รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาการกระจายของยีนที่เกี่ยวข้องกับการสร้างสารพิษใน *Bacillus cereus* และ *Bacillus* สปีชีส์อื่นที่แยกได้จากอาหาร และการพัฒนาวิธีที่รวดเร็วในการวิเคราะห์หายีนที่เกี่ยวข้องกับสารพิษชนิดต่าง ๆ

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ตุลาคม 2548

สัญญาเลขที่ MRG 4680182

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สนับสนุนโดย

สำนักงานคณะกรรมการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

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Abbreviations

°C	degree Celsius
µg	microgram
bp	base pair
DNA	deoxyribonucleic acid
g	gram
h	hour
kb	kilobase pair
l	liter
M	molar
mg	milligram
min	minuit
ml	milliliter
ng	nanogram
PCR	polymerase chain reaction
pM	picomolar
s	second
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate EDTA buffer
Tris	Tris (hydroxymethyl) aminomethane
U	unit
v/v	volume by volume
w/v	weight by volume

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บทคัดย่อ

Bacillus cereus เป็นแบคทีเรียที่สามารถก่อให้เกิดโรคอันเนื่องมาจากการบริโภคอาหาร ซึ่งสามารถสร้างสารพิษที่เกี่ยวข้องกับระบบทางเดินอาหารหลายชนิด ได้แก่ ฮีโมไลซิน บี แอล, นอนฮีโมไลติก เอนเทอโรทอกซิน, ไซโตทอกซิน เค และ เอนเทอโรทอกซิน เอฟ เอ็ม การศึกษานี้มุ่งที่จะสำรวจการกระจายของยีนที่เกี่ยวข้องกับสารพิษเหล่านี้ในเชื้อ *Bacillus cereus* รวมทั้ง *Bacillus* สปีชีส์อื่นที่แยกได้จากอาหาร และมุ่งที่จะพัฒนาวิธีที่รวดเร็วและมีประสิทธิภาพในการตรวจวิเคราะห์หายีนที่เกี่ยวข้องกับสารพิษเหล่านี้ จากการสำรวจการกระจายของยีนเหล่านี้ในเชื้อ *Bacillus* spp. จำนวนรวม 257 ไอโซเลตโดยใช้เทคนิคการเพิ่มจำนวนสารพันธุกรรมในหลอดทดลอง (polymerase chain reaction, PCR) พบว่า ยีนที่เกี่ยวข้องกับสารพิษเหล่านี้ ได้แก่ *hblA, hblC, hblD, nheA, nheB, nheC, entFM, cytK* ปรากฏอยู่ใน *B. cereus* ด้วยอัตราส่วนร้อยละ 64.0, 61.6, 64.0, 59.2, 70.4, 68.8, 60.0, และ 70.4 ของจำนวนไอโซเลตที่ทดสอบ ตามลำดับ และพบว่าทุกยีนปรากฏใน *Bacillus* สปีชีส์อื่นด้วยคือใน *B. mycoides*, *B. thuringiensis*, และ *B. licheniformis* โดยยีนที่เกี่ยวข้องกับสารพิษทุกยีน (ยกเว้น *entFM* และ *nhe* ในบางกรณี) มีการกระจายอยู่ในไอโซเลตของ *B. mycoides* และ *B. thuringiensis* ซึ่งมีความสัมพันธ์ใกล้ชิดกับ *B. cereus* ด้วยอัตราส่วนที่สูงกว่าที่กระจายอยู่ใน *B. licheniformis* ข้อมูลดังกล่าวทำให้เห็นว่าเชื้อในจีนัส *Bacillus* ที่แยกได้จากอาหารโดยทั่วไปนั้นอาจมีความสามารถที่จะสร้างสารพิษได้และควรได้รับการเฝ้าระวังควบคู่ไปกับ *B. cereus* ด้วยในงานที่เกี่ยวข้องกับการป้องกัน การควบคุม และการตรวจวินิจฉัยโรค นอกจากนั้น วิธีที่รวดเร็วและมีประสิทธิภาพในการตรวจวิเคราะห์หายีนที่เกี่ยวข้องกับสารพิษเหล่านี้ได้ถูกพัฒนาขึ้นโดยใช้เทคนิคการเพิ่มจำนวนสารพันธุกรรมจากส่วนของทั้ง 8 ยีนในปฏิกิริยาเดียว หรือวิธี Multiplex PCR ซึ่งสามารถให้ข้อมูลการตรวจสอบไม่เพียงเชื้อ *B. cereus* หรือสารพิษชนิดใดชนิดหนึ่งตามวิธีที่มีใช้อยู่ในปัจจุบันเท่านั้น แต่จะสามารถให้ข้อมูลการตรวจสอบเชื้อ *Bacillus* species ที่มีศักยภาพในการสร้างสารพิษได้

Abstract

Bacillus cereus, a bacterium recognized as foodborne pathogen, is known to produce many kinds of enterotoxins, such as Hemolysin BL, Non-Hemolytic Enterotoxin, Cytotoxin K, and Enterotoxin FM. This study aimed to investigate frequencies of distribution of genes associated with these enterotoxins in *B. cereus* and other *Bacillus* species isolated from foods and to develop a rapid and efficient method for detection of these genes. Distribution of the genes was investigated among 257 food isolates of *Bacillus cereus* and other *Bacillus* species, using PCR. Gene *hblA, hblC, hblD, nheA, nheB, nheC, entFM,* and *cytK* were found to present among isolates of *B. cereus* with a frequency of 64.0, 61.6, 64.0, 59.2, 70.4, 68.8, 60.0, and 70.4 %, respectively. These enterotoxin genes were also found present in *Bacillus* species other than *B. cereus*. All genes, except *entFM* and *nheB* in some cases, are distributed wider in *B. mycoides* and *B. thuringiensis*, species that are closely related to *B. cereus*, than in *B. licheniformis*. These data indicate potential toxigenicity of these *Bacillus* species which are normally recovered from foods and should be noted by those involved in prevention, disease control, and diagnosis of enteropathogenic *B. cereus*. We also developed a rapid and efficient detection method for these 8 enterotoxin genes using a multiplex PCR, which is applicable in analysis of toxigenic *Bacillus* species and can be used as an alternative to the currently-available traditional *B. cereus* agar plate method or methods detecting some individual toxins.

Executive summary

1. Problems underlying this research

Bacillus cereus, heat-tolerant, spore-forming bacterial species widely distribute among the environment and therefore can be easily transferred into foods. This microorganism is held significant for the food industry since it is a cause of some particular type of food spoilage and is a cause of foodborne illnesses. In causing foodborne illnesses, there are two types of enterotoxin, emetic and diarrheagenic. Molecular identification of the diarrheagenic type of toxins has revealed that there are more than one enterotoxic molecules involved, which are Hemolysin BL (HBL) (Heinrichs *et al*., 1993; Ryan *et al*., 1997), Non-Hemolytic Enterotoxin (NHE) (Granum *et al*., 1999), Enterotoxin FM (Asano *et al*., 1997), and Cytotoxin K (Lund *et al*., 2000).

B. cereus is required to be monitored and detected in many food criteria and standards, such as milk formulae, spices, cereal products, etc. Detection method that is most commonly used at the present is through selective plating followed by traditional biochemical tests. These isolation and confirmation procedures take a long time (4-5 days) to complete and are labor intensive. However, there is an increasing need in the food industry for rapid and efficient microbiological techniques. Reasons for this are concern for the health and economy. A rapid and efficient microbiological technique can, for examples, support food quality assurance systems and reduce holding time for food products ready to be distributed, which in turn benefits consumers and food suppliers in both direct and indirect ways. Since the diarrheal type of illness caused by *B. cereus* can be associated with many toxin identities as mentioned above, therefore a detection method for this microorganism directed to the factors related to toxigenicity should be considered. Such factors can be toxin-related genetic materials, or toxin proteins. Considering the multi-toxins involved in the illnesses, the genetic method seems to be a good approach.

2. Research objectives

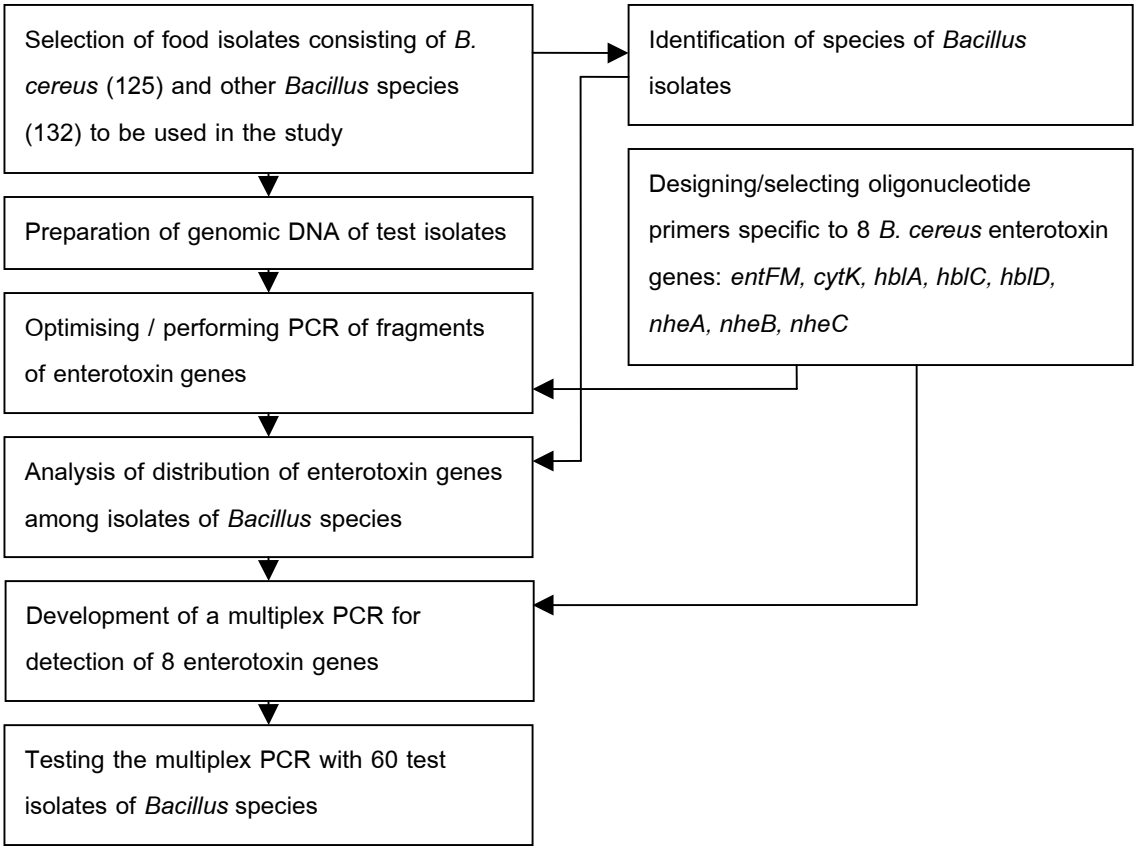
As mentioned above, the genetic method would allow efficient detection of enterotoxigenic factors of *B. cereus*. This study, therefore, was proposed with two main objectives:

