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Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance

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Abstract

A group-specific primer pair was designed to amplify the 16S rRNA gene of representative reference strains from environmentally sourced, mesophilic aerobic spore-forming *Bacillus* taxa. The PCR generated a 1114 bp amplicon but did not do so with DNA extracted from 16 other Eubacterial species. When amplicons were digested with restriction enzymes *Alu*I or *Taq*I, different profiles containing between 2 and 5 fragments ranging in size from 76 to 804 base pairs were seen with different *Bacillus* species. This procedure, known otherwise as amplified ribosomal DNA restriction analysis or ARDRA, produced unique and distinguishable patterns to differentiate between 15 ATCC reference strains (10 *Bacillus*, 3 *Paenibacillus* and 2 *Brevibacillus* member species) as well as 3 misidentified *Bacillus* probiotic strains in a commercial collection. Our simplified PCR-ARDRA protocol provides a facile method for the identification of most environmentally important species of *Bacillus*.

Keywords: *Bacillus*-specific primers; ARDRA; 16S rDNA gene; *Bacillus* identification

1. Introduction

Members of the genus *Bacillus* have played a significant dual role in many human activities. On the one hand, species such as *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* are used industrially for the production of enzymes, antibiotics, solvents and other molecules (Gerhartz, 1990). Others such as *B. thuringiensis* and *B. sphaericus*, because of their insecticidal activity, are used in crop protection (Bourque et al., 1995) while *B. mycoides* has the ability to promote plant growth (Petersen et al., 1995). In countries like Italy and Vietnam, *B. subtilis*, *B. clausii* or *B. alcalophilus* have been used as an oral bacteriotherapeutic for the treatment of gastrointestinal disorders (Casula and Cutting, 2002; Hoa et al., 2000; Senesi et al., 2001). On the other hand, some strains of *B. anthracis* and *B. cereus* are pathogenic to humans and/or animals (Shangkuan et al., 2000) and honeybees are susceptible to American foulbrood, a disease

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caused by *Paenibacillus larvae* subsp. *larvae* (formerly *Bacillus larvae*) (White, 1920).

In its original classification, the genus *Bacillus* contained a heterogeneous assembly of aerobic, or facultatively anaerobic, Gram-positive, rod-shaped, spore-forming bacteria widely distributed in the environment. Traditionally, *Bacillus* spp. are identified in the laboratory by biochemical tests and fatty acid methyl ester (FAME) profiling (Bobbie and White, 1980; Vaerewijck et al., 2001). Alternatively, the API (Analytab Products, Inc.) system of identification has been shown to be more reproducible than classical methods (Logan and Berkeley, 1984) and is capable of speciating bacilli using a combination of the 12 tests in the API 20E strip, and 49 tests in the API 50CHB strips. These phenotyping protocols are laborious and time-consuming to undertake and cannot provide a rapid screening system (Wattiau et al., 2001). The shortcomings of phenotypically-based identification methods have led to the development of molecular alternatives based on the microbial genotype or DNA sequence. This approach minimizes problems associated with typability and reproducibility, and importantly, facilitates the assembly of large reference databases (Olive and Bean, 1999).

Comparisons of the 16S rRNA sequence is one of the most powerful tools for the classification of microorganisms (Wang et al., 2003; Woese, 1987; Yamada et al., 1997) and have provided sequence specific primers as gold standards for the identification of pure cultures of *Bacillus* species such as *B. subtilis* (Wattiau et al., 2001), *B. cereus* and *B. thuringiensis* (Hansen et al., 2001), and *Paenibacillus alvei* (formerly *Bacillus alvei*) (Djordjevic et al., 2000). However, environmental samples such as those from sewage, water, soil, feces and even beehives, usually contain mixtures of *Bacillus* species. The presence of such combinations can be better detected with the use of a group-specific primer that distinguishes as many member species as possible within the genus. Individual species representative of different *Bacillus* genera can then be characterised by subjecting the amplicons to restriction enzyme digest. Although genus-specific primers have been successfully developed for lactobacilli (Dubernet et al., 2002), mycoplasmas (van Kuppeveld et al., 1992), *Bifidobacterium* (Matsuki et al., 1999), *Pandoraea* (Coenye et al., 2001) and *Clostridium* (Van Dyke and McCarthy, 2002), a group-specific primer pair capable of amplifying a specific sequence of 16S rDNA from all *Bacillus* taxa has not been developed in accompaniment with restriction digest mapping.

In this study, we have focused on a subset of environmental (probiotic and gut-associated) *Bacillus* strains that are primarily mesophilic spore-forming bacteria capable of growing aerobically on nutrient agar at neutral pH, and between 25–45 °C. The strains of interest to us, included species from related genera such as *Bacillus*, *Brevibacillus*, *Paenibacillus* and *Geobacillus* but not *Alicyclobacillus* (thermophiles and acidophiles), *Amphibacillus* and *Ureibacillus* (alkaliphiles), *Halobacillus*, *Filobacillus*, *Gracilibacillus*, *Virgibacillus* and *Salibacillus* (halotolerant or halophilic bacteria) and even several extremophilic, asporogenous and nutritionally fastidious species present in *Bacillus*, *Geobacillus* and *Paenibacillus*. The specificity of our target PCR primers was also validated against related Gram-positive but non-*Bacillus* species that frequently co-exist with *Bacillus* in the environment. Species identification of *Bacillus*, based on amplicons generated by our group-specific PCR primer pair, was accomplished by amplified ribosomal DNA restriction analysis (ARDRA) with *Alu*I and *Taq*I. The utility of our assay was then used to check for mislabelled identifications if any, in a commercial collection of probiotic *Bacillus* strains.

2. Materials and methods

2.1. Reference strains and culture conditions

Reference strains used in this study are listed in Table 1 including 15 reference *Bacillus* strains; 18 reference non-*Bacillus* strains; 17 reference probiotic strains; 50 reference honeybee strains. Reference strains are defined as those strains that have been speciated using standard taxonomical protocols by ATCC, commercial probiotic companies and diagnostic laboratories.

