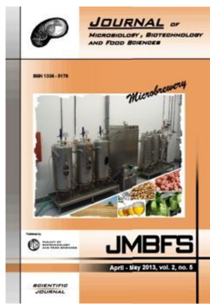

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POLYPHASIC IDENTIFICATION OF CLOSELY RELATED *BACILLUS SUBTILIS* AND *BACILLUS AMYLOLIQUEFACIENS* ISOLATED FROM DAIRY FARMS AND MILK POWDER

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ABSTRACT

A polyphasic approach was done to definitively distinguish isolates into two closely related species of the *Bacillus subtilis* group collected from the dairy farm environment or commercial powdered milk. A hundred and fifty six isolates and type strains *B. subtilis* (1A337) and *B. amyloliquefaciens* (10A18) were classified according to phenotypic and molecular characteristics. Only differences in growth temperature could be used to distinguish isolates among the phenotypic traits tested, and these distinctions were supported by molecular analysis. Randomly amplified polymorphic DNA analysis (RAPD) analysis was shown to be a friendly, technically simple and accurate method for rapid screening and identification of *B. subtilis* and *B. amyloliquefaciens*. Further analysis of 16S rRNA, *rpo*B and *gyr*A gene sequences of the isolates was done to confirm species identification. Sequences from the isolates and type strains showed between 96.5-100% (16S rRNA), 94.8-100% (*rpo*B) and 80.6-99.6% (*gyr*A) similarity, thus allowing for more refined distinction using the *rpo*B and *gyr*A genes. In addition, *gyr*A gene sequences had greater discrimination potential in having higher divergence between species (18.2 ± 0.7 %) than did *rpo*B sequences (4.9 ± 0.3 %). BOX-PCR fingerprinting was shown to have the potential for analysis of genotypic diversity of these species at the strain level.

Keywords: Dairy, polyphasic identification, *Bacillus subtilis* group, RAPD-PCR

INTRODUCTION

Raw milk provides a very suitable medium for bacterial growth, and milk quality is largely dependent on its microflora. *Bacillus* species and their spores, often present in raw milk (**Coorevits *et al.*, 2008**), play an important role in the dairy industry in being responsible for spoilage of raw and pasteurized milk as well as other dairy products (**Burgess *et al.*, 2010**; **Scott *et al.*, 2007**). These spore-forming bacteria contaminate raw milk both as the vegetative form and as heat resistant spores. Contamination sources in the dairy farm environment are very diverse and include water, soil, air, feces, udder and milking equipment. Industrial processes such as ultra-high temperature treatment and milk powder manufacture are designed to result in sterile products, however spoilage frequently occurs because of microbial heat resistance, spore-formation and growth and biofilm formation on processing lines (**Scott *et al.*, 2007**). Bacterial production of extracellular enzymes such as proteases, lipases and lecithinases can contribute to spoilage. The majority of aerobic spore-forming contaminants in milk belong to the genus *Bacillus* and nearly 70% of these are in the *Bacillus subtilis* group. This group includes primarily *B. subtilis, B. licheniformis, B. amyloliquefaciens* and *B. pumilus*, which are also generally considered the representative species (**Coorevits *et al.*, 2008**).

