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KIYMET GÜVEN

M. BURÇİN MUTLU

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Properties of *Bacillus cereus* Collected from Different Food Sources

Kıymet GÜVEN, M. Burçin MUTLU

Department of Biology, Faculty of Science, Anadolu University, 26470 Eskişehir - TURKEY

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Abstract: Forty-nine *Bacillus cereus* isolates were obtained from 10 different food samples consumed in Turkey. The isolates were identified as *B. cereus* based on colonial, cellular morphology, and biochemical characters, including fatty acid methyl ester (FAME) analysis. The potentials of 53 isolates including a reference strain, *B. cereus* NRRL B-3711, and 3 strains from Japan were tested to produce diarrhoeal enterotoxin (by the BCET-RPLA test) in BHI culture and 35 isolates (66%) were positive. Fatty acid composition varied for all *B. cereus* isolates, with 74 different fatty acids being detected. Grouping of *B. cereus* isolates by fatty acid composition suggested the existence of 4 groups: A, B, C, and D. The major difference between the groups was found in the following FAME: 15:0 ISO. Automated riboprinting was applied to the 53 isolates of *B. cereus* to assess the genetic similarity of the isolates obtained from different food sources. The automatic ribotyping of the total genomic DNA with *Eco*RI yielded a total of 8 band patterns among the isolates. Grouping of the 53 randomly selected *B. cereus* isolates based on their genotypic and phenotypic characteristics showed that these characteristics were independent of the food source of the various strains. The use of this polyphasic approach to strain differentiation enabled all the *B. cereus* isolates to be distinguished from each other. However, there was no relation between the groups and food of origin.

Key Words: *Bacillus cereus*, food, diarrhoeal toxin, FAME, ribotyping

Çeşitli Gıdalardan Toplanmış *Bacillus cereus*'ların Özellikleri

Özet: Türkiye'de tüketilen 10 farklı gıda çeşidinden 49 *Bacillus cereus* izolatı toplanmıştır. İzolatlar koloni yapıları, hücre morfolojileri ve yağ asitleri metal esterlerinin analizi de dahil biyokimyasal karakterlerine göre tanımlanmışlardır. Bir tane referans strain ve Japonya'dan sağlanmış 3 strain dahil toplam 53 izolatın BHI kültüründe diyare enterotoksini üretme potansiyelleri (BCET-RPLA testi ile) test edilmiş ve 35 izolatın (% 66) toksin ürettiği saptanmıştır. Yağ asit bileşimleri tüm *B. cereus* izolatlarında farklı olup, 74 farklı yağ asidi tespit edilmiştir. Yağ asitleri bileşimlerine gore A, B, C ve D olmak üzere 4 farklı grup oluşmuştur. Gruplar arasındaki temel farklılık FAME, 15:0 ISO yağ asidinde gözlenmiştir. Farklı gıda kaynaklarından toplanmış 53 *B. cereus* izolatının genetik benzerliğini ortaya koymak için otomatik ribotiplendirme yöntemi uygulanmıştır. Toplam genomik DNA'nın *Eco*RI ile otomatik ribotiplendirmesi sonucu izolatların yedi farklı bant profili oluşturduğu gözlenmiştir. Gıdalardan rastgele seçilmiş 53 *B. cereus* izolatının genotipik ve fenotipik özelliklerine dayalı gruplandırma, bu karakterlerin izolatların toplandığı gıda çeşidinden bağımsız özellikler olduğunu göstermiştir. Strain farklılığını gösteren bu polifazik yaklaşımın kullanımı test edilen her bir *B. cereus* izolatının diğerinden ayırdedilmesini sağlamıştır. Ancak gruplar ve gıda kaynakları arasında bir ilişki bulunamamıştır.

Anahtar Sözcükler: *Bacillus cereus*, gıda, diyare toksini, FAME, ribotiplendirme

Introduction

Bacillus cereus spores and vegetative forms are frequent inhabitants of a wide range of environments, including soils and clays, sediment, dust, mineral water, vegetation, and many food types, especially cereals and cereal derivatives, milk and dairy products, meat, and raw and processed foods and vegetables (1-4).

