UDC 579.852.11:57.083.3
ISSN 1330-9862 *original scientific paper*

(FTB-1023)

Differentiation of *Bacillus cereus* Isolates from Milk and Milk Products with Biochemical, Immunological, AP-PCR and PCR-RFLP Methods

Karmen Godič Torkar[1]* *and Sonja Smole Možina*[2]

[1]Institute of Dairying, Zootechnical Dept., Biotechnical Faculty, University of Ljubljana, Groblje 3, SI-1230 Domžale, Slovenia

[2]Dept. of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

Received: February 11, 2000
Accepted: April 25, 2000

Summary

Physiological features including lecithinase and haemolytic activity, API biotyping and immunodetection of diarrhoeal enterotoxin were compared with AP-PCR genotyping and PCR-RFLP analysis of *hbl*A and *cer*AB gene fragments for differentiation of 82 *Bacillus cereus* isolates from raw milk and milk products. The amplification of the *cer*AB gene with selected primers was successful in 78 out of 82 (95 %) of lecithinase positive strains. An *hbl*A amplification product was obtained in 66 (80.5 %) strains. By using BCET-RPLA immunoassay kit the same result was achieved in 97.5 % of isolates tested. A comparative analysis of phenotypic expression and PCR amplification of genes coding for lecithinase and diarrhoeal enterotoxin synthesis in *Bacillus cereus* milk isolates reveal a high level of correlation and confirm the usefulness of rapid molecular detection and/or identification methods for toxinogenic *Bacillus cereus* strains from milk and milk products. Furthermore, restriction analysis of toxin coding gene sequences in *Bacillus cereus* strains revealed a very high heterogeneity and thus the usefulness of PCR-RFLP typing of strains on the basis of these sequences. No correlation was found between the clustering of strains on the basis of API biotyping and AP-PCR genotyping. However, high discrimination indexes were calculated for both typing methods, so they could be successfully used for differentiation of *Bacillus cereus* isolated from milk and milk products. We found PCR-RFLP analysis of toxin coding gene sequences as a preferable method for detection, identification and/or typing and therefore tracing the repositories and distribution routes of toxinogenic *Bacillus cereus* strains at raw milk supply and manufacturing process in a dairy plant.

Key words: Bacillus cereus, milk, genotyping, biotyping, enterotoxigenity

Introduction

Bacillus cereus is an ubiquitous gram-positive, spore-forming, motile rod, commonly found in soil, plant material, hay, raw and processed food. It is also frequently found in pasteurised milk, causing spoilage because of the production of lipases and proteases (*1*).

Present routine detection methods for *Bacillus cereus* rely on standard plate counting which require, up to 4 days to be performed, including confirmatory testing. This is too much time-consuming when inspecting products with short shelf-live. The presence of *Bacillus cereus*

* Corresponding author; Phone.: ++386 (0)1 7217 906; Fax: ++386 (0)1 7214 074; E-mail: karmen.torkar@bfro.uni-lj.si

strains that cause food poisoning can also be indicated by detection of their toxins. This microorganism causes two different types of food poisoning: the diarrhoeal type and the emetic type. The diarrhoeal type of food poisoning is caused by different enterotoxin complexes (*2–4*), produced during the growth of *Bacillus cereus* in the small intestine (*5*), while the emetic toxin is produced by the growing cells in food (*6*). There are two available commercial immunoassays for quantification of the toxicity of the enterotoxins from *Bacillus cereus*. The BCET-RPLA-assay (Oxoid), measuring the L2 component of haemolytic diarrhoeal enterotoxin called haemolysin BL (HBL) complex, does not detect strains containing only the nonhaemolytic diarrhoeal enterotoxin NHE. The kit BDEVIA (Tecra) detecting the protein(s) of NHE, will leave out the HBL complex (*7*).

The aim of the present study was a comparative analysis of specific physiological features (sugar fermentation pattern and other enzymatic activities in API test system, lecithinase activity, haemolytic activity and enterotoxigenity) and amplification of arbitrary and specific DNA regions (cereolysin *cer*AB and haemolysin *hbl*A genes) of *Bacillus cereus* isolates from raw milk, pasteurised milk, some milk products and selected reference strains. Our aim was to evaluate the potential of the rapid molecular typing for characterisation, identification and tracing the source of *Bacillus cereus* strains in raw milk and milk products. A correlation between enterotoxicity of the strains and PCR amplification of their *hbl*A genes was in focus to evaluate the usefulness of the PCR-based technique for quicker detection of enterotoxigenic isolates from milk and milk products. The cytolytic determinant cereolysin AB, a coupled DNA sequence encoding for phospholipase C (*cer*A) and sphingomyelinase (*cer*B), has been chosen to design primers that could be useful for detection of *Bacillus cereus* in food because the phospholipase C is responsible for lecithin degradation, a major conventional criterion for detection and identification of *Bacillus cereus* (*8*).