Phenotypic grouping of these closely related species based on colony morphology, fatty acid composition and physiological characteristics such as carbohydrate fermentation is very often misleading particularly in efforts to distinguish *B. subtilis* and *B. amyloliquefaciens* (**Coorevits *et al.*, 2008; Logan and Berkeley, 1984**). Bacterial identification and distinction of these species using 16S rDNA sequencing also has major drawbacks. Analysis of 16S rRNA gene sequences could not be used to distinguish *B. subtilis* and *B. amyloliquefaciens* because sequence similarities greater than 99% were found (**Ash *et al.*, 1991; Nakamura *et al.*, 1999**). However, restriction fragment length polymorphism (RFLP) analysis of this sequence distinctly differentiated closely related *B. amyloliquefaciens, B. licheniformis* and *B. pumilus* from *B. subtilis* sensu stricto (**Jeyaram *et al.*, 2011**). Alternative phylogenetic markers have recently been described for differentiating closely related *Bacillus* species using protein-encoding genes (i.e., *rpoB, gyrA, gyrB, vrrA*) (**Blackwood *et al.*, 2004; Chun and Sook Bae, 2000; Ki *et al.*, 2009; Wang *et al.*, 2007**). The gene encoding beta subunit of DNA-directed RNA polymerase, *rpoB*, was demonstrated to be useful for identification of *Bacillus* species (**Ki *et al.*, 2009**). An advantage using the *rpoB* gene instead of the 16S rRNA gene is its occurrence in genomes as a single copy. Comparative sequence analysis of other genes universally distributed among bacterial species, i.e., *gyrA* and *gyrB*, which encode protein subunits A and B of DNA gyrase and that play an essential role in DNA replication, were shown to provide a solid framework for a rapid and accurate identification of *B. subtilis* and related species (**Chun and Sook Bae, 2000; Wang *et al.*, 2007**). To investigate simple methods for distinguishing *Bacillus* species and for study of intraspecies diversity, a number of DNA fingerprinting analysis methods have been reported. Repetitive element primed genomic fingerprinting (REP-PCR, BOX-PCR and $(GTG)_5$-PCR) allows for differentiation of species to the subspecies and strain level because individual strains typically have variable distances between genomic repetitive sequences. Rep-PCR methods have been used to determine intraspecies variation rather than for species identification purposes (**De Clerck and De Vos, 2004; Velezmoro *et al.*, 2012**), whereas randomly amplified polymorphic DNA analysis (RAPD) and 16S-23S internal spacer region (ISR) polymorphism has been used for *Bacillus* species identification (**Flint *et al.*, 2001; Kwon *et al.*, 2009; Ronimus *et al.*, 2003**).

Not all fingerprinting methods are equally effective for distinguishing species within the *B. subtilis* group (i.e., REP-PCR, **De Jonghe *et al.*, 2008;** ISR, **Miranda et al, 2008**), and so the development of more useful methods for distinguishing species within *Bacillus* genera remains a challenge. The RAPD-PCR method using the OPR13 primer (**Ronimus *et al.*, 1997**) enabled identification of large numbers of thermophilic and mesophilic *Bacillus* species from dairy industry and farm environment. The potential of this method as an identification tool is well documented (**Ronimus *et al.*, 2003; Rüeckert *et al.*, 2004**), but identification of *B. subtilis* and *B. amyloliquefaciens* OPR13 profiles was not resolved at species level. **Reginensi *et al.* (2011)** was able to distinguish most thermophilic *Bacillus* contaminants in commercial powdered milk using OPR13 profile analysis and 16S rDNA sequencing. As reported in the study of **Reginensi *et al.* (2011)**, two different OPR13 RAPD patterns with similar occurrence were identified as *B. subtilis* group isolates, but they could not be further differentiated by 16S rRNA gene BLAST homology analysis.

Bacteria of the *B. subtilis* group are widely recognized as one of the most important microbial contaminants that affect dairy production, however this group also includes strains with activities that are useful for a range of industrial applications. A greater understanding of contamination hazard and potential benefit within this bacterial group will require improved methods to identify isolates accurately and definitively (**Ruiz-García *et al.*, 2005a; Reva *et al.*,**

2004). Our study explored routine physiological and biochemical tests, genomic fingerprinting techniques (rep-PCR and RAPD) and gene sequencing (16S rRNA, *rpoB* and *gyrA*) to develop an easy, inexpensive and widely accesible method for distinguishing *B. subtilis* and *B. amyloliquefaciens* isolates.

MATERIAL AND METHODS

Bacterial isolates and culture conditions

A hundred and fifty six bacteria identified as members of the *B. subtilis* group isolated from commercial milk powder and dairy environment sources (soil, feces, cow udder swab, feed concentrate and raw milk) belonging to our laboratory collection were used in this study. *B. subtilis* 1A337 and *B. amyloliquefaciens* 10A18 from the *Bacillus* Genetic Store Center (BGSC) were also included as type strain. Working cultures were prepared from isolate and type strain frozen stocks after growth and transfer twice in fresh Triptic Soy Broth (TSB, Oxoid Ltd., UK) at 37 °C for 24 h prior to growth at different temperatures on Plate Count Agar (PCA, Oxoid Ltd., UK). A 100-fold dilution of each overnight culture was plated in duplicate on PCA and incubated at 37 °C, 50 °C or 55 °C for 24-48 h.

