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NOVEL IDENTIFICATION METHODS INCLUDING A SPECIES-SPECIFIC PCR FOR HAZARDOUS *BACILLUS* SPECIES

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Fifteen strains of *Bacillus* spp. (three *B. cereus* strains, one *B. subtilis*, *B. macerans*, *B. pumilus*, and *B. mycoides* strains, and eight unknown isolates from the food-industry) were identified on species level with three designed pairs of primers for *B. cereus*, *B. licheniformis*, and *B. subtilis*. Primers designed for *B. licheniformis* (BlichF and BlichR) and *B. subtilis* (BsubF and BsubR) allowed specific species identification, whereas the designed pair of primers for *B. cereus* (BcerF2 and BcerR2) showed specificity for *B. cereus sensu lato*, because both *B. cereus* strains and *B. mycoides* gave positive reaction. Bacilli identification was also carried out with routine API method with unsatisfactory results. The $(GTG)_5$-PCR method was used for strain characterization, enabling bacilli classification into separate clusters according to their taxonomic designations.

Keywords: *Bacillus*, hazardness, species-specific PCR

Bacilli have been traditionally classified into different species based on phenotypic characteristic, pathogenicity, clinical symptoms, host preference, and ecological niche (Rasko et al., 2005). Most of them are of economic, environmental, medical, and biodefence importance. *B. cereus* is associated mainly with food poisoning and is also increasingly reported to be a cause of serious and potentially fatal non-gastrointestinal infections. The pathogenicity of *B. cereus*, whether intestinal or non-intestinal, is intimately associated with the production of tissue-destructive exoenzymes, e.g four hemolysins, three distinct phospholipases, an emesis-inducing toxin, and proteases (Bottone, 2010). *B. subtilis* and *B. licheniformis* are involved in ropiness, which cause technological spoilage (Pepe et al., 2003; Sorokulova et al., 2003), and also have been associated with several human infections that cause a range of diseases and incidents of foodborne gastroenteritis (Rowan et al., 2001). On the other hand, species such as *B. subtilis*, *B. amyloliquefaciens*, and *B. licheniformis* are used industrially for the production of enzymes, antibiotics, solvents, and other molecules (Gupta et al., 2002; Tran et al., 2010).

The species within the *B. subtilis* and *B. cereus* groups are not easily differentiated. Most situations only demand identification to the species level, for which techniques such as biochemical characterization, RAPD-PCR, the 16S rRNA sequencing, or DNA-DNA hybridization are used. Often more than one method is needed to distinguish these species (Wang et al., 2007; Thorsen et al., 2011). If species-specific primers or probes are available, these offer a very fast way to detect the target microorganism(s). For routine quality control,

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commercial easy-to-perform phenotypic tests may provide satisfying results if done under standardized conditions (Temmerman et al., 2004).

The aim of this work is the design of the species-specific primers, which will be able to discriminate between *B. cereus* and *B. subtilis* groups and allow their rapid identifications.

1. Materials and methods

1.1. Cultivation of strains used

All bacilli species were cultivated aerobically in BHI broth at 30 °C/20 h, with the exception of the *Geobacillus stearothermophilus* strain, which was incubated at 55 °C. Strains *Lactobacillus rhamnosus*, *Clostridium sporogenes*, *Cl. beijerinckii*, and *Cl. butyricum* were cultivated anaerobically at 37 °C/16 h in MRS broth for *Lactobacillus* and RCM broth for clostridia. All bacterial strains used in this work and their origin are shown in Table 1.