Arbitrary primed polymerase chain reaction (AP--PCR) can be used to amplify certain segments of a genome by using short arbitrary primers (*9*). This technique provides good discrimination, especially if multiple primers are used (*10*). To allow direct comparison, API-biotyping, immunodetection of diarrhoeal enterotoxin by BCET-RPLA method and genotyping by AP-PCR and PCR - restriction fragment length polymorphism (PCR- RFLP) of *cer*AB and *hbl*A PCR products for characterisation of *Bacillus cereus* isolates from milk and milk products were applied.

Materials and Methods

Bacterial strains and media

A total of 82 *Bacillus cereus* strains were isolated from raw milk, pasteurised milk and some milk products taken from one dairy in the period of one year. The isolation of the strains has taken place from samples before and after the heat treatment in water bath at 80 °C for 12 minutes. *Bacillus cereus* ATCC 11778 was received from Oxoid, the other reference strains *Bacillus cereus* S1 RZS, *Bacillus mycoides* LMG 7128^T, *Bacillus brevis* S12 RZS and *Bacillus firmus* LMG 7125 were kindly provided by Government Dairy Research Station, Melle, Belgium (RZS). Colony morphology and cell morphological and physiological characteristics were determined using conventional procedures (*11*). Hydrolysis of lecithin was detected on *Bacillus cereus* selective agar (PEMBA, Oxoid) supplemented with egg yolk emulsion and polymyxin B. Haemolytic activity was determined on blood agar (tryptic soy agar supplemented with 5 % of defibrinated sheep blood). The plates were incubated at 30 °C 24–48 hours. Milk isolates had been identified with the API 50 CHB and API 20 E test system using the identification programme V.2.0. (BioMerieux, France). The procedures were performed according to manufacturer's instructions.

Immunoassay procedure

All of the 82 strains of *Bacillus cereus* isolated from milk and milk products as well as *Bacillus cereus* ATCC 11778 were analysed by using BCET-RPLA (Oxoid) immunoassay kit. The assay was performed according to the manufacturer's instructions.

DNA isolation procedure

A single isolated colony of each strain was streaked onto a plate of the Brain-heart infussion agar, supplemented with 0.1 % glucose. The plates were incubated at 30 °C 24–48 h. DNA from bacterial biomass was isolated as described previously (*12,13*).

AP-PCR

PCR amplifications were carried out in 10 μL reaction volume containing 50 ng of bacterial DNA, 2.5 mmolL^{-1} $MgCl_2$, 0.5–1 μmolL^{-1} of primer, 0.5 U of Ampli*Taq* DNA polymerase (Pharmacia Biotech), 0.2 mmolL^{-1} of each dNTP and 1 μL of 10x PCR Buffer II (Perkin-Elmer: 500 mmolL^{-1} KCl, 100 mmolL^{-1} Tris – HCl, pH= 8.3). Decamer primers with GC content 60–80 % and microsatellite primer $(GACA)_{4x}$ (Codon Genetic System) were used. On the basis of the number and reproducibility of the amplified bands, the primers with 70 % GC (5' - AGC GGG CGT A- 3') and 80 % GC (5' - CGC GTG CCC A- 3') were selected for the differentia-

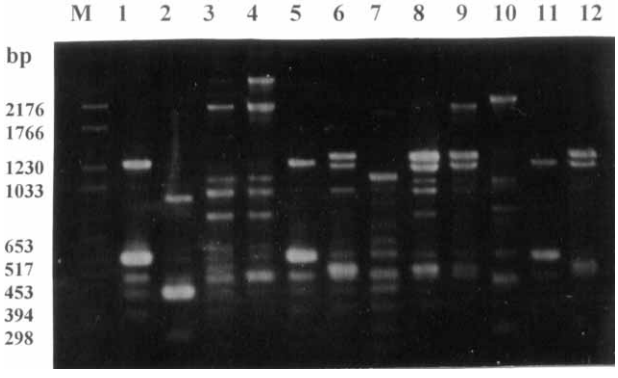

Fig. 1. The sample of AP-PCR amplification of some *Bacillus cereus* isolates from milk and milk products with the primer **5'-CGCGTGCCC -3'; lane 9**: *Bacillus cereus* ATCC 11778; **M**: molecular weight marker Boehringer VI

tion of *Bacillus cereus* strains. A Perkin-Elmer Gene Amp PCR System 2400 was used for all amplifications in this study. The cycling programme of AP-PCR started with the initial denaturation of DNA at 94 °C for 5 min and continued with 35 cycles at 94 °C for 30 s, at 38 or 42 °C (depending on the primer) for 45 s and at 72 °C for 45 s. Final extension at 72 °C for 7 min was performed, following by cooling to 4 °C. The PCR products were analysed by electrophoresis on 1.5 % (w/v) agarose gels in 1 x TAE. DNA fragments were stained with ethidium bromide, visualised by transillumination and photographed with a Polaroid camera on Polaroid 665 films (see Fig. 1).