(1) to survey distribution of enterotoxin genes among *B. cereus* and other *Bacillus* species isolated from food, in order to provide a basis for developing a rapid and efficient genetic-based method for detection of potentially toxigenic *B. cereus* and other *Bacillus* species, and to provide beneficial data for epidemiology and disease control, and

(2) to develop a rapid and efficient genetic-based method that will enable detection of *B. cereus*-enterotoxin genes or potentially toxigenic *Bacillus* species, which in turn will increase the efficiency of food quality assurance and food safety systems of the food industry.

3. Research methods

The research procedures carried out in this study can be summarized as shown in the following diagram.



4. Results and Discussions

Food isolates of *Bacillus* species used in this study consisted of 125, 15, 28, and 89 isolates of *B. cereus, B. thuringiensis, B. mycoides,* and *B. licheniformis*, respectively. Detection of 8 enterotoxin genes in these isolates by PCR revealed that up to 97.28 % of the isolates harbor at least one of the enterotoxin genes. Gene *hblA, hblC, hblD, nheA, nheB, nheC, entFM,* and *cytK* were found to present in isolates of *B. cereus* with a frequency of 64.0, 61.6, 64.0, 59.2, 70.4, 68.8, 60.0, and 70.4 %, respectively. The frequency of distribution of the genes is found to be at different percentages for different genes, both within one species and in different species. In general, isolates belonging to *B. cereus* group (including *B. cereus, B. thuringiensis, B. mycoides*) are observed to have all genes (except *entFM*, and *nheB* in some cases) distributed among them at higher percentages than among *B. licheniformis* isolates. Moreover, it was found that the co-

occurrence of the three genes in the *hbl* and *nhe* operon (*nhe*, in particular) are not conserved, indicating possible variation in nucleotide sequence within this operon which require further study. These findings had formed a basis for development of a multiplex PCR for detection of *B. cereus*-enterotoxin genes, which all 8 genes, including each individual gene in the operons, should be taken into account when developing the method. Therefore, a set of 8 pairs of primers for 8 enterotoxin genes were selected to be used in the multiplex PCR and other factors involved in the PCR were optimized. Finally a multiplex PCR was successfully developed. The multiplex PCR was tested with 60 test isolates of *Bacillus* species and the results were found to be well correlated with those of single PCRs, giving identical results in 86.66 % of the isolates tested. For those that were not identical, the multiplex PCR was more sensitive than single PCRs in 6.67 %, and less sensitive in 6.67 % of the isolates tested. False negative result (no single enterotoxin gene observed from a multiplex PCR while at least one enterotoxin gene observed from single PCR) occurred with a frequency of only 1.67 % of the test isolates. Considering the fact that there are only 2.72 % of the *Bacillus* test isolates harbor none of these enterotoxin genes, the chance of occurring of a false negative result is very low. Currently, this method is available as an in-house rapid-and-efficient method for food laboratories to screen or enumerate potentially toxigenic *Bacillus* species. Further development of this multiplex PCR in terms of increasing convenience for operation and transport should be carried out for wider application in food laboratories.

5. Conclusions

From the study, it can be concluded that:

(1) As many as 97.28 % of *Bacillus* isolates tested harbor at least one of the enterotoxin genes and the genes are found distributed among *B. cereus, B. thuriengiensis, B. mycoides,* and *B. licheniformis* at different percentages for different genes and in different species, with no exception for the genes in *hbl* and *nhe* operon in which non conservative occurrence of the three genes were observed with the method used.

(2) A multiplex PCR for detection of 8 *B. cereus*-enterotoxin genes has been successfully developed with satisfactory performance with test *Bacillus* isolates. The method is now available as a more advantageous alternative to the existing methods, allowing rapid and efficient detection of potentially toxigenic *Bacillus* species.

6. Outputs

6.1 Manuscript of research paper entitled: Occurrence Profiles and distribution of *Bacillus cereus*-enterotoxin genes among food isolates of *Bacillus cereus* and other *Bacillus* species (in final revision before submission - see appendix)

6.2 Manuscript of research paper entitled: Development of a multiplex PCR system for detection of potentially enterotoxigenic *Bacillus* species (in preparation)

6.3 Proposal for patenting: Multiplex PCR system for detection of potentially enterotoxigenic *Bacillus* species (in process)

Distribution of enterotoxin genes among food isolates of *Bacillus cereus* and other *Bacillus* species and development of a rapid method for detection of these genes

1. General Introduction and Literature Review

1.1 General characteristics and significance of *B. cereus* in foods

B. cereus is a bacterium that has been well recognized to be associated with food spoilage and food pathogen. The key characteristics of this organism can be observed easily microscopically as spore-forming, Gram-positive rods. Spores of *B. cereus* are ellipsoidal, formed after the exponential growth phase in a central position of the cells and the sporulating cells are not swollen (Claus and Berkely, 1986). The microorganism is a facultative anaerobe, however, sporulation occurred under aerated conditions (Bergere, 1992). *B. cereus* is generally mesophilic, having the temperature range for growth at 10-50^{O}C (Claus and Berkely, 1986). However, some strains are psychrotrophic and capable to grow at 7^{O}C (te Giffel *et al*., 1997). The pH range for growth of *B. cereus* varies according to laboratory or food media, but in general it can grow at pH range 4.9-9.3 (Kramer and Gilbert, 1989). It forms acid from D-glucose and fructose, but not from L-arabinose, D-xylose, and D-mannitol. However, the ability to utilize sucrose, salicin, maltose, mannose, glycerol, m-inositol, and lactose depends on strains. The majority of *B. cereus* strains hydrolyzes casein, gelatin, and starch, utilize citrate, and reduce nitrate to nitrite. It is positive in the catalase test, Voges-Proskauer test, egg-yolk lecithinase test, and a small percentage of strains are urease-positive (Cause and Berkeley, 1986). These characteristics are used as general criteria in isolation and identification of *B. cereus* through selective plating and biochemical methods (Gordon *et al.*, 1973; Rhodehamel and Harmon, 2001).

1.2 *B. cereus* and related species

Genus *Bacillus* is arranged in the group of the spore-forming Gram-positive rod-shaped bacteria in Bergey's Manual of Systematic Bacteriology, together with genera *Sporolactobacillus*, *Clostridium*, *Desulfotomaculum*, and *Oscillospira* (Claus and Berkeley, 1986). Gordon *et al.* (1973) suggested a primary classification of the genus *Bacillus* into three groups according to their cellular morphology and physiological properties. The species *B. subtilis*, *B. anthracis*, *B. cereus*, *B. firmus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, *B. pumilus*, and *B. thuringiensis* were arranged into "group 1" by their common characteristics to have unswollen sporangia and ellipsoidal spores. Within "group 1," there are some characteristics that *B. cereus, B. thuringiensis*, *B. mycoides*, *B. anthracis* share in common, especially the ability to produce egg-yolk lecithinase and ability to produce acid from D-mannitol (Stadhouders, 1992). These four species are designated as "*B. cereus* group" (as *B. cereus* is the best known species in this group). In fact, many selective/differentiative plating media for *B. cereus* used at the present are based on these properties, with addition of an antibiotic. The species of this group can be differentiated by some simple biochemical tests including motility, staining for parasporal crystal inclusion, susceptibility to penicillin, and hemolysis (Turnbull and Kramer, 1991).

1.3 Ecology of *B. cereus*

B. cereus can be found naturally in soil, groundwater, on plants and animals at field and in farm environment. The organism, therefore, can be easily introduced into foods as it was reported to be present in milk and milk products (Phillips and Griffiths, 1986; van Heddeghem and Vlaemynck, 1992), beans, spices, and cereal products (Fang *et al*., 1999; Rusul and Yaacob, 1995), cooked and processed foods, such as curry, salads, chilli sauce, etc. (Suksuwan, 1983), and in meat products and bakeries, as reviewed by Kramer & Gilbert (1989). In general, *B. cereus* is found in various kinds of foods, both raw and processed foods. The presence of this organism in moderately heated or pasteurized food is of concern as its ability to survive pasteurization and tolerate low storage temperature

would enable this organism to be predominant in foods, thus easily increase in numbers to the point that causes food spoilage or sets off food poisoning.

1.4 Pathogenicity of *B. cereus*

B. cereus can cause two distinct forms of gastrointestinal illnesses which are the "diarrheal" and "vomiting" (or "emetic") syndromes, as being classified by the main symptoms. The main symptoms of the diarrheal type of illness are diarrhea and acute abdominal pain, usually without fever. The symptoms usually occur between 8-16 hours after ingestion of food contaminated with *B. cereus* and the duration of illness is usually 12-24 hours. The main symptoms of the emetic type of illness are mainly nausea and vomiting. Diarrhea may occur but it is not the major symptom. The incubation time is usually less than 4 hours, and duration of illness is between 12-48 hours (Christiansson, 1992).

