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The design and analysis of unique DNA primer sets and probes to identify and distinguish the bacillus cereus group species: developing a real-time DNA-biosensor

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UNIVERSITY *of* LIMERICK

OLLSCOIL LUIMNIGH

The design and analysis of unique DNA primer sets and probes to identify and distinguish the *Bacillus cereus* group species: developing a real-time DNA-biosensor

PhD Thesis

Kamila Oliwa-Stasiak

Chemical and Environmental Sciences Department

University of Limerick

Supervisors: Dr. C. Adley and Prof. K. Arshak

Submitted to the University of Limerick, November 2010

Declaration

I hereby declare that this work is the result of my own investigations and that this thesis has not been submitted previously in this form or any other form to this or any other university in candidature for a higher degree.

____

Kamila Oliwa-Stasiak

Date:

<u>The design and analysis of unique DNA primer sets and probes to identify and distinguish the *Bacillus cereus* group species: developing a real-time DNA-biosensor</u>

Candidate: Kamila Oliwa-Stasiak

Bacillus cereus from the *Bacillus cereus* group species, which consist of: *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomycoides* is one of the most frequently isolated bacterial foodborne pathogens. Growth of *B. cereus* results in production of several highly active toxins therefore, consumption of food containing 10^{5}-10^{6} bacteria (spores)/g or toxins, is sufficient to cause emetic and diarrhoeal syndromes. The most common source of this bacterium is milk and mixed food products that include milk powder, thus is of particular concern in the baby formula industry.

In this study 138 strains of *B. cereus* group spp. were characterized based on their phenotypic and genotypic features. The study developed unique DNA primers for use in PCR and these were then tested via real-time PCR (RT-PCR): (i) the *motB* gene encoding the flagellar motor protein MotB was used as a PCR primer target. (ii) New primers and probes, targeting a hypothetical protein, unique only for *B. pseudomycoides* strains were then developed. (iii) A RT-PCR assay developed together with species specific TaqMan probes were able to differentiate *B. weihenstephanensis* and *B. pseudomycoides* strains. (iv) In addition multiplex PCR with primers targeting *motB* and a hypothetical protein proved successful in identification of the *B. cereus* group spp. with differentiation of *B. pseudomycoides*. This is the first description of a molecular technique able to distinguish *B. pseudomycoides* from other members belonging to the *B. cereus* group spp. and the first RT-PCR protocol to use the *motB* gene as a diagnostic target. The assays performed well with milk samples artificially contaminated with bacteria belonging to the *B. cereus* group spp. To analyze the ability to detect bacterial spores, fat and nonfat milk was contaminated and treated to destroy spores or allow germination.

The design of a hybridization probe for use in a biosensor was then undertaken. The two probes designed were optimised for a hybridisation reaction using dot blot analysis: one for the detection of all species belonging to the *B. cereus* group spp., and the second for detection only of *B. pseudomycoides* strains. Currently DNA must be initially extracted for analysis in a biosensor and the study tested the efficiency of two commercial DNA extraction kits. To be able to receive a signal via the biosensor from bacterial spores present in milk, each sample should be pre-treated by incubation of the milk for the proper time and temperature.

To my husband and son

I would like to thank everybody who helped me to find a needle in a haystack

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Abbreviations

aa	Amino acid	**GC**	Gas chromatography
ATCC	American Type Culture Collection	**LB**	Luria-Bertani
BGSC	*Bacillus* Genetic Stock Center	**$MgCl_2$**	Magnesium chloride
bp	Base pair	**MIHE**	Military Institute of Hygiene and Epidemiology
CFU	Colony forming units	**MWRH**	Mid-West Regional Hospital
Cp	Crossing point	**MYP**	Mannitol egg yolk polymyxin agar
CSPD	Disodium 3-(4-methoxy-spiro {1,2-dixetane-3,2'-(5'-chloro) tricycle [3.3.1.$1^{3,7}$] decan}-4-yl) phenyl phosphate)	**NA**	Nutrient agar
DIG	Digoxygenin	**NaCl**	Sodium chloride
Dig Easy Hyb	Granules for reconstitution used in dot blotting	**NCTC**	National Collection of Type Cultures
DMSO	Dimethyl sulphoxide	**OD**	Optical density
DNA	Deoxyribonucleic Acid	**PCA**	Plate count agar
DSMZ	German Collection of Microorganisms	**PCR**	Polymerase Chain Reaction
dNTP	Deoxynucleoside triphosphate	**PEMBA**	Polymyxin egg yolk mannitol bromothyl blue agar
dH_2O	Distilled water	**RT-PCR**	Real-time PCR
EtBr	Ethidium bromide	**SDS**	Sodium Dodecyl Sulfate
FA	Fatty acid	**SEM**	Scanning electron microscope
FAME	Fatty acid methyl ester	**TBE**	Tris Borate EDTA
FRET	Fluorescence resonance energy transfer	**Tm**	Melting temperature

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1. INTRODUCTION

1.1. Foodborne pathogens

Foodborne diseases, which are caused by foodborne pathogens, viruses and toxins, are of serious public health concern (Wallace *et al.* 1979). The World Human Organization (WHO) defined foodborne disease as: *'Any disease of an infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water'* (WHO 2007).
Published on 26 April 2010 "The Community Summary Report on Foodborne Outbreaks in the European Union in 2008" reported 5,332 wide spectrum of foodborne outbreaks, involving 45,622 people, 6,230 hospitalizations and 32 deaths in the 27 member states. Most of the outbreaks were caused by *Salmonella* sp., viruses and bacterial toxins (EFSA 2010). Contamination with bacterial toxins includes toxins produced by *Bacillus* sp., *Clostridium* sp. and *Staphylococcus* sp. *Bacillus cereus* may produce emetic and diarrhoeal toxins, and depending on the type, the bacteria cause severe nausea, vomiting and diarrhoea. Bacteria are found in approximately 25% food products including milk and its products, meat, spices, rice and noodles.

1.2. The genus *Bacillus*

The genus *Bacillus* is an aerobic or facultatively anaerobic part of the family *Bacillaceae*. It is a large and heterogeneous collection of rod shaped, endospore-forming bacteria widely distributed in the environment (Topley and Wilson 1990). The anaerobic part of the family includes the genus *Clostridium* which species does not resume growth and cannot form spores if oxygen is present. Strains of the genus *Bacillus*, with a few exceptions, produce catalase which also distinguishes bacilli from clostridia. The endospores of bacilli are more resistant than the vegetative cells to heat, drying and disinfectant, therefore endospore-forming species (*B. anthracis* and *B. subtilis*) are more viable. The number of species belonging to this genus (Fritze 2004) increased to more than 222 recognized species distributed across terrestrial and aquatic habitats, including marine sediments (Ki *et al.* 2009). There are two reasons of this rapid increase in named species: first, the development of more diverse methods for

enrichment and isolation of organisms. The second was the development of sophisticated genotyping (Fritze 2004). Amplification and sequencing, in particular 16*S* rRNA sequence, made the detection and identification of specific bacteria simpler. It overcame traditional biochemical tests and fatty acids methyl ester profiling, especially for specific bacteria lacking distinguishable phenotypic characteristics (Ki *et al.* 2009).

Bacteria belonging to the genus *Bacillus* are widely distributed in water, soil, and air and because of their resistant spores present problems in the food processing industry in controlling and preparation sterile and non sterile products.

Bergey's Manual of Determinative Bacteriology is a widely used international reference work for bacterial taxonomy. Analysis of full length high quality 16*S* rRNA sequences presented the phylogenetic subclusters within the genus *Bacillus* (Ludwig *et al.* 2009):

a) *Bacillus subtilis*, *amyloliquefaciens*, *atrophaeus*, *mojavensis*, *licheniformis*, *sonorensis*, *vallismortis*, including the very likely misclassified *Paenibacillus popilliae*

b) *Bacillus farraginis*, *fordii*, *fortis*, *lentus*, *galactosidilyticus*

c) *Bacillus asahii*, *bataviensis*, *benzoevorans*, *circulans*, *cohnii*, *firmus*, *flexus*, *fumarioli*, *infernus*, *jeotgali*, *luciferensis*, *megaterium*, *methanolicus*, *niacini*, *novalis*, *psychrosaccharolyticus*, *simplex*, *soli*, *vireti*

d) *Bacillus anthracis*, *cereus*, *mycoides*, *thuringiensis*, *weihenstephanensis*

e) *Bacillus aquimaris*, *marisflavi*

f) *Bacillus badius*, *coagulans*, *thermoamylovorans*, *acidicola*, *oleronius*, *sporothermodurans*

g) *Bacillus alcalophilus*, *arsenicoselenatis*, *clausii*, *gibsonii*, *halodurans*, *horikoshii*, *krulwichiae*, *okhensis*, *okuhidensis*, *pseudoalcaliphilus*, *pseudofirmus*

h) *Bacillus arsenicus*, *barnaricus*, *gelatini*, *decolorationis*,

i) *Bacillus carboniphilus*, *endophyticus*, *smithii*,

j) *Bacillus pallidus*,

k) *Bacillus funiculus*, *panaciterrae*

1.2.1. The *Bacillus cereus* group species

The *B. cereus* group spp. is a highly homogenous cluster within the *Bacillus* genus, and consists of six species: *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides* and *B. weihentephanensis*. That group has been differentiated based on their phenotypic characteristics, including pathogenic potential. These bacilli is one of

the most taxonomically ambiguous bacterial group (Priest 1993, pp.3-16). It is controversial and several researchers have suggested that these closely relates species should all be grouped as members of *B. cereus* (Chang *et al.* 2003). The G+C content of the DNA of species within the genus can vary from 32-69% (Drobniewski 1993). Whereas high genetic relatedness of *B. cereus* (*sensu stricto*), *B. thuringiensis* and *B. anthracis* has contributed to the suggestion that these three are a members of a single species, *B. cereus sensu lato*. Although *B. mycoides*, *B. pseudomycoides* and *B. weihentephanensis* differ significantly in their ecological features and/or symbiotic associations with other organisms, they are genetically closely related and often as varieties of the same taxon, *B. cereus sensu lato* (Ash *et al.* 1991; Daffonchio *et al.* 2000; Helgason *et al.* 2000b).

The genetic diversity of the *B. cereus* group has been studied using pulsed field gel electrophoresis (PFGE), multilocus enzyme electrophoresis (MEE) and amplified fragment length polymorphism (AFLP) (Carlson *et al.* 1994; Helgason *et al.* 1998; Helgason *et al.* 2000a; Helgason *et al.* 2000b). However, it is difficult to compare the results because the described methods are difficult to standardize between laboratories (Helgason *et al.* 2004).

In this group are transitional strains named *Bacillus* sp. Ba813^{+}. These strains carry chromosomal marker Ba813 characteristic only for *B. anthracis* strains (Olsen *et al.* 2007; Ramisse *et al.* 1999). However, some *B. cereus* and *B. thuringiensis* strains may carry this chromosomal marker but other features (hemolysis of blood agar, motility, penicillin resistance) are different than for *B. anthracis*.

The *B. cereus* group spp., causing foodborne diseases, are Gram-positive, aerobic and facultatively anaerobic, endospore-forming rod shaped organisms commonly found in water and soil. *B. cereus* has been isolated from a variety of food: milk and milk products, meat, eggs, rice, beans, spices. They often contaminate food during handling, manufacture, processing, distribution or cooking causing decomposition or food poisoning (Shinagawa 1993b). The vegetative cells can grow from pH 4.5-9.3 and temperature range 4ºC-50ºC. Based on temperature strains can be subdivided as follows:

- mesophilic strains: able to grow above 10ºC and at 43ºC with optimal growth at 30ºC-37ºC,
- psychrophilic strains: able to grow at the temperature close to 0ºC with optimal growth below 15ºC, the ultimate growth temperature 20ºC,

- psychrotrophic strains: able to grow below 10ºC and not at 43ºC, the optimal growth temperature is 20ºC-35ºC.

The pathology of *B. cereus* to humans is mostly manifested through gastroenteric disorders. Outbreaks are not common due to the high number of organisms that are necessary to cause infection, but the resistance of its toxins to heat makes it a threat in raw and cooked food.

In 2010 The European Food Safety Authority (EFSA) reported about 124 outbreaks caused by *Bacillus* species in 10 European Union (EU) member states in 2008. Only 45 of the *Bacillus* outbreaks were verified (36,3%) with 1,132 cases, 41 cases were hospitalized. The total number of outbreaks in the EU, including 27 member states, caused by *Bacillus* sp. toxins increased by 18,1% compared to 2007 (105 outbreaks) (EFSA 2010).

***Bacillus cereus*:**

The name *Bacillus* (rod) and *cereus* (wax) was named because bacteria on the solid media have a waxy appearance (Frankland and Frankland 1887). Its endospores can survive heat treatment and may germinate if cooling is too slow. The optimum grow temperature is 30-37ºC however *B. cereus* strains able to grow below 7ºC have been reported. The bacterium is approximately 1-1.2µm wide, 3-5µm long and has a short germination time, usually 20-30 minutes.

It was first recognized as a foodborne pathogen in 1949, after an outbreak of diarrhoeal food poisoning at a hospital in Oslo, Norway (Hauge 1955). *B. cereus* is commonly isolated from soil and has been involved in food-related diseases (Drobniewski 1993). The bacterium causes gastrointestinal illness by several protein toxins (enterotoxins) which elicit diarrhoea and one heat stable cereulide causing the emetic type of disease (Agata *et al.* 1996). The diarrhoeal syndrome is caused by ingestion *B. cereus* cells in the food, followed by toxin production in the small intestine. However, the emetic syndrome is due to ingestion of the emetic toxin produced in food (Granum *et al.* 1993). The diarrhoeal type is mainly associated with soups, sauces, meat products, vegetables and milk products whereas the emetic illness with pasta, noodles, rice and pastry (Shinagawa 1990).

Consumption of food containing 10^5-10^6 bacteria or spores/g, is sufficient to cause infection (EFSA 2005). EU regulation 2073/2005 on the microbiological criteria for foodstuffs details acceptable microbiological levels in food stuffs and sampling plans

required to ensure the microbiological criteria are met (EC 2005). The EFSA summary report on *B. cereus* in food (EFSA 2005) stated the lower numbers in food which resulted in a food poisoning outbreak was 3-4 log per g. Hazardous level of pathogen can develop when food is held at temperatures between 29-49ºC for a long time.

Severe forms of disease caused by *B. cereus* have involved hospitalization or even death (Dierick *et al.* 2005). It has been demonstrated that *B. cereus* was responsible for wound and eye infection (Beecher *et al.* 2000; Drobniewski 1993; Helgason *et al.* 1998), as well as systemic infections and periodontitis (Helgason *et al.* 2000b). The bacterium was identified as a causative agent of serious infections in neutropenic (Henrickson *et al.* 1989) and immunosuppressed patients and neonates (Arnaout *et al.* 1999; Hilliard *et al.* 2003).

Fricker *et al.* (2007) designed a TaqMan based RT-PCR assay for detection of the emetic *B. cereus* strains in food. Amplification targeted a highly specific part of cereulide synthetase (*ces*) genes and was applied to identify the causative agent of emetic food-poisoning outbreaks. Ehling-Schulz *et al.* (2006) reported a simple multiplex PCR assay which allowed detection of all known *B. cereus* toxins. Yamada *et al.* (1999) designed a set of primers which allowed for differentiation the *B. cereus* species from the *B. cereus* group. Primers targeted the *gyrB* gene, however, the authors reported some reference strains which were not positive in the PCR amplification.

***Bacillus thuringiensis*:**

Bacillus thuringiensis is a common soil bacterium which was first identified in 1901 by Ishiwata as a pathogen of silkworm (*Bombyx mori*) which causes sotto disease and was named *Bacillus sotto* (Ishiwata 1901 cited in: Anilkumar 2008). In 1911 it was isolated from dead Mediterranean flour moth larvae *Ephestia kuhniella* and named *B. thuringiensis* by Ernst Berliner (Berliner 1915 cited in: Anilkumar 2008).

B. thuringiensis occurs naturally in the environment and is an insect pathogen usually harmless to human, although human infection may occur (Damgaard *et al.* 1997). The bacteria is extensively used as a biopesticide against insect pests because of the production of the crystal proteins encoded by *cry* genes and makes δ–endotoxins during sporulation (Aronson and Shai 2006), cytolytic proteins, vegetative insecticidal proteins and beta-exotoxin. The most widely group are δ–endotoxins (De Maagd *et al.* 2003; Anilkumar 2008). In 1961 the *B. thuringiensis* was registered in the United States for use as a pesticide and reregistered in 1998 (Anonymous 1998).

B. thuringiensis is the most diverse species in the *B. cereus* group and the current nomenclature and classification can be found on the *B. thuringiensis* Toxin Nomenclature: http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/.

Except for the production of the insecticidal proteins, *B. thuringiensis* cannot be distinguished from *B. cereus*. Classical microbiology methods, phospholipid and fatty acid analysis, 16*S* rRNA sequence comparison (Schnepf *et al.* 1998), multilocus enzyme electrophoresis and PFGE (Carlson *et al.* 1994)have been tested to distinguish *B. thuringiensis* from *B. cereus*. Most of the results failed to make a distinction between them.

Manzano *et al.* (2003) reported amplification of *gyrB* gene and restriction nuclease technique (PCR-RE) that was able to distinguish between *B. thuringiensis*, *B. cereus* and *B. mycoides* species. However, only 3 x *B. cereus*, 2 x *B. thuringiensis* and 2 x *B. mycoides* strains were used in this study.

The presence of *cry* genes on extrachromosomal plasmid (Schnepf *et al.* 1998) and coding the insecticidal toxins is the only established difference of *B. thuringiensis* strains. When plasmids are lost, *B. thuringiensis* can no longer be distinguished from the rest of the *B. cereus* group spp. (Helgason *et al.* 2000b). Many *B. thuringiensis* strains can transfer the plasmids to other *B. thuringiensis* or *B. cereus* strains using conjugation (Gonzalez and Carlton 1982, pp.85-95).

Based on different toxicity to insects, the *B. thuringiensis* subspecies have been identified. The examples of *B. thuringiensis* subspecies and the insects that they affect are: *B. thuringiensis* subsp. *israelensis* (mosquitoes and flies), *B. thuringiensis* subsp. *kurstaki* (moths), *B. thuringiensis* subsp. *aizawai* (moths), and *B. thuringiensis* subsp. *tenebrionis* (beetles) (Anonymous 1998; Tomlin 1997, pp.73-78). *B. thuringiensis* to be effective must be eaten by the insects which die from the infection (Kamrin 1997, pp.535-539). Insecticidal proteins are released in the alkaline gut and hydrolyzed to toxins by proteases (Hofte and Whiteley 1989).

***Bacillus anthracis*:**

B. anthracis is an etiologic agent of an acute disease-anthrax (Hornitzky 2000). It is a spore-forming bacterium therefore in unfavourable or harsh environments it hibernates as spores. After crossing into the human or animals (generally cows, sheeps, goats and ducks) with favourable growth conditions, they germinate and change into the vegetative form which produces different toxins resulting in most cases in death of the

infected organism (Quinn and Turnbull 1998, pp.799-818). On shedding from the host to the environment, they develop spores again which are the source of the disease. However *B. anthracis* life cycle is possible in the soil when environmental conditions are good for bacterial growth. Humans become infected after contact with diseased animal, animal materials, infected soil, or spores used as a bioweapon. Spores can live in the soil for many years and have been isolated from animal burial (bones) over 200 years old (Hudson *et al.* 2008).

Anthrax infection can occur in three different forms: *inhalation* (after inhaling anthrax spores); *cutaneous* (bacterium can infect via injured skin); or *gastrointestinal* (results from the ingestion of undercooked meat from infected animals) (Anonymous 2007). Except for Antarctica, anthrax spores can be found in every continent. In each country there are areas with an increased potential presence of *B. anthracis* spores in the soil. This may be caused through the burial of sick dead animals without disinfection or by artificial contamination caused through deliberate contamination of a region (Van Ert *et al.* 2007). This situation happened in 1942 during the Second World War, in the Scottish Gruinard Island, where the British government tested a biological warfare scenario by releasing *B. anthracis* spores on the small island, to wipe out the sheep population. For many years the island was quarantined and visits to the island were strictly prohibited. Starting in 1986, a determined effort was made to decontaminate the island. On April 24, 1990 after 48 years of quarantine Gruinard Island was approved as safe and the warning signs were removed (Manchee *et al.* 1983; 1981). Unfortunately, anthrax is still known as one of the major biological weapons. In 2001, after the terrorist air attack on the US World Trade Center and The Pentagon, envelopes containing white powder with lyophilized anthrax spores, were sent to media companies and politicians offices. The strain used was a virulent, Ames strain which infected 22 people, with 5 mortalities (Anonymous 2007).

The bacteria produced two pathogenicity factors: a toxin consisting of a protective agent carried on the pXO1 (181kb) plasmid and a capsule encoded by pXO2 plasmid (93.5kb) (Hornitzky 2000). Three genes: *pag*, *lef* and *cya* on pXO1 plasmid, encode the three secreted toxins proteins PA (protective antigen), LF (lethal factor) and EF (oedema factor) (Hutson *et al.* 1993; Reif *et al.* 1994). Virulent strains are always: $pXO1^{+}/pXO2^{+}$, whereas avirulent are characterized as: $pXO1^{-}/pXO2^{+}$, $pXO1^{+}/pXO2^{-}$ or $pXO1^{-}/pXO2^{-}$.

Beside traditional microbiological techniques for identification of *B. anthracis*, different molecular techniques exist. They use specific proteins or genes as a target for identification. The methods include: nested PCR targeting the protective agent *pag* gene on the plasmid pXO1, as well as genes of the capsule (*cap*) on pXO2 (Beyer *et al.* 1996); specific detection of CAP-C gene on the pXO2 plasmid by RT-PCR (Lee *et al.* 1999); ribotyping of 16*S*/23*S* rRNA genes; and detecting chromosomal marker *rpoB* by PCR (Qi *et al.* 2001; Shangkuan *et al.* 2000). Serological procedures include ELISA in plates coated with the protective agent component of the anthrax toxin which appears to be specific for *B. anthracis* (Anonymous 2002). An advanced technique for monitoring *B. anthracis* consists of a handheld nucleic acid analyzer method (HANAA). DNA extracted from three isolates were successfully tested for the *vrrA* and *capA* genes (Higgins *et al.* 2003).

Wang *et al.* (2004) developed a quantitative DNA chip for characterization of *B. anthracis*. They combined multiplex PCR assay with arrayed anchored primer PCR and a biotin-avidin alkaline phosphatase indicator system. The assay based on amplification of *pag*, *cap* genes and Ba813 marker gave a positive result due to a colour reaction of alkaline phosphatase.

***Bacillus weihenstephanensis*:**

In 1998, *Bacillus weihenstephanensis* was suggested as a new species on the basis of the sequence differences in rDNA and cold-shock protein genes (Lechner *et al.* 1998). It is a psychrotolerant (psychrotrophic) bacterium, isolates of this species grow at 4-7ºC but not at 43ºC, and it has become a problem in the refrigerated storage and cold chain distribution for the food industry. Pasteurised milk and milk products are a common source for isolation of *B. weihenstephanensis* (Christiansson *et al.* 1989).

Daffonchio *et al.* (2000) based on genetic relationship within six species of the *B. cereus* group, reported that *B. weihenstephanensis* was closely related to *B. mycoides* and *B. pseudomycoides* in both genotype and phenotype. Whereas, *B. cereus*, *B. anthracis* and *B. thuringiensis* clustered in a separate group.

Lechner *et al.* (1998) designed a PCR assay with primers targeting the gene encoding a major cold shock protein (*cspA*), specific for the differentiation of psychrotolerant strains from mesophilic strains of the *B. cereus* group. The high degree of similarity (96-100%) of *cspA* gene showed phylogenetic relationship between the species. However, analysis of the 171bp coding region of *cspA* from different *Bacillus* sp. strains

indicated differences in two positions what allowed for differentiation of psychrotolerant and mesophilic strains.

Stenfors and Granum (2001) used two different set of primers to differentiate the psychrotolerant and mesophilic strains of the *B. cereus* group spp. Isolates which grew at 4-7ºC were identified using PCR with primers targeting *cspA* gene and 16*S* rDNA. In the 16*S* rDNA assay, the primer pair amplified a 249bp product from mesophilic and a 132bp product from psychrotrophic strains. Primers targeting *cspA* gene amplified a 160bp fragment of psychrotrophic strains. The authors reported problems in differentiation using those criteria, as strains able to grow at 6ºC and not at 43ºC showed three positive results for the PCR reactions. They also found one strain contained both types of rDNA, however, it was PCR-negative for the psychrotophic *cspA*. The authors also reported that there was no correlation between cytotoxicity and growth temperatures and concluded that there are psychrotrophic *B. cereus* strains which cannot be classified as *B. weihenstephanensis*.

***Bacillus mycoides* and *pseudomycoides*:**

Bacillus mycoides and *pseudomycoides* are ubiquitous in soil and water and toxigenic strains can be found within both species. Both species grow as white to cream opaque colonies that show rhizoid growth on solid media. The rhizoid filament interferes in plate counting and makes it difficult to obtain a pure culture. There are aerobic, spore-forming, non motile rods, 1µm wide and 3-5µm long. The optimum temperature to growth is 28-30ºC (Mantynen and Lindstrom 1998). *B. mycoides* was classified as a species in 1886, reclassified in 1946 (Gibson and Gordon 1974, pp.529-550) as *B. cereus* var. *mycoides* and again reclassified in 1986 as *B. mycoides* (Claus and Berkeley 1986, pp.1105-1139).

Von Wintzingerode *et al.* (1997) after analysis of its 16*S* rDNA, defined an oligonucleotide probe specific for the *B. mycoides* strains. Dot blot hybridization allowed for rapid identification of this species from *B. cereus*, *B. thuringiensis* and *B. anthracis*. Hao *et al.* (2006) designed a quick identification of *B. mycoides* from *B. cereus* based on an optimized BioMérieux Vitek32 method. Thirty biochemical reactions could be done within 24 hours using BAC identification cards in the Vitek32 equipment.

B. pseudomycoides was first distinguished from *B. mycoides* by Nakamura in 1998 based on the gas chromatographic fatty acid methyl ester analysis. The species was

distinguished from *B. mycoides* by differences in 12:0 iso and 13:0 anteiso fatty acid levels. Without fatty acid analysis *B. pseudomycoides* cannot be distinguished from *B. mycoides* (Nakamura 1998).

***Bacillus* sp. Ba813$^+$, transitional:**

Patra *et al.* (1996) proposed a 277bp fragment (coding Ba813 marker) as useful for *B. anthracis* identification. This DNA sequence is specific for *B. anthracis* and is missing in other *Bacillus* species and other genus. It is a chromosomal marker helpful for identification avirulent *B. anthracis* strains pXO1$^-$/pXO2$^-$. However during their study some *B. anthracis* strains pXO1$^-$/pXO2$^-$ strains were found that shared other features with *B. cereus* or *B. thuringiensis*. Those strains were named transitional *Bacillus* sp. Ba813$^+$ and were included in the *B. cereus* group spp. These strains carry chromosomal marker Ba813 characteristic for *B. anthracis* strains, however, other features (hemolysis of blood agar, motility, penicillin resistance) are different than for *B. anthracis* (Ramisse *et al.* 1999).

1.2.2. *Bacillus cereus* toxins

There are two principal types of food poisoning caused by the *B. cereus* group spp. Bacterial growth results in production of toxins and ingestion leads to two types of illness: emetic and diarrhoeal syndrome (Borge *et al.* 2001; Dierick *et al.* 2005). Generally in both types of food poisoning, food is heat treated (cooking, pasteurizing) and surviving spores (the source of toxins) germinate and multiply when food is inadequately refrigerated.

The emetic type is caused by a highly stable peptide toxin that is performed in the food (cereulide or emetic toxin). The diarrhoeal type is caused by protein toxin (enterotoxins) which must be produced in the intestinal tract by vegetative growth of bacteria after ingestion of the microorganism.

Enterotoxins are heat-labile proteins causing abdominal pain and diarrhoea after incubation for 8-16 hours with duration time of illness 12-24 hours (Notermans and Batt 1998). The minimum infection dose is 10^5-10^7 bacterial cells (Granum and Lund 1997). Three types of enterotoxins involved in outbreaks have been identified. Two of them are three-component proteins, named hemolysin BL (HBL) (Lund and Granum 1997) and nonhemolytic toxin (NHE) (Lund and Granum 1996) while the last is a one-component

cytotoxin K (CytK) (Lund *et al.* 2000; Święcicka *et al.* 2006). Some bacterial strains can produce both three-component toxins while other bacteria only one (Lund and Granum 1997).

The emetic type is effected due to a preformed small cyclic peptide that is heat-stabile and causes vomiting a few hours after ingestion. This toxin is produced by growing cells in food and consist of the ring structure of three repeats of four amino and/or oxy acids: [D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$. This toxin is very apolar, heat-resistant and pH-resistant (Agata *et al.* 1994). Incubation time of emetic syndromes is 0.5-5 hours with duration 6-24 hours (Granum and Lund 1997). The infection dose for emetic type ranges between 10^5-10^8 bacterial cells/g of food. The amount of expressed cereulide generally depends on the incubation temperature and culture medium. The highest toxicity was reported in 10% skim milk media in compared to others (e.g. BHI) (Finlay *et al.* 1999). There are two known modes of action of cereulide: first when the toxin binds to the 5-HT_3 receptor and through this stimulate the nervus vagus afferent inducing a vomiting reflex (Agata *et al.* 1994); second, when cereulide is toxic to mitochondria by interfering with their metabolic action (Mikkola *et al.* 1999) and is involved in liver failure in humans (Mahler *et al.* 1997). Emetic toxin producers are usually isolated from: rice, noodles and pasta, but also from infant formulas and skim milk powder (Holmes *et al.* 1981; Shinagawa 1993a). The most commonly used technique to test the emetic toxin is HEp-2 vacuolation described by Hughes *et al.* (1988) with colorimetric modifications. The mitochondrial swelling caused by purified cereulide appears as cytoplasmic vacuoles in HEp-2 cells. Sperm-based bioassay is also a popular method for detection emetic toxin. The technique is based on loss of motility of boar sperm cells because of exposure to the *B. cereus* emetic toxin. Cereulide blocks the mitochondrial oxidative phosphorylation which in necessary for motility of boar spermatozoa (Andersson *et al.* 1998). Ehling-Schulz *et al.* (2004) first presented a PCR assay for rapid detection of *B. cereus* emetic toxin. They successfully used the primers targeting the DNA fragment of an unknown function which was specific for emetic toxin producing strains of *B. cereus*. Following this research, Fricker *et al.* (2007) designed the real-time PCR (RT-PCR) assay for the detection of emetic *B. cereus* strains in food. The assay targeted the *ces* gene encoding part of the cereulide synthetase.

For detection of enterotoxic *B. cereus* strains, PCR assays have been designed. Hansen and Hendriksen (2001) reported a PCR analysis for the detection of enterotoxin-

encoding genes in *B. cereus* and *B. thuringiensis*. They successfully used six sets of primers for detection of genes in NHE and HBL operons and primers for the detection of *bceT* gene encoding enterotoxin T. However, Prüβ *et al.* (1999) beside Southern blotting analysis used the PCR assay with primers targeting the *hblA* gene encoding the binding subunit B of HBL. The authors determined that HBL enterotoxin was broadly distributed among all species of *B. cereus* group.

There are also commercially available immunological assays: the BCET-RPLA *B. cereus* Enterotoxin Test Kit (Oxoid) based on reversed passive latex agglutination. The test is specific to the L2 component of the hemolysin BL complex. The Tecra BDE (*Bacillus* Diarrhoeal Enterotoxin) kit (Tecra Diagnostics) visual immunoassay which detects the *B. cereus* NheA subunit of NHE, using the double-sandwich enzyme technique.

1.2.3. *Bacillus cereus* in milk

Milk is a healthy food source for essential vitamins, proteins and minerals. The dairy sector plays an important role for Ireland with 617,000 tonnes of drinking milk and buttermilk used for consumption in 2009 (Anonymous 2010).

Almost all viable bacteria present in milk are killed by pasteurization (Valik *et al.* 2003). This process relies on the principle that most bacteria can be killed by heat. There are three different types of milk pasteurization (Smith 1981):

- High Temperature Short Time Treatment (HTST)-where milk for 15 seconds is treated with 72ºC.
- Low Temperature Long Time Treatment (LTLT)-treating the milk for 30 minutes at 63ºC.
- Ultra High Temperature Pasteurization (UHT)-heating milk to 138-150ºC for 1-2 seconds.

B. cereus is an important spore-forming bacteria, because its spores are ubiquitous in raw milk, survive the pasteurization process and produce enterotoxins causing food poisoning (Granum *et al.* 1993). *B. cereus* spores in milk germinate more effectively than any other bacilli spores (Wilkinson and Davies 1973). Germination of spores before the pasteurization process is reduced but increases after pasteurization. It increases their chances for surviving, growth and milk spoilage during storage. Spores surviving the pasteurization step and growing at 4-7ºC are called psychrotrophs and can

cause the milk to "go off". Organisms associated with defects such as: sweet curdling, bitty cream and off-flavours caused by produced proteinase, lipase and phospholipase enzymes decreasing the shelf-life of the product (Meer *et al.* 1991). Wilkinson and Davies (1973) found that the best heat activation temperature for those strains was 90-94ºC for 15 seconds.

Moreover, the *B. cereus* spores are hydrophobic (Koshikawa *et al.* 1989), and they can adhere to the stainless steel surfaces of equipment of dairy industry (Andersson *et al.* 1995). Attachment of the spores increases their heat resistance (Simmonds *et al.* 2003) and they may germinate, multiply, form biofilms and resporulate (Andersson *et al.* 1995). Those key features were investigated within the Microbial Functionality and Safety Research program of the Wageningen Centre for Food Science (WCFS) including mechanisms of biofilm formation (De Vries *et al.* 2004b). *B. cereus* can contaminate the milk from soil, water and from pumps processing equipment and "milk-stone" residue on bulk tanks. Because *B. cereus* spores are always present in the farm environment (soil, faeces), it is impossible to avoid the presence of *B. cereus* in raw milk.

B. cereus is also of particular concern in the infant formula industry (Becker *et al.* 1994; Reyes *et al.* 2007). For these foods microbial control is achieved by ensuring a low initial level of the bacteria in the product. It can be achieved by using well-designed equipment with effective cleaning methods to prevent biofilm formation. EU legislation on the Microbiological Criteria for Foodstuff (Commision Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005) established microbiological criteria between 50-500 CFU/g for powdered infant (below 6 months of age) formula and formula for special medical purposes (Kyprianou 2007).

Two international norms are recommended for *B. cereus* isolation and enumeration in food: ISO 7932:2004 (ISO 2004) and ISO 21871:2006 (ISO 2006). Apart from *B. cereus*, many different Gram-positive and Gram-negative bacteria have been reported in milk processing lines. Gram-positive microorganisms include: *B. subtilis*, *B. coagulans*, *B. licheniformis*, *B. circulans*, *B. pumilus* (Cosentino *et al.* 1997), *Listeria monocytogenes*, and *Staphylococcus aureus*. Whereas Gram-negative: *Campylobacter jejuni*, *Escherichia coli*, *Salmonella* Typhimurium, and *Yersinia enterocolitica* (Skanderby *et al.* 2009, pp.180-234) have also been reported.

To improve the milk shelf life and food safety, the industry developed different preservation methods to control the microbial milk spoilage. The current industry trends

such as extended refrigerator storage time of raw milk and extended storage of pasteurised milk have served to enhance the importance of thermoduric psychrotrophs, like *B. cereus* group spp. (Christiansson *et al.* 1999; Zhou *et al.* 2008).

1.2.4. *Bacillus cereus* spores

The *B. cereus* group spp. are sporeformers which form highly resistant particles inside the cells, called endospores during sporulation process. It enables bacterium to survive extreme conditions: moist and dry heat, UV radiation, chemical solvents (formaldehyde, nitrous acid) (Setlow 2005) ionizing radiation, mechanical abrasion, antibiotics, pH extremes (De Vries 2006). Each spore is formed with the following components: exosporium, coat, outer membrane, cortex, inner membrane and core (Gerhardt and Ribi 1964; Kozuka and Tochikubo 1985; De Vries 2006). The coat protects the core from UV radiation (Wilson and Russel 1964) the membranes from high pressure (100-200MPa) and low water content in the core makes the spore heat resistant (Stein and Rogers 1945).

Spores of *Bacillus* are present in many foods from harvest through processing. It causes significant problems in the food industry, as it allows them to survive food processing and conservation methods. Spore germination is essential to the proliferation of sporeformers, and their spoilage and poisoning of food. Germination is the process of actively growing and dividing cells together with losing the resistance capacities. Procedures exploiting spore germination may involve exposure to strong germinative compounds, followed by inactivation of germinated spores by a mild heat treatment (De Vries 2006). Spores are equipped with special proteins-sensors to choose the time of germination. Sensors are located at the inner membrane, called germinant or nutrient receptors and recognize conditions suitable for growth. Germination can be enhanced by heat treatment and factors such as nutrients (sugars, amino acids) or physical factors such as high pressure (100MPa-800MPa) (Aertsen *et al.* 2005; Keynan and Evenchik 1969, pp.359-396; Paidhungat and Setlow 2000; Wuytack *et al.* 2000).

The time required to destroy the spores depends on: the strain, temperature and type of food. As Mikolajcik and Koka (1968) reported, heat-treated milk may create the environmental conditions affecting spore germination, outgrowth and proliferation of vegetative cells. They analyzed that the time required for all those processes at 35ºC was 124 minutes for heat-shocked spores in pasteurized milk to 355 minutes for

unheated spores in raw milk. Xu and Côté (2006) described the thermal inactivation of *B. anthracis* spores in cow's milk. They determined that the decimal reduction time to inactivate 90% of spores in milk ranged from 3.4 to 16.7 hours at 72ºC and from 1.6 to 3.3 seconds at 112ºC. Wilkinson and Davies (1973) mentioned that *B. cereus* spores in pasteurized milk usually germinated more than in any other source. However, the nature of the changes occurring in milk on heating and affecting the suitability as germination medium was not clear.

Kramer and Gilbert (1989, pp.22-70) reported the thermal destruction of spores in skim milk to be 100ºC for 2.7-3.1 minutes. However, in rice it was 22-36 minutes. Azanza and Centeno (2004) evaluated the efficiency of boiling on inactivation of *B. cereus* spores in rice. The authors found that heating at 100ºC for 5 minutes could reduce 90% of the spores. The results were obtained by plating of germinated spores, followed by incubation and colony counting. In the first stage of germination, the dipicolinic acid (DPA) was excreted from the spore and water taken up. It caused loss of spore refractility and decreases of absorbence (De Vries 2006; De Vries *et al.* 2005). De Vries *et al.* (2004a) in the real-time quantification of spore germination of *B. cereus* involved fluorescence-based flow cytometry (FCM). They analyzed thousands of spores in germinating suspension and determined the kinetics of the response to the germinants. The use of different fluorescent dyes in combination with FCM allowed for quantification of various specific stadiums of germination. Kuo *et al.* (2006) described the plasma effects on the spores in a wet environment. An arc-seed microwave plasma torch was applied for studying the effects of atomic oxygen on *B. cereus* spores in solution. The results showed that the plasma effluent can penetrate into the water and kill the spores; the kill time was 10 seconds at an exposure distance of 3cm, 24 seconds at 4cm and 31 seconds at 5cm.

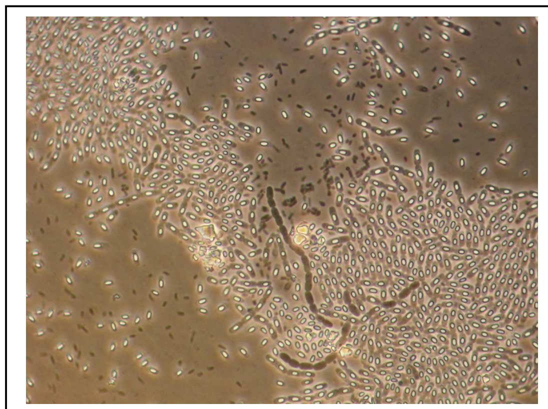

Figure 1. Sporulating cells of *B. cereus* NCTC 7464

Spores are visible as refractile bodies inside the mother cells. Phase contrast microscope model, magnification 1000x (this study)

1.3. Identification of the *Bacillus cereus* group species

Identification and differentiation of the *B. cereus* group spp. can be performed by traditional microbiological assays. This bacterial group consist of six, closely related species which can be distinguished based on phenotypic differences.

Criteria for differentiation the members of the *B. cereus* group are listed in Table 1. Based on properties such as: motility, blood hemolysis, rhizoid growth, penicillin resistance/susceptibility, growth in a psychrotrophic conditions and toxin crystal production.

However, traditional, conventional laboratory techniques are time consuming, require multistep analysis and pre-enrichment of the sample. Furthermore, phenotypic properties may not always be expressed or may be difficult to interpret. Therefore techniques based on molecular biology have advantages. Genome analysis provides better characterizing of the *B. cereus* group spp., it allows for more detailed analysis on bacterial similarities and differences. Hybridization and amplification are one of the most important and fundamental techniques based on the double helix structure of the DNA and complementary base pairing which is the heart of DNA technology (Drlica 2004; Junhui *et al.* 1997).

Table 1. Characteristics of the *B. cereus* group species

	Motility	Hemolysis	Rhizoid growth	Penicillin resistance/ susceptibility	Crystal formation	Growth below 7ºC
B. cereus	+	+	-	R	-	-
B. thuringiensis	+	+	-	R	+	-
B. anthracis	-	-	-	S	-	-
B. mycoides	-	+	+	R	-	-
B. pseudomycoides	-	+	+	R	-	-
B. weihenstephanensis	+	+	-	R	-	+

1.3.1. Traditional techniques for the *B. cereus* group spp. identification

Conventional methods for the identification of *B. cereus* group spp. consist of several selective plating methods, microscopic analysis of cell morphology and biochemical tests. All species hydrolyze starch and reduce nitrates, are mannitol negative and usually egg yolk lecithinase positive. They grow in the presence of lysozyme and fail to produce acid from D-mannitol (Stadhouders 1992). One of the most popular media for testing the *B. cereus* group spp. is PEMBA (polymyxin egg yolk mannitol bromothyl blue) agar which is a selective media developed by Holbrook and Anderson in 1980 for the isolation and enumeration of *B. cereus* in food. The primary diagnostic features of the medium are: colonial appearance, egg yolk hydrolysis resulting in a precipitation zone around colonies and the failure to utilise mannitol (to production acids from mannitol). Polymyxin B was added in order to inhibit Gram-negative organisms. *B. cereus* is unable to metabolise mannitol which is the only carbon source in PEMBA medium. Addition of a pH indicator (bromothyl blue) allows distinguishing between strain which can and cannot metabolise mannitol. Metabolisation of mannitol causes a decrease the pH-value and the colour changes to yellow. *B. cereus* will ferment peptides, proteins, and amino acids instead of mannitol thus the pH will increase and the colour turns blue (Holbrook and Anderson 1980). The second media is MYP (mannitol egg yolk polymyxin) agar developed by Mossel and

Koopman (1967). This plating media differs from PEMBA in base composition but is based on the same detection principles: characteristic colony appearance, precipitation of hydrolysed lecithin and the failure to utilise mannitol. Phenol red is added as a pH indicator. PEMBA together with MYP are recommended agars for the identification and enumeration of *B. cereus* colonies by food authorities such as the International Organization for Standardization (ISO 7932/2004) (ISO 2004) or the Food and Drug Administration (FDA) (Rhodehamel and Harmon 1998).

Traditional biochemical tests for identification and differentiation of the *B. cereus* group spp. are standardized and described in the FDA Bacteriological Analytical Manual and include: test for nitrate reduction, hemolytic activity, protein toxin crystals, Voges-Proskauer reaction (production of acetyl methyl-carbinol), lysozyme resistance (growth in presence 0.001% lysozyme), test on MYP agar (Rhodehamel and Harmon 1998).

One of the most popular, less labour-intensive biochemical test is the BioMérieux API commercial system. It is a standardized and miniaturized version of biochemical techniques. BioMérieux recommends the API 50 CHB test kit for identification of the *B. cereus* group spp. The API 50 CHB strips, used in conjunction with API 50 CHB/E Medium, are biochemical tests used to study fermentation of different substrates. Strips enable the analysis of the metabolism of 49 carbohydrates and are adapted to identification the *B. cereus* group spp. in 48 hours. During incubation carbohydrates are fermented to acids which decrease the pH. This change is detected by pH indicators that change the colour of the used medium. Achieved results make up the bacterial biochemical profile which is analyzed by apiweb™ SOFTWARE V4.0 Version: 1.2.1 (BioMérieux® SA 69280 Marcy l'Etoile/France). This technique was originally developed by Logan and Berkley (1984). The BioMérieux Vitek2, fluorescence-based, microbiology system is a commercial automated, BioMérieux identification system utilizing growth-based technology (Stager and Davis 1992). The system accommodates a colorimetric reagent BCL card which is incubated and automatically interpreted. The BCL reagent card is specific for identification Gram-positive spore-forming bacilli. The BCL card has wells that each contain an individual test substrate; it is based on biochemical methods and newly developed substrates (Pincus 2006, pp.1-32). 46 tests are measured: carbon source utilization, enzymatic activities, resistance to antibiotics (oleandomycin, kanamycin, polymyxin B) and inhibition of growth by 6.5% NaCl. All results are read, based on the growth pattern in the wells, using a computer system. With almost 100% correct identifications of the most commonly isolated species (*B.*

cereus, *B. subtilis*, *B. licheniformis*, *B. pumilus*) and 93% correct identification overall, the BioMérieux Vitek2 system represents an advance in the identification of aerobic endosporeformers (Halket *et al.* 2010).

1.3.2. Gas chromatographic fatty acid methyl ester (FAME) analysis

Bacterial fatty acid methyl ester (FAME) profiles are unique from one species to another, which allows for the creation of microbial FAME libraries. The idea that fatty acids analysis can be useful in identification of bacteria was presented by Abel *et al.* (1963). However, the first results of fatty acid (FA) analysis of the genus *Bacillus* was obtained by Kämpfer (1994). Fatty acid content of bacterial species is highly conserved, its contents is also consistent for particular strains. Therefore the FAME analysis should be performed for every taxon separately. FA can be differentiated based on their chain length, position of double bonds and binding of functional groups. Gram-negative bacteria are composed of straight chains, whereas in Gram-positive branched chain (iso and anteiso acids) are common (Dawyndt *et al.* 2006). Gas chromatographic (GC) FAME analyses are now an automated identification tool routinely used in some microbiology laboratories. The most popular system for microbiological identification based on GC of extracted FAMEs is called MIDI Sherlock Microbial Identification System (MIS, Microbial ID, Inc. (MIDI), Newark, Delaware, USA). The National Institute for Occupational Safety and Health (NIOSH) has validated the MIDI System for the identification of aerobic bacteria (Pendergrass 1998). Moreover, the Association of Official Analytical Chemists (AOAC) approved the technique for *B. anthracis* identification (AOAC Method #2004.11) (Kunitsky *et al.* 2006, pp.1-18). The MIDI research laboratory has found more than 300 fatty acids and related compounds in the bacterial cell membranes (Gluodenis and Kutnitsky 2004). Since 2007, when a new sample preparation technique (Instant FAME^{TM}) was applied, rapid identification of aerobes was possible in 15 minutes from pure culture.

Lawrence *et al.* (1991) published the results on differentiation *B. cereus* and *B. anthracis* by GC whole-cell FA analysis. The authors found significant differences between the fatty acid patterns of those two species. Similarly, Whittaker *et al.* (2005) reported about two unique branched chains fatty acid for *B. anthracis* and *B. cereus*. Analysis allowed for differentiation of those two species based on their vegetative cell and spores using GC of FAME. Adams *et al.* (2005) published the results of cellular FA

analysis of four commercial preparations of *B. thuringiensis* strains var. *kurstaki*. They demonstrated the capability to detect the strain variation in the species due to differentiate strain variants. However, the authors concluded that these analyses identified only commercial products and more study was required to evaluate cellular FA analysis of various *B. thuringiensis* isolates. Nakamura and Jackson (1995) based on differences in fatty acid profiles were able to distinguish the *B. mycoides* species from *B. cereus*. In 1998, Nakamura proposed the new species *B. pseudomycoides* which should be included in the *B. cereus* group spp. This species phenotypically is the same as *B. mycoides* however, FA analysis showed significant differences in acid composition (Nakamura 1998). To date, *B. pseudomycoides* cannot be distinguished from *B. mycoides* without FAME analysis (Luna *et al.* 2007).

1.3.3. Molecular techniques for the *B. cereus* group spp. identification

Alternative approaches for detection and identification of the *B. cereus* group spp. are DNA amplification and hybridization techniques, which include the popular amplification of unique DNA techniques (PCR, RT-PCR) as well as blotting assays. Hybridization and amplification are the most important and fundamental techniques in this area. The techniques which are at the heart of DNA technology are based on the double helix and complementary base pairing structure of DNA (Drlica 2004; Junhui *et al.* 1997). Only when nucleotides are base-paired, the double stranded molecule is formed (Watson and Crick 1953a,b)

Analysis of DNA sequence is the most precise tool for microbial identification. DNA contains more etiologically specific information about evolution than the traditionally used phenotypic techniques. It is also precisely defined and is relatively simple to determine. There are two important details when using DNA for bacterial identification: nucleotide sequences must contain sufficient sequence variation and must be universal in its distribution (Chang *et al.* 2003).

The Polymerase Chain Reaction (PCR):

Developed in 1983 by Kary Mullis, amplification, commonly known as a polymerase chain reaction (PCR) is a widely used DNA technique (Mullis and Faloona 1987, pp.189-204). This molecular assay enables the production of many copies of a specific DNA region between two known flanking base pair sequences (primers)

(Fozdar *et al.* 2006, pp.141-162). Hybridization events are also crucial in this assay. At an optimized temperature two primers hybridize to the complementary fragment of DNA. To amplify the DNA fragments, a heat-stable enzyme (*Taq* polymerase) isolated from *Thermus aquaticus*, thermophilic bacteria that live in hot springs, is required (Leonard *et al.* 2003). Polymerase always initiates amplification at characteristic points on single stranded DNA (ssDNA) template that are defined by primers (Drlica 2004). To achieve the ssDNA the double stranded DNA (dsDNA) is heated at 94-96ºC to denature, and separate into two single strands.

Primers which are polynucleotide fragments of ssDNA hybridize (annealing step) only to complementary ssDNA template at defined temperature (50-60ºC). Following the annealing of primers, the polymerase elongates the new strand by adding in the reaction mix nucleotides complementary to base pairs of template DNA at the temperature 70-72ºC.

PCR was the first nucleic-based assay for detecting pathogens (Leonard *et al.* 2003; Palchetti and Mascini 2008). This reaction allows for rapid and specific detection of a wide range of bacterial species and it is a key procedure for microorganism detection (Carrino and Lee 1995; McPherson and Møller 2006).

The genomes of the *B. cereus* group spp. are closely related and attempts to date to design unique DNA primers allowing differentiation has proved difficult. PCR was able to detect significant differences between the species which was not possible by modern approaches, such as pulsed-field gel electrophoresis (PFGE), and multilocus enzyme electrophoresis (Von Wintzingerode *et al.* 1996). Hansen *et al.* (2001) designed the PCR assay for detection of the *B. cereus* group spp. based on primers targeting the 16*S* rDNA. The PCR analyses were performed with DNA from a number of non-*B. cereus* group bacilli, confirming the specificity of the assay. Group specific PCR assay was also described by Schraft and Griffiths (1995), they designed three primers against the cereolysin AB sequences for detection of egg yolk-hydrolysing *Bacillus* sp. Results demonstrated the high specificity of designed primers for isolates of *B. cereus* group spp. Tsen *et al.* (2000) also designed primers specific for amplification of *B. cereus* group spp. B16S1 and B16S2 primers targeted the 16*S* rRNA for the *B. cereus* group. This assay was successful in detecting all tested *B. cereus*, *B. anthracis*, *B. thuringiensis* and *B. mycoides* strains.

A PCR assay to differentiate the species of the *B. cereus* group was reported by Yamada *et al.* (1999). The authors used a molecular diagnostic marker against the gyrase B gene

(*gyrB*) encoding the subunit B protein of DNA gyrase. Three primer sets were able to amplify and differentiate between *B. cereus*, *B. thuringiensis* and *B. anthracis*.
B. thuringiensis can be discriminated from the other members of the *B. cereus* group by the PCR targeting the *cry* genes. Over 100 *cry* genes sequences encode the endotoxin characteristic only for *B. thuringiensis*. Cerón *et al.* (1995) designed specific primers for amplification conserved regions and identification *cryI* and *cryIII* genes. Bravo *et al.* (1998) characterized the Mexican *B. thuringiensis* strain collection by multiplex PCR. This study included analysis with specific primers which could detect *cry1*, *3*, *5*, *7-9*, *11-14*, *21*. A PCR assay was designed by Aly (2007) which used a PCR assay for the detection of 5 different *cry* (*cry1Aa*, *cry1Ac*, *cry1I*, *cry2*, *cry3*) genes in local *B. thuringiensis* isolates. All authors were able to successfully detect *B. thuringiensis* strains. However, some results (Cerón *et al.* 1995) did not provide information about the insecticidal properties of newly identified *cry* genes. One PCR assay could not distinguish between expressed and silent genes (Bravo *et al.* 1998). The *cry* gene list is still increasing and new primers are needed to identify recently described genes.
Differentiation of *B. anthracis* is generally based on amplification of genes located on two virulence plasmids: pXO1 and pXO2. The only disadvantage is the inability to detect plasmid-free strains. The ability to distinguish *B. anthracis* is important for epidemiological purposes and quality control of vaccine seed cultures (pXO1^{+}/pXO2^{-}) (Henderson *et al.* 1994).

PCR is sensitive, specific and a very useful tool for detecting small amount of bacterial DNA in a sample (Leonard *et al.* 2003; Palchetti and Mascini 2008), but can be limited by problems with the sensitivity of polymerase to environmental contaminants, difficulties in quantification or contamination of the samples.

Real-time PCR:

Real-time PCR (RT-PCR), also known as kinetic PCR, quantitative PCR (qPCR) is a quantitative assay for the determination of gene (template) copy number. qRT-PCR also offers an alternative method for detection of bacterial contamination in food. However, unlike conventional PCR, RT-PCR does not require post-reaction sample handling and use of ethidium bromide, a carcinogen agent although safer stain have been developed, e.g. SYBR®Safe. Moreover, detection of the amplicon during the early phases of the reaction results in increased sensitivity of the RT-PCR over PCR

(Priha *et al.* 2004). This method is performed in a closed tube system which prevents the potential for PCR contamination in the laboratory (Heid *et al.* 1996).
In the reaction mix, different chemistries allow for detection of the PCR product via the generation of a fluorescent signal: SYBR Green or sequence specific labelled probes. SYBR Green is a fluorogenic dye intercalating into the double-stranded PCR product (Mackay 2004). This dye is very useful in detecting low concentration of double-stranded DNA, and exhibits fluorescence enhancement upon DNA binding and has high affinity for DNA (Rengarajan *et al.* 2002). The limitation associated with most of the intercalating dyes is their toxicity and cariogenicity properties. Labelled probes offer a major advantage over intercalating dyes: greater specificity because primer-dimer and non-specific PCR products are not detected (Heid *et al.* 1996). Oligonucleotide hybridization probes (i.e. Molecular Beacons, Scorpions) and hydrolysis TaqMan probes give the fluorescence signal only in the presence of the target DNA. The detection of those probes is based on Fluorescence Resonance Energy Transfer (FRET). It is a distance-dependent interaction event between two dye molecules occurring when a donor fluorophore and an acceptor chromophore (quencher) are in close proximity. In every technique a fluorescent signal is generated when a fluorophore (attached to one end of ssDNA) is in a certain distance from the quencher (attached to opposite end of ssDNA). When these two chromophores are in proximity, the characteristic emission spectrum-generated by the donor, is transferred to the acceptor which returns the fluorophore to its ground state without emission. However, when they are separated, the donor is able to emit fluorescence uninhibited (Cady and Batt 2006, pp.413-425; Epstein *et al.* 2002).

During work with SYBR green, analysis of the melting (dissociation) curve is necessary. It ensures that no other dsDNA, such as: primer-dimer or contaminating DNA is included in the analysis. The melting curve presents a sudden decrease in fluorescence, and occurs when 50% of dsDNA dissociate to ssDNA after enriching the melting point (Tm) and release of the SYBR green. This temperature depends on G+C content, length of DNA fragments, and sequence order. Single peak indicate a single product.

For the real-time PCR assay, sensitivity refers to the number of copies of a target sequence that must be present in a sample in order to positively identify the presence of the pathogen (Johnson *et al.* 2005). The quantification arises by measuring the amount of amplified product at each stage during the PCR cycle. The establishment of a

standard curve using the quantitative real-time PCR (qRT-PCR) process is the key step in determining the copy number of a given target sequence. qRT-PCR technology relies on the ability to monitor fluorescence emitted from specific double-stranded DNA binding dyes or fluorophore-labelled probes that hybridize with target sequences during the exponential phase of the PCR reaction such that quantification is accomplished (Dorak 2006). In the amplification reaction, the crossing point (Cp) in the PCR cycle is the point where the fluorescence of a sample exceeds the background and a significant increase in fluorescence is observed (Higuchi *et al.* 1993; Kontatis and Reed 2006). Cp value corresponds to PCR product accumulation, thus it is correlated with the starting template amount. A lower Cp value implies a higher starting quantity of the nucleic acid target. The standard curve method produces a linear plot of the log of the initial copy number for a set of standards versus Cp value (McPherson and Møller 2006). Quantitation of the target amount in an unknown sample can be estimated by generating a standard curve (Gentle *et al.* 2001; Song *et al.* 2002). The Cp of unknown genes is measured, and the standard curve is used to determine their starting copy number.
A perfect amplification reaction should produce a standard curve with an efficiency of "2", because the amount of target DNA should double with each cycle. Efficiency (E) is an indication of how well the reaction has proceeded (Dorak 2006) and is calculated from the slope of the linear regression line (Kontatis and Reed 2006; Pfaffl 2004, pp.87-112; Rasmussen 2001, pp.21–34):

$$E=10^{[-1/slope]}$$

RT-PCR is a newer technique than PCR, therefore there are limited publications on *B. cereus* analysis. Bell *et al.* (2002) developed the RT-PCR detection assay for *B. anthracis*. Primers and probes have been designed targeting the *pagA* gene on pXO1 plasmid and *capB* gene on pXO2 plasmid. The assay was specific for *B. anthracis* and the detection limit was 1 copy number. Kim *et al.* (2005) reported the multiplex RT-PCR technique for the genotypic detection of the *B. cereus* group and *B. anthracis* based on melting curve analysis. Reactions included 3 sets of primers targeting genes: *sspE* encoding chromosomal sequence motif found within a spore structural gene, *lef* of the pXO1 plasmid, and *capC* of pXO2 plasmid. Efficient amplification of all target genes and significant differences between amplicon's Tm's were necessary for successful melting curves analysis. It allowed for rapid detection *B. anthracis* and *B. cereus* group. RT-PCR assay for *B. anthracis* detection was also described by Antwerpen *et al.* (2008). The authors designed specific primers and TaqMan probe

targeting the chromosomal marker BA_5345 that allowed for efficient differentiation of *B. anthracis* from other *B. cereus* group spp. The limit of detection was 12.7 copies per reaction. To detect emetic *B. cereus* strains in food, Fricker *et al.* (2007) developed a diagnostic RT-PCR assay based on unique primers and TaqMan probe. The assay targeted a highly specific part of the cereulide synthetase gene (*ces*). The TaqMan assay was able to detect 10 CFU/g emetic *B. cereus* in food after 4-6 hours enrichment time.

***The motB* gene as a target to design PCR and RT-PCR for detection *B. cereus* group spp.:**

The most common motility mechanism used by bacteria is the flagellum protein structure attached to the bacteria surface. The arrangement of flagella on the bacterial surface varies between organisms. There can be many different variations, such as peritrichous (or lateral), tufted polar (2-6 flagella) or single polar flagella (Winstanley and Morgan 1997). *B. cereus* is motile due to its peritrichous flagella and consist of three major domain: filament, hook and basal body (Xu and Côté 2006). The filament is powered by a protonmotive force (chemiosmotic potential) (Larsen *et al.* 1974) and rotated by a motor in the plasma membrane. The filament length is 5-10µm, and is a part of the flagellar apparatus that performs hydronamic work. Each flagellum has a conserved N- and C-terminal region, and a central domain which may vary in the size and amino acid sequence.

The central region is build from a large number of subunits encoded by *flaA, flaB* and *fliC* genes which form a helical structure and have different functions, this region is polymorphic and highly diverse (Farfán *et al.* 2009; Kondoh and Hotani 1974).

Rasko *et al.* (2004) reported different numbers of flagellin subunits present in different *B. cereus* group spp. strains. *B. anthracis* contained only one gene whereas *B. cereus* ATCC 14579 four and *B. cereus* ATCC 10987 two. It was possible because different flagellin were expressed under different conditions resulting in structurally, functionally variable flagella. Lövgren *et al.* (1993) and Ankarloo *et al.* (1996) described two different but highly homologous flagellin forms (encoded by *flaA* and *flaB*) incorporated into the flagella of *B. thuringiensis* isolates.

Domainal structure of flagellin genes are good fragments for PCR amplification. Many PCR assays for identification of particular bacteria using flagellin genes are described in the literature. Way *et al.* (1993) designed a multiplex PCR to detect environmental isolates *Salmonella* sp. primers targeted the *H-li* region of the H1 flagellin gene (*fliC*

which is called *flaA* or *flaB* in other species), region flanked by *hin* gene (encoding an invertase enzyme) and H2 flagellin gene (*fliB*). *Listeria* sp. have also been identified based on PCR using primers targeting its *flaA* gene (Gray and Kroll 1995). Oyofo and Rollins (1993) and Rasmussen *et al.* (1996) reported a PCR assay able to detect *Campylobacter coli* and *Campylobacter jejuni* using primers specific for *flaA* and/or *flaB* genes. Detection was possible with a high sensitivity and specificity.

The hook section of the flagellum is a flexible structure between the filament and basal body and is made of about 120 copies of a protein molecule FlgE. Its flexibility permits to transmit torque from the rotor (Samatey *et al.* 2004, pp.1062-1068).

The basal body anchors the flagellum to the bacterial cell membrane and wall and is build from the rings (Patenge *et al.* 2001). It is a bacterial motor which consists of rotor and stator. Two rings of the rotor: M and S reside in the cell membrane and are surrounded by integral cytoplasmic membrane proteins: MotA and MotB (Chun and Parkinson 1988; Kojima and Blair 2004) which form the stator affixed to the peptidoglycan. MotA/MotB complex acts as a proton pathway (ion channel) across the cytoplasmic membrane and couples proton flow with torque generation. MotB is essential for rotation and proton production (Kojima *et al.* 2008) and has a highly conserved peptidoglycan-binding motif in its C-terminal periplasmic domain (Kojima *et al.* 2009; Sowa and Berry 2008). This motif is well conserved among outer membrane proteins such as OmpA, Pal and MotY (Kojima *et al.* 2008). The periplasmic region (residues 149-269 in 309 amino acids fragment) of MotB showed sequence similarity to other OmpA-like proteins (Kojima *et al.* 2009), therefore MotB is classified as an outer membrane protein OmpA.

