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Molecular identification of *Bacillus licheniformis* isolates from Korean traditional fermented soybean by the multilocus phylogenetic analysis

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Abstract

In this study, *Bacillus licheniformis* which has been used as probiotics was isolated from Korean traditional fermented soybean. A total of 69 strains were presumptively identified as *B. licheniformis* by phenotypic methods. Based on PCR amplification and 16S rRNA gene sequencing, the multilocus sequence typing of *gyrA* and *rpoB*, followed by phylogenetic analysis was performed. The isolates were distinctly differentiated and found to be closely related to *B. amyloliquefaciens*, *B. subtilis*, and *B. aerius*. The partial 16S rRNA gene sequences of those strains matched those of *B. sonorensis* (97%) and *B. aerius* (98%) in the phylogenetic tree. In contrast, multilocus phylogenetic analysis (MLPA) showed that only 61 (86.9%) out of 69 strains were *B. licheniformis*. The rest of those strains were found to be *B. subtilis* (5.8%), *B. amyloliquefaciens* (2.9%), and *B. sonorensis* (2.9%), respectively. Therefore, our results suggested that since the 16S rRNA gene sequencing alone was not sufficient to compare and discriminate closely related lineages of *Bacillus* spp., it was required to analyze the MLPA simultaneously to avoid any misleading phenotype-based grouping of these closely related species.

Key words : *Bacillus licheniformis*, Korean fermented soybean, Molecular identification, Multilocus phylogenetic analysis

INTRODUCTION

For many years, microbial adjuncts have been used as dietary supplements for farm animals and humans. They have known as probiotics as functional food with health benefits (Bernardeau and Vernoux, 2013). *Bacillus* spp. is Gram-positive, aerobic, and endospore forming bacterium commonly found in the environment (Lee et al, 2005). Fermented soybean products indigenous to Asian countries including Korea have been traditionally used as nutritious food source, especially as digestible protein source. They contain *Bacillus* spp., including *B. subtilis* and *B. licheniformis* (Lee et al, 2005; Kim et al, 2009; Knap et al, 2010). Recent studies have shown that certain strains of *B. licheniformis* confer significant potential probiotic actions as either food supplements for human or feed additives for animals (Sorokulova et al, 2008; Yun et al, 2014). It has been suggested that *B. licheniformis* strains enhance the potential functional capacity of gut microbiota, promoting the growth and general immune response of laying hens and pigs (Davis et al, 2008; Deng et al, 2012).

Identification of *Bacillus* spp. has become difficult and laborious because conventional phenotypic tests fail to distinguish different strains. In addition, *Bacillus* spp. strains have identical 16S rRNA gene sequences (99.2～99.6% sequence similarity) (Ash et al, 1991; Nakamura et al, 1999). The 16S rRNA gene has been used as a framework for modern bacterial classification. However, it often shows limited variation for members of closely

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related taxa (Fox et al, 1992). Recent taxonomic studies have indicated that *B. licheniformis* is closely related to *B. subtilis* and *B. amyloliquefaciens* according to comparisons of 16S rDNA and 16S—23S internal transcribed spacer (ITS) nucleotide sequences (Xu and Cote, 2003). Sequencing *gyrA* and *rpoB* genes was found to be useful to distinguish species within the *B. subtilis* group (Chun and Bae, 2000; Palmisano et al, 2001) and the *gyrB* gene was studied to discriminate members within the *B. cereus* group (Yamada et al, 1999).

Therefore, the objective of this study is to investigate the molecular identification of 69 *B. licheniformis* isolates by carrying out the multilocus phylogenetic analysis (MLPA) of *gyrA* and *rpoB* sequences, which was isolated from Korean traditional fermented soybean and initially confirmed based on 16S rRNA gene sequencing. Also, the current study could be the basis to suggest using several *B. licheniformis* among those definitely classified isolates for further useful field.

