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Use of novel specific primers targeted to *pheS* and *tuf* gene for species and subspecies identification and differentiation of the *Bacillus subtilis* subsp. *subtilis*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis*

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To clearly delimit the members of the *Bacillus subtilis* group (BSG), is difficult using common phenotypic and genotypic methods. This study described the use of *pheS* and *tuf* gene as targets for interspecies discrimination within the BSG, and also to develop specific PCR and SNP primers for species and subspecies identification and differentiation. The average sequence similarity values of the *pheS* and *tuf* gene among type strains were 85.1 and 94.7%, respectively, and all members of the BSG could be clearly distinguished based on phylogenetic analyses of *pheS* gene sequence. In addition, the specific primers were designed according to *pheS* and *tuf* gene sequence. The primers were shown to specifically identify *B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens* and *Bacillus licheniformis*, and clearly differentiate the subspecies of *B. amyloliquefaciens* using specific-PCR, combined with two-plex minisequencing method. In conclusion, we have successfully established a comparative sequence analysis and rapid molecular diagnosis techniques for determination of interspecies within the BSG.

Key words: *Bacillus subtilis* group (BSG), species and subspecies discrimination, comparative sequence analysis, specific Pcr, two-plex minisequencing.

INTRODUCTION

The *Bacillus subtilis* group (BSG) contains more than 10 closely related taxa (Dunlap et al., 2015), and have some beneficial effects of BSG members as reported, like the production of enzymes, antibiotics, vitamins and fermented foods, which are commonly applied as animal feeds additives (Sorokulova et al., 2013; Kubo et al., 2011). Many studies have demonstrated that BSG strains have beneficial effects on production performance in domestic

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animals (Alexopoulos et al., 2004; Kritas et al., 2006; Knap et al., 2010; Ahmed et al., 2014). However, the species and subspecies determination of these phylogenetically related bacteria has long been problematic.

DNA-DNA hybridization (DDH) is the gold standard for bacterial species delineation (Stackebrandt et al., 2002), but this method is time-consuming, labor intensive, costly and difficult to use routinely in laboratories. To date, a comparative analysis on the 16S rDNA is a commonly used genotypic method for bacterial identification, and the strains that show at least 98.7% sequence similarity between the 16S rDNA are recognized to the same species (Stackebrandt and Ebers, 2006).

Unfortunately, poor discrimination has been observed in BSG, due to the high degree of similarity (reaching 99-100%) of the 16S rRNA gene sequences (Wang et al., 2007). In contrast, DNA sequences of housekeeping genes with a higher resolution seem to be more effective than the 16S rDNA and may act as an alternative to DDH, for species determination (Guo et al., 2012). The *tuf* gene encodes the elongation factor Tu associated with protein biosynthesis, which facilitates the aminoacyl-tRNA to the ribosomes during the translation process. Moreover, *tuf* is universally distributed, and the various copy numbers (one to three) per bacterial genome have been found (Ke et al., 2000). The *tuf* gene is ideally suited for inferring phylogenetic relationships between bacteria (Chavagnat et al., 2004; Picard et al., 2004). Phenylalanyl-tRNA synthase gene (*pheS*) has also been proposed as a useful molecular marker in the closely related species complex (Naser et al., 2005; Naser et al., 2007). In this study, we determined the utility of *pheS* and *tuf* genes sequences for species and subspecies discrimination in BSG, and as targets to develop specific primers for identification and differentiation.

MATERIALS AND METHODS

Bacillus strains and culture conditions

All BSG type strains and isolates were obtained from Bioresource Collection and Research Center (BCRC) and are listed in Table 1. *Bacillus* strains were incubated aerobically on Nutrient agar (NA, Difco) for 24 h at 30°C.

