Journal of Dairy Research (2008) **75** 392–398. © *Proprietors of Journal of Dairy Research 2008*
doi:10.1017/S0022029908003543 *Printed in the United Kingdom*

Molecular methods for identification of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* using methionine biosynthesis and 16S rRNA genes

Aysun Cebeci[1] and G Candan Gürakan[2]*

[1] Department of Biochemistry, Middle East Technical University, Turkey
[2] Department of Food Engineering, Middle East Technical University, Ankara, Turkey

Received 5 July 2007; accepted for publication 14 April 2008; first published online 14 July 2008

Yoghurt and starter culture producers are still searching strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* to produce healthier yogurt with longer shelf life, better texture, taste and quality. However, selective identification of *Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus* from a mixed population using microbiological and biochemical methods is difficult, time consuming and may not be accurate. In this study, a quick, sensitive and accurate method is proposed to identify both *Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus* using PCR. The method is comprised of two parts. In the first part, methionine biosynthesis genes, known to be present in both species were partially amplified by designed primers (cysmet2F and cysmet2R). Partial amplification of the methionine biosynthesis gene which gives 700 bp fragment resulted in selective identification of *Lb. bulgaricus* and *Strep. thermophilus*. All 16 *Lb. bulgaricus* and 6 *Strep. thermophilus* isolates assessed by this method gave the expected amplification. On the other hand, further analysis of other closely related species with the same primers have indicated that the same product was also amplified in two more lactobacilli namely, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* species. Thus, in the second part of the method, further differentiation of *Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus* from each other and these species was achieved using restriction analysis of 16S rRNA gene with *Eco*RI.

Keywords: *Lb. delbrueckii* subsp. *bulgaricus*, *Strep. thermophilus*, yoghurt starter, strain selection, 16S rRNA gene.

Yogurt is a favorite fermented milk product as an important part of people's diet in many countries. Production of yogurt is achieved using starter cultures in industry, namely *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Yogurt quality is highly influenced by the strain differences of these cultures. Therefore, yogurt manufacturers and starter producers are still seeking, identifying, screening, and selecting strains as yogurt cultures from mixed populations. Those strains are mostly isolated from their natural habitats such as raw milk, naturally fermented yogurts (without using commercial starter cultures) (Holzapfel, 2002). However, identification and discrimination of these strains, particularly species of *Lactobacillus* genus has always been a problem because of the heterogeneity of the genus. The divergency among lactobacilli is so great that guanine and cytosine content of the species varies between 32–54%, which is about twice the amount expected in a well-defined genus (Nour, 1998). For identification of *Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus* from a mixed population, as in the case of naturally fermented yoghurt, the situation may even be more difficult, since several species and subspecies of lactic acid bacteria co-exist.

Conventional methods used for identification of yoghurt starter bacteria include growth at different temperatures, and pH values, carbohydrate fermentation tests, API kits, production of lactate isoforms, SDS-PAGE, etc. (Coeuret et al. 2003; Andrighetto et al. 1998). Such procedures are labour intensive, time consuming, and may result in misidentification because of the limitations of the methods, i.e. carbohydrate fermentation tests rely on colour changes, which may be difficult to interpret (Andrighetto et al. 1998).

*For correspondence; e-mail: candan@metu.edu.tr

https://doi.org/10.1017/S0022029908003543 Published online by Cambridge University Press

The number of studies, based on molecular biology, to identify many bacteria including *Lb. bulgaricus* (Tilsala-Timisjarvi & Alatossava, 1997; Giraffa, De Vecchi & Rosetti, 1998; Miteva et al. 2001) and *Strep. thermophilus* (Tilsala-Timisjarvi & Alatossava, 1997; Lick et al. 1996), at species, subspecies level has increased in the last decade. However, our survey of the literature and preliminary studies have revealed the lack of a complete rapid method for accurate selective identification of both *Strep. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* at species and subspecies level from a mixed population and results of trials are either unsatisfactory or ambiguous. As an example, identification of both *Strep. thermophilus* and *Lb. delbruecki* at species level was accomplished by PCR based method using species specific primer sets targeted intergenic spacer region of 16S-23S rRNA gene by Tilsala-Timisjarvi & Alatossava (1997). Yet, the differentiation of *Lb. delbrueckii* strains at subspecies level was not described in their study. On the other hand, identification of two *Lb. delbrueckii* subspecies; subsp. *lactis* and subsp. *bulgaricus* was reported by Delley & Germond (2002), while selective identification of these isolates from a mixed population was not mentioned in their study.