The organism sometimes causes food poisoning and this is due to poor temperature control during the preparation or storage of food, which allows *B. cereus* to reach hazardous levels of $>10^5$ cfu g^{-1}. It can cause 2 different types of illness, the diarrhoeal form and the emetic form, attributed to consumption of foods containing large numbers of this organism. The first and

best known type is characterised by abdominal pain and diarrhoea that occur between 8 and 16 h after ingestion of the contaminated food. The second type of illness is characterised by nausea and vomiting that occur within 30 min to 5 h after the consumption of contaminated food (5). The diarrhoeal type of food-poisoning is caused by an enterotoxin or enterotoxins produced during vegetative growth of *B. cereus* in the small intestine (6) and the emetic toxin, cereulide, is produced by cells growing and sporulating in the food (7,8).

Incidence and biochemical characterisation of *B. cereus* isolated from rice (9), dairy products (10), corn and cassava flour (11), pasta and meat dishes (12,13), on farms in the Netherlands (14), and dried milk products (15) were studied previously. Toxin characterisation of *B. cereus* (16-19), sub-typing by total cell protein patterns and arbitrary primer polymerase chain reaction (20,21), genotyping by multilocus enzyme electrophoresis (MEE), pulsed field gel electrophoresis (PFGE) (22), and 16S rDNA analysis (23,24) were done previously in different studies.

In this study we aimed to subtype and to investigate the correlation between the subtypes of *B. cereus* strains collected from a wide range of food sources consumed in Turkey by the assessment of diarrhoeal enterotoxin production, analysing the whole cell FAME of the bacterium and automatic ribotyping. This is the only study combining both the FAME analysis and ribotyping of *B. cereus* isolates.

Materials and Methods

Bacterial strains

The bacteria were isolated from the following 10 different food sources: milk products (e.g. raw milk, pasteurised milk, cheese, and ice cream), meat products (e.g. ground meat, soudjouk, and pastrami), and boza, which is a typical Turkish fermented beverage. *Bacillus cereus* NRRL 3711 was obtained from USDA, Agricultural Research Service, Peoria, IL, USA. Three strains of *B. cereus* isolated from 5 emetic-syndrome outbreaks were kindly provided by Y. Nishikawa (Osaka City Institute of Public Health and Environmental Sciences, Japan) (18). The isolates and their source of origin are shown in Figure 1.

Samples were obtained from local markets and brought to the laboratory under sanitary conditions in cold bags and processed on the same day (25). The *B. cereus* was isolated by the surface plating method with mannitol egg yolk polymyxin (MYP) agar (5) and the plates were incubated at 30 °C for 24 h for typical colony formation. The *B. cereus* colonies appeared as a red-purple, dry, rough surface, with a white precipitate on MYP agar.

Biochemical characterisation

Typical colonies of *B. cereus* were then transferred to nutrient agar slants and identification was confirmed by microscopic and biochemical characterisation including Gram stain, lecithinase production, acid production from mannitol, anaerobic utilisation of glucose, reduction of nitrate, Voges-Proskauer test, motility, endospore formation, and inability to produce parasporal crystal as suggested by Shinagawa (26), Pirttijarvi et al. (27), Nout et al. (28), and Altayar and Sutherland (29).

Haemolytic activity

Trypticase soy blood agar was prepared (30) with a modification. Five percent defibrinated human blood was added instead of sheep blood. Haemolytic activity was recorded as positive when a zone of complete haemolysis was observed around the growth after incubation at 30 °C for 24 h.

Diarrhoeal toxin production

B. cereus isolates were grown in Brain Heart Infusion (Oxoid CM 225, UK) at 32 °C for 18 h. Then the culture was centrifuged at 4000 ×g for 10 min at 4 °C (31). The production of diarrhoeal toxin was monitored using BCET-RPLA (Oxoid TD950) test according to the recommendations of the manufacturer.