Genomic DNA preparation and design of degenerate primers

The chromosomal DNA was extracted using the DNeasy Kit (Qiagen, Valencia, CA, USA), and the DNA concentration and purity were measured using an absorbance ratio of 260/280 nm and checked by agarose gel electrophoresis. By comparison with the *pheS* and *tuf* genes from the whole genome sequence in BSG species (Accession no: CP002905, AL009126, CP002207, FN597644, CP000560, CP000002), the degenerate primers, pheS-21F: 5'-CAYCCNGCHCGYGAYATGC-3' and BasbpheS-416R: 5'-ARYACRTTCGGRTGAACCAT-3', and Basbtuf-F1: 5'-CAAACTCGTGAGCACATYCT-3' and Basbtuf-R1: 5'-CGTCAGTTGTACGGAARTAG-3', were designed and targeted to the most conserved region of the gene.

Target gene amplification and DNA sequencing

The partial fragments of *pheS* and *tuf* genes of BSG related strains were amplified and sequenced using consensus degenerate primers. The thermal protocol was carried out under the following conditions: 5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; and 7 min at 72°C. The resulting amplicons were purified using the QIAquick PCR purification Kit (Qiagen Inc., Valencia, CA, USA) and sequenced with the BigDye Terminator v3.1 cycle-sequencing Kit on the 3730 DNA sequencer (Applied Biosystems and Hitachi, Foster City, CA, USA).

Intespecific bioinformatic analysis

The *pheS* and *tuf* gene sequences of all strains were aligned using the Clustal X program (version 1.8). The DNA sequence similarities were calculated using the MatGAT (version 2.02) (Campanella et al., 2003). Phylogenetic tree was performed with the PHYLIP (version 3.63) package, using the neighbour-joining method (Felsenstein, 2004; Kimura, 1980; Saitou and Nei, 1987).

Species and subspecies-specific primers design and PCR identification

The PCR oligonucleotide primers is specific for *B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens* and *Bacillus licheniformis* were designed based on the *pheS* and *tuf* gene sequences, and all reference strains were used for specific PCR testing. The thermal protocol was carried out under the following conditions: 5 min at 94°C; 25 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C; and 7 min at 72°C.

Subpecies-specific SNaPshot mini-sequencing assay

The SNP primers specific for *B. amyloliquefaciens* subspecies were designed based on the *pheS* gene sequences. The mini-sequencing protocol and final data analysis were followed as previously described (Huang et al., 2014).

RESULTS AND DISCUSSION

In this study, partial *pheS* and *tuf* gene fragments (approximately 410 bp and 750 bp) were successfully amplified from all BSG strains and were used for sequencing, by using degenerate primers. The average sequence similarity values for *pheS* and *tuf* genes among BSG type strains were mean 85.1 and 94.7%, respectively, which exhibit greater variation than 16S rDNA sequence (98.9%). The topology of the *pheS* tree showed all BSG members that could be discriminated (Figure 1). Nowadays, several molecular targets have been exploited to differentiate BSG members. The three targets (*gyrA*, *gyrB* and *phoR*) showed good resolution with a high discrimination power, and the average

Table 1. Strains used in this study and their detection using each primer pairs.

Bacillus spp.	BCRC strains	Species and subspeices-specific PCR assays			
		Bamy[a]	Blic[b]	Blic[c]	Bsub[d]
Bacillus licheniformis	11702^T, 11718, 11594, 11958, 11978, 14353, 10287, 10259	−	+	+	−
Bacillus sonorensis	17416^T, 17532	−	−	−	−
Bacillus subtilis subsp. *subtilis*	10255^T, 12144, 10872, 10058, 11602, 14717, 17441, 17435, 17436, 17437, 17438, 17439, 17440, 17442, 17443, 12141, 17890, 10613, 10614, 10615, 10616, 10617, 11703, 12142, 14645, 14638, 14639, 14640, 14199, 10258	−	−	−	+
Bacillus subtilis subsp. *inaquosorum*	17984^T, 17975, 17976, 17977, 17978, 17979, 14192, 80075	−	−	−	−
Bacillus subtilis subsp. *spizizenii*	17366^T, 10447, 14643, 14644, 80045, 80046, 80047, 80076	−	−	−	−
Bacillus mojavensis	17124^T, 17501, 17502, 17531, 17653, 17654, 80138, 80139, 80140,	−	−	−	−
Bacillus amyloliquefaciens subsp. *amyloliquefaciens*	11601^T, 11266, 17038, 14710, 14711, 12815, 11199, 10453, 14637	+	−	−	−
Bacillus amyloliquefaciens subsp. *plantarum*	80282^T, 80285, 80604, 80110, 11557, 14193, 17650, 17710	+	−	−	−
Bacillus atrophaeus	17123^T, 17530	−	−	−	−
Bacillus siamensis	80787^T	−	−	−	−
Bacillus vallismortis	17183^T, 80798, 80799	−	−	−	−
Bacillus tequilensis	17634^T	−	−	−	−