Biochemical characterization

Conventional biochemical tests were done as described in Bergey's Manual of Systematic Bacteriology (**Sneath, 1986**). Proteolytic and amylolytic activities were evaluated for isolated colonies on PCA plates supplemented with 1% casein and 1% starch, respectively, and lipolytic activity was evaluated in Spirit Blue Agar (Difco Laboratories, Detroit, MI). The appearance of a clear halo zone around the colony was interpreted as positive for the activity being assayed.

Isolation of total DNA

Bacterial cultures were grown overnight in TSB and cells were harvested at 10,000 rpm for 5 min in a Spectrafuge 7M tabletop centrifuge (Labnet International Inc., USA). Cell pellets were suspended in 200 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA was purified using the Genomic DNA purification kit (Fermentas International Inc., USA) following manufacturer's instructions. Purified DNA was suspended in 40 μL TE buffer and used as template in amplification reactions. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific Incorporation, Wilmington, DE, USA).

RAPD-PCR analysis

RAPD analysis was done in 25 μL reaction mixtures containing 1X Thermo buffer (Fermentas, USA), 2.5 mM $MgCl_2$, 200 μM of each dNTP (Fermentas, USA), 1U Taq polymerase (Fermentas, USA), 1 mM primer OPR13 (5'-GGACGACAAG-3') (**Ronimus *et al.*, 1997**) and 20 ng template DNA. PCR cycling parameters included a denaturation step at 94 ºC for 3 min, 45 s; 35 cycles each consisting of 94 ºC for 15 s, 36 ºC for 15 s and 72 ºC for 2 min; and a final extension step at 72 ºC for 4 min. Control reaction mixtures lacking template DNA were included with each analysis. RAPD-PCR reactions were electrophoresed on 1.8% agarose gels using 0.5 x TBE buffer (45 mM Tris, 45 mM Boric acid, 1 mM EDTA, pH=8.0) as running buffer at 10 V/cm for 1 h, stained with 0.5 μg/mL ethidium bromide and visualized and photographed on a UV transilluminator.

PCR amplification of 16S rRNA, *rpoB* and *gyrA* genes

Eight randomly selected isolates representative of distinct RAPD profiles and type strains were identified by gene sequencing. Universal primers fD1 and rD1 were used to amplify a 1540 bp genome fragment within the 16S rRNA gene sequence (**Weisburg *et al.*, 1991**). Twenty-five µL reaction mixtures contained 1X Thermo buffer (Fermentas, USA), 2.5 mM $MgCl_2$, 200 µM of each dNTP (Fermentas, USA), 1U Taq polymerase (Fermentas, USA), 0.2 mM of each PCR primer and 20 ng template DNA. PCRs were done using an initial denaturation step at 94 ºC for 7 min, then 35 cycles of 94 ºC for 1 min, 56 ºC for 1 min and 72 ºC for 1 min and a final extension at 72 ºC for 10 min. A *B. subtilis rpoB* gene fragment corresponding to *rpoB* positions 6 to 585 in the coding region was PCR-amplified using primers rpoB-f (5'-AGGTCAACTAGTTCAGTATGGAC-3') and rpoB-r (5'-AGAACCGTAACCGGCAACTT-3'). A *gyrA* fragment corresponding to *B. subtilis gyrA* positions 43 to 1070 was amplified using primers gyrA-f (5'-CAGTCAGGAAATGCGTACGTCCTT-3') and gyrA-r (5'-CAAGGTAATGCTCCAGGCATTGCT-3'). Reactions were carried out as described by **De Clerck and De Vos (2004)**. All PCR amplifications were done in a Corbett CG1-96 thermal cycler with a palmtop computer interface (Corbett Research Ltd., Cambridge, UK) and each experiment included a negative control with no added template. Amplified fragments were purified and sequenced by Macrogen Sequencing Service, Korea, using an ABI PRISM 3730XL capillary sequencer (Applied Biosystems, CA, USA). DNA sequences were compared with those of the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and were aligned against each other using Clustal W package (**Thompson *et al.*, 1994**). Phylogenetic trees were constructed using neighbourjoining method (**Saintou and Nei, 1987**) by MEGA4 software (**Tamura et al., 2007**). The sequences of 16S rRNA, *rpoB* and *gyrA* genes determined in this study were deposited in GenBank/NCBI under accession numbers: HM441231 – 32, JQ746561-67 (16s rDNA), JQ746570-79 (*rpoB*) and JQ746582-91 (*gyrA*).