PC-RFLP of cerAB gene fragment

Selected primers for amplification the *cer*AB gene coding for the lecithinase activity of *Bacillus cereus* strains were Pf with the sequence 5'-GAG TTA GAG AAC GGT ATT TAT GCT GC -3' and Cr with the sequence 5'-GCA TCC CAA GTC GCT GTA TGT CCA G -3' (*8*). PCR amplifications were carried out in 10 μL reaction volume containing 50 ng of isolated DNA, 1 μL of a 10x reaction buffer (Perkin Elmer : 100 mmolL^{-1} Tris-HCl, pH=8.3, 15 mmolL^{-1} $MgCl_2$, 500 mmolL^{-1} KCl, 0.01 % (w/v) gelatin), 0.2 mmolL^{-1} of each dNTP (Perkin Elmer), 0.185 μmolL^{-1} of primer Pf, 0.198 μmolL^{-1} of primer Cr and 0.5 U of *Taq* DNA polymerase (TaKaRa).

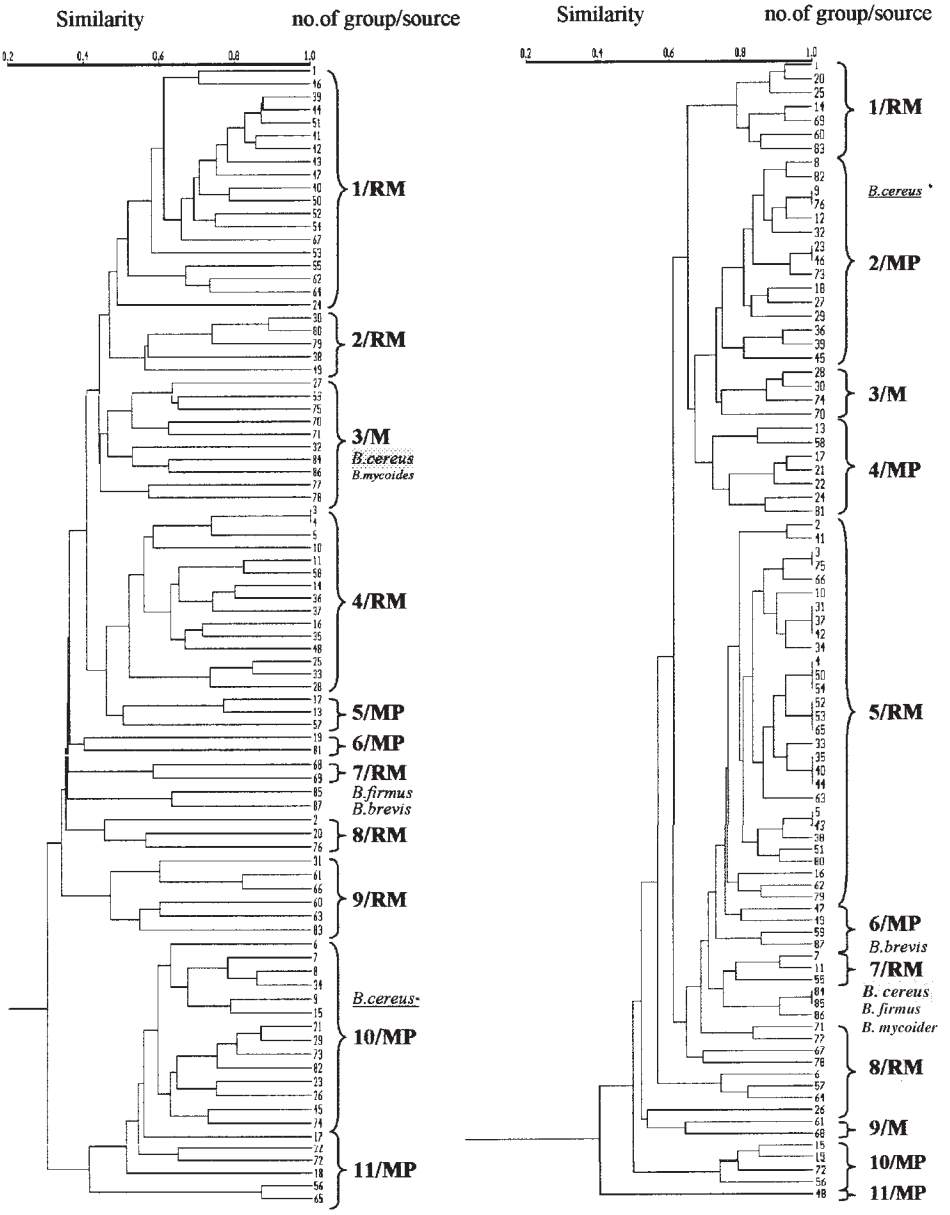

Fig. 2. Similarity dendrograms of 82 *Bacillus cereus* isolates from milk and milk products and 5 reference strains constructed on the basis of AP-PCR genotyping (left) and API biotyping (right) from 34 and 29 binary characteristics, respectively, by using Jaccard's index and UPGMA