BCRC, Bioresource Collection and Research Center at Food Industry Research and Development Institute, Taiwan; +, PCR amplicons with each primer pair detected; −, PCR amplicons with each primer pair not detected. [a], species-specific-PCR amplification with primer spBamyphes-171F1 (5'-TCTCGTCGTSGACCGCAA-3')/353R1 (5'-GATCCAGCCTGTTTTTTTGC-3'); [b], species-specific-PCR amplification with primer spBlichphes-177F1 (5'-CGTTGACCGTGATATCAGCAT-3')/304R1 (5'-GCAGGAAACATCGACTTC-3'). [c], species-specific-PCR amplification with primer spBlichtuf-118F1 (5'-CTTTCTGAGTATGAGTTCC-3')/210R1 (5'-CCKCCATCAGTTCRAAGATT-3'). [d], subspecies-specific-PCR amplification with primer spBsubtuf-190F1 (5'-GACGCTGAGTGGGAAGCTAA-3')/363R1 (5'-ATGATTTCAACTTCGTCACCG-3').

sequence similarity values of these targets were 83.7, 83.3 and 77.9%, respectively (Chun and Bae, 2000; Guo et al., 2012; Wang et al., 2007). Therefore, the *pheS* gene can be as an additional phylogenetic marker for differentiating among the BSG.

On the other hand, all DNA sequences were submitted to GenBank (accession number: KX987658-KX987837), and this accumulated sequence data could be applied to design specific primers for direct identification of particular microbials (Krawczyk et al., 2002). The species-specific primer has been established for *B. subtilis* based on Endo-beta 1,4-glucanase gene and *ytcP* (encoding a hypothetical protein similar to a ABC-type transporter) gene (Ashe et al., 2014; Kwon et al., 2009). To the best of our knowledge, there were no such studies, in the identification of *B. subtilis* at subspecies level. Four primer pairs were designed based on multiple alignments of the *pheS* and *tuf* sequences, and these primers successfully generated a single species and subspecies-specific band (202 bp, 145 bp, 112 bp and 194 bp) when used in PCR reactions with *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* subsp. *subtilis* DNA (data not shown).

Moreover, the amplified fragments were sequenced, and the results demonstrated that the sequence agreed with what were expected. Annealing temperatures and additional PCR amplification cycles may influence PCR specificity (Krawczyk et al., 2002). In the present study, the most appropriate conditions for our primer pairs were an annealing temperature of 65°C and 25 cycles of PCR amplification. The specificity of these primer pairs were tested against the