Table 1. Bacterial strains used in this work

Species	Strain	Origine	Source
Bacillus cereus	LCC218	Pudding dessert, CZE	LCC
	DMF2007	Yoghurt, CZE	UCT
	DMF2008	UHT milk, CZE	UCT
B. subtilis	LCC666	UHT milk	LCC
B. pumilus	CCM2218	Nd	CCM
B. macerans	DMF2011	Nd	UCT
B. mycoides	DMF2010	Nd	UCT
Geob. stearothermophilus	CCM2062	Nd	CCM
Lactobacillus rhamnosus	VT1	Tartar sauce	UCT
Clostridium sporogenes	795	Soil	DSMZ
Cl. beijerinckii	791	Nd	DSMZ
Cl. butyricum	10702	Intestine of pig	DSMZ
Aerobic sporulating bacteria (isolates)	SPA12	Smear from dairy plant, CZE	
	1867C	Edam cheese after salting, CZE	
	1410A	Edam cheese after packaging, CZE	
	1656B	Edam cheese after pressing, CZE	
	SPA9	Raw cow´s milk (batch sample), CZE	
	1646B	Pasteurized milk, CZE	
	1398A	Raw cow´s milk, CZE	
	1044B	Edam cheese, CZE	

Nd: not detected; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DEU; LCC: Culture Collection of Dairy Microorganism Laktoflora®, CZE; CCM: Czech Collection of Microorganisms, CZE; UCT: University of Chemistry and Technology, Prague, CZE.

1.2. API test

Phenotypic identification of strains was performed by the API 50CH test (Biomerieux, Marcy-L´Etoile, FRA) according to the manufacturer's instructions, and the results were analyzed by the APILab software (BioMerieux, Marcy-L´Etoile, FRA).

1.3. In silico analysis of 16S-23S rDNA sequences

All sequences encoding the region 16S-23S rRNA were acquired from the GenBank (NCBI, http://www.ncbi.nlm.nih.gov/sites/gquery) and aligned using the MultAlin software (http://multalin.toulouse.inra.fr/multalin/). Appropriate conditions for the designed primers (Table 2) were determined by the NetPrimer software (http://www.premierbiosoft.com/netprimer/). Type strains used for comparison were: *B. cereus* ATTC10987 (the NCBI reference sequence NC_003909.8; region 9306–14157), *B. cereus* AH820 (the NCBI reference sequence NC_011969.1; region 9309–14161), *B. cereus* Q1 (the NCBI reference sequence NC_011773.1; region 9308–14159), *B. cereus* 033BB102 (the NCBI reference sequence NZ_CP009318.1; region 80057–84908), *B. subtilis* subsp. *subtilis* 168 (the NCBI reference sequence NC_000964.3; region 9810–14636), *B. licheniformis* ATCC14580 (the NCBI reference sequence NC_006270.3; region 9899–14720), and *B. pumilus* SAFR-032 (the NCBI reference sequence NC_009848.3; region 9572–14410). Five pairs of primers were designed. A pair of the BacF and BacR primers was designed for genus *Bacillus*, a pair of the BsubF and BsubR primers for *B. subtilis* and a pair of the BlichF and BlichR primers for *B. licheniformis*. For *B. cereus* two pairs of primers (BcerF1 and BcerR1; BcerF2 and BcerR2) were designed, and one pair of primers (BCFW1 and BCrevnew) was taken from the literature (Manzano et al., 2003).

Table 2. Designed specific primers

Primer		Primer sequence (5′-3′)	T_a (°C)	PCR product length (bp)
Bacillus spp.	BacF	gctggttagagcgcacgcctgata	65	263
	BacR	catccaccgtgcgccctttctaac		
B. subtilis	BsubF	cagaacgttccctgtcttgtttag	46	284
	BsubR	gttactaattgaatgtgatgtcta		
B. licheniformis	BlichF	gacaggtgcgtttggatcttg	50	299
	BlichR	cagaaaattcttgtgaatgtctac		
B. cereus	BcerF1	GTTTCTGGTGGTTTACATGG	46	329
	BcerR1	TTTTGAGCGATTTAAATGC		
	BcerF2	GAGAGTTCAATAAAAAGTATT	45	294
	BcerR2	CACTGTTATCTAGTTTTCAAAGAAC		
	BCFW1*	GTTTCTGGTGGTTTACATGG	46	374
	BCrevnew*	TTTTGAGCGATTTAAATGC		

