuconostoc* species in food analysis laboratories.

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