Bacillus strains were cultivated in tryptic soy broth (TSB) or agar (TSA, DIFCO, Becton Dickinson, Sparks, MD) aerobically at 34 °C. The probiotic *Bacillus* strains were recovered by suspension of dried bacterial preparations in saline followed by plating on TSA. The non-*Bacillus* strains were cultivated on

Table 1
List of bacterial strains used as a source of genomic DNA for the validation of primer pairs K-B1/F and K-B1/R1

Bacillus strains (15)	Source	Non-*Bacillus* strains (18)	Source	Probiotic strains (17)	Source	Honey bee strains
B. subtilis	ATCC 6633	*Clostridium tertium*	ATCC14573	*B. subtilis*	BMS 1003 (PROGEN)	*P. larvae* strains ($n = 50$)
B. licheniformis	ATCC 25972	*Staphylococcus xylosus*	ATCC700404	*B. subtilis*	BS1 (Agtech)	
B. pumilus	ATCC 21356	*E. coli* O157H7	EMAI	*B. subtilis*	3AP4 (Agtech)	
B. cereus	ATCC 14579	*Enterococcus faecium*	ATCC19434	*B. subtilis*	15AP1 (Agtech)	
B. thuringiensis	ATCC 10792	*Bifidobacterium bifidum*	ATCC 29521	*B. subtilis*	22CP1 (Agtech)	
B. laterosporus	ATCC 64	*Fusobacterium mortiferum*	ATCC25557	*B. subtilis*	BS2 (Agtech)	
B. laterosporus	ACM 5117	*Salmonella typhimurium*	ATCC23853	*B. badius* (**B. subtilis*)	BKPM-7090 (PolyMed)	
B. coagulans	ATCC 7050	*L. acidophilus*	ATCC4356	*B. subtilis*	G-18 (PolyMed)	
B. sphaericus	ATCC 14577	*L. reuteri*	LRU008 (NUTRACEUTIX)	*B. licheniformis* (**B. subtilis*)	X-15 (PolyMed)	
B. circulans	ATCC 15518	*Pediococcus damnosus*	ATCC11309	*B. licheniformis*	BMS 1103 (PROGEN)	
B. badius	ATCC14574	*L. casei*	LC-10 (RHODIA)	*B. licheniformis*	BMS 2004 (PROGEN)	
B. clausii	ATCC 700160	*L. delbrueckii*	ATCC 11842	*B. licheniformis*	BL-2 (Agtech)	
P. polymyxa	ATCC 842	*L. rhamnosus*	ATCC7469	*B. licheniformis*	BL-1 (Agtech)	
P. larvae	ATCC 9545	*S. thermophilus*	ATCC19528	*B. circulans*	BMS 2005 (151#) (PROGEN)	
P. lentimorbus	ATCC 14707	*Propionibacterium jensenii*	ATCC 4868	*B. thuringiensis*	BMS 2005 (131#) (PROGEN)	
		Campylobacter faecalis	ATCC33710	*P. polymyxa* (**B. macerans*)	BMS 1004 (PROGEN)	
		Mycoplasma hyopneumoniae	ATCC25934	*B. laterosporus*	BMS 2002 (PROGEN)	
		Enterobacter agglomerans	ATCC27989			

ATCC: American Type Culture Collection, Manassas, USA; ACM: Australian Collection of Microorganisms, Department of Microbiology, University of Queensland; PROGEN: PROGEN Industries Ltd. Australia; Agtech: Agtech Products, Inc, Waukesha, USA; PolyMed: PolyMed Ltd. Russia; RHODIA: Rhodia Pharma Solutions Inc. Cranbury, USA; NUTRACEUTIX: Nutraceutix, Inc. Redmond, USA; EMAI: Elizabeth Macarthur Agriculture Institute, Camden, Australia.

* Originally mislabelled.

Table 2a
List of *Bacillus* taxa providing 16S rDNA gene sequences from GenBank for the design of primers

Phylogroup[a]	Species	Accession No.
I	*B. subtilis*	AF198249, AB018486
I	*B. licheniformis*	AF234841, AB055006
I	*B. pumilus*	AF234856, AB020208
I	*B. atrophaeus*	AB021181, AY121428
I	*B. amyloliquefaciens*	AY055223, AY055224
I	*B. cereus*	AF206326, Z84577
I	*B. thuringiensis*	Z84584, Y18473
I	*B. mycoides*	AB021192, Z84582
I	*B. anthracis*	AF176321, AF290552
I	*B. megaterium*	D16273, AB022310
I	*B. coagulans*	AF466695, AF346895
I	*B. badius*	D78310, X60610
I	*B. firmus*	D16268, D78314
I	*B. lentus*	AB021189, D16272
I	*B. circulans*	X60613, AY043084 Y13064, Y13062
I	*B. clausii*	AJ297492, X76440
I	*B. simplex*	AJ439078, D78478
II	*B. sphaericus*	L14012, AF169495
III	*P. polymyxa*	AJ320493, AF355463
III	*P. l. larvae*	X60619, U86605
III	*P. lentimorbus*	X60622, AB110988
IV	*B. laterosporus*	D16271, D78461
IV	*B. brevis*	AF424048, D78457
V	*G. stearothermophilus*	AY491497, AJ005760

[a] The phylogenetic grouping is based on Ash et al., 1991.

nutrition agar media as recommended by ATCC. Fifty *P. larvae* isolates were obtained from the Elizabeth Macarthur Agricultural Institute, Regional Veterinary Laboratory culture collection. These had been cultured and identified as described previously (Hornitzky and Clark, 1991; Hornitzky and Wilson, 1989). All strains were stored at −80 °C in 15% glycerol.

2.2. Morphological and biochemical identification of *Bacillus* strains

Bacterial isolates were identified as *Bacillus* spp. based on: (i) Gram stain (Gram positive rods); (ii) spore forming capacity, examined using phase contrast microscopy; and (iii) API tests, using the API 20E and API 50 CHB system (BioMerieux).

2.3. DNA isolation

Bacterial genomic DNA was isolated according to the modified method of Mantynen and Lindstrom (Mantynen and Lindstrom, 1998). In brief, 1.5 ml of an overnight bacterial culture grown in TSB were collected and washed with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Bacterial DNA was extracted by lysozyme (Sigma, Australia) and proteinase K (Promega, Australia) treatment, followed by CTAB-chloroform extraction and isopropanol precipitation. The purified DNA was resuspended in TE buffer. The quality of the DNA preparation was verified by measuring the absorbance ratio at 260/280 nm.

2.4. Primer design and PCR amplication

16S rRNA sequences were retrieved from GenBank, EMBL and RDP (the Ribosomal Database Project) databases (Table 2a and 2b). Multiple alignments of 16S rRNA from *Bacillus* spp. and phylogenetically related reference species were constructed with the Pileup program (GCG, University of Wisconsin) accessed through the Australian National Genome Information Service, University of Sydney (ANGIS). Two potential PCR primer sites, starting at position 255 and position 1350 (*E. coli* 16S rRNA gene numbering), respectively, were selected. A 19-mer forward

Table 2b
List of non-*Bacillus* genera providing 16S rDNA gene sequences from GenBank for the design of primers

Species	Accession No.
Clostridium tertium	Y18174
Clostridium difficile	AB075770
Enterococcus faecium	AJ276355
Enterobacter aerogenes	AB004750
Bifidobacterium bifidum	M38018
Staphylococcus aureus	X68417
Campylobacter faecalis	AF372091
Streptococcus thermophilus	AY188354
Salmonella typhimurium	X80681
Pediococcus damnosus	AJ318414
Lactococcus lactis	AJ488173
Lactobacillus acidophilus	M99704
Lactobacillus fermentum	AF477499
Lactobacillus plantarum	M58827
Lactobacillus reuteri	X76328
Listeria monocytogenes	AL591981
E. coli O157H7	AB035925
Propionibacterium jensenii	X53219
Mycoplasma hyopneumoniae	Y00149
Fusobacterium mortiferum	X55414

primer (B-K1/F, 5′-TCACCAAGGCRACGATGCG-3′) and an 18-mer reverse primer (B-K1/R1, 5′-CGTATTCACCGCGGCATG-3′) were designed based on data from these sites. Two 16S rRNA universal primers, 341f and 926r (Liu et al., 1997), were used as a PCR control to ensure the quality of the template DNA. The oligonucleotide primers were synthesized commercially (Sigma, Australia).