RM: raw milk, **MP**: milk products, **M**: samples from raw milk and milk products

*B. cereus**: *Bacillus cereus* ATCC 11778, *B. cereus:* *Bacillus cereus* S1 RZS, *B. mycoides: Bacillus mycoides* LMG 7128^T, *B. firmus: Bacillus firmus* LMG 7125, *B. brevis: Bacillus brevis* S12 RZS

Thirty five amplification cycles were performed with denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min. During the last cycle, the elongation temperature was held for a total of 4 min. The PCR products were analysed electrophoretically as described at AP-PCR analysis. Purity and concentration of PCR products were checked on a gel. According to the DNA concentration, 1–3 μL of the amplified *cer*AB gene fragments of all isolates and reference strains were digested directly with *Taq*I, *Msp*I, *Hae*III, *Cfo*I and *Rsa*I (Boehringer Mannheim). Reaction conditions were set as recommended by the manufacturer. RFLP profiles were analysed by electrophoresis on 1.5 % agarose gels and stained with ethidium bromide as mentioned previously.

PCR-RFLP of hblA gene fragment

The *Bacillus cereus* hemolysin BL *hbl*A gene-specific primers were used. The sequence for the HblA1 primer was 5'-GCT AAT GTA GTT TCA CCT GTA GCA AC-3', and the sequence for HblA2 primer was 5'-AAT CAT GCC ACT GCG TGG ACA TAT AA-3'(*14*).

PCR amplifications were carried out as described for PC-RFLP of *cer* AB. The concentration of primers in PCR mixture was 0.26 $\mu molL^{-1}$ of primer HblA1 and 0.25 $\mu molL^{-1}$ of primer HblA2. The *Taq* DNA polymerase (TaKaRa) was added after the reaction mixture had been heated to 80 °C (»hot start«) (*15*). Thermal cycles for the HblA primers were as follows: 5 cycles at 94 °C for 30 s, at 70 °C for 1 min, and at 72 °C for 1.5 min followed by 30 cycles at 94 °C for 30 s, at 65 °C for 1 min, and at 72 °C for 1.5 min. The amplification products were analysed on 1.5 % agarose gels as mentioned previously. Restriction analyses were made as described for *cer*AB gene fragments with *Taq*I, *Hinf*I and *Rsa*I.

Statistical analysis

From the biochemical patterns obtained with API test systems and AP-PCR fingerprints, a binary profile was established for each isolate. The distances between all pairs of strains were calculated by Jaccard's index $[d=1-c/(p+q+c)]$, where c is the number of variables present in both strains, and p and q are the numbers of variables present in each strain. Dendrograms were constructed from the resulting distance matrix by using the unweighted pair-group method with arithmetic averages (UPGMA) with the software package NTSYS – pc (*16*). The correlation coefficients between the Jaccard's indexes of simmilarity between biotyping and genotyping clustering was calculated by Microsoft software Excel. The Discrimination index (DI) was calculated as recommended by Hunter and Gaston (*17*).

Results

Phenotyping: lecithinase, hemolysin and enterotoxin detection

All of the 82 *Bacillus cereus* isolates, as well as the reference strains except *Bacillus firmus*, showed a positive lecithinase reaction on PEMBA medium. All of them except *Bacillus brevis* showed also a hemolytic reaction on blood agar. By using BCET-RPLA immunoassay kit, 66 (80.1 %) isolates of *Bacillus cereus* from milk and milk products gave a positive reaction. *Bacillus cereus* ATCC 11778 gave negative reaction at BCET-RPLA test.

API biotyping

After the biochemical tests with API 50 CHB and API 20 E, strains differed in 29 characteristics, which in combination offered 71 different profiles with similarity between 40–100 %. The strains were classified in 11 groups. The similarity of isolates inside the groups was in the range from 40 to 78 %. Isolates from raw milk formed four main groups, isolates from milk products formed another five groups, while strains isolated from both sources, raw milk and milk products, formed the last two groups (Fig. 2, right).

The DI (discrimination index) for biotyping analysis with API test systems for discrimination of 82 tested *Bacillus cereus* isolates from milk and milk products and reference strains was 0.995.