MATERIALS AND METHODS

Bacteria

A total of 69 strains of *B. licheniformis* identified by phenotypic tests were provided by the Microbial Institute for Fermentation Industry (Sunchang, Korea). These strains were isolated from Korean traditional fermented soybean including Doenjang, Cheongkookjang, Gochujang, and Soy sauce.

All strains of *B. licheniformis* were cultured on tryptic soy agar (TSA) plate at 37°C for 24 hours. Single colonies were separately recultured in tryptic soy broth (TSB). Cultured *Bacillus* spp. were stored at −70°C with Cryocare Bacteria Preservers (Key Scientific Products, Stamford, USA) until used.

Genomic DNA extraction

Stored bacteria were inoculated to TSA and incubated at 37°C for 24 hours. Single colony was inoculated to TSB and cultured at 37°C for 24 hours. Bacterial culture was centrifuged at 8,000 rpm for 10 min. After discarding the supernatant, cell pellet was subjected to genomic DNA extraction using DNeasy Mini Kit (Qiagen, CA, USA). The concentration of extracted DNA was determined using a Take3 session with Epoch spectrophotometer (Biotek, VT, USA) at 260, 280 and 320 nm.

Analysis of 16S rRNA gene

The 69 strains of *B. licheniformis* were subjected to 16S rRNA gene sequence analysis. Sequencing was conducted through a commercial service (Macrogen, Seoul, Korea). PCR was performed to amplify DNA fragments of 16S rRNA gene using 27 F (5'-AGAGTTTGATCMTGGCTC) and 1492 R (5'-TACGGYTACCTT GTTACGACT T-3') primers. The amplicons were purified using the *Accupower*® gel purification kit (Bioneer, Daejeon, Korea) according to the manufacturer's instruction. Sequencing was performed using Big Dye terminator cycle sequencing kit v.3.1 (Applied Biosystems, CA, USA) and Applied Biosystems model 3730 XL automated DNA sequencing system (Applied Biosystems, USA). Primers 518 F (5'-CCAGCAGCCGCGGTAATACG-3') and 800 R (5'-TACCAGGGTATCTAATCC-3') were used for sequencing. The 16S rRNA sequences of the 69 strains were aligned with reference sequences obtained from GenBank database (NCBI, USA). Phylogenetic trees were constructed using neighbor-joining method with MEGA5 program (Tamura et al, 2001).

Multilocus Phylogenetic analysis (MLPA) of *gyrA* and *rpoB*

Species level identification of the 69 strains was performed through phylogenetic analysis using *gyrA* and *rpoB*. Primer sequences, PCR amplification, and sequencing for both genes were performed as described previously (De Clerck and De Vos, 2004; Rooney et al, 2009). Gene-specific primers used in this study are listed in Table 1. Amplicons were purified using the *Accupower*® gel purification kit and sequenced at the Macrogen Service Center. Partial *gyrA* or *rpoB* sequences of these strains were aligned to those of well-known *Bacillus* strains using ClustalW2 (EBI, UK).

Table 1. Primers used for the phylogenetic analysis of Bacillus licheniformis

Primers	Sequence (5'-3')	Size (bp)	Target gene	Reference
gyrA-42f	CAGTCAGGAAATGCGTACGTCCTT	964	*GyrAse* subunit A	De Clerck and De Vos (2004)
gyrA-1066r	CAAGGTAATGCTCCAGGCATTGCT			
rpoB-2292f	GACGTGGGATGGCTACAACT	875	RNA polymerase subunit B	Rooney et al. (2009)
rpoB-3354r	ATTGTCGCCTTTAACGATGG			

Genetic distances were determined using Kimura's two-parameter model (Kimura, 1980). Neighbor-joining phylogenetic trees were constructed using MEGA5 (Tamura et al, 2011). Branch quality was assessed by bootstrap test using 1,000 replicates.