Amplification was carried out using a PC-960 thermal cycler (Corbett Research, Australia) with a reaction volume of 50 μl. A 2 μl sample of DNA template was added to a mixture containing 0.2 mM of each dATP, dGTP, dCTP and dDTP (Astral Scientific, Australia), 1× buffer solution (QIAGEN, Australia), 1.5 mM $MgCl_2$, 1.0 μM of each primer (B-K1/F and B-K1/R1 or 341f and 926r) and 1.0 unit of *Taq* DNA polymerase (QIAGEN, Australia). Each PCR program was conducted using a denaturation step of 3 min at 94 °C, followed by 25 cycles of 94 °C for 30 s, 63 °C (or 56 °C for primers 341f/926r) for 30 s and 72 °C for 2 min, with an extension step at 72 °C for 10 min. A MQ-H_2O control was included in each PCR batch.

PCR products were separated by agarose gel electrophoresis. A 5 μl sample of PCR product was mixed with 3 μl of loading dye containing 0.25% bromophenol blue in 15% Ficoll solution and loaded onto a 2.0% agarose gel (Ultrapure Agarose, Life Technology, Australia) containing ethidium bromide (1 μg/ml), using 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) as running buffer. DNA in the gel was visualized by exposure to UV light and photographed with a digital capture system (Gel Doc, Bio-Rad, Richmond, California). The sizes of DNA fragments were estimated using a 100 bp+ DNA ladder (Promega, Australia).

2.5. ARDRA analysis of PCR amplicons

The restriction enzymes with a 4-bp recognition site used in this study were *Alu*I (AG′CT), *Taq*I (T′CGA) and *Hha*I (GCG′C) (Fermentas, Hanover, MD), *Msp*I (C′CGG), *Mbo*I (′GATC) and *Rsa*I (GT′AC) (New England Biolabs, Beverly, MA). PCR products were purified using WIZARD PCR purification columns (Promega, Australia) and were eluted in a final volume of 40 μl. A 5 μl sample of purified PCR amplicon was digested with 5 U of individual restriction enzyme in a 20 μl reaction volume for 4 h using the conditions recommended

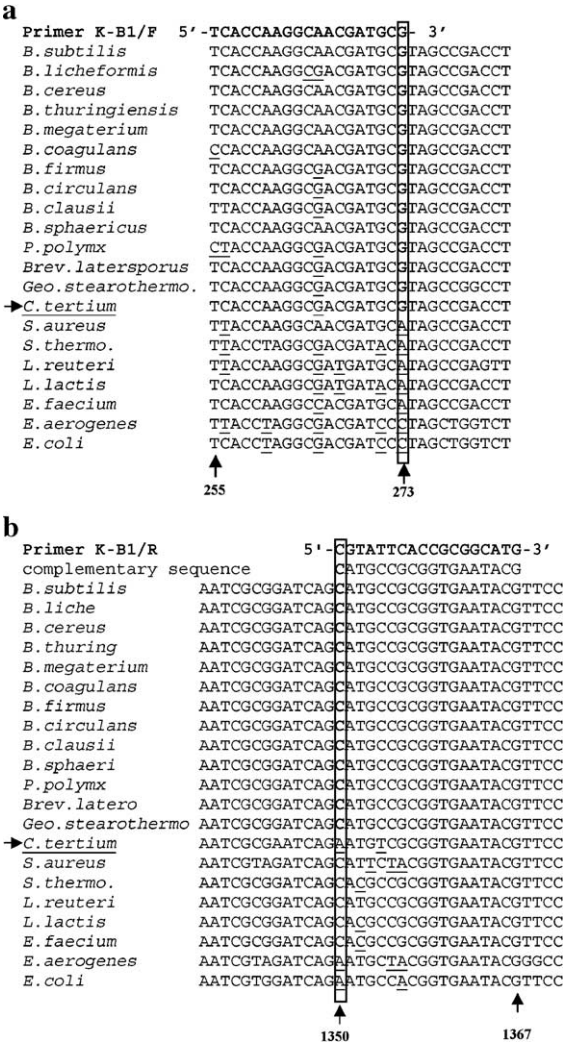


Fig. 1. (a) Alignment of primer K-B1/F with homologous target sequences of 16S rDNA (*E. coli* numbering scheme—nucleotides 255 and 273) from *Bacillus*, *Paenibacillus*, *Brevibacillus* and other genera, sourced from GenBank. Mismatched primer sequences have been underlined. The guanosine nucleotide in position 273 is maintained for all *Bacillus* genus but changes to either adenosine or cytosine for non-*Bacillus* genera (see boxed inset). (b) Alignment of primer K-B1/R1 with homologous target sequences of 16S rDNA (*E. coli* numbering scheme—nucleotides 1350 and 1368) from *Bacillus*, *Paenibacillus*, *Brevibacillus* and other genera, obtained from GenBank. Mismatched primer sequences have been underlined. The box inset shows the different nucleotide in position 1350 between all *Bacillus* and *Clostridium*.

by the manufacturer. The restriction fragments were electrophoresed through a 2% agarose gel (Ultrapure Agarose, Life Technology) followed by ethidium bromide staining. Digitized gel images were analysed with the GelCompar software (Applied Maths, Belgium, version 3.1) to construct ARDRA profiles for *Bacillus* strains. Similarity between fingerprints was determined on the basis of the Dice coefficient and a band position tolerance of 1% was set. The theoretical ARDRA patterns were created by means of a MWTOGEL method in the GelCompar. Basically, the restriction band size data was obtained through a Maplot program (ANGIS) analysis for the 16S rRNA gene sequences obtained from GenBank. The data was then imported into GelCompar through the MWTOGEL method to display the fingerprints.