AP-PCR genotyping

Amplification with the primers with 80 and 70 % GC resulted in 21 and 13 amplified fragments, respectively, from 154 to 2500 bp in length (Fig. 1). The similarity among 82 genotype profiles was 28–100 %. They could be distributed into 11 groups. The similarity of bacterial isolates inside the groups was between 41 and 57 %. Isolates from raw milk mainly formed six groups, isolates from milk products formed four groups, while the last group contained the isolates from both sources (Fig. 2, left). The DI (discrimination index) for AP-PCR analysis of 82 tested *Bacillus cereus* isolates from milk and milk products and reference strains was 0.999.

The correlation coefficient between the Jaccard's indexes of simmilarity between biotyping and genotyping clustering was 0.176 ($P \geq 0.001$).

PCR-RFLP of cerAB gene fragment

With the primer set *Pf – Cr* a fragment of *cer*AB gene was amplified in 78 out of 82 tested *Bacillus cereus* isolates, as well as in strains *Bacillus cereus* S1(RZS), *Bacillus cereus* ATCC 11778 and *Bacillus mycoides* LMG 7128. A weak signal was found also at *Bacillus brevis* S12 (RZS). All PCR products were of the expected size of 1460 bp (not shown).

Among the enzymes used in restriction analysis of the amplified *cer*AB gene fragments, the patterns of *Cfo*I and *Rsa*I endonucleases were the most informative for differentiation of strains (Fig. 3). We found 1 up to 3 restriction sites with *Cfo*I and 4 up to 7 restriction sites with *Rsa*I, *i.e.* four and seven different restriction patterns with *Cfo*I and *Rsa*I, respectively. In combination, we could differentiate 16 different groups among 72 strains analysed.

The largest heterogeneity was found among strains of *Bacillus cereus* isolated from raw bulk milk from transport tanks on the dairy entrance. These strains were classified in 14 groups while isolates from milk products formed only 1 to 3 different groups (Table 1).

PCR – RFLP of hblA gene fragment

A *hbl*A amplification product of expected length of 873 bp was obtained from 66 out of 82 tested strains from milk and milk products, as well as from *Bacillus cereus* S1 (RZS) and *Bacillus mycoides* LMG 7128 (not shown). The *hbl*A gene fragment of *Bacillus cereus* ATCC 11778 was not amplified.

A restriction with *Taq*I gave two fragments of 62 bp and 812 bp and with *Rsa*I two fragments of 227 bp and 647 bp at all strains tested, in accordance with EMBL data bank by using hemolysin sequence from *Bacillus cereus* F837 / 76 (accession no. L20441). After restriction with *Hinf*I we obtained five different digestion patterns (Fig. 4). The majority (74.1 %) shared the same pattern,

Table 1. The origin of *Bacillus cereus* strains and their grouping on the basis of PCR – RFLP of *cer*AB amplification products after restriction with *Cfo*I and *Rsa*I endonucleases

Origin / Group	RBM	RMU	PM	MP	CU	PSC	IC	ST	PU	No. of strains in the group
1	11									11
2	3			2						5
3	6		2						1	9
4	8			5		1	1			15
5	3	2	3		3		2	1		14
6	1						1			2
7	1									1
8	1									1
9	4									4
10	1									1
11	2									2
12	1									1
13	2									2
14					1					1
15	1									1
16			1			1				2
NA/NR	2/4	1/0	0/1	1/0	0/1	0/0	0/0	0/0	0/0	4/6
Total no. of groups/ strains	**15/51**	**2/3**	**3/7**	**3/8**	**2/5**	**2/2**	**3/4**	**1/1**	**1/1**	**Σ 82**

RBM: raw bulk milk from transporter tanks, **RMU:** raw milk from udders with higher number of somatic cells, **PM**: pasteurised milk, **MP**: milk powder, **CU:** curd, **PSC**: pasteurised sweet cream, **IC**: ice cream, **ST**: UHT sterilised milk and cream, **PU**: puddings, **NA/NR**: the *cer*AB sequence was not amplified/restriction pattern could not be determined

Table 2. The origin of *Bacillus cereus* strains and their grouping on the basis of restriction patterns of *hbl*A amplification products after restriction with *Hinf*I endonuclease

Origin / Group	RBM	RMU	PM	MP	CU	PSC	IC	ST	PU	No. of strains in the group
1	27	3	5	4	2		1	1		43
2			2						1	3
3	4									6
4	4							1		5
5	1									1
NA/NR	11/4	0/0	0/0	3/1	0/1	1/1	1/1	0/0	0/0	Σ 16/8
Total no. of groups/ strains	**5/51**	**1/3**	**2/7**	**2/8**	**2/5**	**1/2**	**3/4**	**1/1**	**1/1**	**Σ 82**

RBM: raw bulk milk from transporter tanks, **RMU:** raw milk from udders with higher number of somatic cells, **PM**: pasteurised milk and cream, **MP**: milk powder, **CU:** curd, **PSC**: pasteurised sweet cream, **IC**: ice cream, **ST**: UHT sterilised milk and cream, **PU**: puddings, **NA/NR**: the *hbl*A sequence was not amplified/restriction pattern could not be analysed