Outer membrane proteins are located at the surface of the cell of Gram-negative bacteria. Their main role is to provide integrity to the membrane by ensuring physical linkage between the outer membrane and the underlying peptidoglycan layer. Moreover they play a role in bacterial conjugation (Skurray and Reeves 1974). OmpA shows homology to the peptidoglycan domain of MotB. The *ompA* genes were found to be useful for species identification. Nair and Venkitanarayanan (2006) developed the PCR assay based on the region *ompA* gene unique for *Enterobacter sakazakii* (now *Cronobacter sakazakii*). Two specific primers amplified 469bp product and the limit of detection was 10^3 CFU/ml in infant formula. Stevens *et al.* (2010) designed and evaluated a quantitative real-time PCR targeting the *ompA* gene for detection of *Chlamydia trachomatis*. Cell culture isolates from 15 prototypic *C. trachomatis*

serovars were successfully identified. The authors applied the assay to test five hundred clinical specimens. Kidd *et al.* (2008) reported of conventional and real-time PCR assays for detection and differentiation of the spotted fever group *Rickettsia* in dog blood. Specific primers targeted a small region of the *ompA* gene and the limit of detection was 15-30 copies of DNA for traditional PCR and 5 copies for RT-PCR. The species of infecting *Rickettsia* were identified, concluding that the above assays could be used to differentiate the species.

MotB gene was found to be highly conserved within the *B. cereus* group spp., therefore it was chosen in this study. The flagellin gene was targeted to design unique and specific primers and probes for detection, identification and differentiation of the *B. cereus* group spp. Newly designed DNA fragments were checked by molecular techniques including: PCR, RT-PCR and blotting techniques (dot blot).

Hybridization technique – dot blotting:

Blotting of nucleic acids is a general technique for hybridization studies for detection of specific DNA sequences. DNA is immobilized on nitrocellulose or nylon membranes and incubated with a labelled probe (single stranded) to detect the hybridization sites with the target. There are different analysis types used i.e. Southern blot, dot blot and slot blot. Southern blotting is the transfer of DNA fragments (enzymatically digested) from an electrophoresis gel to the membrane. After immobilization the labelled nucleic acid probe is allowed to hybridize to the target on the membrane. Dot and slot blots differ only in the geometry of the blot. Dot and slot blotting are simple techniques for immobilizing bulk unfractionated target DNA on the membrane. Hybridization analysis with a labelled probe can be carried out to determine the relative abundance of target (Oto *et al.* 1995; Pividori *et al.* 2001).

The probe is a nucleic acid molecule (ssDNA) with an affinity to specific target (DNA). The probe binds to complementary DNA only, by base-pairing rules (Brown 1993a,b). There are two different types of probes: gene probes (about 500 bases and comprise all or most of the target gene) and oligonucleotide probes (18-50 targeting a specific fragment within gene). The probes can be labelled with radioactive and nonradioactive labels. Radioactive labelling includes isotopes as: ^{32}P, ^{35}S, ^{125}I, ^{3}H and detection is based on autoradiography. Nonradioactively labels are as follow (De Muro 2005, pp.13-23):

- biotin detected with avidin or streptavidin followed by chemiluminescent reaction with peroxidase,
- enzymes, such as horseradish peroxidise detected by reaction with a substrate that changes colour,
- chemiluminescent chemicals detected by their light emission,
- fluorescence chemicals which fluoresce under UV light
- digoxigenin (DIG), the most effective system for labelling; anti-DIG antibody linked with alkaline phosphatase is detected with colorimetric or chemiluminescent alkaline phosphatase substrates (e.g. CSDP).

The nucleic acid probes are widely used in detection of microorganisms. Detection of unique target sequences allows for identification of pathogens in food, specimens or confirms a disease. Oligonucleotide probes targeting small/large ribosomal subunits and internal transcribed spacer regions have been developed for microorganisms, such as: *Borrelia* sp., *Clostridium* sp., *Campylobacter* sp., *Candida* sp., *Helicobacter* sp., *Streptococcus* sp., and *Rickettsia* sp. (Ward *et al.* 1992, pp.219-286). Von Wintzingerode *et al.* (1997) designed the 16*S* rDNA oligonucleotide probe specific for identification *B. mycoides* strains. The specificity of the probe was analyzed by dot blot hybridization with PCR products. An assay allowed for rapid identification *B. mycoides* strains in a large group of isolates. Giffel *et al.* (1997) based on Southern blot hybridization and oligonucleotide probe targeting the variable region V1 of 16*S* rRNA were able to discriminate *B. cereus* and *B. thuringiensis* strains. Probes hybridized to PCR products were specific for those species and allowed for their differentiation.

In blotting techniques, the specificity of the probe is extremely important. If over 5% of the base pairs are not complementary, the probe will hybridize loosely to the target sequence. This causes the probe to be washed out during the washing steps (Childs 2000, pp.131-141). Therefore it is very important to design target specific probe and optimize the hybridization assay. Blotting also allows for the detection of interesting DNA sequence without false positive and/or negative results.

1.3.4. Standardization and validation PCR-based techniques

In designing a new diagnostic PCR/RT-PCR for the detection of foodborne pathogens the food industry requires and expects standardization and validation of those techniques. Standardization aims at development of single standard PCR protocol.
A standardized PCR-based assay for the detection of foodborne pathogens should satisfy the following criteria (Malorny *et al.* 2003):

1. *Analytical and diagnostic accuracy*: false-negative and/or false-positive results should be low. A high degree of diagnostic accuracy is to precisely detect the target microorganism in the presence of a biological matrix without interference from nontarget components.
2. *The detection limit*: for pre-enriched samples the detection limit should be 10^3-10^4 cells per ml of sample.
3. *High robustness*: the method should be tolerant to chemical and physical parameters as: quality of a template DNA, purity of reagents, pippeting errors, accuracy of PCR temperatures, and duration time.
4. *Amplification controls*: in each reaction, positive and negative (reagent control) controls should be included.
5. *Low carry-over contamination risk*: to minimize the risk, separated working areas, decreasing of pippeting steps and filtered tips should be used.
6. *Flexibility with respect to various sample matrices*: sample preparation techniques should be uncomplicated and not-time consuming.
7. *Acceptance by end-user*.
8. *Other requirement*: simplicity of the method and rapidity.

Validation of a standardized assay demonstrates that the new method generates results which are comparable and aims to confirm the specificity and reproducibility when used by different laboratories. Validation can be accepted by international organizations, such as: the Association of American Chemists (AOAC), European Committee for Standardization (CEN) or International Organization for Standardization (ISO).

1.4. Biosensors

A biosensor is an analytical device that detects, transmits and records information about a biochemical change (Kintzios 2006, pp.75-89). It has two main building elements: a biorecognition layer and a transducer. The biorecognition layer is generally biological based, which consist of biological materials needed for biomolecular recognition. Different materials have been used as recognition elements: antibodies, antigens, receptors, enzymes, cell and nucleic acids (Dominguez and Acros 2006, pp.1-22; Rivas and Pedano 2006). The recognition layer is also responsible for the generation of the bio-chemical signal. The signal is generated when the sensor layer and test analyte interact. A nucleic acid recognition layer, in comparison with enzyme or antibodies, can be readily synthesized and regenerated for multiple use (Wang 2000). The transducer is responsible for transforming the generated signal after the biological interaction. The response may be an electrical signal that is amplified and converted into a measurable response (Dominguez and Acros 2006). The intensity of the signal produced is usually proportional to the concentration of the analyte to be detected (Fung *et al.* 2006, pp.451-461). This signal can be further amplified, processed or stored for later analysis (Junhui *et al.* 1997; Velusamy *et al.* 2010). Transducers used can measure the signal by: optical, piezoelectrical, mass based or electrochemical methods. Optical transduction was widely used in the past because fluorescence demonstrated inherent high sensitivity; however this technique requires instrumentation and numerical algorithms for the interpretation of the results. In current devices, electrochemical transduction is the most popular because of their high sensitivity, simple design and portability (Rivas and Pedano 2006).

The biorecognition layer, if using enzymes results in a biocatalytic reaction, while affinity-based biosensors are devices which use DNA sequence, a receptor protein or an antibody and measures chemical binding events (Rivas and Pedano 2006; Rogers and Mulchandani 1998).

Biosensor technology will in time replace the traditional, conventional laboratory techniques which are labour intensive and time consuming, require multistep analysis and pre-enrichment of the sample in the case of microbial detection. PCR technology will also be replaced as it requires specialized equipment and personnel training. The miniaturizing of analytic sensors allows for lap-on-chip and point-of-care feasibility.

1.5. DNA based biosensors

A basic DNA biosensor is designed by immobilizing a single strand of nucleic acid on a special transducer. The recognition of complementary molecule is based on hybridization (Kerman *et al.* 2004). High specificity of nucleic acid base pairings between homologous strands of ssDNA is the basic principle for DNA biosensors (Figure 2).

The hybridization relies on specific complementarity between the “DNA-probe” which is a specific sequence of single-stranded DNA and the “DNA-target” – the sequence of the sample to be detected by the sensor developer (Epstein *et al.* 2002).

The strength of oligo immobilization and probe orientation play an important role in the performance of a DNA biosensor (Wang 2000). It is necessary to consider the physical-chemical properties of the analyte and the nature of the immobilized bio-element. The immobilization methods depend on the type of transducer and their application which include:

a) adsorption (non-covalent binding) – simple adsorption of the biocomponent due to the hydrophobic interactions or Van Der Waals forces onto carbon, silica gel, alumina or cellulose electrodes (Labuda *et al.* 2006, pp.201-227; Wang 2000),

b) use of thiol-labeled DNA for self-assembly onto gold transducer (covalent binding; gold electrodes or gold-coated piezoelectric crystals),

c) covalent linkage to the gold surface via functional alkanethiol-based monolayers (Wang 2000),

d) use of biotylated DNA for complex formation with a surface-confined avidin/streptavidin (Wang 2000). Avidin can be attached directly to a carbon surface (Marrazza *et al.* 1999).

Sensitivity (to detect the lowest quantity of DNA) and selectivity (to detect point mutation) are important issues in the development of DNA biosensors (Rivas and Pedano 2006).

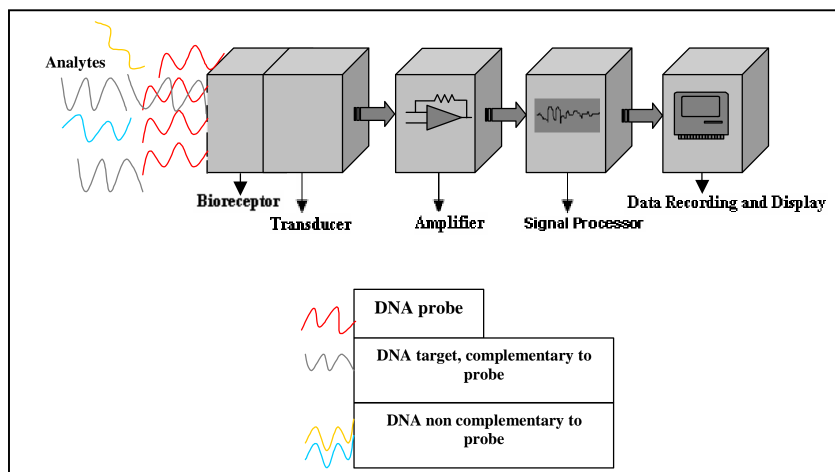

Figure 2. Schematic diagram of a DNA biosensor

The real advance of DNA technology can be observed in the detection and identification of foodborne pathogens. The need for a rapid and specific detection of foodborne microorganisms leads to the development of sensitive biosensors for on-site detection of pathogens. Most of the biosensors for bacterial detection use nucleic acid as a biorecognition element attached to a transducer (Muhammad-Tahir and Alocilja 2004). The main advantages of the use of biosensors in the food safety is the short time of analysis, low cost, but there is still a deficiency of portable, hand-hold systems (Palchetti and Mascini 2008). Publications describing the detection of pathogenic bacteria using DNA based biosensors are limited in number.

1.6. Bioinformatics

Bioinformatics is a new area of science that uses computational approaches to answer biological questions. It helps in the design of experimental work in the laboratory. Bioinformatics incorporates expertise from biology, computer science, and mathematics. Growing sequence databases allow for analysing viral, bacterial and eukaryotic organisms.

The National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) maintains one of the world's most comprehensive ranges of molecular databases and is one of the most popular websites. It allowed for creating

public databases, developing software tools for analysis the genomes, and understanding of molecular processing. Tools in NCBI included analysis of data (i.e. BLAST) (Altschul *et al.* 1990), genetic sequences (in GenBank) (Benson *et al.* 2009), index of research articles (PubMed) and other information necessary in biotechnology.
In Europe there are additional centres e.g. European Molecular Biology Laboratory (EMBL) (Williams 1997) established in 1974. The European Bioinformatics Institute (EBI) is an academic organisation and part of EMBL, called EMBL-EBI. It collects and disseminates biological data as: EMBL-Bank (DNA and RNA sequences), UniProt (protein sequences), PDBe (macromolecular structures) and many others.
Another very helpful database providing access to different databases and molecular analytical tools is ExPASY. Generally the ExPASY server includes protein identification and analysis tools (Appel *et al.* 1994; Gasteiger *et al.* 2003). However, some analytical tools, such as Lalign, ClustalW can also be applied in DNA analysis.
All described Bioinformatic Centres provide analytical tools and are irreplaceable in laboratory work. Most experiments can be designed and analyzed on site using virtual programmes and softwares, decreasing possible errors also save time and money.

The aim of this study

1. The phenotypic characterization of 138 strains belonging to the *B. cereus* group spp. and 28 other *Bacillus* and non-*Bacillus* strains, based on traditional microbiological techniques.
2. Molecular characterization of the *B. cereus* group spp. by PCR, multiplex PCR, and RT-PCR. It includes searching for unique sequences for this group and designing specific primers and TaqMan probes.
3. Estimation the limit of detection of designed PCR/RT-PCR methods and calculation of standard curves for RT-PCR assay. It allows for estimation the numbers of bacteria in unknown sample.
4. Spiking of fat and nonfat milk with known amount (CFU) of bacteria belonging to the *B. cereus* group spp., to estimate the efficiency of commercial kits [Genomic mini AX food (A&A Biotechnology) and DNeasy Blood & Tissue Kit (Qiagen)] for genomic DNA extraction.
5. Contamination of fat and nonfat milk with known amount (CFU) of bacilli spores to elucidate the best method to release the DNA for test and quantification by designed assays.
6. Designing a specific probe to be applied in develop a biosensor able to detect the *B. cereus* group spp. in milk. Probes and their specific target hybridization were optimized by existing techniques i.e. blotting technique.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Suppliers

Amersham Bioscience – now GE Healthcare.

A&A Biotechnology, Gdynia, Poland.

BD Difco, Le Pont de Claix, France.

Biolab Inc., Budapest, Hungary.

Bioline, The Edge Business Centre, Humber Rd., London, NW2 6EW, UK.

BioMérieux SA, Marcy-l'Ètoile, France.

Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK.

Fisher Scientific Ireland, Ballycoolin, Dublin, Ireland.

GE Healthcare, Little Chalfont, Buckinghamshire HP7 9NA, UK.

Invitrogen Life Technologies Ltd., Inchinnan Business Park, Paisley, UK.

Fluka, Sigma-Aldrich Ireland Ltd., Dublin, Ireland.

Macherey-Nagel, Neumann-Neander, Düren, Germany.

Millipore Ltd., Watford, Hertfordshire, UK.

MWG Biotech AG, Fraunhoferstr, Martinsried, Germany.

New England Biolabs Ltd, Hitchin, Hertfordshire, UK.

OptiCult Plates-Cruinn Diagnostics Ltd., Dublin, Ireland.

Oxoid, Cambridge, UK.

Promega, Southampton Science Park, UK.

Qiagen Ltd, Fleming Way, Crawley, West Sussex , UK.

Roche Diagnostics Ltd., Burgess Hill, West Sussex, UK.

Sigma Aldrich Ltd, Airton Rd., Tallaght, Dublin 24, Ireland.

TIB Molbiol, Eresburgstrasse, Germany.

TSC Ltd., Lancashire, England, UK.

2.1.2. Media and Supplements

Media and supplements were purchased from:

BD Difco (Le Pont de Claix, France)

Media:

Nutrient broth, per litre: beef extract 3.0g, peptone 5.0g.

Nutrient agar, per litre: beef extract 3.0g, peptone 5.0g, agar 15.0g.

Luria-Bertani (LB) broth, per litre: tryptone 10.0g, yeast extract 5.0g, sodium chloride 10.0g.

Luria-Bertani (LB) agar, per litre: tryptone 10.0g, yeast extract 5.0g, sodium chloride 10.0g, agar 15.0g.

Plate count agar, per litre: pancreatic digest of casein 5.0g, yeast extract 2.5g, dextrose, 1.0g, agar 15.0g.

Starch agar, per litre: beef extract 3.0g, peptone 5.0g, agar 15.0g, soluble starch agar 10g

Oxoid (Cambridge, UK)

Media:

Brain heart infusion broth, per litre: calf infusion solids 12.0g, beef heart infusion solids 5.0g, proteose peptone 10.0g, sodium chloride 5.0g, glucose 2.0g, di-sodium phosphate 2.5g.

Brain heart infusion agar, per litre: calf infusion solids 12.0g, beef heart infusion solids 5.0g, proteose peptone 10.0g, sodium chloride 5.0g, glucose 2.0g, di-sodium phosphate 2.5g, agar 10.0g.

***Bacillus cereus* agar base**, per litre: peptone 1.0g, mannitol 10.0g, sodium chloride 2.0g, magnesium sulphate 0.1g, di-sodium hydrogen phosphate 2.5g, potassium dihydrogen phosphate 0.25g, sodium pyruvate 10.0g, bromothyl blue 0.12g, agar 15.0g.

MYP agar base, per litre: meat extract 1.0g, peptone 10.0g, mannitol 10.0g, sodium chloride 10.0g, phenol red 0.025g, agar 12.0g.

MRS agar, per litre: peptone 10.0g, ‘Lab-Lemco’ powder 8.0g, yeast extract 4.0g, glucose 20.0g, sorbitan mono-oleate 1ml,dipotassium hydrogen phosphate 2.0g, sodium acetate $3H_20$ 5.0g, triammonium citrate 2.0g, magnesium sulphate $7H_20$ 0.2g, manganese sulphate $4H_20$ 0.05g, agar 10.0g.

MRS broth, per litre: peptone 10.0g, 'Lab-Lemco' powder 8.0g, yeast extract 4.0g, glucose 20.0g, sorbitan mono-oleate 1ml,dipotassium hydrogen phosphate 2.0g, sodium acetate $3H_20$ 5.0g, triammonium citrate 2.0g, magnesium sulphate $7H_20$ 0.2g, manganese sulphate $4H_20$ 0.05g

Supplements for media:

Sterile egg yolk emulsion, supplement for *B. cereus* selective agar base. 25ml were mixed with 475ml sterile and cooled to 50ºC agar supplemented with polymyxin B.

Polymyxin B (50.000 IU/vial), the contents of 1 vial (2ml) and was aseptically added to the 500ml of *B. cereus* selective agar base enriched with egg yolk emulsion.

Powder media were dissolved in distilled water and sterilised at 121ºC, 15lbs, for 15 minutes, unless otherwise stated.

Penicillin G disks, 10IU

Fluka, Sigma-Aldrich Ireland Ltd. (Dublin, Ireland)

Nitrate broth, per litre: peptone 5.0g, meat extract, 3.0g, potassium nitrate 1.0g.

OptiCult (Dublin, Ireland)

Columbia blood agar plates contain Nutrient agar and 5% sheep blood.

Biolab Inc. (Budapest, Hungary)

Mueller Hinton agar, per litre: 19.5g peptone, 1.5g starch, 17.0g agar.

Media prepared in the laboratory

ICPM medium, per litre: peptone 6.0g, glucose 5.0g, calcium carbonate ($CaCO_3$) 1.0g, magnesium sulfate ($MgSO_4$) 0.5g, potassium phophate (KH_2PO_4) pH 7.0 0.5g, erythromycin 250µg.

BioMérieux SA (Marcy-l'Ètoile, France)

Media:

API 50 CHB/E medium: ammonium sulphate 2.0g, yeast extract 0.5g, tryptone 1.0g, phenol red 0.18g, mineral base (Cohen-Bazire) 10ml, phosphate buffer pH 7.8 1000ml.

Supplements:

NIT 1 (w/v): sulfanilic acid 0.4g, acetic acid 30g, H_2O 70ml.

NIT 2 (w/v): N,N-dimethyl-1-naphthylamine 0.6g, acetic acid 30g, H_2O 70ml.
Both supplements used in test for reduction of nitrates to nitrogen.

2.1.3. General buffers and reagents

The recipes for solutions were obtained from Sambrook and Russel (2001) and/or Ausubel (1997) unless otherwise indicated. If necessary, solutions were autoclaved at 121˚C, 15lbs, for 15 minutes and stored at room temperature or in the fridge (4ºC). Chemicals were purchased from Sigma-Aldrich Ireland Ltd. (Dublin, Ireland), Oxoid (Cambridge, UK) and Fisher Scientific Ireland (Ballycoolin, Dublin, Ireland).

Agarose gel-loading buffer: 0.25% (w/v) bromophenol blue, 0.25% (w/v) Xylene-Cyanol FF, 30% (w/v) glycerol solution. The solution was filter-sterilized using a 0.22µm Millipore filter.

Ampicillin: 100mg/ml stock was prepared with distilled water and sterilized by filtration (0.22µm Millipore filter). Stored at -20ºC but avoided repeated thaw/freeze.

Crystal violet (Hucker's): solution was prepared by dissolving 2.0g crystal violet (90% dye content) in 20ml ethanol (95%); 0.8g ammonium oxalate was dissolved in 80ml distilled water and two solutions were mixed together.

Ethanol, molecular grade: absolute (99%); other concentrations: 96%, 70%, 50%, 20%, 10% were made by dilution of absolute ethanol with distilled water.

Ethidium bromide, 10mg/ml: 0.1g of ethidium bromide was added to the 10ml of distilled water and stirred to complete dissolving of dye using a magnetic stirrer.
Caution: this chemical is carcinogen.

EDTA (ethylenediaminetetraacetic acid), 0.5M, pH 8.0: 186.1g of disodium EDTA-$2H_2O$ was added to 800ml of distilled water and stirred vigorously on a magnetic stirrer. The pH was adjusted with solid NaOH. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0.

Isopropanol: Propan-2-ol 99.5%.

Lugol's iodine solution: 1g of iodine crystals and 2g of potassium iodine were dissolved in 80ml distilled water and maked up to 300ml.

Maleic acid, 1M: 11.6g of maleic acid was dissolved in 80ml of distilled water. The pH was adjusted to 7.5 and volume adjusted to 100ml.

Malachite green 5%: 5.0g of malachite green was dissolved in 100ml distilled water.

McFarland standards (Table 2): were made up according to Chapin and Lauderdale (2003, pp.729-748) by mixing together the specified amounts of 1% barium chloride ($BaCl_2$) and 1% sulfuric acid (H_2SO_4).

Table 2. McFarland standard

McFarland standard No.	1% $BaCl_2$ (ml)	1% H_2SO_4 (ml)	Corresponding cell density (10^8 CFU/ml)
0.5	0.5	99.5	1.5
1	1	99	3
2	2	98	6
3	3	97	9
4	4	96	12
5	5	95	15
6	6	94	18
7	7	93	21
8	8	92	24
9	9	91	27
10	10	90	30

Phenol:chlorophorm:isoamylalcohol, 25:24:1, saturated with 10mM Tris, pH 8.0, 1mM EDTA.

Ringer's solution, 1/4 strength: 1 tablet was dissolved in 500ml distilled water.

Safranin solution 0.25%: 0.25ml Safranin O was mixed with 10ml ethyl alcohol (95%) and adjusted to 100ml with distilled H_2O.

Saline solution, 0.9%: 0.9g of sodium chloride dissolved in 100ml of distilled water.

Sodium acetate, 3M, pH 5.2: 408.3g of sodium acetate-$3H_2O$ was dissolved in 800ml of distilled water and pH adjusted with glacial acetic acid. Made up to 1 litre with dH_2O.

SDS (Sodium dodecyl sulfate), 10%: 10g SDS was dissolved in 100ml of distilled water and heated to 60˚C to assist dilution.

20xSSC, pH 7.0: 175.5g sodium chloride, 88.2g sodium citrate dissolved in 800ml of distilled water and brought to 1 litre with dH_2O.

Tris-HCl, 1M, pH 8.0: 121g Tris base was dissolved in 800ml distilled H_2O and the pH was adjusted with concentrated HCl. The solution volume was adjusted to 1 litre with dH_2O.

Basic fuchsin (dye content >88%) used in test for crystal formation was purchased in Sigma Aldrich, Ireland.

For milk contamination the water **spore suspension of *B. thuringiensis* ATCC 29730** was used. The suspension was provided by BAG Health Care GmbH (Lich, Germany) at a concentration of 10^6 CFU/0.1ml.

Table 3. Solutions used in dot blotting

Solution	Composition/Preparation	Use
Washing buffer	0.1M Maleic acid, 0.15M NaCl; pH 7.5 (20ºC); 0.3% (V/V) Tween20	Removal of unbounded antibody
Maleic acid buffer	0.1M Maleic acid, 0.15M NaCl; adjust with NaOH (solid) to pH 7.5 (20ºC)	Dilution of blocking solution
Detection buffer	0.1M Tris-HCl, 0.1M NaCl; pH 9.5 (20ºC)	Adjustment of pH to 9.5
Denaturating solution	1.5M NaCl, 0.5M NaOH)	Denaturation the dsDNA
Neutralization buffer	1.5M NaCl, 0.5M Tris-HCl, 1mM EDTA; pH 7.2)	Neutralizing buffer
Post hybridization washing buffers	RI: 2xSSC, 0.1% SDS RII: 0.5xSSC, 0.1% SDS RIII: 0.1xSSC	Post hybridization washes

For developing the dot blot results, Kodak processing chemicals for autoradiography films were used (Sigma-Aldrich Ireland Ltd., Dublin, Ireland):

GBX/Developer and replenisher: 22ml diluted in 78ml of distilled water.

GBX/ Fixer and replenisher: 22ml diluted in 78ml of distilled water.

Amersham Hyper™ ECL High performance chemiluminescence film was purchased from GE Healthcare Lmt (Buckinghamshire, UK)

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Table 4. Molecular biology reagents

Reagent	Content/ Concentration	Supplier
Taq DNA Polymerase	5U/µl	Bioline (London, UK)
PCR buffer	10x	Bioline (London, UK)
Magnesium chloride	50mM	Bioline (London, UK)
dNTP, nucleotide mix	25mM of each dNTP	Bioline (London, UK)
Primers	100µM	MWG (Eurofins MWG Operon, Martinsried, Germany) and TIB Molbiol (Eresburgstrasse, Germany)
Agarose	1.0-2.0% (w/v)	Sigma-Aldrich Ireland Ltd. (Dublin, Ireland)
TaqMan probes	20µM	TIB Molbiol (Eresburgstrasse, Germany)
EcoRI restriction endonuclease	10U/µl	Roche Diagnostics Ltd. (Burgess Hill, West Sussex, UK)
Lysostaphin from *Staphylococcus staphylolyticus*	1U/µl (2mg/ml)	Sigma-Aldrich Ireland Ltd. (Dublin, Ireland)
Lysozyme from chicken egg white	100mg/ml	Sigma-Aldrich Ireland Ltd. (Dublin, Ireland)
Proteinase K from *Engyodontium album*	20mg/ml	Sigma-Aldrich Ireland Ltd. (Dublin, Ireland)
RNase, DNase-free	500µg/ml	Roche Diagnostics Ltd. (Burgess Hill, West Sussex, UK)
Quantitative DNA markers: HyperLadder I, IV, V	200 lanes	Bioline (London, UK)
Lambda phage DNA from *Escherichia coli* host strain	5 units	Sigma-Aldrich Ireland Ltd. (Dublin, Ireland)
Tween20 (endonuclease, exonuclease, Rnase free)		Sigma-Aldrich Ireland Ltd. (Dublin, Ireland)
DIG Easy Hyb Granules	6x100ml	Roche Diagnostics Ltd. (Burgess Hill, West Sussex, UK)

2.1.4. PCR and RT-PCR oligonucleotides and probes

All primers were synthesised and purified by HPLC at Eurofins MWG (Eresburgstrasse, Germany). TaqMan hydrolysis probes by TIB Molbiol (Eresburgstrasse, Germany).

The primers and probes used and designed in this study are presented in Table 5.

Table 5. Primers and probes used in this study

Primer/ Probe	**Sequence**	**Size**	**Target in amplified product**
BCFomp1	5'-ATCGCCTCGTTGGATGACGA-3'	20nt	1-20
BCRomp1	5'-CTGCATATCCTACCGCAGCTA-3'	21nt	575-555
BCFomp2	5'-CGCCTCGTTGGATGACG-3'	17nt	1-17
BCFomp3	5'-CGTTGGATGACGACTTTTACAG-3'	22nt	1-22
BCRomp2	5'-GATATACATTCACTTGACTAATACCG-3'	26nt	288-263
BCRomp3	5'-CATCTACTTGCTCCGTATCACTAAAC-3'	26nt	288-263
BpmF	5'-TAATTTAGGGGGGCATCTTTACTTTTC-3'	27nt	1-27
BpmF2	5'-GTACATCAATTCAATCATTCAATAGA-3'	26nt	87-112
BpmR	5'-TTTCTATACCCAAAACTTAGATATGCTCATG-3'	31nt	220-190
BpmR2	5'-CTATACCCAAAACTTAGATATGCTC-3'	25nt	217-193
S-S-Bc-200-a-S-18	5'-TCGAAATTGAAAGGCGGC-3'	18nt	1-18
S-S-Bc-470-a-S-18	5'-GGTGCCAGCTTATTCAAC-3'	18nt	288-271
BcF2	5'-CGAATTTGATAATGTGTGGATTC-3'	23nt	1-23
CSPU3	5'-CCCGGATCCGGTTACGTTA(G/C)C(A/T)GCT-3'	25nt	171-147
BC1	5'-ATTGGTGACACCGATCAAACA-3'	21nt	1-21
BC2r	5'-TCATACGTATGGATGTTATTC-3'	21nt	365-345
BT1	5'-ATCGGTGATACAGATAAGACT-3'	21nt	1-21
BT2r	5'-CCTTCATACGTATGAATATTATTT-3'	24nt	368-345
R1	5'-TTAATTCACTTGCAACTGATGGG-3'	23nt	1-23
R2	5'-AACGATAGCTCCTACATTTGGAG-3'	23nt	152-130
MotB-FAM-1	FAM-TTCAAGCATCTTTGACAATTTTACTGCAT-BBQ	29nt	114-86
MotB-FAM-2	5'-FAM-TTCAAGCATCTTYGATAATTTTACTGTAT-BBQ-3' Y=CT	29nt	114-86
Bpm-FAM-1	5'-FAM-CTGAGAAGGTAGTCATACGCTATACATG-BBQ-3'	28nt	161-134
DIG-BCRomp2b	5'-DIG-TAAYGGTRTTAGTCAAGTGAATGTATATCGAGAGGATACAGGGG-3' Y=CT, R=A/G	44nt	No amplification
DIG-Bpm	5'-DIG-TAGCGTATGACTACCTTCTCAGCTTAATATATACCTA-3'	37nt	No amplification

2.1.5. Molecular weight markers

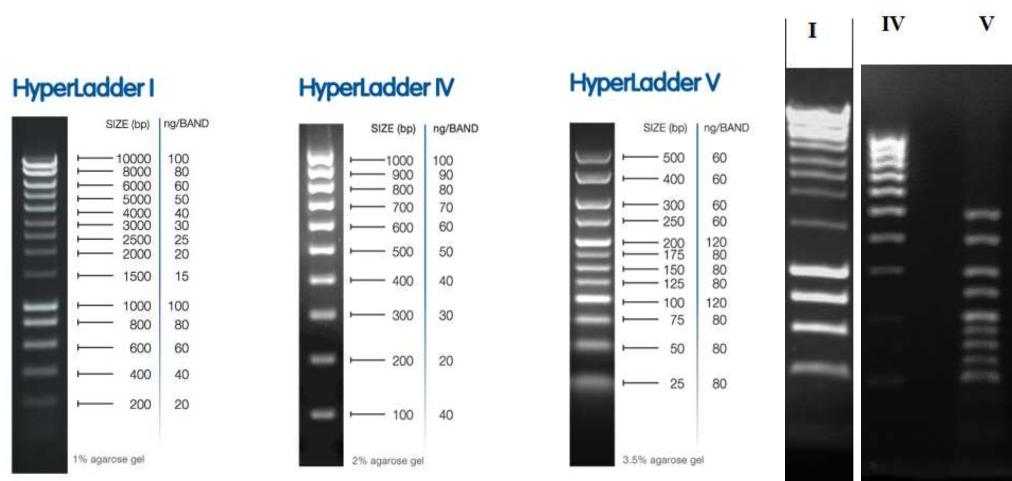
DNA molecular weight markers (Bioline) were as follows:

HyperLadder I produces 14 regularly spaced bands: 10.000, 8.000, 6.000, 5.000, 4.000, 3.000, 2.500, 2.000, 1.500, 1.000, 800, 600, 400, 200 base pairs.

HyperLadder IV produces 10 regularly spaced bands: 1.000, 900, 800, 700, 600, 500, 400, 300, 200, 100 base pairs.

HyperLadder V produces 12 regularly spaced bands: 500, 400, 300, 250, 200, 175, 150, 125, 100, 75, 50, 25 base pairs.

Each band corresponds to quantity of DNA. The ideal banding patterns are outlined in Figure 3a).



a) **b)**

Figure 3. Bioline quantitative DNA markers

a) shows the ideal pattern; b) shows the pattern observed with 1% (HyperLadder I) and 2% (HyperLadder IV and V) agarose gel in the laboratory

2.1.6. System used in bacterial cloning

To obtain the RT-PCR standard curves based on gene copy numbers, the PCR products were cloned into the commercial vector and multiplied in *E. coli* strain (Table 6).

Table 6. System used in bacterial cloning

pGEM-T Easy Vector System	Promega (Southampton, UK)
Bacterial strain *E. coli* JM109	Promega (Southampton, UK)

2.1.7. Commercial kits used in this study

A selection of commercial kits were used in experimental analysis and are outlined in Table 7:

Table 7. Commercial kits used in this study

Kit	Use	Supplier
Genomic mini	for universal genomic DNA isolation	A&A Biotechnology (Gdynia, Poland)
Isolate Genomic DNA mini Kit	for universal genomic DNA isolation	Bioline (London, UK)
Genomic mini AX food	for DNA isolation from food samples	A&A Biotechnology (Gdynia, Poland)
DNeasy Blood & Tissue Kit	for DNA isolation from food samples	Qiagen (West Sussex, UK)
Wizard Plus SV Minipreps	DNA purification system	Promega (Southampton, UK)
Nucleospin Extract II	for direct purification of PCR product (PCR clean-up, gel extraction)	Macherey-Nagel (Neumann-Neander, Düren, Germany)
LightCycler® TaqMan® Master	For PCR on LightCycler® using hydrolysis (TaqMan®) probes	Roche Diagnostics Ltd. (Burgess Hill, West Sussex, UK)
DIG luminescence detection kit	for detection of DIG-labelled nucleic acids by enzyme immunoassay	Roche Diagnostics Ltd. (Burgess Hill, West Sussex, UK)

Buffers and reagents supplied by **Genomic mini** for universal genomic DNA isolation (for 50 isolations):

Wash solution	50ml
Total lysis solution	12.5ml
Tris buffer, 10mM, pH 8.0	25ml
Proteinase K	1.1ml
Minicolumns	hold up to 1ml
Collecting tubes	hold up to 2ml

Buffers and reagents supplied by **Isolate Genomic DNA mini Kit** for universal genomic DNA isolation (for 10 isolations):

Lysis buffer D	5ml
Binding buffer D	2x2ml
Proteinase K	0.3ml
Wash buffer	6ml
Elution buffer	2x2ml
Spin column D	hold up to 1ml
Collection tube	hold up to 2ml

Buffers and reagents supplied by **Genomic mini AX Food** for universal genomic DNA isolation (for 60 isolations):

Wash solution	190ml
Lysis suspension	100ml
Elution solution	90ml
Equilibrating solution	55ml
Precipitation mix	55ml
Protease	1.3ml
Columns	hold up to 5ml
Precipitation tubes	hold up to 2ml

Buffers and reagents supplied by **DNeasy Blood & Tissue Kit** for DNA isolation from food samples (for 50 isolations):

Tissue lysis buffer	10ml
Lysis buffer	12ml

Wash buffer (1)	19ml
Wash buffer (2)	13ml
Elution buffer	22ml
Proteinase K	1.25ml
Columns	hold up to 1ml
Collecting tubes	hold up to 2ml

Buffers and reagents supplied by **Wizard Plus SV Minipreps** DNA purification system (for 50 isolations):

Cell resuspension solution	20ml
Cell lysis solution	20ml
Neutralization solution	30ml
Column wash solution	20ml
Alkaline protease solution	550µl
Nuclease free water	13ml
Wizard minicolumn	hold up to 1ml
Collecting tubes	hold up to 2ml

Buffers and reagents supplied by **Nucleospin Extract II** for direct purification of PCR product (for 50 preparations):

Binding buffer	30ml
Washing buffer	2x7ml
Tris buffer, 5mM, pH 8.5	25ml
Extract columns	hold up to 1ml
Collecting tubes	hold up to 2ml

Buffers and reagents supplied by **Lightcycler® TaqMan® Master** for PCR on LightCycler® using hydrolysis (TaqMan®) probes (for 480 reactions)

Enzyme	5x30µl
Reaction mix	15x118µl
Water, PCR grade	7x1ml

Buffers and reagents supplied by **DIG luminescence detection kit** for detection of DIG-labeled nucleic acids by enzyme immunoassay (for 50 blots of $100cm^2$):

Labeled control DNA 50µl
DNA dilution buffer 1ml
Anti-digoxigenin-AP
(Fab fragments) 100µl
Blocking reagent 2x50g
CSPD (Disodium 3-
(4-methoxy-spiro
{1,2-dixetane-3,2'-
(5'-chloro) tricycle
[3.3.1.$1^{3,7}$] decan}-4-yl)
phenyl phosphate) 1ml

2.1.8. Composition of the BioMérieux API 50 CHB test used for biochemical identification of the *B. cereus* group strains

Table 8. API 50 CHB test strip

Strip No. 1	Strip No. 2	Strip No. 3	Strip No. 4	Strip No. 5
0.Control 1.Glycerol 2.Erythritol 3.D-Arabinose 4.L- Arabinose 5.Ribose 6.D-Xylose 7.L-Xylose 8.Adonitol 9.ß-Methyl-D-Xyloside	10.Galactose 11.Glucose 12.Fructose 13.Mannose 14.Sorbose 15.Rhamnose 16.Dulcitol 17.Inositol 18.Mannitol 19.Sorbitol	20.α-Methyl- 20.D-Mannoside 21.α-Methyl-D-Glucoside 22.N-Acetyl-Glucosamine 23.Amygdalin 24.Arbutin 25.Esculin 26.Salicin 27.Celiobiose 28.Maltose 29.Lactose	30.Melibiose 31.Sucrose 32.Trehalose 33.Inulin 34.Melezitose 35.Raffinose 36.Starch 37.Glycogen 38.Xylitol 3.Gentibiose	40.Turanose 41.D-Lyxose 42.D-Tagatose 43.D-Fucose 44.L-Fucose 45.D-Arabitol 46.L-Arabitol 47.Gluconate 48.2-Keto-Gluconate 49.5-Keto-Gluconate

2.1.9. General laboratory equipment used in this study

Table 9. Laboratory equipment used in this study

Item	Model and/or Manufacturer
-20°C freezer unit	Whirlpool Italy
-85°C freezer unit	NUAIRE MN USA
Autoclave	OT 012 Nuve
Biofuge centrifuge	Heraeus
Block Heater	Stuart SBH130
Camera	Canon Power Shot A570 IS; Kodak digital science, electrophoresis documentation and analysis system, version 2.0.3. Scientific Imaging
Centrifuge	Heraeus Biofuge Stratos Centrifuge
Eppendorf pipettes	P10, P20,P200, P1000, Eppendorf
Exposure cassette	Kodak BioMax (8'x10'), Sigma-Aldrich
Gel Electrophoresis system	Mini-Plus, Horizon 58
Gel documentation System	Gene Genius Bio Imaging System (GelDoc), Syngene
Gilson pipettes	P10, P20, P100, P1000, Gilson Ltd
Incubator/Oven (hybridization/shaking)	Stuart SI30H
Labtop cooler	Nalgene
LightCycler®, Carousel-Based System	LightCycler®1.2, Roche
LightCycler®, Well Plate-Based System	LightCycler®480, Roche
Magnetic ministirrer	230V, 50Hz, Fisherbrand, Fisher Scientific
Magnetic stirrer hotplate	230V, 50Hz, 630W, Fisherbrand, Fisher Scientific
Microscope	Olympus system microscope model CX41
Microwave	Samsung TDS
Microcentrifuge	Hettich Mikro 120, ALC Inc.
Microcentrifuge, refrigerated	FRESCO 17, Thermo Scientific Heraeus
Miximatic vortex	Press-To-Mix 220V/60Hz, Snijders
Orbital Shaker Incubator	GFL Shaking incubator 3033
Oxoid Disk Dispenser	Oxoid
pH Meter	pH/mV, 230V, 50Hz, Hanna Instruments
Protect bacterial Preservers	Technical Service Consultants Ltd
Rocker	230V, 50Hz, 235mm x 235mm, Stuart
Scanning Electron Microscope	JEOL Carryscope JCM-5700
Shaker/Rocker	Stuart SSL4
Shaking incubator	Stuart SI500
Spectrophotometer UV-VIS	Spectronic Genesys 20
Spectrophotometer	NanoDrop-1000, Fisherbrand, Fisher Scientific
Sputter coater for SEM sample preparation	Emitech K550
Thermal cycler	Perkin Elmer GeneAmp 2400 Applied Biosystem

Thermal cycler	Techne TC-312, Techne Endurance
UV Crosslinker	HL-2000 HybriLinker, UVP Upland, CA 91786 USA
UV Lamp, Compact	UVGL-55, UVP,LLC
UV Transilluminator	UVP Benchtop 3UV^{TM}, Ultra Violet Products, Inc.
Water bath	Clifton, Bennett-Scientific

2.1.10. List of the bacterial strains

The list of bacterial strains used in this study are described in Table 10. They were obtained from the National Collection of Type Cultures (NCTC), London, UK; the American Type Culture Collection (ATCC, Middlesex, UK); Mid Western Regional Hospital, Limerick (MWRH), Ireland; the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany; the *Bacillus* Genetic Stock Center (BGSC), Ohio State University, USA;

Some strains, particularly described in Supplementary Table A and Table 10 , were kindly provided by: Dr. Stenfors Arnesen (Norwegian School of Veterinary Medicine, Oslo, Norway), Dr. A. H. Bishop, University of Greenwich, UK, Dr. Ehling-Schulz (Technische Universität München, Germany), Dr. Noura Raddadi (Milano, Italy), Dr. Kieran Jordan (Moorepark Food Research Centre, Cork, Ireland); all the *B. mycoides/pseudomycoides* strains were provided by Dr. I. Święcicka, University of Białystok, Poland; Bacteria with rhizoid growth classified as *B. mycoides/pseudomycoides* without further discrimination (Święcicka and Mahillon 2006).

B. anthracis strains were provided and tested in the laboratory with biosafety level 3 containment facilities in the Military Institute of Hygiene and Epidemiology, Puławy, Poland. *B. thuringiensis* and transitional strains namely *Bacillus* sp. Ba813^{+} were also tested. These strains carry chromosomal marker Ba813^{+} characteristic for *B. anthracis* strains however other features (i.e. motility, hemolyse of blood agar plates, penicillin resistance) are similar to other strains of the *B. cereus* group spp. and not to *B. anthracis*.

Table 10. List of *B. cereus* group strains used in this study

Bacterial strain	Species	Source	Collection Source
NCTC 7464/DSM 9378/ATCC 10876	***B. cereus***	Contaminated flask	NCTC/DSMZ
BCSUL1	***B. cereus***	Soil	UL laboratory strain
BCSUL2	***B. cereus***	Soil	UL laboratory strain
6A1	***B. cereus***	BGSC	BGSC
6A2	***B. cereus***	BGSC	BGSC
6A3	***B. cereus***	BGSC	BGSC
6A4	***B. cereus***	BGSC	BGSC
6A6	***B. cereus***	BGSC	BGSC
6A7	***B. cereus***	BGSC	BGSC
6A15/ATCC 10987	***B. cereus***	BGSC	BGSC
6E1	***B. cereus***	BGSC	BGSC
BMeSUL1	***B. cereus***	Soil	UL laboratory strain
BCFUL1	***B. cereus***	Food (curry powder)	UL laboratory strain
BCFUL2	***B. cereus***	Garlic mayonnaise	UL laboratory strain
BCFUL4	***B. cereus***	Food	UL laboratory strain
BCFUL6	***B. cereus***	Whipped cream	UL laboratory strain
BCFUL7	***B. cereus***	Food	UL laboratory strain
BCFUL8	***B. cereus***	Food	UL laboratory strain
BCFUL9	***B. cereus***	Sea food powder	UL laboratory strain
BCFUL10	***B. cereus***	Powder milk	UL laboratory strain
BCFUL11	***B. cereus***	Powder milk	UL laboratory strain
DSM 31/6A5/ATCC 14579	***B. cereus***	Type strain	DSMZ/BGSC
DSM 4312	***B. cereus***	Food poisoning incident	DSMZ
6A48/WSBC 10312	***B. cereus***	Kurkuma root	BGSC

6A51/ 4/1(m1278)	***B. cereus***	Dairy products/milk products	BGSC
ATCC 13472/6A10	***B. cereus***	N/A	ATCC
BCMUL1	***B. cereus***	Milk	This study
BCMUL2	***B. cereus***	Milk	This study
T7-019	***B. thuringiensis***	N/A	MIHE
T7-030	***B. thuringiensis***	N/A	MIHE
T7-055	***B. thuringiensis***	N/A	MIHE
T7-101	***B. thuringiensis***	N/A	MIHE
T07-001	***B. thuringiensis***	IEBC T07-001	MIHE
T07-005	***B. thuringiensis***	IEBC T07-005	MIHE
T07-113	***B. thuringiensis***	N/A	MIHE
T07-128	***B. thuringiensis***	N/A	MIHE
T07-146	***B. thuringiensis***	IEBC T07-146	MIHE
T07-148	***B. thuringiensis***	N/A	MIHE
T07-151	***B. thuringiensis***	N/A	MIHE
T07-153	***B. thuringiensis***	N/A	MIHE
T07-202	***B. thuringiensis***	IEBC T07-202	MIHE
H36 (T36 001)	***B. thuringiensis***	IEBC T36-001	MIHE
ATCC 33679	***B. thuringiensis***	Insect (diseased insect larvae)	MIHE
ATCC 35646	***B. thuringiensis***	Sewage Israel	MIHE
BTSUL7	***B. thuringiensis***	Soil	UL laboratory strain
BTFUL1	***B. thuringiensis***	Food	UL laboratory strain
DSM 2046/ATCC 10792	***B. thuringiensis*** (Berliner)	Mediterranean flour moth	MIHE
DSM 6029	***B. thuringiensis*** (Berliner)	Type strain	DSMZ
DSM 6032	***B. thuringiensis*** (Berliner)	Type strain	DSMZ
DSM 6017	***B. thuringiensis*** (Berliner)	*Spodoptera litura* on sweet potato	DSMZ
DSM 6025	***B. thuringiensis*** (Berliner)	*Cadra figulilella*	DSMZ
DSM 6094	***B. thuringiensis*** (Berliner)	Airborne contaminant isolated from a plate culture of *Bacillus thuringiensis*	DSMZ

DSM 6102	***B. thuringiensis*** (Berliner)	Diseased *Pectinophora gossypiella* in mass-rearing program at Brownsville, TX	DSMZ
DSM 6107	***B. thuringiensis*** (Berliner)	*Notodonta ancera* moth	DSMZ
BT1	***Bt* subsp. *israelensis***	Soil	Dr. A.H. Bishop
BT2	***Bt* subsp. *morrisoni***	Soil	Dr. A.H. Bishop
BT3	***Bt* subsp. *kurstaki***	Soil	Dr. A.H. Bishop
BMFUL1	***B. mycoides***	Raw milk	UL laboratory strain
BMSUL1	***B. mycoides***	Soil	UL laboratory strain
BMSUL2	***B. mycoides***	Soil	UL laboratory strain
6A11/ F95/1883	***B. mycoides***	N/A	BGSC
6A13/NRS 306/ATCC 6463	***B. mycoides***	N/A	BGSC
6A14/Gibson 71	***B. mycoides***	N/A	BGSC
6A20/ATCC 11986	***B. mycoides***	N/A	BGSC
6A12/ F96/3308	***B. mycoides***	N/A	BGSC
6A19/ATCC 31101	***B. mycoides***	Soil	BGSC
6A47/WSBC 10277	***B. mycoides***	Dairy product	BGSC
6A49/WSBC 10360	***B. mycoides***	N/A	BGSC
6A68	***B. mycoides***	Soil	BGSC
A81	***B. mycoides***	Soil	Dr. N Raddadi
Nov1	***B. mycoides***	Maize rhizosphere	Dr. N Raddadi
Nov2	***B. mycoides***	Maize rhizosphere	Dr. N Raddadi
BiF	***B. mycoides***	Soil	Dr. N Raddadi
BmF	***B. mycoides***	N/A	Dr. N Raddadi
DSM 307	***B. mycoides***	Soil	DSMZ
DSM 309	***B. mycoides***	Corn leaf	DSMZ
DSM 384	***B. mycoides***	Onion roots	DSMZ
Bm/Bpm PID 1/21	***B. mycoides /pseudomycoides***	Milk	Dr. I. Święcicka
Bm/Bpm PID 2/43	***B. mycoides/ pseudomycoides***	Milk	Dr. I. Święcicka
Bm/Bpm PID 3/2	***B. mycoides/ pseudomycoides***	Milk	Dr. I. Święcicka

Bm/Bpm GRD 1/17	***B. mycoides/ pseudomycoides***	Milk	Dr. I. Święcicka
Bm/Bpm GRD 2/71	***B. mycoides/ pseudomycoides***	Milk	Dr. I. Święcicka
Bm/Bpm 1/1	***B. mycoides/ pseudomycoides***	Sow bugs	Dr. I. Święcicka
Bm/Bpm 1/2	***B. mycoides/ pseudomycoides***	Sow bugs	Dr. I. Święcicka
Bm/Bpm 17/3	***B. mycoides/ pseudomycoides***	Sow bugs	Dr. I. Święcicka
Bm/Bpm 22/2	***B. mycoides /pseudomycoides***	Sow bugs	Dr. I. Święcicka
Bm/Bpm 29/2	***B. mycoides /pseudomycoides***	Sow bugs	Dr. I. Święcicka
WS 3118 [T]	***B. pseudomycoides***	Soil	Dr. M. Ehling-Schulz
WS 3119	***B. pseudomycoides***	Soil	Dr. M. Ehling-Schulz
WS 3120	***B. pseudomycoides***	Soil	Dr. M. Ehling-Schulz
DSM 12442	***B. pseudomycoides***	Soil	DSMZ
DSM 12443	***B. pseudomycoides***	N/A	DSMZ
B346	***B. pseudomycoides***	Soil	Dr. N Raddadi
B618	***B. pseudomycoides***	Soil	Dr. N Raddadi
A82	***B. pseudomycoides***	Soil	Dr. N Raddadi
TP1	***B. pseudomycoides***	Soil	Dr. N Raddadi
DA	***B. pseudomycoides***	Soil	Dr. N Raddadi
DSM 11821/WSBC 10204	***B. weihenstephanensis***	Pasteurised milk	DSMZ
WSBC 10389	***B. weihenstephanensis***	Pasteurised milk	Dr. L.P. Stenfors Arnesen
WSBC 10392	***B. weihenstephanensis***	Pasteurised milk	Dr. L.P. Stenfors Arnesen
WSBC 10405	***B. weihenstephanensis***	Pasteurised milk	Dr. L.P. Stenfors Arnesen
WSBC 10416	***B. weihenstephanensis***	Pasteurised milk	Dr. L.P. Stenfors Arnesen
WSBC 10201	***B. weihenstephanensis***	Pasteurised milk	Dr. L.P. Stenfors Arnesen
WSBC 10045	***B. weihenstephanensis***	Pasteurised milk	Dr. Monika Ehling-Schulz
WSBC 10206	***B. weihenstephanensis***	Pasteurised milk	Dr. Monika Ehling-Schulz
WSBC 10364/6A50	***B. weihenstephanensis***	Soil	Dr. Monika Ehling-Schulz
WSBC10202/6A46	***B. weihenstephanensis***	Pasteurised milk	BGSC

BWMUL1	***B. weihenstephanensis***	Pasteurised milk	This study
BWMUL2	***B. weihenstephanensis***	Pasteurised milk	This study
BWMUL3	***B. weihenstephanensis***	Pasteurised milk	This study
BWMUL4	***B. weihenstephanensis***	Pasteurised milk	This study
BWMUL5	***B. weihenstephanensis***	Raw milk	This study
BWMUL6	***B. weihenstephanensis***	Raw milk	This study
BWMUL7	***B. weihenstephanensis***	Raw milk	This study
Sterne 34F2	***B. anthracis***	Sterne vaccine	MIHE
211	***B. anthracis***	cow spleen	MIHE
1583	***B. anthracis***	N/A	MIHE
1584	***B. anthracis***	N/A	MIHE
#6 (I/2)	***Bacillus* sp. Ba813+**	Soil	MIHE
#7 (II/3)	***Bacillus* sp. Ba813+**	Soil	MIHE
#11 (9594/3)	***Bacillus* sp. Ba813+**	Station effluent	MIHE
#13 (PC1)	***Bacillus* sp. Ba813+**	N/A	MIHE
#15 (11614-2)	***Bacillus* sp. Ba813+**	N/A	MIHE
#16 (PJ572)	***Bacillus* sp. Ba813+**	N/A	MIHE
#17 (094)	***Bacillus* sp. Ba813+**	N/A	MIHE
#18 (T2 97-76)	***Bacillus* sp. Ba813+**	Soil	MIHE
#19 (T5 97-77)	***Bacillus* sp. Ba813+**	Soil	MIHE
#21 (T11 97-79)	***Bacillus* sp. Ba813+**	Soil	MIHE
#22 (BU-1B)	***Bacillus* sp. Ba813+**	N/A	MIHE
#23 (III-BL)	***Bacillus* sp. Ba813+**	N/A	MIHE
#24 (III-BS)	***Bacillus* sp. Ba813+**	N/A	MIHE
#25 (97-27)	***Bacillus* sp. Ba813+**	Human wound	MIHE
#28 (III)	***Bacillus* sp. Ba813+**	N/A	MIHE
#29 (IV)	***Bacillus* sp. Ba813+**	N/A	MIHE
#31	***Bacillus* sp. Ba813+**	N/A	MIHE
#3403	***Bacillus* sp. Ba813+**	N/A	MIHE
ZZ5	***Bacillus* sp. Ba813+**	Dental pulp of buried cow	MIHE
ZL2	***Bacillus* sp. Ba813+**	Soil under buried cow	MIHE
BSFUL1	***B. subtilis***	Food (cheese)	UL laboratory strain

BSFUL2	***B. subtilis***	Food (tuna sandwich)	UL laboratory strain
BCiFUL1	***B. circulans***	Food	UL laboratory strain
BPFUL1	***B. pumilus***	Food (chicken+ham)	UL laboratory strain
ATCC 12759	***B. licheniformis***	Type strain	MWRH
BLFUL1	***B. licheniformis***	Food (chicken+ham)	UL laboratory strain
BLMUL1	***B. licheniformis***	Milk	This study
10A6/FZB42	***B. amyloliquefaciens***	Plant-pathogen-infested soil	BGSC
BRFUL1	***Brevibacillus brevis***	Food	UL laboratory strain
LMFUL1	***Listeria monocytogenes***	Food	UL laboratory strain
YEFUL1	***Yersinia enterocolitica***	Food	UL laboratory strain
ATCC 29213	***Staphylococcus aureus***	Clinical isolate	MWRH
ATCC 25923	***Staphylococcus aureus***	Clinical isolate	MWRH
SAUL1	***Staphylococcus aureus***	Clinical isolate	UL laboratory strain
ULCV48	***Campylobacter jejuni***	Food	UL laboratory strain
ULCH2	***Campylobacter jejuni***	Food	UL laboratory strain
ULCV38	***Campylobacter jejuni***	Food	UL laboratory strain
ULCE1	***Campylobacter jejuni***	Food	UL laboratory strain
ULM001	***Ralstonia pickettii***	Laboratory purified water	UL laboratory strain
ULM003	***Ralstonia pickettii***	Laboratory purified water	UL laboratory strain
NCTC 11149/ATCC 27511	***Ralstonia pickettii***	Clinical isolate	NCTC
ATCC 25922	***Escherichia coli***	Clinical isolate	ATCC
NCTC 74	***Salmonella* Typhimurium**	N/A	NCTC
3054	***Lactococcus lactis***	N/A	Dr. Kieran Jordan
HP	***Lactococcus lactis***	N/A	Dr. Kieran Jordan
5378	***Lactobacillus acidophilus***	N/A	Dr. Kieran Jordan
DPC 6060	***Lactobacillus acidophilus***	N/A	Dr. Kieran Jordan

DPC 6059	***Lactobacillus casei***	N/A	Dr. Kieran Jordan

NCTC-National Collection of Type Cultures, London, UK; BGSC-*Bacillus* Genetic Stock Center; ATCC-American Type Collection Culture; DSMZ-German Collection of Microorganisms and Cell Cultures; WS-General Collection of Bacteria, Technische Universität München, Germany; WSBC-Research Collection of *Bacillus cereus* group, Technische Universität München, Germany; MWRH-Mid Western Regional Hospital, Limerick, Ireland; MIHE-Military Institute of Hygiene and Epidemiology, Puławy, Poland; N/A-Not Available.

2.2. Methods

2.2.1. Growth and different techniques used in the preparation and preservation of bacterial strains

B. cereus strains were growing overnight at 30-34ºC and stored frozen in 15%, 40% (v/v) glycerol and on treated beads in cryopreservation fluid.

a) 15% and 40% (v/v) glycerol bacterial stocks

Duplicate cultures in nutrient broth-glycerol (15% v/v) and LB-glycerol (40%) were prepared and stored at –20ºC, which served as a working stock, and at –85ºC.
Working cultures were prepared by dipping or scrapping (if frozen) with a sterile loop a small amount of bacterial culture and streaked for a single colony on solid media.

b) Protect^{TM} bacterial storage system using treated beads in cryopreservation fluid (TSC Ltd., Lancashire, England, UK)

The cryotube was aseptically inoculated with colonies from fresh culture to a density equivalent to McFarland 3 or 4 standards. Then the culture was mixed by inverting the tube to completely distribute the organism. With a sterile pipette the cryopreservative fluid was removed and the tube was stored at –85ºC or –20ºC as was demanded.
Organism was recovered by removing a single bead from the cryotube and dropped into a liquid medium or streaked over the surface of solid medium and incubated under suitable conditions.

2.2.2. Identification and characterization procedures of the *Bacillus cereus* group species

Gram stain:

The Gram stain was carried out by preparing a bacterial smear on a microbiology glass slide and fixed with heat. 1ml of crystal violet solution was poured on. Washed briefly in water and flooded with Lugol's iodine. After washing with water was decolourized with 95% ethanol and washed with tap water again. The bacterial smear was then counterstained with safranin and after washing with tap water, blot dried with bibulous paper. Results were observed under light microscope or phase contrast microscope using the oil immersion lens (Olympus system microscope model CX41; magnification 400-1000x).

Endospore stain by Schaeffer – Fulton's method modified by Ashby:

The Schaeffer – Fulton (1933) stain was a method to visualizate endospores with using malachite green and the vegetative cells with red safranin. The modification given by Ashby (1938) replaces the direct heating by steam heating.

Prepared bacterial smear on a microscope slide was allowed to air dry and fixed by heat. The smear was covered by malachite green and heat-fixed over the flame for few seconds to observe first steam. The slide was washed well with water, covered with safranin and left for 1 minute. After rinsed with water and dried the prepared slide was observed with the oil immersion lens at 1000x phase contrast microscope (Schaeffer and Fulton 1933).

For a simple spore stain a bacterial smear on a glass slide was mixed with malachite green and fixed over the flame to observe first steam. Washed with water and observed in microscope (1000x phase contrast microscope, Olympus system microscope model CX41).

Growth on PEMBA medium:

A single colony of each *B. cereus* isolate was streaked onto fresh PEMBA plates and incubated at 35ºC for 24 hours following with 24 hours at room temperature.

The primary diagnostic features of the medium are: colonial appearance, egg yolk hydrolysis resulting in a precipitation zone around colonies and the failure to utilise mannitol (to production acids from mannitol).

Growth on MYP medium:

A single colony of each *B. cereus* isolate was streaked onto fresh MYP plates and incubated at 35ºC for 24 hours following with 24 hours at room temperature.
The primary diagnostic features of the medium are: colonial appearance, egg yolk hydrolysis resulting in a precipitation zone around colonies and the failure to utilise mannitol (to production acids from mannitol).

Nitrate reduction test:

Nitrate reduction was examined by the cultivation of isolates in Nitrate broth (Fluka) and the addition of NIT 1 (sulfanilic acid, acetic acid) and NIT 2 (N, N-dimethyl-1-naphthylamine) after overnight incubation at 30°C-35°C (Blazevic and Ederer 1975).
5ml Nitrate broth was inoculated with loopful of culture and incubated 24 hours at 30°C-35°C. To test for nitrate, 2 drop of each: NIT 1 and NIT 2 were added. For *B. cereus* group spp. a pink colour should develop in 10 minutes which indicates that nitrate has been reduced to nitrite.

Starch hydrolysis:

To demonstrate the production of amylase and maltase enzymes by bacterium, a single colony of *B. cereus* isolate was streaked onto Starch agar plates contained 1% soluble starch and incubated for 24 hours at 30ºC. Lugol's iodine was then added to the surface of the agar. Positive starch colonies show yellow-gold/colourless zone around the growth.

Motility (hanging-drop) test:

To detect bacterial motility, a single colony of fresh *Bacillu*s culture (after overnight incubation on solid media) was smeared with a drop of distilled water on a clean microscope cavity slide (hanging-drop slide), covered with a cover slip and viewed with oil immersion lens at 1000x using phase contrast microscope (Olympus system microscope model CX41). If overnight bacteria incubation was performed in liquid media, the drop of bacteria was taken directly on clean cavity slide.

Blood hemolysis test:

The hemolysis test distinguish *B. anthracis* from other species belongs to the *B. cereus* group. To distinguish this species, a single colony of a bacilli strain from Nutrient agar (NA) plate was streaked on Columbia blood agar plates enriched with 5% sheep blood. Plates were incubated at 33ºC for 24 hours after which they were examined for hemolysis (Cappucino and Sherman 2001, pp.93-98).