*: published by Manzano and co-workers (2003); T_a: annealing temperature

1.4. PCR method with specific primers

DNA was isolated by the Gen-Elute™ Bacterial Genomic DNA Kit (Sigma Aldrich, USA) according to the manufacturer's instructions. The composition of the PCR reaction mix (25.0 µl) was as follows: 5.0 µl of genomic DNA, 10.0 µl of demineralized water, 2.5 µl of $MgCl_2$ (25 mM, Sigma Aldrich, USA), 2.5 µl of dNTP Mix (2 mM, Sigma Aldrich, USA), 2.5 µl of 10xPCR buffer (Sigma Aldrich, USA), 1.0 µl of each primer (25 µM; Generi Biotech, CZE), and 0.5 µl of Taq DNA polymerase (5 U μl^{-1}, Sigma Aldrich, USA). PCR was performed using the Labcycler (SensoQuest, Göttingen, DEU). The PCR conditions were: denaturation

step at 95 °C/1 min, followed by 30 cycles of denaturation at 95 °C/15 s, annealing at temperature mentioned in Table 2 for 15 s, and extension at 72 °C/30 s. A final extension step was performed at 72 °C/8 min. All PCR products were analyzed by electrophoresis in 0.8% (w/v) agarose gel (Sigma Aldrich, USA) in the Horizont 11-14 (Gibco Laboratories, USA) instrument. After electrophoresis (100 V/1 h/1×TBE), the gels were stained with ethidium bromide and visualized by UV light (λ=365 nm).

1.5. $(GTG)_5$-PCR fingerprinting

DNA was isolated with the Gen-Elute™ Bacterial Genomic DNA Kit (Sigma Aldrich, USA) according to the manufacturer's instructions, and the $(GTG)_5$-PCR mixture was prepared using the FastStart™ Taq DNA Polymerase, dNTPack (Roche, CHE). The composition of the PCR reaction mix (25.0 µl) was as follows: 5.0 µl of genomic DNA (adjusted to 10 ng μl^{-1}), 13.3 µl of demineralized water, 2.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTP Mix (10 mM), 2.5 µl of 10xPCR buffer, 1.0 µl of $(GTG)_5$ primer (5′-GTGGTGGTGGTGGTG-3′, 25 µM; Generi Biotech, CZE), and 0.2 µl of Fast start Taq DNA polymerase (5 U μl^{-1}). PCR was performed using the Labcycler (SensoQuest, Göttingen, DEU). The PCR conditions were: denaturation step at 94 °C/4 min, followed by 35 cycles of denaturation at 94 °C/30 s, annealing at 40 °C/30 s and extension at 72 °C/2 min. A final extension step was performed at 72 °C/16 min. All PCR products were analyzed by electrophoresis under the same conditions mentioned in part 1.4. The resulting fingerprints were analyzed by the BioNumerics V7.6 software package (Applied Maths, BEL). The similarity among digitized profiles was calculated using the Dice coefficient, and an average linkage (UPGMA) dendrogram was derived from the profiles.

2. Results and discussion

Fifteen strains of *Bacillus* spp. (three *B. cereus* strains, one *B. subtilis*, *B. macerans*, *B. pumilus*, and *B. mycoides* strains, and eight unknown aerobic sporulating isolates of food-industrial origin) were identified phenotypically by the API 50CH test and by species-specific PCR method with newly designed primers as well. A few of the primers applied in this study were previously used for identification of isolates from raw milk samples (Němečková et al., 2011) but with unsatisfactory results. Thus, primers and conditions for PCR reactions were optimized and new primers were designed as well.

That all fifteen tested strains belong to the genus *Bacillus* was at first confirmed by PCR with primers for *Bacillus* spp. The expected PCR product (263 bp) was detected for all of them. Other strains used as negative controls gave no PCR product (*Lbc. rhamnosus* VT1 and *Cl. butyricum* 10702) or product with size 380 bp (*Geob. stearothermophilus* CCM2062, *Cl. sporogenes* 795, and *Cl. beijerinckii* 791). The difference in the expected PCR product (263 bp) and the product of negative controls (380 bp) was about 120 bp, and so these could clearly be distinguished on agarose gel.