BOX and REP-PCR analysis

The REP2I (5'-ICGICTTATCIGGCCTAC- 3'), REP1R (5'-IIIICGICGICATCIGGC-3'), and BOX A1R (5'-CTACGGCAAGGCGACGCTGACG-3') primers were used as described previously by **Versalovic *et al.* (1994)**. The PCR products were electrophoresed on 1.5% agarose gels and analyzed as described above.

RESULTS AND DISCUSSION

A hundred and fifty six isolates previously classified as members of the *B. subtilis* group obtained from dairy farm environment and commercial powdered milk and two type strains (*B. subtilis* 1A337 and *B. amyloliquefaciens* 10A18) obtained from the *Bacillus* Genetic Stock Center, Ohio State University, were analyzed using biochemical assays and genotyped by fingerprinting and sequencing methods. The isolates were initially classified as members of the *B. subtilis* group because their colonies were of rod shaped Gram-positive cells often in chains and had the ability to form heat-resistant endospores. All isolates were motile, catalase positive, Voges-Proskauer positive, nitrate reduction test positive, and urease test negative. All isolates fermented D-glucose, D-mannitol and lactose, and had moderate growth with 10% NaCl included in the medium. There was strain-dependent variation in starch hydrolysis and in proteolytic and lipolytic activities. All isolates and type strains were able to grow at 37 and 50 ºC, but only half of the isolates and the *B. subtilis* type strain1A337 were able to grow at 55 ºC. Maximum growth temperature for *B. amyloliquefaciens* and *B. subtilis* isolates found in this study was in agreement with a number of literature reports (**Huang *et al.*, 2005; Priest *et al.* 1987; Montorsi and Lorenzetti, 1993; Rooney *et al.*, 2009**).

Historically, *B. amyloliquefaciens* and *B. subtilis* are at best very difficult to distinguish using the limited number of phenotypic tests available (**Logan and Berkeley, 1984; Ruiz-García *et al.*, 2005b**). **Welker and Campbell (1967)** found that these two species could be distinguished by the ability to growth in 10% NaCl, lactose fermentation with acid production and growth above 50 ºC. We found, however, that only maximum growth temperature could be used to categorize isolates and type strains. Other phenotypic tests could not be used to distinguish any of these strains as proposed by **Welker and Campbell (1967).**

RAPD typing

Primer OPR13 based RAPD analysis is described as a very useful way for identifying *Bacillus* species as contaminants in dairy products (**Reginensi *et al.*, 2011; Ronimus *et al.*, 2003; Rüeckert *et al.*, 2004**). Representative OPR13 RAPD profiles of thermophilic *Bacillus* isolates were initially defined by **Ronimus *et al.* (2003)**. *B. subtilis* isolate profiles shared bands of 1900, 1700, 1500, 950 and 700 bp, however a *B. amyloliquefaciens* profile pattern was not described in that study. RAPD profile analysis using the OPR13 primer allowed for identification and for distinguishing of the isolates and type strains by virtue of characteristic DNA fragment banding patterns obtained (see Figure 1). Isolates identified as either *B. subtilis* or *B. amyloliquefaciens*, based on growth temperature, were clearly related to one another and distinct for the isolates of the two species. There was a distinct banding pattern for isolates identified as *B. subtilis* and a different pattern for isolates designated *B. amyloliquefaciens*. The banding patterns obtained for type strains used in our study were somewhat different compared with corresponding group of dairy isolates, but shared some features in common in banding patterns. *B. subtilis* isolates shared representative bands of 1700, 1500, 1000, 950 and 500 bp (Figure 1, lanes 6 to 9), *B. subtilis* 1A337 type strain banding profile was slightly different, having a clear band at 700 bp as described by **Ronimus *et al.* (2003)**, but bands at 1000 and 500 bp were absent (Figure 1, lane 11). *B. amyloliquefaciens* isolate profiles showed representative bands at 2300, 1250, 1150 and 850 bp (Figure 1, lanes 2 to 5), but *B. amyloliquefaciens* 10A18 type strain lacked a 1150 bp band and had an additional band at 3000 bp (Figure 1, lane 10). To our knowledge, a *B. amyloliquefaciens* OPR13 RAPD profile banding pattern has not been reported previously. The RAPD profiles in lanes 2 and 8, however, was previously generated from an isolate from commercial powdered milk and provisionally identified within *B subtilis* group isolates (**Reginensi *et al.*, 2011**) because it could not be further discriminated by 16S rDNA BLAST analysis. Results from the current study reveal that this isolates should be assigned to *B. amyloliquefaciens* (UY976) and *B. subtilis* (UY1067).