RESULTS

Identification of *Bacillus licheniformis*

The phylogenetic tree showed that most of strains were closely related to each other belonging to *B. licheniformis* among the 69 strains isolated from Korea traditional fermented soybean food. However, some of strains were closely associated with other *Bacillus* species. In addition, a couple of were confirmed to be very closely related *to B. licheniformis* (Fig. 1). The partial 16S rRNA gene sequences (1,052 nt) of strain *B. licheniformis* matched those of *B. sonorensis* (97%) and *B. aerius* (98%) in the phylogenetic tree.

Partial sequence analysis of *rpoB* and *gyrA*

Detailed discrimination was performed through MLPA using partial *gyrA* and *rpoB* gene sequences. The MLPA tree showed that those strains were distinctly separated from independent lines of *B. sonorensis* due to high levels of sequence divergence (Fig. 2). It did not seem to be possible to discriminate these strains since *gyrA* and *rpoB* gene sequences of *B. aerius* were not available in public databases. Based on the MLPA results, three subgroups of *B. licheniformis* were found. The first group comprised of 45 strains, including the type strain American Type Culture Collection (ATCC) 14580. The second group contained eight strains. The last group had one strain (Fig. 2). Both *gyrA* and *rpoB* sequences of 59 strains shared 99.8～100% identities with *B. licheniformis* ATCC 14580 type strain. The MLPA results revealed that the 69 strains contained two strains of *B. sonorensis* (2.9%), two strains of *B. amyloliquefaciens* (2.9%), four strains of *B. subtilis* (5.8%), and 61 strains of *B. licheniformis* (86.9%, Fig. 2).

DISCUSSION

In general, to delineate strains for the *Bacillus* classification as well as for typing other numerous bacteria, the 16S rRNA gene sequencing has been used as a framework (Heyndrickx et al, 1996). According to the 16S rRNA gene sequence of *B. licheniformis* was found to be closely related to other *Bacillus* spp. (Xu and Cote, 2003). Sequencing *gyrA*, *rpoB*, and gyrB genes has been found to be a useful tool in discriminating species within the *B. subtilis* group (Chun and Bae, 2000; Palmisano et al, 2001) and the *B. cereus* group (Yamada et al, 1999). In this study, 69 isolates originated from Korean traditional fermented soybean food were identified by the PCR amplification and the 16S rRNA gene sequencing and the MLPA of *gyrA* and *rpoB* genes followed by phylogenetic analysis. Our results revealed that the 69 strains were closely related to *B. amyloliquefaciens*, *B. subtilis*, and *B. aerius*. The partial 16S rRNA gene sequences (1,052 nt) of these strains matched those of *B. sonorensis* (97%) and *B. aerius* (98%) in the phylogenetic tree. On the other hand, the result of the MLPA showed that only 86.9% (n=61) of those strains belongs to *B. licheniformis*. The rest of strains was found to belong to *B. subtilis* (5.8%; n=4), *B. amyloliquefaciens* (2.9%; n=2), and *B. sonorensis* (2.9%; n=2). Our results suggested that the MLPA were valuable methods to differentiate *B. licheniformnis* from closely related *Bacillus* species.

Fig. 1. Phylogenetic tree constructed by aligning 16S rRNA sequences derived from currently known *Bacillus* spp. and the 69 strains isolated from Korean fermented soybean food. Numbers at nodes indicate bootstrap values (Percentages of 1,000 replicates). Bar: 0.002 substitutions per nucleotide position.