Fatty acid composition

B. cereus isolates were inoculated onto Trypticase Soy Broth (TSB) agar and grown for 24 h at 28 °C. Cellular fatty acids were extracted and derivatised to their fatty acid methyl esters (FAMEs) as described by Sasser (32). FAMEs were separated by the MIDI (Microbial Identification System) (Microbial ID, Inc. Newark, DE, USA) utilising an Agilent Technologies 6890N gas liquid chromatograph with a G2614A auto sampler and a 6783 injector. After flame ionisation, FAME peaks were analysed by MIDI Microbial Identification System, software version TSBA 50. Cluster analysis techniques were used to produce unweighted pair matching based on fatty acid composition. Strains within 6 Euclidian distances or distance units, the value determined for subspecies, and 2 Euclidian distances, the value determined for strain according to the MIDI protocol (33), were grouped in the same cluster.

SOURCE	ENTEROTOXIN PRODUCTION	RIBOGROUP	ISOLATE
Pasteurised milk	+	8	3M
Pasteurised milk	+	8	3BA
Raw meat	+	4	E8
Ground meat	+	8	K24
Raw meat	+	6	E5
Raw meat	+	4	E6
Ground meat	+	8	K15
Poultry	+	8	TP23
Ground meat	+	8	K5
Boza	+	1	B10
Raw meat	+	1	E17
Pasteurised milk	-	6	3HA
REFERENCE	-	3	91-97-21
Soudjouck	+	2	ES16
Boza	-	1	B7
Cheese	-	2	4E
Ice cream	+	3	1GSB
Raw meat	+	3	E20
Pasteurised milk	-	1	EP6
Pasteurised milk	+	4	3Y
Raw milk	+	5	2WS
Raw milk	+	2	2ES
Raw milk	-	3	2FSA
Ground meat	-	1	K7
Raw milk	-	3	2XS
Pasteurised milk	+	2	3L
Raw milk	-	3	2KSA
Pasteurised milk	+	7	3K
Pasteurised milk	-	3	3EC
Pasteurised milk	-	5	3VB
Ice cream	+	1	1G
Pasteurised milk	-	3	3EB
Boza	+	1	B8
Pasteurised milk	+	2	3R
Raw milk	-	7	2LSB
Ground meat	+	8	K22
Raw milk	+	6	2Y
Pasteurised milk	+	7	3IB
Raw milk	+	2	2FSB
Raw milk	+	1	2ISB
Pasteurised milk	+	3	3CA
Cheese	-	2	4F
REFERENCE	-	5	94-35-15B
REFERENCE	+	1	NRRL 3711
REFERENCE	-	3	91-97-22B
Ice cream	+	6	1C
Pasteurised milk	+	7	3BB
Raw milk	+	1	E10
Soudjouck	+	1	ES17
Poultry	-	1	TP24
Boza	+	1	B9
Boza	+	8	B4
Pasteurised milk	-	3	3EA