Hemolytic positive strains were classified as produced beta (β) hemolysin and alpha (α) hemolysin. Strains presented no lysis of red blood cells resulting in no changes in the appearance of medium around colony are called gamma γ hemolytic strains.

Rhizoid growth test:

Rhizoid growth was examined by inoculating, using 3mm loop a bacteria strain onto solid media and incubated in and upright position at at 33ºC for 24 hours. Then left for 24-48 hours at room temperature.

The rhizoid growth test can distinguish *B. mycoides* and *B. pseudomycoides* from other *B. cereus* group spp.

Penicillin susceptibility test:

The test was performed using the National Committee for Clinical Laboratory Standards disk diffusion susceptibility assay. In the penicillin susceptibility test, a small paper disks containing a standard amount of antibiotic (10U of Penicillin G) were used.

A few colonies of the tested bacteria were inoculated into 5ml saline solution (0.9%) and the turbidity of suspension was adjusted to 0.5 McFarland standards. By using a sterile swab saline suspension was swabbed onto Mueller-Hinton agar medium. Plates were allowed to dry for 5 minutes and then antibiotic disks were placed (use sterile forceps) on the agar. All plates were incubated for 18 hours at 30-35ºC. Following overnight incubation the diameters of zones of growth inhibition were measured using a vernier caliper.

The breakpoints were determined by previous work (Molnar 2005) where inhibition zones were measured using the Osiris system (Bio-Rad, France). This semiautomated system was used to read and interpret the inhibition results. Susceptibility breakpoints were determined as:

>= 29mm diameter, susceptible

<= 28mm diameter, resistant

The penicillin susceptibility test distinguished *B. anthracis* from other species belong to the *B. cereus* group.

Crystal formation:

B. thuringiensis is distinguished from the *B. cereus* group spp. by producing protein crystal during the sporulation phase. They are bipyramidal, cuboidal, spherical, or diamond-shaped and have pesticidal properties. Two different techniques were used to observe the crystals with (I) phase contrast microscope and (II) scanning electron microscope (SEM).

(I) The first technique was performed according to the instructions of the Food and Drug Administration (FDA) (Rhodehamel and Harmon 1998). Bacteria after incubation on Nutrient agar for 3-4 days were smeared with sterile distilled water on a microscope slide. Slides were left to air dry, then gently heat fixed with a flame, and flooded with methanol and allowed to stand for 30 seconds. After this time methanol was poured off and the slides were left to air dry. 0.5% aqueous solution of basic fuchsin was flooded onto the slide with the flame until steam was seen. After 2 minutes the last step was repeated. The stain was poured off after 30 seconds and the slide rinsed with tap water. Slides were examined under oil immersion lens with phase contrast microscope (Olympus system microscope model CX41), magnification 1000x. Free spores and darker stained toxin crystals should be observed. Toxin crystals can be observed when the cells have lysed and free spores are observed.

(II) For SEM observation of bacterial toxin crystals, the technique of Shao *et al.* (2001) was used. Bacteria were cultivated in a liquid ICPM medium for 36 hours at 28ºC and 0.03 x g shaking. Spores and crystals were collected by centrifugation and washed three times with 1M NaCl. After washing three times with water the spores and crystals mixture was resuspended in water and placed onto 2x2cm glass slide and left to air dry. After coating the slide with gold using a Sputter coater for SEM sample preparation, the spores were observed using different magnification ranging from 2500-7000 and at 6-10kV.

Microscopic analysis of crystalline protein toxin produced by *B. thuringiensis* is necessary to distinguish this species from the group.

Growth below 7ºC:

To detect psychrotrophic strains of the *B. cereus* group spp. 30ml of LB broth was inoculated with single colony and left in the fridge (4ºC) for up to 21 days. After 14 and 21 days bacterial growth was tested. Non-inoculated LB media was tested as a control in each test.

Biochemical identification using BioMérieux API 50 CHB commercial system:

The BioMérieux API 50 CHB system (BioMérieux UK Limited, Hampshire, UK) is a standardized system with 49 biochemical tests for the study of carbohydrate metabolism of microorganisms. The test was used in conjunction with API 50 CHB/E Medium for the identification of the *B. cereus* group spp. The test was prepared according to the manufacturer's instruction, using young cultures which has grown in Nutrient agar medium and incubated overnight at 30ºC.

A few colonies were used for inoculation the API 50 CHB/E Medium to achieve a suspension with the turbidity adjusted to 2 McFarland standard. Suspension was used to fill the API 50 CHB strips. The strips were read after incubation at 30ºC after 24 and 48 hours.

Biochemical reactions were read as either positive or negative, and interpreted using apiweb^{TM} SOFTWARE V4.0 Version: 1.2.1 (BioMérieux® SA 69280 Marcy l'Etoile, France).

2.2.3. Molecular techniques for the *Bacillus cereus* group spp. identification

DNA isolation:

Two different methods and three commercial kits were used to extract genomic DNA from bacteria. This DNA was used as a template for the PCR and RT-PCR reactions and was spotted on positively charged nylon membrane in dot blot assays.

Heating method:

An isolated colony, picked from solid media with a sterile loop, was used to inoculate 100-150µl of water or 10mM Tris-HCl buffer (pH 8.0). It was preheated for 15 minutes at 95ºC and spinned at 7.8 x g for 1 minute. The achieved supernatant was used as a template in PCR reactions (Gussow and Clackson 1989). DNA extracted by this assay was not sufficiently pure to use in the RT-PCR and dot blot assay. The efficiency of the reactions were reduced or did not proceed.

Genomic DNA purification:

To achieve pure genomic DNA, the phenol-chlorophorm technique described by Schraft and Griffiths (1995) with modification was used. Basically, the *Bacillus* strains were grown overnight at 30ºC-33ºC in 10ml of nutrient broth media. The cells were pelleted at 5.0 x g for 10 minutes, resuspended in 200µl lysis buffer (100mM Tris–HCl pH 8.0, 100mM EDTA, 0.75M sucrose, 10U lysostaphin, 10mg/ml lysozyme) and incubated at 37ºC for 30 minutes. 20µl of proteinase K solution (20mg/ml) and 1% SDS were added to each sample, followed by incubation at 37ºC for 2 hours. The lysates were extracted with phenol:chlorophorm:isoamylalcohol (25:24:1) and precipitated with 3M sodium acetate solution 1⁄10 volume and 0.9 volume isopropanol. After washing with 600µl 70% ethanol, the DNA was dissolved in 50µl water of 10mM Tris-HCl pH 8.0 buffer. For the optimization of the PCR, genomic DNA was purified and used as a template (Schraft and Griffiths 1995).

Commercial kits:

DNeasy Blood & Tissue Kit (Qiagen), Genomic mini (A&A Biotechnology) and Isolate Genomic DNA mini (Bioline) according to the manufacturer's instructions were used. Briefly, the DNA was extracted from 2.5-4.5ml of overnight culture grown in liquid media. After centrifugation the pellet was suspended in 100µl Tris-HCl buffer. Gram-positive bacteria were pretreated with 10U of lysostaphin and incubated 30 minutes at 37ºC (Genomic mini and Isolate Genomic DNA mini kits) or resuspended in enzymatic lysis buffer containing 100mM Tris–HCl pH 8.0, 100mM EDTA, 0.75M sucrose, 10U lysostaphin, 10mg/ml lysozyme and incubated at the same conditions (DNeasy Blood & Tissue Kit). Further steps included: mixing with lysis buffer and proteinase K, binding the DNA with the spin column and washing. Pure DNA was eluted from the column with nuclease free water, 10mM Tris-HCl pH 8.0 or buffer included in the kit.

To quantify the DNA concentration two techniques were used. The first, based on spectrophotometric quantification using the DNA absorbance of UV light at 260nm. DNA absorbs UV light in the 260nm range, therefore to estimate the DNA concentration the UV-VIS NanoDrop (Fisherbrand, Fisher Scientific, Ireland) spectrophotometer was used. After analyzing the absorbance of DNA at 260nm the software of the NanoDrop calculated the concentration of nucleic acid presented in ng/µl. The purity of analyzed DNA was also checked by analyzing the ratio 260/280nm

absorbance. Proteins absorb the light at 280nm therefore DNA contamination with proteins results in the ratio being 1.8 for pure DNA. If the ratio was lower, it indicates the presence of proteins or phenol contamination that absorbs strongly the UV light at 280nm.
The second DNA quantification method was based on comparison the DNA separated by electrophoresis in agarose gel with standard molecular weight marker bands. Every band of the DNA molecular weight marker has a known mass expressed in ng/band and shows different intensity under UV light. Based on these standards, 5µl of DNA solution was separated in an agarose gel and stained with EtBr. The intensity of analyzed bands observed using UV transilluminator were compared with standards on molecular weight markers and adequately calculated to achieve the mass in ng/µl.

Precipitation of DNA:

To precipitate the extracted DNA or PCR product, 3 volumes of 96% ethanol was added. The product was stored at -20ºC for minimum 90 minutes and centrifuged at 13.0 x g for 30 minutes at 4ºC. The pellet was dissolved in 200µl of 80% ethanol and centrifuged at the same conditions for 10 minutes. After drying the pellet was dissolved in nuclease free water or 10mM Tris-HCl pH 8.0 buffer and stored at -20ºC.

Agarose gel electrophoresis:

Agarose was dissolved by boiling in 0.5xTBE buffer in microwave. 1µl of EtBr (0.3µg/ml) was added when cooled to approximately 50ºC agarose. The agarose was poured into the gel casting tray equipped with special combs and left to solidation. The DNA samples were mixed with 1µl loading buffer and loaded to the gel. DNA fragments were separated in the gel according to their size after applying an electric field (90 V) for about 45-60 minutes at room temperature. After the electrophoresis was completed, the DNA fragments were visualised under UV lamp using a transilluminator. Pictures were taken using the Gene Genius Bio Imaging System (GelDoc).

DNA extraction from agarose gel:

The PCR products used for sequencing or cloning were analyzed by electrophoresis in 1-1.5% agarose gel and extracted using the Nucleospin Extract II Kit according to the manufacturer's instruction. Briefly, the DNA band was cut with a

sterile razor, placed in a 1.5ml tube, weighted and dissolved with two volumes of binding buffer. After placing in the extract column and spun (7.8-11.2 x g) the product was washed with washing buffer and eluted with nuclease free water, 5mM Tris-HCl buffer pH 8.5 or 10mM Tris-HCl buffer pH 8.0.
The DNA was aliquoted and stored at - 20ºC.

2.2.4. PCR specific detection of 16*S* rDNA of the *B. cereus* group spp. (Hansen *et al.* 2001)

PCR with S-S-Bc-200-a-S-18 and S-S-Bc-470-a-S-18 primers targeting the 16*S* rDNA is specific for identification of the *B. cereus* group spp. (Hansen *et al.* 2001). The primers sequences are presented in Table 11, and the expected product size was 288bp.

Table 11. Primers for amplification of 16*S* rDNA of *B. cereus* group spp. (Hansen *et al.* 2001)

Specificity	Primers	Oligonucleotide sequences (5'-3')	Target	Product size (bp)
The *Bacillus cereus* group spp.	S-S-Bc-200-a-S-18 S-S-Bc-470-a-S-18	TCGAAATTGAAAGGCGGC GGTGCCAGCTTATTCAAC	16*S* rDNA	288

Random strains belonging to the *B. cereus* group spp. were tested with those primers using the Perkin Elmer Applied Biosystem GeneAmp 2400 Thermal cycler. The reaction mix and PCR conditions were as follows:

DNA	3µl	94ºC 5min
Buffer (10x)	2.5µl	
$MgCl_2$ (50mM)	1µl	30 cycles:
dNTPs (25mM each)	0.2µl	94ºC 15sec
S-S-Bc-200-a-S-18 (10µM)	1.5µl	63ºC 45sec
S-S-Bc-470-a-S-18 (10µM)	1.5µl	72ºC 2min
Polymerase (5U/µl)	0.3µl	
H_2O	To 25µl	72ºC 7min

Each PCR product was further analyzed by electrophoresis (90 V, 60 minutes) in 1% agarose gel stained with ethidium bromide and visualised under UV light with transilluminator.

2.2.5. PCR for specific detection of *B. weihenstephanensis* (Lechner *et al.* 1998)

Psychrotrophic *B. weihenstephanensis* produces a cold shock protein (c*spA* gene) that enables it to survive and grow at low temperatures. The presence of this gene can be determined by PCR reaction using BcF2/CSPU3 primers (Lechner *et al.* 1998). It is a *B. weihenstephanensis* specific PCR and generated a 171bp product. The sequences of the *cspA* specific primers are outlined in Table 12.

Table 12. Primers specific for amplification of *cspA* gene of *B. weihenstephanensis* (Lechner *et al.* 1998)

Specificity	Primers	Oligonucleotide sequences (5'-3')	Target	Product size (bp)
Bacillus weihenstephanensis	BcF2	CGAATTTGATAATGTGTGGATTC	*cspA*	171
	CSPU3	CCCGGATCCGGTTACGTTA(G/C)C(A/T)GCT		

A PCR reaction was carried out for all *B. weihenstephanensis* strains used in this study including 11 milk isolates, in a total volume of 25µl reaction. The reaction mix and PCR conditions were as follows:

DNA	3µl	94ºC 5min
Buffer (10x)	2.5µl	
$MgCl_2$ (50mM)	1µl	30 cycles:
dNTPs (25mM each)	0.2µl	94ºC 15sec
BcF2 (10µM)	1.25µl	51.5ºC 30sec
CSPU3 (10µM)	1.25µl	72ºC 30sec
Polymerase (5U/µl)	0.3µl	
H_2O	To 25µl	72ºC 5min

Amplified products were analyzed by electrophoresis (90 V, 60 minutes) in 1% agarose gel stained with ethidium bromide and visualised under UV light.

2.2.6. Differentiation of *B. cereus* and *B. thuringiensis* based on PCR assay targeting the *gyrB* gene (Yamada *et al.* 1999)

To differentiate the *B. cereus* strains from *B. thuringiensis*, the PCR assays with BC1/BC2r and BT1/BT2r primers were performed (Yamada *et al.* 1999). Both sets of primers target the gyrase B gene (*gyrB*) with expected products: 365bp and 368bp, respectively. Primers were designed by the authors based on single nucleotide polymorphisms of the gene and their sequences are outlined in Table 13.

Table 13. Primers specific for amplification of *gyrB* gene of *B. cereus* and *B. thuringiensis* strains (Yamada *et al.* 1999)

Specificity	Primers	Oligonucleotide sequences (5'-3')	Target	Product size (bp)
Bacillus cereus	BC1 BC2r	ATTGGTGACACCGATCAAACA TCATACGTATGGATGTTATTC	*gyrB*	365
Bacillus thuringiensis	BT1 BT2r	ATCGGTGATACAGATAAGACTCCTTCAT ACGTATGAATATTATTT	*gyrB*	368

The reaction conditions including the amplification programme are outlined below:

DNA	3µl	94°C 5min
Buffer (10x)	2.5µl	
$MgCl_2$ (50mM)	1µl	30 cycles:
dNTPs (25mM each)	0.2µl	94°C 1min
BC1 or BT1 (10µM)	2µl	58°C 1.5min
BC2r or BT2r (10µM)	2µl	72°C 2min
Polymerase (5U/µl)	0.3µl	
H_2O	To 25µl	72°C 7min

2.2.7. PCR assay for specific detection of *Bacillus* sp. Ba813$^+$ strains and *B. anthracis* (Patra *et al.* 1996)

R1 and R2 are the PCR primers specific for amplification of Ba813 which is a chromosomal DNA sequence specific for *B. anthracis* and missing in other *Bacillus* species (Patra *et al.* 1996). *Bacillus* sp. Ba813$^+$ strains named transitional strains are

PCR positive when using primers outlined in Table 14. However, other features (i.e. blood hemolysis) are different than *B. anthracis*.

Table 14. Primers specific for amplification of Ba813 of *Bacillus* transitional and *B. anthracis* strains (Patra *et al.* 1996)

Specificity	Primers	Oligonucleotide sequences (5'-3')	Target	Product size (bp)
Bacillus sp. Ba813+ *B. anthracis*	R1 R2	TTAATTCACTTGCAACTGATGGG AACGATAGCTCCTACATTTGGAG	Ba813	152

The product amplified with R1 and R2 primers generated a 152bp product. The reaction conditions and mix used in the amplification were as follows:

DNA	3µl	94ºC 5min
Buffer (10x)	2.5µl	
$MgCl_2$ (50mM)	1µl	40 cycles:
dNTPs (25mM each)	0.25µl	94ºC 1min
R1 (10µM)	1.25µl	60ºC 1min
R2 (10µM)	1.25µl	72ºC 1min
Polymerase (5U/µl)	0.3µl	
H_2O	To 25µl	72ºC 7min

Each PCR product was further analyzed in 1% agarose gel in 0.5xTBE buffer (electrophoresis: 90 V, 60 minutes). Ethidium bromide intercalated in the product was visualised under UV lamp showing the PCR product.

2.2.8. PCR with BCFomp1/BCRomp1 primers for specific detection of the *B. cereus* group spp. (Molnar 2005)

In a previous study in our laboratory BCFomp1 and BCRomp1 primers were designed to detect the *B. cereus* group spp. There are species specific primers generating a 575bp product during PCR reaction. Primers targeted the *motB* gene which encodes flagellar motor protein MotB and classified as an outer membrane protein (OmpA). The sequences of the unique primers were as follows:

Table 15. Primers specific for amplification of fragment of *motB* gene of *B. cereus* group spp. (Molnar 2005)

Specificity	Primers	Oligonucleotide sequences (5'-3')	Target	Product size (bp)
The *B. cereus* group spp.	BCFomp1 BCRomp1	ATCGCCTCGTTGGATGACGA CTGCATATCCTACCGCAGCTA	*motB*	575

The amplification cycles programme and reaction mix in a total volume of 25µl were as follows:

DNA	3µl	94ºC 5min
Buffer (10x)	2.5µl	
$MgCl_2$ (50mM)	1µl	30 cycles:
dNTPs (25mM each)	0.2µl	94ºC 30sec
BCFomp1 (10µM)	2µl	55ºC 30sec
BCRomp1 (10µM)	2µl	72ºC 1min
Polymerase (5U/µl)	0.3µl	
H_2O	To 25µl	72ºC 7min

Electrophoresis (90 V, 60 minutes) in 1% agarose gel was used to analyze the PCR products. Amplification products were viewed in the agarose gel using staining with EtBr.

2.2.9. Primer and probe design

Unique primers for PCR and RT-PCR used in this study were designed following the standard rules (Dorak 2006; McPherson and Møller 2006):

- the optimal length of the primer should be between 16-30 nucleotides long, which provides good specificity for unique target sequence.
- contain approximately equal numbers of each nucleotide.
- base composition (GC content) should be between 40-60%.
- primers should end with G, C, GC or CG bases within the last five bases at the 3'-end of primer what increases efficiency of priming due to stronger bonding of

G and C bases. However, avoid three or more Gs or Cs at the 3'-end, because it may promote mispriming at G or C-rich sequences.

- primers with melting temperature (Tm) between 52-60ºC give the best results.

To rough calculate the Tm of the primer the Wallace formula was used (Wallace *et al.* 1979):

$$Tm\ (^{\circ}C) = 2(A+T)+4(G+C)$$

(A+T) – the sum of A and T residues in the oligonucleotide

(G+C) – the sum of G and C residues in the oligonucleotide

Two primers designed for one PCR experiment must have similar Tm's (be within 5ºC of each other). Annealing temperature should be below the Tm of the primers.

- primers secondary structures should be avoided:
    - hairpin: is formed by intramolecular interaction of complementary sequences within the primer
    - self-dimer: is formed by base-pairing between just one of the two primers
    - heterodimer (cross-dimer): is formed by base-pairing between the forward and reverse primers

When the 3'-end of the primer is involved in the base-pairing, the other primer can be used as a DNA template and be amplified. Primers before use must be checked if they form the secondary structures with the programme IDT DNA OligoAnalyzer 3.1 available at: http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx (Owczarzy *et al.* 2008).

The software analyzing the Gibbs Free Energy G of the primers shows if the secondary structures can be formed spontaneously:

$$\Delta G = \Delta H - T\Delta S$$

ΔG – Gibbs Free Energy

ΔH – Enthalpy

T- Temperature in Kelvin

ΔS – Entropy

Gibbs Free Energy is the measure of the amount of work that can be extracted from a process operating at a constant pressure.

If $\Delta G < 0$, the reaction is spontaneous

If $\Delta G > 0$, the reaction is nonspontaneous

If $\Delta G = 0$, the reaction is at equilibrium

For hairpins, 3'-end with ΔG of –2 kcal/mol and internal hairpin with ΔG of –3 kcal/mol is tolerated. For self-dimers, 3'-end with ΔG of –5 kcal/mol and internal self-dimers with ΔG of –6 kcal/mol are accepted. 3'-end heterodimer with ΔG of –5 kcal/mol and internal heterodimer with ΔG of –6 kcal/mol are also tolerated.

In designing the TaqMan probes it is recommended that (Dorak 2006):

- the probe should be longer than primers and have 20-30 nucleotides.
- the GC content is between 35-65%.
- melting temperature should be 10ºC higher than Tm of forward or reverse primer what allows for the hybridization with the target DNA during the extension. The Tm of both primers should be equal.
- 5'-end cannot finish with G as it causes quenching of reporter fluorescence.
- avoid forming the secondary structure of the probe by self-anneal.
- the probe should be as close as possible to both primers, therefore the amplicon size must be between 100-250bp.
- the reporter dye should be located at 5'-end and the quencher dye at 3'-end.

Primers and probes were designed manually and/or by using the online analysis programme Primer3 (Rozen and Skaletsky 2000, pp.365-386). The programme helps to use the primer/probe according to the conditions specified by the user.

In designing hybridization probes the important considerations for an oligonucleotide probe are its specificity and sensitivity. Oligonucleotide probes are the preferable probes because they are produced synthetically by an automated chemical synthesis and allows for modification at defined positions.

The specificity of the probe must be determined by the ability to bind to the target sequence and no other sequence. The probe must match perfectly with the target. Under experimental conditions the probe after binding to the target sequence must give a

strong signal. An oligonucleotide probe cannot bind to itself (self-annealing) because it reduces probe's sensitivity. In addition, oligonucleotide probes should have:

- 18-50 nucleotides, short probes allow for penetration in the genomic DNA during hybridization. Smaller probes lack the specificity and have longer hybridization time and a low synthesis yield.
- the optimum GC content should be between 40-60%, higher GC content produces the high non specific binding.
- avoid more than four single bases repeating in a row.
- after comparison with non target DNA available in public databases, the homologies of the probe (and its inverted version) should not be higher than 70% or have eight or more bases in a row.

To obtain the optimal hybridization condition for probes, the hybridization to specific and non specific DNA, was determined. In this study oligonucleotide probes used in hybridization technique were labelled with digoxigenin (DIG). It is a non-radioactive method for the detection and remains one of the most sensitive.
PCR primers and hybridization probes were manufactured by MWG (Germany), whereas TaqMan probes were synthesized by TIB Molbiol (Germany).

Sequencing:

DNA fragments that required sequencing were prepared according to the Eurofins MWG Operon requirements for their Sequencing Service à la Carte. For sequencing, the PCR products and plasmid DNA with the insert were posted to the facilitator. Each DNA had to be purified and dissolved in double distilled water or 5mM Tris-HCl (pH 8.0-9.0). The concentration of plasmid DNA was 50-100ng/µl (minimum 1µg per reaction) and PCR products:

<300bp:	2ng/µl in a minimum volume of 15µl
300-1000bp:	5ng/µl in a minimum volume of 15µl
>1000bp:	10ng/µl in a minimum volume of 15µl

Primer concentration was 2pmol/µl and minimum volume per reaction was 10µl.

2.2.10. Bioinformatics tools

Bioinformatics tools are the software programmes for analysis of nucleic acid and protein data. In this study different online available programmes were used. DNA sequences necessary for designing the primers and probes were taken from NCBI (National Center for Biotechnology Information). This public database in accessed at http://www.ncbi.nlm.nih.gov/.

The bioinformatics tool kits used included:

GeneBank, accessible through NCBI system - ENTREZ, is the collection of all publicly available nucleotide sequences available at http://www.ncbi.nlm.nih.gov/genbank/index.html (Benson *et al.* 2009).

The BLAST (Basic Local Alignment Search Tool) programme http://blast.ncbi.nlm.nih.gov/Blast.cgi was used to compare the degree of similarity sequences/genes against others available in database (Altschul *et al.* 1990).

Two programmes were used to compare sequences of specific genome region from different bacteria strains, available from databases or after sequencing of the PCR product. ClustalW available at http://align.genome.jp/ (Thompson *et al.* 1994) where multiple sequence alignments were compared. LALING at http://www.ch.embnet.org/software/LALIGN_form.html, where only 2 alignments of sequences could be compared (Huang and Miller 1991). Those programmes calculate the best match and show the similarities and differences between selected sequences.

ClustalW results were further analyzed in GeneDoc, downloaded from http://www.nrbsc.org/gfx/genedoc/. This programme was used to visually represent the alignments (Nicholas *et al.* 1997).

GENtle software was used as a general tool for DNA editing, database management, plasmid maps, restriction, ligation, for analysis sequenced PCR products or sequences of inserts cloned into the vector and is available to download from http://gentle.magnusmanske.de/.

For designing PCR/RT-PCR primers and probes from DNA sequence, Primer3 software was used (http://frodo.wi.mit.edu/primer3/). It is a very powerful design programme which allows for analyzing product and primers size, Tm range, GC content and presence/absence of a 3'-GC clamp (Rozen and Skaletsky 2000, pp.365-386).

Further, all designed primers were analyzed with IDT OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx) where Tm,

GC content and propensity to form self- or hetero-dimers were also checked (Owczarzy *et al.* 2008).

New designed primers were always checked *in silico*. '*In silico* PCR' (http://insilico.ehu.es/PCR/) is a virtual PCR tool for simulation of amplification assay against particular prokaryotic genomes. This service allowed for only 2 mismatches between primers and template, therefore the stringency of the PCR was high. *In silico* PCR could be performed against prokaryota with complete genome sequencing only (Bikandi *et al.* 2004).

In 2009, a total of 26 and in 2010, a total of 31 sequenced *Bacillus* genera were available in '*in silico* PCR':

- *B. cereus* ATCC 14579 (NC_004722), ATCC 10987 (NC_003909), ZK/E33L (NC_006274), AH187 (NC_011658), B4264 (NC_011725), G9842 (NC_011772), AH820 (NC_011773), Q1 (NC_011969), 03BB102 (NC_012472).
- *B. anthracis* str. Ames (NC_003997), str. Ames 0581 (Ancestor) (NC_007530), str. Sterne (NC_005945), str. CDC 684 (NC_012581), str. A0248 (NC_012659).
- *B. thuringiensis* 97-27 (NC_005957), str. Al Hakam (NC_008600), BMB171 (NC_014171 - in 2010).
- *B. weihenstephanensis* KBAB4 (NC_010184).
- *B. cereus* subsp. *cytotoxis* NVH 391-98 (NC_009674).
- *B. subtilis* subsp. *subtilis str. 168* (NC_000964)
- *B. halodurans C-125* (NC_002570).
- *B. licheniformis* ATCC 14580 (NC_006270), DSM 13 (NC_006322).
- *B. clausii* KSM-K16 (NC_006582).
- *B. amyloliquefaciens* FZB42 (NC_009725).
- *B. pumilus* SAFR-032 (NC_009848).
- *B. pseudofirmus* OF4 (NC_013791).
- *B. megaterium* QM B1551 (NC_014019), DSM 319 (NC_014103) (both in 2010).
- *B. tusciae* DSM 2912 (NC_014098 - in 2010).
- *B. selenitireducens* MLS10 (NC_014219 - in 2010).

2.2.11. Isolation procedure of *Bacillus* sp. strains from milk samples

Pasteurized fat/nonfat and raw milk samples were used to isolate the *B. cereus* group strains. For isolation of *B. weihenstephanensis* strains, the milk was preheated for 10 minutes at 80ºC to kill all vegetative cells and stored at 4ºC. After 2-3 weeks sample was diluted in 1/4 Ringer's solution (1:10, 1:100) and 100µl was spread onto PEMBA or MYP agar plates. For isolation of other *B. cereus* group strains, the milk was also diluted and streaked onto agar plates without thermal pretreatment.

2.2.12. Preparation of standard curve

The establishment of a standard curve using the quantitative real-time PCR (qRT-PCR) process is the key step in determining the copy number of a given target sequence. The concentration/quantity of unknown samples can be determined from the standard curve prepared, using diluted template with known concentration. Using the defined template, such as plasmid with insert which is the gene of interest, 10-fold dilutions must be prepared where the amount of template should be expressed in molecules (gene copy numbers) (Dorak 2006). Each dilution was tied to a specific Cp value and the standard curve was generated.

Plasmid construction and analysis:

pGEM-T Easy Vector is high-copy-number linearized vector with 3'Thymidine-tailed ends. This fact improved the efficiency of ligation of a PCR product that was generated with *Taq* polymerase which left the 3'–terminal Adenine at both ends. It also prevents recircularization of the plasmid. The vector contains T7 and SP6 polymerase promoters flanking a cloning region (Figure 4). The plasmid contains the ampicillin resistance gene which allowed for selection of transformants on agar plates enriched with ampicillin.

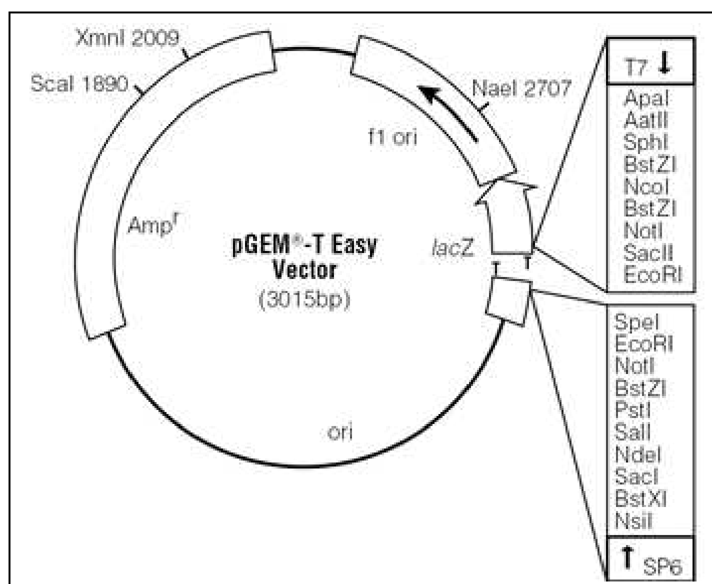


Figure 4. Scheme of pGEM-T Easy Vector (Promega-Corporation 2007)

Preparation of PCR product for cloning into the vector:

125µl of PCR reaction mix containing required primers was amplified with *Taq* polymerase to achieve the product with 3'-Adenine-tailed ends. All PCR products were analyzed by electrophoresis in 1% agarose gel and extracted using the Nucleospin Extract II Kit to remove primer-dimers or other undesired reaction products. PCR product was stored at -20ºC in 100 µl water or 10mM Tris-HCl pH 8.0 buffer.

Competent cells preparation:

Electrocompetent cells of *E. coli* JM 109 were prepared according to Sambrok and Russel (2001). Overnight colony of *E. coli* JM109 was refreshed (0.2ml culture with 40ml LB medium) and allowed to grow at 37ºC with shaking to OD_{600nm}=0.4. The culture was placed on ice, divided into two prechilled tubes and spinned at 1.9 x g for 10 minutes at 4ºC. After carefully removing the supernatant, 5ml of cold water was added and spinned at the same conditions. The step was repeated and the pellet resuspended in 1ml of cold water and spinned at 1.9 x g for 5 minutes at 4ºC. The pellets were collected together using 250µl of 10% glycerol, aliquoted and frozen in liquid nitrogen for about 7 minutes. Competent cells were stored at -80ºC.

Ligation:

T4 DNA Ligase supplied with the pGEM-T Easy Vector was used during the ligation process. Because ligase was free from exonuclease activities, it did not remove the terminal deoxythymidines from the plasmid, improving the efficiency of the ligation. The ligation reaction was performed according to the manufacturer's recommendation and is presented as outlined:

Reaction components	**Standard reaction**
2x Rapid Ligation Buffer, T4 DNA Ligase	5µl
pGEM-T Easy Vector (50ng/µl)	1µl
PCR product	Xµl*
T4 DNA Ligase (3 Weiss units/µl)	1µl
Nuclease-free water to a final volume	10µl

*Molar ratio of PCR product:vector

The molar ration of PCR product:vector was 3:1. Ligation was performed overnight at 4ºC as these conditions were recommended by the manufacturer to produce the maximum number of transformants.

Electrotransformation of competent cells and selection of transformants:

To transform the product of the ligation with earlier prepared competent cells the electroshock method was used. 5µl aliquot from a 10µl ligation was mixed with 50µl of competent *E. coli* JM 109 cells and transferred to the electroporation cuvette. Then cuvette was placed in an electroporator (Easyjet Prima, Equibio) and submitted to a pulse of 1800V for 2 seconds. 1ml of pre-warmed (at 37ºC) LB media was pipetted into the cuvette and then all content was transferred to 30ml tube which was incubated for 1 hour at 37ºC with low shaking. After incubation, 10µl, 50µl and 100µl bacteria were plated onto LB agar plates supplemented with 50µg/ml and 100µg/ml ampicillin to select from transformants. Remaining bacteria were spinned at 1.2 x g for 5 minutes, suspended in 100µl of supernatant and spread onto one of the prepared plates. Plates were incubated at 37ºC for about 15-17 hours.

Isolation of plasmid with insert:

E. coli transformants grew on LB agar plates enriched with ampicillin were used to inoculate liquid media (LB+100µg/ml ampicillin) and grown overnight at 37ºC with shaking. 1.5ml of overnight culture was centrifuged at 7.8 x g for 3 minutes and the pellet dissolved in 100-150ml of 10mM Tris-HCl pH 8.0 buffer. The DNA was extracted using the heating method. 3µl of supernatant was used as a template in the PCR assay to confirm the presence of the insert. Afterwards the recombinant plasmid was extracted using Wizard Plus SV Minipreps kit (Promega).

Enzymatic digestion of plasmid with insert:

The cloning region of pGEM-T Easy Vector was flanked by recognition sites for the *EcoRI* restriction enzyme. The digestion of DNA was carried out to determine the size of insert and plasmid DNA. The conditions used for enzymatic digestion were as follows:

Enzyme buffer	1.5µl
10U enzyme	2µl
DNA	5µl (100-130ng/µl)
H_2O	to 15µl

The reaction was pipetted in a 200µl tube and incubated in the thermal cycler at the recommended temperature 37ºC for 3 to 6 hours. After the incubation time, the enzyme was inactivated at 65ºC for 15 minutes and the products of digestion checked by electrophoresis (90 V, 45-60 minutes) in 1.5% agarose gel.

DNA sequencing:

Following enzymatic digestion, plasmids contained insert with expected size were prepared according to MWG instructions for sequencing. Universal primers were used for sequencing because the vector contained T7 and SP6 polymerase promoters flanking the cloning region. Concentration of plasmid was between 50-100ng/µl (minimum 1µg per reaction). The product was suspended in double distilled water or 5mM Tris-HCl buffer pH 8.0.

2.2.13. Dot blot hybridization technique

To obtain the probes specific for identification of the *B. cereus* group spp. dot blot hybridization technique was performed. DNA used for spotting onto positively charged membranes had to be extracted by a kit, such as Genomic mini (A&A Biotechnology), Isolate Genomic DNA mini (Bioline), DNeasy Blood & Tissue Kit (Qiagen) or by phenol-chloroform assay.

1-3µl of DNA was spotted onto the membrane and washed in denaturing solution for 5 minutes. The membrane was transferred to neutralization buffer and washed for 1 minute. After washings the 3MM Whatman filter was soaked with 10xSSC and the membrane with DNA was placed onto the Whatman filter. To fix DNA to the blot, the UV crosslinking for 12 seconds was performed and the membrane was left to air dry. In this time 6.5ml of working solution of DIG Easy Hyb Granules was preheated to probe hybridization temperature. The hybridization temperature was 10ºC below the Tm of the probe and was increased in case of non-specific binding.

The dry membrane was prehybridized with preheated DIG Easy Hyb working solution for 30 minutes at the hybridization temperature with gentle agitation. The probe was denatured by five-minute heating at 95ºC and rapidly cooled on ice. The denatured probe (1-10pmol per 1ml of hybridization solution) was added to DIG Easy Hyb (4ml/100cm^2 of membrane) preheated to hybridization temperature. The 6.5ml of DIG Easy Hyb from the membrane was poured off and immediately the hybridization solution was added with the probe. Hybridization was performed overnight at the calculated probe hybridization temperature. Following hybridization, sequentially, stringency washes in 3 different post hybridization washing buffers RI, RII and RIII were performed. First the membrane was washed twice in RI buffer for 5 minutes and twice for 15 minutes in RII buffer. The last wash was in RIII for 10 minutes. All stringent washes were performed at the hybridization temperature.

After hybridization and stringency washes the membrane was rinsed briefly (for 1-5 minutes) in washing buffer. After 30 minutes incubation in 18ml of blocking solution the membrane was incubated for further 30 minutes in 5ml antibody solution (1x blocking solution+anti-digoxigenin-AP). Afterwards, the membrane was washed twice in washing buffer for 15 minutes and equilibrated in detection buffer for 2-5 minutes. Next, a few drops of CDPS solution was applied onto the membrane surface and the membrane was placed in a Kodak cassette (8x10 inches). Alkaline phosphatase causes

enzymatic dephosphorylation of CSPD which finally emits light at maximum wavelength 477nm.
To develop the picture in the dark room, the membrane was covered with high performance chemiluminescence film and left for 1-20 minutes in the exposure cassette. Afterwards, the membrane was placed in Kodak developer for a few seconds to a few minutes with gentle agitation and then rinsed with water. It developed the picture of dots showing places where the DNA hybridized with the probe. In the next step the developed film was fixed with a Kodak fixer solution.

2.2.14. Artificial milk contamination

Fat and nonfat pasteurised milk was purchased from local shops in Limerick. Milk was transported to the laboratory in refrigeration conditions. Each milk sample (50ml) was autoclaved for 15 minutes at 121ºC and treated with UV light (254nm) for 30 minutes. Short wave UV light (254nm) can destroy DNA by inducing the formation of covalently bonded Thymine-dimers within the DNA strand. In each stage (before, after autoclaving and after UV treatment) 100µl of milk was spread onto PEMBA and/or MYP.
Milk was contaminated with *B. cereus* ATCC 14579, *B. weihenstephanensis* DSM 11821 or *B. pseudomycoides* DSM 12442. An overnight colony was diluted and every dilution spread onto PCA to estimate the CFU/ml. After estimation the CFU of the overnight culture this data was used to calculate how many µl was used to contaminate the milk. After contamination with known bacterial counts, 100µl was taken for genomic DNA extraction by two commercially available kits: Genomic mini AX food (A&A Biotechnology) and DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's instructions.
Example of calculations:

Overnight culture – $4x10^7$ CFU/ml
Amount of bacteria expected in 5µl of DNA eluent taken to RT-PCR reaction -10^5 CFU
Amount of DNA eluent after extraction by kit -100µl

10^5 CFU - 5µl
X CFU - 100µl
X = $2x10^6$ CFU/100µl

$2x10^6$ CFU - 100µl
Y CFU - 1000µl
Y = $2x10^7$ CFU/ml

$4x10^7$ CFU/ml x 1ml = $2x10^7$ CFU/ml x Z
Z = 2ml
1ml milk + 1ml overnight culture

In this case 1ml of milk was contaminated with 1ml of overnight culture ($4x10^7$ CFU/ml) and 100µl was taken for extraction the genomic DNA using the kits. 5µl of extracted DNA was used in RT-PCR reactions to check the efficiency of the used kits.

3. RESULTS AND DISCUSSION

The *Bacillus cereus* group species consist of 6 species and the transitional strains named Ba813^{+}. In this study 166 strains were tested: 28 x *B. cereus*, 29 x *B. thuringiensis*, 4 x B. a*nthracis*, 17 x *B. weihenstephanensis*, 20 x *B. mycoides*, 10 x *B. pseudomycoides*, 10 x *B. mycoides/pseudomycoides* and 20 x transitional strains. In addition every experiment included control strains which were both *Bacillus* and non-*Bacillus* strains, in total number 28 strains. The majority of those strains were purchased from bacterial collections (DSM, ATCC, NCTC, BGSC) or gifts from researchers all over the world. The list of strains included 31 in house laboratory strains and 11 raw and pasteurized milk isolates. An overall profile of the strains and isolates are presented in Supplementary Table A.

3.1. Isolation of *Bacillus* sp. strains from pasteurized and non pasteurized milk

B. cereus can enter milk at the farm during production, and as a contaminant in the dairy plant (Svensson *et al.* 1999). The bacteria can contaminate the milk from soil, water and from pumps processing equipment and ”milkstone” residue on bulk tanks. The raw milk in this study was obtained from a local farm in Limerick. The raw milk was preheated for 10 minutes at 80ºC diluted in 1/4 Ringer’s solution and spread on PEMBA/MYP agar plates outlined in materials and methods. Colonies with blue (peacock)/pink (respectively) in colour with precipitation zone were selected and identified, in totally 2 x *B. cereus* and 3 x *B. weihenstephanensis*. Pasteurized milk was diluted and spread on agar plates as described above. 1 x *B. cereus*, 4 x *B. weihenstephanensis* and 1 x *B. licheniformis* were isolated. All *B. weihenstephanensis* strains were isolated after the milk was stored for 2-3 weeks at 4ºC. None of the isolates had filamentous growth typical for *B. mycoides* and *B. pseudomycoides* strains.

3.2. Phenotypic characterization of *B. cereus* group strains

Tests for the confirmation and differentiation of all *B. cereus* group strains used in this study were performed following a Dichotomous tree (Figure 5) flow chart.

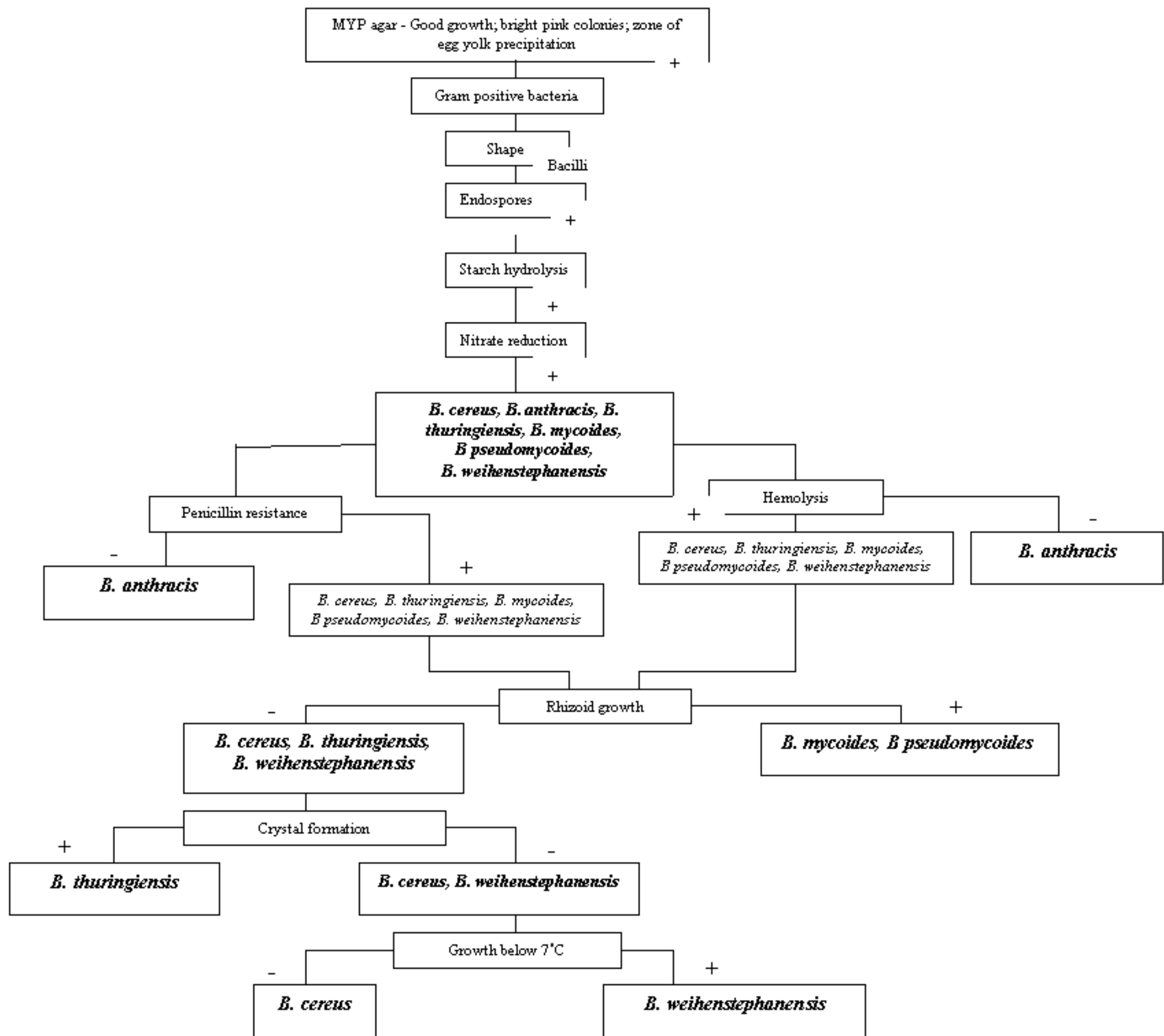
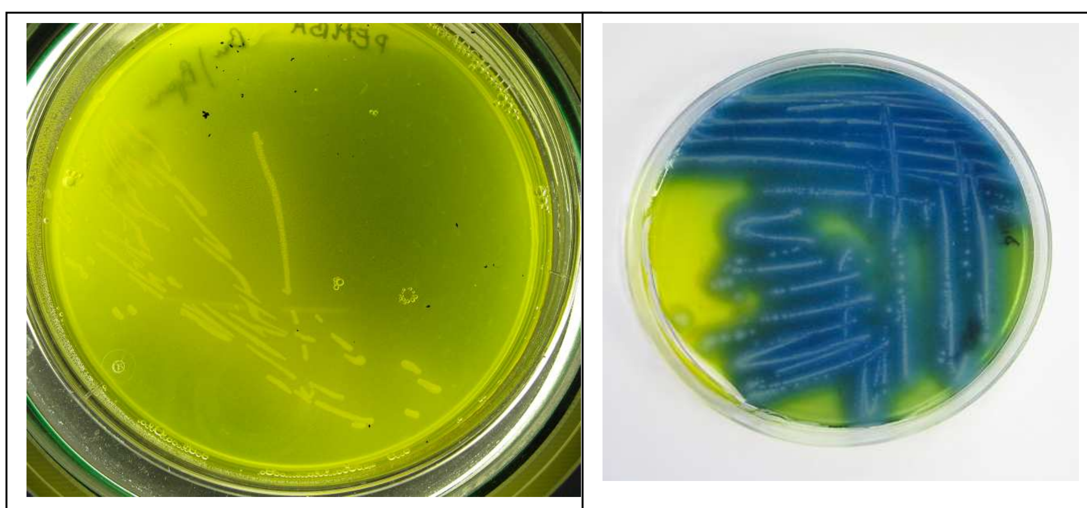


Figure 5. Dichotomous tree to differentiate the *B. cereus* group spp.

The techniques included: growth on selective media (PEMBA, MYP), Gram and spore staining, starch hydrolysis, nitrate reduction test, hemolysis on blood agar, penicillin resistance/susceptibility, a rhizoid growth colony morphology, crystal formation, and growth below 7ºC. In addition the motility test was performed. 4 x *B. anthracis* and 20 x Ba813^{+} were tested only for hemolysis on blood agar plates. 16 x *B. thuringiensis* strains provided by Military Institute of Hygiene and Epidemiology (MIHE, Poland) were tested only for rhizoid growth. In every test positive control and non-*Bacillus* and other *Bacillus* strains as negative control were included.

Growth on PEMBA agar:

B. cereus selective agar media (PEMBA) was developed by Holbrook and Anderson (1980) for the isolation and enumeration of *B. cereus* in food. The medium uses polymyxin B supplement to inhibit growth of Gram-negative bacteria. A low level of peptone promotes sporulation. The diagnostic features of the media are the colony appearance, precipitation of egg yolk due to the presence of enzymes that hydrolyse lecithin and failure to utilise mannitol by *B. cereus*. The bromothyl blue is a pH indicator. Mannitol as the only carbon source in the media that can be utilized which causes a decrease in pH and leads to yellow colonies. *B. cereus* cannot ferment mannitol, therefore the pH increases and colonies turn to blue/peacock colour (Figure 6b).



a) b)

Figure 6. Results of bacterial growth on PEMBA agar

a) *B. subtilis* BSFUL2; b) *B. cereus* ATCC 14579

Observation of colonies, vegetative cells of 98 strains and their growth on PEMBA confirmed or not the affiliation to the *B. cereus* group species. Major strains belong to the *B. cereus* group spp. presented results as expected. Summarized results are presented in Supplementary Table A.

However, 12 tested strains belonging to the *B. cereus* group spp. showed variable results: very light blue colony appearance and/or lack of egg yolk precipitation. One *B. cereus* ATCC 13472 strain presented straw coloured colonies and no precipitation zone. One *B. mycoides* 6A47 strains did not grow on PEMBA gar.

Identification of those strains was verified by other techniques included in the Dichotomous tree flow chart. Care was taken to distinguish the *B. cereus* group spp. from other *Bacillus* strains, and *Staphylococcus aureus* and *Listeria monocytogenes* which also grows on PEMBA. These colonies were differentiated from the *B. cereus* group spp. by colony morphology and colour.

B. subtilis, *B. brevis*, *B. circulans*, *B. amyloliquefaciens*, *L. monocytogenes* and one *S. aureus* strain grew as straw coloured colonies presenting none or very small precipitation zone (Figure 6a). Another two *S. aureus* presented white to light blue in colour colonies without precipitation zone (data not shown). *B. pumilus* and *B. licheniformis* strains grew with white or light blue colonies with small precipitation zones. *L. casei* DPC 6059 strain grew as slight, transparent colonies without egg yolk precipitation. Gram-negative *Escherichia coli*, *Campylobacter jejuni* and *Ralstonia pickettii* did not grew on PEMBA agar plates. A summarize of strains with unexpected results are summarized in Table 16.

Table 16. Summarize of *B. cereus* group spp. strains with unexpected growth and control strains on PEMBA agar

Bacterial strain	Collection source	Colony appearance	EYP
B. cereus NCTC 7464	NCTC	light blue	+
B. cereus BCSUL1	UL laboratory strain	light blue	+
B. cereus BMeSUL1	UL laboratory strain	light blue	-
B. cereus ATCC 13472	ATCC	straw	-
B. mycoides/pseudomycoides 1/1	Dr. I. Święcicka	light blue	+
B. weihenstephanensis WSBC 10389	Dr. L.P. Stenfors Arnesen	white to light blue	+
B. weihenstephanensis WSBC 10392	Dr. L.P. Stenfors Arnesen	white to light blue	+
B. weihenstephanensis WSBC 10416	Dr. L.P. Stenfors Arnesen	very light blue	+
B. weihenstephanensis WSBC 10202	Dr. L.P. Stenfors Arnesen	white to light blue	+/very small
B. weihenstephanensis BWMUL5	UL laboratory strain	light blue	+
B. mycoides 6A19	BGSC	white to light blue	+
B. mycoides 6A49	BGSC	blue	-
B. subtilis BSFUL1	UL laboratory strain	straw	-
B. subtilis BSFUL2	UL laboratory strain	straw	-
B. circulans BCiFUL1	UL laboratory strain	straw	-
B. amyloliquefaciens 10A6	BCGS	straw	+/very small
B. brevis BRFUL1	UL laboratory strain	straw	-
L. monocytogenes LMFUL1	UL laboratory strain	straw	-
S. aureus ATCC 29213	ATCC	straw	-
S. aureus ATCC 25923	ATCC	white to light blue	-
S. aureus SAUL1	MWRH	white to light blue	-
L. casei DPC 6059	Dr. K. Jordan	transparent	-

EYP – zone of egg yolk precipitation

The percentage of bacteria that grew on PEMBA and confirmed as belonging to the *B. cereus* group strains are reported in Table 17 (page 99). The percents of bacteria that present peacock blue/turquoise colonies on PEMBA agar were as follows: *B. cereus* 86% (24 from 28 strains), *B. thuringiensis* 100% (13 strains), *B. weihenstephanensis* 71% (12 from 17 strains), *B. mycoides* 90% (18 from 20 strains), *B. pseudomycoides* 100% (10 strains), *B. mycoides/pseudomycoides* 90% (9 from 10 strains). 4 x *B. anthracis*, 20 x Ba813^{+} and 16 x *B. thuringiensis* strains (from MIHE, Poland) were not tested.

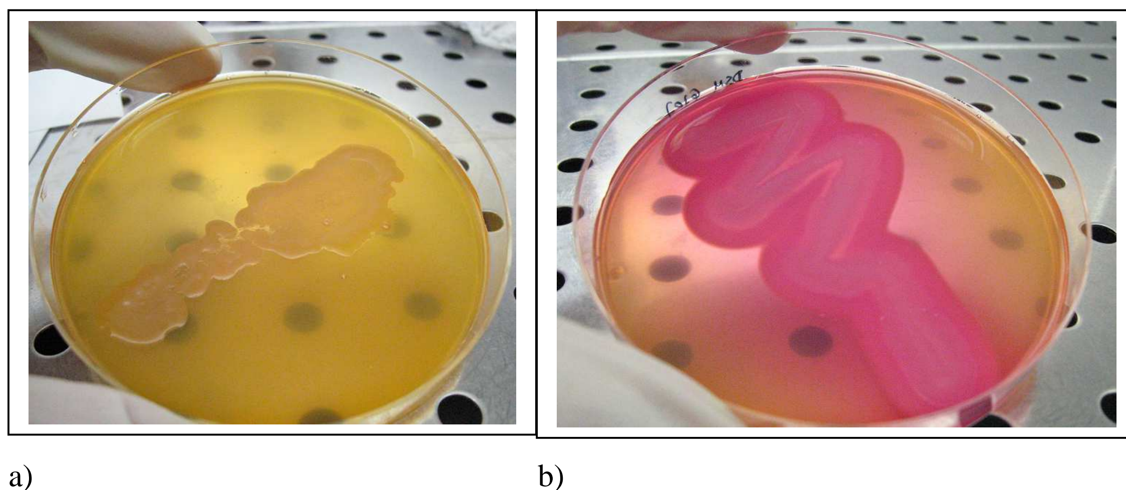
B. cereus, *B. thuringiensis* and *B. weihenstephanensis* strains grew on PEMBA agar with colonies size 2-6mm in diameter. *B. mycoides* and *B. pseudomycoides* presented markedly rhizoid growth with >20mm in diameter.

Growth on MYP agar:

MYP medium differentiates *B. cereus* group spp. from other bacteria based on its resistance to polymyxin, lack of mannitol fermentation and the presence of lecithinase.

Polymyxin B inhibits the growth of most other bacteria (generally Gram-negative). The mannitol content allows for differentiation *B. cereus* group spp. from other Gram-positive bacteria with ability to grow on MYP media. Mannitol, the only carbon source in the media can be utilized which causes a decrease in pH and leads to yellow colonies. *B. cereus* cannot ferment mannitol therefore the pH increases and colonies turn to pink (Figure 7b). Phenol red is the pH indicator in the medium.

The diagnostic features of the media were based on the colony appearance, precipitation of egg yolk due to the presence of enzymes that hydrolyse lecithin and failure to utilise mannitol by *B. cereus*.



a) b)

Figure 7. Results of bacterial growth on MYP agar

a) *S. aureus* ATCC 25923, negative control; b) *B. cereus* ATCC 14579

Ninety eight strains belonging to the *B. cereus* group spp. were tested and results are summarized in Supplementary Table A. Three strains showed different phenotypes than expected. The colony appearance of two *B. weihenstephanensis*: WSBC 10202 and BWMUL5 were light pink and presented the very small egg yolk precipitation. *B. cereus* BMeSUL1 grew as light pink to orange colonies without the precipitation zone, which indicates that lecithinase, is not produced. Those strains were further analyzed as belonging to the *B. cereus* group spp. with molecular techniques. The percentage number of strains with expected phenotype on MYP agar plates was calculated and

outlined in Table 17 (page 99). The results were as follow: *B. cereus* 93% (26 from 28 strains), *B. thuringiensis* 100% (13 strains), *B. weihenstephanensis* 88% (15 from 17 strains), *B. mycoides* 100% (20 strains), *B. pseudomycoides* 100% (10 strains), and *B. mycoides/pseudomycoides* 100% (10 strains). 4 x *B. anthracis*, 20 x Ba813$^+$ and 16 x *B. thuringiensis* strains (from MIHE, Poland) were not tested.
Analysis of control strains showed that 3 x *S. aureus* and 7 other *Bacillus* sp. strains (see Supplementary Table A) fermented mannitol and were able to grow on MYP plates as yellow colonies (Figure 7a). In addition *B. amyloliquefaciens* 10A6 presented a zone of egg yolk precipitation. Two other control strains: *B. licheniformis* BLFUL1 and BLMUL1 grew as light pink colonies without the precipitation zone around the colonies. Two Gram-positive strains: *L. casei* DPC 6059 and *L. acidophilus* DPC 6060 presented straw and transparent colonies on MYP medium, however then also had no precipitation zone.

Gram and spore staining:

The Gram stain is the most popular differential stain, named after its inventor Dr. Christian Gram (Cappucino and Sherman 2001, pp.63-66). It divides bacteria into two groups: Gram-positive and Gram-negative based on differentially coloured bacterial cells. The different colour depends on the different chemical composition of the cell wall. Gram-positive bacteria stain purple because of the presence of a thick peptidoglycan layer. It causes the crystal violet to be retained during staining. Gram-negative bacteria have a much thinner peptidoglycan layer and lies between the outer and inner membrane. Because crystal violet is not retained during the decolorization process, Gram-negative bacteria stain red/pink.

Morphological observation of colonies and microscopy analyzing of Gram and spore staining presented large Gram-positive and rod shaped bacteria with visible spores belongs to the *Bacillus* group. All Gram-positive bacteria presented purple colonies. Whereas Gram-negative bacteria, such as: *Y. enterocolitica*, *C. jejuni*, *R. pickettii*, *E. coli* and *S.* Typhimurium were pink. In this study all tested members of the *B. cereus* group showed positive Gram staining with purple cells. Cells were viewed with oil immersion lens at magnification 400-1000x using a light or phase contrast microscope (Olympus system microscope model CX41). Figure 8 demonstrates example of a Gram stain of a mixed culture of *E. coli* ATCC 25922 and *B. thuringiensis* DSM 6017.

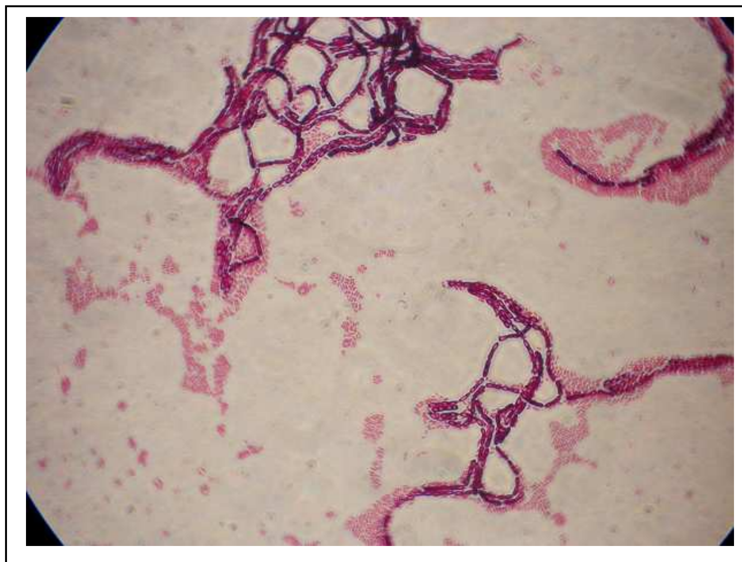


Figure 8. Gram staining of *B. thuringiensis* DSM 6017 (Gram-positive, purple colonies) and *E. coli* ATCC 25922 (Gram-negative, pink colonies)
Phase contrast microscope, magnification 400x

Spores are the inactive form of the bacterium that allows survival in unfavourable environmental conditions. The Schaffer-Fulton staining method used malachite green which penetrates the walls of endospores, whereas safranin stains the vegetative cells in red (Figure 9). Each tested strain belonging to the *B. cereus* group spp. presented green spores usually after 24-hour incubation at 30-33°C. Spores were viewed with oil immersion lens at magnification 400-1000x using phase contrast microscope (Olympus system microscope model CX41). A representative example of spore stain of *B. weihenstephanensis* is presented in Figure 9.

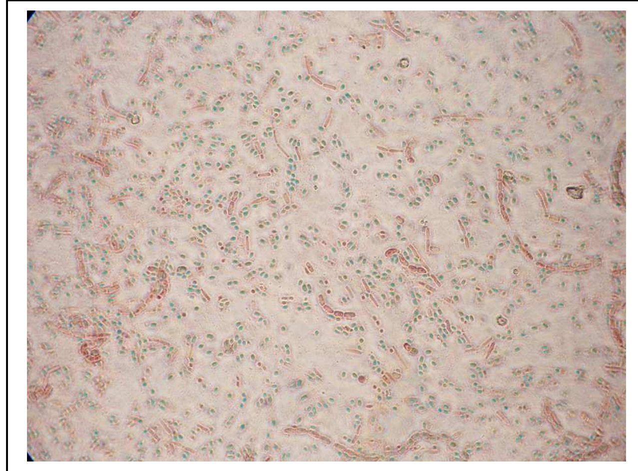
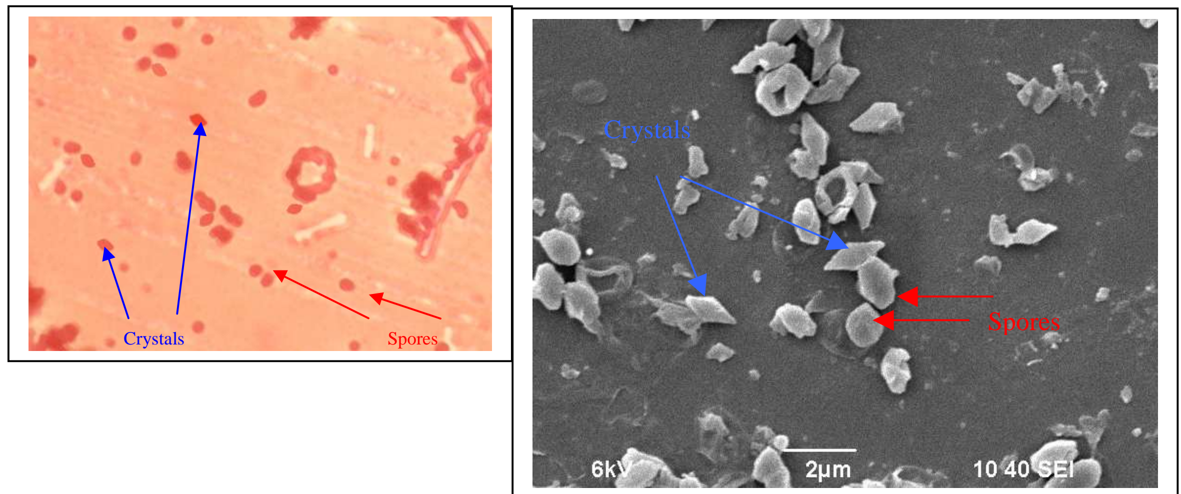


Figure 9. Spore staining of *B. weihenstephanensis* WSBC 10389 by the Schaffer-Fulton method
Phase contrast microscope, magnification 400x

Crystal formation:

The natural toxins produced by *B. thuringiensis* are used for the control of insect pests. It is also a popular phenotypic difference, allows the distinction of the *B. thuringiensis* strains from other members of the *B. cereus* group spp. The intracellular protein crystals are produced by sporulating cells and are accumulated in the cytoplasm of the cells. Two visualization assays were performed to determine the crystal formation in our study: (I) staining with basic fuchsin, where after a few days of cultivation, the cells sporulated and started to lyse. The results showed either intracellular spores and crystals or free spores and crystals (Figure 10a) under phase contrast microscope (Olympus system microscope model CX41). (II) Incubation in ICPM medium where free spores and crystals were collected washed and detected during Scanning Electro Microscope (SEM) observation (Figure 10b).