Our study was carried out to present a new method for selective identification of *Strep. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* from mixed cultures. It has been reported by Bolotin et al. (2004), that *Strep. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* have 95% identical methionine biosynthesis genes. Thus, the proposed method is based on partial amplification of methionine biosynthesis gene region to differentiate yoghurt starter cultures, *Strep. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* from mixed population.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. These include, strains isolated from commercial starter cultures and from rural yoghurts, produced traditionally, by back-slopping (Holtzapfel, 2002), in rural areas of Turkey and reference strains. The strains were provided as freeze dried cultures, activated 3 times in MRS and M17 broth (Merck KGaA, Darmstadt, Germany) at 37 °C and 42 °C for use, and maintained on Microbanks (Pro-Lab Diagnostics) and 20% glycerol stocks at −80 °C.

Phenotypic identification of isolates

Isolates were identified according to Randazzo et al. 2002. Briefly; colony morphology on agar, microscopic examination, gram staining, catalase production, reduction of nitrate, gas production, hydrolysis of arginine, hydrolysis of esculin, growth at different pH values (pH 2, pH 10) and different temperatures (10 and 45 °C) were tested. Carbohydrate fermentation profiles were performed using microtiter plates according to Bulut et al. (2004). The carbohydrates tested were arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, melibiose, ribose, saccharose, salicin, sorbitol, trehalose and xylose. Fermentation patterns were also screened using appropriate API galleries (Biomerieux® S.A., Marcy-l Etoile, France). The results of API system were analysed with API identification software.

DNA isolation

Preparation of genomic DNA was performed according to modified method of Luchansky et al. (1991) as follows: 2 ml of overnight grown culture was centrifuged at 25 000 ***g*** (Andreas Hettich GmbH, Tuttlingen, Germany) for 2 min, and washed twice in TE buffer (10 mM-TrisHCl (AppliChem GmbH, Darmstadt, Germany), 1 mM-EDTA (pH 8·0; Merck KGaA, Darmstadt, Germany). The pellet was dissolved in 300 μl TE buffer and 5 μl lysozyme (50 mg/ml; AppliChem GmbH) was added to lyse the cells. The solution is incubated at 37 °C for 45 min. Thereafter, 20 μl EDTA (0·25 M; Merck KGaA), 25 μl SDS (10%; Merck KGaA, Darmstadt, Germany) and 4 μl Proteinase K (20 mg/ml; Fermentas, Vilnius, Lithuania) was added to the solution and incubated at 60 °C for 60 min. Once digestion was complete, samples were extracted with phenol-chloroform three times, and ethanol precipitated with the addition of 0·1 V 3 M-sodium acetate (pH 5·5; Merck KGaA). DNA was precipitated; samples were washed with 70% ethanol (Merck KGaA), air-dried for 20 min and dissolved in distilled H_2O. Rnase (Fermentas) was used to digest RNA, and samples were stored at −20 °C until use. The quality of DNA isolations were tested both with A_{260} /A_{280} ratios and on agarose gels with comparison to decreasing concentrations of bacteriophage λ (Fermentas).

Primers and PCR conditions

For the methionine biosynthesis gene, primers cysmet2F (forward): 5′-GGAACCTGAAGGCTCAAT-3′, cysmet2R (reverse): 5′-GTCAACCACGGTAAAGGTC-3′ were designed using on-line tools, primer3 and oligocalc program. Nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) was made to confirm primers' selectivity. Genbank accession numbers of methionine biosynthesis genes were; YP_141251 and YP_141252. For the amplification of 16S rRNA gene, 9699–9700 primer pair was used (Delley & Germond, 2002). PCR amplifications were made on MJMini thermal cycler (BioRad, Hercules, USA). Reaction mixture and amplification conditions for the analysis of methionine biosynthesis gene were as follows: an initial denaturation at 94 °C for 2 min, 45 cycles of denaturation at 94 °C for 30 sec for 10 min. The reaction mixture was 30 μl, and consisted of 1·5 mM-$MgCl_2$, annealing at 54 °C for 40 sec, extension at 72 °C for 45 sec,

https://doi.org/10.1017/S0022029908003543 Published online by Cambridge University Press

