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Enterotoxigenic Profiles of psychrotolerant and mesophilic strains of the *Bacillus cereus* group isolated from food in Morocco

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ABSTRACT

The species *Bacillus cereus*, known for its ability to cause food borne disease, consists of a large variety of strains. An important property for discrimination of strains is their growth temperature range. Fifty-two strains of *Bacillus cereus* isolated from different sources of food (milk, dairy product, spices and rice salad) for two years were determined to be either mesophilic or psychrotrophic by growth at 6 °C and at 43° C on optimal agar medium. The strains were also screened by real time polymerase chain reaction to discriminate between mesophilic and psychrotrophic types. The result obtained allowed highlighting eight profiles. Thirty seven of the 52 strains were able to grow at 6°C, but only thirteen conformed to the new psychrotolerant species *Bacillus weihenstephanensis*. The presence of the gene components encoding production of enterotoxins Nhe, Hbl, EntT and a recently described cytotoxin K was determined by PCR. All the strains possessed genes for at least one of these toxins. The *nhe* genes were detected in a higher proportion than *hbl* genes. Haemolytic enterotoxin was detected in 71.1 per cent of the isolates. Results of this study indicate that there are intermediate forms between *B. cereus* and *B. weihenstephanensis*, these results might be of importance for gaining further understanding of the growth properties of *B. weihenstephanensis* and psychrotolerant *B. cereus* as well as their contribution to food poisoning. However, no relationship among haemolysis test, enterotoxin genes and growth temperatures of the strains was found.

Keywords: *Bacillus cereus group, mesophilic, Morocco, psychrotolerant, virulence genes*

I. INTRODUCTION

Bacillus cereus is one of the pathogens responsible for human diarrhoea, and source of infestation is mainly due to consumption of contaminated food. There are two types of *B. cereus* food poisoning syndromes caused by two independent toxins. The emetic toxin (<5kDa) is resistant to heat, proteolytic enzyme and low *p*H. This toxin causes nausea and vomiting within 1-5 h after the consumption of contaminated food. The diarrhoeal toxin is a 50 kDa heat-labile protein, which is sensitive to proteolytic enzymes and expressed during the late exponential phase of growth. The onset of *B. cereus* mediated infection is about 8-16 h, lasts for 12-24 h, and mostly associated with abdominal pain, profuse watery diarrhoea and tenesmus than nausea and vomiting [1]. Four different enterotoxins have been characterized: two protein complexes, hemolysin BL (HBL) and nonhemolytic enterotoxin (NHE), and two enterotoxic proteins, enterotoxin T (bc-D-ENT) and cytotoxin K [2][3]. HBL complex is composed of three proteins, B, L1, and L2 [4] transcribed from the genes *hblC* (encoding L2), *hblD* (encoding L1), and *hblA* (encoding B), organized in one operon together with a fourth gene, *hblB* (encoding the B' protein) [5][6] . NHE complex is also composed of three different proteins, NheA, NheB, and NheC encoded by the three genes *nheA*, *nheB*, and *nheC*, and it is also organized in one operon [7]. Among the strains of *B. cereus* a great diversity exists, for instance with respect to the presence of enterotoxin genes, with respect to the ability to produce emetic toxin and with respect to the ability to grow at various temperatures. *B. cereus* was not considered as a psychrotolerant species until some cold-tolerant isolates had been identified in the 1990. A new species *B. weihenstephanensis* was studied which was distinctive from mesophilic *B. cereus* [8]. Considering the heat resistance of their spores, the vegetative production of emetic and enterotoxic toxins at refrigeration temperatures, the occurrence of psychrotolerant *B. cereus* strains and their consequent impact on the safety of chilled foods are needed to be investigated.

B. weihenstephanensis was initially defined as "an isolate of a new species growing at 4–7 °C but not at 43 °C and which can be identified rapidly using rDNA or *cspA* targeted PCR". However, some research indicated that the growth temperature of *B.*

weihenstephanensis should be limited from 5 °C to 37 °C [9] or 7 °C to 38 °C [10], and that some *B. cereus* strains also contained both rDNA operons with psychrotolerant and mesophilic signatures [11], suggesting that the psychrotrophic *B. cereus* strains should not be classified as *B. weihenstephanensis* and that intermediate forms between the two species might exist.

The objective of the present work was to evaluate the biodiversity of psychrotrophic and mesophilic bacteria of the *B. cereus* group, and to analyze the distribution of enterotoxin genes and the haemolysis activity in food strains of various origins (isolated from milk, dairy product, spices and rice salad).