From 98 tested *B. cereus* group spp. strains only 13 tested *B. thuringiensis* strains (100%) showed crystal formation (Table 17, page 99). In most cases the crystals were diamond shaped, cuboidal or spherical, however sometimes they were much smaller and irregular in size. Beside the crystals, free spores were observed. It establishing the crystal test as good for differentiation this species from other belonging to the *B. cereus* group.



a) b)

Figure 10. Crystal formation by *B. thuringiensis*

a) phase contrast microscope graph of parasporal crystals (diamond shape) and spores from the *B. thuringiensis* DSM 6029 after staining with basic fuchsin, magnification 1000x

b) crystals and spores of *B. thuringiensis* DSM 6017, SEM microscope, magnification 6500x, 6kV

Starch hydrolysis:

Starch is a high-molecular-weight polymer of glucose units linked together by glycosidic bonds. To hydrolyse the starch to small, soluble molecules, two exoenzymes (extracellular) are necessary: amylase and maltase. Starch hydrolysis was used to demonstrate the hydrolytic activities of exoenzymes produced by bacteria. The test was performed to determine whether the bacteria can hydrolyse starch enzymatically. To visualise the hydrolysis, plates after incubation were flooded with Lugol's iodine. In the presence of starch iodine turns blue-black. If bacteria produce exoenzymes a yellow-gold/colourless zone around the growth was observed, demonstrating starch hydrolysis. In the absence of the enzymes the medium and growth are dark blue (Figure 11).

Figure 11. Results of hydrolysed starch by *B. thuringiensis* DSM 6025 (left) and unhydrolyzed starch by *B. licheniformis* ATCC 12759 (right)

The summarized results of bacterial growth on Starch medium of 98 tested *B. cereus* group spp. strains are outlined in Supplementary Table A. In Table 17 (page 99) is the outline of the percentage of each species with expected growth on analyzed medium: *B. cereus* 82% (23 from 28 strains), *B. thuringiensis* 92% (12 from 13 strains), *B. weihenstephanensis* 100% (17 strains), *B. mycoides* 85% (17 from 20 strains), *B. pseudomycoides* 60% (6 from 10 strains), *B. mycoides/pseudomycoides* 100% (10 strains).

Five *B. cereus* strains: 6A6, BCFUL4, BCFUL8, BCFUL9, DSM 4312 did not show the expected results. In addition: *B. thuringiensis* DSM 6107, *B. mycoides* 6A49, Nov1, Nov2, *B. pseudomycoides* B618, DA and A82 were negative for starch hydrolysis. This may suggest these strains produce emetic toxin. Agata *et al*. (1996) reported that none of the *B. cereus* group spp. that hydrolyzed starch produced cereulide. *B. pseudomycoides* TP1 did not grow on the test Starch agar.

The control strains which grew on Starch agar and were able to hydrolyze starch were: *B. subtilis*, *B. circulans*, *B. pumilus*, *B. licheniformis* BLFUL1, *Brevibacillus brevis* and *E. coli*. Not tested strains from MIHE (Poland) included: 4 x *B. anthracis*, 20 x $Ba813^+$ and 16 x *B. thuringiensis*.

Nitrate reduction test:

The nitrate reduction test was carried out using the Blazevic and Ederer (1975) method. All 98 tested members of *B. cereus* group spp. (100%) were able to reduce nitrates (NO_3^-) to nitrites (NO_2^-) (Table 17, page 99 and Supplementary Table A). After overnight incubation in Nitrate broth (supplemented with 0.1% potassium nitrate KNO_3) the ability of the organisms to reduce nitrate was determined by the addition of two reagents: NIT 1 (sulfanilic acid, acetic acid) and NIT 2 (N, N-dimethyl-1-naphthylamine) which produced intensive pink colour (sample 1 on Figure 12). Strains could also show less reduction of nitrate than others what resulted in a light pink colour (sample 2 on Figure 12). Two strains: *B. cereus* BMeSUL1 and *B. weihenstephanensis* WSBC 10201 reduced nitrate to nitrite after 24 hours incubation at room temperature. Not tested strains from MIHE (Poland) included: 4 x *B. anthracis*, 20 x Ba813$^+$ and 16 x *B. thuringiensis.*

L. monocytogenes LMUL1 was used as a negative control and did not reduce the nitrate (sample 3 on Figure 12). 11 from 28 control strains presented positive nitrate reduction test. There included: 1 x *E. coli*, 2 x *B. subtilis*, 3 x *B. licheniformis*, 1 x *B. amyloliquefaciens*, 3 x *S. aureus* and 1 x *Y. enterocolitica*. The results show that the nitrate reduction test performed as a single test is not sufficient for identification of the *B. cereus* group spp. and further tests are necessary.

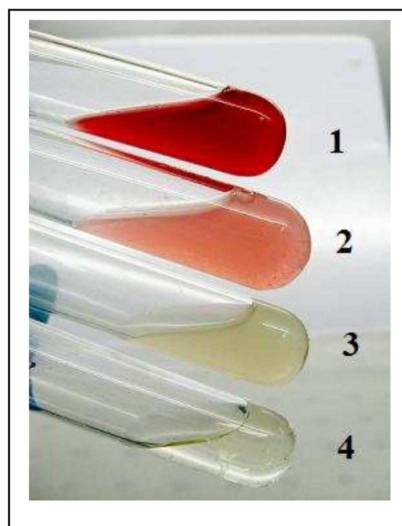


Figure 12. Nitrate reduction test

1) *B. mycoides* 6A49; 2) *B. weihenstephanensis* WSBC 10201; 3) *L. monocytogenes* LMUL1; d) control-not inoculated media

Penicillin susceptibility/resistance:

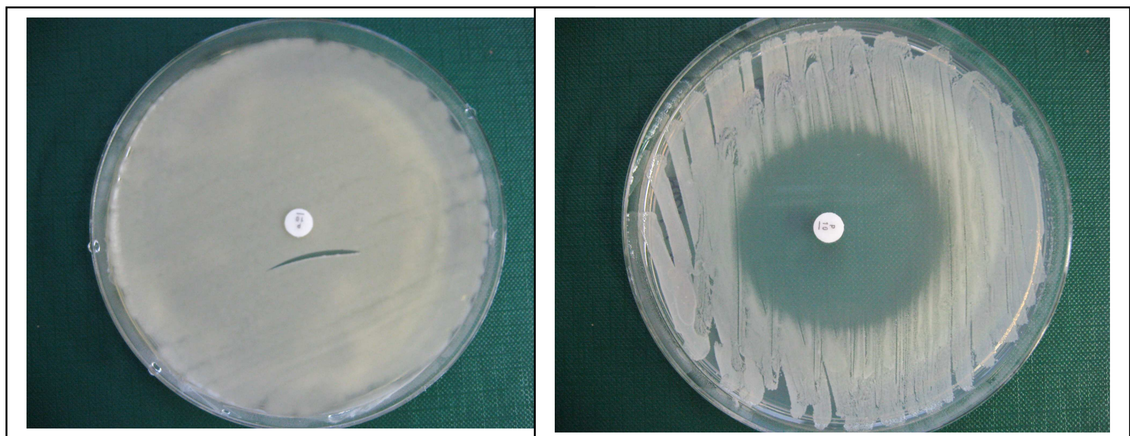
Based on the Kirby-Bauer paper disc agar diffusion procedure, the susceptibility/resistance to penicillin of the *B. cereus* group spp. was determined. As *B. anthracis* is the only member of the group susceptible to the antibiotic, it is an easy test to differentiate this species from *B. cereus* group.

Penicillin belongs to the β-lactam antibiotic group which are characterized by a β-lactam ring. Resistance to β-lactams is due to the hydrolysis of the ring by a β-lactamase. Bacteria are resistant to penicillin due to the production of one or more types of β-lactamases.

Chen *et al.* (2003) reported two β-lactamase genes (*bla1* and *bla2*) present in penicillin susceptible *B. anthracis*. Those two genes are weakly expressed in *B. anthracis* therefore the resistance to β-lactam antibiotics test is not sufficient for identification purposes.

In our study all 98 in total: *B. cereus*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides* and *B. pseudomycoides* strains presented as resistant to penicillin (Supplementary Table A and Table 17, page 99). Zone of inhibition growth was not observed around 10U penicillin disk for those species. These results confirmed previous reports in the literature about *B. cereus* resistance to penicillin (Andrews and Wise 2002; Drobniewski 1993; Luna *et al.* 2007).

S. aureus ATCC 25923 was one of the control strains which presented the penicillin susceptibility zone (Figure 13b). Not tested strains from MIHE (Poland) included: 4 x *B. anthracis*, 20 x Ba813^{+} and 16 x *B. thuringiensis*.



a) b)

Figure 13. Penicillin resistance/susceptibility test

a) *B. cereus* NCTC 7464 penicillin resistant; b) *S. aureus* ATCC 25923 penicillin susceptible

Blood hemolysis:

Hemolysis is the breakdown of erythrocytes and all *B. cereus* group spp. were tested for hemolysis on Columbia blood agar plates. This test allows for classification the strains as to one of three groups: alpha (α), beta (β) and gamma (γ) hemolytic (Figure 14).

Hemolysin BL (HBL) consists of three components: B, L_1 and L_2. For *B. cereus* group spp. strains all three must combine to produce hemolytic activity. Beside the penicillin resistance test, blood hemolysis test is a good technique for differentiation of *B. anthracis* strains.

Generally the *B. cereus* group strains are beta hemolytic, which confirms the presence of a hemolytic enterotoxin. The red cells in the media around the colonies are completely lysed (Figure 14b). However, all *B. anthracis* are considered to be non hemolytic, which results in white colonies on agar plates without the hemolysis zone (image not presented as a camera could not be taken into the laboratory with biosafety level 3 containment facilities). The example of gamma (γ) hemolysis of *B. subtilis* BSFUL2 is presented in Figure 14c. As Klichko *et al.* (2003) reported, no ortholog of the *B. cereus* HBL to be present in the *B. anthracis* genome, which can explain the low activity of the *B. anthracis* hemolytic system. Detailed results of the test and type of blood agar hemolysis for particular strains are outlined in Supplementary Table A. In Table 17 (page 99) are presented the percentage results of blood hemolysis test for the 122 tested species. Except for 4 x *B. anthracis*, 118 (including 20 x Ba813$^+$) *B. cereus* group spp. strains tested, were hemolytic.

In our study *B. mycoides* and *B. pseudomycoides* strains presented alpha, beta or weak beta hemolysis with grey-green colonies. Alpha hemolysis showed incomplete lysis of red blood cells (Figure 14a). The bacterial growth was greenish as the result of reduction of hemoglobin to methemoglobin. In many cases it was difficult to differentiate those two activities because of their markedly rhizoid growth. Prüβ *et al.* (1999) found the same problem, they considered that hemolytic activity of many *B. mycoides* strains was weaker than for other strains due to the rhizoid growth, yielding lower cell densities on the surface of the agar plates.

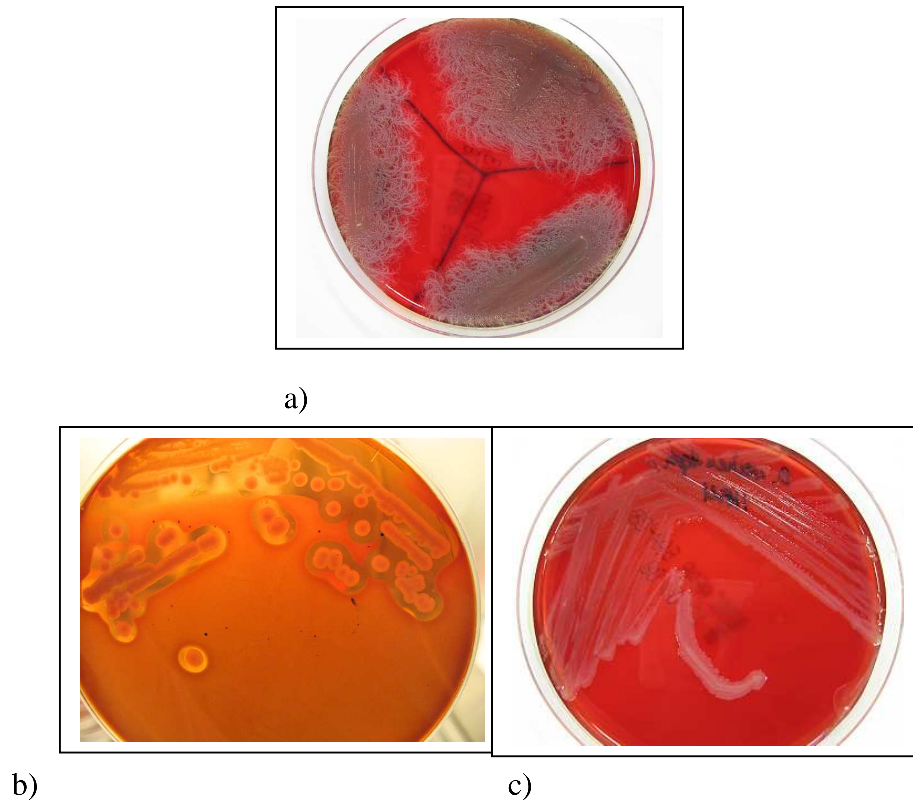


Figure 14. Blood agar hemolysis on Columbia blood agar plates

a) *B. mycoides/pseudomycoides* 1/2-alpha (α) hemolysis; b) *B. cereus* 6A48-beta (β) hemolysis; c) *B. subtilis* BSFUL2-gamma (γ) hemolysis

Rhizoid growth:

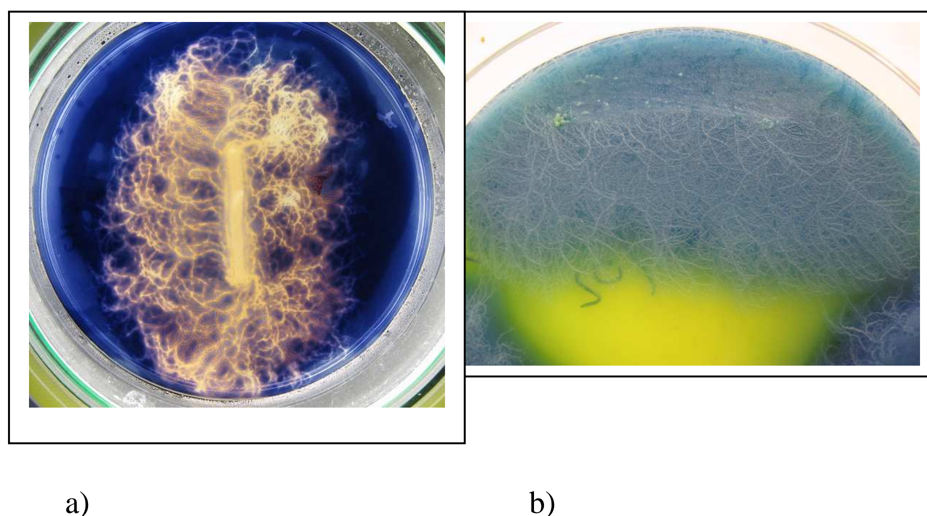
B. mycoides and *B. pseudomycoides* strains can be distinguished from the *B. cereus* group spp. by colony morphology on solid media. They present characteristic rhizoid growth which looks like chains of cells linked end to end forming radial filaments.

In our study each *B. mycoides* and *B. pseudomycoides* strain grew on solid media with characteristic spiral colony pattern (Figure 15a). During the study it was also observed that inoculated plates left for an additional 24-48 hours at room temperature after overnight incubation at 30-33ºC presented bacterial growth with longer filaments. None of the other member of the *B. cereus* group spp. presented this type of growth. This confirmed the previous results of rhizoid growth of *B. mycoides* and *B. pseudomycoides* published in the literature (Bell and Friedman 1994; Buyer 1995; Di Franco *et al.* 2002). In many cases it was very difficult to obtain a single

culture, when plate counting or in the analyze of the zone of penicillin resistance/susceptibility (Figure 15b), due to the rhizoid growth on an agar plate.

Our results (Supplementary Table A) of 114 tested isolates (including 16 x *B. thuringiensis* from MIHE, Poland) of *B. cereus* group spp., presented that only 20 x *B. mycoides*, 10 x *B. pseudomycoides* and 10 x *B. mycoides/pseudomycoides* grew with rhizoid colonies.

4 x *B. anthracis* and 20 x *Bacillus* Ba813$^+$ were analyzed after 24 hours and were not left for additional 24-48 hours at room temperature therefore results are not included in Supplementay Table A. After 24-hour incubation strains did not present rhizoid growth.



a) b)

Figure 15. Rhizoid growth of *B. mycoides* and *B. pseudomycoides*

a) *B. mycoides* 6A14 on Starch agar; b) *B. pseudomycoides* WS 3118 on PEMBA agar

Growth below 7ºC:

Detection of psychrotrophic strains of the *B. cereus* group, able to grow below 7ºC, is slow and takes 7-21 days.

Lechner *et al.* (1998) proposed to accommodate psychrotolerant *B. cereus* strains as new *B. weihenstephanensis* species. Isolates of this new species grow at 4-7ºC but not at 43ºC. It was an easy way to differentiate the *B. weihenstephanensis* strains from the other members belonging to the *B. cereus* group spp. In this study all *B. weihenstephanensis* strains were grown in liquid media at 4ºC for up to 21 days. Also five milk isolates were isolated and distinguished based on their ability to grow below 7ºC. However, two of our laboratory isolates of *B. cereus* (BCFUL6, BMeSUL1) and one *B. thuringiensis* BTSUL7 were able to grow at this temperature. Additional

microbiological and molecular techniques did not confirm those strains as *B. weihenstephanensis*. Similar observations were reported by Stenfors and Granum (2001), based on two PCR assays (targeting *cspA* and 16*S* rDNA gene), they concluded that there are psychrotrophic *B. cereus* strains which cannot be classified as *B. weihenstephanensis*. They also declared that intermediate forms between the two species exist.

Percentage results of 98 strains tested for their ability to grow below 7ºC are presented in Table 17 (page 99). It includes 2 (7%) from 28 x *B. cereus* strains (BCFUL6 and BMeSUL1) and 1 (8%) from 13 x *B. thuringiensis* (BTSUL7) and 17 x *B. weihenstephanensis* (100%) isolates.

Motility test:

B. anthracis, *B. mycoides* and *B. pseudomycoides* are non motile strains and can be differentiate from other strains belong to the *B. cereus* group spp. However, the motility test was not included in the Dichotomous tree flow chart, because according to the US Food and Drug Administration (FDA) report (Rhodehamel and Harmon 1998) 50% of *B. cereus* and *B. thuringiensis* cannot present the expected result, which is a significant percentage. In our study including 98 tested strains, 7% (2 from 28 strains) of *B. cereus* and 8% (1 from 13 strains) *B. thuringiensis* were not motile (Table 17, page 99). Not tested strains from MIHE (Poland) included: 4 x *B. anthracis*, 20 x Ba813^{+} and 16 x *B. thuringiensis*.

Beside *B. anthracis* the two: *B. mycoides* and *B. pseudomycoides* are the members of the *B. cereus* group which do not present motility. In this study 20 x *B. mycoides*, 10 x B. *pseudomycoides* and 10 x *B. mycoides/pseudomycoides* strains showed expected result and were not motile.

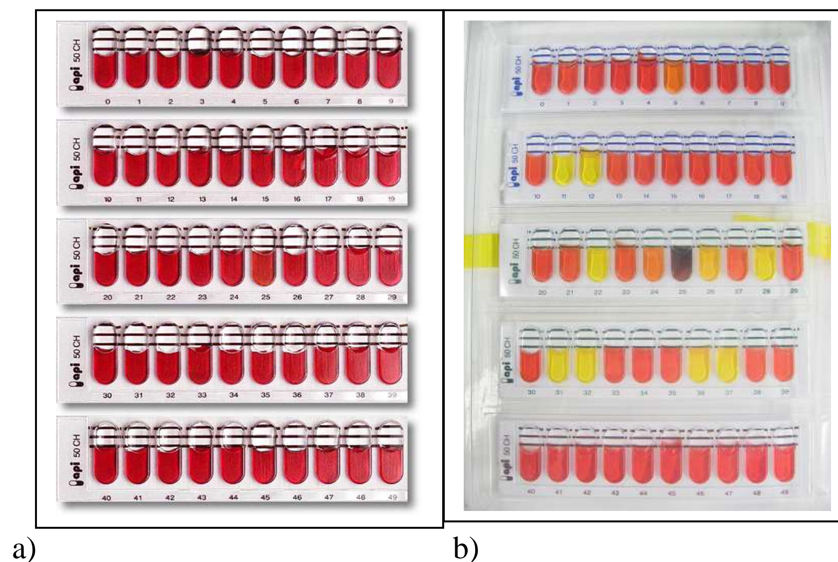
Table 17. Phenotypic characterization of the *B. cereus* group spp. Strains
The numbers of strains with positive results for particular test are presented in percentage

	Number of tested strains	*B. c*	*B. t*	*B. a*	*B. w*	*B. m*	*B. p*	*B. m/p*	Ba813+
PEMBA-PC blue/T colonies	98	86% (24/28)	100% (13/13)	N/T	71% (12/17)	90% (18/20)	100% (10/10)	90% (9/10)	N/T
PEMBA-EYP	98	93% (26/28)	100% (13/13)	N/T	94% (16/17)	90% (18/20)	100% (10/10)	100% (10/10)	N/T
MYP-pink colonies	98	96% (27/28)	100% (13/13)	N/T	88% (15/17)	100% (20/20)	100% (10/10)	100% (10/10)	N/T
MYP-EYP	98	93% (26/28)	100% (13/13)	N/T	88% (15/17)	90% (18/20)	100% (10/10)	100% (10/10)	N/T
Crystal formation	98	0% (0/28)	100% (13/13)	N/T	0% (0/17)	0% (0/20)	0% (0/10)	0% (0/10)	N/T
Starch hydrolysis	98	82% (23/28)	92% (12/13)	N/T	100% (17/17)	85% (17/20)	60% (6/10)	100% (10/10)	N/T
Nitrate reduction	98	100% (28/28)	100% (13/13)	N/T	100% (17/17)	100% (20/20)	100% (10/10)	100% (10/10)	N/T
Penicillin resistance	98	100% (28/28)	100% (13/13)	N/T	100% (17/17)	100% (20/20)	100% (10/10)	100% (10/10)	N/T
Hemolysis	122	100% (28/28)	100% (13/13)	0% (0/4)	100% (17/17)	100% (20/20)	100% (10/10)	100% (10/10)	100% (20/20)
Rhizoid growth	114	0% (0/28)	0% (0/29)	N/T	0% (0/17)	100% (20/20)	100% (10/10)	100% (10/10)	N/T
Growth under 7°C	98	7% (2/28)	8% (1/13)	N/T	100% (17/17)	0% (0/20)	0% (0/10)	0% (0/10)	N/T
Motility	98	93% (26/28)	92% (12/13)	N/T	100% (17/17)	0% (0/20)	0% (0/10)	0% (0/10)	N/T

B. c – B. cereus, B. t – B. thuringiensis, B. a – B. anthracis, B. m – B. mycoides, B. p – B. pseudomycoides, B. m/p – B. mycoides/pseudomycoides, Ba813+ – transitional strain; EYP – zone of egg yolk precipitation; PC blue/T – Peacock blue/Turquoise colonies; N/T – Not Tested;
(number of strains with positive result/number of tested strains)

BioMérieux API 50 CHB analysis:

Milk isolates were further characterized using the BioMérieux API 50 CHB test. Identification of strains by API 50 CHB test are presented in Table 18. The API 50 CHB strips are biochemical tests used to study fermentation of different substrates. During 48 hours of incubation at 30ºC fermentation was revealed by a colour changes in a 49 tubes. The anaerobic production of acid was detected by changes (decrease in the pH) in colour of the pH indicator (phenol red) in the API 50 CHB/E Medium. The first tube did not contain any active ingredient therefore, it acted as a negative control. A positive test corresponds to acidification and the phenol red changes the colour to yellow. For the esculin test (tube number 25) the result was positive if the colour was changed from red to black (Figure 16).



a) b)

Figure 16. BioMérieux API 50 CHB inoculated with *B. mycoides/pseudomycoides* 29/2

a) before incubation, b) after 24 hours incubation at 30ºC

The API was not able to distinguish between *B. mycoides* and *B. pseudomycoides*, between *B. cereus* and *B. thuringiensis*, *B. weihenstephanensis* and *B. anthracis*. One milk isolate was identified as *B. licheniformis* which is not a member of *B. cereus* group spp.

Table 18. API 50 CHB results of milk isolates from raw and pasteurized milk

Bacterial strains	Species	Source	API identification
BCMUL1	*B. cereus*	Pasteurised milk	Good identification/ *B. cereus* 96.6%
BCMUL2	*B. cereus*	Raw milk	Good identification/ *B. cereus* 80.5%
BCMUL3	*B. cereus*	Raw milk	Acceptable identification / *B. cereus* 57.3%
BWMUL1	*B. weihenstephanensis*	Pasteurised milk	Very good identification / *B. cereus* 52.8%
BWMUL2	*B. weihenstephanensis*	Pasteurised milk	Good identification / *B. mycoides* 52.8%
BWMUL3	*B. weihenstephanensis*	Pasteurised milk	Low discrimination/ *B. cereus* 53.3%
BWMUL4	*B. weihenstephanensis*	Pasteurised milk	Good identification / *B. cereus* 61.3%
BWMUL5	*B. weihenstephanensis*	Raw milk	Very good identification / *B. cereus* 47.4%
BWMUL6	*B. weihenstephanensis*	Raw milk	Doubtful profile/ *B. anthracis* 79.9%
BWMUL7	*B. weihenstephanensis*	Raw milk	Good identification / *B. cereus* 80.5%
BLMUL1	*B. licheniformis*	Pasteurised milk	Good identification/ *B. licheniformis* 35%

3.3. Characterization of *B. cereus* group spp. based on molecular techniques

Confirmatory tests of *B. cereus* group species based on molecular biology techniques were performed using PCR and real-time PCR detection with specific DNA primers from the literature or designed in this study.

The following assays available in the literature were used:

(I) PCR with primers targeting the 16*S* rDNA sequence (Hansen *et al.* 2001).

(II) PCR with primers targeting the *gyrB* gene (Yamada *et al.* 1999).

(III) PCR with primers targeting the *cspA* gene (Lechner *et al.* 1998).

(IV) PCR with primers targeting the Ba813 DNA sequence (Patra *et al.* 1996).

(V) PCR with primers targeting the *motB* gene (Molnar 2005).

(I) Confirmation of the *B. cereus* group spp. using 16*S* rDNA primers (Hansen *et al.* 2001):

Analysis of the 16*S* rDNA sequence is a commonly used method for identification of microorganisms. Because *Bacillus* species exhibit a high degree of similarity in their 16*S* rDNA the PCR method with primers S-S-Bc-200-a-S-18 and S-S-Bc-470-a-S-18 designed by Hansen *et al.* (2001) was selected to confirm strains belong to the *B. cereus* group species.

The DNA from a random selected 38 bacterial strains was extracted and tested with primers specific to these 16*S* rDNA sequence. Twenty nine tested strains belonging to the *B. cereus* group species including all raw and pasteurized milk isolates demonstrated the 288bp predicted amplified product. Table 19 and Figure 17 show the results obtained for selected isolates. These data confirmed the BioMérieux API 50 CHB results that 10 milk isolates were members of the *B. cereus* group spp. The API 50 CHB showed 1 isolate, BLMUL1, to be *B. licheniformis*. The PCR confirmed this result, the 288bp product was absent. The 8 negative controls, presented in Table 19 similarly did not display the 288bp amplicon.

Table 19. Strains tested with PCR using S-S-Bc-200-a-S-18 and S-S-Bc-470-a-S-18 primers

Strain tested	**Results of PCR with S-S-Bc-200-a-S-18 and S-S-Bc-470-a-S-18 primers**
B. cereus NCTC 74	+
B. cereus ATCC 14579	+
B. cereus 6A48	+
B. cereus BCMUL1	+
B. cereus BCMUL2	+
B. thuringiensis DSM 6017	+
B. thuringiensis DSM 6025	+
B. thuringiensis DSM 6094	+
B. weihenstephanensis DSM 11821	+
B. weihenstephanensis WSBC 10392	+
B. weihenstephanensis WSBC 10202	+
B. weihenstephanensis BWMUL1	+
B. weihenstephanensis BWMUL2	+
B. weihenstephanensis BWMUL3	+

B. weihenstephanensis BWMUL4	+
B. weihenstephanensis BWMUL5	+
B. weihenstephanensis BWMUL6	+
B. weihenstephanensis BWMUL7	+
B. mycoides 6A11	+
B. mycoides 6A14	+
B. mycoides WS 3120	+
B. mycoides 6A49	+
B. mycoides BMFUL1	+
B. pseudomycoides DSM 12442	+
B. pseudomycoides DSM 12443	+
B. anthracis 1583	+
B. anthracis 1584	+
B. sp. Ba813^{+} #19 (T5 97-77)	+
B. sp. Ba813^{+} #25 (97-27)	+
B. licheniformis ATCC 12759	-
B. licheniformis BLMUL1	-
B. circulans BCiFUL1	-
B. amyloliquefaciens 10A6	-
B. brevis BRFUL1	-
B. pumilus BPFUL1	-
S. Typhimurium NCTC 74	-
S. aureus ATCC 29213	-
E.coli ATCC 25922	-

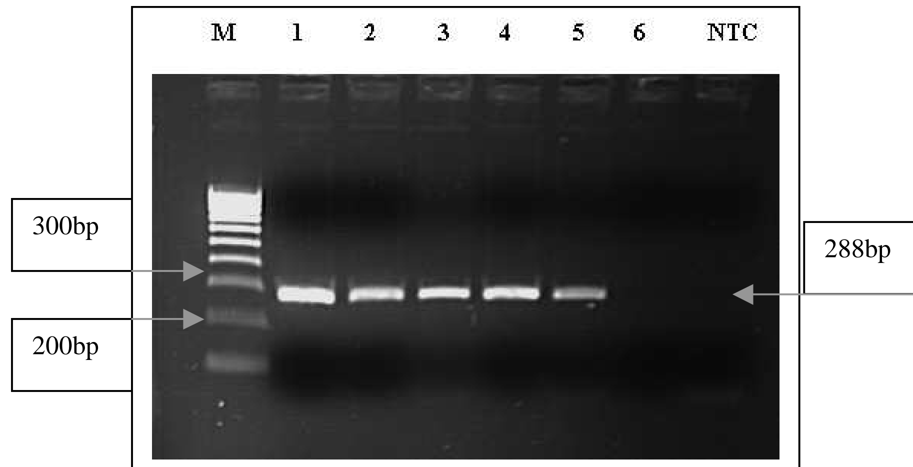


Figure 17. 1% agarose gel showing 288bp PCR product generated with 16*S* rDNA primers: S-S-Bc-200-a-S-18 and S-S-Bc-470-a-S-18

1. *B. cereus* ATCC 14579; 2. *B. pseudomycoides* DSM 12442; 3. *B. mycoides* 6A49; 4. *B. anthracis* 34F2; 5. *B. anthracis* 1583; 6. *B. licheniformis* BLMUL1; NTC-No Template Control; M-Molecular marker (HyperLadder IV)

In the published results of Hansen *et al.* (2001) they tested only 4 x *B. mycoides*, 1 x *B. pseudomycoides* and 1 x *B. weihenstephanensis* strains. None of the *B. anthracis*, transitional Ba813^{+} strains and wild-type isolates was tested. In our hands 38 tested strains gave consistent results.

The method by Hansen *et al.* (2001) using the 16*S* rDNA primers is useful in initial bacteria screening however the authors did not test a large collection of isolates.

(II) Differentiation of *B. cereus* and *B. thuringiensis* by PCR amplification of *gyrB* gene (Yamada *et al.* 1999):

The *gyrB* gene encoding the gyrase B gene was employed by Yamada *et al.* (1999) to differentiate the *B. cereus* group species. A pair-specific primer was designed to differentiate between the species: *B. cereus*, *B. thuringiensis*, and *B. anthracis*. In this study the DNA from random *B. cereus* group species was extracted and tested with BC1/BC2r primers specific for identification of *B. cereus* strains. The expected product was 365bp. For differentiation *B. thuringiensis* from the group, a 368bp PCR product with BT1/BT2r primers was generated.

In our study 11 milk isolates were included in these tests. Moreover, 5 x *B. cereus* strains (3 milk isolates) showed positive reaction with BC1/BC2r and no product with

BT1/BT2r. None of the 8 tested *B. weihenstephanensis* strains (7 milk isolates) 1 x *B. pseudomycoides* and 1 x *B. licheniformis* showed the PCR product with both sets of primers. It confirmed that none of those strains was *B. cereus* and *B. thuringiensis*. However, amplification with BC1/BC2r showed false positive results for 1 x *B. mycoides*, 1 x *B. anthracis* and 3 x *B. thuringiensis* strains. For the same strains, PCR with BT1/BT2r gave no product. It was one of the two major limitations that the authors described. They also found false positive results for some of *B. cereus*, *B. thuringiensis* and *B. mycoides* strains from ATCC collection.

The second described limitation was false-negative reactions that can occur from a number of substances found in samples that inhibit PCR. In this study no false-negative reaction was found.

The *B. anthracis* BA1/BA2r primers designed by the authors were not tested in this study however a false-positive reaction for *B. anthracis* 34F2 strains with *B. cereus* BC1/BC2r primers showed that differentiation using BA1/BA2r primers is limited. The numbers of strains tested by the authors were also small; none of the *B. weihenstephanensis*, *B. pseudomycoides* and no wild-type isolates was tested. The results of PCR using BC1/BC2r and BT1/BT2r primers for particular strains in our study are outlined in Table 20 and Figure 18.

The amplification with primers designed by Yamada *et al*. (1999) is not a good enough system to differentiate the species as irregularities with some isolates were present.

Table 20. Strains tested with PCR using BC1/BC2r and BT1/BT2r primers

Strains tested	Results of PCR with BC1/BC2r primers	Results of PCR with BT1/BT2r primers
B. cereus ATCC 14579	+	–
B. cereus NCTC 7464	+	–
B. cereus BCMUL1	+	–
B. cereus BCMUL2	+	–
B. cereus BCMUL3	+	–
B. thuringiensis DSM 6017	+	–
B. thuringiensis DSM 6025	+	–
B. thuringiensis DSM 6029	–	+
B. thuringiensis DSM 6032	–	+
B. thuringiensis DSM 6094	+	–
B. thuringiensis DSM 2046	–	+
B. weihenstephanensis WSBC 10389	–	–
B. weihenstephanensis BWMUL1	–	–
B. weihenstephanensis BWMUL2	–	–
B. weihenstephanensis BWMUL3	–	–
B. weihenstephanensis BWMUL4	–	–
B. weihenstephanensis BWMUL5	–	–
B. weihenstephanensis BWMUL6	–	–
B. weihenstephanensis BWMUL7	–	–
B. mycoides 6A11	+	–
B. mycoides BMFUL1	–	–
B. pseudomycoides WS 3118	–	–
B. anthracis 34F2	+	–
B. licheniformis BLMUL1	–	–

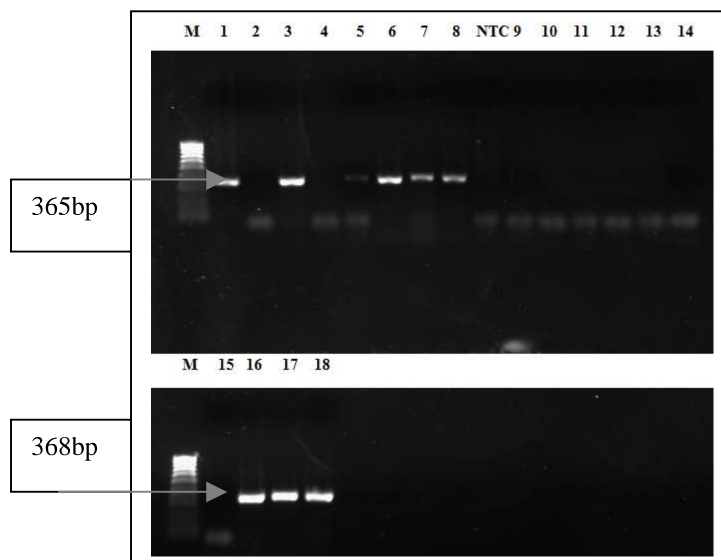


Figure 18. 1.5% Agarose gel showing 365 and 368bp PCR product generated with the *gyrB* gene primers: BC1/BC2r and BT1/BT2r

Lanes 1-8) 365bp product with BC1/BC2r: 1. *B. cereus* ATCC 14579; 2. *B. thuringiensis* DSM 6029; 3. *B. mycoides* 6A11; 4. *B. weihenstephanensis* BWMUL3; 5. *B. anthracis* 34F2; 6. *B. thuringiensis* DSM 6017; 7. *B. thuringiensis* DSM 6025; 8. *B. thuringiensis* DSM 6094;
Lanes 9-18) 368bp product with BT1/BT2r: 9. *B. cereus* ATCC 14579; 10. *B. weihenstephanensis* BWMUL5; 11. *B. anthracis* 34F2; 12. *B. mycoides* 6A11; 13. *B. thuringiensis* DSM 6017; 14. *B. thuringiensis* DSM 6025; 15. *B. thuringiensis* DSM 6094; 16. *B. thuringiensis* DSM 6029; 17. *B. thuringiensis* DSM 6032; 18. *B. thuringiensis* DSM 2046;
NTC-No Template Control; M-Molecular marker (HyperLadder IV)

(III) Differentiation of *B. weihenstephanensis* by PCR amplification of the *cspA* gene (Lechner *et al.* 1998):

Gene *cspA* encodes the major cold-shock protein of psychrotolerant strains and is present its homologue in mesophilic strains of *B. cereus* group (Lechner *et al.* 1998). The high degree of similarity of the *cspA* genes indicated the close phylogenetic relationship between all strains. However, single nucleotide polymorphisms in the gene allowed for simple differentiation of psychrotolerant strains from mesophilic strains. Differentiation was based on the PCR reaction using BcF2 and CSPU3 primers, the expected product of the reaction was 171bp.

The isolates listed in Table 21, including 11 milk isolates from this study, were tested. Based on this experiment 7 of the milk isolates were classified as *B. weihenstephanensis*

strains. Two control *B. weihenstephanensis* strains (type strain DSM 11821 and WSBC 10389) also showed the 171bp product. None of the strains: 4 x *B. cereus*, 1 x *B. thuringiensis*, 1 x *B. mycoides*, 1 x *B. pseudomycoides*, 1 x *B. anthracis* and 1 x *B. licheniformis* gave a positive signal using the described assay.

Lechner *et al.* (1998) tested a small number of mesophilic (21 strains) and psychrotolerant (10 strains) *B. cereus* strains. Moreover, the limit of detection using BcF2 and CSPU3 primers was not estimated by the authors.

The conclusion from our analysis is that the Lechner *et al.* (1998) method worked well in our hands for the detection of *B. weihenstephanensis*.

Table 21. Strains tested with PCR using BcF2/CSPU3 primers

Strain tested	**Results of PCR with BcF2/CSPU3 primers**
B. weihenstephanensis DSM 11821 (type strain)	+
B. weihenstephanensis WSBC 10389	+
B. weihenstephanensis BWMUL1	+
B. weihenstephanensis BWMUL2	+
B. weihenstephanensis BWMUL3	+
B. weihenstephanensis BWMUL4	+
B. weihenstephanensis BWMUL5	+
B. weihenstephanensis BWMUL6	+
B. weihenstephanensis BWMUL7	+
B. cereus BCMUL1	–
B. cereus BCMUL2	–
B. cereus BCMUL3	–
B. licheniformis BLMUL1	–
B. cereus ATCC 14579	–
B. thuringiensis DSM 6017	–
B. mycoides 6A11	–
B. pseudomycoides DSM 12442	–
B. anthracis 34F2	–

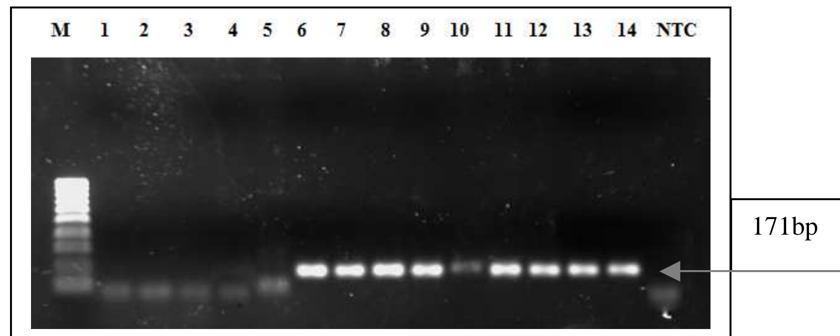


Figure 19. 1% agarose gel showing 171bp PCR product generated with BcF2/CSPU3 primers

1. *B. cereus* ATCC 14579; 2. *B. thuringiensis* DSM 6017; 3. *B. mycoides* 6A11;

4. *B. pseudomycoides* DSM 12442; 5. *B. anthracis* 34F2; 6. *B. weihenstephanensis* WSBC 10389;

7. *B. weihenstephanensis* DSM 11821; 8. *B. weihenstephanensis* BWMUL1;

9. *B. weihenstephanensis* BWMUL2; 10. *B. weihenstephanensis* BWMUL3;

11. *B. weihenstephanensis* BWMUL4; 12. *B. weihenstephanensis* BWMUL5;

13. *B. weihenstephanensis* BWMUL6; 14. *B. weihenstephanensis* BWMUL7;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

(IV) Differentiation of *Bacillus* sp. Ba813$^+$ by PCR amplification of a 152bp fragment of Ba813 sequence (Patra *et al.* 1996):

The virulent strains of *B. anthracis* have two virulence plasmids: pXO1 encoding toxins and pXO2 which codes for capsule production. Avirulent strains of *B. anthracis* can be: pXO1$^-$/ pXO2$^+$, pXO1$^+$/ pXO2$^-$ or pXO1$^-$/ pXO2$^-$, however each strain carries the Ba813 chromosomal DNA sequence. This sequence is specific for *B. anthracis* and is missing in other *Bacillus* species. R1 and R2 are the PCR primers specific for amplification the 152bp Ba813 marker designed by Patra *et al.* (1996).
Avirulent pXO1$^-$/ pXO2$^-$ strains, positive for amplification with R1/R2 can have other features similar to the *B. cereus* or *B. thuringiensis* (e.g. blood hemolysis), in this case the Ba813$^+$ strains are named transitional strains, *Bacillus* sp. Ba813$^+$.
In this study DNA extracted from 20 x transitional strains and 4 x *B. anthracis* provided by the Military Institute of Hygiene and Epidemiology (MIHE, Poland) were tested. Amplification was also performed against random picked 17 x *B. cereus* group spp. strains including 11 milk isolates from this study. Each *Bacillus* sp. Ba813$^+$ and *B. anthracis* strain presented the expected size product (152bp). The results are outlined in Table 22; one strain *B. mycoides* 6A11 (outlined with an asterisk) gave an

unexpected positive signal during amplification using R1 and R2 primers. Other features of the strain (resistance to penicillin, blood hemolysis) were different than *B. anthracis*.

B. mycoides 6A11 strain could be deemed a transitional strain. None of the milk isolates from this study presented the 152bp product.

The 152bp PCR product generated with R1 and R2 primers for transitional strains and the results for *B. cereus* group spp. strains are presented in Figure 20 and Figure 21.

The conclusion from our study is that the amplification with primers designed by Patra *et al.* (1999a) is a good system for differentiation of *Bacillus* sp. Ba813$^+$.

Table 22. Strains tested with PCR using R1 and R2 primers

Strain tested	Results of PCR with R1/R2 primers
Bacillus sp. Ba813$^+$ #6 (I/2)	+
Bacillus sp. Ba813$^+$ #7 (II/3)	+
Bacillus sp. Ba813$^+$ #11 (959/4/3)	+
Bacillus sp. Ba813$^+$ #13 (PC1)	+
Bacillus sp. Ba813$^+$ #15 (11614-2)	+
Bacillus sp. Ba813$^+$ #16 (PJ572)	+
Bacillus sp. Ba813$^+$ #17 (094)	+
Bacillus sp. Ba813$^+$ #18 (T2 97-76)	+
Bacillus sp. Ba813$^+$ #19 (T5 97-77)	+
Bacillus sp. Ba813$^+$ #21 (T11 97-79)	+
Bacillus sp. Ba813$^+$ #22 (BU-1B)	+
Bacillus sp. Ba813$^+$ #23 (III-BL)	+
Bacillus sp. Ba813$^+$ #24 (III-BS)	+
Bacillus sp. Ba813$^+$ #25 (97-27)	+
Bacillus sp. Ba813$^+$ #28 (III)	+
Bacillus sp. Ba813$^+$ #29 (IV)	+
Bacillus sp. Ba813$^+$ #31	+
Bacillus sp. Ba813$^+$ #3403	+
Bacillus sp. Ba813$^+$ ZZ5	+
Bacillus sp. Ba813$^+$ ZL2	+
B. anthracis Sterne 34F2	+
B. anthracis 211	+

B. anthracis 1583	+
B. anthracis 1584	+
B. cereus ATCC 14579	-
B. cereus BCMUL1	-
B. cereus BCMUL2	-
B. cereus BCMUL3	-
B. thuringiensis DSM 6017	-
B. weihenstephanensis DSM 11821	-
B. weihenstephanensis BWMUL1	-
B. weihenstephanensis BWMUL2	-
B. weihenstephanensis BWMUL3	-
B. weihenstephanensis BWMUL4	-
B. weihenstephanensis BWMUL5	-
B. weihenstephanensis BWMUL6	-
B. weihenstephanensis BWMUL7	-
B. mycoides 6A11 (*)	+ (*)
B. mycoides BMSUL1	-
B. pseudomycoides DSM 12442	-
B. licheniformis BLMUL1	-

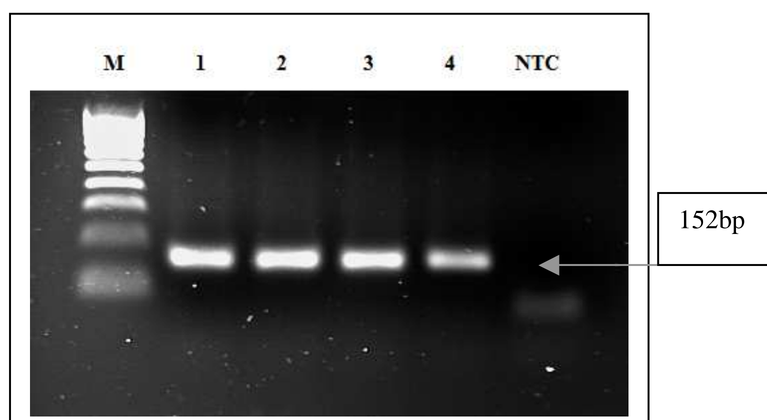


Figure 20. 1% agarose gel showing the 152bp PCR product generated with R1 and R2 primers for *Bacillus* transitional strains

1. *Bacillus* sp. Ba813$^+$ #6 (I/2); 2. *Bacillus* sp. Ba813$^+$ #16 (PJ572); 3. *Bacillus* sp. Ba813$^+$ #25 (97-27); 4. *Bacillus* sp. Ba813$^+$ #3403;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

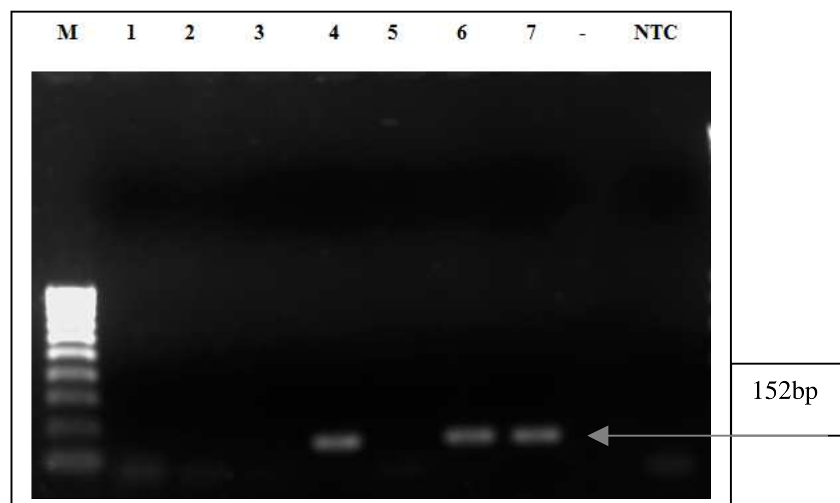


Figure 21. 1% Agarose gel showing the 152bp PCR product generated with R1 and R2 primers for *B. cereus* group spp. strains

1. *B. cereus* ATCC 14579; 2. *B. thuringiensis* DSM 6017; 3. *B. weihenstephanensis* DSM 11821;

4. *B. mycoides* 6A11; 5. *B. pseudomycoides* DSM 12442; 6. *B. anthracis* 34F2;

7. *Bacillus* sp. Ba813^{+} #15 (11614-2);

NTC-No Template Control; M-Molecular marker (HyperLadder IV);

(V) the *motB* gene as a target for identification of *B. cereus* group species by PCR amplification with BCFomp1/BCRomp1 primers (Molnar 2005):

The gene *motB* encoding flagellar motor protein MotB, classified as an outer membrane protein (OmpA) was identified in this study as a suitable target gene for the identification of the *B. cereus* group spp. *OmpA* genes were found to be useful for species identification of *Chlamydia pneumoniae* (Apfalter *et al.* 2003; Everett *et al.* 1999), *Vibrio cholerae* (Martinez-Govea *et al.* 2001), and *Rickettsia* sp. (Fournier *et al.* 2003). In the National Centre for Biotechnology Information (NCBI) database the *motB* gene is also named *motS* and is a homolog to *motB* which is involved in motility on surfaces.

Comparisons of the complete genome sequences of the 7 x *B. cereus* group spp. strains available in NCBI: *B. anthracis* str. Ames Ancestor, str. Sterne, str. Ames, *B. thuringiensis* str. 92-27, *B. cereus* strains ZK (E33L), ATCC 14579, ATCC 10987 (Figure 22) were performed. The *motB* gene was found to be highly conserved within this group and was chosen as the PCR target for the *B. cereus* group spp. identification by a previous member of the laboratory (Molnar 2005). The designed primers were named BCFomp1/BCRomp1 (Figure 23).

B. anthracis str. Ames Ancestor ATGATAAAACGACCGCAAAAGGGATCGCCTCGTTGGATGACGACTTTTAC 50
B. anthracis str. Sterne ATGATAAAACGACCGCAAAAGGGATCGCCTCGTTGGATGACGACTTTTAC 50
B. anthracis str. Ames ATGATAAAACGACCGCAAAAGGGATCGCCTCGTTGGATGACGACTTTTAC 50
B. thuringiensis str. 97-27 ATGATGAAACGACCGCAAAAGGGATCGCCTCGTTGGATGACGACTTTTAC 50
B. cereus E33L ATGATGAAACGACCGCAAAAGGGATCGCCTCGTTGGATGACGACTTTTAC 50
B. cereus ATCC 14579 ATGAGTAAAGGACCGCAAAAGGGATCGCCTCGTTGGATGACGACTTTTAC 50
B. cereus ATCC 10987 ATGATAAAACGACCGCAAAAGGGATCGCCTCGTTGGATGACGACTTTTAC 50
B. cereus G9241 ATGATAAAACGACCGCAAAAGGGATCGCCTCGTTGGATGACGACTTTTAC 50

Figure 22. Cross-species primer (BCFomp1) design in the conserved region of multiple sequences alignment from *motB* gene

The designed primers generated a 575bp PCR product that is a fragment of *motB* gene and encodes part of the motor that rotate the flagella. It was a group specific PCR and rapid identification of the *B. cereus* group species was possible.

Primer	Primer sequence (5'-3')	Target in amplified product	GC content (%)	Tm (°C)
BCFomp1	ATCGCCTCGTTGGATGACGA	1-20	55	58.7
BCRomp1	CTGCATATCCTACCGCAGCTA	575-555	52.4	56.4

Figure 23. The details of BCFomp1 and BCRomp1 primers (Molnar 2005)

In this study the *motB* targeting primers were experimentally examined against an additional 74 strains including: 21 x *B. thuringiensis*, 2 x *B. pseudomycoides*, 12 x *B. mycoides/pseudomycoides*, 9 x *B. weihenstephanensis*, 4 x *B. anthracis*, 20 x transitional Ba813^{+}; six other *Bacillus* and non-*Bacillus* strains were also tested.
The PCR assay was applied to the identification of 117 strains in total with positive result. 19 strains from other microbial species with special emphasis on foodborne pathogens were tested and the 575bp amplicon was not amplified. The summarized results are presented in Table 23.

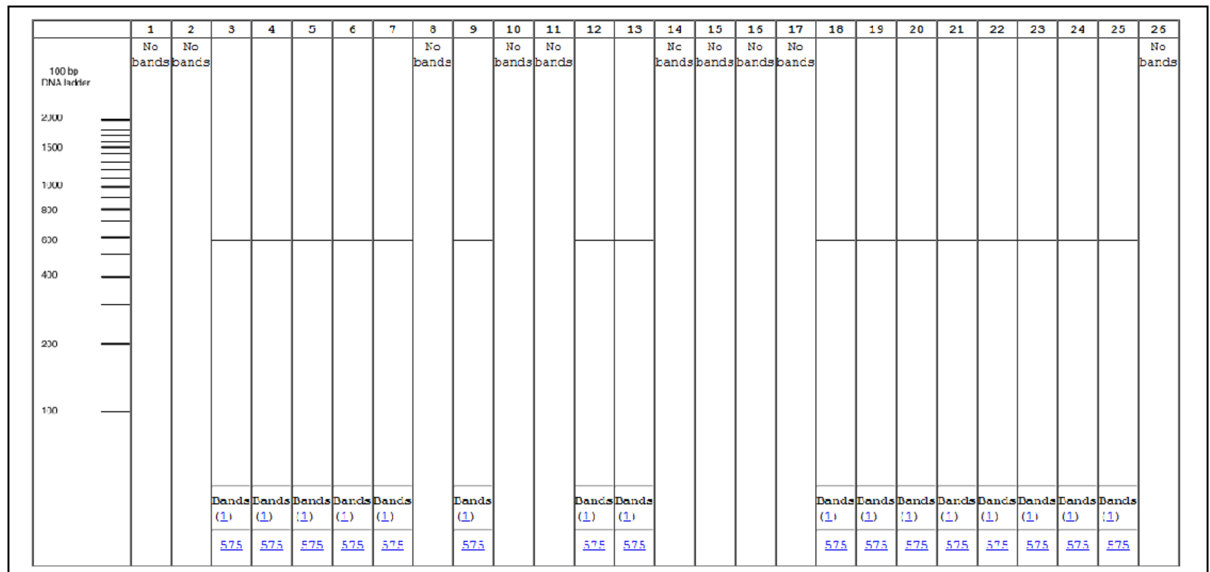
Table 23. Strains tested with PCR using BCFomp1/BCRopm1 *motB* primers

	Number of testes strains	**Result of PCR with BCFomp1/BCRomp1 primers**
B. cereus	35	+
B. thuringiensis	29	+
B. anthracis	4	+
B. mycoides	4	+
B. pseudomycoides	4	+
B. mycoides/pseudomycoides	12	+
B. weihenstephanensis	9	+
Bacillus sp. Ba813^{+}	20	+
Other *Bacillus* strains and foodborne pathogens	19	-

The primers for the fragment of the *motB* gene are group specific and did not react with DNA from other *Bacillus* and non-*Bacillus* species. Using a software programme for '*in silico* PCR', amplification with BCFomp1⁄BCRomp1 was performed. The results are presented in Figure 24. Positive results were obtained for 17 from 26 of the total sequenced *Bacillus* strains available in 2009 in NCBI, while *B. weihenstephanensis* KBAB4 (number 17 in Figure 24) gave a positive result with two allowed mismatches (data not shown).

In 2000, a rare *Bacillus* strain NVH391-98 (number 14 in Figure 24) was isolated and added to the *B. cereus* group spp. (Lund *et al.* 2000); the nucleotide sequences of the conserved genes of NVH391-98 varied greatly from the conserved genes of other strains from the *B. cereus* group spp. The NCBI reported that this strain although identified as a *B. cereus* was very different from other representatives of the group (Genome Project: 13624) and the sequencing revealed that this strain should be assigned a new species status, the name *Bacillus cytotoxis* or *Bacillus cytotoxicus* was proposed (Lapidus *et al.* 2008). We were unable to obtain a culture of this isolate (after numerous attempts) to test it experimentally in our study. However, our *in silico* analysis with primers against the *motB* gene demonstrated that *Bacillus* NVH391-98 strain does not belong to the *B. cereus* group.

Our studies against a large selection of isolates plus *in silico* analysis showed that newly designed BCFomp1/BCRomp1 primers are good for the *B. cereus* group spp. identification.



Selected strains

1 - *Bacillus subtilis* subsp. *subtilis str.* 168; 2 - *Bacillus halodurans C-125*; 3 - *Bacillus cereus* ATCC 14579;

4 - *Bacillus anthracis* str. Ames; 5 - *Bacillus cereus* ATCC 10987; 6 - *Bacillus anthracis* str. Ames 0581

7 - *Bacillus thuringiensis* 97-27; 8 - *Bacillus licheniformis* ATCC 14580; 9 - *Bacillus cereus* ZK

10 - *Bacillus licheniformis* DSM 13; 11 - *Bacillus clausii* KSM-K16;

12 - *Bacillus thuringiensis* str. Al Hakam; 13 - *Bacillus anthracis* str. Sterne;

14 - *Bacillus cereus* subsp. cytotoxis NVH 391-98; 15 - *Bacillus amyloliquefaciens* FZB42;

16 - *Bacillus pumilus* SAFR-032; 17 – *Bacillus weihenstephanensis* KBAB4; 18 - *Bacillus cereus* AH187;

19 - *Bacillus cereus* B4264; 20 - *Bacillus cereus* G9842; 21 – *Bacillus cereus* AH820;

22 - *Bacillus cereus* Q1; 23 - *Bacillus cereus* 03BB102; 24 - *Bacillus anthracis* str. CDC 68

25 - *Bacillus anthracis* str. A0248; 26 - *Bacillus pseudofirmus* OF4

Figure 24. *In silico* PCR results with BCFomp1/BCRomp1 primers against 26 members of *Bacillus* genera with completed genomic sequence

The *motB* PCR assay was analyzed for its sensitivity. To evaluate the sensitivity of the PCR assay, serial dilutions were performed to obtain bacterial numbers using freshly cultured cells of the emetic *B. cereus* DSM 4312. Dilution aliquots were used as templates for PCR amplification using the BCFomp1 and BCRomp1 primers. The results with these primers showed the minimum level of detection was 10^3 CFU/ml. The results of the gel demonstrating the PCR limit of detection is presented in Figure 25.

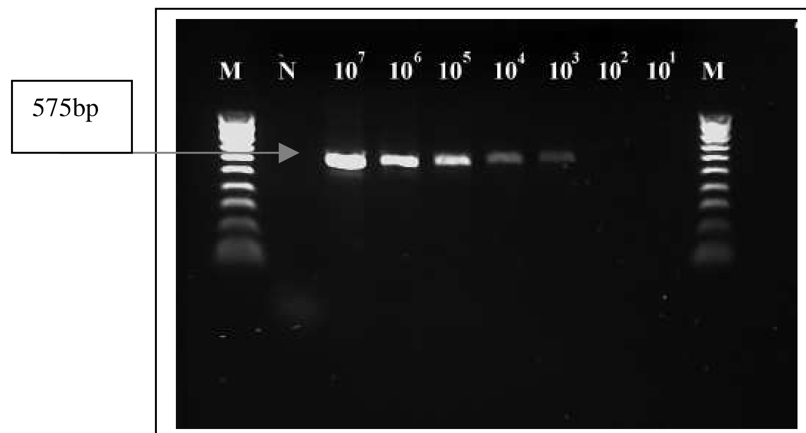


Figure 25. Sensitivity of the PCR detection of *Bacillus cereus* DSM 4312 with primers BCFomp1/BCRomp1

Numbers above lanes indicate the level of inoculation as confirmed by conventional plate count enumeration;

N – No Template Control; M - Molecular marker (HyperLadder IV)

The multiple attempts as seen above with five published PCR systems have been carried out to develop DNA primers allowing the detection of all strains within the *B. cereus* group spp. In addition Schraft and Griffiths (1995) designed three primers against the cereolysin AB sequences for detection of egg yolk-hydrolyzing *Bacillus* sp. However, strains of *B. weihenstephanensis, B. pseudomycoides* and *B. anthracis* species were not tested. Hansen *et al.* (2001) in addition to using universal 16*S* rDNA primers (to avoid false negative reactions) developed 16*S* rDNA-targetic primers (S-S-Bc-200-a-S-18 and S-S-Bc-470-a-A-18) specific for identification of all six species within the *B. cereus* group spp. Their results did not test *B. cereus* spp. wild-type isolates or *B. anthracis* strains and only one isolate each of *B. pseudomycoides* and *B. weihenstephanensis* strains were tested. PCR primers B16S1/B16S2 which were deemed specific to all the *B. cereus* group spp. were also designed by Tsen *et al.* (2000). However, these authors also did not test *B. weihenstephanensis*, *B. pseudomycoides* and wild-type isolates. Moreover, all the *B. mycoides* and *B. anthracis* strains were culture collection types. A 1.2 kb of the *gyrB* genes of *B. cereus* spp. encoding the subunit B protein of the enzyme DNA gyrase was amplified, cloned and sequenced by Yamada *et al.* (1999). The pair-specific primer was designed to differentiation between the species: *B. cereus*, *B. thuringiensis* and *B. anthracis*. These primers can be used to detect the *B. cereus* group spp., however, some of the strains of both *B. cereus* and *B.*

thuringiensis presented false positive or did not give a positive result; no *B. weihenstephanensis* strains were tested. Similarly in our results amplification with *gyrB* primers showed false positive results for 1 x *B. mycoides*, 1 x *B. anthracis* and 3 x *B. thuringiensis* strains.