Table 1. The bacterial strains used in the study

Species or subsp.	Strain or isolate number	Source
Lb. fermentum	NRRL-B1840	ARSCC[1]
Lb. paraplantarum	NRRL-B23115	ARSCC[1]
Lb. casei subsp. *casei*	NRRL-B1922	ARSCC[1]
Lb. gasseri	NRRL-B4240	ARSCC[1]
Lb. rhamnosus	NRRL-B442	ARSCC[1]
Lb. helveticus	NRRL-B4526	ARSCC[1]
Lb. paracesi subsp. *paracasei*	NRRL-B4560	ARSCC[1]
Lb. reuteri	NRRL-B14171	ARSCC[1]
Lb. salivarius subsp. *salivarius*	NRRL-B1949	ARSCC[1]
Lb. johnsonii	NRRL-B2178	ARSCC[1]
Lb. pentosus	NRRL-B227	ARSCC[1]
Lb. amylovorous	NRRL-B4540	ARSCC[1]
Lb. brevis	NRRL-B4527	ARSCC[1]
Lb. acidophilus	ATCC 4356	ATCC[2]
Lb. delbrueckii subsp. *delbruekii*	NRRL-B763	ARSCC[1]
Lb. delbrueckii subsp. *lactis*	NRRL-B4525	ARSCC[1]
Lb. delbrueckii subsp. *bulgaricus*	DSM20081	DSMZ[3]
Lb. plantarum	DSM20174	DSMZ
Lb. delbrueckii subsp. *bulgaricus*	ATCC BAA-365	University of Wisconsin, Madison
Strep. dysgalactiae	NRRL-B688	ARSCC[1]
Strep. equinus	NRRL-B3573	ARSCC[1]
Strep. infantarius	NRRL-B41208	ARSCC[1]
Strep. thermophilus	LMG18311	INRA[4]
Strep. thermophilus	CNRZ1066	INRA[4]
Lc. lactis[6]	3113	JC17[5]
Lc. lactis	2088	—
Lc. lactis	2910	—
Lc. lactis	2911	—
Lb. delbrueckii subsp. *bulgaricus*	B1000-1	Visby
Lb. delbrueckii subsp. *bulgaricus*	B1000-2	Visby
Lb. delbrueckii subsp. *bulgaricus*	B1000-3	Visby
Lb. delbrueckii subsp. *bulgaricus*	Yo-mix 410-1	Danisco
Strep. thermophilus	Ta 040-1	Danisco
Strep. thermophilus	Ta 040-2	Danisco
Strep. thermophilus	B1000-3	Visby
Strep. thermophilus	Yo-mix 410-3	Danisco
Lb. delbrueckii subsp. *bulgaricus*	M2-5	rural yoghurt
Lb. delbrueckii subsp. *bulgaricus*	M2-14	rural yoghurt
Lb. delbrueckii subsp. *bulgaricus*	M2-18	rural yoghurt
Lb. delbrueckii subsp. *bulgaricus*	K1-10	rural yoghurt
Lb. delbrueckii subsp. *bulgaricus*	K1-16	rural yoghurt
Lb. delbrueckii subsp. *bulgaricus*	K1-29	rural yoghurt
Lb. delbrueckii subsp. *bulgaricus*	K1-33	rural yoghurt
Lb. delbrueckii subsp. *bulgaricus*	K1-38	rural yoghurt
Lb. delbrueckii subsp. *bulgaricus*	K1-43	rural yoghurt
Lb. delbrueckii subsp. *bulgaricus*	K1-44	rural yoghurt

[1] Agricultural Research Service Culture Collection, USA
[2] American Type Culture Collections
[3] German Culture Collection Center
[4] National Institute of Agronomical Research, France
[5] Piard et al. 1993
[6] *Lc.–Lactococcus*

and final annealing of 72 °C, 200 μM of each dNTP (Fermentas), 7 μM of each primer, 0·5 U Taq DNA polymerase (BIORON GmbH, Ludwigshafen, Germany), and 500 ng DNA.

For 16S rRNA gene restriction analysis and sequencing, the reaction mixture was 50 μl, and consisted of 1·5 mM-$MgCl_2$, 200 μM of each dNTP (Fermentas), 1 μM of each primer, 0·5 U Taq DNA polymerase (BIORON), and

https://doi.org/10.1017/S0022029908003543 Published online by Cambridge University Press