1.5 *B. cereus* food poisoning toxins and the encoding genes

The different types of illness occurred through different mechanism and associated with different classes of toxins. The emetic type of gastrointestinal illness is caused by the "emetic toxin," a cyclic peptide called Cereulide (Agata *et al*., 1995b), through food intoxication mechanism (Garbutt, 1997). The diarrheal type of illness is believed to occur through food toxicoinfection mechanism (Garbutt, 1997) and is associated with many molecular entities. To date, at least 4 entities have been identified with sufficient evidences to have enterotxic activity. These include Hemolysin BL (HBL) (Beecher & Macmillan, 1990, Beecher & Macmillan, 1991), Non Hemolytic Enterotoxin (NHE) (Granum *et al*., 1996; Granum *et al*., 1999), Enterotoxin FM (Asano *et al*., 1997), and Cytotoxin K (Lund *et al.*, 2000). A protein called Enterotoxin T was also identified (Agata *et al*., 1995a), however, the enterotoxic and molecular properties of this protein are proposed to be reevaluated (Hansen *et al*., 2003). Hemolysin BL comprises of three components, one

binding component (designated “B”) and two lytic components (designated L_1 and L_2), according to their cooperating mechanism for hemolysis. The B, L_1 and L_2 component are encoded by the genes *hblA*, *hblD*, and *hblC*, respectively, which are adjacent to each other (Heinrichs *et al*, 1993; Ryan, *et al*, 1997). Part of a structural gene following the *hblA* gene, named *hblB*, was also found but the function of this gene has not yet been clarified. Non Hemolytic Enterotoxin is another enterotoxin composed of 3 components designated “NheA”, “NheB”, and “NheC”, which are encoded by the genes *nheA*, *nheB*, and *nheC*, respectively. Part of the NHE complex is cytotoxic to CaCo-2 cells (Lund and Granum, 1996). Enterotoxin FM was a protein previously reported to show cytotoxic activity (Shinagawa *et al*., 1991) and eventually was found to be encoded by the gene *entFM* (Asano *et al.*, 1997). Cytotoxin K, a protein isolated from a strain that caused a serious gastrointestinal illness and was later reported to be cytotoxic to CaCo-2 cells, is encoded by the gene *cytK* (Hardy *et al.,* 2001). These enterotoxin encoding genes are summarized in Table 1.1

Table 1.1 *B. cereus* enterotoxins and their encoding genes (Source: Granum *et al*., 1996; Granum *et al*., 1999; Asano *et al*., 1997; Lund *et al.*, 2000; Heinrichs *et al.*, 1993; Ryan *et al.*, 1997)

toxin	**gene**	**Database accession no. / Reference**
Enterotoxin FM	*entFM*	Asano *et al*., 1997
Hemolysin BL (HBL)		
- B component	*hblA*	Genbank/L20441
- L_1 component	*hblD*	Genbank/U63928
- L_2 component	*hblC*	Genbank/U63928
Non Hemolytic Enterotoxin (NHE)		Genbank/Y19005
- A component	*nheA*	
- B component	*nheB*	
- C component	*nheC*	
Cytotoxin K	*cytK*	Genbank/AJ277962

1.6 Methods for detection of *B. cereus* and/or its enteropathogenic factors

The methods for detection of *B. cereus* and/or its pathogenic factors are currently based on cultural and immunological method. The cultural method includes an isolation step, usually through antibiotic selection and enzymatic differentiation (Rusul and Yaacob, 1995; Reissbrodt *et al*., 2004). Confirmation of *B. cereus* is done through a series of biochemical tests (Rhodehamel and Harmon, 2001). Another approach is immunological method detecting *B. cereus* enterotoxins. Currently there are two methods that are available commercially. One is a test kit based on an enzyme-linked immunosorbent assay called "BDE-VIA," manufactured by Bioenterprises Pty (Australia) and supplied by TECRA diagnosis (UK), which was later proved to detect the NheA component of the NHE complex (Granum *et al*., 1996). The other method, "BCET-RPLA," is a test kit based on a reversed passive latex agglutination assay manufactured by Denka Seiken (Japan) and supplied by Oxoid (UK). The BCET-RPLA test kit was reported to be specific to the L_2 component of HBL (Beecher and Wong, 1994).

1.7 Problems underlying this research

B. cereus is required to be monitored and detected in many food criteria and standards, such as milk formulae, spices, cereal products. Therefore, there is an increasing need for a rapid and efficient method to detect this organism and/or its enteropathogenic factors. Reasons for this are concern for the health and economy. A rapid and efficient microbiological technique can, for examples, support food quality assurance systems and reduce holding time for food products ready for distribution, which I turn benefits consumers and food suppliers in both direct and indirect ways. The currently available detection methods mentioned above (1.6) have some disadvantages. The traditional procedure involving plate isolation and biochemical confirmation takes a long time (4-5 days) to complete and labor intensive. The method is also not informative in terms of potential pathogenicity of the isolates. Moreover, it is reported that some enterotoxin-encoding genes have been found in other species beside *B. cereus*. In this traditional procedure, such species are disregarded their significance. The immunological method

also has some disadvantages. Since *B. cereus* are known to be capable to produce many enterotoxins and different strains of *B. cereus* have been reported to produce or harbor different enterotoxin-related factors (Ombui *et al*., 1997; Rowan *et al*., 2001), the currently available immunological methods (1.6) can therefore give insufficient and misleading information. In reponse to the multi-toxins involved in *B. cereus* enteropathogenicity, the genetic method seems to be a good approach. This study, therefore, aimed to develop a rapid and efficient genetic-based method to resolve the disadvantages of the currently available methods. Through this, we hope to provide a better alternative for determination of food safety relating to *Bacillus*, which detects not only *B. cereus* or some particular enterotoxic molecules but all “potentially toxigenic *Bacillus* species.”

1.8 Research objectives

As mentioned above, the genetic method would allow efficient detection of enterotoxigenic factors of *B. cereus*. This study, therefore, was proposed with two main objectives:

(1) to survey distribution of enterotoxin genes among *B. cereus* and other *Bacillus* species isolated from food, in order to provide a basis for developing a rapid and efficient genetic-based method for detection of potentially toxigenic *B. cereus* and other *Bacillus* species, and to provide beneficial data for epidemiology and disease control, and

(2) to develop a rapid and efficient genetic-based method that will enable detection of *B. cereus*-enterotoxin genes or potentially toxigenic *Bacillus* species, which in turn will increase the efficiency of food quality assurance and food safety systems of the food industry.

(a) Polymyxin Pyruvate Egg Yolk Mannitol Bromothymol blue Agar (PEMBA)

Composition per liter: 0.1 g Magnesium sulphate, 2.5 g Di-sodium hydrogen phosphate, 0.25 g Potasium dihydrogen phosphate, 10.0 g Sodium pyruvate, 0.12 g Bromothymol blue, 15.0 g agar, egg-yolk from 2.5 eggs, 100,000 U polymyxin (egg yolk and polymyxin B were sterilized separartely and added into medium at 50 $^{\circ}$C just before pouring into plates), 1000 ml distilled water. In this study PEMBA was prepared from a commercially prepared medium base (*Bacillus cereus* selective agar CM617, Oxoid), into which polymyxin and egg-yolk were separately added.

(b) Buffered peptone water (pH 7.2)

Composition per liter: 10.0 g Peptone, 5.0 g Sodium chloride, 3.5 g Disodium hydrogen phosphate, 1.5 g Potassium dihydrogen phosphate, 1000 ml distilled water.

(c) Nutrient broth (pH 7.0)

Composition per liter: 10.0 g Peptone, 5.0 g Sodium chloride, 10.0 g Meat extract, 1000 ml distilled water.

(d) Lysozyme broth

Composition per liter: 1000 ml Nutrient broth, 10.0 ml Lysozyme solution (0.1 g lysozyme in 100 ml distilled water). Lysozyme solution was sterilized separately by filtration through 0.2 μm membrane and added to nutrient broth at temperature less than 50 $^{\circ}$C just before use.

(e) Motility medium for *B. cereus* (pH 7.4)

Composition per liter: 10.0 g Trypticase, 2.5 g Yeast extract, 5.0 g glucose, 2.5 g Na_2HPO_4, 3.0 g Agar, 1000 ml distilled water.

(f) Nitrate broth (pH 7.0)

Composition per liter: 3.0 g Beef extract, 10.0 g Peptone, 1.0 g KNO_3, 1000 ml distilled water.

(g) Phenol red glucose broth (pH 7.4)

Composition per liter: 10.0 g Proteose peptone, 5.0 g NaCl, 1.0 g Beef extract, 5.0 g Glucose, 0.018 g Phenol red, 1000 ml distilled water.

(h) Tyrosine agar

Composition per liter: 1000 ml Nutrient broth, 100 ml Tyrosine suspension (5 g L-tyrosine, 100 ml distilled water)

(i) Tryptone soy yeast extract agar (pH 7.2)

Composition per liter: 30.0 g Tryptic soy broth, 6.0 g Yeast extract, 15.0 g agar, 1000 ml distilled water.

(j) Voges-Proskauer (VP) medium (pH 6.5)

Composition per liter: 7.0 g Proteose peptone, 5.0 g NaCl, 5.0 g glucose, 1000 ml distilled water.

(k) Tryptone soy-sheep blood agar (pH 7.2)

Composition per liter: 400 ml Tryptone soy yeast extract agar, 20 ml sheep blood. Sheep blood was added to Tryptone soy yeast extract agar at temperature between 50-60^{O}C just before pouring into plates.

2.3.2 Reagents used in biochemical tests

(a) Alpha-naphthol solution

Composition: 5.0 g α-Naphthol, 100 ml Ethanol. The solution was stored at 4 °C.