2.6. DNA sequence analysis

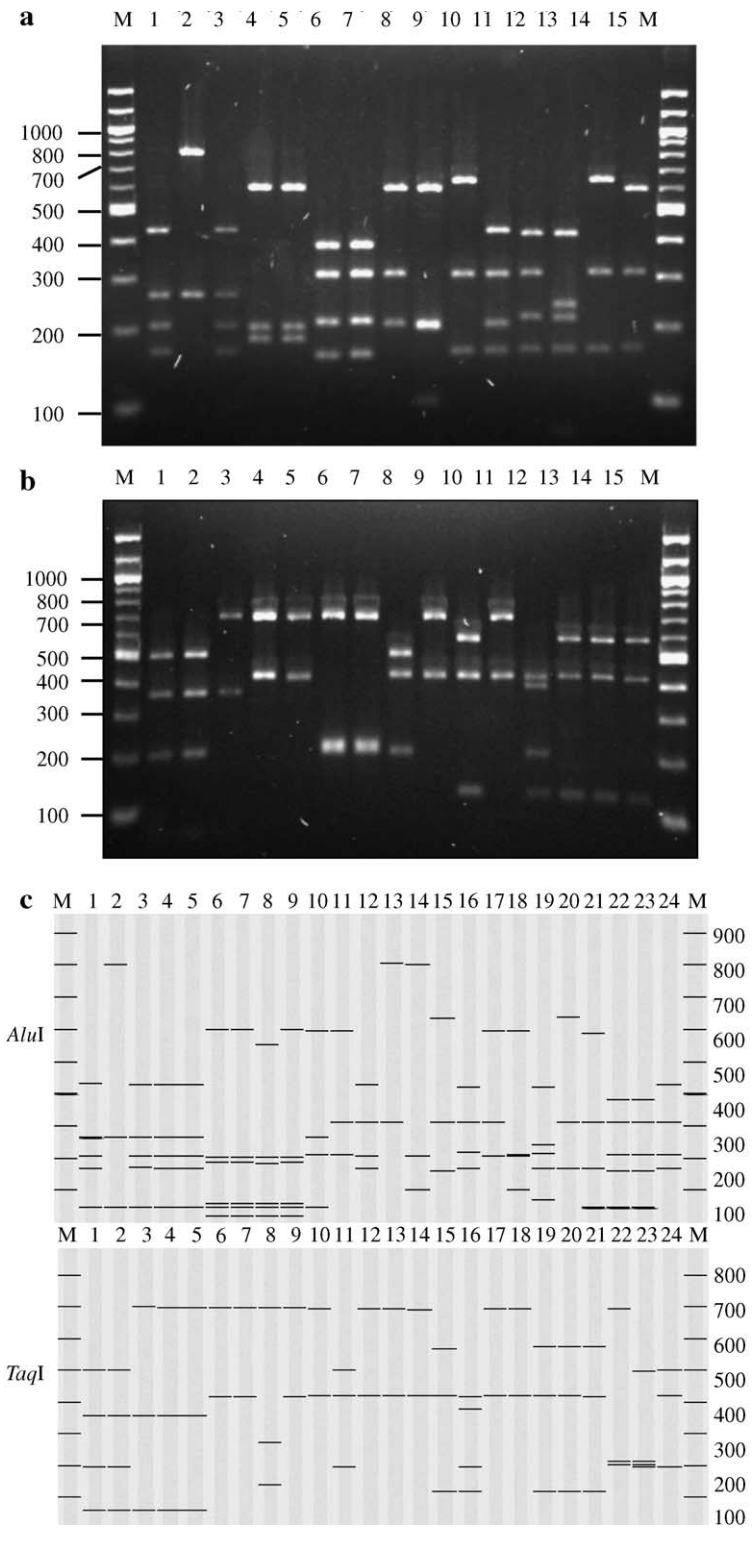
PCR product was purified using the QIAGEN Gel Purification kit (QIAGEN, Australia). A total of 70 ng of purified DNA was subjected to AmpliTaq cycle-sequencing reactions using the BigDye® terminator cycle-sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Electrophoresis of DNA sequencing reaction products was performed through 7% polyacrylamide gels with an automated sequencer (model 377, Applied Biosystems). The nucleotide sequences were analysed with the ANALYSIS program (Applied Biosystems) and the ANGIS program.

3. Results

3.1. Selection and validation of a Bacillus specific forward and reverse primer set

Three criteria were imposed in the search for the forward and reverse primers capable of amplifying a specific 16S rDNA sequence of 3 *Bacillus* genotypes representing 24 species (Table 2a), but not other non-*Bacillus* genera such as *Clostridium*, *Enterococcus*, *Bifidobacterium*, *Staphylococcus*, *Campylobacter*, *Streptococcus*, *Salmonella*, *Pediococcus*, *Lactococcus*, *Lactobacillus*, *Listeria*, *E. coli*, *Propionibacterium*, *Mycoplasma and Fusobacterium* (Table 2b) and normally found associated with environmental samples. Firstly, both primers must have a G+C content >50%. Secondly, one of the primer set had a deliberate nucleotide mismatch for non-*Bacillus* species. Using alignment refined from ANGIS (as shown in Fig. 1a), these conditions were fulfilled by primer K-B1/F corresponding to nucleotide position 255–273 of the reference *E. coli* 16S rDNA. At position 273 (boxed inset), the guanosine nucleotide (bolded) was retained for all bacilli but is replaced by adenosine or cytosine in non-*Bacillus* genera. The specificity of this primer for *Bacillus* was further checked with a BLAST program. However, primer suitability was confounded by *Clostridium tertium* (arrowed) having a guanosine in position 273. To overcome this problem, a third criterion was applied in the design of the reverse primer so that the corresponding *Clostridi-*