Chang *et al.* (2003) explored the *groEL* as a PCR assay with 78 isolates and showed some success with further restriction enzyme digestion analysis of the product. They did not test *B. weihenstephanensis* and only one *B. pseudomycoides.* Multilocus sequence typing (MLST) has been used in evolutionary analysis of the *B. cereus* group species and the BA5510 chromosomal gene marker from *B. anthracis* showed some promise in the identification of *B. anthracis* specifically (Olsen *et al.* 2007).

Therefore, we conclude that the PCR assay based on identification of fragment of *motB* gene is a more reliable test system and its sensitivity and detecting 10^3 CFU/ml is adequate than those previously described.

3.3.1. The design of a real-time PCR (RT-PCR) reaction with the *motB* gene-targeted primers for identification of the *B. cereus* group spp.

RT-PCR is one of the most powerful and sensitive gene analysis assay available on the market today. In this study RT-PCR was used for pathogen detection, identification and gene copy number analysis. The method is a continuous collection of fluorescent signal from PCR throughout the cycle (Dorak 2006). Accumulation of PCR product is detected directly by online monitoring the increase in fluorescence of the reporter dye.

In the design of a RT-PCR reaction for identification of *B. cereus* group spp., the BCFomp1 and BCRomp1 primers (Molnar 2005) were used. We concluded from our study that those primers were most suited for the RT-PCR study. The reaction was performed using the Roche LightCycler®480 and LightCycler®1.2 instruments and based on SYBR Green dye.

The reaction was optimized using different concentration of primers (0.5µM, 0.8µM) and magnesium chloride (2mM, 2.25mM, 2.5mM, and 3mM of Mg^{2+}) which are important, as in incorrect concentration reduces or prevents amplification. Various annealing temperatures and the number of cycles with or without dimetyl suphoxide (DMSO) were also applied. The achieve results were unacceptable as the primer-dimer formation and amplicon that was too long, were obtained. The results of RT-PCR with

varied primer, magnesium chloride and DNA concentration (1, 1x10, $1x10^2$, $1x10^3$, $1x10^7$, $1x10^8$ gene copy numbers), are presented in Appendix A, Appendix B, Appendix C.

Working with SYBR Green as a fluorophore it is possible to analyze temperature-dependent dissociation between amplified products by analyzing the melting curve. These analyses begin with heating the product at the end of amplification. PCR products melt and SYBR Green is released resulting in decrease of fluorescence. The end point melting analysis of the achieved product showed multiple peaks in the melting curve which resulted because of self-priming of one or both primers (primer-dimer). It generated small products that were very efficiently amplified. Primer-dimer occurs when primer ends of the each individual primers are complementary or when ends are complementary to each other. To overcome this problem in RT-PCR the optimal primer concentration, increasing the annealing temperature, adding dimethyl sulfoxide (DMSO) to the reaction should be performed. DMSO increases the specificity of the template to the primers and decreases the primer-dimer formation. When added to the reaction mix before the amplification it interferes with the self-complementarity of the DNA. After changing the parameters of the reaction using BCFomp1 and BCRomp1 primers no appropriate results were achieved.

PCR efficiency was also affected by amplicon size. Long amplicons generate a very strong fluorescence signal, which saturates the camera inside the LightCycler® and leads to decreased PCR efficiency. The optimal size of the amplified product should be 100-250bp. Although the *motB* primers (BCFomp1/BCRomp1) worked well with standard PCR, they were not good enough for RT-PCR.

To overcome the primer-dimer formation and amplicon size generation new primers and TaqMan hydrolysis probes were designed.

3.3.2. Design of new primers and sequence-specific TaqMan probe for real-time PCR identification and quantification of the *B. cereus* group spp.

New primers and probe were designed based on comparison of the 575bp product generated with BCFomp1/BCRomp1 (Molnar 2005) of four strains whose genome sequences were available in public databases in 2009: *B. cereus* ATCC 14579 (GenBank accession number: NC_004722), *B. cereus* ZK (NC_006274), *B.*

thuringiensis serovar konkukian str. 97-27 (NC_005957) and *B. anthracis* str. Ames (NC_003997). Figure 26 shows the genome sequences used in this study.

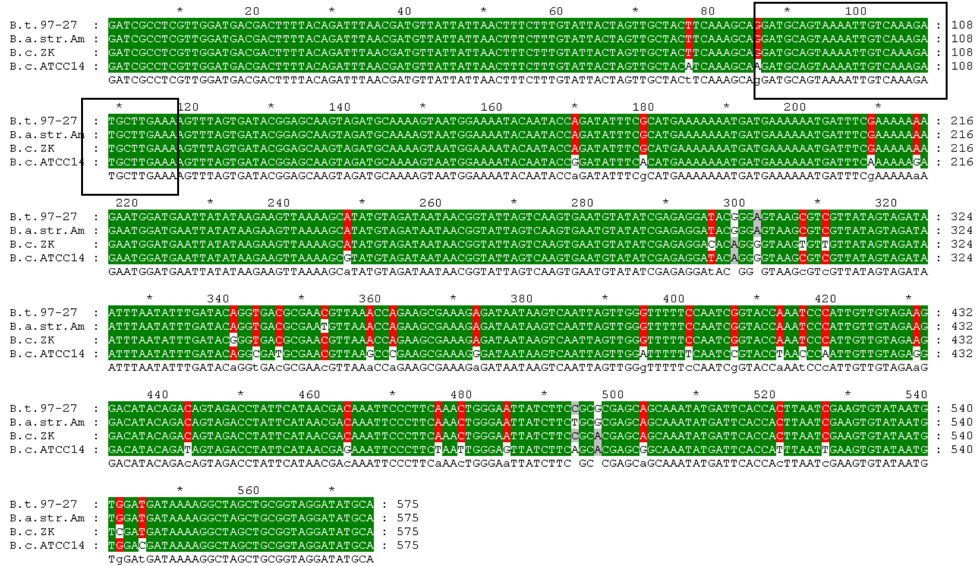


Figure 26. Comparison of the 575bp PCR product generated using BCFomp1/BCRomp1 primers with selected conserved sequence for TaqMan probe hybridization

B. thuringiensis 97-27, *B. anthracis* str. Ames, *B. cereus* ZK, *B. cereus* ATCC 14579

TaqMan probe targeting *motB* gene was designed using Primer3 software (Rozen and Skaletsky 2000, pp.365-386). The designed probe sequence was as follows:

Table 24. The MotB-FAM-1 probe details

Probe	Probe sequence (5'-3')	Target in amplified product	GC content (%)	Tm (ºC)
MotB-FAM-1	FAM-TTCAAGCATCTTTGACAATTTTAC TGCAT-BBQ	114-86	31	56.3

The position of the designed TaqMan probe in the PCR amplicon is demonstrated in Figure 26.

Because the 3'-end of the primer BCFomp1 had multiple binding sites within the generated amplicon, two new forward primers were designed using Primer3 software

and named BCFomp2 and BCFomp3. The reverse primer, BCRomp2, was design to achieve the smaller amplicon size: 285bp with primer BCFomp2 and 280bp with BCFomp3 primer. The new primer sequences are outlined in Table 25 and Box 1. Their sequence positions in the 575bp amplicon, together with the new designed TaqMan probe MotB-FAM-1 are outlined in Figure 27.

Table 25. The details of BCFomp2, BCFomp3 and BCRomp2 primers

Primer	Primer sequence (5'-3')	Target in amplified product	GC content (%)	Tm (°C)
BCFomp2	CGCCTCGTTGGATGACG	4-20	64.7	56.4
BCFomp3	CGTTGGATGACGACTTTTACAG	9-30	53.8	45.5
BCRomp2	GATATACATTCACTTGACTAATACCG	288-263	34.6	51.3

Primer combinations	Size of amplified product
BCFomp2/BCRomp2	285bp
BCFomp3/BCRomp2	280bp

Box 1. Size of PCR product generated with the new primers

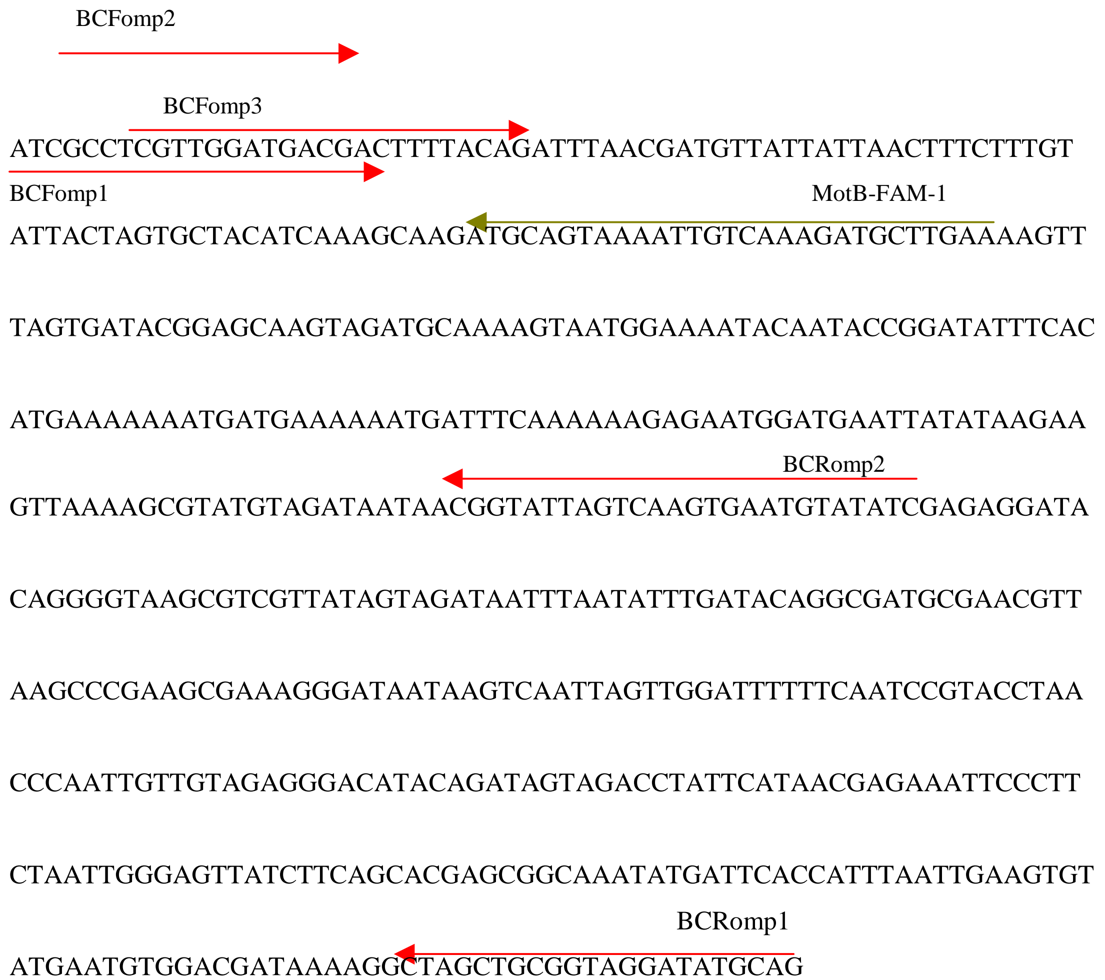
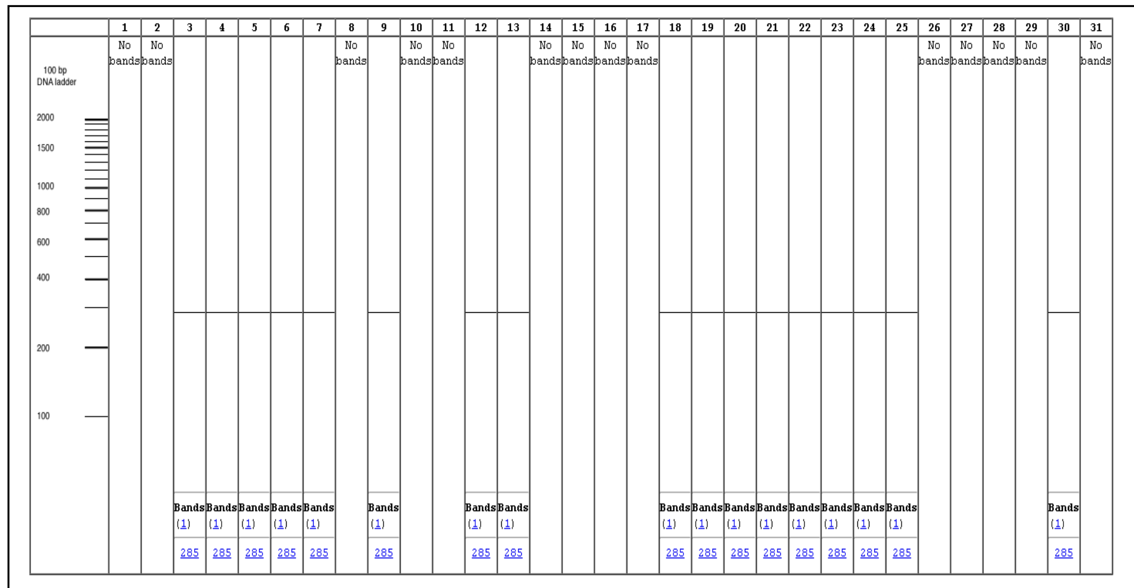


Figure 27. The designed forward BCFomp2, BCFomp3 and reverse BCRomp2 primers and MotB-FAM-1 probe targeting the *motB* gene (575bp) of *B. cereus* ATCC 14579

The *in silico* analysis of BCFomp2/BCRomp2 primers are presented in Figure 28. The *in silico* analysis of BCFomp3/BCRomp2 primers are presented in Appendix D.



Selected strains

1 - *Bacillus subtilis* subsp. *subtilis str.* 168; 2 - *Bacillus halodurans C-125*; 3 - *Bacillus cereus* ATCC 14579;
4 - *Bacillus anthracis* str. Ames; 5 - *Bacillus cereus* ATCC 10987; 6 - *Bacillus anthracis* str. Ames 0581
7 - *Bacillus thuringiensis* 97-27; 8 - *Bacillus licheniformis* ATCC 14580; 9 - *Bacillus cereus* ZK
10 - *Bacillus licheniformis* DSM 13; 11 - *Bacillus clausii* KSM-K16; 12 - *Bacillus thuringiensis* str. Al Hakam;
13 - *Bacillus anthracis* str. Sterne; 14 - *Bacillus cereus* subsp. cytotoxis NVH 391-98
15 - *Bacillus amyloliquefaciens* FZB42; 16 - *Bacillus pumilus* SAFR-032;
17 – *Bacillus weihenstephanensis* KBAB4; 18 - *Bacillus cereus* AH187; 19 - *Bacillus cereus* B4264;
20 - *Bacillus cereus* G9842; 21 – *Bacillus cereus* AH820; 22 - *Bacillus cereus* Q1;
23 - *Bacillus cereus* 03BB102; 24 - *Bacillus anthracis* str. CDC 684; 25 - *Bacillus anthracis* str. A0248;
26- *Bacillus pseudofirmus* OF4; 27 - *Bacillus megaterium* QM B1551; 28 - *Bacillus tusciae* DSM 2912;
29 – *Bacillus megaterium* DSM 319; 30 – *Bacillus thuringiensis* BMB171;
31 – *Bacillus selenitireducens* MLS10

Figure 28. Results of *in silico* PCR with BCFomp2/BCRomp2 primers against 31 members of *Bacillus* group with completed genomic sequences

The *in silico* PCR analysis of the new set of primers BCFomp2/BCRomp2 and BCFomp3/BCRomp2 gave positive results for 18 of the 18 total sequenced *B. cereus* group spp. strains, while *B. weihenstephanensis* KBAB4 (number 17 in Figure 28) gave a positive result with two allowed mismatches but none of them were located in 3’ end (data not shown). The *B. cereus* subsp. *cytotoxis* NVH 391-98 (number 14 in Figure 28) where the nucleotide sequences of the conserved genes vary greatly from the conserved

genes of other strains from the *B. cereus* group showed negative result for both pair of primers also with allowed mismatches. These results confirmed our earlier results published in Oliwa-Stasiak *et al.* (2010) that this strain although identified as a *B. cereus* is different from other members of the *B. cereus* group spp. This strain should be representatived as a novel bacterial species.

The designed TaqMan probe (Table 24) labelled with fluorescent dye FAM (6-carboxyfluorescein) and quenched with Black Berry Quencher (BBQ) was detectable in channel F1 of the LightCycler®1.2. Working with the hydrolysis probe, analysis of efficiency and sensitivity of the reaction was based on one standard curve (described later).

To design the optimized reaction, the assay validation parameters model should be used. The validation was as follow:

- primer pairs were tested in all combinations with a known template (plasmid clone, genomic DNA).
- different assay conditions (primers, magnesium and probe concentrations, annealing temperature) were used.
- the primer pairs that give the lowest Cp values and the best limit of detection were chosen.
- a dilutions of templates were made.
- only primers specific for the *B. cereus* group spp. were chosen.
- the standard curve giving the best efficiency was prepared.

3.3.3. Results of RT-PCR with new designed primers and MotB-FAM-1 TaqMan probe

Two primer combinations were used to achieve the expected products by RT-PCR: BCFomp2 with BCRomp2, and BCFomp3 with BCRomp2. The reaction was optimized with different final primer concentration: 0.5µM, 0.8µM, 1µM of each primer in the reaction and various annealing temperatures (55ºC, 57ºC, 59ºC).

The 0.07µM final probe concentration was experimentally found as optimal for the reaction. Magnesium chloride (Mg^{2+}) was included in the TaqMan MasterMix and its concentration was optimized for almost all primer combinations by the manufacturer. As a DNA template, the 575bp fragment of the *motB* gene cloned into the pGEM-T Easy Vector was used. The generation of the *motB* gene cloned into pGEM-T Easy

Vector is described later. The experimental analyses to optimize the reaction are presented in Figure 29. The DNA amount was 1x10^{8} gene copy numbers in each reaction.

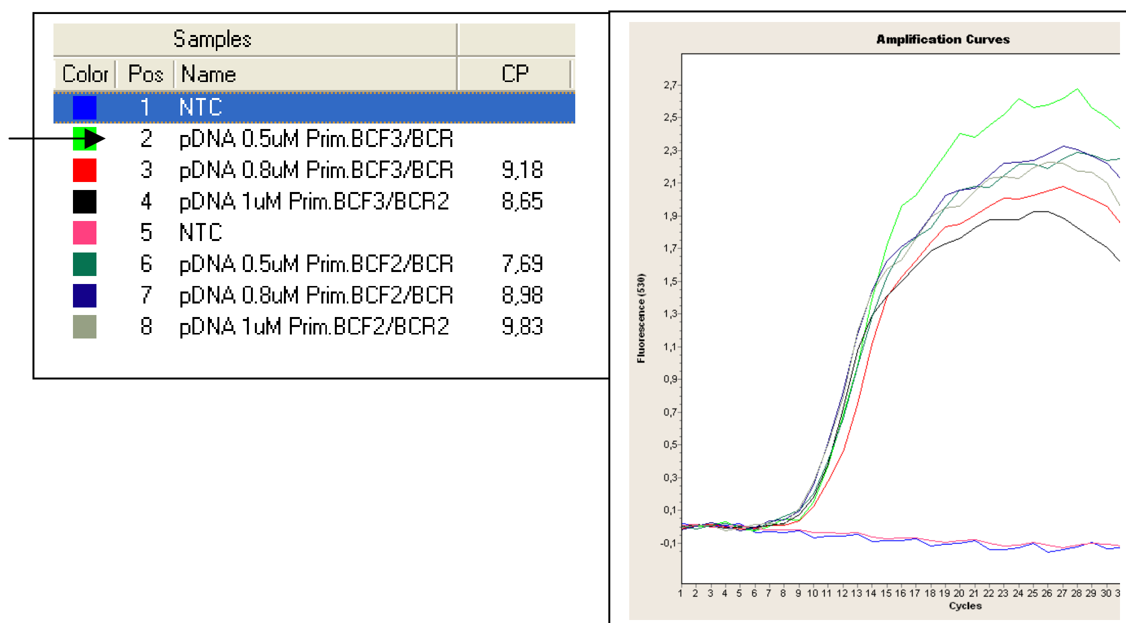


Figure 29. Results of RT-PCR reactions with MotB-FAM-1 probe using different concentration of primers: BCFomp3/BCRomp2 (samples 1-4) and BCFomp2/BCRomp2 (samples 5-8)

1. NTC (1µM of BCFomp3/BCRomp2); 2. 0.5µM of BCFomp3/BCRomp2;

3. 0.8µM of BCFomp3/BCRomp2; 4. 1µM of BCFomp3/BCRomp2;

5. NTC (1µM of BCFomp2/BCRomp2); 6. 0.5µM of BCFomp2/BCRomp2;

7. 0.8µM of BCFomp2/BCRomp2; 8. 1µM of BCFomp2/BCRomp2;

DNA template: 1x10^{8} gene copy numbers; annealing temperature: 59ºC; NTC-No Template Control

Better results were achieved for the reaction with BCFomp2 and BCRomp2 primers. Reaction with 0.8µM of each primer at 59ºC annealing temperature showed lower Cp values (in compare with 0.8µM BCFomp3/BCRomp2) which responded with improved sensitivity. In addition results could also be observed when the RT-PCR products were run in 1% agarose gel (Figure 30).

As seen from the graph (Figure 29) the crossing points (Cp) for 1x10^{8} gene copy numbers were lower for BCFomp2/BCRomp2 primer combination. For 0.5µM of BCFomp3/BCRomp2 (sample 2) no Cp value was observed. The peak for this reaction could be observed on the graph, however the LightCycler® software (version 4.1) did

not include that result (marked with arrow) to be analyzed. For 0.5μM of BCFomp2/BCRomp2 the reaction was included in the results.

The 1% agarose gel picture (Figure 30) with products of the RT-PCR assay showed brighter and stronger bands when BCFomp2/BCRomp2 primers were used.

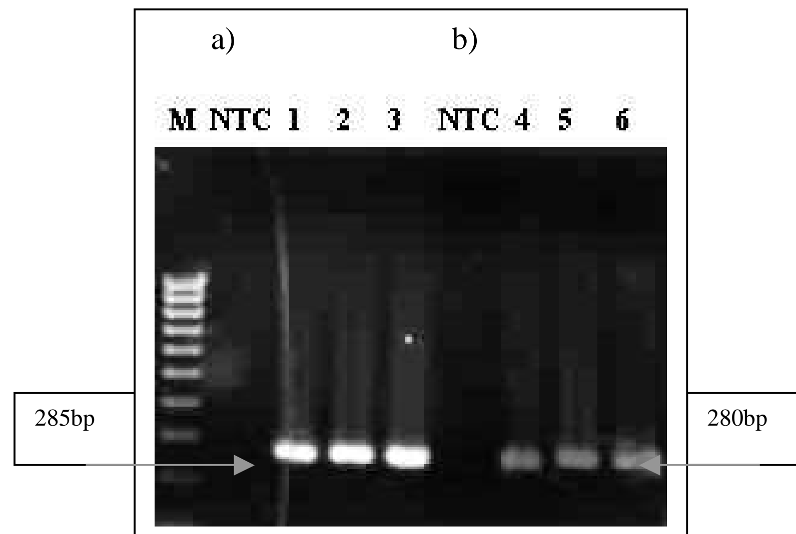


Figure 30. The RT-PCR products run on 1% agarose gel with (a) BCFomp2/BCRomp2 and (b) BCFomp3/BCRomp2 primers

(a): 1. $1x10^8$ gene copy numbers, 0.5μM each primer per reaction; 2. $1x10^8$ gene copy numbers, 0.8μM each primer per reaction; 3. $1x10^8$ gene copy numbers, 1μM each primer per reaction;

(b): 4. $1x10^8$ gene copy numbers, 0.5μM each primer per reaction; 5. $1x10^8$ gene copy numbers, 0.8μM each primer per reaction; 6. $1x10^8$ gene copy numbers, 1μM each primer per reaction;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

In an assay validation all primer combinations should be checked, therefore the reaction with initial primers BCFomp1/BCRomp1 and BCFomp2/BCRomp1 were also tested and compared (see also Appendix E). Different DNA dilutions, ranging from 1 to $1x10^7$ gene copy numbers per reaction were amplified in the same conditions including 0.8μM of each primer and 59ºC annealing temperature. Reaction with the BCFomp2/BCRomp1 primers was able to detect $1x10^2$ gene copy numbers whereas reaction using BCFomp1/BCRomp1 showed detection of $1x10^3$ gene copy numbers and was less sensitive during comparison of Cp values for different dilutions (Figure 31). In the same conditions the reaction with primers BCFomp2/BCRomp1 showed lower Cp values (Figure 31, samples 2-9) and better limit of detection than reactions using initial BCFomp1/BCRomp1 primers (Figure 31, samples 12-19).

These differences were easily to observe on the RT-PCR graphs and also on agarose gels where the final products were run after each reaction. The product of amplification with initial BCFomp1/BCRomp1 primers and $1x10^3$ gene copy numbers was not visible on the agarose gel (Figure 32).

After all analysis in the assay validation the reaction with BCFomp2/BCRomp2 satisfied the major requirements (described earlier). Amplification with the new BCFomp2/BCRomp2 showed the best efficiency and sensitivity among all tested primers combinations. Reaction with these primers was able to detect 1x10 gene copy numbers in RT-PCR assay.

Primers BCFomp2/BCRomp2 were chosen for future work.

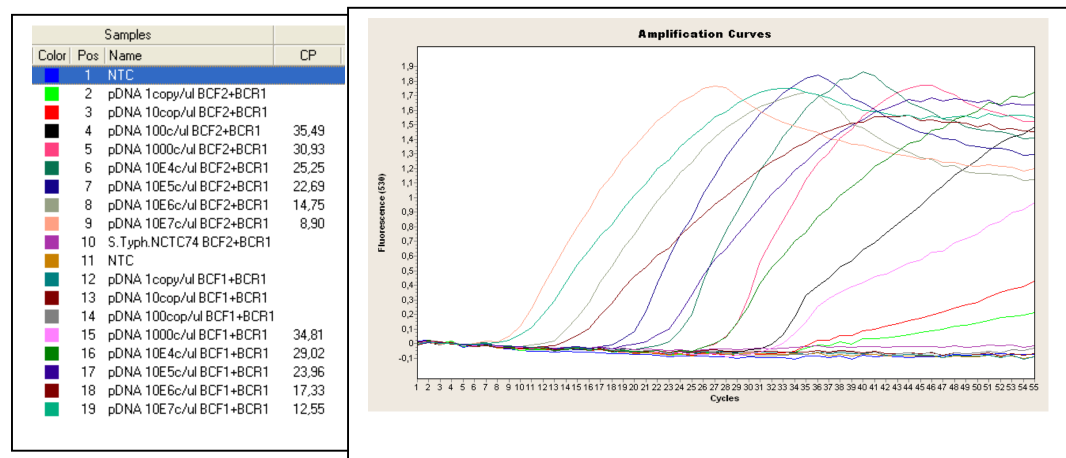


Figure 31. Results of RT-PCR reactions using 0.8µM BCFomp2/BCRomp1 (samples 1-9) and 0.8µM BCFomp1/BCRomp1 (samples 11-19) primers at the same reaction conditions

1. NTC; 2. 1 gene copy number; 3. 1x10 gene copy numbers; 4. $1x10^2$ gene copy numbers;

5. $1x10^3$ gene copy numbers; 6. $1x10^4$ gene copy numbers; 7. $1x10^5$ gene copy numbers;

8. $1x10^7$ gene copy numbers; 9. $1x10^8$ gene copy numbers; 10. *S.* Typhimurium NCTC 74;

11. NTC; 12. 1 gene copy number; 13. 1x10 gene copy numbers; 14. $1x10^2$ gene copy numbers;

15. $1x10^3$ gene copy numbers; 16. $1x10^4$ gene copy numbers; 17. $1x10^5$ gene copy numbers;

18. $1x10^6$ gene copy numbers; 19. $1x10^7$ gene copy numbers;

Annealing temperature: 59°C; *S.* Typhimurium NCTC 74-negative control; NTC-No Template Control

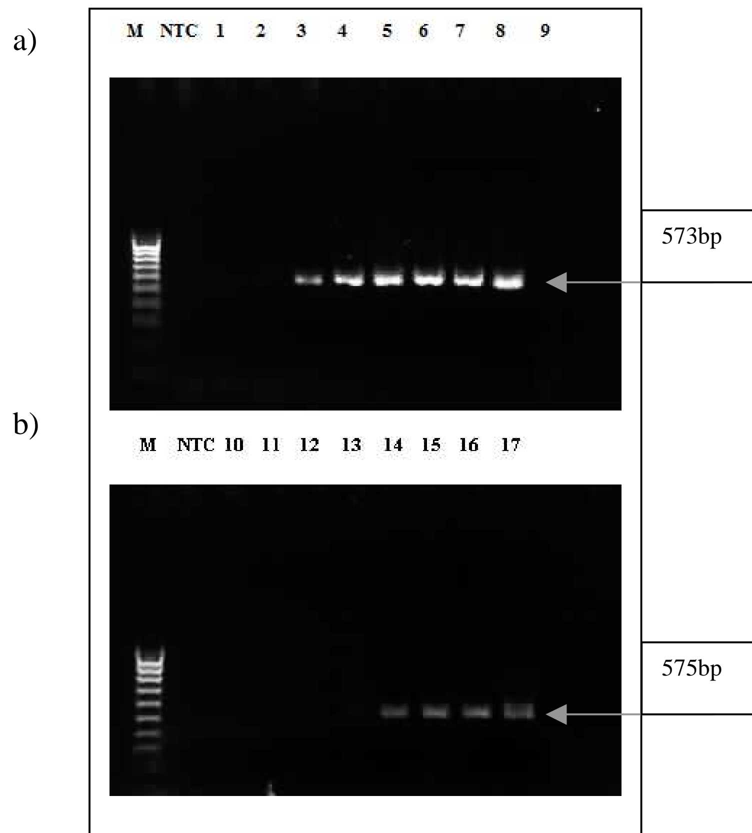


Figure 32. RT-PCR products run on 1% agarose gel with BCFomp2/BCRomp1 (a) and BCFomp1/BCRomp1 (b) primers

(a): 1. 1 gene copy number; 2. 1x10 gene copy numbers; 3. $1x10^2$ gene copy numbers; 4. $1x10^3$ gene copy numbers; 5. $1x10^4$ gene copy numbers; 6. $1x10^5$ gene copy numbers; 7. $1x10^6$ gene copy numbers; 8. $1x10^7$ gene copy numbers; 9. gDNA *Salmonella* Typhimurium NCTC 74; (b): 10. 1 gene copy number; 11. 1x10 gene copy numbers; 12. $1x10^2$ gene copy numbers; 13. $1x10^3$ gene copy numbers; 14. $1x10^4$ gene copy numbers; 15. $1x10^5$ gene copy numbers; 16. $1x10^6$ gene copy numbers; 17. $1x10^7$ gene copy numbers;

Every reaction was run with 0.8µM of each primer;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

The optimized RT-PCR reaction conditions were as follows:

DNA	5µl	95ºC 10min
LightCycler® TaqMan® Master	4µl	30 cycles:
MotB-FAM-1 (2µM)	0.7µl	95ºC 10sec
BCFomp2 (10µM)	1.6µl	59ºC 40sec
BCRomp2 (10µM)	1.6µl	72ºC 1sec (single slop)
H_2O	To 20µl	
		40ºC 30sec

Primers BCFomp2/BCRomp2 were analyzed for their specificity, selectivity and ability to detect 130 strains of *B. cereus* group species by RT-PCR reaction. The completed results are shown in a Supplementary Table A. Under described conditions all tested strains belonging to *B. cereus*, *B. thuringiensis*, *B. anthracis* and transitional strains showed positive results when MotB-FAM-1 probe was used in the reaction. The fluorescence signal from the probe was detectable by channel F1 in the LightCycler®1.2 and the product size checked on 1% agarose was as expected. None of the *B. weihenstephanensis* and *B. pseudomycoides* gave a fluorescence signal which shows the equivalent to a negative result when working with RT-PCR. As seen from the Figure 33, *B. weihenstephanensis* DSM 11821 and *B. pseudomycoides* DSM 12442 together with no template control (NTC) did not present peaks of fluorescence. The rest of the five strains belonging to the *B. cereus* group spp. and *Bacillus* sp. Ba813$^+$ amplified and the following Cp values and quantity of gene copy numbers calculated by LightCycler® software were displayed:

Strain	**Cp value**	**Calculated quantity of gene copy numbers**
B. cereus ATCC 14579	13,65	$2.71x10^7$
B. thuringiensis DSM 6017	12,06	$8.08x10^7$
B. anthracis 34F2	18,25	$1.12x10^6$
Bacillus Ba813$^+$ #19 (T5 97-77)	14,23	$1.82x10^7$
B. mycoides 6A68	16,15	$4.84x10^6$
B. weihenstephanensis DSM 11821	-	-
B. pseudomycoides DSM 12442	-	-

Despite the RT-PCR with MotB-FAM-1 probe did not work for all *B. weihenstephanensis*, the product run on 1% agarose gel after the assay showed the expected product (285bp). The reason for the negative result with the RT-PCR was that the fluorescence signal was not emitted during the real-time reaction from all *B. weihenstephanensis* strains because of single nucleotide polymorphisms (SNP) within the sequence for MotB-FAM-1 probe hybridization resulting in lack of probe hybridization (Appendix F). This result was detected after the sequencing of the PCR product. *B. pseudomycoides* strains were not detected during work with the BCFomp2/BCRomp2 primers and MotB-FAM-1 probe. Moreover, the RT-PCR products were not visible on agarose gel what will be described later.

Amplification with extracted DNA from *B. mycoides* strains showed various results. Only 2 (6A49 and 6A68) from 20 x *B. mycoides* and 4 (PID 1/21, PID 2/43, PID 3/2, 1/1) from 10 x *B. mycoides/pseudomycoides* strains showed positive fluorescence signal and expected product size (285bp) using MotB-FAM-1 probe. Amplicon for the rest of the strains was not detected using MotB-FAM-1 probe however was visible on 1% agarose gel for: 13 x *B. mycoides* and 2 x *B. mycoides/pseudomycoides* strains (Supplementary Table A).

The conclusion to date shows that the new MotB-FAM-1 probe and redesigned BCFomp2/BCRomp2 primers could identify all *B. cereus*, *B. thuringiensis*, *B. anthracis*, *Bacillus* sp. Ba813$^+$ and only 2 from 20 x *B. mycoides* and 4 from 10 x *B. mycoides/pseudomycoides* strains. *B. weihenstephanensis* and *B. pseudomycoides* strains were not detectable by RT-PCR using MotB-FAM-1 probe.

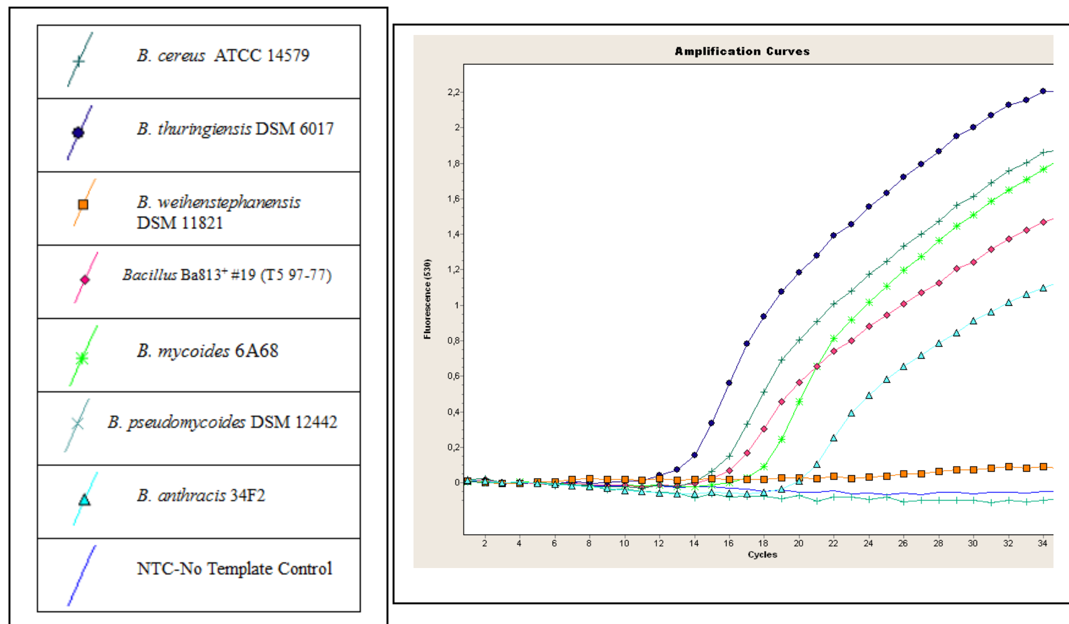


Figure 33. Results of RT-PCR assay with BCFomp2/BCRomp2 primers and MotB-FAM-1 probe

Cp values: 13,65 for *B. cereus* ATCC 14579; 12,06 for *B. thuringiensis* DSM 6017; 18,25 for *B. anthracis* 34F2; 14,23 for *Bacillus* Ba813$^+$ #19 (T5 97-77); 16,15 for *B. mycoides* 6A68

3.3.4. Differentiation of *B. weihenstephanensis* from other members of *Bacillus cereus* group with non rhizoid growth

Working with real-time PCR using BCFomp2/BCRomp2 primers and MotB-FAM-1 TaqMan probe, no positive fluorescence signal was achieved for 17 x *B. weihenstephanensis* strains used in this study. The LightCycler®1.2 did not report the signal from the MotB-FAM-1 probe which meant lack of hybridization between probe and the target DNA. However, the products of RT-PCR checked by 1% agarose gel electrophoresis in 0.5xTBE buffer showed the 285bp product amplified with BCFomp2/BCRomp2.

Four strains of *B. weihenstephanensis*: WSBC 10392, WSBC 10416, WSBC 10201 and WSBC 10389 were used as a target to amplifying and sequencing the 575bp product generated with BCFomp1/BCRomp1 primers. Sequences analyzed by MWG were compared against two strains with complete genome sequences available in NCBI (National Center for Biotechnology Information): *B. weihenstephanensis* KBAB4 (GenBank accession number: NC_010184) and *B. cereus* ATCC 14579 (NC_004722) in 2010. The results are presented in Figure 34.

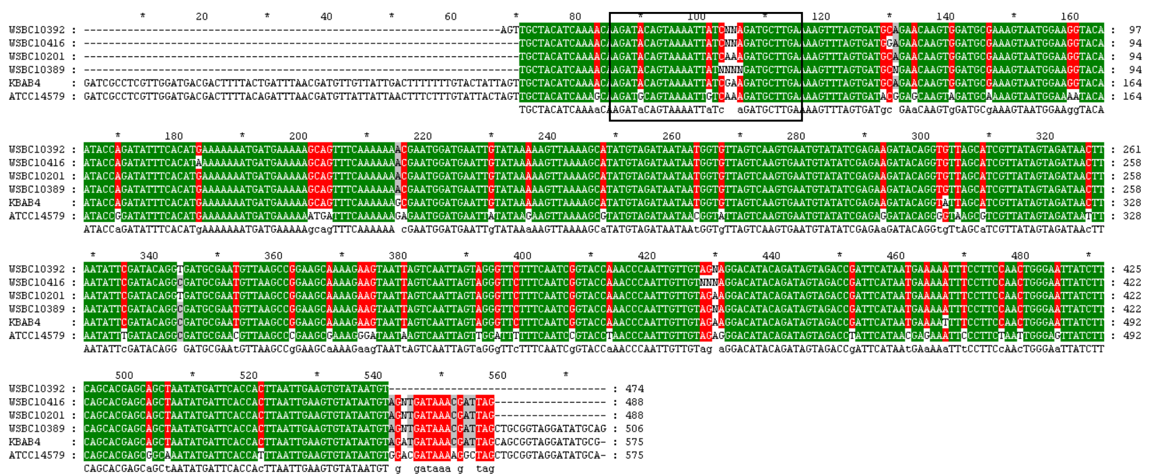


Figure 34. Comparison of 575bp PCR products generated using BCFomp1/BCRomp1 primers with selected conserved sequence for MotB-FAM-1 TaqMan probe hybridization

Comparised strains: *B. weihenstephanensis* WSBC 10392, *B. weihenstephanensis* WSBC 10416, *B. weihenstephanensis* WSBC10201, *B. weihenstephanensis* WSBC 10389, *B. weihenstephanensis* KBAB4, *B. cereus* ATCC 14579

Analyzes of the sequenced fragments with special emphasis on the MotB-FAM-1 probe hybridization sequence demonstrated a single nucleotide polymorphisms (SNPs) in this fragment. Because SNPs were similar in all analyzed *B. weihenstephanensis* strains and located at the same positions in the DNA sequence (Figure 35), the TaqMan probe was redesigned and used for the identification of *B. weihenstephanensis* strains with BCFomp2/BCRomp2 primers.

```
WSBC_10201 : ATACAGTAAAATTATCAAAGATGCTTGAA : 29
WSBC_10416 : ATACAGTAAAATTATCNNAGATGCTTGAA : 29
WSBC_10392 : ATACAGTAAAATTATCNNAGATGCTTGAA : 29
WSBC_10389 : ATACAGTAAAATTATNNNNGATGCTTGAA : 29
B.w._KBAB4 : ATACAGTAAAATTATCGAAGATGCTTGAA : 29
ATCC_14579 : ATGCAGTAAAATTGTCAAAGATGCTTGAA : 29
             ATaCAGTAAAATTaTc   aGATGCTTGAA
```

Figure 35. Comparison of DNA fragments of *B. weihenstephanensis* and *B. cereus* for hybridization of TaqMan probes

In a 29 nucleotide sequence, 3 nucleotides were significant. First and second nucleotides (position 3rd and 14th) presented Adenine for 5 x *B. weihenstephanensis* strains instead of Guanine for *B. cereus* ATCC 14579. The third nucleotide (position 17th) for some *B. weihenstephanensis* was the same as for analyzed *B. cereus*, and it was Adenine. For the rest *B. weihenstephanensis* strains the nucleotide in this position was Guanine. From the sequencing results obtained from MWG for 3 x *B. weihenstephanensis* strains (WSBC 10416, WSBC 10392, and WSBC 10389) the nucleotide in position 17th was not clear and was not defined. However, analyzes of the peaks received after sequencing showed that the result was between Adenine and Guanine. The new, redesigned probe was named MotB-FAM-2 and its sequence 5'-3' is shown in Box 2 and Table 26. The 5'-end was labelled with fluoresceine (FAM), 3'-end was quenched with Black Berry Quencher (BBQ).

MotB-FAM-1: FAM-TTC AAG CAT CTT TGA CAA TTT TAC TGC AT--BBQ
MotB-FAM-2: FAM-TTC AAG CAT CTT YGA TAA TTT TAC TGT AT--BBQ
(Y=T/C)

Box 2. Comparison of the sequences of two TaqMan probes: MotB-FAM-1 and MotB-FAM-2

Table 26. Details of MotB-FAM-2 probe

Probe	**Probe sequence (5'-3')**	**Target in amplified product**	**GC content (%)**	**Tm (ºC)**
MotB-FAM-2	FAM-TTCAAGCATCTT(T/C)GATAATTTTACTGTAT-BBQ	114-86	24	52.1

The nucleotide in position 13th of the MotB-FAM-2 probe was wobbled (Y=T/C) because *B. weihenstephanensis* strains presented a double profile in this position. The next two nucleotides (position 16th and 27th) of the probe were changed from Cytosine into Thymine. The described changes in the probe were sufficient to achieve the fluorescence signal during work with *B. weihenstephanensis* in RT-PCR with BCFomp2/BCRomp2 primers (Appendix G). From the Figure 36, it can be seen that for 7 strains which represent the *B. cereus* group spp. only *B. weihenstephanensis* DSM 11821 was detectable by MotB-FAM-2 probe and the Cp value was 13,32 which corresponded with $3.40x10^7$ gene copy numbers.

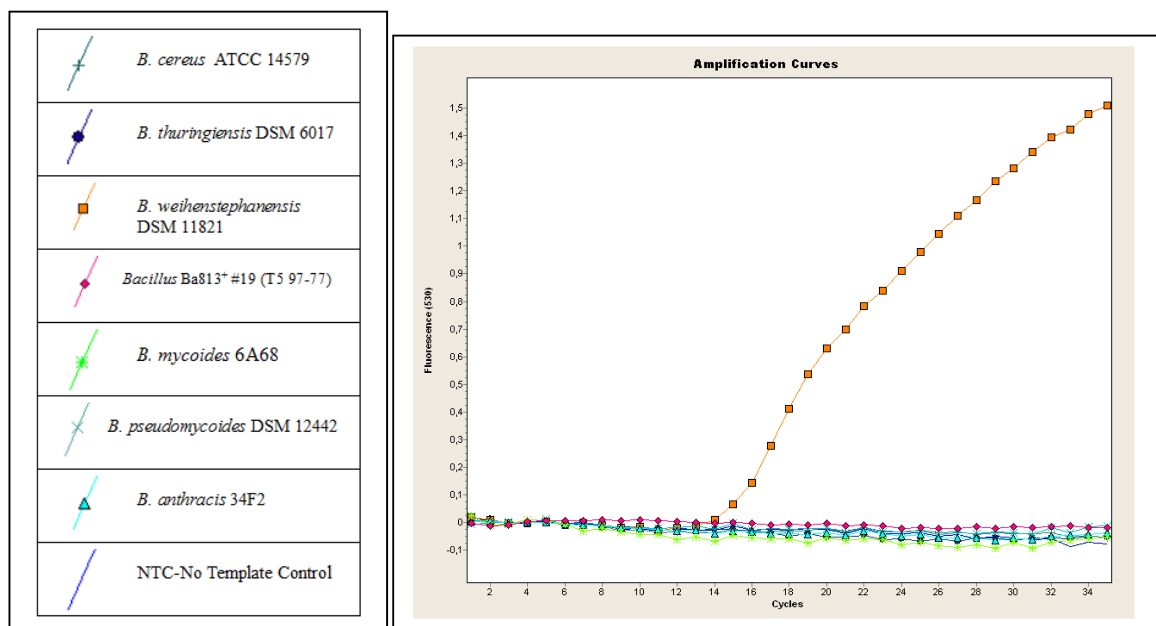


Figure 36. Results of RT-PCR with BCFomp2/BCRomp2 primers and MotB-FAM-2 probe

The optimized RT-PCR reaction conditions were as follows:

DNA	5µl	95ºC 10min
LightCycler® TaqMan® Master	4µl	30 cycles:
MotB-FAM-2 (2µM)	0.7µl	95ºC 10sec
BCFomp2 (10µM)	1.6µl	59ºC 40sec
BCRomp2 (10µM)	1.6µl	72ºC 1sec (single slop)
H_2O	To 20µl	
		40ºC 30sec

MotB-FAM-2 emitted the signal from 17 x *B. weihenstephanensis* strains. None of the tested *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. pseudomycoides* and transitional strains gave the positive RT-PCR result. Amplification with DNA extracted from 15 x *B. mycoides* and 6 x *B. mycoides/pseudomycoides* strains generated the product and fluorescence signal using either probe MotB-FAM-1 or MotB-FAM-2 (Supplementary Table A). However, 5 x *B. mycoides* and 4 x *B. mycoides/pseudomycoides* did not react with both probes and amplification product was not generated. Those strains were further discriminated as *B. pseudomycoides*, and are described later.

The conclusion from this work section showed that the real-time analysis with BCFomp2/BCRomp2 and probe MotB-FAM-2 allowed for simple differentiation *B. weihenstephanensis* strains from other strains belongs to *B. cereus* group with non

rhizoid growth. *B. mycoides* that were identified in reactions using probe MotB-FAM-2 were easily discriminated from *B. weihenstephanensis* by their rhizoid growth on agar plates. *B. weihenstephanensis* as a psychrotolerant strain able to grow at 4ºC and can be major problem in milk product contamination. Pasteurised milk is a common source for isolating of this bacterium.

The assay was tested with an environmental medium e.g. milk. Overall our results showed that in the bacterial identification the milk samples were always plated on PEMBA/MYP media first and any *B. mycoides* or *B. pseudomycoides* strain which grew on the agar presented rhizoid colonies. Colonies without rhizoid growth were identified as *B. cereus*, *B. thuringiensis*, *B. anthracis* or *B. weihenstephanensis* strains and tested by RT-PCR using probe MotB-FAM-1 and/or MotB-FAM-2. Moreover *B. weihenstephanensis* strains were differentiated by amplification with MotB-FAM-2. RT-PCR assay applying MotB-FAM-2 in analysis allowed for rapid identification and differentiation of the *B. weihenstephanensis* strains isolated from milk.

Those results were confirmed in traditional PCR reaction with primers BcF2 and CSPU3 designed by Lechner *et al.* (1998) against the *cspA* gene encodes a major cold shock protein. The primers allowed for differentiation the *B. weihenstephanensis* strains by PCR. Based on their results we could compare our results in *B. weihenstephanensis* differentiation. However, their results did not demonstrate the quantification and limit of detection which were analyzed in our study.

The genomes of the various *Bacillus* species are highly conserved, which makes the differentiation of species highly challenging. However, in this study based on a single nucleotide polymorphisms (SNPs) the RT-PCR assay was found to differentiate *B. weihenstephanensis* from other *B. cereus* group spp. with non rhizoid growth. SNPs are individual base positions in the genome that show natural variation in the population. As Wattiau and Fretin (2006) mentioned, the RT-PCR based on 5' exonuclease activity of *Taq* polymerase (TaqMan assay) is the most common method used for SNPs detection. The advantage of this assay is that it only requires a one step enzymatic reaction. Twyman (2005, pp.1202-1207) reported that the discriminating between alleles at SNP locus is called allele-specific hybridization and is based on hybridization to genomic regions that differ at the SNP site using probes specific to each allele. A single-base mismatch is sufficient to prevent hybridization of the nonmatching probe. Primer binds upstream of the targeted SNP and *Taq* polymerase initiated DNA extension. If the probe matched the target DNA, then the DNA is

extended from the forward primer to the probe and polymerase cleaves and releases the 5'-end of the probe attached with a fluorescent dye. This release results in a fluorescence. Mismatches between probe and target reduce the efficiency of probe hybridization; the probe does not hybridize to the target and the extension of the forward primer does not cut the fluorescent molecule from the probe.

The conclusion from this work section showed that the new redesigned MotB-FAM-2 probe made possible the detection and differentiation of the *B. weihenstephanensis* strains from other strains with non rhizoid growth belonging to the *B. cereus* group spp. This ability was especially helpful in detection of *B. weihenstephanensis*, which is psychrotolerant species, from milk samples stored at 4℃.

3.3.5. Identification of the *B. cereus* group species, excluding *B. pseudomycoides*, by RT-PCR reaction using two TaqMan probes

RT-PCR amplification using BCFomp2/BCRomp2 primers and probe MotB-FAM-1 could identify *B. cereus*, *B. thuringiensis*, *B. anthracis*, transitional Ba813^{+} and some *B. mycoides* strains. With the same condition but with the second newly designed probe MotB-FAM-2, it was possible to identify all tested *B. weihenstephanensis* and the remaining *B. mycoides* strains not identified by the MotB-FAM-1 probe. None of the *B. pseudomycoides* strains were identified using the two probes.

To research the possibility of identify all those strains in one multiplex reaction, both probes were used in the same RT-PCR master mix. The reaction was optimized using different probe concentration: 0.025µM, 0.035µM and 0.07µM of each probe per reaction. DNA extracted from *B. cereus* ATCC 14579 and *B. weihenstephanensis* WSBC 10389 with unknown concentration was used as a template. In the reactions the DNA presenting $1x10^{6}$ *motB* gene copy numbers was included as a control and as standard dilution, furthermore used as a template to estimate the gene copy numbers of particular unknown samples based on standard curve saved in a software. Results of the RT-PCR using different concentrations of two probes in one reaction are outlined in Table 27.

Table 27. RT-PCR results with different concentrations of MotB-FAM-1 and MotB-FAM-2 probes used in one reaction

	B. cereus **ATCC 14579**	***B. weihenstephanensis*** **WSBC 10389**	**$1x10^6$ *motB* gene copy numbers (*B. cereus* ATCC 14579)**	**$1x10^6$ *motB* gene copy numbers (*B. weihenstephanensis* WSBC 10389)**
MotB-FAM-1 (0.07µM)	12.03 ($1.19x10^8$)	–	18.97 ($1x10^6$)	–
MotB-FAM-2 (0.07µM)	–	16.96 ($5.65x10^6$)	–	18.77 ($1x10^6$)
MotB-FAM-1 + MotB-FAM-2 (0.025µM each)	<u>11.77 ($1.21x10^8$)</u>	15.28 ($1.81x10^7$)	N/T	N/T
MotB-FAM-1 + MotB-FAM-2 (0.035µM each)	13.62 ($3.98x10^7$)	<u>17.04 ($5.34x10^6$)</u>	N/T	N/T
MotB-FAM-1 + MotB-FAM-2 (0.07µM each)	12.46 ($8.88x10^7$)	N/T	N/T	N/T

The best results are underlined; N/T-Not Tested; "– "-Negative reaction

Two probes: MotB-FAM-1 and MotB-FAM-2 used in one RT-PCR reaction with BCFomp2/BCRomp2 primers were able to give a fluorescence signal and identify the *B. cereus* group spp. except the *B. pseudomycoides* strains. Those two probes used together in a final concentration: 0.025µM MotB-FAM-1 and 0.035µM MotB-FAM-2 gave the same sensitivity as when used separately with the final concentration 0.07µM. The optimized reaction conditions were as follows:

DNA	5µl	95ºC 10min
LightCycler® TaqMan® Master	4µl	<u>30 cycles:</u>
MotB-FAM-1 (2µM)	0.25µl	95ºC 10sec
MotB-FAM-2 (2µM)	0.35µl	59ºC 40sec
BCFomp2 (10µM)	1.6µl	72ºC 1sec (single slop)
BCRomp2 (10µM)	1.6µl	
H_2O	To 20µl	40ºC 30sec

These probes concentrations were used in RT-PCR with DNA extracted from strains representing each species belonging to the *B. cereus* group spp.: *B. cereus* ATCC 14579, *B. thuringiensis* DSM 6017, *B. anthracis* 34F2, *Bacillus* Ba813$^+$ #19 (T5 97-77),

B. mycoides 6A68, *B. weihenstephanensis* DSM 11821 and *B. pseudomycoides* DSM 12442 (Figure 37).

Achieved results were as follows:

Strain	**Cp value**	**Calculated quantity of gene copy numbers**
B. cereus ATCC 14579	13,77	$3.19x10^7$
B. thuringiensis DSM 6017	12,40	$8.28x10^7$
B. anthracis 34F2	18,57	$1.44x10^6$
Bacillus Ba813$^+$ #19 (T5 97-77)	14,20	$2.24x10^7$
B. mycoides 6A68	16,07	$5.08x10^6$
B. weihenstephanensis DSM 11821	13,54	$3.30x10^7$
B. pseudomycoides DSM 12442	-	-

The achieved values are comparable to results of RT-PCR with 0.07µM probe for the same strains (Figure 33, Figure 36).

B. pseudomycoides DSM 12442 did not present the positive reaction in each assay.

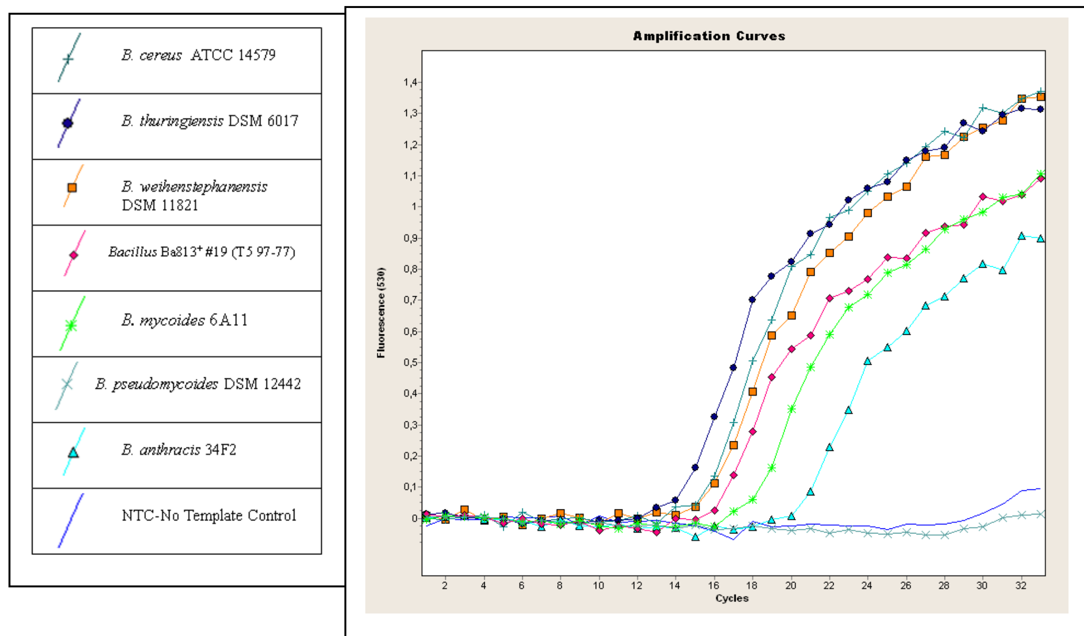


Figure 37. Results of RT-PCR assay with BCFomp2/BCRomp2 primers and 0.025µM MotB-FAM-1 and 0.035µM MotB-FAM-2

Cp values: 13,77 for *B. cereus* ATCC 14579; 12,40 for *B. thuringiensis* DSM 6017;

13,54 for *B. weihenstephanensis* DSM 11821; 14,20 for *Bacillus* Ba813$^+$ #19 (T5 97-77);

16,07 for *B. mycoides* 6A68; 18,57 for *B. anthracis* 34F2

3.3.6. Creation of a RT-PCR standard curve and limit of detection for BCFomp2/BCRomp2 primers and MotB-FAM-1/MotB-FAM-2 probes

To determine the quantitative information of the unknown sample a standard curve using DNA of known concentration is required. When the standard curve has been generated it can be used as a reference standard in further assay.

The fragment of *motB* amplicon (575bp) of *B. cereus* ATCC 14579, *B. cereus* NCTC 7464 and *B. weihenstephanensis* WSBC 10389 was generated by PCR using the primer set BCFomp1/BCRomp1 and cloned into pGEM-T Easy Vector system (Figure 38).

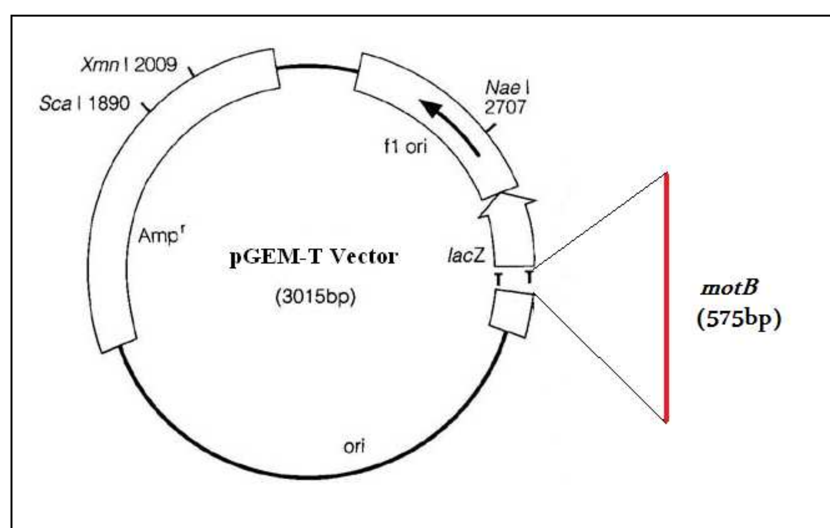


Figure 38. Scheme of 575bp fragment of *motB* gene cloned into pGEM-T Easy Vector

The PCR product was ligated with 50ng of vector DNA at 1:3 molar ratio of vector:insert. The ligated product was transformed into *E. coli* JM 109 cells by electro shock. Possible transformants that grew on LB plates with addition of ampicillin (100µg/ml) were analyzed by PCR for the target gene with BCFomp1/BCRomp1 or BCFomp2/BCRomp2 primers specific to the cloned fragment *motB* gene to confirm the correct insert. DNA was extracted by heating method and 5µl of supernatant was used as DNA template. Only clones positive for insert were used to extract the recombinant plasmid (pGEM-*motB*) using the Promega plasmid kit (Wizard Plus SV Minipreps) and confirming PCR reactions with the same set primers were carried out. A second confirming test which included the enzymatic digestion of recombinant plasmid was

carried out. pGEM-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzyme *EcoRI* providing a single enzyme digestion for release of the insert. After incubation of the plasmid in optimal for enzyme activity conditions the result of the digestion was checked on 1.5% agarose gel. Recombinant clones presented two bands: 601bp (insert+flanking sequence) and 2989bp (plasmid). pGEM-T Easy Vector without an insert presented 3015bp band. The results are presented in Figure 39.

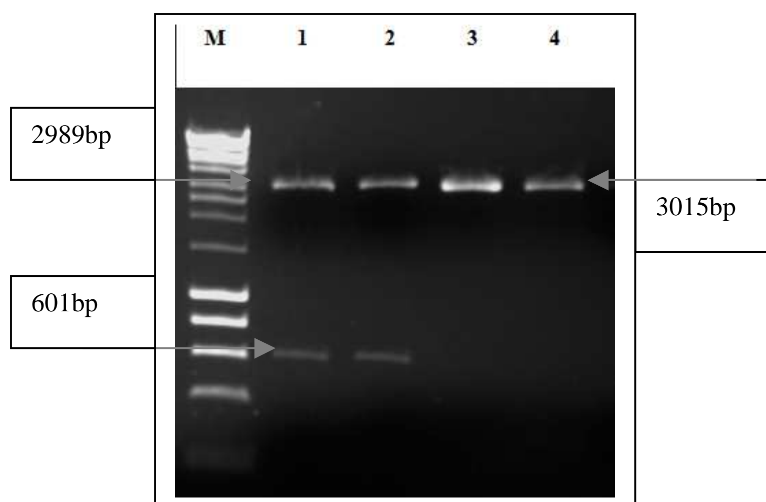


Figure 39. Results of enzymatic digestion of pGEM-*motB* with *EcoRI*

1-2) pGEM-*motB*; 3-4) pGEM-T Easy Vector without an insert;
M-Molecular marker (HyperLadder I)

Two positive clones from each cloning were sequenced by Eurofins MWG Operon. Both, T7 (forward) and SP6 (reverse) primers were used for sequencing.

The DNA concentration was determined by measuring the absorbance at 260nm. DNA purity was measured by calculating the ratio of absorbance at 260-280nm (Vis NanoDrop-1000, Fisherbrand, Fisher Scientific, Ireland). Calculation of the gene copy numbers was based on the assumption that the average weight of a base pair (bp) is 650 Da. The amount of the template should be expressed in molecules (copies) of DNA. Conversion the mass to molecules was done by using the formula (Dorak 2006, p.41):

$$\frac{\text{Mass (in grams) x Avogadro's Number}}{\text{Average mol. wt. of a base x template length}} = \text{molecules (copies) of DNA}$$

Ten-fold serial dilutions of the standard (1– $1x10^{10}$ gene copy numbers) were made in sterile water or 10mM Tris-HCl pH 8.0, aliquoted and stored at -20°C until use. Each dilution was thawed only once immediately before use.