(b) Sulfanilic acid reagent

Composition: 1.0 g Sulfanilic acid, 125 ml 5 N Acetic acid (5 N acetic acid was prepared by mixing 28.75 ml glacial acetic acid in 71.25 ml distilled water). The solution was stored at 4 °C.

(c) α-Naphthol reagent C

Composition: 1.0 g α-Naphthol, 200 ml 5 N Acetic acid. The solution was stored at 4 °C.

(d) 40 % potassium hydroxide

Composition: 40.0 g Potassium hydroxide, 100 ml distilled water.

(e) Crystal violet solution (for Gram staining)

Composition: 2.0 g Crystal violet, 20.0 ml 95 % Ethyl alcohol, 0.8 g Ammonium oxalate, 80.0 ml distilled water. The reagent was filtered through a filter paper and stored in a light-protected bottle at room temperature.

(f) Safranin O (10×stock solution) (for Gram and spore staining)

Composition: 2.5 g Safranin O, 100 ml ethyl alcohol. The reagent was filtered through a filter paper and stored in a light-protected bottle at room temperature. The reagent was diluted to 1× for use as working solution.

(g) Iodine solution

Composition: 1.0 g Iodine (crystal), 2.0 g Potassium iodide, 300 ml distilled water. The reagent was stored in a light-protected bottle at room temperature.

(h) Alcohol-acetone

Composition: 250 ml 95 % (v/v) Ethyl alcohol, 250 ml Acetone.

(i) Malachite green

Composition: 5.0 g Malachite green, 100 ml Distilled water. The reagent was filtered through a filter paper and stored in a light-protected bottle at room temperature.

(j) Basic fuchsin staining solution

Composition: 0.5 g Basic fuchsin dye, 100 ml 95 % (v/v) ethanol. The reagent was filtered through a filter paper and stored in a light-protected bottle at room temperature.

2.3.3 reagents for DNA extraction

The reagents used in this section were supplied by Research Organics, Fisher Scientific, or Fisher Chemicals. Solutions were prepared according to the compositions given below, and sterilized by autoclaving at 121 $^{\circ}$C for 15 min, unless stated otherwise.

(a) TE buffer (pH 8.0)

Composition: 1 ml 1 M TrisCl (pH 8.0), 0.2 ml 0.5 M EDTA (pH 8.0), adjusted to 100 ml with distilled water.

(b) 10 % (w/v) SDS

Composition: 10 g SDS, adjusted to 100 ml with Distilled water. SDS was dissolved using low heat (no autoclaving).

(c) Phosphate buffer solution (PBS)

Composition: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1000 ml distilled water.

(d) Phenol:Chloroform:isoamylalcohol (24:24:1)

Composition: TE saturated Phenol (pH 8.0), mixed with chloroform and isoamylalcohol at ratio 25:24:1 (v/v/v).

(e) 2 M Sodium acetate (pH 5.2)

Composition: 13.608 g Sodium acetate, 50 ml distilled water.

(f) 3 M Sodium acetate (pH 5.2)

Composition: 20.412 g Sodium acetate, 50 ml distilled water.

(g) 1 M Tris-Cl (pH 8.0)

Composition: 121 g Tris base, 1000 ml distilled water. Approximately 800 ml of water was initially added into Tris base and the pH was adjusted to 8.0 with conc HCl. After obtaining the required pH, the solution was adjusted to 1000 ml with distilled water.

(h) 0.5 M EDTA (pH 8.0)

Composition: 18.6 g EDTA, 100 ml distilled water. Approximately 80 ml of water was initially added into EDTA and the pH was adjusted to 8.0 with NaOH. After obtaining the required pH, the solution was adjusted to 100 ml with distilled water.

2.3.4 PCR reagents

(a) 10×Mg-free buffer (DynaZyme)

Preparation and use: ready-to-use form, no preparation required. Used at 1× in PCR.

(b) 50 mM $MgCl_2$ (DynaZyme)

Preparation and use: ready-to-use form, no preparation required. Used at concentration 1.0-3.0 mM in PCR.

(c) 1.25 mM dNTPs (Bio Basic)

Preparation and use: ready-to-use form, no preparation required. Used at concentration 0.2-0.4 mM in PCR.

(d) DNA polymerase (DynaZyme)

Preparation and use: ready-to-use form, no preparation required. Used at 0.5 unit/gene/ PCR

(e) Oligonucleotide primer (Bio Basic)

Preparation and use: Supplied as lyophilized form. Prepared to 100 μM (as main stock solution) in TE buffer (pH 8.0), and 10 μM (as working stock solution) in nuclease-free water. Used at final concentration of 1 pM in PCR.

2.3.5 Agarose gel eletrophoresis reagents

Molecular reagents in this section were supplied by Research Organics, unless stated otherwise.

(a) 50× TAE electrophoresis running buffer

Composition per liter: 242 g Tris base, 57.1 ml glacial acetic acid, and 100 ml 0.5 M EDTA. Final pH was adjusted to 8.0 with acetic acid and made up to 1 liter with water. The solution was sterilized by autoclaving at 121°C for 15 min.

(b) DNA loading buffer

DNA loading buffer type I: 0.25 % (w/v) bromophenol blue, 40 % (w/v) sucrose in water.

DNA loading buffer type II: 40 % (w/v) sucrose in water. (used in analysis of PCR product of size 400-600 bp).

DNA loading buffer was autoclaved at 121°C for 15 min and stored at 4°C.

(c) λ-*Hind*III marker (250 ng/10 μl)

Composition per millilitre: 25 μg λ-*hind*III fragments (Promega), 100 μl DNA loading buffer type I, 10 μl 5 M NaCl, made up to 1 ml with TE buffer. The solution was mixed thoroughly and made into aliquots of 250-500 μl. It was heated at 70 °C for 10 min and chilled immediately on ice afterward. The solution was stored at 4 °C.

(d) DNA marker (100 bp-1.5 kb) (0.2 mg/ml, supplied by SibEnzyme)
DNA marker (100 bp-1.5 kb) was supplied as ready-to-use form, including 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp DNA fragments.

(e) Agarose Gel
Agarose gel was prepared at desired percentages in TAE buffer.

2.4 Growth conditions and physiological/biochemical tests for *Bacillus* isolates

2.4.1 General growth condition

Bacillus isolates were cultured at 37 °C. A liquid culture subject to DNA extraction or maintenance in glycerol was grown in a shaking condition at 150 rpm for 18-20 h before the harvest. Agar plates and slant cultures were grown at 37 °C for 20-48 h as indicated in each protocol.

2.4.2 Physiological and biochemical tests (Rhodehamel and Harmon, 2001)

Bacillus cultures (20-24 h) from TSYE agar slant was subject to different physiological and biochemical tests as followed (positive and negative controls were included for each test).

(a) Anaerobic utilization of glucose

A loopful of culture was inoculated in 3 ml Phenol red glucose broth and incubated at 35° C for 24 h in Gaspack anerobic jar. Anaerobic utilization of glucose was observed by increased turbidity of the medium and color change from red to yellow, indicating growth and anaerobic acid production from glucose, respectively.

(b) Reduction of nitrate

A loopful of culture was inoculated in 5 ml of Nitrate broth and incubated at 35°C. After incubation for 24 h, 0.25 ml of Sulfanilic acid reagent and 0.25 ml of α-Naphthol reagent were added into the broth culture. An orange color, which develops within 10 min, indicating reduction of nitrate to nitrite was observed.

(c) Gram reaction

Gram staining was carried out on cultures aged between 18-24 h, according to the standard procedure described in the Bacteriological Analytical Manual, U.S. Food and Drug Administration. *Bacillus* spp. is rod-shaped, Gram-positive which is stained purple.

(d) Position of spore and shape of sporangia

The position of spore and shape of sporangia were observed through spore staining. A culture was smeared on a slide, air-dried, and heat–fixed on flame for 20 times. The slide-fixed culture was stained with malachite green for 15 min, and rinsed with running tap water afterward for 20 s. It was then counterstained with safranin O for 1 min and rinsed with running tap water. The slide was blot-dried and subject to microscopic examination using oil immersion objective (100×). The position of green-stained spores and the shape of sporangia (swollen/no swollen) were observed.

(e) acetylmethylcarbinol activity

A loopful of culture was inoculated in 5 ml VP medium and incubated at 35 °C for 48 ± 2 h. After incubation, 1 ml of culture was transferred to a tube, and was added with 0.6 ml α-Naphthol solution, 0.2 ml 40 % (v/v) Potassium hydroxide, followed by few crystals of Creatine. Pink or purple color was observed after 1 h incubation for the presence of acetylmethylcarbinol.

(f) Decomposition of tyrosine

A loopful of culture was streaked on the entire surface of Tyrosine agar slant and incubated at 35 °C for 48 h. Tyrosine decomposition was observed by a clear zone surrounding growth of the culture.

(g) Resistance to lysozyme

A loopful of culture was inoculated in 2.5 ml Lysozyme broth and incubated at 35 °C for 24-48 h. Resistance to lysozyme was observed by increased turbidity indicating growth of the culture.

(h) Growth at 50 ºC และ 60 ºC

A loopful of culture was streaked on the surface of Tryptone soy yeast extract agar and incubated at 50 and 60 °C for 24 h. Growth of the culture at each temperature was observed.

(i) Motility

A culture was inoculated into Motility medium (*B. cereus*) by stabbing down the center. After incubation at 30 °C for 18-24 h, the type of growth along the stab line was observed. Motility is noted when growth appeared out of and to the sides of the stab line.

(j) Rhizoid growth

A culture was spot-inoculated onto the center of nutrient agar plate and incubated at 30 °C for 48-72 h. Rhizoid growth was observed by production of colonies with long hair or root-like structure that extend from the site of inoculation.