Fig. 2. (a) *Alu*I restriction profiles of amplified regions of the 16S rRNA genes of *Bacillus* reference strains. Lane M, 100 bp+ DNA ladder (size are indicated on the left in bp); lane 1, *B. subtilis* ATCC6633; lane 2, *B. licheniformis* ATCC25972; lane 3, *B. pumilus* ATCC21356; lane 4, *B. cereus* ATCC14579; lane 5, *B. thuringiensis* ATCC10792; lane 6, *B. laterosporus* ATCC64; lane 7, *B. laterosporus* ACM5117; lane 8, *B. coagulans* ATCC7050; lane 9, *B. sphaericus* ATCC14577; lane 10, *B. circulans* ATCC15518; lane 11, *B. badius* ATCC14574; lane 12, *B. clausii* ATCC700160; lane 13, *P. polymyxa* ATCC842; lane 14, *P. larvae* ATCC9545; lane 15, *P. lentimorbus* ATCC 14707. (b) *Taq*I restriction profiles of *Bacillus* reference strains. Lane M, 100 bp+ DNA ladder (size are indicated on the left in bp); lane 1, *B. subtilis* ATCC6633; lane 2, *B. licheniformis* ATCC25972; lane 3, *B. pumilus* ATCC21356; lane 4, *B. cereus* ATCC14579; lane 5, *B. thuringiensis* ATCC10792; lane 6, *B. laterosporus* ATCC64; lane 7, *B. laterosporus* ACM5117; lane 8, *B. coagulans* ATCC7050; lane 9, *B. sphaericus* ATCC14577; lane 10, *B. circulans* ATCC15518; lane 11, *B. badius* ATCC14574; lane 12, *B. clausii* ATCC700160; lane 13, *P. polymyxa* ATCC842; lane 14, *P. larvae* ATCC9545; lane 15, *P. lentimorbus* ATCC 14707. (c) Theoretical prediction of ARDRA profiles generated with restriction enzymes *Alu*I and *Taq*I based on published 16S rDNA sequences from GenBank. Lane M, DNA ladder (size are indicated on the right in bp); lane 1, *B. subtilis* (accession no. AF198249); lane 2, *B. licheniformis* (AF234841); lane 3, *B. pumilus* (AF234856); lane 4, *B. atrophaeus* (AB021181); lane 5, *B. amyloliquefaciens* (AY055223); lane 6, *B. cereus* (AF206326); lane 7, *B. thuringiensis* (Z84584); lane 8, *B. mycoides* (AB021192); lane 9, *B. anthracis* (AF176321); lane 10, *B. megaterium* (AB022310); lane 11, *B. coagulans* (AF466695); lane 12, *B. badius* (D78310); lane 13, *B. firmus* (D16268); lane 14, *B. lentus* (AB021189); lane 15, *B. circulans* (Y13062); lane 16, *B. clausii* (AJ297492); lane 17, *B. simplex* (AJ439078); lane 18, *B. sphaericus* (AF169495); lane 19, *P. polymyxa* (AJ320493); lane 20, *P. larvae* (X60619); lane 21, *P. lentimorbus* (X60622); lane 22, *B. laterosporus* (D16271); lane 23, *B. brevis* (AF424048); lane 24, *G. stearothermophilus* (AY491497).


a
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
1000
800
700
500
400
300
200
100
b
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
1000
800
700
500
400
300
200
100
c
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 M
AluI
900
800
700
600
500
400
300
200
100
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 M
TaqI
800
700
600
500
400
300
200
100

um rDNA sequence would not be amplified when used in conjunction with K-B1/F. As shown in Fig. 1b, the reverse primer B1-K/R1 spanning the reference *E. coli* nucleotide positions 1350–1367 contained an adenosine nucleotide for *Clostridium*, *E. aerogenes* and *E. coli* at position 1350 (arrowed; boxed inset) while all *Bacillus* species contained a cytosine residue. The presence of cytosine at position 1350 for *S. aureus*, *S. thermophilus*, *L. reuteri*, *L. lactis* and *E. faecium* would not generate amplicons because of a mismatched forward primer for these species. The rationale for stringency in the selection of a single mismatch at the 3′ end of a primer is known to interfere strongly with the PCR reaction, while there is a higher degree of tolerance for mismatches at the 5′ end (Kwok et al., 1990).

3.2. Genus-specific amplification of reference Bacillus strains

The specificity of this primer pair for *Bacillus* was confirmed by PCR using genomic DNA extracted from 33 reference strains (Table 1). All *Bacillus*, *Paenibacillus* and *Brevibacillus* reference strains yielded a PCR product with an amplicon sized around 1114 bp, in agreement with theoretical prediction. The 18 non-*Bacillus* strains (Table 1) were not amplified. PCR amplicons were also generated with 17 probiotic strains as well as all 50 *P. larvae* honeybee larvae isolates. From all these analyses, it was determined that a 25 cycle amplification required a minimum of 0.5 ng *Bacillus* genomic DNA for successful amplification.

3.3. Speciation of reference Bacillus strains by ARDRA

To determine whether genus-specific amplicons from the *Bacillus*, *Paenibacillus* and *Brevibacillus* reference strains could be speciated, restriction digests were carried out with *Alu*I, *Taq*I, *Hha*I, *Msp*I, *Mbo*I and *Rsa*I. Restriction fragments were generated by all 6 restriction enzymes for amplicons from each of the 15 reference *Bacillus* strain. However, only *Alu*I and *Taq*I produced clear ARDRA patterns capable of distinguishing species members in each of the 3 genera-*Bacillus*, *Paenibacillus* and *Brevibacillus* (Fig. 2a and b). The ARDRA patterns also matched the theoretical digestion maps generated by simulated restriction digests of published GenBank sequences (Fig. 2c).

With *Alu*I and *Taq*I, most *Bacillus* species could be readily speciated using this ARDRA system by comparing either with the theoretical ARDRA pattern or with actual fragment size profiles as shown in Fig. 2a, b and c. The utility of this procedure was restricted (Fig. 2c) by its inability to distinguish between three members of the *B. subtilis* cluster (*B. pumilus, B. amyloliquefaciens* and *B. atrophaeus*), and three species in the *B. cereus* cluster *(B. cereus*, *B. thuringiensis* and *B. anthracis*).

The ARDRA assay could not differentiate between *B. circulans* from *P. larvae*. This should not present a problem because they can be simply differentiated by their catalase reaction and growth characteristics. For instance, *P. larvae* only grows under facultative anaerobic conditions as described by Hornitzky and Clark (1991).

3.4. Identification of test Bacillus isolates from environmental sources

The ARDRA assay and API identification kits were used to screen 17 probiotic and 50 honeybee isolates (Table 1). The species identity of each of the probiotic strain has been previously labelled by the manufacturer. Of the 17 probiotic strains examined, 3 were found to be misclassified based on ARDRA profiles (Fig. 3) and API CHB 50 results (data not shown). *B. subtilis* strain X-15 (from PolyMed, Russia) was observed to possess a typical ARDRA pattern for *B. licheniformis*. API testing identified *Bacillus* strain X-15 as being maltose positive (*B. subtilis* is maltose negative) with 99% similarity to *B. licheniformis.* Another *B. subtilis* strain from PolyMed, BKPM-7090, was identified as *B. badius* based on ARDRA and API test. The probiotic *B. macerans* strain BMS 1004 (from PROGEN, Australia) displayed an ARDRA pattern for *P. polymyxa*. API testing confirmed the identification of this strain as a *P. polymyxa* isolate. Sequence analysis of the 16S rDNA gene and BLAST sequence comparison confirmed that strain X-15 is a *B. licheniformis* isolate, BKPM-7090 is a *B. badius* isolate and strain BMS 1004 is a *P. polymyxa* isolate