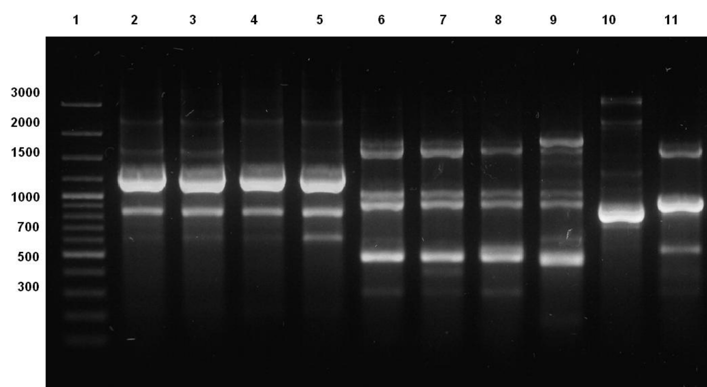

Figure 1 OPR13 RAPD fingerprint profile of isolates of *B. subtilis* group and type strains. Lane1, DNA molecular mass standard*; lanes 2-5, *B. amyloliquefaciens* isolates UY976, UY1091, T11, T144; lanes 6-9, *B. subtilis* isolates T46, UY907, UY1067, UY628; lane 10, *B. amyloliquefaciens* 10A18; lane 11, *B. subtilis* 1A337. * Molecular sizes in bp of the DNA standard (Generuler 100bp DNA Ladder Plus, Fermentas) are indicated in the left-hand margin.

Strain identification using 16S rRNA gene sequence

Eight randomly selected isolates representative of distinct RAPD profiles and type strains were identified by gene sequencing. A 1540 bp 16S rRNA gene fragment was amplified and sequenced from isolates and type strains. BLAST analysis of sequences obtained were highly similar to each other and could not be used to distinguish *B. subtilis* and *B. amyloliquefaciens* type strains or isolates. Analysis based on pairwise alignment of 16S rDNA sequences showed limited variation, and sequence similarities between isolates and type strains range from 96.5 to 100% (mean 98.5%). The high percentage of interspecies similarity for isolates and type strain sequences reflects the limited usefulness of 16S rRNA gene as molecular marker. These limitations for identification of species of *Bacillus* were discussed in detail previously (**Fox *et al.*, 1992; Hutsebaut *et al.*, 2006; Maughan and Van der Auwera, 2011**), and the 16S rRNA genes sequences of species within the *B. subtilis* group were found to be too highly conserved to enable discrimination among species and subspecies (**Daffonchio *et al.*, 1998; Shaver *et al.* 2002**). This restriction prompted the search for and development of alternative conserved genetic markers having a greater degree of variability among strains compared with the 16S rRNA gene. The use of essential housekeeping genes that evolve more quickly than the 16S rDNA gene have proven to be useful for taxonomic classification (**Palys *et al.*, 2000**).