After genus confirmation, all bacilli strains were identified phenotypically. The API test allowed identification at level 99.9% for *B. subtilis* LCC666, *B. pumilus* CCM2218, and also for four *Bacillus* spp. isolates (SPA9, 1646B, 1398A, and 1044B) as *B. licheniformis* strains. Members of *B. cereus* group (*B. cereus* and *B. mycoides*; 75.9−97.0% of certainty) were not satisfactorily identified by this method due to insufficient level of certainty. The rest of the isolates were identified as follows: 1656B as *B. licheniformis* species (99.3% of certainty),

SPA12 as *B. mycoides* (97.2% of certainty), 1867C as *B. cereus* 1 (63.2% of certainty), and 1410A as *B. cereus* 2 (60.9% of certainty). Identification of the strain *B. macerans* DMF2011 was not possible by the API test at all, though the genus *Bacilli* was confirmed by PCR for this strain. Phenotypic grouping of these closely related *Bacillus* species, based on morphology, physiology, fatty acid composition, and carbohydrate fermentation, is very often misleading (Logan & Berkley, 1984; Wunschel et al., 1995) and must be confirmed by further methods.

The species-specific primers for *B. licheniformis* gave the expected PCR product (299 bp) only for four isolates SPA9, 1646B, 1398A, and 1044B, which were identified by the API test as *B. licheniformis* as well. The rest of the bacilli did not provide positive product, nor did *B. subtilis* LCC666, which belongs to the *B. subtilis* group (*B. subtilis sensu stricto, B. licheniformis, B. vallismortis, B. atrophaeus, B. mojavensis, B.amyloliquefaciens*, and *B. sonorensis*) (Thorsen et al., 2011). The designed primers for *B. subtilis* (BsubF and BsubR) gave positive reaction (the PCR product 284 bp) only for the *B. subtilis* LCC666 strain. These results suggest that these two pairs of primers could be able to identify *B. licheniformis* and *B. subtilis sensu stricto* at species level, but more extensive research should be done with a wider collection of strains to confirm this statement.

Identification of the group *B. cereus* (so-called *B. cereus sensu lato*) is complicated, because it includes a closely related group of species *B. anthracis*, *B. cereus sensu stricto*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. weihanstephanensis* (Ludwig et al., 2009). The API test did not allow identification of the tested strains with satisfactory certainty. According to the obtained results, the specificity of the two pairs of primers, a designed pair BcerF1 and BcerR1 and a pair BCFW1 and BCrevnew designed by Manzano and co-workers (2003), proved to be inconvenient as well (data not shown).

Thus, a new pair of primers was designed. This pair (BCerF2 and BCerR2) provided the expected PCR product (294 bp) for all tested *B. cereus* and *B. mycoides* strains, and positive reaction was detected for isolates SPA12, 1867C, and 1410A, which were preliminarily identified by the API test as species *B. mycoides* (SPA12) or *B. cereus* (1867C and 1410A), and also for the isolate 1656B, which was identified as *B. licheniformis* by the API test (99.3% of certainty). None of the remaining seven strains (*B. macerans* DMF2011, *B. subtilis* LCC666, *B. pumilus* CCM2218, and the four isolates SPA9, 1646B, 1398A, and 1044B, preliminary identified by the API test as *B. licheniformis*) gave positive reaction. This pair of primer showed satisfactory discriminatory power for *B. cereus sensu lato* strains, although it was not able to discriminate between *B. cereus sensu stricto* and *B. mycoides*.

For precise species identification of bacilli, a combination of at least two methods that are predominantly equipment-, skill-, and financially demanding and are suitable for epidemiological and phylogenetic purposes, is usually needed. Methods, used for *Bacillus* species identification, include e.g. RAPD (Lee et al., 2011), ARDRA (Wu et al., 2006), the 16S rRNA gene sequencing, or *gyr*B combined with various phenotypic tests (Wang et al., 2007), and RFLP (Jeyaram et al., 2011).