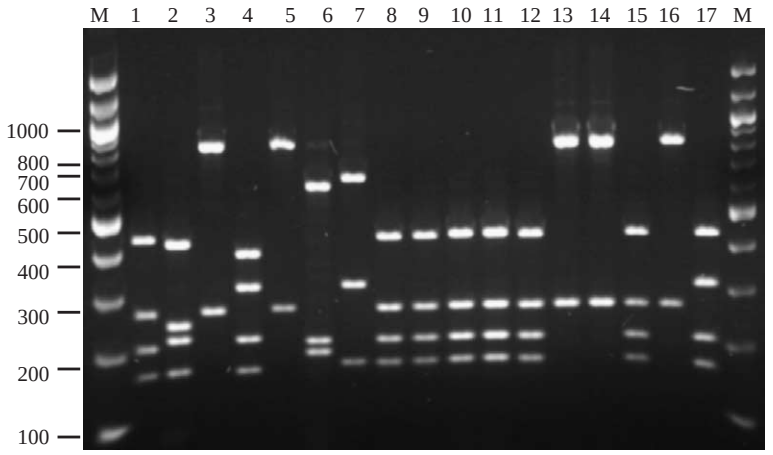


Fig. 3. ARDRA analysis of 17 commercial probiotics strains corresponding to *Alu*I digestion. Lane M, 100 bp+ DNA ladder (size are indicated on the left in bp); lane 1, *B. subtilis* BMS1003; lane 2, *P. polymyxa* BMS1004 (originally mislabelled as *B. macerans*); lane 3, *B. licheniformis* BMS1103; lane 4, *B. laterosporus* BMS2002; lane 5, *B. licheniformis* BMS2004; lane 6, *B. thuringiensis* BMS2005; lane 7, *B. circulans* BMS151; lane 8, *B. subtilis* BS1; lane 9, *B. subtilis* BS2; lane 10, *B. subtilis* 3AP4; lane 11, *B. subtilis* 15AP1; lane 12, *B. subtilis* 22CP1; lane 13, *B. licheniformis* BL-1; lane 14, *B. licheniformis* BL-2; lane 15, *B. subtilis* G-18; lane 16, *B. licheniformis* X-15 (originally mislabelled as *B. subtilis*); lane 17, *B. badius* BKPM-7090 (originally mislabelled as *B. subtilis*).

(data not shown), validating the ARDRA and API results.

4. Discussion

In recent years, the use of molecular techniques has greatly changed the original taxonomic classification of the *Bacillus* taxa. Taking advantage of 16S rRNA sequence information, Ash et al. (1991) re-organised the genus into five phylogenetically distinct groups (group 1–5). Subsequent re-classifications have created eight or more genera: *Alicyclobacillus* (Wisotzkey et al., 1992), *Aneurinibacillus* (Shida et al., 1996), *Bacillus*, *Brevibacillus* (Shida et al., 1996, 1995), *Gracilibacillus* (Waino et al., 1999), *Paenibacillus* (Ash et al., 1991, 1993), *Salibacillus* (Waino et al., 1999), and *Virgibacillus* (Heyndrickx et al., 1998). Under this new scheme, many previous members of the genus *Bacillus* have been either renamed or regrouped but this re-organised scheme is yet to be accommodated in current assay kits used in the laboratory for the identification of *Bacillus* species. In this new classification, only a subset of species is environmentally significant, either of use to, and in the service of mankind, or represent a threat as pathogens. Our ultimate goal was to develop a simplified and rapid method for the detection of these environmentally important *Bacillus* species. Using available information from GenBank, the 16S rDNA sequences of 24 reference *Bacillus* strains representing all 5 phylogroups (Ash et al., 1991) were aligned. A 19-mer forward primer K-B1/F corresponding to nucleotide position 255–273 of the reference *E. coli* 16S rDNA and a 18-mer reverse primer K-B1/R1 (nucleotides 1350–1367 of *E. coli* numbering) proved to be selective for *Bacillus*, *Brevibacillus* and *Paenibacillus* genera.

Based on 16S rDNA alignments, the primer set used in this study also showed sequence matches with these new genera such as *Gracilibacillus* and *Geobacillus*. It is, however, beyond the scope of this paper since strains from the above genera grow under different conditions from probiotic and the gut-associated bacilli. When PCR of communal DNA (e.g. from feces) was analysed using universal bacterial primers, only numerically dominant species were amplified whilst minority bacilli were not easily detected. Prior to this study there was no PCR assay for *Bacillus* group members, although genus-specific PCR primers have been reported for other genera such as *Clostridium*, *Bifidobacterium*, *Lactobacillus* and *Mycoplasma* (Dubernet et al., 2002; Matsuki et al., 1999; Van Dyke and McCarthy, 2002; van Kuppeveld et al., 1992).

As a 16S rDNA gene based molecular technique, ARDRA has been used for phylogenetic and taxonomic studies in *Mycobacetrium* (De Baere et al., 2002), *Brevibacillus* (Logan et al., 2002), *Clostridium* (Gurtler et al., 1991), *Streptococcus* (Jayaro et al., 1991) and *Acinetobacter* species (Vaneechoutte et al., 1995). Heyndrickx and colleagues have developed an ARDRA method for identification of strains of the genera *Alcaligenes, Bordetella*, *Bacillus* and *Paenibacillus* (Heyndrickx et al., 1996). In that study, a universal bacterial 16S rDNA gene primer set was used to amplify a 1500 bp amplicon, followed by construction of ARDRA patterns using 5 restriction enzymes. The obtained ARDRA patterns for each species were combined to form a database for strain identification in each genus. However, there are several limitations to this ARDRA assay. Firstly, universal bacterial rather than *Bacillus*-specific primers were used for PCR amplication. Thus subsequent species identification relied on prior genus identification by phenotypic and biochemical methods, FAME analysis and SDS-PAGE profiling. Secondly, compared to the use of *Bacillus*-specific primers, ARDRA using universal bacterial primers is difficult to assess changes in *Bacillus* composition. Thirdly, employing 5 restriction enzymes in the ARDRA assay is costly in time, labour and expense, although theoretically the more restriction enzyme used in ARDRA, the more accurate result will be obtained. Heyndrickx also tested different combinations of restriction enzymes in the ARDRA assay and found that 3 enzymes yielded similar results in terms of species identification as did 5 enzymes (Heyndrickx et al., 1996). Some authors have suggested that two restriction enzymes are adequate for ARDRA, especially in analysing species composition changes in complex communities (Gich et al., 2000; Koschinsky et al., 2000; Moyer et al., 1996). In our study, we have found that the ARDRA assay using *Bacillus*-specific primers and two restriction enzymes, *Alu*I and *Taq*I, was quite adequate in differentiating most reference bacilli strains.

It was previously reported that ARDRA using universal primers could not separate *B. licheniformis* from *B. pumilus* and *B. subtilis* from *B. amyloliquefaciens* (Vaerewijck et al., 2001). However, the ARDRA assay developed in this study was able to differentiate *B. subtilis* and *B. licheniformis* from the other species of the "*B. subtilis* phylogenetic cluster" (*B. pumilus*, *B. amyloliquefaciens* and *B. atrophaeus*). Two species within the "*B. cereus* phylogenetic cluster", *B. cereus* and *B. thuringiensis*, cannot be differentiated on the basis of the 16S rDNA sequence, and therefore cannot also be differentiated by ARDRA assay. On the other hand, one member of this cluster, *B. mycoides*, showed a high degree of similarity with *B. cereus* (99.5%) with only 7–9 scattered nucleotides differences in the 16S rRNA gene sequence (Ash et al., 1991; Daffonchio et al., 1998). This was sufficient to allow ARDRA identification in our assay.