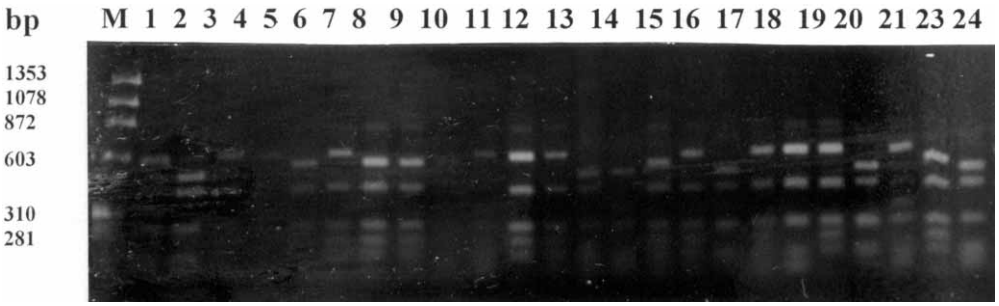

Fig. 3. Restriction analysis of *cer*AB amplification product of 24 out of 82 strains with *Rsa*I endonuclease; **lanes 1–6, 8–24**: *Bacillus cereus* strains isolated from milk and milk products. lane **7**: *Bacillus cereus* ATCC 11778; **M**: Molecular weight marker Boehringer IX

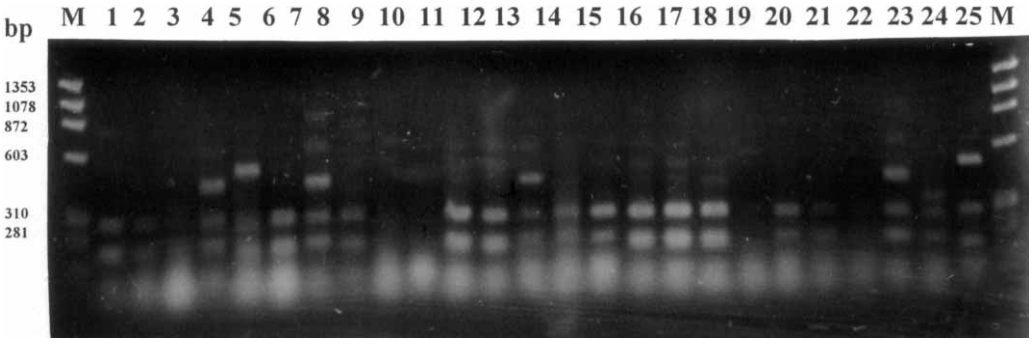

Fig. 4. Restriction analysis of *hbl*A amplification product of 25 out of 66 strains with *Hinf*I endonuclease; **lanes 1–25**: *Bacillus cereus* strains isolated from milk and milk products; **M**: Molecular weight marker Boehringer IX

while the other groups included 10.3, 8.6, 5.2 and 1.7 % of isolates.

Discussion

In this paper, the cytolytic determinant cereolysin AB, a coupled DNA sequence encoding for phospholipase C (*cer*A) and sphingomyelinase (*cer*B) has been amplified with specific primers Pr and Cr (*8*). PCR product of the expected size of 1460 bp was amplified at 78 out of 82 (95 %) lecithinase positive *Bacillus cereus* strains (not shown). Therefore, we could find the amplification of *cer*AB gene with selected primers as a useful method for detection/identification of *Bacillus cereus*, comparable to official detection method, based on phenotypic determination of lecithinase activity of strains. One of the possible explanations why there is no *cer*AB gene amplification at four other strains of *Bacillus cereus* is that these strains were lecithinase negative (*18*). The positive result (precipitation cone) on PEMBA medium could be the consequence of proteolytic and not lecithinase activity on egg yolk supplement (*19*). Such atypical *Bacillus cereus* strains which may be lecithinase negative but toxigenic (*18*) remain hidden food poisoning hazard when traditional (phenotypic) or genotypic method based on *cer*AB gene is used for identification. The importance of such atypical strains in dairy processing should be further studied as well.

Cereolysin AB is not the only multicomponent hemolysin of *Bacillus cereus*. Hemolysin BL consists of three components which work synergetically to lyse human erythrocytes (*20*). In this study, an *hbl*A amplification product was obtained in 66 (80.5 %) out of 82 tested *Bacillus cereus* strains, isolated from milk and milk products. The same percentage of enterotoxigenic strains has been proven after using the BCET-RPLA immunoassay. One isolate was PCR negative but gave a positive reaction with BCET-RPLA immunoassay kit. Another isolate gave the *hbl*A amplification product and negative result in BCET-RPLA testing in limits of its sensitivity. The same result was therefore achieved in 97.5 % of cases tested. It could be concluded that the comparative analysis of phenotypic expression and PCR amplification of genes coding for lecithinase and diarrhoeal enterotoxin synthesis in *Bacillus cereus* milk isolates revealed a very high level of correlation and confirmed the usefulness of more rapid molecular detection and/or identification methods for toxinogenic *Bacillus cereus* strains from milk and milk products.