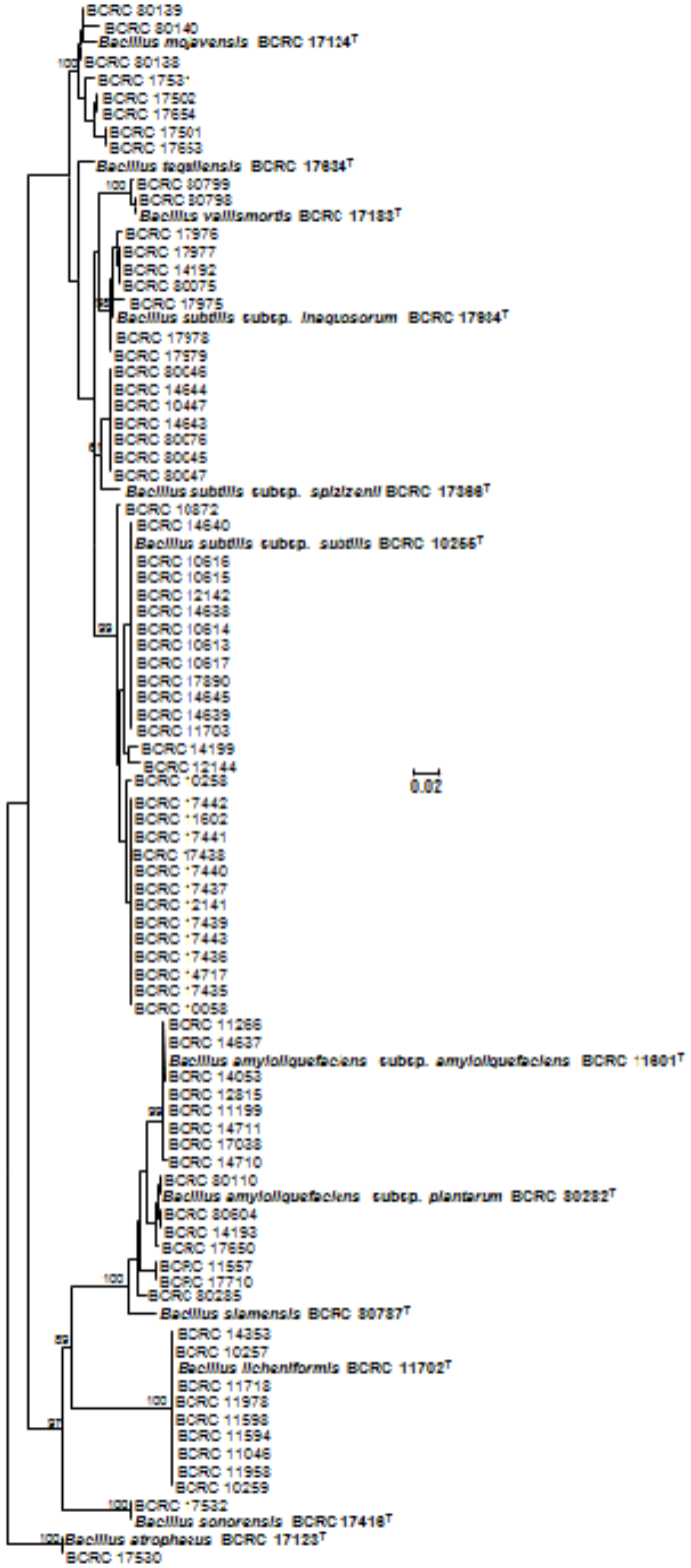

Figure 1. Phylogenetic tree of the BSG strains based on *pheS* gene sequences. The tree was constructed using the neighbor-joining method. Only bootstrap percentages above 60% are indicated (based on 1,000 replications). The scale bar represents 0.02% sequence divergence.

Table 2. Single nucleotide polymorphisms in *pheS* gene sequences of the subspecies within the *B. amyloliquefaciens* strains.

***B. amyloliquefaciens* subspecies**	**Sequence at the indicated position:**	
	231	**306**
amyloliquefaciens (no.=9)	G	A
plantarum (no.=8)	C	G