II. MATERIALS AND METHODS

II. 1 *Bacillus cereus* group strains

The collection comprised 52 isolates of the *B. cereus* group that were isolated from milk and dairy products, rice salad and spices (Table 3). The food samples were collected from hotels, restaurants, and private companies in several cities in Morocco (mainly from Casablanca, Tanger, Rabat and Marrakech). All strains were stored at -80°C in a meat broth with glycerol.

II. 2 Growth profiles of isolates

The strains were search for specific sequences by real time PCR (SYBR Green chemistry, iCycler optical module 584BR, BioRad, Marnes-la-Coquette, France), allowing to the discrimination between psychrotrophic and mesophilic strains in the group. The PCR conditions described by Von Stetten et al. (1998) [12] and Francis et al. (1998) [13] were adapted to real time PCR, by using the same published primers, mf and ur primers for the 16S rDNA1-m sequence, pr and uf primers for the 16S rDNA-2 p sequence, and BcAPF1 and BcAPR1 for the psychrotrophic *cspA* sequence.

The isolates were also propagated in Mossel agar at 30 °C for 24 h for the determination of the ability to grow at 6 °C and 43 °C on solid medium.

II. 3 Molecular characterization to detect virulence genes

The 52 strains of the *B. cereus* group were tested for the presence of the virulence genes *hbl* (C, D, A, and B), *nhe* (A, B, and C), *bceT* (encoding the bc-D-ENT enterotoxin), and *cytK* (encoding cytotoxin K). Genomic DNA was purified with the DNeasy 96 Tissue kit (Quiagen, Courtaboeuf, France) as recommended by the manufacturer.

The primers used are shown in Table 1. PCR amplification was performed in a 25 µl reaction volume. Each reaction mixture contained 100 ng of DNA template, primers (primers and concentration used are described in Table 1), 1.5 U of *Taq* polymerase (BioLabs, Ipswich, Mass.), 200 µM deoxyrebonuleoside triphsphate (BioLabs), and 1.5 mM $MgCl_2$ in a PCR buffer (2.5 µl of a 10X PCR buffer). The amplification reactions were carried out in a Biorad- iCycler version 1.280 (Biorad, Marnes la coquette, France) as follows: 4 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s for annealing (annealing temperatures are given in Table 2), and 1 min at 72°C. The program finished with an additional 7 min extension step at 72°C.

For the *hblB* amplification reaction, parameters used were 2 min at 94°C, followed by 10 cycles of 10 s at 94°C, 30 s at 58°C, and 2 min at 68°C; 20 cycles of 10 s at 94°C, 30 s at 58°C, 2 min (plus 20 s per cycle) at 68°C; and a final extension at 68°C for 7 min. For each run, the whole PCR mix without DNA template was used as a negative control. After the amplification, 5 µl of reaction mixture was analyzed by electrophoresis on a 1 % agarose gel in Tris-borate-EDTA buffer (Tris, 89 mM; boric acid, 89 mM; EDTA, 2 mM) at 90 V for 45 min. The gel was stained by ethidium bromide to check the size of the PCR products.

II. 4 Haemolysin assay

Data for the hemolytic activity and production on blood agar plates were detailed in previous studies [14]. Briefly, culture of individual isolates of *B. cereus* obtained from overnight culture grown in brain heart infusion broth (BHI) for 24h at 30°C assessed for haemolytic activity by agar well plate assay using sheep blood agar (SBA) (bioMeriux, France). Some microlitres of culture were added in the SBA plates and incubated at 30°C and monitored for haemolytic pattern [15].

III. RESULTS

III. 1 Classification of the 52 isolates on the basis of genotypic and phenotypic characteristics

All strains were separated into psychrotrophic/psychrotolerant or mesophilic groups by their ability to grow at 6 and 43°C (Table 2). In this study, the two PCR-based methods described by Von Stetten et al. (1998) and Francis et al. (1998) were adapted to real time PCR, to discriminate mesophilic strains from psychrotrophic (*B. weihenstephanensis*). One is based on the amplification of a segment of the cold-shock protein A gene (*cspA*), in which only psychrotrophic strains have the specific signature sequence that makes amplification possible. The other method takes advantage of specific sequence differences between mesophilic and psychrotolerant strains in the 16S rDNA. When screening our strains according to these methods, eight profiles were highlighted (Table 2). The profile 1 represented 25% of the collection. It comprised isolates able to grow at 6 °C, unable to