Beside their rapidness, there is another advantage of PCR-based methods in comparison with the conventional phenotyping of toxinogenic *Bacillus cereus* isolates. Amplified regions could be a basis for the rapid differentiation of strains that can not be identified by available classical tests. Table 1 presents the origin of *Bacillus cereus* strains and their grouping on the basis of restriction patterns of *cer*AB amplification products. It is obvious that population is very heterogeneous in raw bulk milk, which was sampled from transport tanks at dairy entrance, whilst a few groups of *Bacillus cereus* strains were isolated from different milk products. The *Bacillus cereus* strains isolated from milk that was sampled directly from the udders with higher number of somatic cells (Group *5*) were classified in one group, with the ex-

ception of one isolate which did not give the amplification product. The strains from pasteurised milk and milk products were classified into one to three different groups. It is interesting that no strains from the largest group of isolates from raw milk (Group *1*) were isolated from pasteurised milk or milk products. In fact, a majority of the strains isolated from pasteurised milk and milk products belonged to a small number of groups, i.e. they share only a few restriction patterns of *cer*AB amplification product (Groups 2–*5*). This could be an indication that strains from different groups had different origin (raw milk, dairy plant with its equipment) or resistance to technological (heat) treatment during manufacturing process which varies a lot among the strains from different groups. This indication should be tested further.

With the selected primers HblA1 and HblA2 we amplified relatively short fragment of hemolysin BL gene of *cc*. 870 bp. This fact resulted in a small number of restriction sites and therefore in a difficulty to find the proper enzymes to show differences in restriction patterns. The majority of the strains shared the same restriction pattern with all the enzymes tested. Consequently, it was difficult to make any correlation between the origin of the isolates and restriction patterns of *hbl*A amplification products (Table 2). However, when we combined the results of restriction analysis of *cer*AB and *hbl*A amplification products, we could differentiate up to 30 groups among 82 strains analysed. These results reveal high heterogeneity of toxin coding regions in *Bacillus cereus* strains isolated from milk and milk products and thus the usefulness of PCR-RFLP typing of strains on the basis of these sequences. From the practical point of view, it is also important that the restriction analysis can be performed very quickly (in a few hours) after detection/identification of toxinogenic *Bacillus cereus* in milk and/or milk product on the basis of PCR amplification of *cer*AB and *hbl*A gene fragments.

Concerning the results of API biotyping and AP--PCR genotyping, no correlation was found between the clustering of strains on the basis of selected typing methods (Fig. 2). These results were somehow expected while completely different data were included into analysis: phenotypic expression of many different biochemical features in case of API biotyping and amplification of non-specific, arbitrary chosen DNA sequences in case of AP-PCR biotyping. Similar results were reported also by Schraft *et al*. (*21*), who compared the clustering of 62 *Bacillus cereus* isolates on the basis of twenty-one biochemical variables, growth characteristics, PCR-RFLP and fatty acid profiles. We also noticed that a distribution of the enterotoxigenic strains inside the groups of closely related strains formed on the basis of biotyping and genotyping was totally different. Certain correlation could be observed among the strain clusters and the source of isolates (raw milk, milk products) while the isolates of the same source predominated in the majority of clusters formed with both typing methods. However, it is not possible to predict the source of the isolate on the basis of any typing method used. On the other hand, relatively similar discrimination indexes were calculated for both typing methods. It can be concluded that both methods could be successfully used for the determination of heterogeneity of *Bacillus cereus* contaminants in milk and milk products, which is extremely high, but PCR-RFLP of toxin coding gene sequences is a preferable method for detection, identification and tracing the repositories and distribution routes of toxinogenic *Bacillus cereus* strains at raw milk supply and manufacturing process in a dairy plant.