The establishment of a standard curve using quantitative real-time PCR (qRT-PCR) process is the key step in determining the copy number of a given target sequence. A dilution series ($1x10^{10}$-1 gene copy numbers/20µl reaction) of recombinant plasmid were tested in triplicates by the real-time assay using LightCycler® system. The obtained Cp values were plotted against log-transformed concentrations of serial ten-fold dilutions of the target. After each amplification the LightCycler® software (version 4.1) produced the standard curve by measuring the crossing point of each standard and plotting them against the logarithmic values of construction (Figure 40).

Cp values from each reaction were calculated to achieve one average value for every dilution. The results are presented in Table 28. The standard deviation for each value of gene copy number was evaluated.

The results showed that significant signals were detected between $1x10^{10}$- 1x10 gene copy numbers. For the target sequence, in assay using BCFomp2/BCRomp2 primers, as little as 1x10 copies per reaction could be detected. Whereas during standard PCR reaction with the same primers, the limit of detection was $1x10^3$ gene copy numbers (data not shown) per reaction (25µl). Data analysis showed that in all experiments with BCFomp2/BCRomp2 primers and MotB-FAM-1 probe, the mean amplification average efficiency was E=2.0489 ± 0.078. It corresponds to 104.8% efficiency where the average slope was -3.21. Obtained average efficiency of the reaction showed the results were exceptional.

The correlation (R^2) between Cp value and $\log_{\text{gene copy number}}$ was 0,99. This linear relationship makes the Cp value a reliable way to estimate gene copy number.

The efficiency of MotB-FAM-2 probe was compared with the efficiency of MotB-FAM-1 probe. Serial dilutions of recombinant plasmid with fragment of *motB* gene of *B. weihenstephanensis* WSBC 10389 showed the same averaged Cp values and the limit of detection was 1x10 gene copy numbers per reaction.

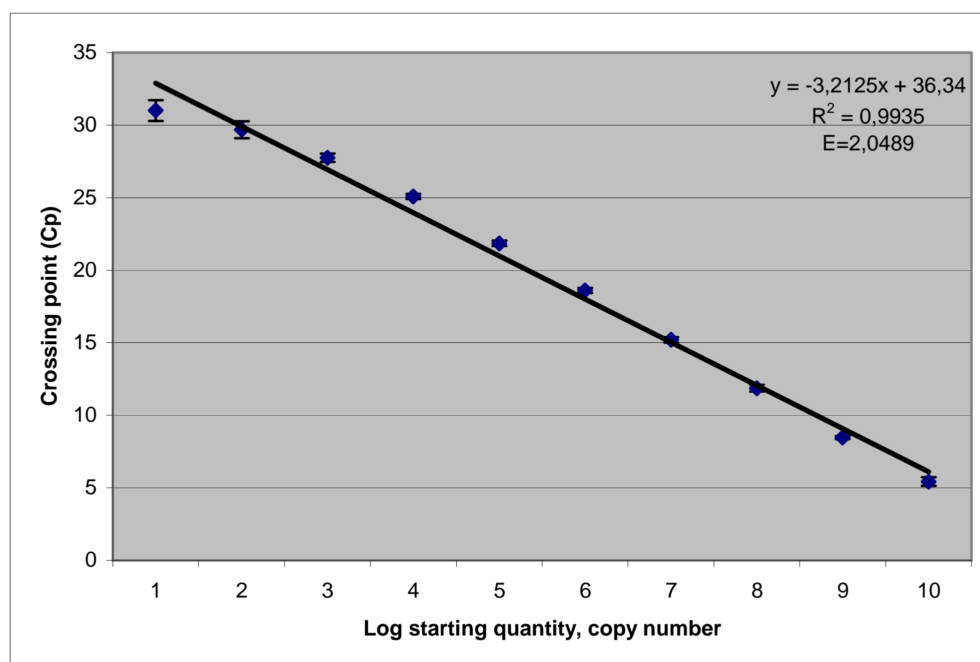


Figure 40. Standard curve for determination of *motB* gene copy numbers using BCFomp2/BCRomp2 primers and MotB-FAM-1/MotB-FAM-2 probes

Crossing point values plotted against the log of the initial template DNA concentration. ± One standard deviation is indicated for each gene copy number. Average efficiency of every RT-PCR amplification was E=2.0489 ± 0.078

Table 28. Average Cp values for RT-PCR assay with BCFomp2/BCRomp2 and MotB-FAM-1/MotB-FAM-2 probes

Gene copy numbers	Average Cp value of TaqMan assay with BCFomp2/BCRomp2 primers and MotB-FAM-1/MotB-FAM-2 probes
$1x10^{10}$	6,31 ± 0,59
$1x10^{9}$	8,46 ± 0,21
$1x10^{8}$	11,86 ± 0,46
$1x10^{7}$	15,2 ± 0,37
$1x10^{6}$	18,61 ± 0,35
$1x10^{5}$	21,85 ± 0,38
$1x10^{4}$	25,08 ± 0,36
$1x10^{3}$	27,75 ± 0,56
$1x10^{2}$	29,68 ± 1,18
1x10	31,02 ± 1,42

3.3.7. New primers for *B. pseudomycoides* identification and differentiation from the *B. cereus* group spp.

Because *B. pseudomycoides* was not detectable by PCR and RT-PCR using BCFomp2/BCRomp2 primers targeting the *motB* gene, the new gene target was selected for this species. Unique DNA regions for primers hybridization were identified after blasting the genome sequence draft of *B. pseudomycoides* DSM 12442 (GenBank accession number: NZ_CM000745) against other *B. cereus* group species sequences available on: http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi in 2010. A 220bp fragment was selected for *B. pseudomycoides* as unique for PCR analysis. In this study the selected fragment was called the *Bpm* gene. The result of blasting this sequence against other *B. cereus* group spp. available on line is presented in Appendix I. This DNA fragment encodes the hypothetical protein whose structure, function and protein family is unknown. Based on the 220bp unique sequence the selection of primers was done manually using generally known guidelines described earlier. Four different primers were designed and tested in various combinations: BpmF/BpmR, BpmF/BpmR2 and BpmF2/BpmR2. The details of the primers are outlined in Table 29. The product sizes generated with particular sets of primers are outlined in Box 3.

Table 29. The details of BpmF, BpmR, BpmF2 and BpmR2 primers

Primer	Primer sequence (5'-3')	Target in amplified product	GC content (%)	Tm (˚C)
BpmF	TAATTTAGGGGGGCATCTTTACTTTTC	1-27	37	55.3
BpmR	TTTCTATACCCAAAACTTAGATATGCTCATG	220-190	32.3	55.2
BpmF2	GTACATCAATTCAATCATTCAATAGA	87-112	26.9	49.8
BpmR2	CTATACCCAAAACTTAGATATGCTC	217-193	34.6	52.2

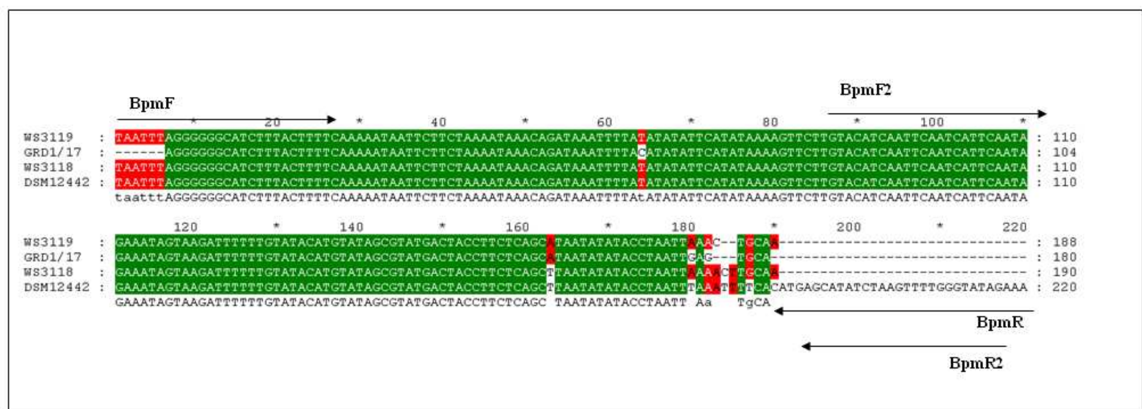
Primer combinations	Size of amplified product
BpmF/BpmR	220bp
BpmF/BpmR2	217bp
BpmF2/BpmR2	134bp

Box 3. Size of PCR product generated with different primers for *B. pseudomycoides* detection

When this study was underway (2007-2010) there were no *B. pseudomycoides* strains with complete genome sequences available for '*in silico* PCR'. *In silico* positive testing of the BpmF/BpmR and BpmF/BpmR2 could not be determined and were checked only for negative results against the 31 available in 2010 sequenced strains belonging to *Bacillus* genera. None of those strains presented a 220bp and 217bp, respectively, amplification product, even with two allowed mismatches (Appendix J). Our results therefore demonstrated that the newly designed primers are presently specific for *B. pseudomycoides*.

Using BpmF/BpmR primers the three 220bp fragments of *B. pseudomycoides* WS 3118, *B. pseudomycoides* WS 3119 and *B. mycoides/pseudomycoides* GRD 1/17 were sequenced and compared with the draft sequence of *B. pseudomycoides* DSM 12442 available on line in NCBI.

The results are outlined in Box 4. The positions of different primers (BpmF, BpmR, BpmF2, and BpmR2) in the PCR amplicon are also demonstrated.



Box 4. Comparison of 220bp PCR products generated with BpmF/BpmR primers

The places of hybridization of particular primers are marked with arrows

After optimization the reaction parameters with BpmF/BpmR primers, the BpmF/BpmR2 primers were further checked. Using the same *B. pseudomycoides* strains the expected 217bp product was observed. The reaction conditions were optimized and both sets of primers were applied for the reaction with DNA extracted from different *B. cereus* group species. In each case the described primers showed the *B. pseudomycoides* specificity and selectivity. Nine tested *B. pseudomycoides* strains showed expected products. None of the other *B. cereus* group spp. strains presented the amplicon. It also included 15 x *B. mycoides* and 6 x *B. mycoides/pseudomycoides* strains tested (Supplementary Table A).

By date, the only technique that can differentiate *B. mycoides* and *B. pseudomycoides* strains is gas chromatographic (GC) fatty acid methyl ester analysis (Nakamura 1998). With GC species can be distinguished from *B. mycoides* by differences in fatty acid levels. It is the only existing technique for differentiation those species.

Our results also showed that 4 x *B. mycoides/pseudomycoides* strains: GRD 1/17, GRD 2/71, 1/2, 17/3 which did not present the product with earlier described BCFomp2/BCRomp2 primers showed the expected 220bp and 217bp products with the new unique for *B. pseudomycoides* primers (BpmF/BpmR and BpmF/BpmR2).

The similar results were obtained with 5 strains defined as *B. mycoides*: Nov1, Nov2, 6A19, A81, DSM 307 (Figure 41). Because strains presented product with BpmF/BpmR and BpmF/BpmR2 primers, this result concludes that those five strains are *B. pseudomycoides* strains and not *B. mycoides*.

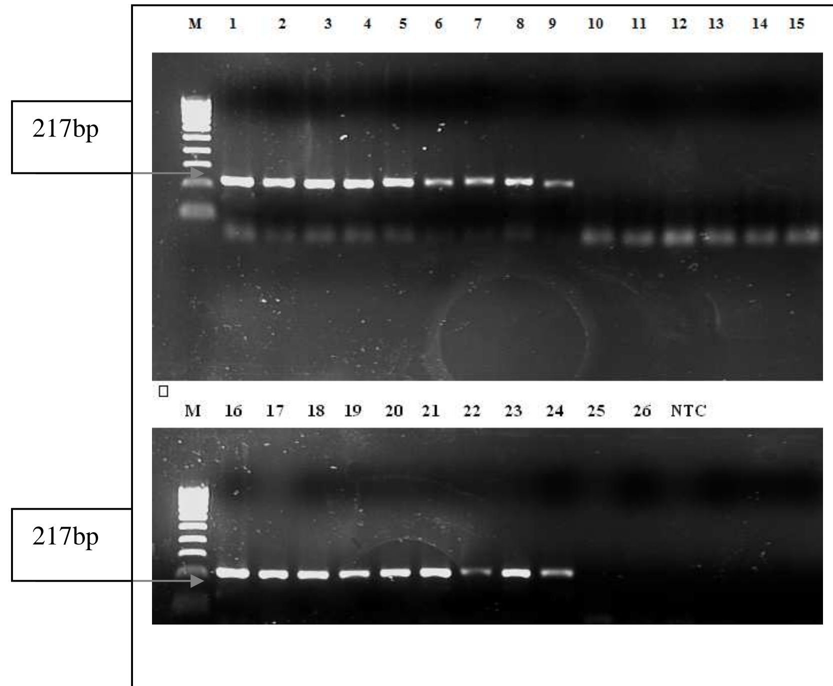


Figure 41. 1% agarose gel showing 217bp PCR products generated with BpmF/BpmR2 primers

1. *B. pseudomycoides* DSM 12442; 2. *B. pseudomycoides* DSM 12443; 3. *B. pseudomycoides* WS 3118; 4. *B. pseudomycoides* WS 3119; 5. *B. pseudomycoides* B346; 6. *B. pseudomycoides* B618; 7. *B. pseudomycoides* TP1; 8. *B. pseudomycoides* DA; 9. *B. pseudomycoides* A82; 10. *B. cereus* ATCC 14579; 11. *B. thuringiensis* DSM 6017; 12. *B. weihenstephanensis* DSM 11821; 13. *B. anthracis* 34F2; 14. *B. mycoides* 6A11; 15. *Bacillus* Ba813$^+$ #25 (97-27); 16. *B. mycoides* Nov1; 17. *B. mycoides* 6A19; 18. *B. mycoides* Nov2; 19. *B. mycoides* DSM 307; 20. *B. mycoides/pseudomycoides* GRD 1/17; 21. *B. mycoides/pseudomycoides* 1/2; 22. *B. mycoides/pseudomycoides* GRD 2/71; 23. *B. mycoides/pseudomycoides* 17/3; 24. *B. mycoides* A81; 25. *B. licheniformis* BLMUL1; 26. *B. subtilis* BSFUL1;
NTC-No Template Control; M-Molecular marker (HyperLadder IV)

For the two new unique set of primers (BpmF/BpmR and BpmF/BpmR2) the limit of detection was compared using DNA extracted from an overnight colony of *B. pseudomycoides* DSM 12442 as a template. Serial dilutions were made to evaluate the CFU/ml and DNA was extracted from the dilutions ranging from 1 to 10^5 CFU/ml using the method of Hansen *et al.* (2001). The achieved limit of detection for BpmF/BpmR was 10^4 CFU/ml (Figure 42, lane 2) whereas for BpmF/BpmR2 10^3 CFU/ml (Figure 43, lane 3). Because the reaction with BpmF/BpmR2 primers presented higher sensitivity, those primers were used in RT-PCR for species specific identification of *B. pseudomycoides* strains. There were also used in multiplex PCR for identification of the *B. cereus* group species. Both assays are described later.

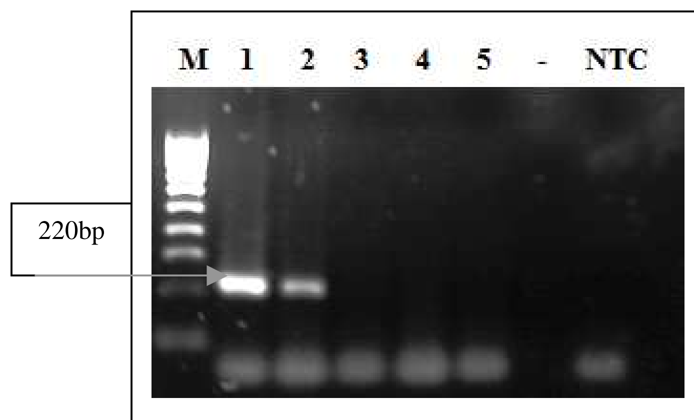


Figure 42. Sensitivity of the PCR reaction with BpmF/BpmR primers
1. 10^5 CFU/ml per reaction; 2. 10^4 CFU/ml per reaction; 3. 10^3 CFU/ml per reaction;

4. 10^2 CFU/ml per reaction; 5. 10 CFU/ml per reaction;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

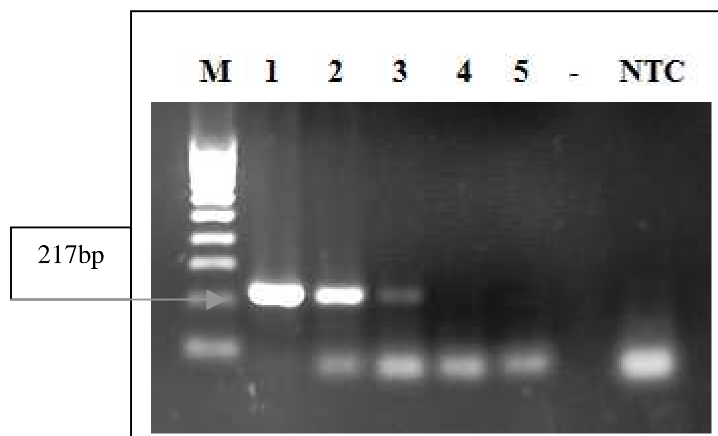


Figure 43. Sensitivity of the PCR reaction with BpmF/BpmR2 primers
1. 10^5 CFU/ml per reaction; 2. 10^4 CFU/ml per reaction; 3. 10^3 CFU/ml per reaction;

4. 10^2 CFU/ml per reaction; 5. 10 CFU/ml per reaction;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

The reaction conditions of PCR with BpmF/Bpmr2 primers targeting the hypothetical protein of *B. pseudomycoides* were as follows:

DNA	3µl	94ºC 5min
Buffer (10x)	2.5µl	
$MgCl_2$ (50mM)	1.2µl	30 cycles:
dNTPs (25mM each)	0.25µl	94ºC 30sec
BpmF (10µM)	1.6µl	58ºC 30sec
BpmR2 (10µM)	1.6µl	72ºC 30sec
Polymerase (5U/µl)	0.3µl	
H_2O	To 25µl	72ºC 7min

The only exception in the PCR using *B. pseudomycoides* specific primers was one strains: *B. pseudomycoides* WS 3120 which did not present the expected products: 217bp and 220bp. The PCR with primers targeting the 16*S* rDNA (Hansen *et al.* 2001) presented expected product with DNA extracted with *B. pseudomycoides* WS 3120 (Appendix K). This result included the strain to the *B. cereus* group spp., however was not detectable in assays using BpmF/BpmR, BpmF/BpmR2 and BCFomp2/BCRomp2 primers designed in this study. The reason of this result might be due to single nucleotide polymorphisms (SNPs) causing lack of primer annealing during the PCR reaction.

The BpmF2/BpmR2, the third, set of primers were tested against *B. pseudomycoides* and other *B. cereus* group species. PCR using these primers generated 134bp product and obtained results showed the same selectivity as the two previously described unique primer sets: BpmF/BpmR and BpmF/BpmR2. However amplification with BpmF2/BpmR2 primers showed lower sensitivity as observed on Figure 44. A few strains generated PCR product which was weaker on an agarose gel in comparison with PCR product generated using BpmF/BpmR and BpmF/BpmR2 primers. Nevertheless, PCR with the third set of primers confirmed that 5 x *B. mycoides* strains: Nov1, Nov2, 6A19, A81, DSM 307 can be classified as *B. pseudomycoides*.

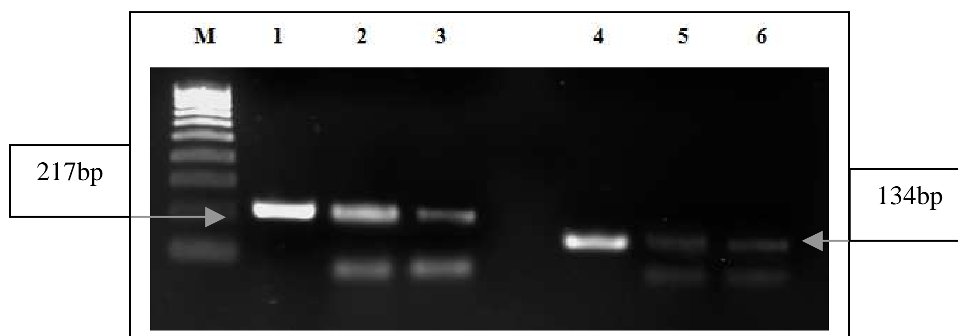


Figure 44. Agarose gel showing 217bp product generated with BpmF/BpmR2 primers and 134bp product generated with BpmF2/BpmR2 primers

1-3) PCR with BpmF/BpmR2 primers: 1. *B. pseudomycoides* DSM 12442; 2. *B. pseudomycoides* A82; 3. *B. mycoides/pseudomycoides* GRD 2/71

4-6) PCR with BpmF2/BpmR2 primers: 4. *B. pseudomycoides* DSM 12442; 5. *B. pseudomycoides* A82; 6. *B. mycoides/pseudomycoides* GRD 2/71;

M-Molecular marker (HyperLadder IV)

3.3.8. RT-PCR identification of *B. pseudomycoides* strains

Based on comparison of 220bp sequences (generated in amplification with BpmF/BpmR primers) of four *B. pseudomycoides* strains: DSM 12442, WS 3118, WS 3119 and GRD 1/17, the unique site for probe hybridization was identified. The selected site (28 nucleotides) was the same for the four strains therefore no wobbled nucleotides were used. The locus of new designed probe Bpm-FAM-1 in the 220bp sequence is presented in Figure 45. The 5'-end of the probe was labelled with fluoresceine (FAM), the 3'-end was quenched with Black Berry Quencher (BBQ). The probe sequence and its details are summarized in Table 30.

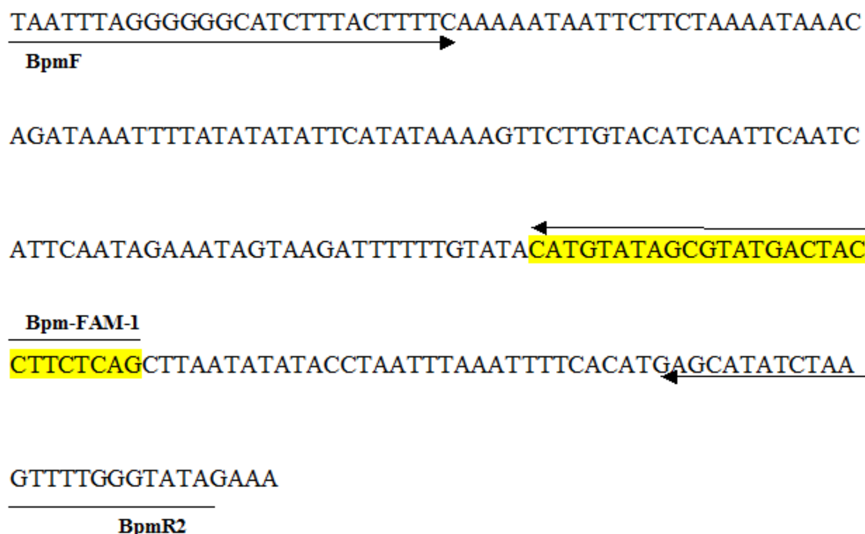


Figure 45. PCR product amplified with BpmF/BpmR2 primers with highlighted locus for Bpm-FAM-1 probe hybridization

Table 30. The Bpm-FAM-1 TaqMan probe details

Probe	**Probe sequence (5'-3')**	**Target in amplified product**	**GC content (%)**	**Tm (˚C)**
Bpm-FAM-1	FAM-CTGAGAAGGTAGTCATACGCTATACATG-BBQ	161-134	42.9	56.1

Because BpmF/BpmR2 primers presented higher sensitivity in traditional PCR reaction, those primers were selected to RT-PCR assay. The final concentration 0.07µM of the probe in the reaction mix was experimentally found as optimal. After optimization of the reaction conditions, the assay was applied to detect the *B. pseudomycoides* strains based on generated by LightCycler®1.2 fluorescence signal. All *B. pseudomycoides*, *B. mycoides*, *B. mycoides/pseudomycoides* and 90 other strains belong to the *B. cereus* group spp. used in this study were analyzed.

Optimized RT-PCR conditions with BpmF/BpmR2 primers and Bpm-FAM-1 probe were as follows:

DNA	5µl	95ºC 10min
LightCycler® TaqMan® Master	4µl	30 cycles:
Bpm-FAM-1 (2µM)	0.7µl	95ºC 10sec
BpmF (10µM)	1.6µl	59ºC 40sec
BpmR2 (10µM)	1.6µl	72ºC 1sec (single slop)
H_2O	To 20µl	
		40ºC 30sec

Figure 46 presents the results of the RT-PCR with DNA from the 6 species belonging to the *B. cereus* group spp. and *Bacillus* sp. Ba813$^+$. From 7 analyzed strains, only *B. pseudomycoides* DSM 12442 amplified and showed the fluorescence signal. The achieved Cp value was 15,56 and the amount of DNA calculated based on standard curve was 7.27x10^6. The other strains: *B. cereus* ATCC 14579, *B. thuringiensis* DSM 6017, *B. anthracis* 34F2, *Bacillus* Ba813$^+$ #19 (T5 97-77), *B. weihenstephanensis* DSM 11821 and *B. mycoides* 6A68 did not amplify using BpmF/BpmR2 primers which confirmed achieved earlier PCR results.

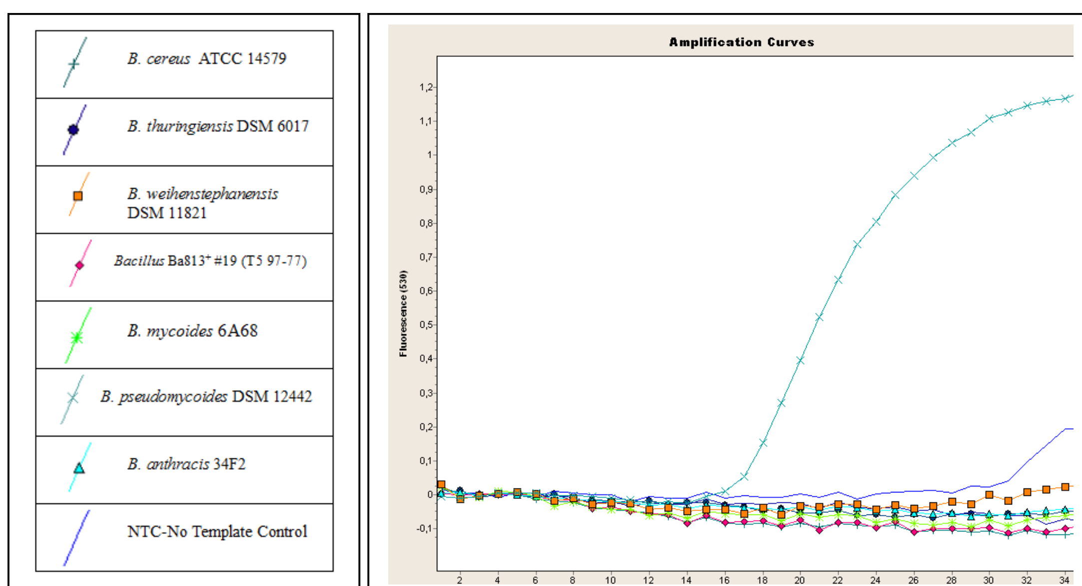


Figure 46. Results of RT-PCR with BpmF/BpmR2 primers using 0.07µM Bpm-FAM-1 probe

Bacterial strains used in the reaction: *B. pseudomycoides* DSM 12442, *B. cereus* ATCC 14579, *B. thuringiensis* DSM 6017, *B. weihenstephanensis* DSM 11821, *Bacillus* Ba813$^+$ #19 (T5 97-77), *B. mycoides* 6A68 and *B. anthracis* 34F2

The results confirmed those obtained in traditional PCR reaction using BpmF/BpmR2 primers, which generated a 217bp product. Nine from ten tested *B. pseudomycoides* strains showed the fluorescence signal and peak illustrating a positive reaction and the presence of the PCR product. None of the *B. cereus*, *B. thuringiensis*, *B. weihenstephanensis*, *B. anthracis* and *Bacillus* sp. Ba813$^+$ strains presented the amplicon. The same results were obtained for 15 x *B. mycoides* and 6 x *B. mycoides/pseudomycoides* strains tested. Five strains defined as *B. mycoides*: Nov1, Nov2, 6A19, A81, DSM 307 showed positive reaction in RT-PCR with Bpm-FAM-1 probe, what confirmed that those strains should be classify as *B. pseudomycoides* not *B. mycoides* (Table 31, outlined with an astrix). 4 from 10 not discriminated *B. mycoides/pseudomycoides* (GRD 1/17, GRD 2/71, 1/2, 17/3) strains which did not present the positive amplification with BCFomp2/BCRomp2 primers, showed positive result when working with Bpm-FAM-1 probe and BpmF/BpmR2 primers (Table 31, marked with hash symbol). This result showed that the described assay can be used for differentiation *B. pseudomycoides* from *B. mycoides* strains what was possible only by fatty acid analysis, to date.

The results of RT-PCR assay for *B. pseudomycoides*, *B. mycoides* and *B. mycoides/pseudomycoides* strains are summarized in Table 31.

Table 31. Summarized results of RT-PCR assay with Bpm-FAM-1 probe and BpmF/BpmR2 primers for *B. pseudomycoides*, *B. mycoides* and *B. mycoides/pseudomycoides* strains

Species	No. of strains	Bacterial strains	RT-PCR BCFomp2/BCRomp2		RT-PCR BpmF/BpmR2 and Bpm-FAM-1
			MotB-FAM-1	MotB-FAM-2	
Bacillus pseudomycoides	10	WS 3118	-	-	+
		WS 3119	-	-	+
		WS 3120	-	-	-
		DSM 12442	-	-	+
		DSM 12443	-	-	+
		B346	-	-	+
		B618	-	-	+
		TP1	-	-	+
		DA	-	-	+
		A82	-	-	+
Bacillus mycoides	20	BMFUL1	-	+	-
		BMSUL1	-	+	-
		BMSUL2	-	+	-
		6A11	-	+	-
		6A12	-	+	-
		6A13	-	+	-
		6A14	-	+	-
		6A19 *	-	-	+
		6A20	-	+	-
		6A47	-	+	-
		6A49	+	-	-
		6A68	+	-	-
		DSM 307*	-	-	+
		DSM 309	-	+	-
		DSM 384	-	+	-
		Nov1*	-	-	+
		Nov2 *	-	-	+
		A81*	-	-	+
		BiF	-	+	-
		BmF	-	+	-
Bacillus mycoides/ pseudomycoides	10	PID 1/21	+	-	-
		PID 2/43	+	-	-
		PID3/2	+	-	-
		GRD 1/17[#]	-	-	+
		GRD 2/71[#]	-	-	+
		1/1	+	-	-
		1/2[#]	-	-	+
		17/3[#]	-	-	+
		22/2	-	+	-
		29/2	-	+	-

3.3.9. Determination of RT-PCR standard curve and limit of detection for BpmF/BpmR2 primers and Bpm-FAM-1 probe

The fragment of *Bpm* amplicon (217bp) of *B. pseudomycoides* WS 3118 was generated by PCR using the primer set BpmF/BpmR2 and cloned into pGEM-T Easy Vector system (Figure 47). The PCR product was ligated with 50ng of vector DNA at 1:3 molar ratio of vector:insert and ligated product was transformed into *E. coli* JM 109 strain.

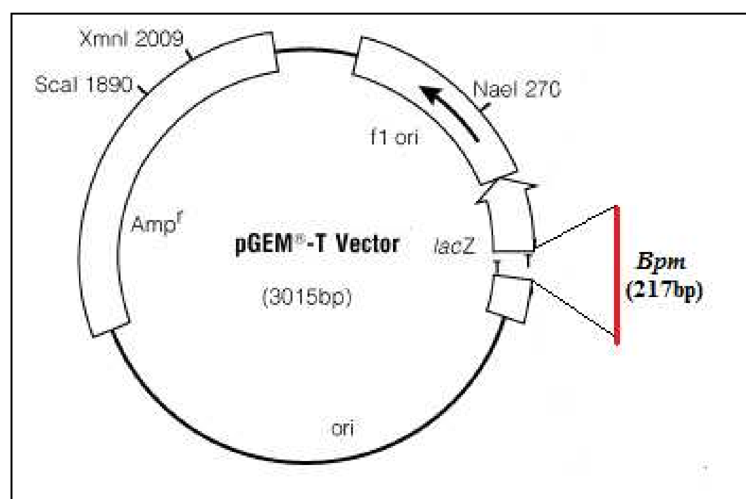


Figure 47. Scheme of 217bp fragment of the *Bpm* gene cloned into pGEM-T Easy Vector

Possible transformants were confirmed by PCR reaction where the DNA was extracted by the heating method. Three vectors with 217bp insert were extracted from *E. coli* by Promega plasmid kit (Wizard Plus SV Minipreps) and checked by enzymatic digestion with *EcoRI* enzyme. Positive recombinant plasmids presented two bands on 1.5% agarose gel: 243bp (insert+flanking sequence) and a 2989bp (plasmid) which are illustrated in Figure 48.

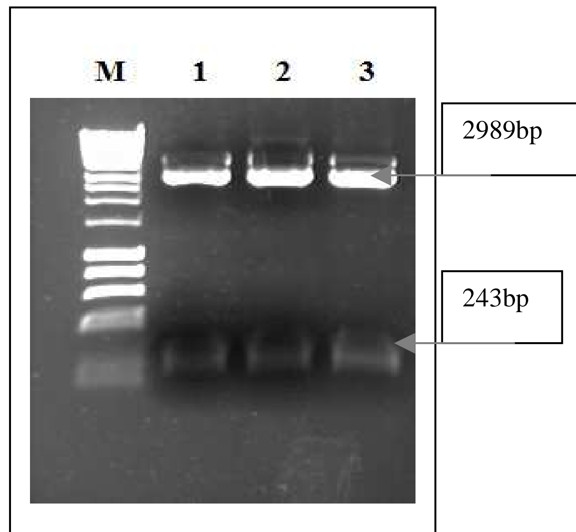


Figure 48. Results of enzymatic digestion of pGEM-*Bpm* recombinant plasmids with *EcoRI*
Three positive clones showing 2989bp plasmid and 243bp insert with flanking sequence;
M-Molecular marker (HyperLadder I)

Two positive clones were sequenced by Eurofins MWG Operon using T7 (forward) and SP6 (reverse) primers. After determining the DNA concentration by A UV-Vis NanoDrop-1000 spectrophotometer, the conversion of the mass to molecules was carried out using the formula described earlier in this work (page 139).

A dilution series ($1x10^{10}$-1 gene copy numbers/20µl reaction) of recombinant plasmid (pGEM-*Bpm*) were tested in triplicates by the real-time PCR and Cp values were plotted against log-transformed concentrations of serial ten-fold dilutions of the target. Cp values from each reaction were calculated to achieve one average value for every dilution. The standard deviation for each value of gene copy number was evaluated.

Significant signals were detected between $1x10^9$-1 gene copy numbers per reaction with Bpm-FAM-1 probe and BpmF/BpmR2 primers. Using the same primers in ordinary PCR, the limit of detection was $1x10^3$ CFU/ml per reaction (25µl) (Figure 43, page 146) whereas in the real-time assay it was 1 gene copy number.

The average slope was –3.45. The efficiency of amplifications was E=1.95 ± 0.007 which corresponded with 95%. The correlation (R^2) between Cp value and $\log_{\text{gene copy number}}$ was 0,97 (Figure 49).

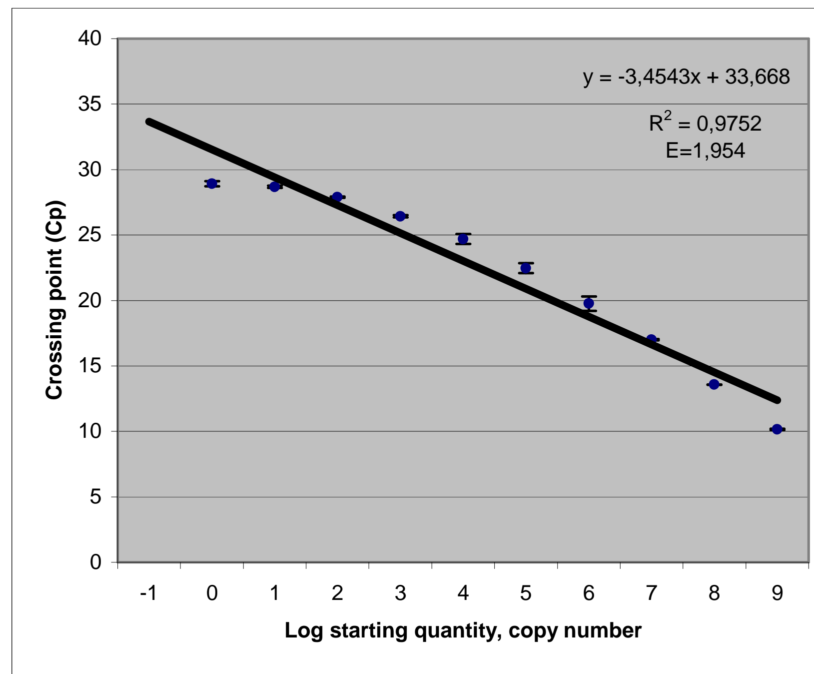


Figure 49. Standard curve for determination of gene copy numbers of *B. pseudomycoides* based on hypothetical gene using BpmF/BpmR2 primers and Bpm-FAM-1 probe

Crossing point values plotted against the log of the initial template DNA concentration. ± One standard deviation is indicated for each gene copy number. Average efficiency of every RT-PCR amplification was 1.95 ± 0.007

The average main crossing points values of serially diluted DNA of recombinant plasmid DNA (pGEM-*Bpm*) in the real-time PCR with BpmF/BpmR2 primers and Bpm-FAM-1 probe are presented in Table 32. They were calculated and summarized from every RT-PCR. One standard deviation (±) was indicated for each average Cp value.

Table 32. Average Cp values for RT-PCR assay with BpmF/BpmR2 primers and Bpm-FAM-1 probe

Gene copy number	Average Cp value of TaqMan assay with BpmF/BpmR2 primers And Bpm-FAM-1 probe
$1x10^9$	10.14 ± 0.047
$1x10^8$	13.56 ± 0.007
$1x10^7$	17.00 ± 0.056
$1x10^6$	19.76 ± 0.55
$1x10^5$	22.47 ± 0.37
$1x10^4$	24.69 ± 0.37
$1x10^3$	26.42 ± 0.085
$1x10^2$	27.89 ± 0.039
1x10	28.68 ± 0.086
1	28.91 ± 0.2

3.3.10. Optimization of RT-PCR with three probes to detect *B. cereus* group species

When three designed TaqMan probes were used in one reaction mix, it was possible to detect and identify 129 from 130 tested *B. cereus* group sp strains. The reaction was optimized using different probe concentration: 0.025µM, 0.035µM and 0.07µM of each probe per reaction. The primers concentration was the same as when used separately and was 16pmol of each primer (BCFomp2/BCRomp2/BpmF/BpmR2) per reaction mix. The reaction conditions used in previous RT-PCR assays were not changed.

To optimize the probe concentration the DNA extracted from three strains: *B. thuringiensis* DSM 6017, *B. weihenstephanensis* WSBC 10389 and *B. pseudomycoides* WS 3118 were used. First, the 5µl of DNA from each strain was amplified with 0.07µM probe and then the exact amount of DNA was calculated based on earlier prepared standard curve imported from the LightCycler® software. A standard dilution of DNA representing $1x10^6$ gene copy numbers (*motB* and *Bpm* genes) per reaction was determined as necessary when external standard curve is used to define the concentration/quantity of unknown sample. Based on achieved results of the reaction

with 0.07μM each probe: MotB-FAM-1, MotB-FAM-2, Bpm-FAM-1, similar results were obtained for three probes used in one reaction mix. Probes used in a concentration: 0.035μM MotB-FAM-1, 0.035μM MotB-FAM-2, 0.035μM Bpm-FAM-1 showed the same efficiency as when used separately with a final concentration 0.07μM. Detailed results presenting particular Cp values and calculated gene copy numbers are outlined in Table 33, Table 34 and Table 35.

Table 33. Results of RT-PCR with BCFomp2/BCRomp2/BpmF/BpmR2 primers and three probes for *B. thuringiensis* DSM 6017

MotB-FAM-1 probe concentration (+0.035μM MotB-FAM-2, 0.035μM Bpm-FAM-1)	Cp value	Calculated gene copy numbers
0.025μM	13,10	$1.35x10^8$
0.035μM	12,65	$1.59x10^8$
0.07μM	13,24	$1.05x10^8$
0.07μM (when used separately)	12,53	$2x10^8$

Table 34. Results of RT-PCR with BCFomp2/BCRomp2/BpmF/BpmR2 primers and three probes for *B. weihenstephanensis* WSBC 10389

MotB-FAM-2 probe concentration (+0.035μM MotB-FAM-1, 0.035μM Bpm-FAM-1)	Cp value	Calculated gene copy numbers
0.025μM	17,52	$9.63x10^6$
0.035μM	16,43	$1.02x10^7$
0.07μM	17,10	$9.52x10^6$
0.07μM (when used separately)	16,66	$9.84x10^6$

Table 35. Results of RT-PCR with BCFomp2/BCRomp2/BpmF/BpmR2 primers and three probes for *B. pseudomycoides* WS 3118

Bpm-FAM-1 probe concentration (+0.035μM MotB-FAM-1, 0.035μM MotB-FAM-2)	Cp value	Calculated gene copy numbers
0.025μM	17,86	$3.12x10^6$
0.035μM	16,67	$4.15x10^6$
0.07μM	16,76	$3.74x10^6$
0.07μM (when used separately)	16,30	$5.14x10^6$

After optimization the probes concentration in one reaction mix, the assay was applied to amplify the DNA extracted from strains representing each species belonging

to the *B. cereus* group spp.: *B. cereus* ATCC 14579, *B. thuringiensis* DSM 6017, *B. anthracis* 34F2, *Bacillus* Ba813^{+} #19 (T5 97-77), *B. mycoides* 6A68, *B. weihenstephanensis* DSM 11821 and *B. pseudomycoides* DSM 12442. The concentration of BCFomp2/BCRomp2/BpmF/BpmR2 primers was equal (0.8µM each); the parameters of the RT-PCR reaction were not changed and were as follows:

DNA	5µl	95ºC 10min
LightCycler® TaqMan® Master	4µl	
MotB-FAM-1 (2µM)	0.35µl	<u>30 cycles:</u>
MotB-FAM-2 (2µM)	0.35µl	95ºC 10sec
Bpm-FAM-1 (2µM)	0.35µl	59ºC 40sec
BCFomp2 (10µM)	1.6µl	72ºC 1sec (single slop)
BCRomp2 (10µM)	1.6µl	
BpmF (10µM)	1.6µl	40ºC 30sec
BpmR2 (10µM)	1.6µl	
H_2O	To 20µl	

The Figure 50 presents results where achieved Cp values and calculated DNA quantity (gene copy numbers) were as follow:

Strain	**Cp value**	**Calculated quantity of gene copy numbers**
B. cereus ATCC 14579	13,49	$3.90x10^{7}$
B. thuringiensis DSM 6017	12,20	$1.16x10^{8}$
B. anthracis 34F2	18,96	$1.10x10^{6}$
Bacillus Ba813^{+} #19 (T5 97-77)	14,64	$2.17x10^{7}$
B. mycoides 6A68	16,07	$5.15x10^{6}$
B. weihenstephanensis DSM 11821	13,99	$3.38x10^{7}$
B. pseudomycoides DSM 12442	15,54	$7.63x10^{6}$

In the RT-PCR assay using three probes (0.035µM MotB-FAM-1, 0.035µM MotB-FAM-2, 0.035µM Bpm-FAM-1) in one reaction mix, seven tested strains gave positive signal. The achieved values are comparable to the results of RT-PCR with the same strains, where probes were used separately in 0.07µM final concentration (Figure 33, page 129; Figure 36, page 133 and Figure 46, page 150).

Products of RT-PCR run on 1.5% agarose gel showed expected sizes: 217bp and 285bp (Figure 51).

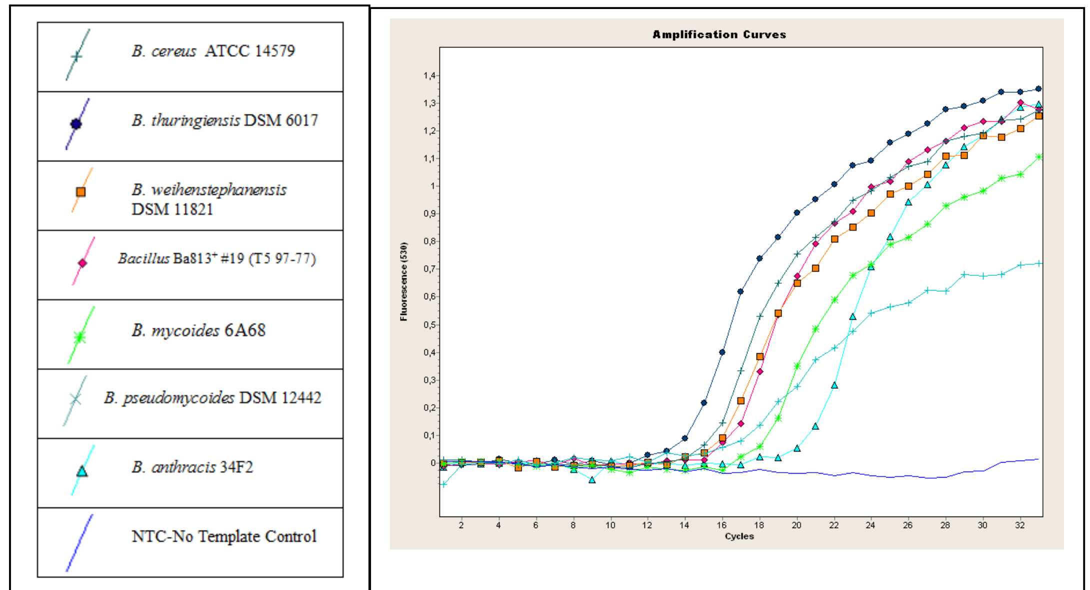


Figure 50. Results of RT-PCR assay with BCFomp2/BCRomp2/BpmF/BpmR2 primers and 0.035µM MotB-FAM-1, 0.035µM MotB-FAM-2, 0.035µM Bpm-FAM-1

Cp values (calculated DNA quantity-gene copy numbers): 13,49 ($3.90x10^7$) for *B. cereus* ATCC 14579; 12,20 ($1.16x10^8$) for *B. thuringiensis* DSM 6017; 13,99 ($3.38x10^7$) for *B. weihenstephanensis* DSM 11821; 14,64 ($2.17x10^7$) for *Bacillus* Ba813$^+$ #19 (T5 97-77); 16,07 ($5.15x10^6$) for *B. mycoides* 6A11; 15,54 ($7.63x10^6$) for *B. pseudomycoides* DSM 12442 and 18,96 ($1.10x10^6$) for *B. anthracis* 34F2

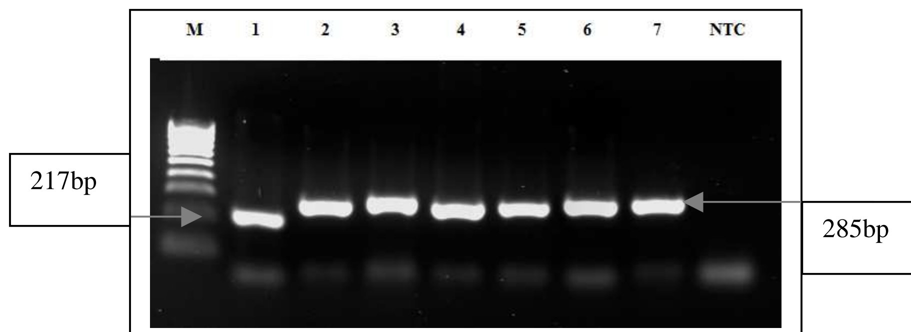


Figure 51. Products of RT-PCR on 1.5% agarose gel. Amplification with BCFomp2/BCRomp2/BpmF/BpmR2 primers and 0.035µM MotB-FAM-1, 0.035µM MotB-FAM-2, 0.035µM Bpm-FAM-1

1. *B. pseudomycoides* DSM 12442; 2. *B. cereus* ATCC 14579; 3. *B. thuringiensis* DSM 6017;

4. *B. weihenstephanensis* DSM 11821; 5. *B. mycoides* 6A68; 6. *B. anthracis* 34F2;

7. *Bacillus* Ba813$^+$ #19 (T5 97-77);

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

3.3.11. Multiplex PCR to detect *B. cereus* group spp. and differentiation of *B. pseudomycoides*

Amplification with BCFomp2/BCRomp2 primers was able to detect and identified the *B. cereus* group species strains except 10 x *B. pseudomycoides* and 5 x *B. mycoides* and 4 x *B. mycoides/pseudomycoides* strains. Those strains were detected by PCR using new *B. pseudomycoides* specific BpmF/BpmR2 primers.

To achieve the possibility for detection the *B. cereus* group species and differentiate *B. pseudomycoides* strains in one reaction, a multiplex PCR was tested. Two combinations of multiplex amplifications using BCFomp2/BCRomp2/BpmF/BpmR and BCFomp2/BCRomp2/BpmF/BpmR2 were tested. To select the PCR with better sensitivity the limit of detection for both reactions were checked. In both cases the limit of detection for *B. cereus* ATCC 14579 was 10^4 CFU/ml (Figure 52, lane 4). The detection of *B. pseudomycoides* DSM 12442 was different in amplifications with these two primers combinations. PCR with BCFomp2/BCRomp2/BpmF/BpmR primers was able to detect 10^4 CFU/ml (Figure 53, lane 2) whereas with BCFomp2/BCRomp2/BpmF/BpmR2 primers – 10^3 CFU/ml for *B. pseudomycoides* DSM 12442 (Figure 54, lane 2).

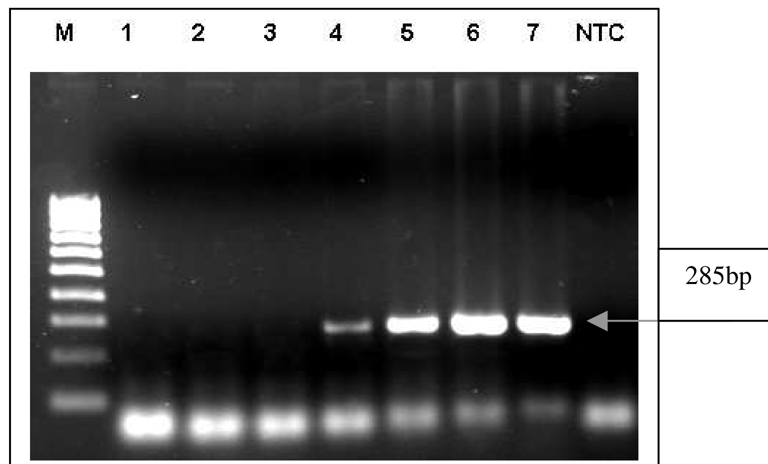


Figure 52. Sensitivity of the multiplex PCR reaction with BCFomp2/BCRomp2/BpmF/BpmR primers for *B. cereus* ATCC 14579

1. 10 CFU/ml per reaction; 2. 10^2 CFU/ml per reaction; 3. 10^3 CFU/ml per reaction;

4. 10^4 CFU/ml per reaction; 5. 10^5 CFU/ml per reaction; 6. 10^6 CFU/ml per reaction;

7. 10^7 CFU/ml per reaction;

annealing temperature: 58°C;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

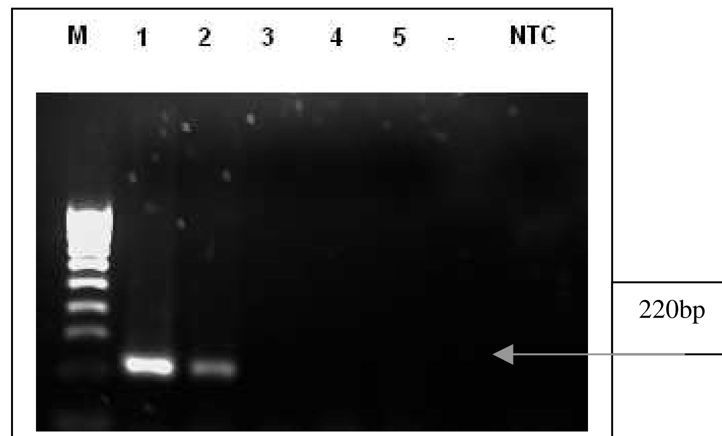


Figure 53. Sensitivity of the PCR reaction with BCFomp2/BCRomp2/BpmF/BpmR primers for *B. pseudomycoides* DSM 12442

1. 10^5 CFU/ml per reaction; 2. 10^4 CFU/ml per reaction; 3. 10^3 CFU/ml per reaction;

4. 10^2 CFU/ml per reaction; 5. 10 CFU/ml per reaction;

annealing temperature: 58°C;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

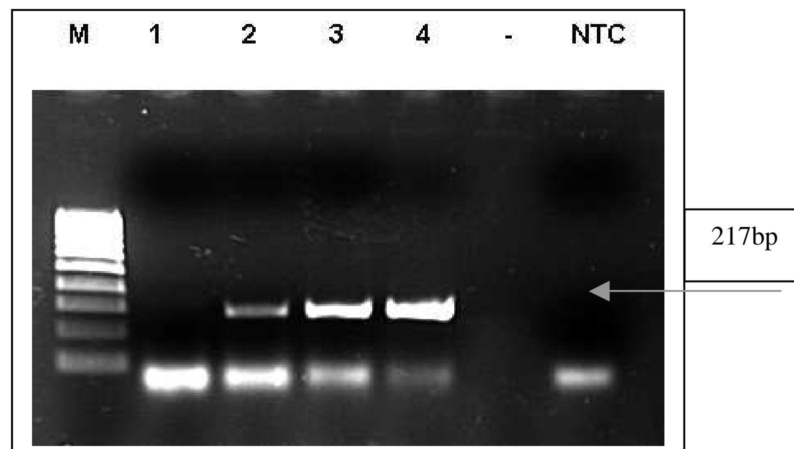


Figure 54. Sensitivity of the multiplex PCR reaction with BCFomp2/BCRomp2/BpmF/BpmR2 primers for *B. pseudomycoides* DSM 12442

1. 10^2 CFU/ml per reaction; 2. 10^3 CFU/ml per reaction; 3. 10^4 CFU/ml per reaction;

4. 10^5 CFU/ml per reaction;

annealing temperature: 58°C;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

The PCR conditions were optimized using different primer concentration where the optimal was 16pmol of each primer used in PCR reaction mix. The optimal annealing temperature was also experimentally chosen for these conditions. The described four primers had different melting temperature: BCFomp2 56.4ºC; BCRomp2 51.3ºC; BpmF 55.3ºC; BpmR2 52.2ºC. The multiplex PCR was performed using the following annealing temperatures: 53ºC, 56ºC and 58ºC. For *B. cereus* ATCC 14579 the reaction presented the same sensitivity at each annealing temperature. Only *B. pseudomycoides* specific primers (BpmF/BpmR2) showed double specificity. At 53ºC, beside the expected 217bp product, an unspecific ~380bp amplicon was observed (Figure 55). At 56ºC the unspecific product was less visible whereas at 58ºC this product was not present (Figure 56).

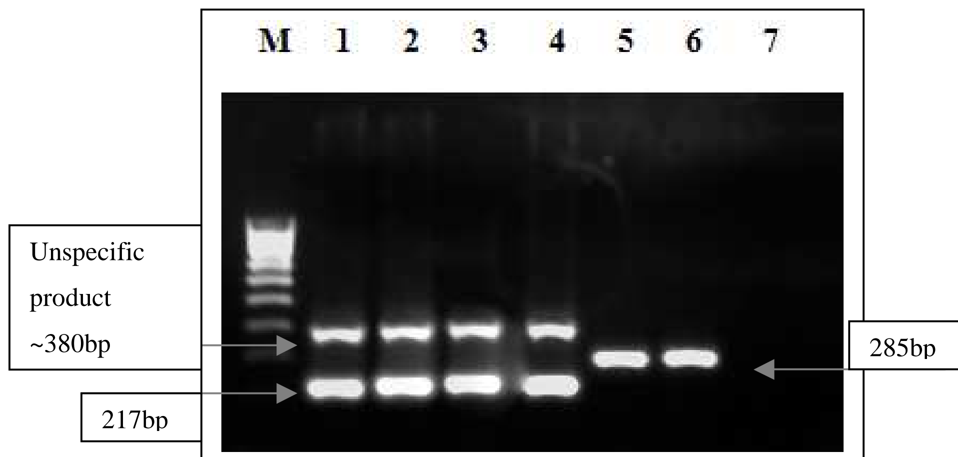


Figure 55. 2% agarose gel showing 285bp and 217bp multiplex PCR products generated with primers BCFomp2/BCRomp2 and BpmF/BpmR2 respectively, annealing temperature 53°C

1. *B. pseudomycoides* DSM 12442; 2. *B. pseudomycoides* DSM 12443; 3. *B. pseudomycoides* WS 3118; 4. *B. pseudomycoides* WS 3119; 5. *B. mycoides* 6A1; 6. *B. mycoides* 6A13;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

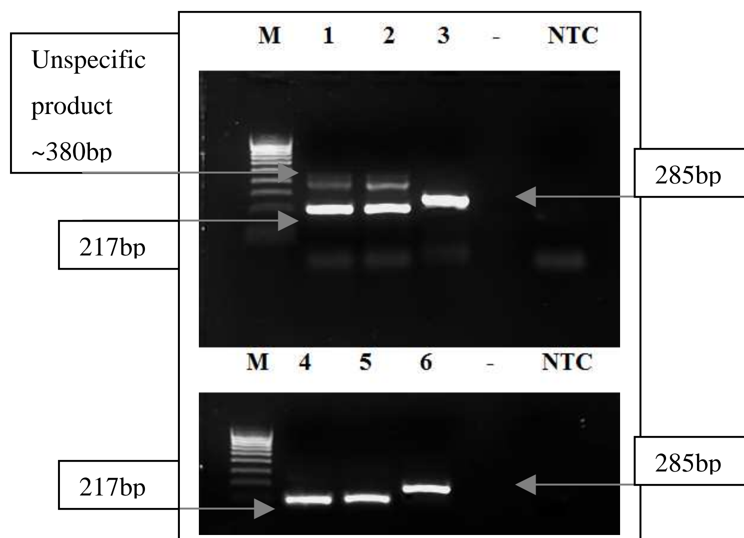


Figure 56. 2% agarose gel showing 285bp and 217bp multiplex PCR products generated with primers BCFomp2/BCRomp2 and BpmF/BpmR2 respectively, with annealing temperature 56°C (1-3) and 58°C (4-6)

1. *B. pseudomycoides* DSM 12442; 2. *B. pseudomycoides* WS 3118; 3. *B. mycoides* 6A11;

4. *B. pseudomycoides* DSM 12442; 5. *B. pseudomycoides* WS 3118; 6. *B. mycoides* 6A11;

NTC-No Template Control; M-Molecular marker (HyperLadder IV)

Optimized conditions of multiplex PCR with BCFomp2/BCRomp2 and BpmF/BpmR2 primers were as follows:

DNA	3µl	94ºC 5min
Buffer (10x)	2.5µl	
$MgCl_2$ (50mM)	1.2µl	40 cycles:
dNTPs (25mM each)	0.25µl	94ºC 30sec
BCFomp2 (10µM)	1.6µl	58ºC 30sec
BCRomp2 (10µM)	1.6µl	72ºC 40sec
BpmF (10µM)	1.6µl	
BpmR2 (10µM)	1.6µl	72ºC 7min
Polymerase (5U/µl)	0.3µl	
H_2O	To 25µl	

After optimization all reaction parameters, the multiplex PCR with primers designed in this study was applied in testing 130 strains belongs to the *B. cereus* group species and 28 other *Bacillus* and non-*Bacillus* species. Each time the reaction showed 217bp product for *B. pseudomycoides* strains or 285bp product for the other *B. cereus* group species.

Also in multiplex PCR reaction, 4 x *B. mycoides/pseudomycoides* strains: GRD 1/17, GRD 2/71, 1/2, 17/3 showed only the 217bp product. The same situation was repeated for 5 x *B. mycoides*: Nov1, Nov2, 6A19, A81, DSM 307, which confirmed that these strains belong to the *B. pseudomycoides* group. Only *B. pseudomycoides* WS 3120 did not present the expected product.

None of the control strains including 28 other *Bacillus* and non-*Bacillus* species showed products in the multiplex PCR assay. The results of multiplex PCR for selected strains are presented in Appendix L.

3.4. Analysis of milk samples artificially contaminated with *B. cereus*

The assay for detecting the *B. cereus* group spp. was further evaluated in milk. Milk samples, inoculated with bacteria as described in material and methods, were used for DNA extraction directly by using two different column-based systems: DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and Genomic Mini AX Food kit (A&A Biotechnology, Gdynia, Poland). Both kits worked with the same efficiency. Genomic DNA extracted from 100µl of spiked milk (spiked with known amount of CFUs) gave similar results during the RT-PCR reaction with both sets of primers (BCFomp2/BCRomp2 and BpmF/BpmR2) and three designed probes (MotB-FAM-1, MotB-FAM-2, Bpm-FAM-1). Achieved Cp values for the DNA extracted from artificially contaminated milk samples had similar results after using both extraction kits.

The accuracy of the assay using DNA extracted by both kits was assessed by comparing the values of the gene copy numbers for DNA extracted from spiked samples with the extrapolated values using earlier generated standard curves (Figure 40, page 141 and Figure 49, page 155).

After contamination with bacterial culture, which CFU/ml was calculated to achieve 1×10^5 gene copy numbers in 5µl, spiked milk (100µl) was used for bacterial genomic DNA extraction. Kits were able to extract $5.74 \times 10^4 \pm 0.08$ to $7.78 \times 10^4 \pm 0.14$ gene copy numbers from fat milk and $8.87 \times 10^4 \pm 0.12$ to $1.05 \times 10^5 \pm 0.19$ gene copy numbers from nonfat milk. Relative accuracy values ranged from 57.4% to 105% was observed in milk samples (Table 36).

Table 36. Accuracy of the real-time PCR assays for the quantification of *B. cereus* group spp. in milk after extraction of DNA with column-based kits

Quantity of DNA used for milk contamination (gene copy numbers)	Estimated quantity of DNA after kit extraction (gene copy numbers)	Milk type used for contamination	Relative accuracy (%)
$1x10^5$	$7.78x10^4 \pm 0.14$ [b)]	Fat milk	77.8
$1x10^5$	$7.58x10^4 \pm 0.14$ [a)]	Fat milk	75.8
$1x10^5$	$6.12x10^4 \pm 0.08$ [a)]	Fat milk	61.2
$1x10^5$	$5.74x10^4 \pm 0.08$ [b)]	Fat milk	57.4
$1x10^5$	$1.05x10^5 \pm 0.19$ [a)]	Nonfat milk	105
$1x10^5$	$9.84x10^4 \pm 0.09$ [b)]	Nonfat milk	98.4
$1x10^5$	$9.05\ x10^4 \pm 0.08$ [b)]	Nonfat milk	90.5
$1x10^5$	$8.87x10^4 \pm 0.12$ [a)]	Nonfat milk	88.7

a) Genomic Mini AX Food kit (A&A Biotechnology, Gdynia, Poland)
b) DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany)

The comparison of Cp values for DNA extracted from particular milk samples are presented in Appendix M.