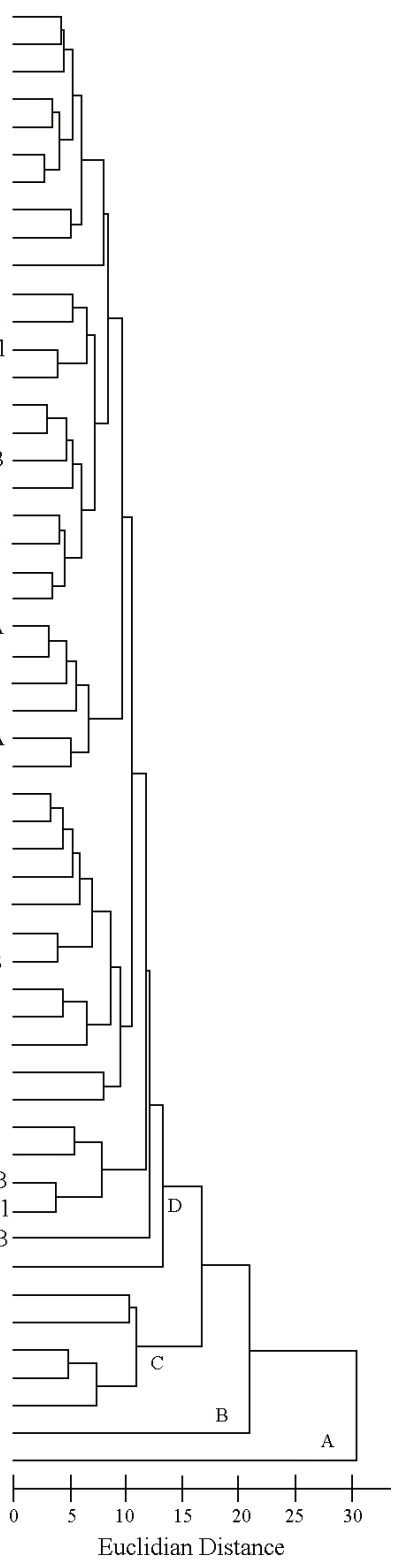

Figure 1. Relationship among strains of B. cereus on the basis of source, enterotoxin production, ribogroup, and cellular composition in fatty acids. The letters represent the designated groups for clusters.

Ribotyping

The automated ribotyping was performed using a robotised instrument (Riboprinter™ Microbial Characterization System, Qualicon, Du Pont, Wilmington, DE, USA) and the Riboprinter™ System Data Analysis Program. The procedure used for processing each sample is described in detail by the manufacturer. Briefly, the isolates were grown overnight at 28 °C, suspended in buffer, heated at 80 °C for 10 min, and lysed. The total DNA was restricted with *Eco*RI, electrophoretically separated, and transferred to a membrane followed by hybridisation. A combined dendrogram was prepared using SPSS (SPSS for Windows Release 10.0 SPSS INC., 1999).

Results and Discussion

This paper describes the genetic and phenotypic diversities among food originated strains of *B. cereus.* A total of 53 isolates were examined for this aim and except for 4 reference isolates 49 of them were isolated from different food sources including milk products, meat products, poultry, and boza.

The use of MYP agar for the detection of *B. cereus* is based on a double diagnostic system: nonfermentation of mannitol and lecithinase production. Polymyxin B sulphate was included as a selective agent to inhibit gram-negative organisms (34). Anaerobic utilisation of glucose, reduction of nitrate, positive Voges-Proskauer test, motility, endospore formation, haemolysis, and parasporal crystal formation tests confirmed the identity of the isolates as *B. cereus.*

In this study, presumptive *B. cereus* isolates were considered positively identified when they were shown to be motile and haemolytic, and unable to produce parasporal crystals and FAME analysis by MIDI not only gave fatty acid profiles of the strains tested but also recognised all the strains as *B. cereus*, confirming the biochemical identification.

Fifty-three isolates characterised as *B. cereus* were tested for diarrhoeal enterotoxin production and 35 strains (66%) were positive (+) while 18 strains (34%) were negative (-) with the BCET-RPLA test including emetic toxin producer strains obtained from Japan (18). These enterotoxin negative strains were isolated from raw milk, pasteurised milk, cheese, raw and ground meat, poultry, and boza samples. The proportion of positive enterotoxigenic strains of *B. cereus* isolated from different food samples investigated in this study is shown in Table 1. Various studies detecting the diarrhoeal enterotoxin production of *Bacillus cereus* isolates showed that the toxin production rate was 84% for cooked food isolates (17), 28.9% for dried milk products (15), and 71.9% for fresh vegetable and refrigerated food isolates (19). It seems impossible to compare enterotoxin production rates of *Bacillus cereus* isolates in different studies. However, it is clear that the enterotoxin production rate of Turkish isolates of the bacterium is higher than that of Chilean isolates.