Sequence variations in 16S rRNA genes may exist between some *Bacillus* strains within species. In order to ensure a typical ARDRA pattern correctly represented a particular species, we have retrieved 2 or more 16S rDNA sequences from GenBank for each *Bacillus* species. The experimentally obtained ARDRA patterns for the *Bacillus* reference strains matched the theoretical ARDRA patterns in almost every case. There was one exception, *B. circulans* species, in which the 16S rRNA gene sequences of four individual *B. circulans* strains selected from GenBank were dispersed at a low similarity level (88.1%). Due to the variant gene sequences of *B. circulans* and the presence of undetermined nucleotides (represented by *n*) in the 16S rRNA gene sequences deposited in GenBank, each strain produced a unique ARDRA pattern. The *B. circulans* reference strain ATCC15518 only matched with one of these (*B. circulans* strain WSBC 2003, accession no. Y13062). Logan and Berkeley reported that the species *B. circulans* forms a very diffuse group phenotypically and displays wide biochemical variations between strains (Logan and Berkeley, 1984). Other authors have also found this species to be genetically heterogeneous by 16S rDNA gene analysis (Heyndrickx et al., 1996; Nakamura and Swezey, 1983).

Consumption of certain live microorganisms has been shown to have a beneficial health impact on both humans and animals. A diverse group of *Bacillus* strains has been evaluated as probiotics and been sold worldwide including *subtilis*, *licheniformis*, *coagulans*, *clausii* and *cereus* (Sanders et al., 2003). However, the inaccurate species labelling on

some commercial *Bacillus* probiotic products have been reported recently due to misclassification of some species (Green et al., 1999; Hoa et al., 2000; Sanders et al., 2003). Our results have shown that 3 out of 17 commercial *Bacillus* probiotic strains were wrongly identified based on ARDRA assay. This was confirmed by additional API testing and 16S rDNA sequencing. Two misclassifications were in relation to closely related species (e.g. *B. licheniformis* and *B. subtilis*; and *P. polymyxa* and *B. macerans*). The other misclassification was *B. badius* and *B. subtilis*, both these species are in phylogenetic group 1. An important aspect of establishing safety of *Bacillus* probiotics is proper taxonomic characterization of the bacteria in the product. Moreover, accurate species labelling is essential for responsible quality control, and to build consumer confidence in the products.

ARDRA has been used in the analysis of mixed bacterial populations from different environments such as activated sludge, compost samples, marine bacterioplancton and hipersaline environments (Acinas et al., 1999; Gich et al., 2000; Koschinsky et al., 2000; Martinez-Murcia et al., 1995). It has been proven to be sensitive for quick assessment of genotypic changes in the community over time, and successfully used to compare communities subject to different environmental conditions. Comparably, a current molecular community profiling method, denaturing gradient gel electrophoresis (DGGE), represent more powerful tool for studying bacterial diversities in complex environments (Heilig et al., 2002; Muyzer et al., 1993; Satokari et al., 2001). The employment of DGGE in association with our group specific *Bacillus* PCR/ARDRA technique may provide even better definition between species and such a combined approach would provide a new way to investigating *Bacillus* diversity in gut microflora as well as in soil, water and sewage samples.

In conclusion, we have developed and validated a *Bacillus*-specific PCR-ARDRA system, which was successfully applied to identify *Bacillus* strains from different environmental niches. This simplified PCR-ARDRA methodology targets the environmentally important *Bacillus* species such as probiotic and gut-associated bacilli. It is easy to conduct and has inherently a high level of discriminatory specificity.

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References

Acinas, S.G., Anton, J., Rodriguez-Valera, F., 1999. Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. Appl. Environ. Microbiol. 65, 514–522.

Ash, C., Farrow, J., Wallbanks, S., Collins, M., 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Lett. Appl. Microbiol. 13 (4), 202–206.

Ash, C., Priest, F.G., Collins, M.D. 1993. Molecular identification of rRNA group 3 bacilli (ash, farrow, wallbanks and collins) using a PCR probe test. SO-Antonie van Leeuwenhoek. 64(3–4). 1993–1994. 253–260.

Bobbie, R.J., White, D.C., 1980. Characterization of benthic microbial community structure by high solution gas chromatography of fatty acid methyl easters. Appl. Environ. Microbiol. 39, 1212–1222.

Bourque, S.N., Valero, J.R., Lavoie, M.C., Levesque, R.C., 1995. Comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences of *Bacillus thuringiensis* strains and subspecies and of closely related species. Appl. Environ. Microbiol. 61, 1623–1626.

Casula, G., Cutting, S.M., 2002. *Bacillus* probiotics: spore germination in the gastrointestinal tract. Appl. Environ. Microbiol. 68, 2344–2352.

Coenye, T., Liu, L., Vandamme, P., LiPuma, J.J., 2001. Identification of *Pandoraea* species by 16S ribosomal DNA-based PCR assays. J. Clin. Microbiol. 39, 4452–4455.

Daffonchio, D., et al., 1998. 16S–23S rRNA internal transcribed spacers as molecular markers for the species of the 16S rRNA group I of the genus *Bacillus*. FEMS Microbiol. Lett. 163, 229–236.

De Baere, T., et al., 2002. Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of cultured mycobacteria in a diagnostic laboratory. BMC Microbiol. 2, 4 (Electronic publication 2002 Mar 2001).

Djordjevic, S.P., Forbes, W.A., Smith, L.A., Hornitzky, M.A., et al., 2000. Genetic and biochemical diversity among isolates of *Paenibacillus alvei* cultured from Australian honeybee (*Apis mellifera*) colonies. Appl. Environ. Microbiol. 66, 1098–1106.

Dubernet, S., Desmasures, N., Gueguen, M., 2002. A PCR-based method for identification of lactobacilli at the genus level. FEMS Microbiol. Lett. 214, 271–275.

Gerhartz, W., 1990. Enzymes in Industry: Production and Applications. Weinheim, F.R.D., New York, NY, USA.

Gich, F.B., et al., 2000. Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA). Int. Microbiol. 3, 103–106.

Green, D.H., et al., 1999. Characterization of two *Bacillus* probiotics. Appl. Environ. Microbiol. 65, 4288–4291.

Gurtler, V., Wilson, V.A., Mayall, B.C., 1991. Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. J. Gen. Microbiol. 137, 2673–2679.

Hansen, B.M., Leser, T.D., Hendriksen, N.B., 2001. Polymerase chain reaction assay for the detection of *Bacillus cereus* group cells. FEMS Microbiol. Lett. 202, 209–213.

Heilig, H.G.H.J., et al., 2002. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. 68, 114–123.