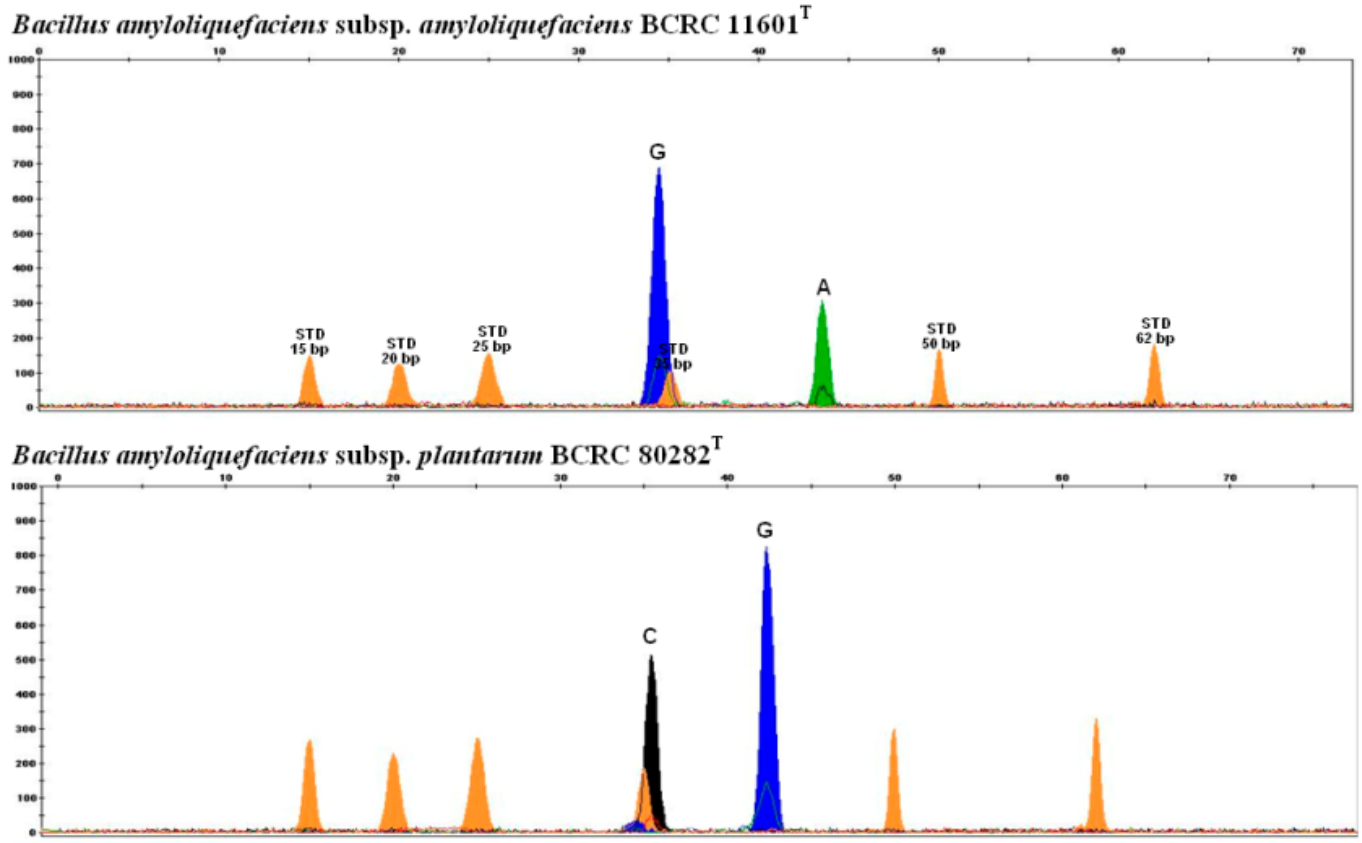

Figure 2. Electropherograms obtained from *B. amyloliquefaciens* strains by mini-sequencing assay. The *X*-axis represents the size of the mini-sequencing products (nucleotides); the *Y*-axis represents relative fluorescence units (RFUs). STD: GS120 LIZ size standard.

organisms indicated in Table 1.

In addition, five strains of *B. subtilis* subsp. *subtilis* were isolated from probiotic and feed additive samples, which were identified based on the DNA sequencing and specific-PCR method (data not shown).