All bacilli strains were subjected to the $(GTG)_5$-PCR fingerprinting as well. The results of a numerical analysis of the generated $(GTG)_5$-PCR banding patterns are shown in the dendrogram (Fig. 1). All strains can be clearly grouped in separate clusters according to their taxonomic designations. *B. cereus sensu lato* strains were subdivided into two sub-clusters (1a and 1b). In cluster 1a belonged isolates SPA12 and 1656B. Cluster 1b was formed by strains DMF2007, DMF2008, LCC218, and isolates 1867C and 1410A. *B. licheniformis* isolates 1646B, 1398A, SPA9, 1044B created their own cluster (number 2). Profiles of

individual strains *B. subtilis* LCC666, *B. macerans* DMF2011, *B. mycoides* DMF2010, and *B. pumilus* CCM2218 were evaluated as single clusters. Taking into consideration all achieved results above, isolates 1646B, 1398A, SPA9, and 1044B were identified as *B. licheniformis* and isolates SPA12 1867C, 1410A, and 1656B as *B. cereus sensu lato*.

A fairly high discriminatory power up to the strain level was found for the tested collection, as all strains could be differentiated from each other on the basis of at least one band difference in their $(GTG)_5$-PCR fingerprints (Fig. 1). This technique, thus, can be applied as supplementary method for strain characterization of bacilli.

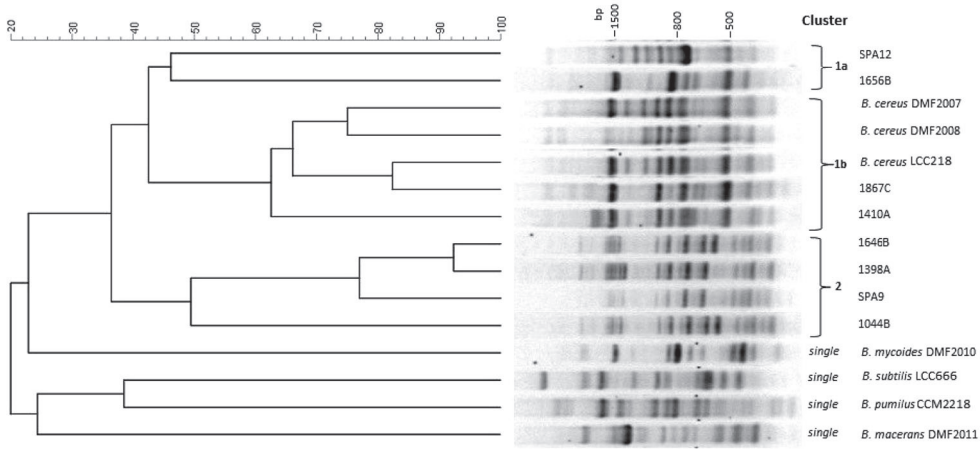

Fig. 1. Dendrogram generated after a cluster analysis of the digitized $(GTG)_5$-PCR fingerprints of bacilli strains. The dendrogram was constructed using the UPGMA method with the Dice coefficient (tolerance 0.5% and optimization 0.5%)

The Rep-PCR method with different primers was previously successfully used for bacilli characterization and allowed revealing novel species as well (Scheldeman et al., 2004; Heyrman et al., 2005).

3. Conclusions

Bacilli as causative agents of food spoilage and foodborne illnesses represent a serious issue to the food industry, thus, their rapid and simple detection and identification is essential. The two newly designed pairs of primers for *B. licheniformis* (BlichF and BlichR) and *B. subtilis* (BsubF and BsubR) were successfully used for species identification of a group of fifteen *Bacillus* spp. strains (three *B. cereus* strains, one *B. subtilis*, *B. macerans*, *B. pumilus*, and *B. mycoides* strains, and eight food-industrial isolates). The designed pair of primers for *B. cereus* (BcerF2 and BcerR2) showed specificity for *B. cereus sensu lato*, because both *B. cereus* and *B. mycoides* strains gave positive reaction. By $(GTG)_5$-PCR genomic fingerprinting, the tested bacilli could be classified into separate clusters according to their taxonomic designations. The designed collection of primers seems to be promising tool for quick, simple, and highly reliable identification of selected bacilli species. Obtained results of this work can be used both in scientific research laboratories and control laboratories during

screening of *Bacillus* spp. for the effective assurance of health safety and standard quality of various food raw materials and products using modern identification methods.

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