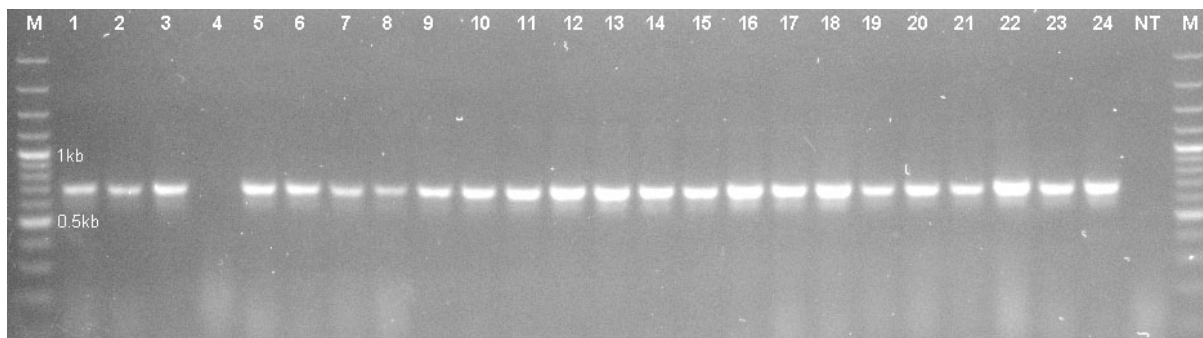

Fig. 1. Partial amplification of the methionine biosynthesis gene in commercial, rural yogurt isolates, some reference strains of *Lb. delbrueckii* subsp. *bulgaricus* and closely related lactobacilli and *Strep. thermophilus*. Lanes 1–2, 5–18: *Lb. delbrueckii* subsp. *bulgaricus* (1: DSM20081, 2: ATCC BAA-365, 5: Visby B1000-1, 6: Visby B1000-2, 7: Visby B1000-3, 8: Danisco Yo-mix 410-1, 9: M2-5, 10: M2-14, 11: M2-18, 12: K1-10, 13: K1-16, 14: K1-29, 15: K1-33, 16: K1-38, 17: K1-43, 18: K1-44), 3: *Lb. delbrueckii* subsp. *lactis* NRRL-B4525, 4: *Lb. delbrueckii* subsp. *delbrueckii* NRRL-B763, 19: *Lb. helveticus* NRRL B-4526, lanes 20–24: *Strep. thermophilus* (20: LMG18311, 21: CNRZ1066, 22: Visby B1000-3, 23: Danisco Yo-mix 410-3, 24: Danisco Ta 040-1), NT: Negative control (no DNA control), M: 100 bp DNA ladder.

500 ng DNA. Amplification conditions were, an initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min, and final annealing of 72 °C for 10 min.

The PCR products were run on 1·5% agarose gels, by gel electrophoresis for 1 h at 80 V in 1× TAE (40 mM-Tris-acetate and 1 mM-EDTA, pH 8·0) and post-stained with ethidium bromide (1 μg/ml), and visualized under UV light in a gel documentation system (BioRad, Hercules, USA). DNA ladder of 100 bp plus (Fermentas) was used as a DNA molecular weight marker.

Restriction analysis of 16S rRNA gene product

Restriction of 16S rRNA gene product was carried on using *Eco*RI as the enzyme (Delley & Germond, 2002). The reaction mixture contained 1× of the corresponding buffer, 8·5 μl of PCR product, and 0·5 μl *Eco*RI (Fermentas). The restriction products were run on 1·5% agarose gels, post-stained with ethidium bromide, and visualized under UV light.

Genotypic identification by 16S rRNA gene sequence analysis

For sequencing of the 16S rRNA gene, PCR product was amplified using forward primer 5′-AGAGTTTGATCC-TGGCTCAG-3′ (Mora et al. 1998) and reverse primer U926 (CCGTCAATTCCTTTRAGTTT) (Baker et al. 2003). PCR amplicons were recovered from PCR mixtures using DNA Extraction Kit (Fermentas) and further subjected to sequencing analysis (Iontek, Istanbul, Turkey). The sequences of each isolate were compared with those reported in the basic BLAST database (Altschul et al. 1997; http://www.ncbi.nlm.nih.gov/BLAST/). Strains showing homology of at least 97% were considered to belong to the same species (Stackebrandt & Goebel, 1994).

Results and Discussion

The aim of this study was to identify yogurt starter bacteria (*Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus*) rapidly and selectively from mixed populations containing other natural inhabitants of dairy products, a new PCR-based method has been tested. To achieve this goal, 16 *Lb. bulgaricus* and 6 *Strep. thermophilus* strains were used. The strains were initially identified by conventional methods. Confirmation of identification results for those strains was performed by genotypic identification using sequencing of V1-V3 region of 16S rRNA gene. Comparisons with 16S rRNA sequences held in Genbank verified that all of the studied strains, including commercial starter isolates and isolates from rural yoghurts, belonged to *Strep. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*.

In order to achieve selective identification of *Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus* strains by methionine biosynthesis gene, more than 10 primer pairs were designed and primer pair, cysmet2F and cysmet2R, was chosen to identify both yoghurt starters. Partial amplification of the methionine biosynthesis gene using these primers revealed a 700 bp product in all *Lb. delbrueckii* subsp. *bulgaricus* (n=16) and *Strep. thermophilus* isolates (n=6) as expected and also in two closely related bacteria, *Lb. delbrueckii* subsp. *lactis* NRRL-B4535 and *Lb. helveticus* NRRL-B442 (Fig. 1). All other species (n=20) including other lactobacilli, lactococci and streptococci species (Table 1) used in this study gave no PCR reaction (Fig. 2).