Fatty acid composition varied for the 53 *B. cereus* isolates, with 74 different fatty acids being detected. Eight different fatty acids, namely 13:0 ISO, 13:0 ANTEISO, 14:0 ISO, 15:0 ANTEISO, 16:0 ISO, Sum in feature 3 (16:1 w7c/15 iso 2OH), 16:00, and 17:0 ISO, were detected in all isolates analysed. Grouping of *B. cereus* isolates by fatty acid composition suggested the existence of 4 groups: A, B, C, and D (Figure 1). Group A included 1 isolate obtained from pasteurised milk. Group B contained 1 isolate obtained from boza, while group C contained 5 isolates obtained from boza, chicken, soudjouk, meat, and pasteurised milk. Group D included majority of the isolates (45 isolates) including the reference strain. The relative percentages of each major fatty acid occurring in each group (A-D) are shown in Table 2. Minor fatty acids, which were present at lower values (< 1%), were omitted. The major difference

Table 1. The proportion of enterotoxigenic strains on the basis of BCET-RPLA test.

Food products	Number of positive isolates	Proportion (%) of positive isolates
Raw milk	5/9	55.5
Pasteurised milk	9/14	64.2
Cheese	0/2	0
Ice cream	3/3	100
Raw meat	6/6	100
Ground meat	4/5	80
Soudjouk	2/2	100
Poultry meat	1/2	50
Pastrami	0/1	0
Boza	4/5	80

between the groups was found in the following FAME: 15:0 ISO.

Fatty acid analysis is a useful tool for identifying new bacterial species including *Bacillus* (35). Pirttijärvi et al. (27) examined a cheese dairy and its whey manufacturing line for *B. cereus* and compared the whole cell fatty acids of the bacterium. They found that the composition of whole cell fatty acids of *B. cereus* isolates originating from the whey part of the process was different from that of the type strain and of the isolates originating from the raw material of the cheese. Pirttijärvi et al. (36) reported that *B. cereus* was not or only poorly recognised by a commercial whole cell fatty acid library as in the case of biomaterial-based industrial processes. Haque and Russell (23) grouped *B. cereus* collected from Bangladeshi rice by the fatty acid composition and our results agree that the predominant fatty acid group is 15:0 ISO. All of the strains tested in this study were also recognised as *B. cereus* by the TSBA 50.

Automated riboprinting was applied to the 53 strains of the *B. cereus* to assess the genetic similarity of the strains isolated from different food sources. Restriction of the total DNA with *Eco*RI yielded about 13 fragments of 2-13 kb in size (Figure 2). A total of 8 banding patterns were obtained among the isolates. All the isolates tested were identified as *B. cereus* with the ribotyping system. The differences in the ribopatterns were mostly located in bands between 3 and 10 kb in size (Figure 2). The number of ribotypes determined and the number of isolates representing them are shown in Figure 2. There was no relation between the sources of the isolates and ribotype patterns (Figure 1). A combined dendrogram was prepared using UPGMA of the fragments obtained with the *Eco*RI enzyme (Figure 3).

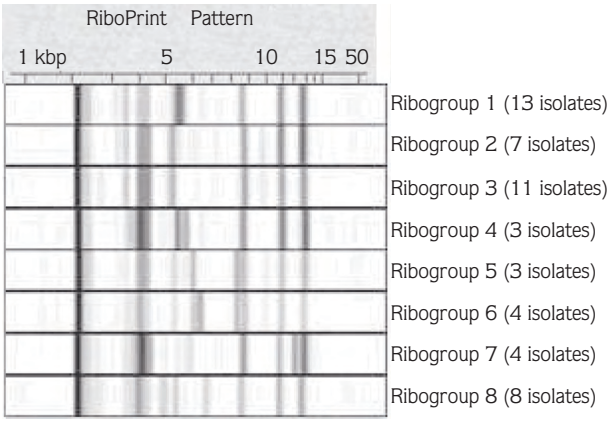

Figure 2. Ribotype patterns of B. cereus isolates with EcoRI. enzyme.

Table 2. Proportion of major (>2%) fatty acid in cluster (groups A-D) of B. cereus strains.