(k) Hemolytic activity

A loopful of 24 h culture was inoculated on the surface of Trypticase soy-sheep blood agar and incubated at 35 °C for 24 h. Clear zone surrounding growth, indicating the hemolytic activity was observed. Complete (β) hemolysis from *B. cereus* is usually noticed by 2-4 mm clear zone. Most *B. thuringiensis* and *B. mycoides* are also β-hemolytic, while *B. anthracis* strains are usually nonhemolytic after 24 h incubation.

(l) Protein toxin crystals

A culture was inoculated on nutrient agar and incubated at 30 °C for 48-72 h. The culture was then smeared on a slide, air-dried, and heat-fixed. The slide was flooded with methanol, left to stand for 30 s, drained and allowed to air-dry. The slide was flooded with 0.5 % basic fuchsin and heated gently with a small flame until steam is noticed. It was left to stand for 2 min and the heating step was repeated. Finally, the slide was left to stand for 3 min and rinsed with running tap water. Protein toxin crystals which are produced by *B. thuringiensis* were observed microscopically as darkly-stained tetragonal (diamond-shaped) crystals.

(m) Anaerobic growth

A culture was inoculated on Nutrient agar and incubated at 35 °C in a GasPak anaerobic jar. Growth after 24 h was observed.

2.5 Maintenance of bacterial strains and isolates

Strains and isolates of *Bacillus* spp. were maintained on TSYE agar slant at 4°C and subcultured every 6 months or alternatively, in 70 % (v/v) glycerol at -70°C. To prepare a glycerol stock culture, 10 ml overnight culture grown in TSYE broth was collected by centrifugation (1 min at 10,000 g) in a microtube. A volume of fresh TSYE broth was added to the cell pellet so that the total volume of the cell suspension was made up to 300 μl, and mixed using pipette tips into a suspension. The cell suspension was then transferred into a cryovial containing 700 μl sterilized glycerol, mixed thoroughly and stored at -70°C.

2.6 Chromosomal DNA preparation

To isolate chromosomal DNA from a *Bacillus* culture, the isolate was grown in Tryptone soy yeast extract broth (TSYEB) at 37 °C for 24 h, and the cell pellet from a 3 ml (1.5 ml × 2 times) culture was collected in a microtube by centrifugation at 6000 g for 30 s. The cell pellet was resuspended in 500 μl Phosphate buffer solution (PBS, pH 7.2) by pipetting up and down, and centrifuged at 6000 g for 30 s. This step was repeated another 2 times. After last washing with PBS, 120 μl of 2 M sodium acetate (pH 5.2) and 160 μl of 10 % SDS was added to the cell pellet and mixed by converting the tube. After that, 700 μl of phenol:chloroform:isoamylalcohol (25:24:1) was added to the suspension and mixed by converting the tube for 5 min. The mixture was then subject to centrifugation (12,000 g, 5 min) and the aqueous phase was transferred to a fresh tube, leaving white interphase behind. To the aqueous, 1 ml of isopropanol and 120 μl of 3 M sodium acetate (pH 5.2) was added. The mixture was placed at -70 °C for 30 min before being centrifuged at 12,000 g for 20 min. After the supernatant was discarded, 500 μl of 70 % (v/v) ethanol was added to the DNA pellet, which was then left to stand for 1-2 min before centrifugation (12,000 g, 10 min). Ethanol was drained off completely and the DNA pellet was left to dry at room temperature for 30 min or until dried. The DNA was

resuspended in 50 µl of TE buffer and incubated at 37 °C until dissolved or overnight. The DNA was stored at 4°C. Throughout the preparation, filtered pipette tips were used.

2.7 Polymerase Chain Reaction (PCR) conditions

PCR was performed in a thermal cycler (Mastercycler, Eppendorf). Filtered pipette tips were used throughout the preparartion of PCR mixtures. The programs for PCR used in this study are listed below.

Program C (Ta Gradient)

Step 1) 94 °C, 5 min

Step 2) 94 °C, 30 s

Step 3) 50 °C, 30 s

G=10 °C

(giving temperature gradient from 40-60 °C across the rows of the thermal cycler)

Step 4) 72 °C, 1 min 30 s

Step 5) GOTO 2 REPEAT 34

Step 6) 72 °C, 10 min

Step 7) END

Program D (Ta 59)

Step 1) 94 °C, 5 min

Step 2) 94 °C, 30 s

Step 3) 59 °C, 30 s

Step 4) 72 °C, 1 min 30 s

Step 5) GOTO 2 REPEAT 34

Step 6) 72 °C, 10 min

Step 7) END

Program G (2-step-down)

Step 1) 94 °C, 5 min

Step 2) 94 °C, 30 s

Step 3) 60 °C, 30 s

Step 4) 72 °C, 1 min 30 s

Step 5) GOTO 2 REPEAT 20

Step 6) 94 °C, 30 s

Step 7) 45 °C, 30 s

Step 8) 72 °C, 1 min 30 s

Step 9) GOTO 6 REPEAT 20

Step 10) 72 °C, 10 min

Step 11) END

Program H (Ta 46.7)

Step 1) 94 °C, 5 min

Step 2) 94 °C, 30 s

Step 3) 46.7 °C, 30 s

Step 4) 72 °C, 1 min 30 s

Step 5) GOTO 2 REPEAT 34

Step 6) 72 °C, 10 min

Step 7) END

Program I (2-step-up)

Step 1) 94 °C, 5 min

Step 2) 94 °C, 30 s

Step 3) 45 °C, 30 s

Step 4) 72 °C, 1 min 30 s

Step 5) GOTO 2 REPEAT 20

Step 6) 94 °C, 30 s

Step 7) 60 °C, 30 s

Step 8) 72 °C, 1 min 30 s

Step 9) GOTO 6 REPEAT 20

Step 10) 72 ^{O}C, 10 min

Step 11) END

2.8 Agarose gel eletrophoresis and visualization of nucleic acid

DNA and PCR products were analyzed using agarose gel electrophoresis in 1 × Tris-Acetate-EDTA (TAE) buffer to a desired concentration. Throughout this study, 0.8-1.5 % (w/v) agarose was used when analyzing DNA. The agarose gel after electrophoresis was stained with ethidium bromide solution (500 μg/l in TAE buffer). Nucleic acid was visualized on a UV transilluminator (Ultra LUM) and photographed by a digital camera attached to the chamber of the UV transilluminator which links to a gel documentation system (DigiDoc, Biorad).

2.9 Experiments

Two main experiments were carried out in this study, a study on distribution of enterotoxin genes in food isolates of *B. cereus* and other *Bacillus* species, and development of a multiplex PCR. The orderly scheme of the main and side experiments is summarized in Fig. 2.1.

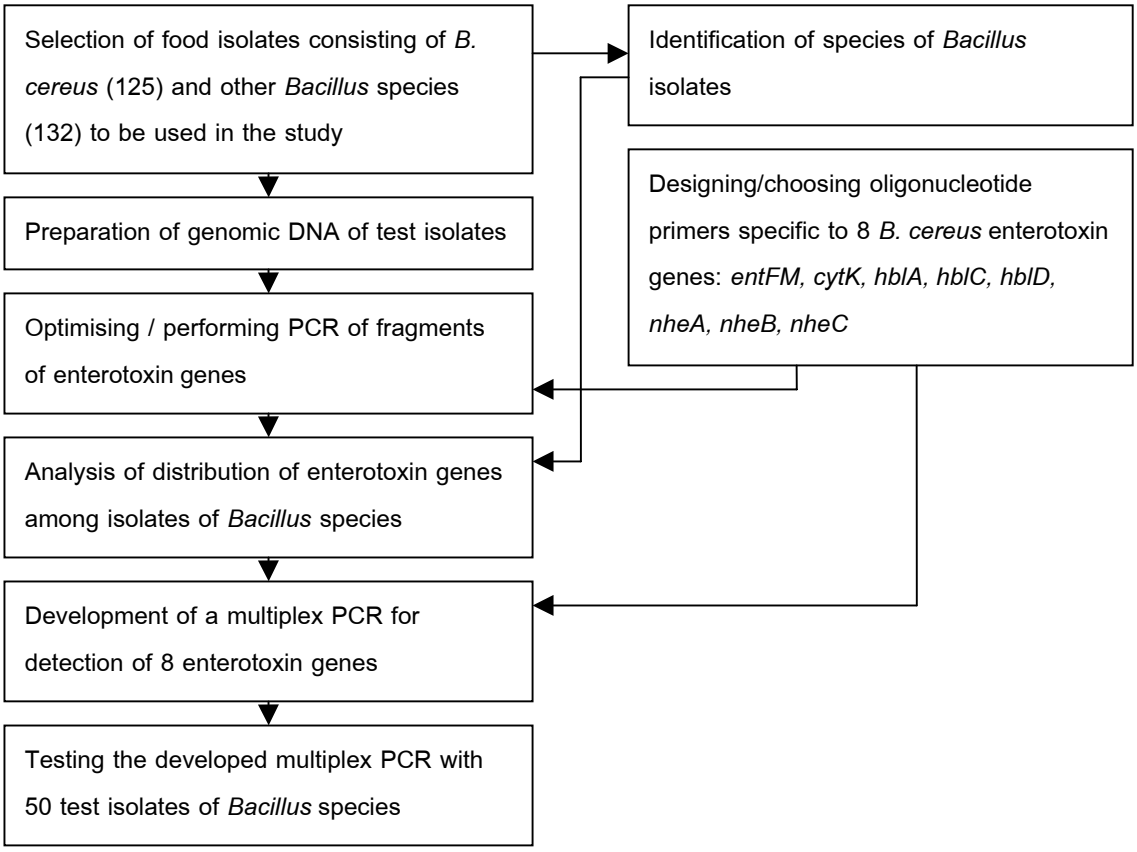


Fig. 2.1 Diagram showing experimental procedures

3. Results and Discussions

3.1 Identification of *Bacillus* species

Bacillus species obtained from various foods through polymyxin Egg-yolk Mannitol Bromothymol blue (PEMB) selective agar (Dispan, 2003) were identified at species level using physiological and biochemical tests as listed in 2.4.2. Typical results for each species are given in Table 3.1. The identification of each isolate can be found in section 2.1. In total, 257 isolates were used in this study, consisting of 125, 15, 28, and 89 isolates of *B. cereus*, *B. thuringiensis*, *B. mycoides,* and *B. licheniformis,* respectively.