grow at 43°C and showing the following genetic signatures: presence of the *cspA* and 16S rDNA-2 p signatures and absence of the 16S rDNA-1 m signature. This profile corresponds to the characteristics of the species *B. weihenstephanensis* described by Lechner et al. (1998) [8]. The two isolates of the profile 2 (3.8%) had the same features except for the growth at 43°C. The two isolates of the profile 3 (3.8%) possessing the *cspA* and 16S rDNA-2 p signatures, was able to grow at 43 °C but not at 6 °C. We also observed the presence of isolates exhibiting both mesophilic and psychotrophic 16S rDNA signatures, corresponding to the profiles 4, 5, 6, and 7, representing 17.3, 19.2, 11.5, and 5.8% of the collection, respectively. Only seven strains constituting the profile 8 (13.5%) were strictly mesophilic in the sense that just the mesophilic of 16S rDNA was amplified and that they were negative in the *cspA* PCR.

III. 2 Detection of virulence genes

The four genes, *hblC, hblD, hblA* and *hblB* were detected in 20 isolates (38.5%). Six (11.5%) isolates possessed three of the foor *hbl* genes, ten (19.2%) isolates possessed two of the foor *hbl* genes and two (3.8%) had only one gene coding the HBL complex. Fourteen (26.9%) *B. cereus* isolates had none of HBL complex.

All the three genes *nheA, nheB* and *nheC*, were detected in 35 (67.3%) of 52 *B. cereus* isolates. Fourteen (26.9%) isolates harboured two *nhe* genes and two (3.8%) isolates harboured one *nhe* genes, whereas only one (1.9%) isolate lacked all three genes of NHE complex (Table 3). The nonhaemolytic enterotoxin (NHE) genes *nheA, nheB,* and *nheC* (98, 82.7 and 78.8%, respectively) were frequently detected than haemolytic enterotoxin (HBL) genes, *hblC, hblD, hblA and hblB* (73.1, 55.8, 51.9 and 50%, respectively). The newly described CytK, which might cause necrotic enteritis, was detected in eleven of the strains (21.1%). The possible significance of the enterotoxin T in *B. cereus* foodborne illness has not been established. This toxin is a single protein, which was identified by Agata et al [2]. In our assay, 29 of 52 strains (55.8%) possessed the *bceT* gene (Table 3).

No significant occurrence in difference of enterotoxic genes between the psychrotolerant and mesophilic strains was observed in this study.

III. 3 Haemolysin assay

Majority (71.1%) of the *B. cereus* isolates exhibited haemolysis (Table 3). The expression of haemolysin was associated with presence of any of the *hbl* genes. Haemolytic activity was not detected in 15 isolates, which did not harbour any *hbl* genes (Table 3).

IV. DISCUSSION

B. cereus associated food poisoning is underreported as the types of illnesses witches were relatively mild and usually last for less than 24 h. Nevertheless, occasional reports of more severe form of diarrhoeal type of illnesses, ubiquitous presence and heat-stable endospore forming nature of the organism underscore the significance of the organism. The unique properties such as heat resistance, endospore forming ability, toxin production and psychrotrophic nature give ample scope for this organism to be a prime cause of public health hazard [16].

In this study, we have basically established correlation between our strains and a number of already known profiles [17]. These profiles comprised pure psychrotrophic ones characterized by the presence of both 16S rDNA-2 p and *cspA* sequences, pure mesophilic ones characterized by the presence of 16S rDNA-1 m, intermediate ones exhibiting either the psychrotrophic sequences together with the mesophilic phenotypic profile described by Von Stetten et al. (1999), or both the 16S rDNA-2 p and 16S rDNA-1 m sequences, probably due to the coexistence of mesophilic and psychrotolerant 16S rDNA operon copies within a single strain [12]. The profile 1, identified as the species *B. weihenstephanensis*, represented 25% of the collection. These results are in agreement with those of Bartoszewicz et al. (2008) [18] who mainly collected *B. weihenstephanensis* isolates from milk. In 1998 the species *B. weihenstephanensis* was suggested as the sixth species within the *B. cereus* group comprising psychrotolerant, but not mesophilic, *B. cereus* strains [8]. Isolates of this new species grow at 4-7°C but not at 43°C and can be identified rapidly using rDNA or *cspA* targeted PCR. The main problem seems to be that the almost half of the strains that we have looked at in this study are positive for both a mesophilic and a psychotropic PCR product using the 16S rDNA primers (Table 2), as pointed out before by Pruss et al. Only seven of our 52 strains were strict mesophilic. The thirteen strains that gave a psychrotrophic PCR rDNA product were all positive for the psychrotrophic *cspA*, and grew at 6°C but not at 43°C and should, according to the definition, be *B. weihenstephanensis*. However, the main problem is that the three strains (Profile 7) that grew at 6°C but not at 43°C were positive for the psychrotrophic *cspA* and positive for both the mesophilic and psychrotrophic rDNA. For the time being we will still call these three strains *B. cereus*. We can therefore found that there are intermediate forms between *B. cereus* and *B. weihenstephanensis* based on the suggested criteria.