Strain identification using *rpoB* and *gyrA* gene sequences

DNA fragments containing portions of *rpoB* and *gyrA* genes of selected isolates and type strains were amplified using specific primers. The amplicons were then sequenced and subjected to BLAST analysis. Unlike the high level of 16S rRNA gene sequence similarity among the isolates and type strains, sequences of the *gyrA* and *rpoB* genes had greater variation and could be used to distinguish these two species. Thus isolates UY628, UY1067, UY907 and T46 were unequivocally identified as *B. subtilis*, and isolates UY1091, UY976, T11 and T144 as *B. amyloliquefaciens.* The ability of the isolates and type strains to grow at 55 ºC was consistent with these assignments; isolates identified as *B. subtilis* were able to grow at this temperature whereas those identified as *B. amyloliquefaciens* were unable to grow at this temperature. This classification also agreed with the species-specific RAPD profile described.

Pairwise sequence similarity relationships between isolates and type strains were inferred from comparisons of the *rpoB* and *gyrA* gene sequences (Table 1). The *rpo*B-derived amplicons were of approximately 400 bp in length, and their sequence similarities between the eight isolates and type strains were between 94.8 to 100% (mean 97.1 %). Average (x ± sd) similarity of sequences from *B. amyloliquefaciens* and *B. subtilis* isolates were 99.5 ± 0.4% and 99.8 ± 0.2%, respectively, while the average divergence between species was 4.9 ± 0.3%. The partial *gyrA* gene sequences (835-950 bp) showed similarities between 80.6 to 99.6% (mean 88.9%). The *gyr*A gene had much greater variation between isolates of the same species with means of 98.7 ± 2.0% for *B. subtilis* isolates and 97.6 ± 2.0% for *B. amyloliquefaciens* isolates, and with a significantly higher degree of divergence between the two species 18.2 ± 0.7%. The partial *rpoB* and *gyrA* genes sequences both demonstrated potential as informative taxonomic markers. Highly species-specific sequences were evident in both genes thereby allowing for distinguishing type strains and thus the experimental isolates. Phylogentic analysis (Figure 2) supported pairwise sequence relationships previously discussed and confirmed that *rpoB* and *gyrA* genes allow species discrimination in contrast to 16S rRNA gene. Because of the higher levels of divergence and greater intraspecies variation of *gyr*A gene sequences compared with the *rpo*B gene, *gyrA* gene sequences enabled an accurate identification of the isolates by BLAST analysis.

Table 1 *B. subtilis* group isolates and type strains used in this study and percent similarity between *rpoB* and *gyrA* gene sequences

	rpoB									
	1[*]	2	3	4	5	6	7	8	9	10
1. *B. subtilis* 1A337[T**]		**99,7**	**100**	**100**	**100**	**95,8**	**95,1**	**95**	**95**	**95**
2. *B. subtilis* UY1067	98,6		**99,7**	**99,7**	**99,5**	**95,6**	**94,8**	**94,8**	**94,9**	**94,8**
3. *B. subtilis* UY907	98,7	98,7		**100**	**100**	**95,9**	**95,1**	**95,1**	**95,1**	**95,1**
4. *B. subtilis* T46	98,6	98,4	98,9		**99,7**	**95,9**	**95,1**	**95,1**	**95,2**	**95,2**
5. *B. subtilis* UY628	98,7	98,4	92,2	99,4		**95,7**	**95,1**	**95,1**	**94,9**	**94,9**
6. *B. amyloliquefaciens* 10A18[T**]	82,2	82,4	83,1	82,5	81,7		**99,2**	**99,2**	**99,2**	**99,2**
7. *B. amyloliquefaciens* T11	82,1	81,6	82,3	82,2	81,4	95,6		**99,5**	**99,5**	**99,5**
8. *B. amyloliquefaciens* UY1091	82,5	81	81,8	81,7	80,9	95,4	99,3		**100**	**100**
9. *B. amyloliquefaciens* UY976	81,9	80,6	82,1	82,1	81	95,1	99	99,2		**100**
10. *B. amyloliquefaciens* T144	83,1	81,5	81,3	81,2	81,2	95,1	98,8	99,6	98,8	
	gyrA									

[*] The numbers across the top correspond to the species on the left. ** [T]. Type strains