Although *B. amyloliquefaciens* strains could be preliminary identified using species-specific PCR, but this method was not able to provide an accurate discrimination at the subspecies level. Afterward, a mini-sequencing assay was applied. The SNP specific primers spBamyphes-231f1 (5'-GCACGCTTGAATTGGTYGC-3') and spBamyphes-306f1 (5'-GACTGACTGACTCRTTCACAGAGCCTTCTGTCGA-3') were designed to anneal immediately, adjacent to the nucleotide at two subspecies-specific SNPs found at positions 231 and 306 in the alignment of all *B. amyloliquefaciens pheS* gene sequences (Table 2). Following the above, *B. amyloliquefaciens* species-specific amplicons containing two diagnosis sites were purified and subjected to a duplex mini-sequencing reaction. The results showed the presence of two peaks of the expected color and position in all samples (Figure 2; Table 1). Compared to other genotypic methods for strain differentiation of *Bacillus* spp. such as repetitive element palindromic PCR (rep-PCR), PCR restriction fragment length polymorphism (RFLP) and DNA

sequencing (Banyko and Vyletelova, 2009; Freitas et al., 2008; Jeyaram et al., 2011; Shaver et al., 2002), the mini-sequencing method is more direct and rapid due to its determined exact single nucleotide polymorphism at diagnosis sites.

Conclusion

All members of BSG can be clearly discriminated by housekeeping gene sequencing, and the developed specific primers can be successfully applied to quickly and accurately identify the *B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens* subspecies and *B. licheniformis* using specific PCR combined with mini-sequencing assay.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Ahmed ST, Islam M, Mun HS, Sim HJ, Kim YJ, Yang CJ (2014). Effect of *Bacillus amyloliquefaciens* as a probiotic strain on growth performance, cecal microflora, and fecal noxious gas emissions of broiler chickens. Poult. Sci. 93:1963-1971.

Alexopoulos C, Georgoulakis IE, Tzivara A, Kyriakis CS, Govaris A, Kyriakis SC (2004). Field evaluation of the effect of a probiotic-containing *Bacillus licheniformis* and *Bacillus subtilis* spores on the health status, performance, and carcass quality of grower and finisher pigs. J. Vet. Med. A. Physiol. Pathol. Clin. Med. 51:306-312.

Ashe A, Maji UJ, Sen R, Mohanty S, Maiti NK (2014). Specific oligonucleotide primers for detection of endoglucanase positive *Bacillus subtilis* by PCR. 3 Biotech. 4:461-465.

Banyko J, Vyletelova M (2009). Determining the source of *Bacillus cereus* and *Bacillus licheniformis* isolated from raw milk, pasteurized milk and yoghurt. Lett. Appl. Microbiol. 48:318-323.

Campanella JJ, Bitincka L, Smalley J (2003). MatGAT: an application that generate similarity/identity matrices using protein or DNA sequences. BMC Bioinform. 4:23.

Chavagnat F, Haueter M, Jimeno J, Casey MG (2002). Comparison of partial tuf gene sequences for the identification of lactobacilli. FEMS Microbiol. Lett. 17:177-183.

Chun J, Bae KS (2000). Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* gene sequences. Antonie Van Leeuwenhoek 78:123-127.

Dunlap CA, Kim SJ, Kwon SW, Rooney AP (2015). Phylogenomic analysis shows that *Bacillus amyloliquefaciens* subsp. *plantarum* is a later heterotypic synonym of *Bacillus methylotrophicus*. Int. J. Syst. Evol. Microbiol. 65:2104-2109.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 39:783-791.

Felsenstein J (2004). PHYLIP (phylogenetic inference package), version 3.6. Distributed by the author. Department of Genome Sciences and Department of Biology, University of Washington, Seattle.

Freitas DB, Reis MP, Lima-Bittencourt CI, Costa PS, Assis PS, Chartone-Souza E, Nascimento AM (2008). Genotypic and phenotypic diversity of *Bacillus* spp. isolated from steel plant waste. BMC Res. Notes 1:92.

Guo Q, Li S, Lu X, Li B, Stummer B, Dong W, Ma P (2012). *phoR* sequences as a phylogenetic marker to differentiate the species in the *Bacillus subtilis* group. Can. J. Microbiol. 58:1295-1305.