The different enterotoxic protein complexes have been characterized in this study, *nhe* genes found in most of the strains in a higher

proportion than *hbl* genes. Distribution of these genes in *B. cereus* was in this study compared to other reports [19]. Hansen and Hendriksen reported that polymorphism among the genes is the likely explanation of the inability to detect all genes in some *B. cereus* isolates by PCR [20]. The result obtained for the *cytK* gene (21.1%) is in accordance with the observation of other authors, who founded few strains positive for *cytK* [7]. It's interesting to point out that the presence of the *cytK* gene was almost independent of the presence of the rest of virulence factors analyzed here. The less studied *bceT* gene appears widely distributed or at low frequencies according to yang et al [21]. In this study, 55.8% of the strains possessed the *bceT* gene. A recently published study indicates that the *bceT* gene is widely distributed among *B. cereus* strains, and that there is strain variation in the gene sequence [20].

Indeed presence of the psychrotolerant rDNA and specific gene are not unique characteristics of psychrotolerant strains. Furthermore, our results showed that no significant difference was found between psychrotolerant *B. cereus* and *B. weihenstephanensis* in enterotoxic genes, rDNA or *cspA* targeted PCR, and growth characteristics at low temperature.

All the isolates that were positive for *hbl* genes in PCR exhibited haemolysis in SBA. Similar to the finding of Thaenthanee et al [22], we have detected higher number (71.1%) of *B. cereus* isolates that displayed high haemolysis activity.

There is no correlation between haemolysis test, enterotoxin genes and growth temperatures. Four of the seven strict mesophilic strains were highly haemolytic while the last three were negative in the haemolysis test. Same remarks for the others strains. The reason for lack of heamolysis in some strains may be due to missing components of *Hbl* and/or *Nhe*, or mutations in the genes that might have dramatically reduced biological activity.

V. TABLES

Table 1. Characteristics of primers used in the simplex PCR for the detection of virulence genes in *B. cereus*

Primer name	Gene	Annealing temp (°C)	Product size (bp)	Primer concn (µM)	Sequence (5'→3')
HC F HC R	*hblC*	50	740	0.5	GATAC(T, C)AATGTGGCAACTGC TTGAGACTGCTCG(T, C)TAGTTG
HD F HD R	*hblD*	50	829	0.5	ACCGGTAACACTATTCATGC GAGTCCATATGCTTAGATGC
HA F HA R	*hblA*	50	1.154	0.5	AAGCAATGGAATACAATGGG AGAATCTAAATCATGCCACTGC
HA F HB R	*hblB*	50	2.684	0.5	AAGCAATGGAATACAATGGG AATATGTCCCAGTACACCCG
NA F NA R	*nheA*	55	755	0.2	GTTAGGATCACAATCACCGC ACGAATGTAATTTGAGTCGC
NB F NB R	*nheB*	55	743	0.2	TTTAGTAGTGGATCTGTACGC TTAATGTTCGTTAATCCTGC
NC F NC R	*nheC*	54	683	0.4	TGGATTCCAAGATGTAACG ATTACGACTTCTGCTTGTGC
TF1 TR1	*bceT*	52	407	0.5	TTACATTACCAGGACGAGCTT TGTTTGTGATTGTAATTCAGG
CK F CK R	cytK	50	809	0.5	ACAGATATCGG(G, T)CAAAATGC GAACTG(G,) (AT)AACTGGGTTGGA

Table 2. Profiles assigned to the isolates of the *Bacillus cereus* group strains (52 isolates) as a function of the presence (+)/absence (-) of the *cspA* signature defined by Francis et al. (1998), and the 16S rDNA-1 m and the16S rDNA-2 p signatures defined by Von Stetten et al. (1998), and the ability (+)/inability (-) to grow at 43°C and 6°C on Mossel agar.