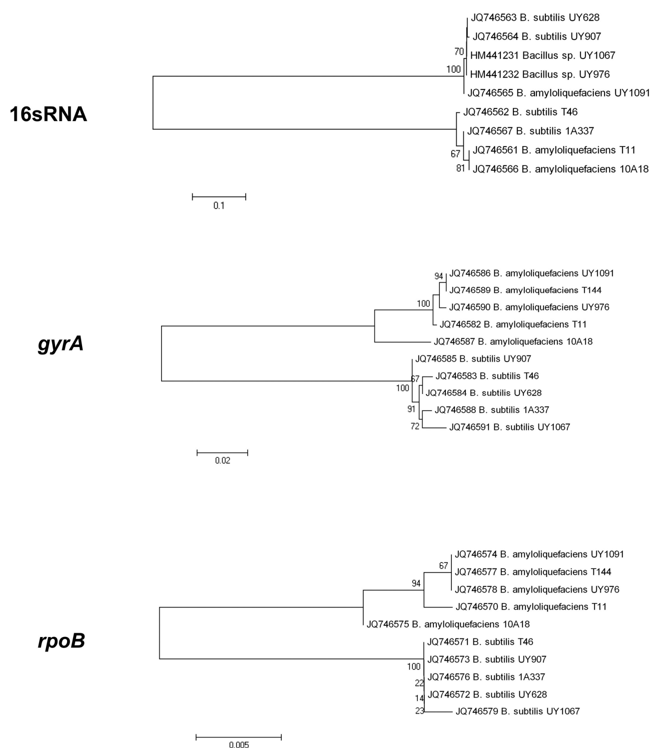

Figure 2 Phylogenetic trees obtained by Neighbor-joining analysis based on 16S rRNA, *gyrA* and *rpoB* genes sequences showing the phylogenetic position of isolates and type strains. Bootstrap values expressed as percentages of 1000 replications are shown at the branch points. Scale bar shows estimated substitutions per nucleotide position.

Our results confirm that molecular phylogenetic studies based on sequences of *gyrA* and *rpoB* can be used to distinguish species and potentially subspecies of the *B. subtilis* group in accordance with previous studies (**Ki *et al.*, 2009; Ko *et al.*, 2003; Nakamura *et al.*, 1999**). The *rpoB* gene sequences were less informative for distinguishing *Bacillus* isolates, however this gene was previously found to be more useful for strain discrimination in *Paenibacillus* and *Geobacillus* (**Da Mota *et al.*, 2004; Meintanis *et al.*, 2008**).

BOX-PCR and REP-PCR amplification

BOX-PCR amplification conditions were optimized using genomic template from isolates and type strains to obtain fingerprints with well-defined informative bands (see Figure 3). Variation in size and number of the BOX-PCR bands generated resulted in 9 different banding patterns or fingerprinting profiles from the isolates and type strains. Despite this general variability, two major groups could be distinguished (Figure 3), corresponding to *B. subtilis* and *B. amyloliquefaciens* isolates (Figure 3, lanes 2 to 6, and 7 to 11, respectively). The REP-PCR based profiles generated, however, contained a limited number of bands (<5) and were consistent within each of the species studied. Although the two species groups could be distinguished using this method, informative bands obtained did not contribute to distinction of internal group diversity (data not shown). The limited potential of this method for assessing genotypic diversity of *Bacillus* species from raw milk was previously reported by **De Jonghe *et al.* (2008)**. Through comparison of the two methods, we found that the BOX-PCR method generated more informative results that REP-PCR for the strains analyzed. We found BOX-PCR profiles to be more complex and with clearly identifiable banding patterns, each showing group-specific bands as well as variable bands that enabled discrimination of the different isolates. In agreement with **Köberl *et al.* (2011)**, results show that genotypic BOX-PCR fingerprinting can be a tool to study genotypic diversity within *Bacillus* populations.