3.5. Spores in milk

The *B. cereus* group spp. is one of the most popular sporeformer present in many food products from harvest through processing. Psychrotrophic strains of this pathogen were shown to grow at a temperature below 7ºC in milk. Germinating bacteria can grow to large numbers, releasing toxins during growth in food or in the intestinal track. Therefore is very important to detect the presence of spores in a food product. Based on traditional plating techniques, results can be obtained after 24-48 hours incubation. Molecular techniques cannot be used for identification and detection without pretreatment and/or germination of spores to release the DNA. However, spores are highly resistant to a variety of treatment including: heat, pressure, UV.

In this study the spore treatment was also important in designing a DNA biosensor and in testing the designed RT-PCR assay. Because the study was focused on isolation *B. cereus* group spp. from milk, this food product was used in spore testing.

The purchased spores of *B. thuringiensis* ATCC 29730 with known concentration (10^6 CFU/0.1ml) were diluted in water and used for artificial contamination of milk. Each milk sample (50ml) was autoclaved for 15 minutes at 121ºC to kill all vegetative cells and treated with a UV lamp for 30 minutes. Short wave UV light (254nm) can damage DNA by inducing the formation of covalently bonded Thymine-dimers within the DNA

strand. In each stage (before, after autoclaving and after UV treatment) 100µl of milk was spread onto PEMBA and/or MYP medium to check the presence of *B. cereus* group spp. isolates.

Each 90µl of fat and nonfat milk was inoculated with 10µl (10^5 CFU) of spores. After treatment using different methods, the spiked milk sample (100µl) was used to extract the DNA by a column-based kit (Genomic mini AX Food, A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instruction. The final product was eluted from the column with 50µl of eluent what should give 10^4 CFU of spores/bacteria per every 5µl used for RT-PCR analysis. In each extraction, 100µl of non spiked milk was used as a control.

Different treatment of milk spiked with spores are described in the literature and include the thermal destruction as outlined by Kramer and Gilbert (1989, pp.22-70). They reported that the thermal destruction time for spores suspended in skimmed milk at 100ºC is 2.7 to 3.1 minutes. Thermal inactivation of *B. anthracis* spores in cow's milk was also described by Xu *et al.* (2006). In this paper the decimal reduction time to inactivate 90% of the spores in milk ranged from 3.4 to 16.7 hours at 72ºC and from 1.6 to 3.3 seconds at 112ºC. The authors inoculated whole and skim milk with the spores to obtain a concentration of 10^7 CFU/ml. After heat treatment samples were serially diluted in peptone water, plated onto TSA and incubated overnight at 30°C before colonies were counted. Novak *et al.* (2005) reported inactivation of *B. anthracis* and *B. cereus* spores in skim milk. After heat treatment the samples were plated onto BHI agar base medium with a spiral plates. Plates were incubated at 37ºC for 16-20 hours before colonies were counted. The log reduction (log CFU/ml) for viable spores of *B. cereus* ATCC 9818 in skim milk was: 0.39 log CFU/ml after 90 minutes at 72ºC, 0.21 log CFU/ml after 60 minutes at 78ºC, 7.6 log CFU/ml after 60 minutes at 100ºC and 7.37 log CFU/ml after 2 minutes at 130ºC. A different technique to destroy spores was reported by Kim *et al.* (2009). They used microwave irradiation to destroy the *B. licheniformis* spores suspended in PB buffer. Modified output power of a microwave oven (from 0.5kW to 2.0kW) allowed for shorter sterilization. To investigate cell membrane damage caused by irradiation they measured the release of intracellular proteins (at 595nm by Bradford technique) and nucleic acids (by UV spectrophotometer at 260nm) of the spores. The spores were irradiated at 0.5kW and 2.0kW for 0, 20, 40 and 60 seconds. They reported that spore inactivation rate was faster with 2.0kW irradiation than with 0.5kW. After microwave treatment for 1 minute, the survival

exhibited a 0.17-log reduction of 0.5kW microwave and a 1.97-log reduction of 2.0kW (Kim *et al.* 2009).

Based on these literature reviews different spore treatment of fat and nonfat milk was examined in this study. Contaminated milk was heat treated as follow:

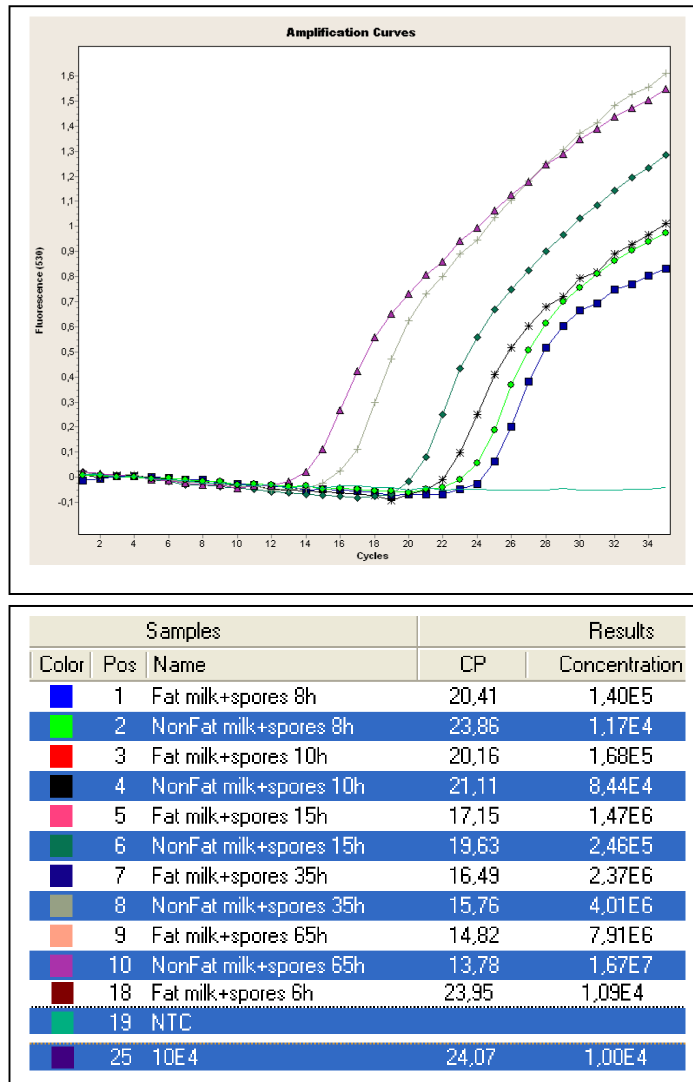
a) 100ºC for 7 minutes
b) 100ºC for 60 minutes
c) 112ºC for 4 seconds and 5 minutes
d) 120ºC for 5 and 8 minutes

and by 0.8kW (maximum output power for standard microwave) irradiation for 1, 5 and 8 minutes.

After each treatment the DNA from the milk sample was extracted. In each assay the control, no contaminated milk sample was included. None of the described techniques worked for milk contamination with *B. thuringiensis* ATCC 29730 spores used in this study. The RT-PCR assay did not present the Cp peaks showing the amplification process, which means that the spores did not release the DNA. They also did not start to germinate and vegetative cells were not broken by the lysis buffer from the kit.

To overcome this problem, another spore heat treatment reported by Mikolajcik and Koka (1968) was used. They described the influence of heat treatment of spores and of the milk upon germination of *B. cereus* 7 in skim milk. Following 2-hour incubation at 35ºC the percentage of germination was based on total counts on Standard Plate Count Agar containing 0.1% soluble starch after 20-24-hour incubation at 35ºC. The results of germination after 2 hours were: 27.3%, 89.7% and 65.2%.

Based on this study, contaminated fat and nonfat milk was incubated at 35ºC for 6, 8, 10, 15, 35, 65 hours. After each incubation time 100µl of spiked milk was used to extract the genomic DNA to observe the necessary time for the pre-treatment of the milk to obtain 10^5 CFU, the initial milk contamination quantity. The results were checked by RT-PCR assay with BCFomp2/BCRomp2 primers using MotB-FAM-1 probe. As a control, the standard dilution of 10^4/5µl gene copy numbers of recombinant plasmid (pGEM-*motB*) was used. Based on this dilution it was possible to calculate the number of copy numbers of unknown samples. The results are outlined in Figure 57 and Figure 58.

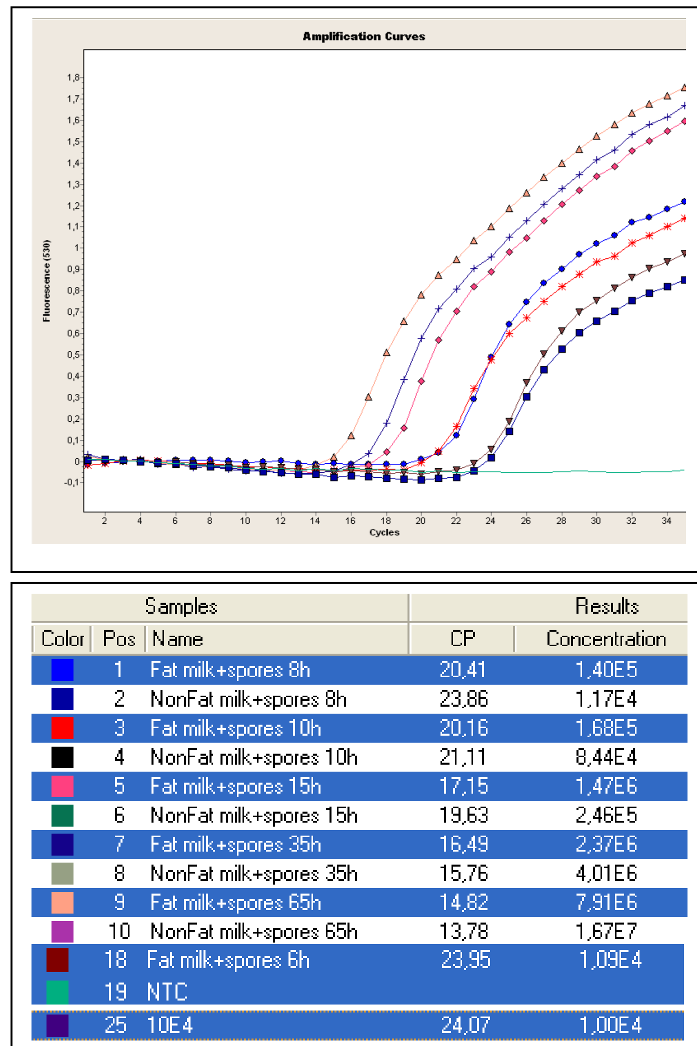


Samples			Results	
Color	Pos	Name	CP	Concentration
	1	Fat milk+spores 8h	20,41	1,40E5
	2	NonFat milk+spores 8h	23,86	1,17E4
	3	Fat milk+spores 10h	20,16	1,68E5
	4	NonFat milk+spores 10h	21,11	8,44E4
	5	Fat milk+spores 15h	17,15	1,47E6
	6	NonFat milk+spores 15h	19,63	2,46E5
	7	Fat milk+spores 35h	16,49	2,37E6
	8	NonFat milk+spores 35h	15,76	4,01E6
	9	Fat milk+spores 65h	14,82	7,91E6
	10	NonFat milk+spores 65h	13,78	1,67E7
	18	Fat milk+spores 6h	23,95	1,09E4
	19	NTC		
	25	10E4	24,07	1,00E4

Figure 57. Results of RT-PCR for contamination the nonfat milk with spores (highlighted samples)

Incubation for 8h-circles (sample 2), 10h-stars (sample 4), 15h-diamond (sample 6), 35h-cross (sample 8), 65h-triangle (sample 10), $1x10^4$ gene copy numbers of pGEM-*motB*-rectangle (sample 25);

NTC-No Template Control (sample 19)



Samples			Results	
Color	Pos	Name	CP	Concentration
	1	Fat milk+spores 8h	20,41	1,40E5
	2	NonFat milk+spores 8h	23,86	1,17E4
	3	Fat milk+spores 10h	20,16	1,68E5
	4	NonFat milk+spores 10h	21,11	8,44E4
	5	Fat milk+spores 15h	17,15	1,47E6
	6	NonFat milk+spores 15h	19,63	2,46E5
	7	Fat milk+spores 35h	16,49	2,37E6
	8	NonFat milk+spores 35h	15,76	4,01E6
	9	Fat milk+spores 65h	14,82	7,91E6
	10	NonFat milk+spores 65h	13,78	1,67E7
	18	Fat milk+spores 6h	23,95	1,09E4
	19	NTC		
	25	10E4	24,07	1,00E4

Figure 58. Results of RT-PCR for contamination the fat milk with spores (highlighted samples)

Incubation for 6h-down triangle (sample 18), 8h-circles (sample 1), 10h-stars (sample 3), 15h-diamond (sample 5), 35h-cross (sample 7), 65h-triangle (sample 9), $1x10^4$ gene copy numbers of pGEM-*motB*-rectangle (sample 25);

NTC-No Template Control (sample 19)

As seen from the figures (Figure 57 and Figure 58), germination occurred in each incubation time of the spores in milk and DNA was successfully extracted.

To achieve the $1x10^5$ gene copy numbers (CFU in 100µl) which were used for contamination the milk with spores, the milk had to be incubated as follow: fat milk for 5.5-6 hours and nonfat milk for 7.5-8 hours. After this time the Cp values of particular samples were very similar to the Cp value of standard dilution ($1x10^4$ gene copy numbers) of recombinant plasmid used as a control. Also calculated concentration of

unknown samples (contaminated milk samples) showed that the best efficiency presented below incubation time.

Spores in fat milk were low heat resistant rather than in nonfat milk. The same result was reported by Novak *et al.* (2005) who observed that spores of *B. cereus* in cream had low heat resistant (20%) compared with skim milk.

Those results showed that pretreatment of milk by incubation at 35ºC gives the best result of examination the milk based on presence of spores. After appropriate time of incubation, the spores germinated to achieve the number of vegetative cells equal to spores that were used to contaminate the milk. To avoid multiplying the vegetative cells already existing in milk, the sample before incubation at 35ºC should be treated at 80ºC for 10 minutes to kill all vegetative cells.

3.6. Dot blot – optimization of probe hybridization for biosensor use

The long term objective of the differentiation of the *B. cereus* group spp. is in the development of a handheld sensor to determine the optimal hybridization conditions for DNA. Therefore, the synthesized probe was hybridized to specific and nonspecific target DNA by dot blot assay in different hybridization conditions.

Designing of *B. cereus* group spp. unique probe:

The newly designed BCFomp2 primer modified with digoxigenin was used as a probe in the dot blot assay. The 1µl of DNA spotted onto the membrane included: genomic DNA of *B. cereus* ACTC 14579 (0.01, 1 and 10ng/µl), *B. weihenstephanensis* WSBC 10392 (30ng/µl), WSBC 10416 (20ng/µl), WSBC 10405 (20ng/µl), PCR product amplified with BCFomp2/BCRomp2 primers (0.01 and 0.1ng/µl), recombinant plasmid with fragment of *motB* gene (pGEM-*motB*; $1x10^5$ gene copy numbers/µl). To detect the hybridized probe by DIG-luminescence detection kit, the hybridization was performed 10ºC below the Tm of the probe, according to the manufacturer's instruction. It was carried out for 5 hours at 46ºC. In these conditions only 0.1ng/µl of PCR product (1C and 2C on Figure 59) and $1x10^5$ gene copy numbers of pGEM-*motB* was detectable (1F and 2F on Figure 59). None of the genomic DNA was detected and the conclusion was that the concentration of the spotted nucleic acid was too low (3A-3C on Figure 59).

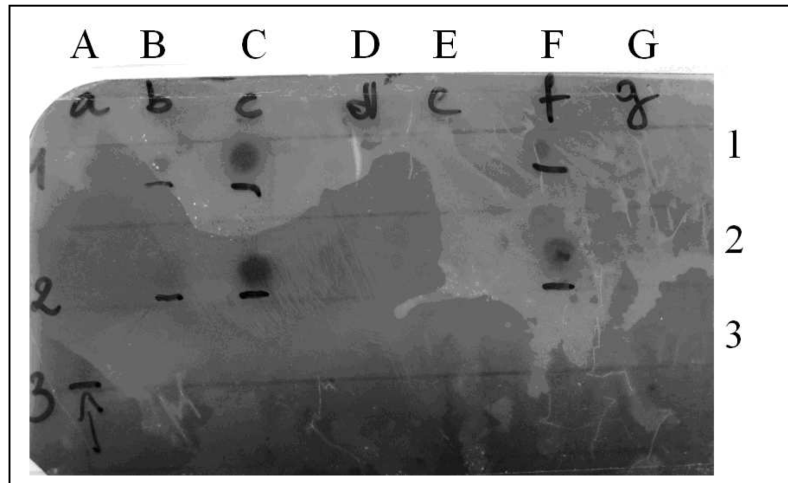


Figure 59. Results of dot blot of DIG labelled BCRomp2 primer after hybridization with various DNA spotted onto nylon membrane

1-A) NTC-No Template Control, B) 0.01ng/µl PCR product, C) 0.1ng/µl PCR product,
D) 0.01ng/µl *B. cereus* ATCC 14579, E) 1ng/µl *B. cereus* ATCC 14579, F) 1x10^5 gene copy numbers of pGEM-*motB*, G) 10ng/µl PCR product *B. cereus* ATCC 14579 ;
2-A) NTC-No Template Control, B) 0.01ng/µl PCR product, C) 0.1ng/µl PCR product,
D) 0.01ng/µl *B. cereus* ATCC 14579, E) 1ng/µl *B. cereus* ATCC 14579, F) 1x10^5 gene copy numbers/µl of pGEM-*motB*, G) empty
3-A) 30ng/µl *B. weihenstephanensis* WSBC 10392, B) 20ng/µl *B. weihenstephanensis* WSBC 10416,
C) 20ng/µl *B. weihenstephanensis* WSBC 10405

To improve the result, 150ng/µl of pure genomic DNA of 7 x *B. cereus* group spp. and 13 other *Bacillus* and non-*Bacillus* species were spotted onto nylon membrane. Those bacteria included: *B. cereus* ATCC 14579, *B. thuringiensis* DSM 6017, *B. weihenstephanensis* DSM 11821, *B. mycoides* 6A12, *B. pseudomycoides* DSM 12442, *B. anthracis* 34F2, *Bacillus* sp. Ba813$^+$ #19 (T5 97-77). Non-*B. cereus* group spp. strains included: *B. subtilis* BSF1, *B. licheniformis* ATCC 12759, *B. amyloliquefaciens* 10A6, *Listeria monocytogenes* LMFUL1, *Yersinia enterocolitica* 27729, *Escherichia coli* ATCC 25922, *Campylobacter jejuni* ULCV48, *Salmonella* Typhimurium NCTC 74, *Lactobacillus acidophilus* DPC 6060 and 5378, *Lactobacillus casei* DPC 6059 *and Lactococcus lactis* 3054 and HP.

After hybridization at the same conditions but for 20 hours each spotted DNA presented the hybridized probe. The 17-nucleotide probe was too short which resulted in lack of

specificity and hybridization to DNA of other *Bacillus* and non-*Bacillus* species with the same efficiency as to *B. cereus* group spp.

The selection of a second probe based on the BCRomp2 primer was designed manually from a known gene sequence. BCRomp2 primer (and inverted version) with different amount of flanking nucleotides were blasted against other *Bacillus* and non-*Bacillus* species with special emphasis on bacteria that are found in milk (Appendix N). It showed the best region in the analyzed sequence to be in the greater part unique only for *B. cereus* group spp. The size of the new DIG-BCRomp2b selected probe was 44 nucleotides (Table 37) and hybridization with DNA extracted from 7 strains of the *B. cereus* group was carried out. The DNA extracted from 13 other *Bacillus* strains (as described above) that could be found in milk were used as a control in each dot blot assay.

Table 37. Details of DIG-BCRomp2b probe

Probe	Sequence (5'-3')	Size	GC content (%)	Tm (ºC)
DIG-BCRomp2b	DIG-TAAYGGTRTTAGTCAAGTGAATGTATATCGAGAGGATACAGGGG Y=CT, R=A/G	44nt	40.9	63

The hybridization temperature always started from 10ºC below the melting temperature (Tm) of the probe and was performed overnight. In the case of non specific hybridization the temperature was increased. The Tm of DIG-BCRomp2b was 63ºC therefore the initial hybridization temperature was 53ºC. In this condition either DNA extracted from the *B. cereus* group spp. or from 13 other *Bacillus* and non-*Bacillus* species hybridized.

To minimalize the non specific hybridization, additional steps were included: the hybridization temperature was increased, the antibodies were added to the 1 x blocking solution about 30 minutes before use, and increased concentration of the probe for the hybridization was used. To avoid non specific hybridization the stringency washes were optimized. The lower the salt concentration (in SSC) and the higher the wash temperature, the more stringent the wash.

The probe concentration was increased from 4.5pmol per 1ml of DIG Easy hybridization buffer to 8.5-10pmol/1ml and hybridization temperature increased to 56ºC. To determine the limit of detection the dilutions of DNA extracted from *B. cereus* ATCC 14579 were spotted onto nylon membrane (5ng, 10ng, 30ng, 50ng, 80ng, 100ng, 150ng, 200ng, 250ng, 300ng).

During stringency washes where the first wash was at room temperature and the second at 56ºC the limit of detection was 30ng of DNA. Also the hybridization of the probe with DNA of 13 other *Bacillus* and non-*Bacillus* species was still visible. Only *S.* Typhimurium NCTC 74 and *L. lactis* 3054 did not present the hybridization signal (Figure 60, sample 3F and 3G).

After applying additional stringency wash (10 minutes in 0.1xSSC) where three washes were performed at the hybridization temperature the limit of detection was 50ng. Moreover, 13 strains not belonging to the *B. cereus* group spp. did not present the hybridization results (Figure 61). The possible unspecific hybridization was washed out during stringency washes.

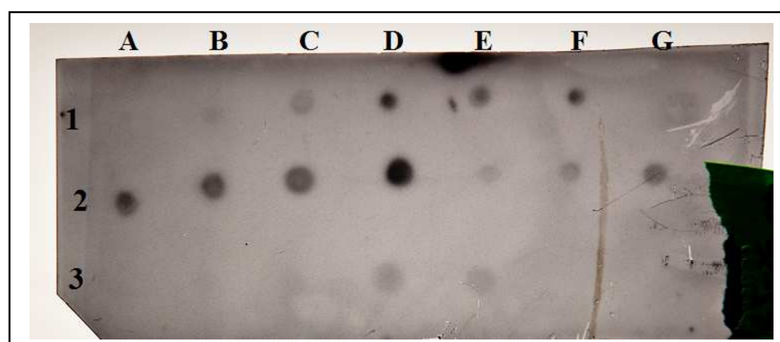


Figure 60. Results of dot blot at 56ºC using DIG-BCRomp2b probe (first stringency wash at room temperature)

1-*B. cereus* ATCC 14579 A) 5ng, B) 10ng, C) 30ng, D) 50ng, E) 80ng, F)100ng, G) no template control

2-*B. cereus* ATCC 14579 A) 150ng, B) 200ng, C) 250ng, D) 300ng; E) *B. licheniformis* ATCC 12759 (150ng), F) *B. amyloliquefaciens* 10A6 (150ng), G) *B. subtilis* BSFUL1 (150ng)

3-A) *L. casei* DPC 6059 (150ng), B) *L. acidophilus* DPC 6060 (150ng), C) *C. jejuni* ULCV48 (150ng), D) *L. monocytogenes* LMFUL1 (150ng), E) *E. coli* ATCC 25922 (150ng), F) *Salmonella* Typhimurium NCTC 74 (150ng), G) *L. lactis* 3054 (150ng)

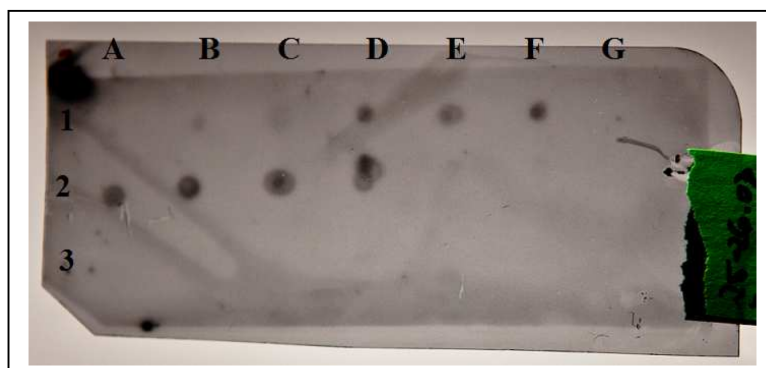


Figure 61. Results of dot blot at 56ºC using DIG-BCRomp2b probe (stringency washes at hybridization temperature)

1-*B. cereus* ATCC 14579 A) 5ng, B) 10ng, C) 30ng, D) 50ng, E) 80ng, F) 100ng, G) no template control

2-*B. cereus* ATCC 14579 A) 150ng, B) 200ng, C) 250ng, D) 300ng; E) *B. licheniformis* ATCC 12759 (150ng), F) *B. amyloliquefaciens* 10A6 (150ng), G) *B. subtilis* BSFUL1 (150ng)

3-A) *L. casei* DPC 6059 (150ng), B) *L. acidophilus* DPC 6060 (150ng), C) *C. jejuni* ULCV48 (150ng), D) *L. monocytogenes* LMFUL1 (150ng), E) *E. coli* ATCC 25922 (150ng), F) *Salmonella* Typhimurium NCTC 74 (150ng), G) *L. lactis* 3054 (150ng)

After optimization of the temperature and other conditions for specific hybridization of DIG-BCRomp2b probe, the dot blot assay for 7 strains representing each species of *B. cereus* group spp. was performed.

The DNA used in the assay was extracted from: *Bacillus* sp. Ba813^{+} #19 (T5 97-77) (1A), *B. thuringiensis* DSM 6017 (1B), *B. weihenstephanensis* DSM 11821 (1C), *B. mycoides* 6A12 (1D), *B. pseudomycoides* DSM 12442 (1E), *B. anthracis* 34F2 (1F), *B. cereus* ATCC 14579 (2A). DNA was spotted in amount 200ng/µl and the results are outlined in Figure 62.

In each dot blot assay a no template control was included, instead of DNA 10mM Tris-HCl pH 8.0 was spotted onto nylon membrane.

The results demonstrated that newly designed DIG-BCRomp2b probe at appropriate temperature of hybridization is specific to each member of *B. cereus* group spp. and, as described earlier, at the same temperature does not hybridize to any other *Bacillus* and non-*Bacillus* species.

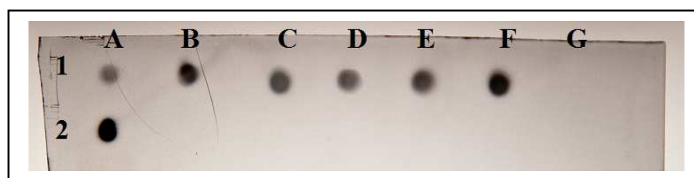


Figure 62. Results of dot blot at 56ºC using DIG-BCRomp2b probe for species belong to the *B. cereus* group

1-A) *Bacillus* sp. Ba813^{+} #19 (T5 97-77), B) *B. thuringiensis* DSM 6017,

C) *B. weihenstephanensis* DSM 11821, D) *B. mycoides* 6A12, E) *B. pseudomycoides* DSM 12442,

F) *B. anthracis* 34F2, G) no template control; (200ng DNA per dot);

2-A) *B. cereus* ATCC 14579; (200ng DNA per dot)

The limit of detection using DIG-BCRomp2b probe at hybridization temperature 56ºC was 50ng. The detection limit using the dilutions (1x10-1x10^8 gene copy numbers) of recombinant plasmid (pGEM-*motB*) was also checked at the same hybridization conditions and was 1x10^4 gene copy numbers (data not shown).

The designed DIG-BCRomp2b probe with optimized hybridization conditions is an ideal DNA fragment for specific identification of the *B. cereus* group spp. based on hybridization techniques. The probe can also differentiate the *B. cereus* group spp. from other, more closely related, bacterial groups. As our research was focused on detection and identification this bacterial group in milk, the specificity of the probe hybridization was checked against both: milk pathogens (e.g. *Salmonella*, *Campylobacter jejuni*, *and Listeria monocytogenes*) and microorganisms that naturally occur in milk (e.g. *L. casei*, *L. acidophilus*, *L. lactis*).

Designing of *B. pseudomycoides* unique probe:

Analysis of the 217bp PCR product (and inverted version) amplified with BpmF/BpmR2 primers by blasting the sequence against *B. cereus* group spp., and other *Bacillus* and non-*Bacillus* species presented the possible sequence for *B. pseudomycoides* differentiation. The sequence of the probe has been chosen based on requirements in designing the oligonucleotide probes for dot blot hybridization Table 38.

Table 38. Details of DIG-Bpm probe

Probe	Sequence (5'-3')	Size	GC content (%)	Tm (ºC)
DIG-Bpm	DIG-TAGCGTATGACTACCTTCTCAGCTTAATATATACCTA	37nt	35.1	58.6

Hybridization was performed for the same strains belong to the *B. cereus* group spp., and other *Bacillus* and non-*Bacillus*, which were used during hybridization using DIG-BCRomp2b probe. Also the procedure, except hybridization temperature, was performed at the same conditions as for the previous probe (DIG-BCRomp2b). Hybridization temperatures used in this experiment were: 49ºC and 53.5ºC. To check the efficiency of the hybridization with DIG-Bpm probe, the dilutions of DNA extracted from *B. pseudomycoides* DSM 12442, and recombinant clone ($1x10$-$1x10^8$ gene copy numbers of pGEM-*Bpm*) were spotted onto the nylon membrane. After overnight incubation, the results for both temperatures were the same. Only *B. pseudomycoides* strain DSM 12442 showed a positive result. None of the other strains belonging to the *B. cereus* group spp.: *B. cereus* ATCC 14579, *B. thuringiensis* DSM 6017, *B. weihenstephanensis* DSM 11821, *B. anthracis* 34F2, *Bacillus* sp. Ba813$^+$ #19 (T5 97-77), *B. mycoides* 6A12 showed the hybridization dot. The same results presented with other tested *Bacillus* and non-*Bacillus* strains: *B. subtilis* BSF1, *B. licheniformis* ATCC 12759, *B. amyloliquefaciens* 10A6, *Listeria monocytogenes* LMFUL1, *Yersinia enterocolitica* 27729, *Escherichia coli* ATCC 25922, *Campylobacter jejuni* ULCV48, *Salmonella* Typhimurium NCTC 74, *Lactobacillus acidophilus* DPC 6060 and 5378, *Lactobacillus casei* DPC 6059, *Lactococcus lactis* 3054 and *Lactococcus lactis* HP. Each DNA was spotted in the amount of 150ng/µl.

The limit of detection was 150ng and $1x10^6$ gene copy numbers for *B. pseudomycoides* strains.

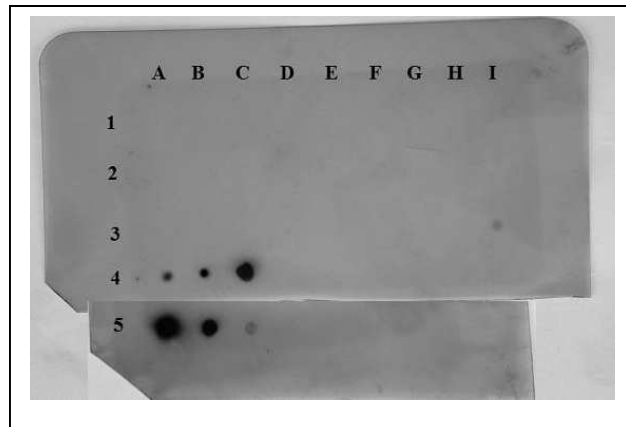


Figure 63. Results of dot blot at 49ºC using DIG-Bpm probe

1-A) *B. subtilis* BSF1, B) *B. licheniformis* ATCC 12759, C) *B. amyloliquefaciens* 10A6, D) *Listeria monocytogenes* LMFUL1, E) *Yersinia enterocolitica* 27729, F) *Escherichia coli* ATCC 225922, G) *Campylobacter jejuni* ULCV48, H) *Salmonella* Typhimurium NCTC 74, I) no template control; (150ng DNA per dot);

2-A) *Lactobacillus acidophilus* DPC 6060, B) *Lactobacillus acidophilus* DPC 5378, C) *Lactobacillus casei* DPC 6059, D) *Lactococcus lactis* 3054, E) *Lactococcus lactis* HP, F) -, G) *B. cereus* ATCC 14579, H) *B. thuringiensis* DSM 6017, I) *B. weihenstephanensis* DSM 11821; (150ng DNA per dot);

3-A) *B. anthracis* 34F2, B) *Bacillus* sp. Ba813$^+$ #19 (T5 97-77), C) *B. mycoides* 6A12,

3-D) to 4-C) *B. pseudomycoides* DSM 12442: 3-D) 20ng, E) 30ng, F) 40ng, G) 50ng, H) 100ng, I) 150ng;

4-A) 200ng, B) 250ng, C) 300ng;

5-A) to 5-I) pGEM-*Bpm*: A) 1x10^8 gene copy numbers, B) 1x10^7 gene copy numbers, C) 1x10^6 gene copy numbers, D) 1x10^5 gene copy numbers, E) 1x10^4 gene copy numbers, F) 1x10^3 gene copy numbers, G) 1x10^2 gene copy numbers, H) 1x10 gene copy numbers, I) 1 gene copy number

4. CONCLUSIONS AND FUTURE WORK

This study focused on identification the *B. cereus* group spp. frequently found in milk and food products. A total 138 strains were characterized by traditional microbiological techniques to confirm their phenotypic features. Strains were also confirmed with PCR methods targeting different DNA fragments as published in the literature i.e.: 16*S* rDNA (Hansen *et al.* 2001), *cspA* (Lechner *et al.* 1998), *gyrB* (Yamada *et al.* 1999), Ba813 (Patra *et al.* 1996), *motB* (Molnar 2005). The authors of those methods did not test a large collection of isolates. Moreover, often the quantification and limit of detection were not obtained. Therefore, new primers (BCFomp2/BCRomp2) targeting the *motB* gene were designed to investigate the use of this gene sequence in the *B. cereus* group spp. identification. In addition, newly designed primers (BpmF/BpmR2) targeting a hypothetical gene with unknown function were able to identify and differentiate *B. pseudomycoides* strains from other members belonging to the *B. cereus* group spp. Both sets of primers (BCFomp2/BCRomp2 and BpmF/BpmR2) used in one multiplex PCR reaction were able to identify the *B. cereus* group spp. and differentiate *B. pseudomycoides* strains. This is the first description of a molecular method to differentiate *B. pseudomycoides* from the *B. cereus* group.
Estimation of the limit of detection for each assays allowed for better quantification of bacteria isolated from an environmental medium e.g. milk.

A RT-PCR is less labour intensive and automated, therefore our studies looked at using this technology. The evaluation of RT-PCR with LightCycler® for detection the *B. cereus* group spp. allowed for reliable measurement of *B. cereus* group spp. in milk sample. Two different sets of primers (BCFomp2/BCRomp2 and BpmF/BpmR2) and three TaqMan probes (MotB-FAM-1, MotB-FAM-2 and Bpm-FAM-1) successfully identified the bacterial group and distinguished *B. weihenstephanensis* and *B. pseudomycoides* strains. Differentiation of *B. weihenstephanensis* was very significant in this study because the bacterium as a psychrotrophic organism is able to grow below 7ºC where milk is stored. Attempts to design DNA primers and probe allowing differentiation of *B. pseudomycoides* had been successful. *B. pseudomycoides* could only be differentiated from *B. mycoides* strains with gas chromatographic fatty acid methyl ester analysis. This study is the first description of molecular technique able to distinguish *B. pseudomycoides* from *B. mycoides* and other members belonging to the *B.*

cereus group spp. The estimated limits of detection and standard curves showed very good sensitivity of the designed primers and probes. Evaluation of the specificity of the new PCR, multiplex PCR and RT-PCR assays was performed by including DNA from a wide range of bacterial strains. Assays presented excellent selectivity and performed only with *B. cereus* group spp.

A parallel study to develop of a portable/handheld biological affinity sensor to detect and monitor the microbial quality of milk was also carried out in the laboratory. Attention was focused on *B. cereus* group spp. Electrochemical DNA-based Nano-Biosensors should detect specific nucleic acid sequences by specific hybridization of complementary DNA fragments. High specificity of nucleic acid base pairings between homologous strands of ssDNA is the basic principle for DNA biosensors. Single-stranded nucleid acid (ssDNA) immobilized on a specific surface can recognize its complementary nucleotides, generate and transfer physical-chemical signals.

The objective of the study was to develop the sensor for the milk industry. The sequences of our newly designed probes, primers and amplified products were analyzes against genomes of other microorganism with special emphasis to milk pathogens. Hybridization of selected ssDNA probes was optimized by dot blot assay. It allowed for selection of optimal probes and the temperature of hybridization in which the probe will hybridize only with DNA of *B. cereus* group spp. Through this mechanism, bacteria can be identified and a hand held devices developed to exhibit the response.

DIG-BCRomp2b probe, as an ideal fragment of DNA identifying the whole *B. cereus* group spp., and the sequence of DIG-Bpm probe for detection and differentiation only *B. pseudomycoides* can be immobilized to the biorecognition layer of the DNA-based biosensor. The recognition of complementary DNA would be based on hybridization process at experimentally found optimal temperature.

Estimated standard curves for each RT-PCR assay allows for applying those result in designing the specific biosenor software able to show the results in real time. To receive the biosensing signal from the milk containing bacilli spores, the samples should be pre-treated by incubation at appropriate temperature for specific time for fat and nonfat milk.

In conclusion:

(i) PCR primers targeting the *motB* gene for identification of the *B. cereus* group spp. were tested against 136 strains (BCFomp1/BCRomp1).

(ii) New primers and probes targeting the *motB* and *Bpm* genes were designed and tested using RT-PCR

- detection of *B. cereus* group spp. (BCFomp2/BCRomp2/BpmF/BpmR2 primers and MotB-FAM-1/MotB-FAM-2/Bpm-FAM-1 probes),
- differentiation of *B. weihenstephanensis* strains (BCFomp2/BCRomp2 primers and MotB-FAM-2 probe),
- differentiation of *B. pseudomycoides* strains (BpmF/BpmR2 primers and Bpm-FAM-1 probe).

(iii) RT-PCR assays were validated against 158 strains.

(iv) RT-PCR standard curves to detect gene copy numbers and limit of detection were generated.

(v) Multiplex PCR for identification of the *B. cereus* group spp. and differentiation of *B. pseudomycoides* was optimized.

(vi) Probes for biosensor adaptation were designed and hybridization of probes was optimized using the dot blot assay.

The future work in the laboratory will concentrate on designing specific and unique probes able to distinguish other species belong to the *B. cereus* group.

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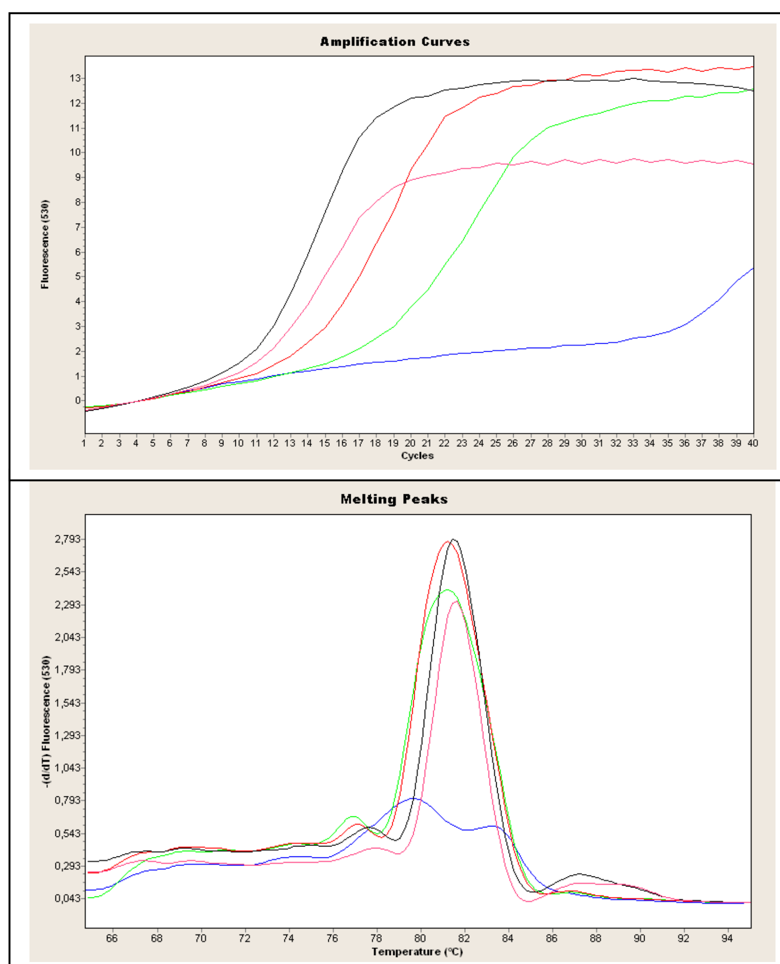
7. APPENDICES

Appendix A. RT-PCR with 0.8µM BCFomp1/BCRomp1 primers using detection based on SYBR Green fluorescence signal.

3µl of DNA ($1x10^7$ gene copy numbers) were amplified with the same primer concentration (0.8µM each) and different concentration of $MgCl_2$: 2mM, 2.25mM, 2.5mM and 3mM.

NTC- No Template Control

	a)	b)	c)	d)
$MgCl_2$ (50mM)	2mM	2.25mM	2.5mM	3mM
BCFomp1 (10µM)	0.8µM	0.8µM	0.8µM	0.8µM
BCRomp1 (10µM)	0.8µM	0.8µM	0.8µM	0.8µM
DNA	$1x10^7$ gene copy numbers	$1x10^7$ gene copy numbers	$1x10^7$ gene copy numbers	$1x10^7$ gene copy numbers



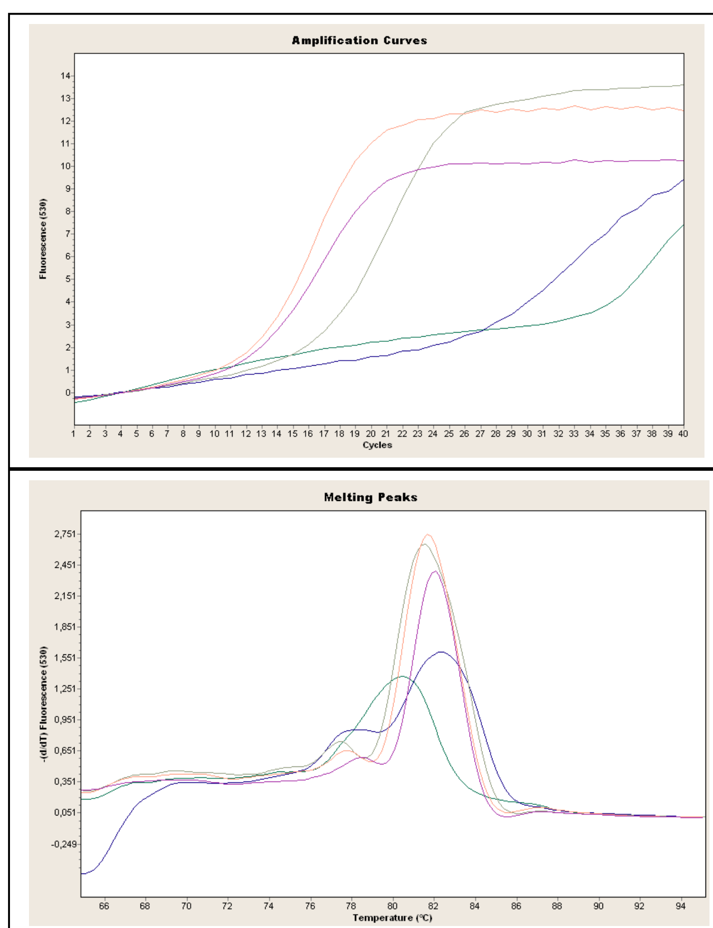
Samples			
Color	Pos	Name	CP
	1	NTC 2.5mM MgCl	>35,00
a)	2	pDNA(10E7) 2mM Mg,0.8uMp	[18,55]
b)	3	pDNA 2.25mM Mg,0.8uMpr	13,92
c)	4	pDNA 2.5mM Mg,0.8uMpr	10,11
d)	5	pDNA(10E7) 3mM Mg,0.8uMp	10,56

Appendix B. RT-PCR with 0.5µM BCFomp1/BCRomp1 primers using detection based on SYBR Green fluorescence signal.

3µl of DNA ($1x10^7$ gene copy numbers) were amplified with the same primer concentration (0.5µM each) and different concentration of $MgCl_2$: 2mM, 2.25mM, 2.5mM and 3mM.

NTC-No Template Control

	a)	b)	c)	d)
$MgCl_2$ (50mM)	2mM	2.25mM	2.5mM	3mM
BCFomp1 (10µM)	0.5µM	0.5µM	0.5µM	0.5µM
BCRomp1 (10µM)	0.5µM	0.5µM	0.5µM	0.5µM
DNA	$1x10^7$ gene copy numbers	$1x10^7$ gene copy numbers	$1x10^7$ gene copy numbers	$1x10^7$ gene copy numbers



	6	NTC 2.5mM MgCl	>35,00
a)	7	pDNA(10E7) 2mM Mg,0.5uMp	27,39
b)	8	pDNA,2.25mM Mg,0.5uMpr	16,50
c)	9	pDNA,2.5mM Mg,0.5uMpr	11,83
d)	10	pDNA(10E7) 3mM Mg,0.5uMp	11,92

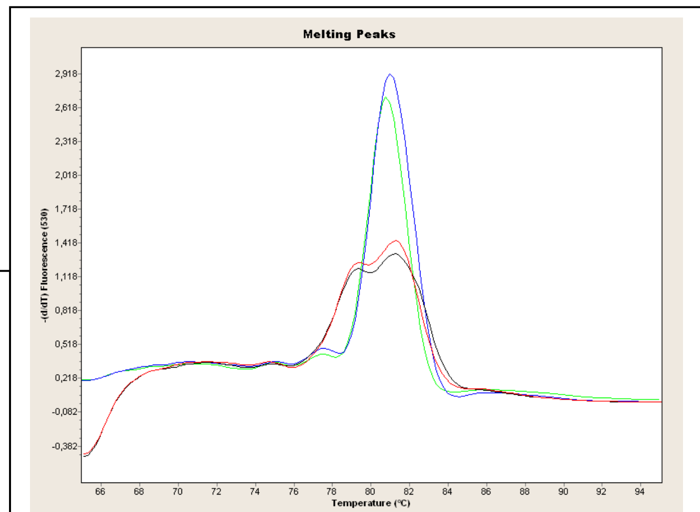
Appendix C. RT-PCR with 0.8µM BCFomp1/BCRomp1 primers and varied DNA concentration using detection based on SYBR Green fluorescence signal.

The same primer (0.8µM each) and $MgCl_2$ concentration (2.5mM) were used to amplify different DNA target concentration: $1x10^8$, $1x10^7$, $1x10^2$ gene copy numbers per reaction at various annealing temperature: a) 56˚C, b) 57˚C and c) 59˚C.

NTC- No Template Control

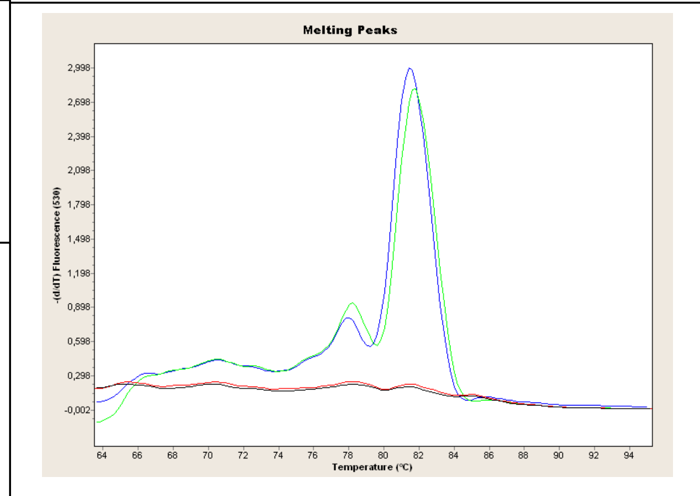
a)

Samples		
Color	Pos	Name
Blue	1	p.DNA, 10E8 copies
Green	2	p.DNA, 10E7 copies
Red	3	p.DNA, 10E2 copies
Black	4	NTC



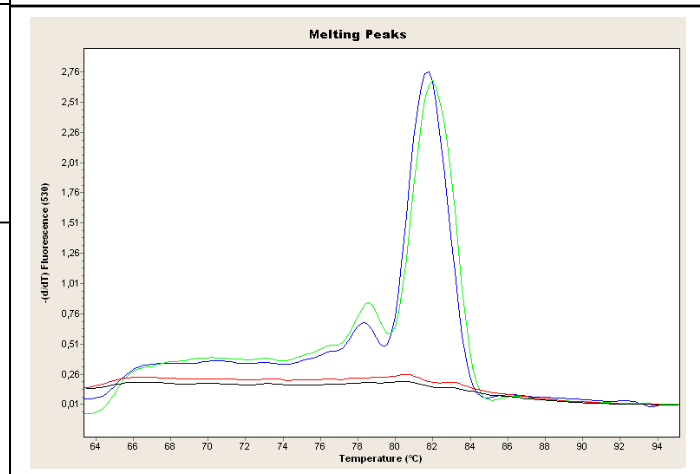
b)

Samples		
Color	Pos	Name
Blue	1	p.DNA, 10E8 copies
Green	2	p.DNA, 10E7 copies
Red	3	p.DNA, 10E2 copies
Black	4	NTC

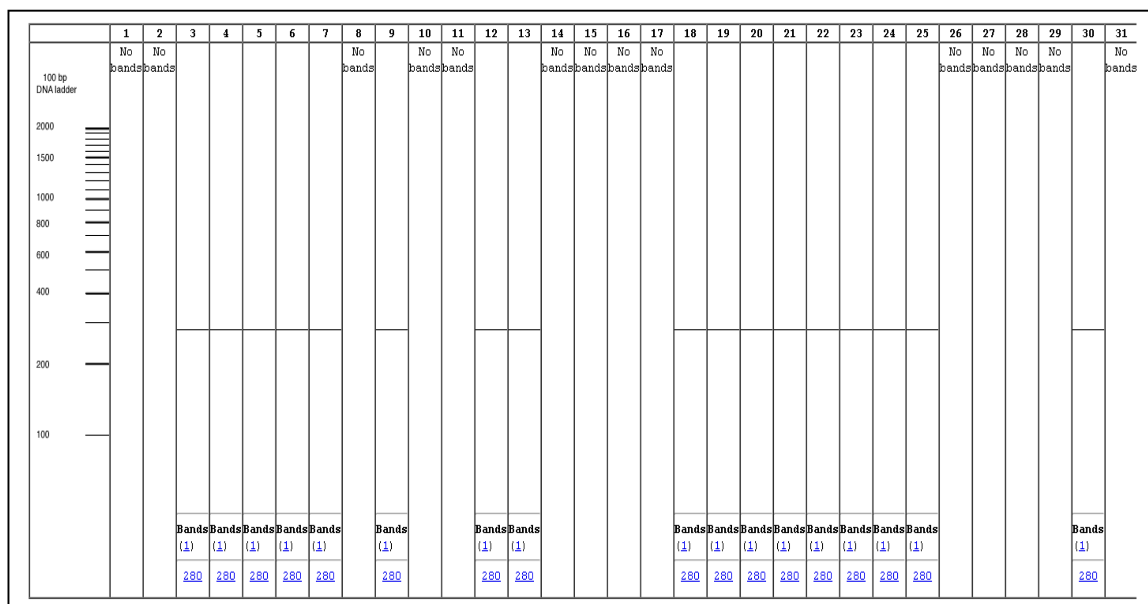


c)

Samples		
Color	Pos	Name
Blue	1	p.DNA, 10E8 copies
Green	2	p.DNA, 10E7 copies
Red	3	p.DNA, 10E2 copies
Black	4	NTC



Appendix D. Results of '*in silico* PCR' with BCFomp3/BCRomp2 primers against 31 members of *Bacillus* group with completed genomic sequences available in 2010.



Selected strains

1 - *Bacillus subtilis* subsp. *subtilis str.* 168; 2 - *Bacillus halodurans C-125*; 3 - *Bacillus cereus* ATCC 14579;
4 - *Bacillus anthracis* str. Ames; 5 - *Bacillus cereus* ATCC 10987; 6 - *Bacillus anthracis* str. Ames 0581
7 - *Bacillus thuringiensis* 97-27; 8 - *Bacillus licheniformis* ATCC 14580; 9 - *Bacillus cereus* ZK
10 - *Bacillus licheniformis* DSM 13; 11 - *Bacillus clausii* KSM-K16; 12 - *Bacillus thuringiensis* str. Al Hakam;
13 - *Bacillus anthracis* str. Sterne; 14 - *Bacillus cereus* subsp. cytotoxis NVH 391-98
15 - *Bacillus amyloliquefaciens* FZB42; 16 - *Bacillus pumilus* SAFR-032;
17 – *Bacillus weihenstephanensis* KBAB4; 18 - *Bacillus cereus* AH187; 19 - *Bacillus cereus* B4264;
20 - *Bacillus cereus* G9842; 21 – *Bacillus cereus* AH820; 22 - *Bacillus cereus* Q1;
23 - *Bacillus cereus* 03BB102; 24 - *Bacillus anthracis* str. CDC 684; 25 - *Bacillus anthracis* str. A0248;
26- *Bacillus pseudofirmus* OF4; 27 - *Bacillus megaterium* QM B1551; 28 - *Bacillus tusciae* DSM 2912;
29 – *Bacillus megaterium* DSM 319; 30 – *Bacillus thuringiensis* BMB171;
31 – *Bacillus selenitireducens* MLS10

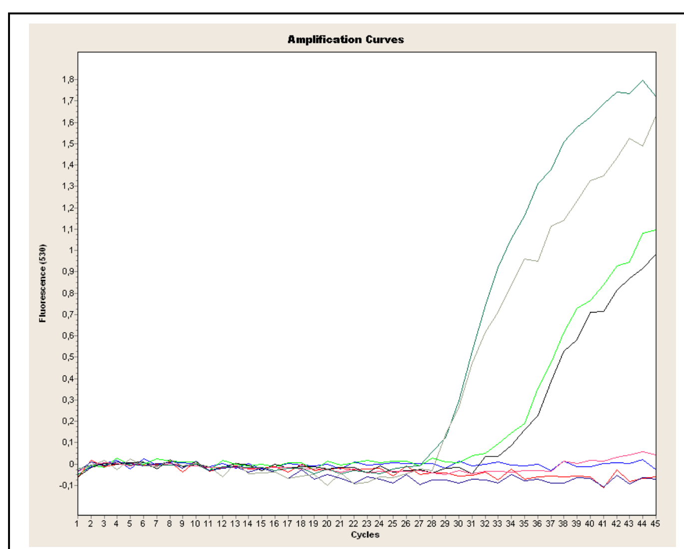
Appendix E. RT-PCR with BCFomp1/BCRomp1 and BCFomp2/BCRomp1 primers using detection based on MotB-FAM-1 probe (0.07µM) fluorescence signal.

The same primer (0.8µM each) and $MgCl_2$ (2.5mM) concentration were used to amplify the same DNA target in concentration $1x10^3$ gene copy numbers.

Samples 1-4: amplification with BCFomp1/BCRomp1 primers.

Samples 5-8: amplification with BCFomp2/BCRomp1 primers.

NTC-No Template Control



Samples			
Color	Pos	Name	CP
Blue	1	NTC	
Green	2	1x10E3 BCFomp1/BCRomp1	29,90
Red	3	NTC	
Black	4	1x10E3 BCFomp1/BCRomp1	31,99
Pink	5	NTC	
Dark green	6	1x10E3 BCFomp2/BCRomp1	27,13
Dark blue	7	NTC	
Grey	8	1x10E3 BCFomp2/BCRomp1	26,36

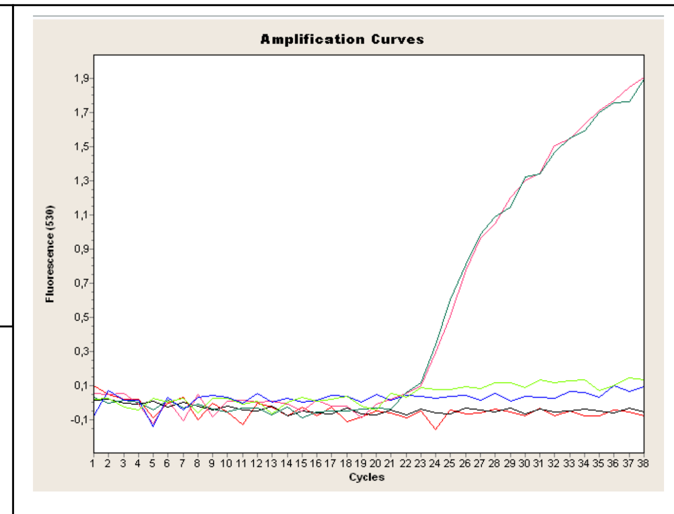
Appendix F. RT-PCR with BCFomp2/BCRomp2 primers (0.8µM) and probe MotB-FAM-1 (0.07µM).

1. NTC-No Template Control; 2. *B. weihenstephanensis* WSBC 10201;

3. *B. weihenstephanensis* WSBC 10392; 4. *B. weihenstephanensis* WSBC 10389;

5. *B. cereus* ATCC 14579; 6. *B. thuringiensis* DSM 6017

Samples			
Color	Pos	Name	CP
	1	NTC	
	2	WSBC10201,MotB-FAM-1	
	3	WSBC10392,MotB-FAM-1	
	4	WSBC10389,MotB-FAM-1	
	5	B.c.ATCC14579,MotB-FAM-1	21,11
	6	B.t.DSM6017,MotB-FAM-1	20,85

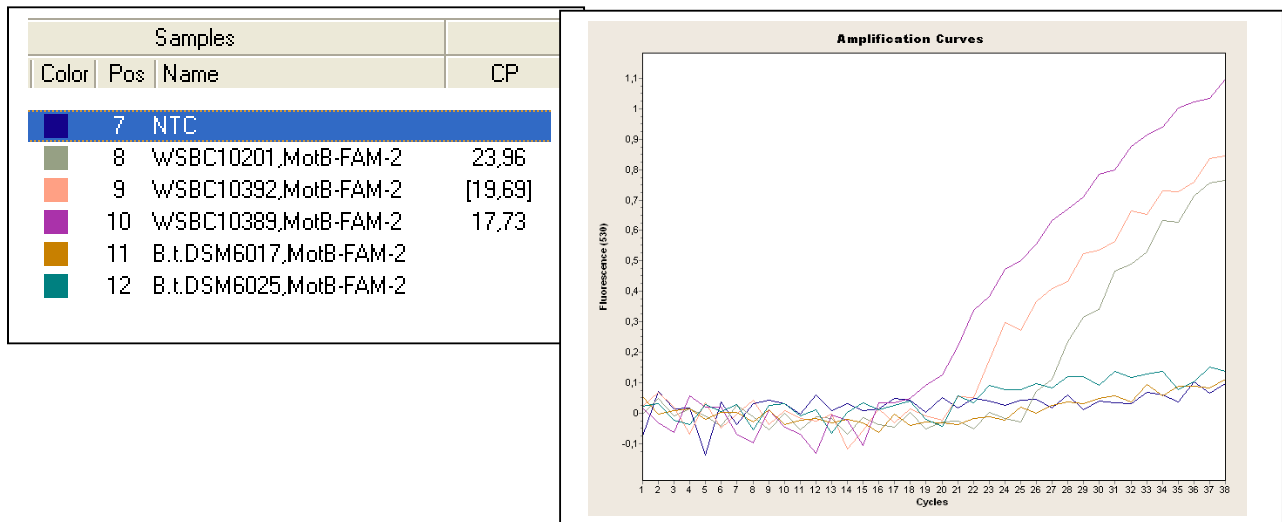


Appendix G. RT-PCR with BCFomp2/BCRomp2 primers (0.8µM) and probe MotB-FAM-2 (0.07µM).