Profile	Growth at 43°C	Growth at 6°C	PCR *cspA*	PCR 16S r DNA-1m	PCR 16S r DNA-2p	Number of isolates	Percentage of isolates
1	-	+	+	-	+	13	25
2	+	+	+	-	+	2	3.8
3	+	-	+	-	+	2	3.8
4	-	+	-	+	+	9	17.3
5	+	+	-	+	+	10	19.2
6	+	-	-	+	+	6	11.5
7	-	+	+	+	+	3	5.8
8	+	-	-	+	-	7	13.5

Table 3. Results of virulence genes and haemolysin assay of *bacillus cereus* isolates from food.

Strain No	Source	*hblC*	*hblD*	*hblA*	*hblB*	*nheA*	*nheB*	*nheC*	*cytK*	*bceT*	Haemolysis
1	milk	+	+	+	+	+	+	+	+	+	+
2	milk	+	+	+	+	+	+	+	-	+	+
3	rice salad	+	+	+	+	+	+	+	-	+	+
4	milk	-	-	-	-	+	+	+	-	+	-
5	spices	+	-	+	-	+	+	+	-	+	+
6	spices	+	+	+	+	+	+	+	-	-	+
7	dairy product	+	+	+	+	+	+	-	-	-	+
8	milk	-	-	-	-	-	-	-	-	-	-
9	dairy product	+	+	-	+	+	+	-	+	-	+
10	rice salad	+	+	+	+	+	-	+	-	-	+
11	spices	+	-	+	+	+	+	+	-	+	+
12	milk	+	-	-	+	+	+	+	-	+	+
13	spices	-	-	-	-	+	+	+	+	+	-
14	milk	+	+	-	-	+	+	-	-	-	+
15	milk	+	+	+	+	+	-	+	+	+	+
16	spices	-	-	-	-	+	+	+	-	-	-
17	rice salad	-	-	-	-	+	+	+	-	+	-
18	spices	+	+	-	+	+	+	-	-	+	+
19	dairy product	+	+	+	+	+	+	+	+	-	+
20	rice salad	+	+	+	+	+	+	+	-	-	+
21	spices	-	-	-	-	+	+	+	-	-	-
22	rice salad	+	+	+	+	+	+	+	-	+	+
23	dairy product	-	-	-	-	+	+	-	+	+	-
24	rice salad	+	+	+	+	+	-	+	-	-	+
25	dairy product	+	-	+	-	+	+	+	-	+	+
26	rice salad	+	-	+	+	+	-	+	-	+	+
27	spices	+	+	+	+	+	-	+	-	-	+
28	milk	-	-	-	-	+	+	+	-	+	-
29	milk	+	+	-	+	+	+	+	-	+	+
30	dairy product	-	-	-	-	+	+	+	+	+	-
31	milk	+	+	+	+	+	+	+	-	-	+
32	dairy product	+	+	+	+	+	+	+	-	-	+
33	milk	+	-	-	-	+	+	+	+	-	+
34	spices	+	+	+	+	+	+	-	-	+	+
35	dairy product	+	-	-	-	+	-	-	-	+	+

36	spices	-	-	-	-	+	+	+	-	+	-
37	rice salad	+	+	-	-	+	+	+	-	-	+
38	spices	+	+	+	+	+	-	-	+	+	+
39	milk	+	+	+	+	+	+	+	-	-	+
40	dairy product	+	+	+	-	+	+	+	-	-	-
41	spices	+	-	+	-	+	-	+	-	+	+
42	dairy product	+	+	+	+	+	+	+	-	-	+
43	spices	+	+	-	-	+	+	-	-	-	+
44	dairy product	-	-	-	-	+	+	+	-	-	-
45	milk	-	-	-	-	+	+	+	-	+	-
46	dairy product	+	+	-	-	+	+	+	-	+	+
47	spices	+	+	+	+	+	+	+	-	+	+
48	milk	+	-	+	-	+	+	-	+	+	+
49	spices	+	+	+	+	+	+	+	-	-	+
50	dairy product	-	-	-	-	+	+	+	-	+	-
51	rice salad	+	+	-	-	+	+	+	+	+	+
52	milk	-	-	-	-	+	+	+	-	-	-

VI. CONCLUSION

In conclusion, the occurrence and behaviour *of bacillus cereus* in food need to be more investigated. A better understanding of the conditions for enterotoxin production and growth temperatures is needed in the future to reduce the food borne infection caused by *B. cereus* in Morocco.

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