Huang CH, Chang MT, Huang L, Chua WS (2014). Molecular discrimination and identification of *Acetobacter* genus based on the partial heat shock protein 60 gene (*hsp60*) sequences. J. Sci. Food Agric. 94:213-218.

Jeyaram K, Romi W, Singh TA, Adewumi GA, Basanti K, Oguntoyinbo FA (2011). Distinct differentiation of closely related species of *Bacillus subtilis* group with industrial importance. J. Microbiol. Methods 87:161-164.

Ke D, Boissinot M, Huletsky A, Picard FJ, Frenette J, Ouellette M, Roy PH, Bergeron MG (2000). Evidence for horizontal gene transfer in evolution of elongation factor Tu in enterococci. J. Bacteriol. 182:6913-6920.

Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111-120.

Knap I, Lund B, Kehlet AB, Hofacre C, Mathis G (2010). *Bacillus licheniformis* prevents necrotic enteritis in broiler chickens. Avian Dis. 54:931-935.

Kubo Y, Rooney AP, Tsukakoshi Y, Nakagawa R, Hasegawa H, Kimura K (2011). Phylogenetic analysis of *Bacillus subtilis* strains applicable to natto (fermented soybean) production. Appl. Environ. Microbiol. 77:6463-6469.

Krawczyk B, Lewandowski K, Kur J (2002). Comparative studies of the *Acinetobacter* genus and the species identification method based on the *recA* sequence. Mol. Cell Probes 16:1-11.

Kritas SK, Govaris A, Christodoulopoulos G, Burriel AR (2006). Effect of *Bacillus licheniformis* and *Bacillus subtilis* supplementation of ewe's feed on sheep milk production and young lamb mortality. J. Vet. Med. A. Physiol. Pathol. Clin. Med. 53:170-173.

Kwon GH, Lee HA, Park JY, Kim JS, Lim J, Park CS, Kwon DY, Kim YS, Kim JH (2009). Development of a RAPD-PCR method for identification of Bacillus species isolated from Cheonggukjang. Int. J. Food Microbiol. 129:282-287.

Picard FJ, Ke D, Boudreau DK, Boissinot M, Huletsky A, Richard D, Ouellette M, Roy PH, Bergeron MG (2004). Use of *tuf* sequences for genus-Specific PCR detection and phylogenetic analysis of 28 Streptococcal species. J. Clin. Microbiol. 42:3686-3695.

Naser SM, Thompson FL, Hoste B, Gevers D, Dawyndt P, Vancanneyt M, Swings J (2005). Application of multilocus sequence analysis (MLSA) for rapid identification of Enterococcus species based on *rpoA* and *pheS* genes. Microbiology, 51:2141-2150.

Naser SM, Dawyndt P, Hoste B, Gevers D, Vandemeulebroecke K, Cleenwerck I, Vancanneyt M, Swings J (2007). Identification of lactobacilli by *pheS* and *rpoA* gene sequence analysis. Int. J. Syst. Evol. Microbiol. 57:2777-2789.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Shaver YJ, Nagpal ML, Rudner R, Nakamura LK, Fox KF, Fox A (2002.) Restriction fragment length polymorphism of rRNA operons for discrimination and intergenic spacer sequences for cataloging of *Bacillus subtilis* sub-groups. J. Microbiol. Methods 50:215-223.

Sorokulova I (2013). Modern Status and Perspectives of *Bacillus* Bacteria as Probiotics. J. Prob. Health 1:e106.

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PA, Kampfer P, Maiden MC, Nesme X, Rossello-Mora R, Swings J, Truper HG, Vauterin L, Ward AC, Whitman WB (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. 52:1043-1047.

Stackebrandt E, Ebers J (2006). Taxonomic parameters revisited: tarnished gold standards. Microbiol. Today 33:152-155.

Wang LT, Lee FL, Tai CJ, Kasai H (2007). Comparison of *gyrB* gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. Int. J. Syst. Microbiol. 57:1846-1850.