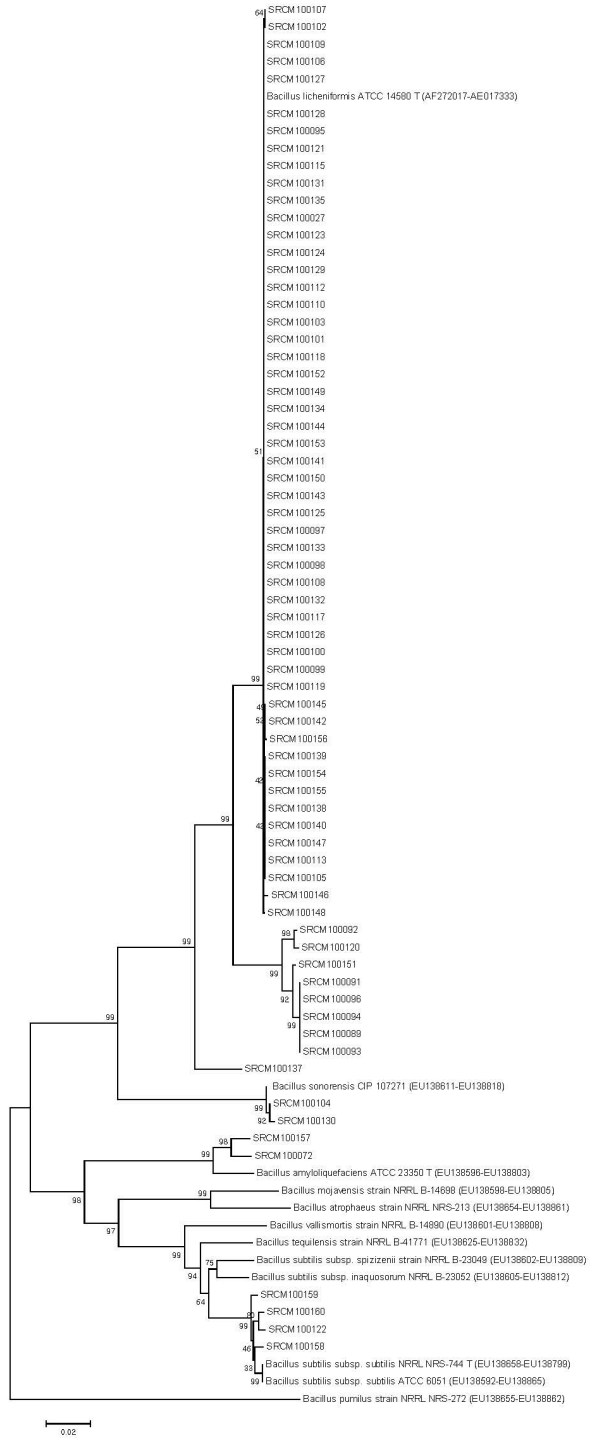

Fig. 2. Multilocus phylogenetic analysis tree constructed from alignments of two different genes (*gyrA* and *rpoB*) derived from the 69 strains and known *Bacillus* spp. Numbers at nodes indicate bootstrap values (Percentages of 1,000 replicates). Bar: 0.02 substitutions per nucleotide position.

The *rpoB* gene sequence is more polymorphic than the 16S rRNA gene sequence (Ki et al, 2009). The high degree of polymorphism is particularly evident for species which cannot be well discriminated using the 16S rRNA gene sequence analysis, such as marine *Bacillus* species (Ki et al, 2009) and bacterial strains belonging to genus *Aeromonas* (Kupfer et al, 2006). In this study, the *rpo*B gene sequencing was presented to have a higher accuracy rate for the identification at species level than the 16S rRNA gene sequencing. Also, the partial *gyrA* sequences (coding for DNA gyrase subunit A) of the representative *B. subtilis* was determined and their taxa were allied to establish the value of our approach in regard of underpinning phylogenetic relationship. Our approach could be used for the routine identification of closely related aerobic endospore-forming bacilli. This is the first step to use.

In conclusion, we demonstrated the potential of *gyrA* and *rpoB* gene sequencing analysis in distinguishing *B. licheniformis* from other closely related *Bacillus* species. Our simple, economical, rapid, and reliable protocol might be used as one of valuable alternatives to misleading phenotype-based grouping of these closely related *Bacillus* species. Accurate strain characterization can be important to step up for further studies such as searching new fields of use. With this clear classification, it will be the basis for further applications of the *Bacillus* spp. isolates such as feed additives for livestock.

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