FATTY ACIDS	GROUP A	GROUP B	GROUP C	GROUP D
13:0 ISO	12.01	1.21	7.17 (1.93)	9.33 (2.36)
14:0 ISO	5.38	0.89	2.92 (0.85)	4.63 (1.47)
14:00	4.88	2.28	3.88 (0.29)	4.25 (0.78)
15:0 ISO	-	43.12	18.65 (1.70)	27.23 (3.60)
15:0 ANTEISO	9.57	4.14	4.73 (0.509)	6.41 (1.24)
16:0 ISO	8.31	4.79	3.57 (0.67)	5.62 (1.29)
SF 3 [a]	11.48	8.63	2.13 (1.39)	4.47 (3.66)
16:00	7.33	3.93	7.22 (2.15)	6.36 (1.31)
ISO 17:1 w10c	2.92	3.19	1.55 (0.7)	1.69 (0.56)
ISO 17:1 w5c	3.9	2.6	1.4	1.71 (0.64)
17:0 ISO	14.72	8.28	4.104	6.27 (1.64)
17:0 ANTEISO	2.95	2.2	1.268 (0.5)	1.83 (0.54)
unknown 14.959	2.23	3.5	6.71	2.80 (1.92)
15:0 3OH	2.22	2.58	6.72 (1.25)	4.08 (1.67)
18:00	2.18	2.18	7.59 (1.37)	2.701(1.68)

[a] summed feature 3 represents 16:1 w7c/15 iso 20H.

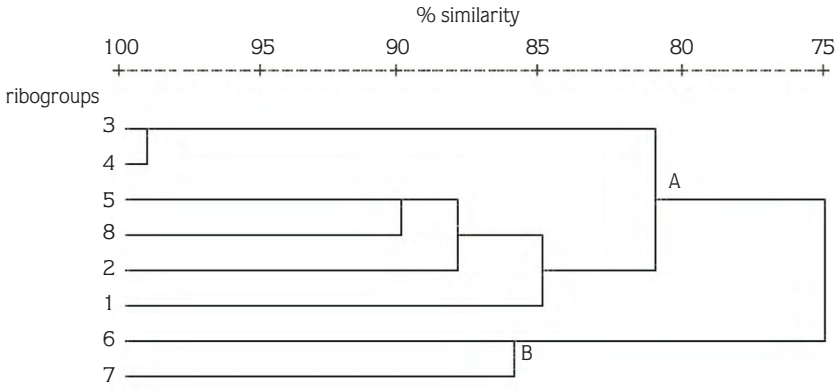

Figure 3. Dendrogram constructed on the basis of ribotype patterns obtained with the EcoRI enzyme.

Ribotyping has long been considered a useful technique (37) but difficulty of performance has limited the widespread application of this method. The automated ribotyping with the Riboprinter™ Microbial Characterization System has simplified the method (38).

Pirttijärvi et al. (39) analysed 11 cereulide-producing *B. cereus* strains, mostly from food and food packaging material, and found that all shared a single specific ribopattern. Andersson et al. (38) found that *B. cereus* strains isolated from dairies were very heterogeneous. However, 17 strains were typed in the same groups and this shows that the method can be used in tracing contamination routes or in epidemiological studies of *B. cereus*. Apetroaie et al. (40) compared the cereulide-producing strains of *B. cereus* by physiological and genetic properties and concluded that they are genetically and biochemically more diverse. They found 3 ribotypes among 24 strains of *B. cereus*.

In our study, 8 ribotypes were obtained among 53 isolates of the bacterium and 2 of 3 emetic strains obtained from a food poisoning outbreak (18) were represented by a single ribotype indicating the same food source. However, some of the diarrhoeal toxin producing strains had the same ribotype in this study. On the other hand, ribogroups were randomly distributed among fatty acid groups A, B, C, and D (Figure 1). Therefore, it is impossible to find a relationship between either enterotoxin type or fatty acid group and ribotyping profiles in this study.

Conclusions

Grouping of the 53 randomly selected *B. cereus* strains based on their genotypic and phenotypic characteristics showed that these characteristics were independent of the food source of the various strains. The use of this polyphasic approach to strain differentiation enabled all the *B. cereus* strains to be distinguished from each other. However, there was no relation between the groups and food of origin.

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Corresponding Author:
Kıymet GÜVEN
Department of Biology,
Faculty of Science,
Anadolu University,
26470 Eskişehir - TURKEY
E-mail: kguven@anadolu.edu.tr

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