Additionally, *Lb. acidophilus* ATCC 4356 did not give an amplicon by those primers (data not shown).

https://doi.org/10.1017/S0022029908003543 Published online by Cambridge University Press

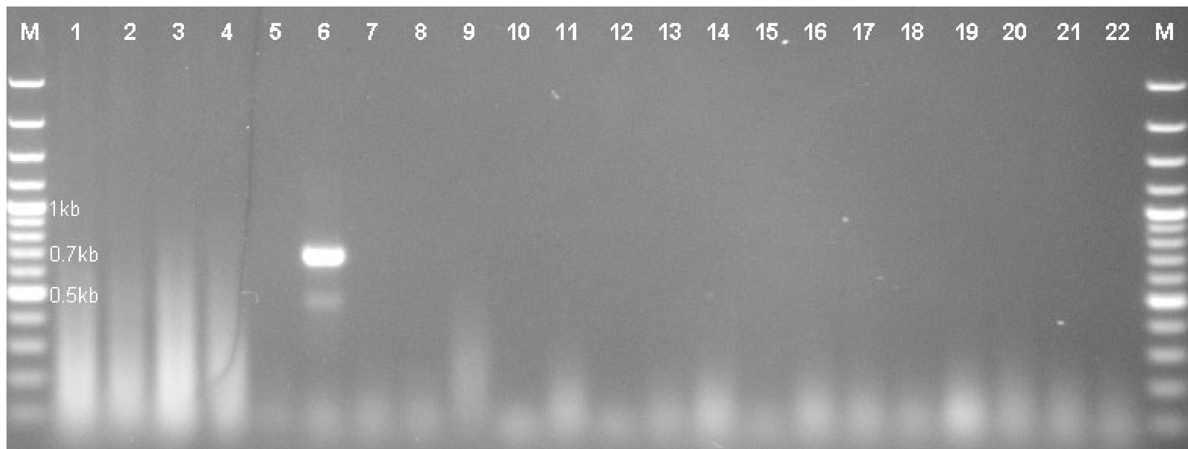

Fig. 2. PCR results for partial amplification of the methionine biosynthesis gene in other reference strains of lactoccocci, lactobacilli and streptococci. Lanes: 1–4: *Lc. lactis* (1: 2910, 2: 2911, 3: 2088, 4: 3113), 5: Negative control (no DNA control), 6: *Lb. delbrueckii* subsp. *bulgaricus* DSM20081, 7: *Lb. reuteri* NRRLB-14171, 8: *Lb. pentosus* NRRLB-227, 9: *Lb. brevis* NRRLB-4527, 10: *Lb. gasseri* NRRLB-4240, 11: *Lb. amylovorous* NRRLB-4540, 12: *Lb. casei* subsp. *casei* NRRLB-1922, 13: *Lb. johnsonii* NRRLB-2178, 14: *Lb. salivarius* subsp. *salivarius* NRRLB-1949, 15: *Lb. paraplantarum* NRRLB-23115, 16: *Lb. fermentum* NRRLB-1840, 17: *Lb. rhamnosus* NRRLB-442, 18: *Lb. paracasei* subsp. *paracasei* NRRLB-4560, 19: *Strep. dysgalactiae* NRRLB 688, 20: *Strep. equinus* NRRLB-3573, 21: *Strep. infantarius* NRRLB-41208, 22: *Lb. delbrueckii* subsp. *delbruekii* NRRLB-763, M: 100 bp DNA ladder.