References

1. P. E. Granum, T. Lund, *FEMS Microbiol. Lett. 157* (1997) 223.
2. D. J. Beecher, A. C. L. Wong, *J. Biol.Chem. 272* (1997) 233.
3. T. Lund, P. E. Granum, *FEMS Microbiol. Lett. 141* (1996) 151.
4. M. C. te Giffel, *Proceedings of the seventh International Conference of the International on Food Microbiology and Hygiene (ICFMH),* Veldhoven (1999) pp. 733–736.
5. P. E. Granum, *J. Appl. Bacteriol. Symp. Suppl. 76* (1994) 61S.
6. J. M. Kramer, R. J. Gilbert: *Bacillus cereus* and other *Bacillus* Species. In: *Foodborne Bacterial Pathogens*, M. P. Doyle (Ed.), Marcel Dekker, New York (1989) pp. 21–70.
7. P. E. Granum, *International Symposium Foodborne Pathogens: Detection and Typing; De Waren(s)-Chemicus, 28* (1998) pp. 24–26.
8. H. Schraft, M. W. Griffiths, *Appl. Environ. Microbiol. 61* (1995) 98.
9. J. Welsh, M. McCleland, *Nucleic Acids Res. 18* (1990) 7213.
10. J. G. K. Williams, A. R. Kubelik, K. J. Livak, J. A. Rafalski, S. V. Tingey, *Nucleic Acids Res. 18* (1990) 6531.
11. *Microbiology – General guidance for the enumeration of Bacillus cereus – Colony-count technique at 30 °C*, 2nd ed. International Organization for Standardization ISO, Geneve, Switzerland (1993) p. 7933.
12. J. Marmur, *J. Mol. Biol. 3* (1961) 208.
13. J. Sambrook, E. F. Fritsch, T. Maniatis: *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press (1989) p. 1659.
14. V. Mäntynen, K. Lindström, *Appl. Environ. Microbiol. 64* (1998) 1634.
15. R. T. D'Acquila, L. J. Bechtel, J. A.Videler, J. J. Eron, P. Gorczyca, J. C. Kaplan, *Nucleic Acids Res. 19* (1991) 3749.
16. F. J. Rohlf: *NTSYS-pc Numerical Taxonomy and Multivariate Analysis System*, version 1.80. Exeter Software, applied Biostatistics Inc., Setauket, New York (1993).
17. P. R. Hunter, M. A. Gaston, *J. Clin. Microbiol. 26* (1988) 2456.
18. T. S. M. Pirttijärvi, M. A. Andersson, A. C. Scoging, M. S. Salkinoja-Salonen, *Proceedings of the seventh International Conference of the International on Food Microbiology and Hygiene (ICFMH),* Veldhoven (1999) pp. 586–590.
19. J. Stadhouders: *Bulletin of the IDF 275*, International Dairy Federation, Brussels, Belgium (1992) pp. 4–8.
20. J. H. Heinrichs, D. J. Beecher, J. D. Macmillan, B. A. Zilinskas, *J. Bacteriol. 175* (1993) 6760.
21. H. Schraft, M. Steele, B. McNab, J. Odumeru, M. W. Griffiths, *Appl. Environ. Microbiol. 62* (1996) 4229.

Raznolikost izolata *Bacillus cereus* iz mlijeka i mliječnih proizvoda utvrđena biokemijskim, imunološkim, AP-PCR i PCR-RFLP postupcima

Sažetak

Fiziološke osobine, uključujući lecitinaznu i hemolitičku aktivnost, API biotipizaciju i imunodetekciju enterotoksina dijareje uspoređeni su s AP-PCR genotipizacijom i PCR--RFLP analizom fragmenata gena *hbl*A i *cer*AB radi razlikovanja 82 izolata *Bacillus cereus* dobivena iz sirovog mlijeka i mliječnih proizvoda. Amplifikacija *cer*AB gena s odabranim »primerima« bila je uspješna u 78 od 82 pozitivna soja za lecitinazu (95 %). U 66 sojeva dobiven je *hbl*A amplifikacijski produkt (80,5 %). Koristeći BCET-RPLA postupak za utvrđivanje imuniteta, postignut je isti rezultat u 97,5 % izolata. Usporedna analiza fenotipske ekspresije i PCR amplifikacije gena, koji kodiraju za lecitinazu i sintezu enterotoksina dijareje u *B. cereus* mliječnih izolata, pokazuju visoki stupanj korelacije i potvrđuju korisnost brze molekularne detekcije i/ili postupaka identifikacije za toksične sojeve *B. cereus* iz mlijeka i mliječnih proizvoda. Nadalje, restrikcijska analiza toksina kodiranog sekvencijom gena u sojevima *B. cereus* otkriva vrlo veliku heterogenost i stoga korisnost PCR-RFLP tipizacije sojeva na osnovi tih sekvencija. Nije nađena korelacija između povećanja broja sojeva na osnovi API biotipizacije i AP-PCR genotipizacije. Međutim, visoki indeksi razlikovanja bili su izračunani za oba postupka tipizacije, pa su se stoga mogli uspješno koristiti za razlikovanje *B. cereus* izoliranih iz mlijeka i mliječnih proizvoda. Prema dobivenim rezultatima PCR-RFLP analiza genskih sekvencija koje kodiraju za toksin, najuspješniji je postupak za detekciju, identifikaciju i/ili tipizaciju, pa se stoga može koristiti za utvrđivanje izvora i putova prenošenja toksikogenih sojeva *B. cereus* pri snabdijevanju sirovim mlijekom i njegove preradbe u mljekari.