Table 3.1 Typical physiological characteristics and biochemical test results of *Bacillus* isolates

Gram reaction	Motility	Reduction of nitrate	Tyrosine decomposition	Lysozyme resistance	Egg-yolk reaction	Anaerobic utilization of glucose	VP reaction	Spore	swollen sporangium	Rhizoid growth	Hemolysis	Toxin crystals	Identification
+	+/-	+	+	+	+	+	+	+	-	-	+	-	*B. cereus*
+	+	-	+	+	+	+	+	+	-	-	+	-	*B. licheniformis*
+	+	+	+	+	+	+	+	+	-	+	+	-	*B. mycoides*
+	+	+	-	+	+	+	+	+	-	+	+	+	*B. .thuringiensis*

3.2 Occurrence and distribution of enterotoxin genes in food isolates of *B. cereus* and other *Bacillus* species

3.2.1 Occurrence and distribution of enterotoxin genes in *B. cereus* and other *Bacillus* species

PCR was carried out using primers listed in Table 2.2 (for single PCRs), in order to examine the occurrence of the enterotoxin genes in *Bacillus* isolates. An optimized PCR mixture contained 1 × reaction buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 1 pM forward and reverse primer, approx. 5-15 ng chromosomal DNA template, and 0.5 U DNA polymerase in 10 µl total reaction volume. The amplification was carried out using program D (2.7) at annealing temperature of 59 $^{\circ}$C, as found to yield satisfactory amplified results for all genes (Fig. 3.1). Occurrence of *B. cereus*-enterotoxin genes in isolates of *B. cereus, B. mycoides, B. thuringiensis,* and *B. licheniformis* are shown in Table 3.2 – 3.5.

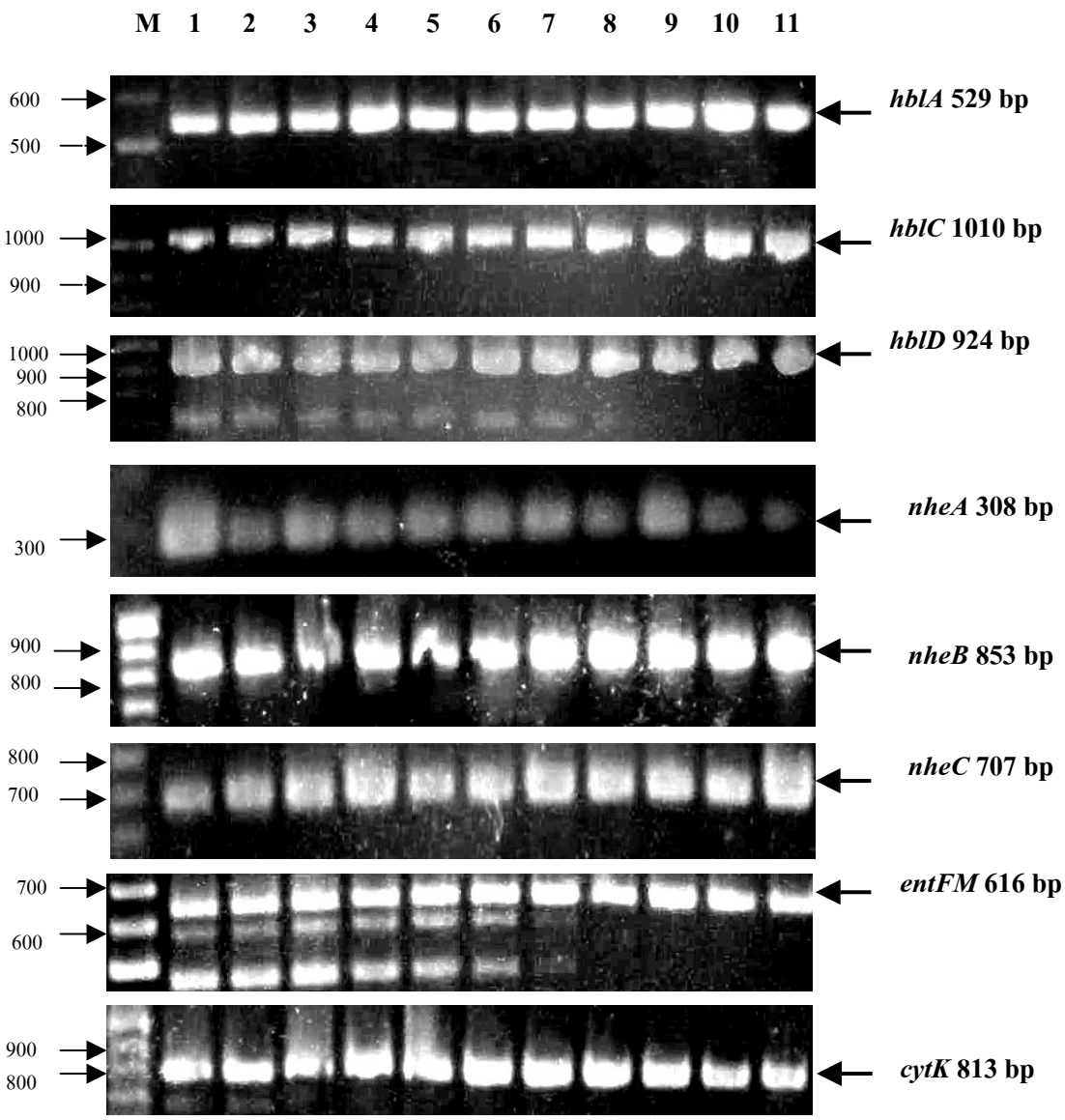


Fig. 3.1 Results from 8 pairs of primers used in single PCRs, amplifying individual enterotoxin genes, performed at different gradient temperatures: **lane (1)** 40.1 °C, **lane (2)** 40.8 °C, **lane (3)** 42.2 °C, **lane (4)** 44.2 °C, **lane (5)** 46.7 °C, **lane (6)** 49.4 °C, **lane (7)** 52.1 °C, **lane (8)** 54.7 °C, **lane (9)** 57.1 °C, **lane (10)** 59.0 °C, **lane (11)** 60.2 °C; **(M)** DNA marker

Table 3.2 PCR amplification of fragments of enterotoxin genes from *B. cereus* isolates

		gene							
No.	**isolate**	***entFM***	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
1	M4	+	+	-	-	-	-	-	-
2	M6	-	-	+	+	+	+	+	+
3	M7	+	+	+	+	+	+	+ (f)	+
4	M8	-	-	+	+	+	+	+	+
5	M10	-	+	+	+	+	+	+	+
6	M11	+	+	+	-	+	+	+ (f)	+
7	M12	+	+	-	-	-	-	-	-
8	M13	+	+	+	-	+	+	+ (f)	+
9	M21	-	+	+	+	+	+	+	-
10	M22	-	+	+	+	+	+	+	-
11	M18	-	+	+	+	+	+	+ (f)	+
12	M19	-	+	+	+	+	+	-	+
13	M33	-	+	-	-	-	-	+ (f)	+
14	M26	+	+	+	+	+	+	+	+
15	M27	-	+	+	+	+	+	-	+
16	M28	+	+	+	+	+	+	+ (f)	+
17	M31	-	+	+	+	+	+	+ (f)	+
18	AB5	-	+	+	+	+	+	+ (f)	+
19	AB8	-	+	+	-	+	+	+ (f)	+
20	AB9	+	+	-	-	-	-	+ (f)	+
21	AB15	-	+	+	+	+	+	+	+
22	AB16	-	-	-	-	-	-	-	-
23	AB34	+	+	-	-	-	-	+ (f)	+
24	AB36	-	+	-	-	-	-	+ (f)	+
25	AB38	-	+	-	-	-	-	-	+

Table 3.2 PCR amplification of fragments of enterotoxin genes from *B. cereus* isolates (cond.)

No.	isolate	gene							
		entFM	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
26	AB40	-	+	+	+	+	+	+	+
27	AB42	-	-	-	-	-	+	+	-
28	AB43	+	+	+	+	+	+	+	+
29	AB46	+	+	+	+	+	+	+	+
30	AB48	+	+ (f)	+	+	+	+	+ (f)	+
31	BC5	-	+	+	+	+	+	+	+
32	BC7	+	+	-	-	-	-	+ (f)	+
33	BC8	-	+	-	-	-	-	+ (f)	+ (f)
34	BC9	-	+	-	-	-	-	+ (f)	+ (f)
35	BC10	+	+	-	-	-	-	+ (f)	+ (f)
36	BC11	-	+	-	-	-	-	+ (f)	+ (f)
37	BC12	+	-	+	+	+	-	+ (f)	+ (f)
38	BC14	-	-	+	+	+	-	-	-
39	BC15	-	+	+	+	+	+	+	+
40	BC16	+	+	+	+	+	+	+	+
41	BC17	-	+	+	+	+	+	+	+
42	BC19	+	+	-	-	-	-	+	+
43	BC26	-	-	-	-	-	+	+	+
44	BC30	+	+	+	+	+	+	+	+
45	BC40	+	+	+	+	+	+	+	+
46	BC41	-	+	+	+	+	+	+	+
47	BC42	+	+	+	+	+	+	-	+
48	BC44	-	+	+	+	+	+ (f)	+	-
49	BC46	+	+	+	+	+	+	+	+
50	BC47	+	+	+	+	+	+	-	+

Table 3.2 PCR amplification of fragments of enterotoxin genes from *B. cereus* isolates (cond.)