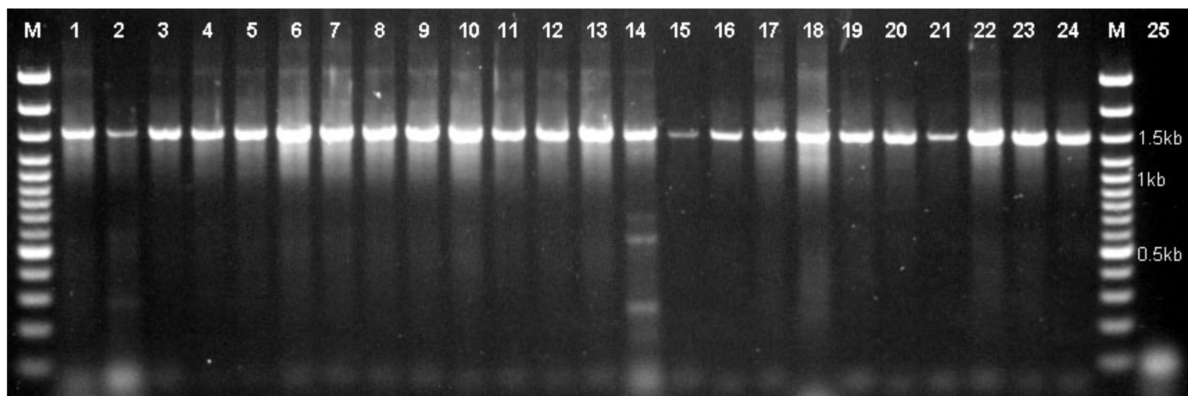

Fig. 3. Amplification of 16S rRNA gene from isolates that amplified methionine biosynthesis gene product. Lanes 1–2, 4–16, 21: *Lb. delbrueckii* subsp. *bulgaricus* (1: DSM20081, 2: ATCC BAA-365, 4: M2-14, 5: M2-18, 6: K1-10, 7: K1-16, 8: K1-29, 9: K1-33, 10: K1-38, 11: K1-43, 12: K1-44, 13: M2-5, 14: Visby B1000-1, 15: Visby B1000-2, 16: Visby B1000-3, 21: Danisco Yo-mix 410-1), 3: *Lb. delbrueckii* subsp. *lactis* NRRL-B4525, 17: *Lb. helveticus* NRRL B-4526, lanes 18–20, 22–24: *Strep. thermophilus* (18: CNRZ1066, 19: LMG18311, 20: Visby B1000-3, 22: Yo-mix 410-3, 23: Danisco Ta 040-1, 24: Danisco Ta 040-2), 25: Negative control (no DNA control). M: 100 bp DNA ladder.

As predicted from BLAST analysis, the primer set synthesized for partial amplification of methionine biosynthesis gene was able to identify *Strep. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* (Fig. 1). This protocol has provided additional discrimination of *Lb. delbrueckii* subsp. *delbrueckii* from yoghurt starters and from *Lb. delbrueckii* subsp. *lactis* (Fig. 1). The unexpected production of 700 bp fragment in *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus*, however, coincides with the horizontal transfer of genes sharing the same niche (Bolotin et al. 2004), since *Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus, Lb. delbrueckii* subsp. *bulgaricus*, and *Strep. thermophilus* do exist in similar environments in dairy products (Hannon et al. 2006). *Lb. helveticus* genome sequence has, lately (03-December-2007), become available in Genbank with accession number CP000517. The analysis of *Lb. helveticus* genome confirmed our result that high homology in methionine biosynthesis gene exists for *Lb. helveticus* as well. However, the same methionine biosynthesis gene product we obtained for *Lb. delbrueckii* subsp. *lactis* remained unconfirmed since the genome sequence of this organism is still unknown.

The aim of the study was to solely identify yoghurt starters. Thus, presence of amplicons of the methionine

https://doi.org/10.1017/S0022029908003543 Published online by Cambridge University Press