Heyndrickx, M., Vauterin, L., Vandamme, P., Kersters, K., De Vos, P., 1996. Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. J. Microbiol. Methods 26, 247–259.

Heyndrickx, M., et al., 1998. *Virgibacillus*: a new genus to accommodate *Bacillus pantothenticus* (proom and knight 1950). Emended description of *Virgibacillus pantothenticus*. SO-Int. J. Syst. Bacteriol. 48 (1), 99–106 (Jan).

Hoa, N.T., et al., 2000. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl. Environ. Microbiol. 66, 5241–5247.

Hornitzky, M.A.Z., Clark, S., 1991. Culture of *Bacillus larvae* from bulk honey samples for the detection of American foulbrood. J. Apic. Res. 30, 13–16.

Hornitzky, M.A.Z., Wilson, S.C., 1989. A system for the diagnosis of the major bacterial brood diseases of honey bees. J. Apic. Res. 28, 191–195.

Jayaro, B.M., Doré, J.J.E., Baumbach, G.A., Matthews, K.R., Oliver, S.P., 1991. Differentiation of *Streptococcus uberis* from *Streptococcus parauberis* by polymerase chain reaction and restriction fragment length polymorphism analysis of 16S ribosomal DNA. J. Clin. Microbiol. 29, 2774–2778.

Koschinsky, S., Peters, S., Schwieger, F., Tebbe, C.C., 2000. Applying molecular techniques to monitor microbial communities in composting processes. Microbial Biosystems: New Frontiers. Proceedings of the 8th International Symposium on Microbial Ecology, Halifax, Canada, pp. 461–468.

Kwok, S.K., et al., 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. 18, 999–1005.

Liu, W., Marsh, T., Cheng, H., Forney, L., 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63, 4516–4522.

Logan, N.A., Berkeley, R.C., 1984. Identification of *Bacillus* strains using the API system. J. Gen. Microbiol. 130 (Pt 7), 1871–1882 (Jul).

Logan, N.A., et al., 2002. Polyphasic identification of *Bacillus* and *Brevibacillus* strains from clinical, dairy and industrial specimens and proposal of *Brevibacillus invocatus* sp. Nov. Int. J. Syst. Evol. Microbiol. 52, 953–966.

Mantynen, V., Lindstrom, K., 1998. A rapid PCR-based DNA test for enterotoxic *Bacillus cereus*. Appl. Environ. Microbiol. 64, 1634–1639.

Martinez-Murcia, A.J., Acinas, S.G., Rodriguez-Valera, F., 1995. Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol. Ecol. 17, 247–255.

Matsuki, T., Watanabe, K., Tanaka, R., Fukuda, M., Oyaizu, H., 1999. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl. Environ. Microbiol. 65, 4506–4512.

Moyer, C., Tiedje, J., Dobbs, F., Karl, D., 1996. A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. Appl. Environ. Microbiol. 62, 2501–2507.

Muyzer, G., de Waal, E., Uitterlinden, A., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59 (3), 695–700.

Nakamura, L.K., Swezey, J., 1983. Oxyribonucleic acid relatedness of *Bacillus circulans* Jordan 1890 strains. Int. J. Syst. Bacteriol. 33, 703–708.

Olive, D.M., Bean, P., 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. 37, 1661–1669.

Petersen, D.J., Shishido, M., Holl, F.B., Chanway, C.P., 1995. Use of species- and strain-specific PCR primers for identification of conifer root-associated *Bacillus* spp. FEMS Microbiol. Lett. 133, 71–76.

Sanders, M.E., Morelli, L., Tompkins, T.A., 2003. Sporeformers as human probiotics: *Bacillus*, *Sporolactobacillus*, and *Brevibacillus*. Compr. Rev. Food Sci. Food Saf. 2, 105–116.

Satokari, R.M., Vaughan, E.E., Akkermans, A.D.L., Saarela, M., de Vos, W.M., 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 67, 504–513.

Senesi, S., Celandroni, F., Tavanti, A., Ghelardi, E., 2001. Molecular characterization and identification of *Bacillus clausii* strains marketed for use in oral bacteriotherapy. Appl. Environ. Microbiol. 67, 834–839.

Shangkuan, Y.H., Yang, J.F., Lin, H.C., Shaio, M.F., 2000. Comparison of PCR-RFLP, ribotyping and ERIC-PCR for typing *Bacillus anthracis* and *Bacillus cereus* strains. J. Appl. Microbiol. 89, 452–462.

Shida, O., et al., 1995. Proposal of *Bacillus reuszeri* sp. Nov., *Bacillus formosus* sp. Nov., nom. Rev., and *Bacillus borstelensis* sp. Nov., nom. Rev. SO-Int. J. Syst. Bacteriol. 45 (1), 93–100.

Shida, O., Takagi, H., Kadowaki, K., Komagata, K., 1996. Proposal for two new genera, *Brevibacillus* gen. Nov. and *Aneurinibacillus* gen. Nov. Int. J. Syst. Bacteriol. 46, 939–946.

Vaerewijck, M.J., et al., 2001. Occurrence of *Bacillus sporothermodurans* and other aerobic spore-forming species in feed concentrate for dairy cattle. J. Appl. Microbiol. 91, 1074–1084.

Van Dyke, M.I., McCarthy, A.J., 2002. Molecular biological detection and characterization of *Clostridium populations* in municipal landfill sites. Appl. Environ. Microbiol. 68, 2049–2053.

Vaneechoutte, M., et al., 1995. Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis. J. Clin. Microbiol. 33, 11–15.

van Kuppeveld, F.J., et al., 1992. Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. Appl. Environ. Microbiol. 58, 2606–2615.

Waino, M., Tindall, B.J., Schumann, P., Ingvorsen, K., 1999. *Gracilibacillus* gen. Nov., with description of *Gracilibacillus halotolerans* gen. Nov., sp. Nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. Nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. Nov., as *Salibacillus salexigens* comb. Nov. Int. J. Syst. Bacteriol. 49, 821–831.

Wang, X., Heazlewood, S.P., Krause, D.O., Florin, T.H.J., 2003. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J. Appl. Microbiol. 95, 508–520.

Wattiau, P., et al., 2001. A PCR test to identify *Bacillus subtilis* and closely related species and its application to the monitoring of wastewater biotreatment. Appl. Microbiol. Biotechnol. 56, 816–819.

White, G.F. 1920. American foulbrood Bulletin, US Department of Agriculture No. 809.

Wisotzkey, J.D., Jurtshuk Jr., P., Fox, G.E., Deinhard, G., Poralla, K., 1992. Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. Nov. Int. J. Syst. Bacteriol. 42, 263–269.

Woese, C.R., 1987. Bacterial evolution. Microbiol. Rev. 51, 221–271.

Yamada, Y., Hoshino, K., Ishikawa, T., 1997. The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *gluconoacetobacter* to the genetic level. Biosci. Biotechnol. Biochem. 61, 1244–1251.