7. NTC-No Template Control; 8. *B. weihenstephanensis* WSBC 10201;

9. *B. weihenstephanensis* WSBC 10392; 10. *B. weihenstephanensis* WSBC 10389;

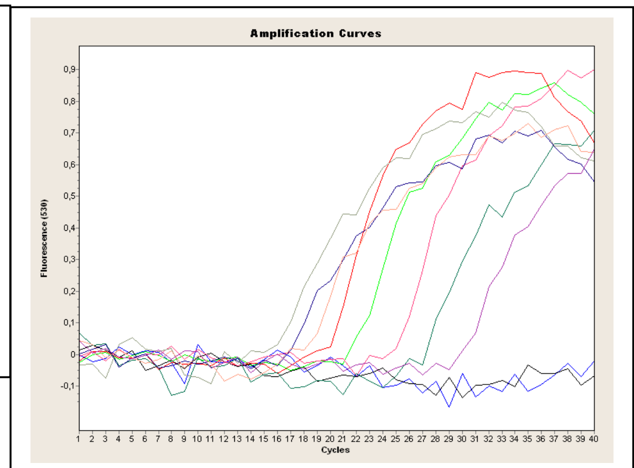
11. *B. thuringiensis* DSM 6017; 12. *B. thuringiensis* DSM 6025



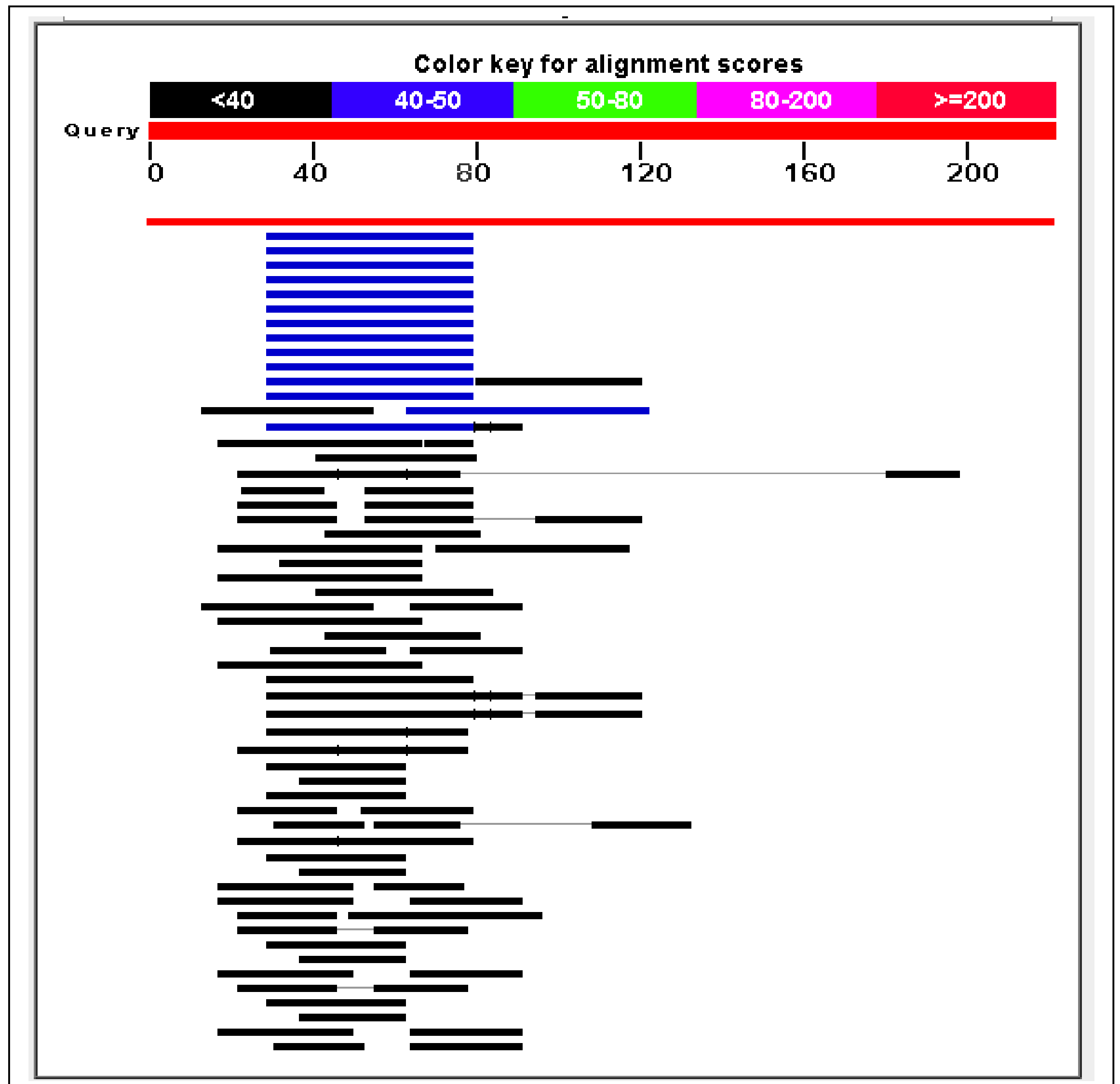
Appendix H. RT-PCR reaction with BCFomp2/BCRomp2 primers (0.8µM) and MotB-FAM-2 probe (0.07µM) presenting different of *B. mycoides* and *B. pseudomycoides* strains.

1. NTC-No Template Control; 2. *B. mycoides* BMFUL1; 3. *B. mycoides* BMSUL1; 4. *B. mycoides/pseudomycoides* GRD 1/17; 5. *B. mycoides/pseudomycoides* 22/2; 6. *B. mycoides/pseudomycoides* 29/2; 7. *B. mycoides* 6A11; 8. *B. mycoides* 6A13; 9. *B. mycoides* 6A14; 10. *B. weihenstephanensis* DSM 11821-positive control

Samples			
Color	Pos	Name	CP
	1	NTC	
	2	BMFUL1	19,98
	3	BMSUL1	18,10
	4	GDR 1/17	
	5	Bm/Bpm 22/2	23,13
	6	Bm/Bpm 29/2	24,85
	7	Bm 6A11	14,98
	8	Bm 6A13	14,45
	9	Bm 6A14	15,68
	10	DSM 11821	28,10

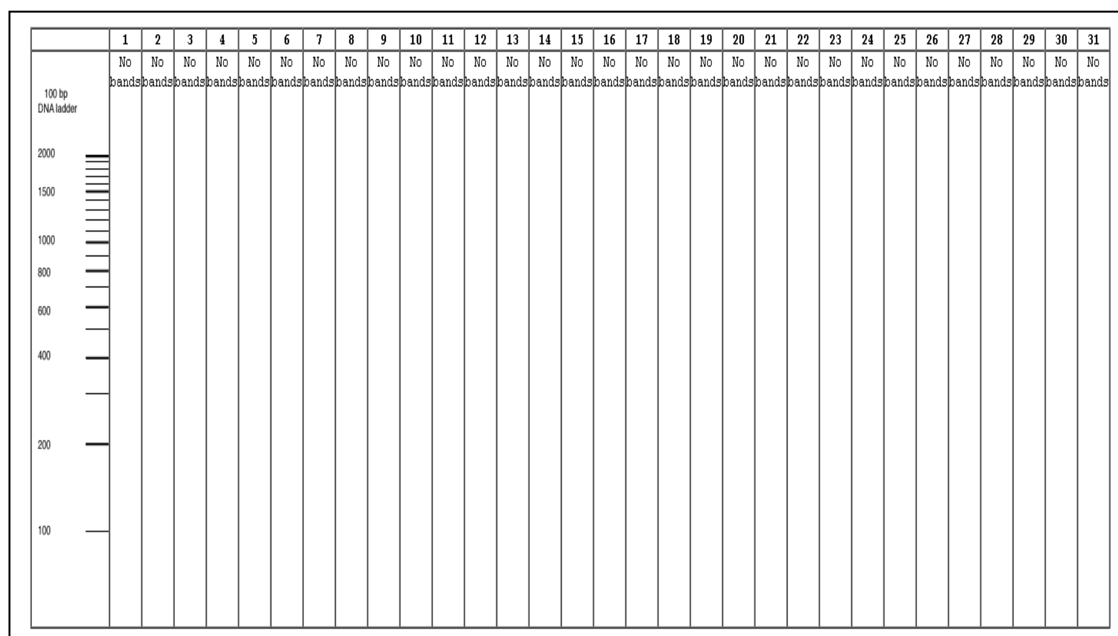


Appendix I. The result of blasting the selected 220bp sequence against other *B. cereus* group spp. available in BLAST databases in 2010.



Sequences producing significant alignments:		(Bits)	Value
ref|NZ_ACMX01000122.1|	Bacillus pseudomycoides DSM 12442 cont...	398	1e-1
ref|NZ_ACNF01000055.1|	Bacillus thuringiensis serovar berline...	42.8	0.01
ref|NZ_ACND01000061.1|	Bacillus thuringiensis serovar kurstak...	42.8	0.01
ref|NZ_ACNC01000078.1|	Bacillus thuringiensis serovar pakista...	42.8	0.01
ref|NZ_ACNA01000048.1|	Bacillus thuringiensis serovar thuring...	42.8	0.01
ref|NZ_ACMZ01000047.1|	Bacillus thuringiensis Bt407 contig000...	42.8	0.01
ref|NZ_ACMQ01000074.1|	Bacillus cereus AH676 contig00121, who...	42.8	0.01
ref|NZ_ACMO01000034.1|	Bacillus cereus F65185 contig00098, wh...	42.8	0.01
ref|NZ_ACMM01000145.1|	Bacillus cereus Rock4-2 contig00228, w...	42.8	0.01
ref|NZ_ACMH01000053.1|	Bacillus cereus Rock1-15 contig00230, ...	42.8	0.01
ref|NZ_ACME01000046.1|	Bacillus cereus BDRD-Cer4 contig00057,...	42.8	0.01
ref|NZ_ACLV01000044.1|	Bacillus cereus 172560W contig00043, w...	42.8	0.01
ref|NZ_ABDA02000009.1|	Bacillus cereus AH1134 gcontig_1113129...	42.8	0.01
ref|NZ_ABDM02000006.1|	Bacillus cereus 03BB108 gcontig_111231...	42.8	0.01
ref|NC_004722.1|	Bacillus cereus ATCC 14579, complete genome	42.8	0.01
ref|NZ_ACMB01000048.1|	Bacillus cereus BDRD-ST24 contig00587,...	39.2	0.15
ref|NZ_ABDA02000035.1|	Bacillus cereus AH1134 plasmid pAH1134...	39.2	0.15
ref|NC_003909.8|	Bacillus cereus ATCC 10987, complete genome	39.2	0.15
ref|NZ_ACNL01000082.1|	Bacillus thuringiensis IBL 4222 contig...	37.4	0.54
gb|ACNK01000052.1|	Bacillus thuringiensis IBL 200 contig00059...	37.4	0.54
ref|NZ_ACNH01000023.1|	Bacillus thuringiensis serovar pondich...	37.4	0.54
ref|NZ_ACNB01000085.1|	Bacillus thuringiensis serovar sotto s...	37.4	0.54
ref|NZ_ACMY01000046.1|	Bacillus thuringiensis serovar tochigi...	37.4	0.54
ref|NZ_ACMU01000045.1|	Bacillus mycoides DSM 2048 contig00081...	37.4	0.54
ref|NZ_ACMP01000063.1|	Bacillus cereus AH603 contig00225, who...	37.4	0.54
ref|NZ_ACMP01000065.1|	Bacillus cereus AH603 contig00227, who...	37.4	0.54
ref|NZ_ACMO01000203.1|	Bacillus cereus F65185 contig00046, wh...	37.4	0.54
ref|NZ_ACMF01000020.1|	Bacillus cereus 95/8201 contig00071, w...	37.4	0.54
ref|NZ_ACMD01000081.1|	Bacillus cereus BDRD-ST196 contig01305...	37.4	0.54
ref|NZ_ACLZ01000023.1|	Bacillus cereus ATCC 4342 contig00070,...	37.4	0.54
ref|NZ_ACLY01000048.1|	Bacillus cereus R309803 contig00937, w...	37.4	0.54
ref|NZ_ACLX01000057.1|	Bacillus cereus AH621 contig00099, who...	37.4	0.54
ref|NZ_ACLW01000046.1|	Bacillus cereus MM3 contig00051, whole...	37.4	0.54
ref|NZ_ACLT01000051.1|	Bacillus cereus ATCC 10876 contig00004...	37.4	0.54
ref|NC_014171.1|	Bacillus thuringiensis BMB171 chromosome, co...	37.4	0.54
ref|NC_011772.1|	Bacillus cereus G9842, complete genome	37.4	0.54
ref|NC_010184.1|	Bacillus weihenstephanensis KBAB4, complete ...	37.4	0.54
ref|NZ_AAEQ01000035.2|	Bacillus anthracis str. Kruger B NZ_AA...	35.6	1.9
ref|NZ_AAEQ01000007.2|	Bacillus anthracis str. Kruger B NZ_AA...	35.6	1.9
ref|NZ_AAER01000039.2|	Bacillus anthracis str. Western North ...	35.6	1.9
ref|NZ_AAEO01000022.2|	Bacillus anthracis str. A1055 NZ_AAE00...	35.6	1.9
ref|NZ_ACNL01000073.1|	Bacillus thuringiensis IBL 4222 contig...	35.6	1.9
ref|NZ_ACNL01000277.1|	Bacillus thuringiensis IBL 4222 contig...	35.6	1.9
ref|NZ_ACNL01000329.1|	Bacillus thuringiensis IBL 4222 contig...	35.6	1.9
gb|ACNK01000049.1|	Bacillus thuringiensis IBL 200 contig00042...	35.6	1.9
gb|ACNK01000068.1|	Bacillus thuringiensis IBL 200 contig00218...	35.6	1.9
gb|ACNK01000093.1|	Bacillus thuringiensis IBL 200 contig00016...	35.6	1.9
gb|ACNK01000120.1|	Bacillus thuringiensis IBL 200 contig00187...	35.6	1.9
ref|NZ_ACNJ01000041.1|	Bacillus thuringiensis serovar pulsien...	35.6	1.9
ref|NZ_ACNJ01000047.1|	Bacillus thuringiensis serovar pulsien...	35.6	1.9
ref|NZ_ACNI01000047.1|	Bacillus thuringiensis serovar huazhon...	35.6	1.9
ref|NZ_ACNI01000070.1|	Bacillus thuringiensis serovar huazhon...	35.6	1.9
ref|NZ_ACNI01000111.1|	Bacillus thuringiensis serovar huazhon...	35.6	1.9
ref|NZ_ACNI01000192.1|	Bacillus thuringiensis serovar huazhon...	35.6	1.9
ref|NZ_ACNH01000037.1|	Bacillus thuringiensis serovar pondich...	35.6	1.9
ref|NZ_ACNG01000056.1|	Bacillus thuringiensis serovar andalou...	35.6	1.9
ref|NZ_ACNG01000064.1|	Bacillus thuringiensis serovar andalou...	35.6	1.9
ref|NZ_ACNF01000058.1|	Bacillus thuringiensis serovar berline...	35.6	1.9
ref|NZ_ACNF01000114.1|	Bacillus thuringiensis serovar berline...	35.6	1.9
ref|NZ_ACNE01000042.1|	Bacillus thuringiensis serovar monterr...	35.6	1.9
ref|NZ_ACNE01000049.1|	Bacillus thuringiensis serovar monterr...	35.6	1.9
ref|NZ_ACND01000063.1|	Bacillus thuringiensis serovar kurstak...	35.6	1.9
ref|NZ_ACND01000068.1|	Bacillus thuringiensis serovar kurstak...	35.6	1.9
ref|NZ_ACND01000142.1|	Bacillus thuringiensis serovar kurstak...	35.6	1.9
ref|NZ_ACNC01000191.1|	Bacillus thuringiensis serovar pakista...	35.6	1.9
ref|NZ_ACNB01000078.1|	Bacillus thuringiensis serovar sotto s...	35.6	1.9
ref|NZ_ACNB01000179.1|	Bacillus thuringiensis serovar sotto s...	35.6	1.9
ref|NZ_ACNB01000182.1|	Bacillus thuringiensis serovar sotto s...	35.6	1.9
ref|NZ_ACNA01000051.1|	Bacillus thuringiensis serovar thuring...	35.6	1.9
ref|NZ_ACNA01000110.1|	Bacillus thuringiensis serovar thuring...	35.6	1.9
ref|NZ_ACMZ01000051.1|	Bacillus thuringiensis Bt407 contig001...	35.6	1.9
ref|NZ_ACMZ01000115.1|	Bacillus thuringiensis Bt407 contig000...	35.6	1.9
ref|NZ_ACMY01000050.1|	Bacillus thuringiensis serovar tochigi...	35.6	1.9
ref|NZ_ACMY01000059.1|	Bacillus thuringiensis serovar tochigi...	35.6	1.9
ref|NZ_ACMW01000090.1|	Bacillus mycoides Rock3-17 contig00056...	35.6	1.9
ref|NZ_ACMV01000224.1|	Bacillus mycoides Rock1-4 contig00108,...	35.6	1.9
ref|NZ_ACMU01000044.1|	Bacillus mycoides DSM 2048 contig00061...	35.6	1.9
ref|NZ_ACMU01000094.1|	Bacillus mycoides DSM 2048 contig00077...	35.6	1.9
ref|NZ_ACMT01000112.1|	Bacillus cereus AH1273 contig00061, wh...	35.6	1.9
ref|NZ_ACMT01000132.1|	Bacillus cereus AH1273 contig00067, wh...	35.6	1.9
ref|NZ_ACMS01000104.1|	Bacillus cereus AH1272 contig00794, wh...	35.6	1.9
ref|NZ_ACMS01000136.1|	Bacillus cereus AH1272 contig00126, wh...	35.6	1.9
ref|NZ_ACMQ01000082.1|	Bacillus cereus AH676 contig00370, who...	35.6	1.9
ref|NZ_ACMQ01000211.1|	Bacillus cereus AH676 contig00039, who...	35.6	1.9
ref|NZ_ACMP01000035.1|	Bacillus cereus AH603 contig00193, who...	35.6	1.9
ref|NZ_ACMP01000064.1|	Bacillus cereus AH603 contig00226, who...	35.6	1.9
ref|NZ_ACMP01000145.1|	Bacillus cereus AH603 contig00061, who...	35.6	1.9
ref|NZ_ACMO01000038.1|	Bacillus cereus F65185 contig00030, wh...	35.6	1.9
ref|NZ_ACMO01000043.1|	Bacillus cereus F65185 contig00043, wh...	35.6	1.9
ref|NZ_ACMO01000122.1|	Bacillus cereus F65185 contig00064, wh...	35.6	1.9
ref|NZ_ACMO01000224.1|	Bacillus cereus F65185 contig00569, wh...	35.6	1.9
ref|NZ_ACMN01000050.1|	Bacillus cereus Rock4-18 contig00043, ...	35.6	1.9
ref|NZ_ACMM01000150.1|	Bacillus cereus Rock4-2 contig00232, w...	35.6	1.9
ref|NZ_ACMM01000154.1|	Bacillus cereus Rock4-2 contig00237, w...	35.6	1.9
ref|NZ_ACMK01000059.1|	Bacillus cereus Rock3-42 contig00025, ...	35.6	1.9
ref|NZ_ACMK01000065.1|	Bacillus cereus Rock3-42 contig00002, ...	35.6	1.9
ref|NZ_ACMH01000054.1|	Bacillus cereus Rock1-15 contig00231, ...	35.6	1.9
ref|NZ_ACMH01000108.1|	Bacillus cereus Rock1-15 contig00294, ...	35.6	1.9
ref|NZ_ACMG01000046.1|	Bacillus cereus Rock1-3 contig00020, w...	35.6	1.9
ref|NZ_ACMF01000040.1|	Bacillus cereus 95/8201 contig00002, w...	35.6	1.9

Appendix J. The results of '*in silico* PCR' with BpmF/BpmR2 primers against members of *Bacillus* group with completed genomic sequences available in 2010.



Selected strains

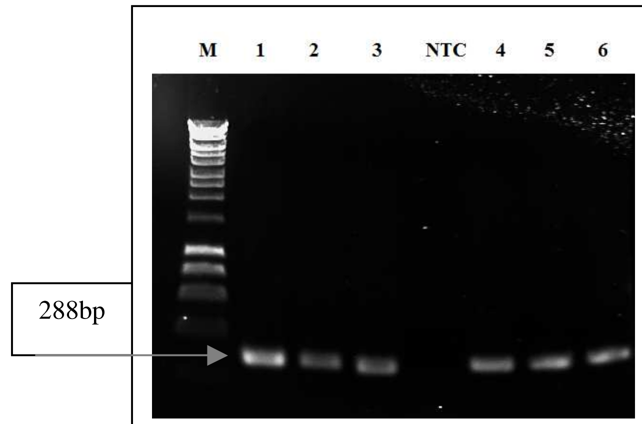
1 - *Bacillus subtilis* subsp. *subtilis str.* 168; 2 - *Bacillus halodurans C-125*; 3 - *Bacillus cereus* ATCC 14579;
4 - *Bacillus anthracis* str. Ames; 5 - *Bacillus cereus* ATCC 10987; 6 - *Bacillus anthracis* str. Ames 0581
7 - *Bacillus thuringiensis* 97-27; 8 - *Bacillus licheniformis* ATCC 14580; 9 - *Bacillus cereus* ZK
10 - *Bacillus licheniformis* DSM 13; 11 - *Bacillus clausii* KSM-K16; 12 - *Bacillus thuringiensis* str. Al Hakam;
13 - *Bacillus anthracis* str. Sterne; 14 - *Bacillus cereus* subsp. cytotoxis NVH 391-98
15 - *Bacillus amyloliquefaciens* FZB42; 16 - *Bacillus pumilus* SAFR-032;
17 – *Bacillus weihenstephanensis* KBAB4; 18 - *Bacillus cereus* AH187; 19 - *Bacillus cereus* B4264;
20 - *Bacillus cereus* G9842; 21 – *Bacillus cereus* AH820; 22 - *Bacillus cereus* Q1;
23 - *Bacillus cereus* 03BB102; 24 - *Bacillus anthracis* str. CDC 684; 25 - *Bacillus anthracis* str. A0248;
26- *Bacillus pseudofirmus* OF4; 27 - *Bacillus megaterium* QM B1551; 28 - *Bacillus tusciae* DSM 2912;
29 – *Bacillus megaterium* DSM 319; 30 – *Bacillus thuringiensis* BMB171;
31 – *Bacillus selenitireducens* MLS10

Appendix K. 1% agarose gel showing 288bp PCR product generated with 16*S* rDNA primers S-S-Bc-200-a-S-18 and S-S-Bc-470-a-S-18 (Hansen *et al.* 2001).

1. *B. pseudomycoides* WS 3120; 2. *B. mycoides* 6A49; 3. *B. cereus* BCMUL1;

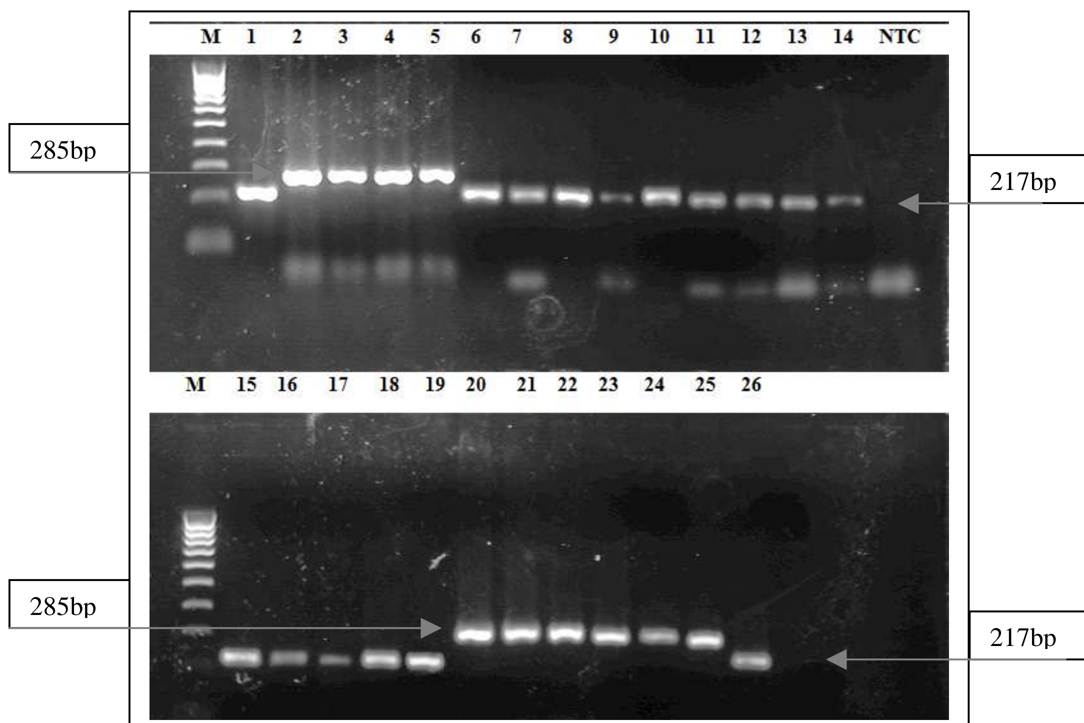
4. *B. thuringiensis* DSM 6017; 5. *B. anthracis* 34F2; 6. *B. mycoides* 6A11;

NTC-No Template Control; M-Molecular marker (HyperLadder I)



Appendix L. 2% agarose gel showing 285bp and 217bp multiplex PCR products generated with primers BCFomp2/BCRomp2 and BpmF/BpmR2, respectively.

1. *B. pseudomycoides* DSM 12442; 2. *B. cereus* ATCC 14579; 3. *B. thuringiensis* DSM 6017;
4. *B. weihenstephanensis* DSM 11821; 5. *B. anthracis* 34F2; 6. *B. mycoides/pseudomycoides* GRD 1/17;
7. *B. mycoides/pseudomycoides* 1/2; 8. *B. mycoides/pseudomycoides* 17/3;
9. *B. mycoides/pseudomycoides* GRD 2/71; 10. *B. mycoides* Nov1; 11. *B. mycoides* 6A19;
12. *B. mycoides* DSM 307; 13. *B. mycoides* Nov2; 14. *B. mycoides* A81;
15. *B. pseudomycoides* WS 3118; 16. *B. pseudomycoides* WS 3119;
17. *B. pseudomycoides* DSM 12443;18. *B. pseudomycoides* B346; 19. *B. pseudomycoides* B618;
20. *B. cereus* BCMUL1; 21. *B. cereus* BCMUL2; 22. *B. cereus* BCMUL3;
23. *B. weihenstephanensis* BWMUL1; 24. *B. weihenstephanensis* BLMUL5;
25. *B. weihenstephanensis* BWMUL7; 26. *B. pseudomycoides* A82;
NTC-No Template Control; M-Molecular marker (HyperLadder IV)



Appendix M. The RT-PCR results of milk contamination with 10^5 CFU/ml of bacterial culture

Fat and nonfat milk was used for the contamination. Two column-based kits were used for the extraction of genomic DNA: DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and Genomic Mini AX Food kit (A&A Biotechnology, Gdynia, Poland).

A) milk contaminated with *B. cereus* ATCC 14579.

Amplification with BCFomp2/BCRomp2 primers and MotB-FAM-1 probe (0.07µM).

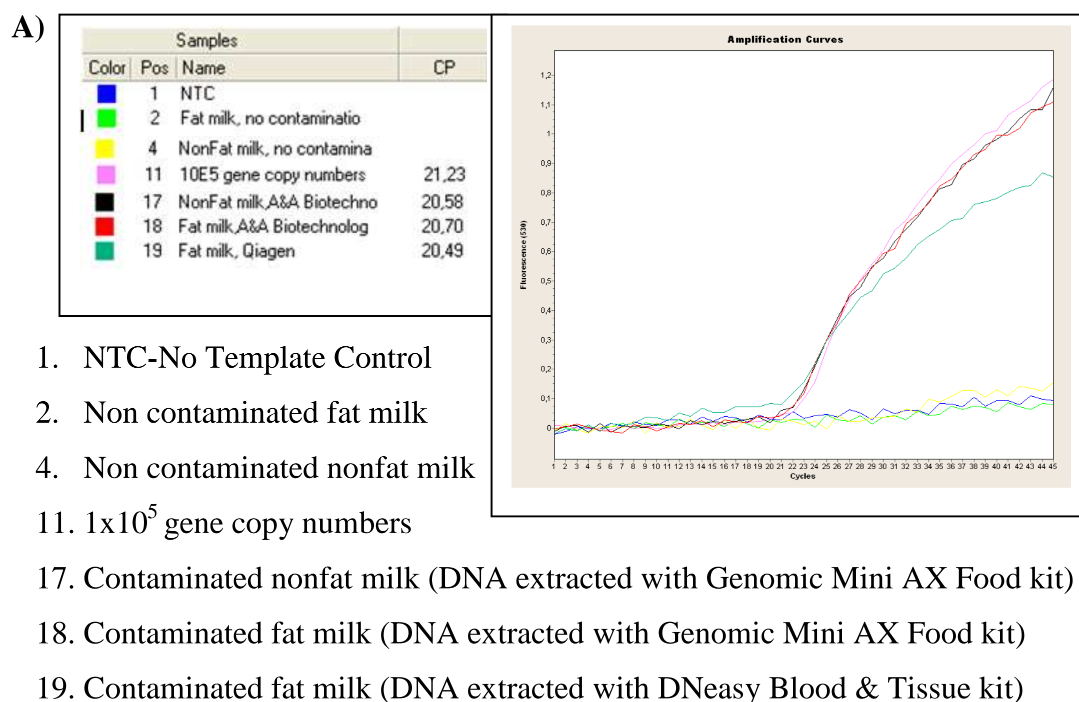
B) milk contaminated with *B. weihenstephanensis* DSM 11821.

Amplification with BCFomp2/BCRomp2 primers and MotB-FAM-2 probe (0.07µM).

C) milk contaminated with *B. pseudomycoides* DSM 12442

Amplification with BpmF/BpmR2 primers and Bpm-FAM-1 probe (0.07µM).

A)

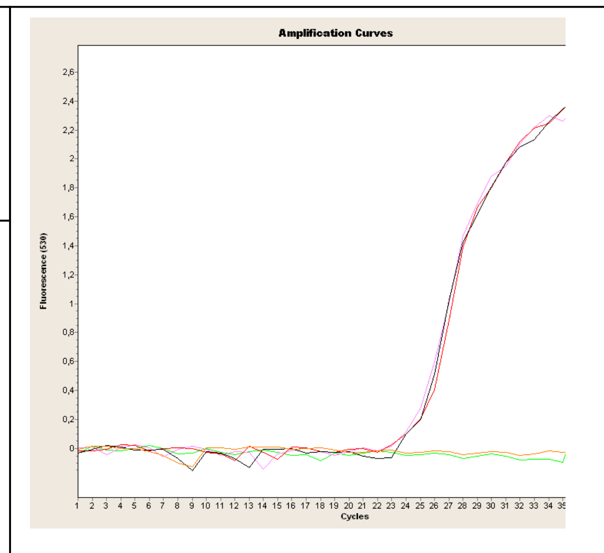


Color	Pos	Name	CP
	1	NTC	
	2	Fat milk, no contaminatio	
	4	NonFat milk, no contamina	
	11	10E5 gene copy numbers	21,23
	17	NonFat milk,A&A Biotechno	20,58
	18	Fat milk,A&A Biotechnolog	20,70
	19	Fat milk, Qiagen	20,49

1. NTC-No Template Control
2. Non contaminated fat milk
4. Non contaminated nonfat milk
11. 1x10^5 gene copy numbers
17. Contaminated nonfat milk (DNA extracted with Genomic Mini AX Food kit)
18. Contaminated fat milk (DNA extracted with Genomic Mini AX Food kit)
19. Contaminated fat milk (DNA extracted with DNeasy Blood & Tissue kit)

B)

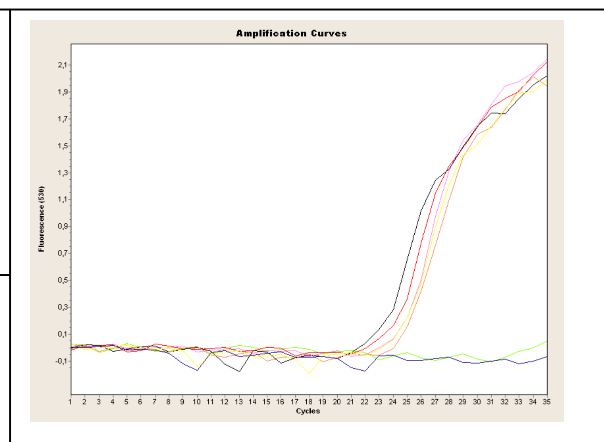
Samples			
Color	Pos	Name	CP
	1	Fat milk-no contamination	32,16
	2	10E5	22,57
	3	Fat milk, Qiagen	22,05
	4	NonFat milk, Qiagen	20,01
	5	NTC	



1. Non contaminated fat milk
2. $1x10^5$ gene copy numbers
3. Contaminated fat milk (DNA extracted with DNeasy Blood & Tissue kit)
4. Contaminated nonfat milk (DNA extracted with DNeasy Blood & Tissue kit)
5. NTC-No Template Control

C)

Samples			
Color	Pos	Name	CP
	1	Fat milk,no contamination	
	2	10E5gene copy numbers	22,78
	3	Fat milk,A&A Biotechnogy	22,08
	4	NonFat milk,A&A Biotechno	21,15
	5	Fat milk, Qiagen	23,04
	6	Nonfat milk, Qiagen	22,82
	7	NTC	



1. Non contaminated fat milk
2. $1x10^5$ gene copy numbers
3. Contaminated fat milk (DNA extracted with Genomic Mini AX Food kit)
4. Contaminated nonfat milk (DNA extracted with Genomic Mini AX Food kit)
5. Contaminated fat milk (DNA extracted with DNeasy Blood & Tissue kit)
6. Contaminated nonfat milk (DNA extracted with DNeasy Blood & Tissue kit)
7. NTC-No Template Control

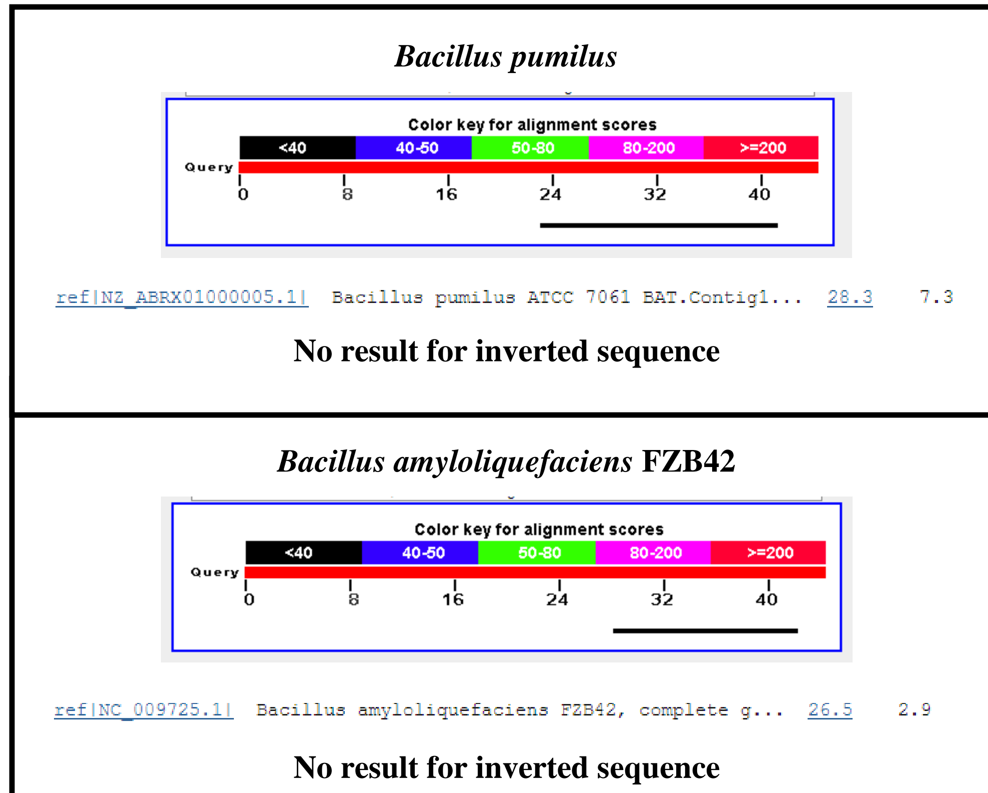
Appendix N. Results of blasting the sequence (in BLAST programme) of DIG-BCRomp2b probe used in dot blot, against other *Bacillus* and non-*Bacillus* species which can occur in milk.

The sequence of the probe was:

5’-TAACGGTATTAGTCAAGTGAATGTATATCGAGAGGATACAGGGG-3’

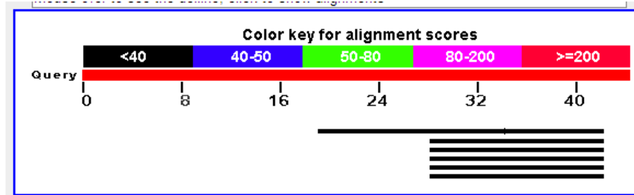
Results for inverted sequence were also reported:

3’-GGGGACATAGGAGAGCTATATGTAAGTGAACTGATTATGGCAAT-5’



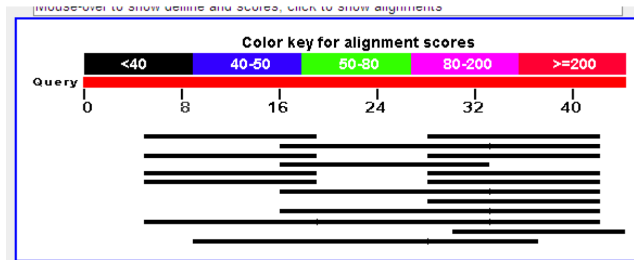
***Bacillus subtilis*, *Brevibacillus brevis* NBRC 100599,**

***Bacillus cereus* subsp. cytotoxis NVH 391-98**



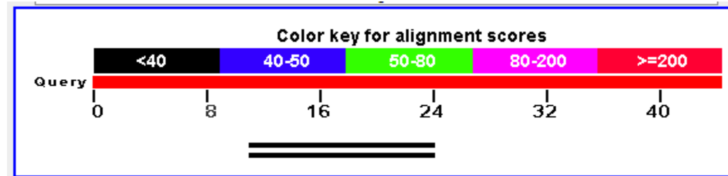
Accession	Description	Score	E-value
ref|NC_012491.1|	Brevibacillus brevis NBRC 100599, complete g...	28.3	2.4
gb|ABQN01000001.1|	Bacillus subtilis subsp. subtilis str. SMY...	26.5	8.3
gb|ABQM01000001.1|	Bacillus subtilis subsp. subtilis str. JH6...	26.5	8.3
gb|ABQL01000001.1|	Bacillus subtilis subsp. subtilis str. NCI...	26.5	8.3
gb|ABQK01000001.1|	Bacillus subtilis subsp. subtilis str. 168...	26.5	8.3
ref|NC_000964.3|	Bacillus subtilis subsp. subtilis str. 168, ...	26.5	8.3

inverted sequence



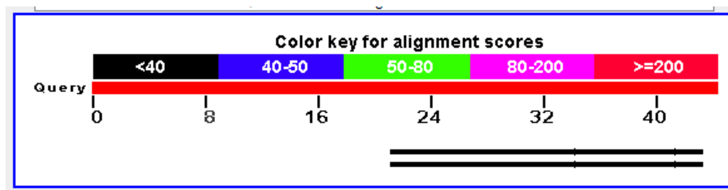
Accession	Description	Score	E-value
gb|ABQN01000001.1|	Bacillus subtilis subsp. subtilis str. SMY...	26.5	8.3
gb|ABQN01000006.1|	Bacillus subtilis subsp. subtilis str. SMY...	26.5	8.3
gb|ABQN01000008.1|	Bacillus subtilis subsp. subtilis str. SMY...	26.5	8.3
gb|ABQM01000001.1|	Bacillus subtilis subsp. subtilis str. JH6...	26.5	8.3
gb|ABQM01000006.1|	Bacillus subtilis subsp. subtilis str. JH6...	26.5	8.3
gb|ABQM01000007.1|	Bacillus subtilis subsp. subtilis str. JH6...	26.5	8.3
gb|ABQM01000008.1|	Bacillus subtilis subsp. subtilis str. JH6...	26.5	8.3
gb|ABQL01000001.1|	Bacillus subtilis subsp. subtilis str. NCI...	26.5	8.3
gb|ABQL01000004.1|	Bacillus subtilis subsp. subtilis str. NCI...	26.5	8.3
gb|ABQL01000005.1|	Bacillus subtilis subsp. subtilis str. NCI...	26.5	8.3
gb|ABQK01000001.1|	Bacillus subtilis subsp. subtilis str. 168...	26.5	8.3
gb|ABQK01000004.1|	Bacillus subtilis subsp. subtilis str. 168...	26.5	8.3
gb|ABQK01000005.1|	Bacillus subtilis subsp. subtilis str. 168...	26.5	8.3
ref|NC_000964.3|	Bacillus subtilis subsp. subtilis str. 168, ...	26.5	8.3
ref|NC_012491.1|	Brevibacillus brevis NBRC 100599, complete g...	26.5	8.3
ref|NC_009674.1|	Bacillus cereus subsp. cytotoxis NVH 391-98,...	26.5	8.3

Lactobacillus casei



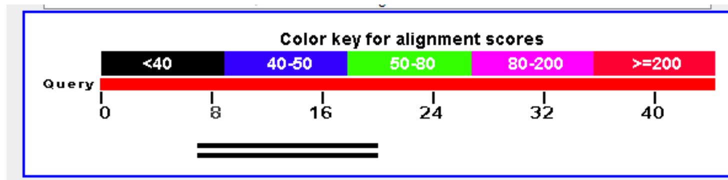
Sequences producing significant alignments:		Score (Bits)	E Value
ref|NC_010999.1|	Lactobacillus casei BL23, complete genome	24.7	5.2
ref|NC_008526.1|	Lactobacillus casei ATCC 334, complete genome	24.7	5.2

inverted sequence



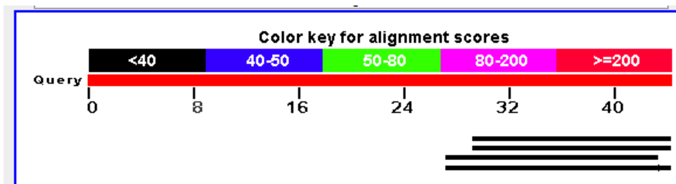
ref|NC_010999.1|	Lactobacillus casei BL23, complete genome	24.7	5.2
ref|NC_008526.1|	Lactobacillus casei ATCC 334, complete genome	24.7	5.2

Lactococcus lactis



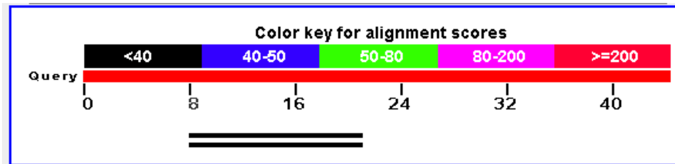
Sequences producing significant alignments:		(Bits)	Value
ref|NC_009004.1|	Lactococcus lactis subsp. cremoris MG1363, c...	24.7	8.4
ref|NC_008527.1|	Lactococcus lactis subsp. cremoris SK11, com...	24.7	8.4

inverted sequence



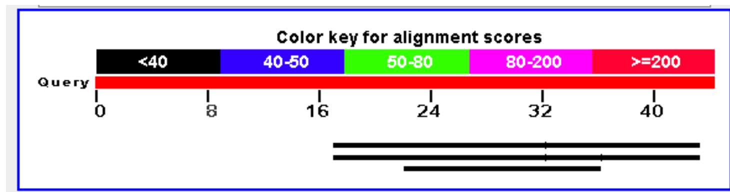
ref|NC_013656.1|	Lactococcus lactis subsp. lactis KF147, comp...	28.3	0.69
ref|NC_002662.1|	Lactococcus lactis subsp. lactis Il1403, com...	28.3	0.69
ref|NC_009004.1|	Lactococcus lactis subsp. cremoris MG1363, c...	24.7	8.4
ref|NC_008527.1|	Lactococcus lactis subsp. cremoris SK11, com...	24.7	8.4

Lactobacillus acidophilus



ref|NZ_ACHN01000047.1| Lactobacillus acidophilus ATCC 4796 co... 24.7 3.4
ref|NC_006814.3| Lactobacillus acidophilus NCFM, complete genome 24.7 3.4

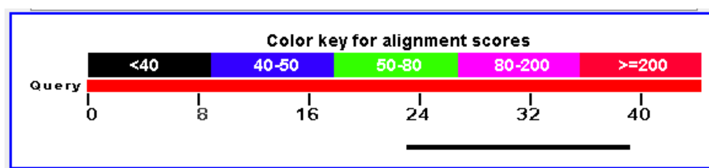
inverted sequence



ref|NZ_ACHN01000005.1| Lactobacillus acidophilus ATCC 4796 co... 28.3 0.42
ref|NC_006814.3| Lactobacillus acidophilus NCFM, complete genome 28.3 0.42
ref|NZ_ACHN01000050.1| Lactobacillus acidophilus ATCC 4796 co... 26.5 1.5

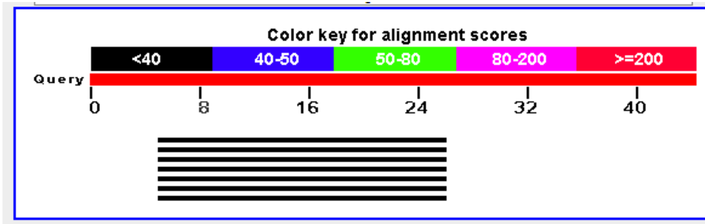
Salmonella enterica

Results only for inverted sequence



Sequences producing significant alignments:	Score (Bits)	E Value
ref|NC_009140.1| Salmonella enterica subsp. enterica serovar ...	30.1	3.9

Escherichia coli

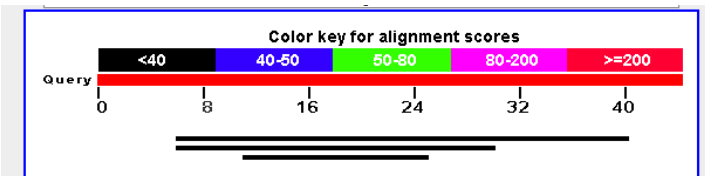


Sequences producing significant alignments:	(Bits)	Value
ref|NZ_ACGN01000126.1| Escherichia coli 83972 contig00155, wh...	30.1	6.3
ref|NC_011745.1| Escherichia coli ED1a, complete genome	30.1	6.3
ref|NC_011742.1| Escherichia coli S88, complete genome	30.1	6.3
ref|NC_011601.1| Escherichia coli O127:H6 str. E2348/69, comp...	30.1	6.3
ref|NC_008563.1| Escherichia coli APEC O1, complete genome	30.1	6.3
ref|NC_007946.1| Escherichia coli UTI89, complete genome	30.1	6.3
ref|NC_004431.1| Escherichia coli CFT073, complete genome	30.1	6.3

No result for inverted sequence

Campylobacter jejuni

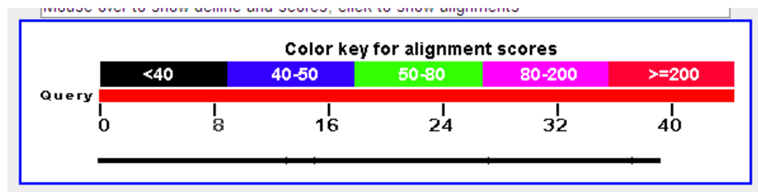
Results only for inverted sequence



Sequences producing significant alignments:	(Bits)	Value
ref|NZ_AASY01000012.1| Campylobacter jejuni subsp. jejuni CG8...	31.9	0.16
gb|ADGM01000012.1| Campylobacter jejuni subsp. jejuni 414 con...	30.1	0.56
ref|NZ_AANQ01000002.1| Campylobacter jejuni subsp. jejuni HB9...	26.5	6.9

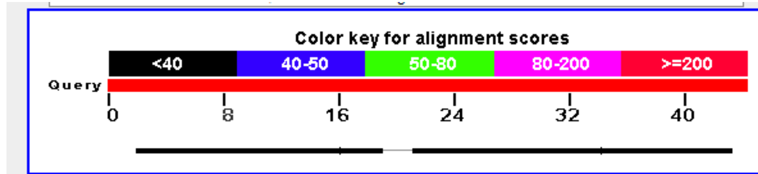
No results for *Listeria monocytogenes*, *Bacillus licheniformis*, and *Bacillus coagulans*

Yersinia enterocolitica subsp. *enterocolitica* 8081



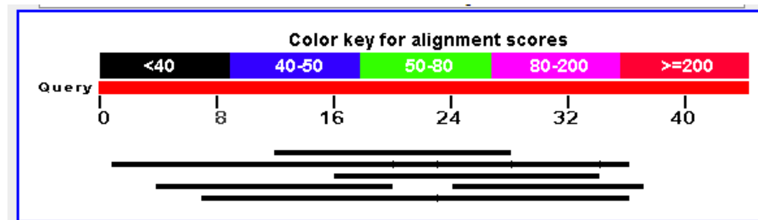
Sequences producing significant alignments:		Score (Bits)	E Value
ref|NC_008800.1|	Yersinia enterocolitica subsp. enterocolitic...	24.7	4.0

inverted sequence



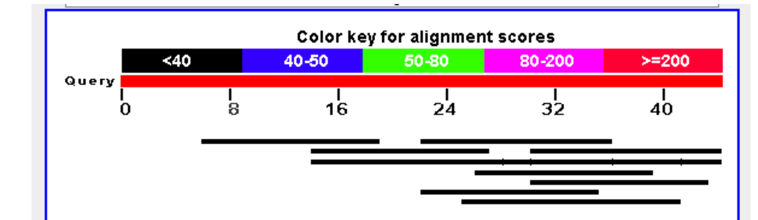
Sequences producing significant alignments:		Score (Bits)	E Value
ref|NC_008800.1|	Yersinia enterocolitica subsp. enterocolitic...	26.5	1.2

Proteus mirabilis



Sequences producing significant alignments:		Score (Bits)	E Value
ref|NZ_ACLE01000068.1|	Proteus mirabilis ATCC 29906 contig000...	30.1	0.23
ref|NC_010554.1|	Proteus mirabilis HI4320, complete genome	30.1	0.23
ref|NZ_ACLE01000010.1|	Proteus mirabilis ATCC 29906 contig000...	24.7	10.0
ref|NZ_ACLE01000023.1|	Proteus mirabilis ATCC 29906 contig000...	24.7	10.0
ref|NZ_ACLE01000025.1|	Proteus mirabilis ATCC 29906 contig000...	24.7	10.0
ref|NZ_ACLE01000029.1|	Proteus mirabilis ATCC 29906 contig000...	24.7	10.0

inverted sequence



Sequences producing significant alignments:		Score (Bits)	E Value
ref|NZ_ACLE01000028.1|	Proteus mirabilis ATCC 29906 contig000...	26.5	2.9
ref|NZ_ACLE01000068.1|	Proteus mirabilis ATCC 29906 contig000...	26.5	2.9
ref|NC_010554.1|	Proteus mirabilis HI4320, complete genome	26.5	2.9
ref|NZ_ACLE01000030.1|	Proteus mirabilis ATCC 29906 contig000...	24.7	10.0
ref|NZ_ACLE01000032.1|	Proteus mirabilis ATCC 29906 contig000...	24.7	10.0
ref|NZ_ACLE01000033.1|	Proteus mirabilis ATCC 29906 contig000...	24.7	10.0
ref|NZ_ACLE01000049.1|	Proteus mirabilis ATCC 29906 contig000...	24.7	10.0
ref|NZ_ACLE01000052.1|	Proteus mirabilis ATCC 29906 contig000...	24.7	10.0
ref|NZ_ACLE01000080.1|	Proteus mirabilis ATCC 29906 contig001...	24.7	10.0

Appendix O. The *motS* gene (homolog to *motB*) of *B. cereus* ATCC 14579 with underlined 575bp fragment used in this study.

```
gi|30018278:c4457817-4457029 Bacillus cereus ATCC 14579, complete genome
DEFINITION  Bacillus cereus ATCC 14579, complete genome.
ACCESSION   NC_004722 REGION: 1582540..1583220
VERSION     NC_004722.1  GI:30018278
DBLINK      Project:384
KEYWORDS    .
SOURCE      Bacillus cereus ATCC 14579
  ORGANISM  Bacillus cereus ATCC 14579

source                  1..681
                        /organism="Bacillus cereus ATCC 14579"
                        /mol_type="genomic DNA"
                        /strain="ATCC 14579"
                        /db_xref="ATCC:14579"
                        /db_xref="taxon:226900"
     gene               1..681
                        /locus_tag="BC1626"
                        /db_xref="GeneID:1203975"
     CDS                1..681
                        /locus_tag="BC1626"
                        /note="Homolog to MotB, appears to be involved in
                        motility on surfaces and under specific ionic
                        conditions. With MotP (a MotA homolog) forms the
                        ion channels that couple flagellar rotation to
                        proton/sodium motive force across the membrane
                        and forms the stator elements of the rotary
                        flagellar machine"
                        /codon_start=1
                        /transl_table=11
                        /product="flagellar motor protein MotS"
                        /protein_id="NP_831404.1"
                        /db_xref="GI:30019773"
                        /db_xref="GeneID:1203975"

     /translation="MMSKGPQKGSPRWMTTFTDLTMLLLTFFVLLVATSKQDAVKLSK
     MLEKFSDTEQVDAKVMENTIPDISHEKNDEKMISKKRMDELYKKLKAYVDNNGISQVN
     VYREDTGVSVVIVDNLIFDTGDANVKPEAKGIISQLVGFFQSVPNPIVVEGHTDSRPI
     HNEKFPSNWELSSARAANMIHHLIEVYNVDDKRLAAVGYADTKPIVPNDSPQNWEKNR
     RVVIYIKE"

ORIGIN
        1 atgatgagta aaggaccgca aaagggatcg cctcgttgga tgacgacttt tacagattta
       61 acgatgttat tattaacttt ctttgtatta ctagttgcta catcaaagca agatgcagta
      121 aaattgtcaa agatgcttga aaagtttagt gatacggagc aagtagatgc aaaagtaatg
      181 gaaaatacaa taccggatat ttcacatgaa aaaaatgatg aaaaaatgat ttcaaaaaag
      241 agaatggatg aattatataa gaagttaaaa gcgtatgtag ataataacgg tattagtcaa
      301 gtgaatgtat atcgagagga tacaggggta agcgtcgtta tagtagataa tttaatattt
      361 gatacaggcg atgcgaacgt taagcccgaa gcgaaaggga taataagtca attagttgga
      421 ttttttcaat ccgtacctaa cccaattgtt gtagagggac atacagatag tagacctatt
      481 cataacgaga aattcccttc taattgggag ttatcttcag cacgagcggc aaatatgatt
      541 caccatttaa ttgaagtgta taatgtggac gataaaaggc tagctgcggt aggatatgca
      601 gacacaaagc caattgtacc aaatgattca ccgcaaaact gggaaaagaa ccgtcgcgtt
      661 gttatttata taaaagagta g
```

Appendix P. Whole genome shotgun sequence of *B. pseudomycoides* DSM 12442 with underlined 220bp sequence used in this study.

```
LOCUS       NZ_ACMX01000122          6425 bp    DNA     linear
DEFINITION   Bacillus  pseudomycoides  DSM  12442  contig00246,  whole
genome shotgun sequence.
ACCESSION   NZ_ACMX01000122 NZ_ACMX00000000
VERSION     NZ_ACMX01000122.1  GI:228994436
DBLINK      Project:29707
KEYWORDS    WGS.
SOURCE      Bacillus pseudomycoides DSM 12442
  ORGANISM  Bacillus pseudomycoides DSM 12442

source                   1..6425
                         /organism="Bacillus pseudomycoides DSM 12442"
                         /mol_type="genomic DNA"
                         /strain="DSM 12442"
                         /db_xref="taxon:527000"
ORIGIN
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       61 atacaaccca aaaaattaaa catgttttta acaatatctt ggatagtcac tgctttaatt
      121 catttaactc ttgcatacat tcttgaacaa gtgtgaccgc ctgactcatg gtagctcccc
      181 cgccaaatgc agctgttacg ccaatgatct caagaatttc ctgctctgaa gctccttgat
      241 ctatacatcc tttagtgtga taaataatgc aatactcatc ctgagagtaa aggcttatcc
      301 ctaaagcaac caattgtttt actttttttg ataatgtacc ttctttaaag cattcctctg
      361 taaaagaatt aaactgttca gccagttttg gcatttttc cgtaaataaa cccaacccat
      421 gtttatattg aagaagtgca gattcagtag tatttctacc ttcaaattcc atatttaatc
      481 aaccccattt ctagtattta taaaacatac gttagtatga gcttaaagta ctttgattaa
      541 tcatgtttga cattatgtaa tttagggggg catctttact tttcaaaaat aattcttcta
      601 aaataaacag ataaatttta tatatattca tataaaagtt cttgtacatc aattcaatca
      661 ttcaatagaa atagtaagat tttttgtata catgtatagc gtatgactac cttctcagct
      721 taatatatac ctaatttaaa ttttcacatg agcatatcta agttttgggt atagaaaagg
      781 caagcattta aatatgcttg ccttcgttct aacgacgatg ctcgactaaa tcaattaccc
      841 ctaaaacaac atgagctaaa ccaaatccaa ataccgtatt agcaatcatc ttattagaac
      901 cacgtgtctg cttaagtgca taacctgcag ctgtgaccgc tgtacctaat gcagttggaa
      961 ttaatccttc acgaatatcc atcgtaatat cctcctagca tcgtagttag taaaacatgt
     1021 agtatttcac aataaaatac taacataaaa attagtatgg tcgcttttta tataaatatg
     1081 taaacaccaa tatcaaggaa atatatataa ctaaaaatcc tgaattaatg tcccaatcca
     1141 ttccaataaa aatctacaat gcttacagct aaatcatcta tattggcaat cagtggcttt
     1201 tgatttacat ctttgtaatc acctaagtat agcaaagcaa caaaggcatg agtacttaaa
     1261 agcgagtgcc ctttagggat ttcacctttt tgcatcgctt tgtgtattgc ttgatccaat
     1321 acttcgtaca ttttatcttc agctttttgc atttgtatta agtgattctc tgaaagagat
     1381 atttttgcat ctttcatgaa actcttgata tcaatgttca gtgttgcggt taaatgaact
     1441 tttgcaacat caagtaatcg ctccttaagc ggcttacttg ttgaaagaat tttatgcata
     1501 ttatctctaa tgcgtgccat catttgaatc attgtatccg taaaaagatc agcttttgta
     1561 gcatagtaat aataaacagt tgcctttgtt acatcgcact cttttgcaac atcttccata
     1621 gagacaattt gataaccttt acttaaaaac agctttgtag caatatcaac aattttatct
     1681 tttgtagatt ttgtattttt attttgaggt ggtcttccta aaggacgttg tttttgttcc
     1741 aatatcattc tctcctgact cttcataatt taaaatatta taacatatca gttgaatagc
     1801 acttgattaa ttaactaacc agtatatata attaattcta aaattagtaa gaaaggtgga
     1861 atatcatgaa aaaacatcct ctttatacat gggggaaata tgtcggtgga ccaaaagcaa
     1921 agtggattac cctttttgta tgggtcttgt tgacacttgt tttatcgttt acttggccag
     1981 ctgtgaatac cgttgaagat gagacagtcc ctaatcttcc tgaaacggct atgtcacaac
     2041 aggctaataa tataattaag aaagaatttc cgaatgactc aggaaaccct gtgcttattg
     2101 tttggcataa agatgaaggt ttagaagaga aggattttaa gaaaattcaa gatacttatc
     2161 aaaaacttag aaattcgcca ttaaaggggc aatcaacgtt accacctttt gataccatac
     2221 cgcaccaagc gttagcaaaa aatgtttcag aagatggtac gtctcttgta actcctgtgt
     2281 tttttgataa aactgtaggg acagatatgc tgcaggagaa tatggctgaa ctgcagattg
     2341 tcataaagaa tacagtggaa gatgatccat ttaaaagaaa gatttctgat tctggtttgc
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2401 atgtacgtct atcagggcca gttggtattc agactgatgc agtaagcata tttagtcagg
2461 ctgatgtgaa attattggtt gcaactgtat tacttgtttt agctttactt attcttcttt
2521 atcgttcacc gctattagca atcttacctt ttattgtagt tgggtttgct tatggaataa
2581 ttagtccaac tttaggtttt ttagctgata aaggatggat tactgctgat gcgcaaggaa
2641 tttcaattat gactgtacta ttatttggag ctggtacaga ttattgtcta tttctaattt
2701 caagatatcg agagtttctt cttgtggagc gaaataaatt taaagcgcta cagcttgcaa
2761 ttaaagaatc aggtggagct atcattatga gtgctcttac cgttgtagta ggattaggta
2821 ctctattact tgctcatata ggttctttcc atagatttgc agtacctttt agcgtggctg
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3001 aaaaaagaaa acaatcaagg taaaggaatc gaaagggttc ttaagcaaaa aacttggaga
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3361 acaaatgttt gaagtttcaa taaaagataa tccgtattct attgagggga tgaataaaat
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5941 gaattaccac ttgatgcacg tgatgatatc acatttgaat ttattcaaca tcgatttaca
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6061 gagaaaagaa gatataaatg ggggaaatat ggaattggaa aatatattta tcaaaaagaa
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6121 gaagaagaag atataaaaag ttatctttat tcttatatgg agaaattctt tcccaatgca
6181 aaattagaat atttcacata ggagatacca ttcactttac aaaaagcata tggaaattta
6241 ttttaaaggc tatatcggtg tttcaaggac gttctgttgc aaagtttttt aaatgagtct
6301 acactctaga aaaaggggct tatccgttgc tcatacaacg attatgcctt gggttcatca
6361 gtatggtcct gaattagaca aaagaatccg tcatcatctc aagcaaatca atgactcttg
6421 tagag
```

Appendix Q. List of publications.

Published papers:

Oliwa-Stasiak, K., Kolaj-Robin, O. and Adley C. C. (2010) 'Development of real-time PCR assay for detection and quantification of the *Bacillus cereus* group spp.: differentiation of *B. pseudomycoides* and *B. weihenstephanensis* from species with non rhizoid growth isolated from milk', *Applied and Environmental Microbiology,* doi:10.1128/AEM.01581-10.

Oliwa-Stasiak, K., Molnar, C. I., Arshak, K., Bartoszcze, M. and Adley, C. C. (2010) 'Development of a PCR assay for identification of the *Bacillus cereus* group species', *Journal of Applied Microbiology,* 108(1), 266-273.

Velusamy, V., Arshak K., Korostynska, O., **Oliwa, K**. and Adley C. C. (2010) 'An overview of foodborne pathogen detection: In the perspective of biosensors', *Biotechnology Advances,* 28(2), 232-254.

Arshak, K., Velusamy, V., Korostynska, O., **Oliwa, K**. and Adley C. C. (2009) 'Conducting polymers and their applications to biosensors: emphasizing on foodborne pathogen detection', *IEEE Sensors Journal,* 9(12), 1942-1951.

Refereed Conference Papers:

Velusamy, V., Arshak, K., Yang, C., Yu, L., Korostynska, O., **Oliwa, K**. and Adley C. (2010) *Label-free detection of Bacillus cereus DNA hybridization using electrochemical impedance spectroscopy for food quality monitoring application.* IEEE, Limerick, Ireland, 135-138.

Velusamy, V., Arshak, K., Korostynska, O., **Oliwa, K.** and Adley. C. C. (2009) *Conducting polymer based DNA biosensor for the detection of the Bacillus cereus group species.* SPIE, Orlando, FL, USA, 731504-731504-8.

Velusamy, V., Arshak, K., Korostynska, O., **Oliwa-Stasiak, K**. and Adley, C. (2009) *Design of a real time biorecognition system to detect foodborne pathogens- DNA Biosensor.* IEEE, New Orleans, LA, USA, 38-42.

Adley, C., Arshak, K., Molnar, C. I., **Oliwa, K**. and Velusamy, V. (2009) *Design of specific DNA primers to detect the Bacillus cereus group species.* SAS, Sensors Application Symposium, New Orleans 17-19 Feb: 236-239,
doi: 10.1109/SAS.2009.4801808

Conference Proceedings:

Oliwa-Stasiak, K., Velusamy, V., Piterina, A., Arshak K. and Adley C. (2009) *Devising a real time DNA-biosensor to detect and monitor the Bacillus cereus group species.* The Irish Division of the Society for General Microbiology (SGM), Cork, Ireland, 59.

Oliwa, K., Arshak K. and Adley C. (2008) *Devising a nano-biosensor platform to detect and monitor foodborne pathogens.* The Irish Division of Society for General Microbiology (SGM), Galway, Ireland, 44.

Magazine Articles:

Oliwa-Stasiak, K., Velusamy, V., Arshak K. and Adley C. (2009) 'SFI Frontiers Grant Awarded to UL researchers to combat anthrax detection', *UL Links. The University of Limerick Magazine, Ireland,* 2(2), 49.

Oliwa-Stasiak, K., Velusamy, V., Arshak K. and Adley C. (2008) 'Designing a nano based platform to detect and monitor food borne pathogens', *National Institute of Health Sciences Research Bulletin, Ireland,* 4(4), 62-63.