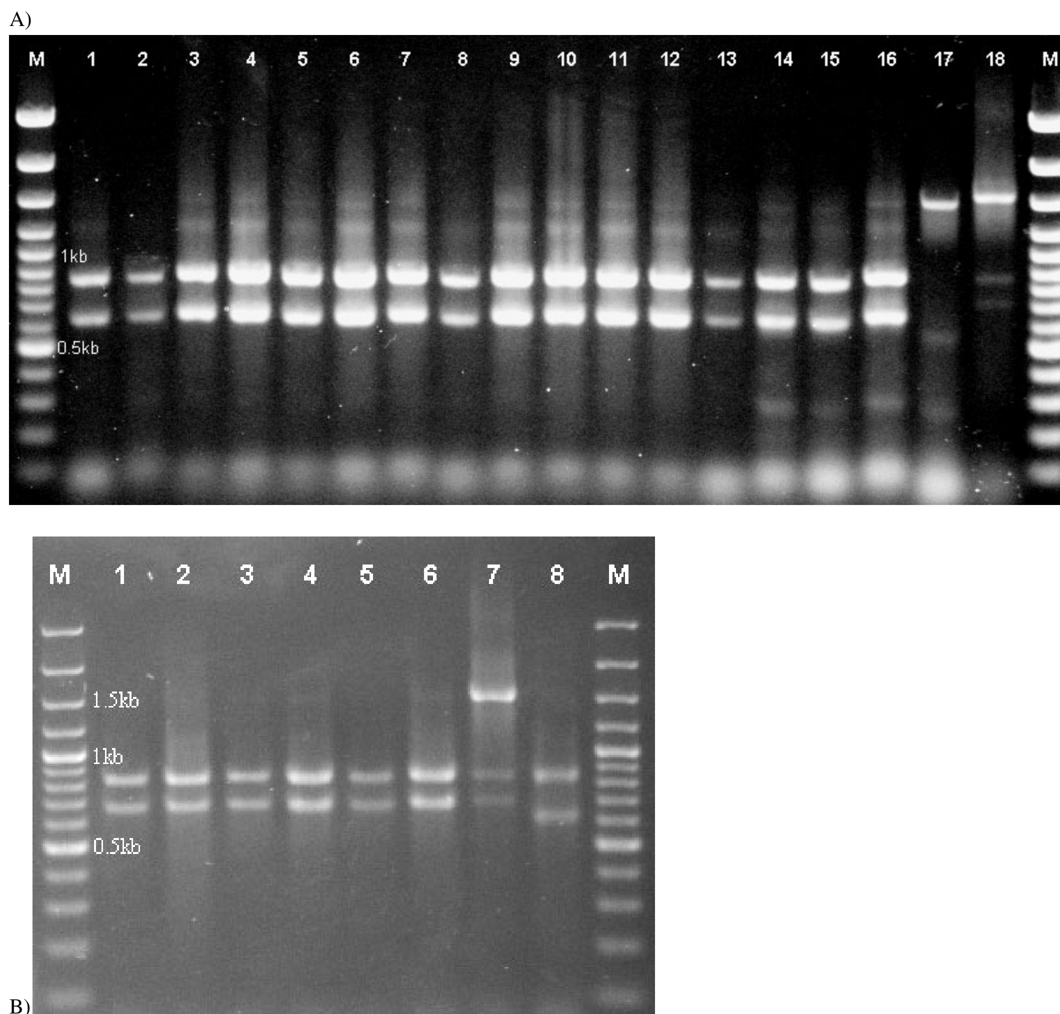

Fig. 4. Restriction analysis of 16S rRNA gene with *Eco*RI. (A) Restriction analysis of 16S rRNA gene with *Eco*RI for differentiation of *Lb. delbrueckii* subsp. *bulgaricus*. Lanes 1–16: *Lb. delbrueckii* subsp. *bulgaricus* (1: DSM20081, 2: ATCC BAA-365, 3: K1-10, 4: K1-16, 5: K1-29, 6: K1-33, 7: K1-38, 8: K1-43, 9: K1-44, 10: M2-5, 11: M2-14, 12: M2-18, 13: Danisco Yo-mix 410-1, 14: Visby B1000-1, 15: Visby B1000-2, 16: Visby B1000-3), 17: *Lb. delbrueckii* subsp. *lactis* NRRL-B4525, 18: *Lb. helveticus* NRRL B-4526, M: 100 bp DNA ladder (B) Restriction analysis of 16S rRNA gene with *Eco*RI for differentiation of *Strep. thermophilus*. Lanes 1–6: *Strep. thermophilus* (1: LMG18311, 2: CNRZ1066, 3: Visby B1000-3, 4: Danisco Yo-mix 410-3, 5: Danisco Ta 040-1, 6: Danisco Ta 040-2), 7: *Lb. helveticus* NRRL B-4526, 8: *Lb. delbrueckii* subsp. *bulgaricus* DSM20081. M: 100 bp DNA ladder.

biosynthesis genes also in *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* necessitated further analysis for differentiation of those organisms. The differentiation of *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus*, was achieved by restriction analysis method of 16S rRNA gene (ARDRA: Amplified Ribosomal DNA Restriction Analysis) by *Eco*RI as reported by Delley & Germond (2002). The amplification sizes of the 16S rRNA gene was approximately 1500 bp in all isolates of *Lb. delbrueckii* subsp. *bulgaricus* (n=16) and *Strep. thermophilus* (n=6) from commercial starter cultures, rural yoghurts and reference strains as well as *Lb. delbrueckii* subsp. *lactis* NRRL-B4525, *Lb. helveticus* NRRL-B4526 (Fig. 3). Restriction endonucleases *Eco*RI digested all *Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus* isolates in two fragments of 650, 850 bp and 700, 850 respectively (Fig. 4a & b). The *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* strains used in our experiment confirmed findings of Delley & Germond (2002) (Fig. 4a). The enzyme digested the PCR product of *Lb. delbrueckii* subsp. *lactis* NRRL-B4525 in one fragment of about 1440 bp (Fig. 4a). However, differing from their finding of complete digestion, *Lb. helveticus* NRRL-B4526 was partially digested. The digestion products were faint bands restricted at the middle of the gene (Fig. 4a & 4b). The results have been confirmed also on another *Lb. helveticus* starter stain from Christian and Hansen (data not shown). The reason for this situation

https://doi.org/10.1017/S0022029908003543 Published online by Cambridge University Press

might result from the presence of several 16S rRNA gene copies in a *Lb. helveticus* genome in some of which complete digestion was not generated by *Eco*RI due to the lack *Eco*RI recognition site (Giraffa et al. 2000). Nevertheless, the method of Delley & Germond (2002) still provided differentiation between *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus*. It is noteworthy that *Strep. thermophilus* has given a different profile than that of *Lb. delbrueckii* subsp. *bulgaricus* when ARDRA with *Eco*RI was performed. Partial digestion of *Lb. helveticus* has differentiated *Strep. thermophilus* from *Lb. helveticus* (Fig. 4b). In case of complete digestion, microscopic examination of cells could be necessary to differentiate *Strep. thermophilus* having the same banding pattern with *Lb. helveticus*.

The study presented here offered rapid, selective identification of *Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus* from a mixed population using a specific PCR method of methionine biosynthesis gene and ARDRA analysis of 16S rRNA gene using *Eco*RI (Delley & Germond, 2002).