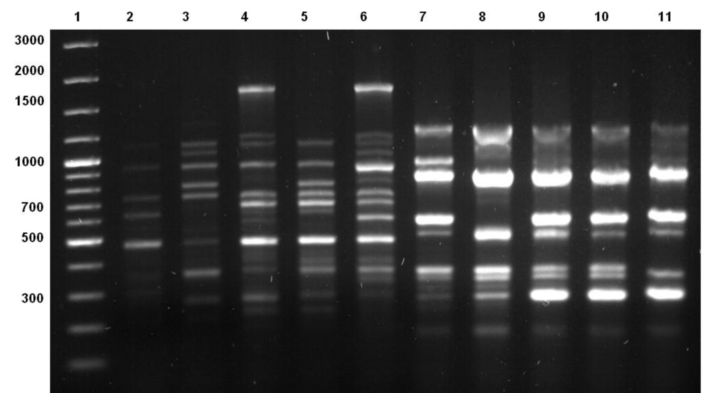

Figure 3 BOX-PCR patterns of isolates of *B. subtilis* group and type strains. Lane1, DNA molecular mass standard*; lane 2, *B. subtilis* 1A337; lanes 3-6, *B. subtilis* isolates UY1067, UY907, T46, UY628; lane 7, *B. amyloliquefaciens* 10A18; lanes 8-11, *B. amyloliquefaciens* isolates T11, UY1091, UY976, T144. * Molecular sizes in bp of the DNA standard (Generuler 100bp DNA Ladder Plus, Fermentas) are indicated in the left-hand margin.

CONCLUSION

B. subtilis and *B. amyloliquefaciens* are very difficult to distinguish from one another (**Reva *et al.*, 2004**), and most studies, including those dealing with dairy aerobic spore-formers, refer to *B. subtilis* isolates without further distinction. The ability to distinguish these species, however, may become relevant as dairy contaminant control processes continue to be improved. In addition, a considerable interest in these two species as resources for various specific biotechnological applications will require that both species and subspecies can be readily distinguished.

In this study, a polyphasic approach based on phenotypic tests including differences in growth at different temperature and a series of different molecular methods was developed for distinguishing identity as either *B. subtilis* or *B. amyloliquefaciens* for dairy isolates grouped previously as *B. subtilis* group in lieu of more specific identification.

The majority of phenotypic surveys report that these *B. subtilis* group members are very difficult to discriminate. A few phenotypic characteristics have been noted that enabled discrimination of these species; in this study, however, only differences in growth temperature allowed for species discrimination consistent with molecular methods. Reliance on phenotypic properties alone for differentiating these species should be avoided, and inconsistencies in strain identification reported in the literature using phenotypic tests only reinforce this point (**Palmisano *et al.*, 2001; Logan and Berkeley, 1984**).

Partial 16S rRNA gene sequence analysis indicated that all strains tested were closely related to *B. subtilis* and *B. amyloliquefaciens*, but provided insufficient resolution for species assignment. Similarity analysis based on *rpo*B and *gyr*A gene sequences indicated that the relatively high *rpo*B and *gyr*A gene sequence dissimilarities present could be used to distinguish isolates as either *B. subtilis* or *B. amyloliquefaciens*. Both the *rpo*B gene and particularly the *gyr*A gene sequences, although conserved, had lower similarity values, i. e., recognizably dissimilar sequences between isolates, compared with 16S rRNA gene sequences. Either *rpoB* or *gyrA* sequences could be used to distinguish isolates as *B. subtilis* or *B. amyloliquefaciens* species, and assignments were consistent with corresponding sequences from the type strains and their characteristic ability to grow at 55 ºC.

OPR13 proved to be useful for rapid screening and identification of both species, and BOX-PCR analysis showed potential for discrimination at the level of individual strains allowing for evaluation of intraspecies diversity. This study also shows that RAPD-PCR using OPR13 is a robust, consistent and accurate method for identification of *Bacillus* isolated from dairy sources including closely related taxa as *B. subtilis* and *B. amyloliquefaciens*.

Although polyphasic approaches are time-consuming, accurate taxonomic data can be obtained to discriminate between microbes that are very similar, i.e., *B. subtilis* and *B. amyloliquefaciens*.Genetically similar dairy isolates can be initially grouped by testing for growth at 55 ºC and a subsequent analysis by RAPD-PCR could be an inexpensive, technically simple and accurate method for species identification. This could be further confirmed by sequence analysis of *rpo*B or *gyr*A genes.

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