		gene							
No.	**isolate**	***entFM***	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
51	BC50	-	+	+	+	+	+	+	+
52	BC51	+	+	+	+	+	-	-	-
53	BC52	+	+	+	+	+	-	+	+
54	BC53	+	-	-	-	-	+	-	+
55	BC54	-	+	+	+	+	-	-	-
56	BC55	+	-	+	+	+	+	+	-
57	BC56	+	+	+	+	+	-	+ (f)	+ (f)
58	BC57	+	+	+	+	+	-	+ (f)	+ (f)
59	BC58	+	+	+	+	+	+	+ (f)	+
60	BC60	+	+	+	+	+	+	+ (f)	+
61	BC64	+	-	-	-	-	-	-	+
62	BC65	-	-	-	-	-	-	+	+
63	BC66	-	+	+	+	+	+	+	+
64	BC67	+	+	+	+	+	+	+	+
65	BC68	-	-	+	+	+	+	+	+
66	BC69	-	+	+	+	+	+	-	+
67	BC70	-	+	+	+	+	+	-	+
68	BC71	-	+	-	-	-	-	+	+
69	BC73	-	+	+	+	+	-	-	-
70	BC75	-	+	+	+	+	+	+	+
71	BC77	-	+	+	+	+	+	+	+
72	BC80	-	-	+	+	+	+	-	+
73	BC81	-	+	+	+	+	+	+	+
74	BC84	+	+	+	+	+	+	+	+
75	BC88	+	+	-	-	-	-	-	-
76	BC89	+	+	+	+	+	+	+	+

Table 3.2 PCR amplification of fragments of enterotoxin genes from *B. cereus* isolates (cond.)

		gene							
No.	**isolate**	***entFM***	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
77	BC90	+	-	+	+	+	+	+	+
78	BC91	+	+	+	+ (f)	+	+	+	+
79	BC94	+	+	+	+	+	+	+	+
80	BC95	+	+	-	-	-	-	+	-
81	BC100	+	+	+	+	+	+	+	+
82	BC102	+	+	+	+ (f)	+	+	+	+
83	BC103	+	+	+	+ (f)	+	+	+	+
84	BC106	-	+	+	+	+	+	+	+
85	BC107	+	+	+	+	+	+	+	-
86	BC108	+	+	+	+	+	+	+	-
87	BC110	+	-	+	+	+	+	+	+
88	BC111	-	-	-	-	-	-	-	-
89	BC112	-	-	+	-	+	-	+ (f)	-
90	BC113	-	-	-	-	-	-	-	-
91	BC114	+	-	+	+	+	-	+ (f)	+ (f)
92	BC115	+	+	+	+	+	+ (f)	+	+
93	BC118	+	+	-	-	-	-	+	+
94	BC119	+	+	+	+	+	+	+	+
95	BC122	+	+	-	-	-	-	-	-
96	BC123	+	-	-	-	-	+	+	+
97	BC124	-	-	+	+	+	-	-	-
98	BC125	+	+	+	+	+	+	+	+
99	BC126	+	+	+	+	+	+	+	+
100	CD2	-	-	-	-	-	-	-	-
101	CD5	+	+	+	+	+	+	+	+

Table 3.2 PCR amplification of fragments of enterotoxin genes from *B. cereus* isolates (cond.)

		gene							
No.	**isolate**	***entFM***	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
102	CD6	+	+	+	+	+	+	+	+
103	CD12	+	-	-	-	-	-	-	-
104	CD13	+	-	-	-	-	-	+	+
105	CD18	+	-	-	-	-	-	-	-
106	CD23	+	-	-	-	-	-	+ (f)	-
107	CD24	+	-	-	-	-	-	-	-
108	CD27	+	-	-	-	-	-	+ (f)	+ (f)
109	CD36	+	-	-	-	-	-	-	-
110	CD39	+	-	-	-	-	-	-	-
111	CD43	+	-	-	-	-	-	-	-
112	CD44	+	-	-	-	-	-	-	-
113	CD48	+	-	-	-	-	-	-	-
114	CD49	+	-	-	-	-	-	-	-
115	CD50	+	-	-	-	-	-	-	-
116	CD52	+	+	+	+	+	+	+	+
117	CD57	+	-	+	+	+	-	-	-
118	CD62	+	+	-	-	-	-	-	+
119	DE18	-	+	-	-	-	+	-	-
120	DE19	-	+	+	+	+	+	+	+
121	DE22	+	-	+	+	+	+	+	+
122	DE23	+	+	+	+	+	+	+	+
123	M20	-	+	+	+	+	+	+	+
124	M32	+	+	-	-	-	-	-	-
125	AB11	+	+	+	+	+	+	+	+

note: +: PCR product observed, +(f): faint signal, indicating low yield of PCR product observed, -: no PCR product observed.

Table 3.3 PCR amplification of enterotoxin gene fragment from *B. mycoides* isolates

		gene							
No.	**isolate**	***entFM***	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
1	M3	+	+	+	+	+	+	+	+
2	M25	+	-	+	+	+	+	+	+
3	AB35	-	+	-	-	-	-	-	+
4	AB41	-	+	-	-	-	-	-	+
5	AB45	+	+	+	+	+	+	+	+
6	BC20	+	+	-	-	-	-	-	+
7	BC32	+	+	+	+	+	+	+	+
8	BC36	+	+	+	+	+	+	-	+
9	BC39	+	+	+	+	+	+	+	+
10	BC43	+	+	+	+	+	+	+	+
11	BC49	+	+	+	+	+	+	+	-
12	BC79	-	+	+	+	+	+	+	+
13	BC86	+	-	-	-	-	-	-	-
14	BC97	-	-	+	+	+	-	-	-
15	BC101	-	+	+	+	+	+	+	+
16	BC104	+	-	+	+ (f)	+	-	-	-
17	BC105	+	-	+	+ (f)	+	-	-	-
18	BC127	-	+	+	+	+	+	+	+
19	CD1	+	-	+	+	+	+	-	+
20	CD8	+	-	+	+	+	+	+	+
21	CD9	+	-	+	+	+	+	-	+
22	CD15	+	-	-	-	-	+	+	+
23	CD17	+	-	-	-	-	-	-	-
24	CD28	-	-	-	-	-	-	-	-
25	CD29	+	-	-	-	-	-	-	-
26	CD31	+	-	-	-	-	-	+ (f)	+ (f)
27	CD42	+	-	-	-	-	-	-	-
28	CD47	+	-	-	-	-	-	-	-

note: +: PCR product observed, +(f): faint signal, indicating low yield of PCR product observed, -: no PCR product observed.

Table 3.4 PCR amplification of enterotoxin gene fragment from *B. thuringiensis* isolates

No.	isolate	gene							
		entFM	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
1	CD3	+	+	+	+	+	+	+	+
2	CD64	+	+	+	+	+	+	+	+
3	CD68	+	+	+	+	+	+	+	+
4	CD71	+	+	+	+	+	+	+	+
5	M24	+	+	+	+	+	+	+	+
6	AB1	+	+	+	+	+	+	+	+
7	AB2	+	+	+	+	+	+	+	+
8	AB3	+	+	+	+	+	+	+	+
9	AB4	+	+	+	+	+	+	+	+
10	AB10	+	+	+	+	+	+	+	+
11	AB13	+	+	+	+	+	+	+	+
12	AB14	+	+	+	+	+	+	+	+
13	AB49	+	+	+	+	+	+	+	+
14	CD69	+	+	+	+	+	+	+	+
15	CD70	+	+	+	+	+	+	+	+

note: +: PCR product observed, +(f): faint signal, indicating low yield of PCR product observed, -: no PCR product observed.

Table 3.5 PCR amplification of enterotoxin gene fragment from *B. licheniformis* isolates

No.	isolate	gene							
		entFM	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
1	M2	-	-	-	-	-	-	-	-
2	M14	+	-	-	-	-	+	+	+ (f)
3	M15	-	-	-	-	-	+	+	+
4	M16	+	-	-	-	-	+	+	+ (f)
5	M17	+	-	-	-	-	+	+	+ (f)
6	M29	+	+	+	+	+	+	+	+
7	M30	+	+	+	+	+	+	+	+
8	AB7	+	+	-	-	-	-	-	-
9	AB19	+	+	-	-	-	-	-	-
10	AB21	+	-	-	-	-	+	+	+
11	AB22	+	+	+	+	+	+	+	+
12	AB25	+	-	-	-	-	+	+	+
13	AB47	+	+	-	-	-	-	+	-
14	BC1	+	-	-	-	-	+	+	+
15	BC2	+	-	-	-	-	+	+	+
16	BC3	+	-	-	-	-	+	+	+
17	BC4	+	+	-	-	-	-	+	-
18	BC6	+	+	-	-	-	-	-	-
19	BC13	+	-	+	+	+	-	-	-
20	BC18	+	-	-	-	-	+	+	+
21	BC21	+	-	-	-	-	-	-	+
22	BC22	+	-	-	-	-	-	+	-
23	BC23	+	-	-	-	-	-	+	-
24	BC24	+	-	-	-	-	-	-	+
25	BC27	+	-	-	-	-	-	-	-
26	BC28	+	-	-	-	-	-	-	-
27	BC29	+	-	-	-	-	+	+	+

Table 3.5 PCR amplification of enterotoxin gene fragment from *B. licheniformis* isolates (cond.)