The authors thank to Agricultural Research Service Culture Collection (USA), National Institute of Agronomical Research (France), and Dr. James L. Steele and Dr. Peter Smeianov from University of Wisconsin, Madison for providing us valuable strains used in this study. This study was partially supported by Scientific Research Projects of METU (Grant no. BAP-2006-03-14-02) and DPT-YUUP (2005 K 120570).

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ 1997 Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* **25** 3389–3402

Andrighetto C, De Dea R, Lombardi A, Neviani E, Rossetti L & Giraffa G 1998 Molecular identification and cluster analysis of homofermentative thermophilic lactobacilli isolated from dairy products. *Research in Microbiology* **149** 631–643

Baker GC, Smith JJ & Cowan DA 2003 Review and re-analysis of domain-specific 16S primers. *Journal of Microbiological Methods* **55** 541–555

Bolotin A, Quinquis B, Renault P, Sorokin A, Ehrlich SD, Kulakauskas S, Lapidus A, Goltsman E, Mazur M, Pusch GD, Fonstein M, Overbeek R, Kyprides N, Purnelle B, Prozzi D, Ngui K, Masuy D, Hancy F, Burteau S, Boutry M, Delcour J, Goffeau A & Hols P 2004 Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. *Nature Biotechnology* **22** 1554–1558

Bulut C, Gunes H, Okuklu B, Harsa S, Kilic S, Coban HS & Yenidunya AF 2004 Homofermentative lactic acid bacteria of a traditional cheese, Comlek peyniri from Cappadocia region. *Journal of Dairy Research* **72** 19–24

Coeuret V, Dubernet S, Bernardeau M, Gueguen M & Vernoux JP 2003 Isolation, characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products. *Lait* **83** 269–306

Delley M & Germond J-E 2002 Differentiation of *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, subsp. *lactis* and subsp. *delbrueckii* using physiological and genetic tools and reclassification of some strains from the ATCC collection system. *Applied Microbiology* **25** 228–231

Giraffa G, De Vecchi P & Rossetti L 1998 Identification of *Lactobacillus delbrueckii* subspecies *bulgaricus* and subspecies *lactis* dairy isolates by amplified rDNA restriction analysis. *Journal of Applied Microbiology* **85** 918–924

Giraffa G, Gatti M, Rossetti L, Senini L & Neviani E 2000 Molecular diversity within *L. helveticus* as revealed by genotypic characterization. *Applied and Environmental Microbiology* **65** 1259–1265

Hannon JA, Deutsch SM, Madec MN, Gassi JY, Chapot-Chartier MP & Lortal S 2006 Lysis of starters in UF cheeses: Behaviour of mesophilic lactococci and thermophilic lactobacilli. *International Dairy Journal* **16** 324–334

Holzapfel WH 2002 Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology* **75** 197–212

Lick S, Keller M, Bockelmann W & Heller KJ 1996 Rapid identification of *Streptococcus thermophilus* by primer-specific PCR amplification based on its *lac*Z gene. *Systematic and Applied Microbiology* **19** 74–77

Luchansky JB, Tennant CM & Klaenhammer TR 1991 Molecular cloning and deoxyribonucleic acid polymorphisms in *Lactobacillus acidophilus* and *Lactobacillus gasseri*. *Journal of Dairy Science* **74** 3293–3302

Miteva V, Boudakov I, Ivanova-Stoyancheva G, Marinova B, Mitev V & Mengaud J 2001 Differentiation of *Lactobacillus delbrueckii* subsp. by ribotyping and amplified ribosomal DNA restriction analysis (ARDRA). *Journal of Applied Microbiology* **90** 909–918

Mora B, Fortina MG, Nicastro G, Parini C & Manachini PL 1998 Genotypic characterisation of thermophilic bacilli: a study on new soil isolates and several reference strains. *Research in Microbiology* **149** 711–722

Nour M 1998 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. *Research in Microbiology* **149** 433–448

Piard J-C, Kuipers OP, Rollema HS, Desmazeaud MJ & de Vos WM 1993 Structure, organization, and expression of the lct gene for Lacticin 481, a novel lantibiotic produced by *Lactococcus lactis*. *Journal of Biological Chemistry* **268** 16361–16368

Randazzo CL, Torriani S, Akkermans ADL, de Vos WM & Vaughan EE 2002 Diversity, dynamics, and activity of bacterial communities during production of an Artisanal Sicilian Cheese as evaluated by 16S rRNA analysis. *Applied and Environmental Microbiology* **68** 1882–1892

Stackebrandt E & Goebel BM 1994 A place for DNA-DNA re-association and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology* **44** 846–849

Tilsala-Timisjarvi A & Alatossava T 1997 Development of oligonucleotide primers from the 16S-23s rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. *International Journal of Food Microbiology* **35** 49–56

https://doi.org/10.1017/S0022029908003543 Published online by Cambridge University Press