No.	isolate	gene							
		entFM	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
28	BC31	+	-	-	-	-	+	+	+
29	BC33	+	-	-	-	-	+ (f)	+	+
30	BC34	+	-	-	-	-	+	+	+
31	BC35	+	-	-	-	-	+	+	+
32	BC37	+	-	-	-	-	-	-	-
33	BC38	-	-	+	+	+	+	+	+
34	BC48	+	+	+	+	+	+	+	+
35	BC59	+ (f)	+	+	+	+	+	+	+
36	BC61	-	+	+	+	+	+	+	+
37	BC63	+	+	+	+	+	+ (f)	+ (f)	+
38	BC72	+	+	+ (f)	+	+	+	+	+
39	BC76	+	+	+	+	+	+	+	+
40	BC78	+	+	+	+	+	+	+	+
41	BC82	+	+	+	+	+	+	+	+
42	BC85	+	+	+	+	+	+	+	+
43	BC92	-	-	+	+ (f)	+ (f)	-	-	-
44	BC93	+	-	+	+	+	+	+	+
45	BC99	-	+	+	+	+	-	-	-
46	BC116	+	+	+	+	+	+	+	+
47	CD4	-	-	+	-	+	+	+ (f)	+
48	CD10	+	+	+	+	+	+	+ (f)	+ (f)
49	CD11	+	-	+	+	+	+	+	+
50	CD14	+	-	-	-	-	-	-	-
51	CD16	+	-	+	+	+	-	+	-
52	CD20	+	-	-	-	-	-	-	-
53	CD21	+	-	-	-	-	-	+	+ (f)
54	CD22	+	-	-	-	-	-	-	-

Table 3.5 PCR amplification of enterotoxin gene fragment from *B. licheniformis* isolates (cond.)

No.	isolate	gene							
		entFM	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
55	CD25	+	-	-	-	-	-	-	-
56	CD26	+	-	-	-	-	-	+	-
57	CD30	+	+	-	-	-	-	+	+
58	CD32	+	-	-	-	-	-	-	-
59	CD34	+	-	-	-	-	-	+	+
60	CD35	+	-	-	-	-	-	-	-
61	CD38	+	-	-	-	-	-	-	-
62	CD40	+	-	-	-	-	-	-	-
63	CD41	+	+	-	-	-	-	+	+
64	CD45	+	-	-	-	-	-	+	-
65	CD46	+	-	-	-	-	-	+	+
66	CD51	+	-	-	-	-	-	+	-
67	CD59	+	+	-	-	-	-	+ (f)	+
68	CD65	+	-	-	-	-	-	-	-
69	CD66	-	-	-	-	-	-	-	-
70	CD67	+	+	-	-	-	-	+	-
71	DE1	+	-	-	-	-	-	+	-
72	DE2	+	-	-	-	-	-	+	-
73	DE3	+	-	-	-	-	-	+	-
74	DE5	+	-	-	-	-	-	+	-
75	DE6	+	-	-	-	-	-	+	-
76	DE7	+	-	-	-	-	-	+	-
77	DE8	+	-	-	-	-	-	+	-
78	DE9	+	+	-	-	-	-	+	-
79	DE11	+	-	-	-	-	-	+	-
80	DE12	+	-	-	-	-	-	+	-
81	DE13	+	-	-	-	-	-	+	-

Table 3.5 PCR amplification of enterotoxin gene fragment from *B. licheniformis* isolates (cond.)

No.	isolate	gene							
		entFM	***cytK***	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***
82	DE14	+	-	-	-	-	-	+	-
83	DE15	+	+	-	-	-	-	+	-
84	DE16	+	-	-	-	-	-	+	-
85	DE21	+	-	-	-	-	-	+	-
86	DE25	+	+	+	+	+	+	+ (f)	+
87	DE26	+	+	+	+	+	+	+	+
88	DE27	+	+	-	-	-	-	+	+
89	DE28	+	-	-	-	-	-	+	-

note: +: PCR product observed, +(f): faint signal, indicating low yield of PCR product observed, -: no PCR product observed.

From Tables 3.2-3.5, it is seen that some amplification yielded comparatively low amount of PCR products, observed as faint signal on agarose gel. This phenomenon occurred most frequently with genes in the *nhe* and *hbl* operons. Frequency of distribution of the enterotoxin genes among isolates of different *Bacillus* species were deduced from Table 3.2-3.5, as shown and illustrated in Table 3.6 and Fig. 3.2, respectively.

Table 3.6 Frequency of distribution of enterotoxin genes among *Bacillus* isolates

species	Distribution of gene in isolates of each species (%)							
(no. of isolates)	***hblA***	***hblC***	***hblD***	***nheA***	***nheB***	***nheC***	***entFM***	***cytK***
B. cereus (125)	64.00	61.60	64.00	59.20	70.40	68.80	60.00	70.40
B. mycoides (28)	60.71	60.71	60.71	50.00	46.43	60.71	75.00	46.43
B. thuringiensis (15)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
B. licheniformis (89)	26.97	25.84	26.97	38.20	75.28	48.31	91.01	32.58

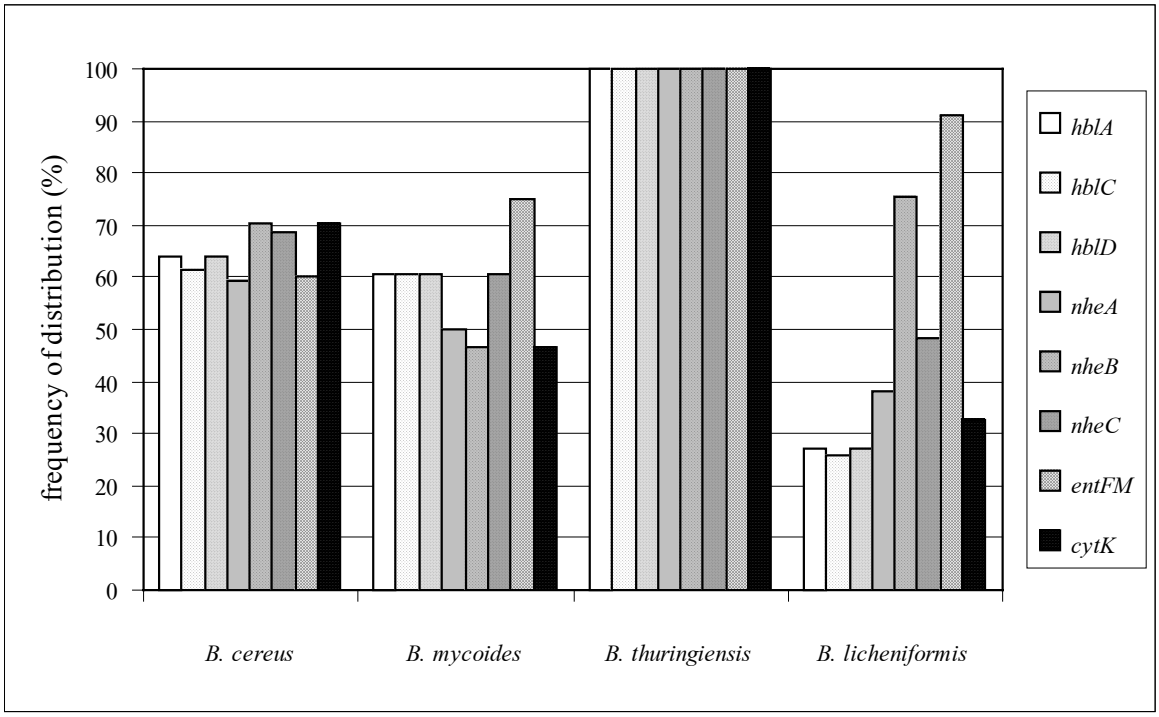


Fig 3.2 Frequency of distribution of *hblA*, *hblD*, *hblC*, *nheA*, *nheB*, *nheC*, *entFM*, and *cytK* in isolates of *Bacillus* species

From Table 3.6 and Fig. 3.2, it is clearly seen that the enterotoxin genes have been found distributed among *Bacillus* isolates with different percentages among different species. In general, isolates belonging to *B. cereus* group (including *B. cereus, B. thuringiensis, B. mycoides*) are observed to have all genes (exept *entFM* in all cases and *nheB* in some cases) distributed among them at higher percentages than those belonging to *B. licheniformis*. Interestingly, in 15 *B. thuringiensis* isolates, all enterotoxin genes were found present. These isolates were not necessarily obtained from the same food source (Table 2.1). Hansen and Hendriksen (2001) examined enterotoxin genes (*hblA, hblC, hblD, nheA, nheB,* and *nheC*) in *B. thuringiensis* isolates and found similar results that these enterotoxin genes are broadlydistributed among the isolates. The possible potential of *B. thuringiensis*, *B. mycoides*, and some other species to be enterotoxigenic was raised by other researchers (Hansen and hendriksen, 2001; Hsieh, *et al.,* 1999). *EntFM* was also found present in other species belonging to *B. cereus* group (Asano *et al*., 1997; Hsieh *et al*., 1999). Our study reveals that *entFM* was found in *B. licheniformis*, a species outside

B. cereus group as well, at a high percentage of 91.01 %. Since various enterotoxin-encoding genes were found in *B. licheniformis*, they could have been associated with toxicity of food-poisoning strains of this species (Salkinoja-Salonen *et al*., 1999) in some cases. As for *cytK*, the gene encoding Cytotoxin K, a highly virulent entertoxin, it was found present in food-borne *B. cereus* isolates with a higher frequency (70.4 %) than what has been reported (37.2 %) in a previous study by Guinebretière *et al*. (2002). The role of these genes associated with food toxicoinfection in the species other than *B. cereus* that are not recognized as food pathogens is still not conclusive and require further study. However, the occurrence of these genes in such species indicate the potential of these species to be enterotoxigenic and should be noted by those involved in pathogen diagnosis, epidemiology, disease control and prevention, and food quality assurance. We therefore strongly recommend that this problem be investigated, as all isolates used in this study were naturally present in food.

3.2.2 Occurrence of genes in *hbl* and *nhe* operons

From Table 3.2 to Table 3.5, it is seen that enterotoxin genes are not necessarily present together in isolates of *B. cereus* and other *Bacillus* spp. It can also be seen that the genes that are traditionally found to be arranged in adjacent to each other in an operon (*nheA, nheB, nheC* in the *nhe* operon, and *hblA, hblC, hblD* in the *hbl* operon) did not always occur together in some of the isolates, using this method. This is more obvious with the *nhe* operon than with the *hbl* operon (Table 3.6 and Fig. 3.2). The profiles of occurrence of these genes are summarized in Table 3.7 and 3.8.

Table 3.7 Profiles of occurrence of genes in the *hbl* operon

Occurrence of gene			Percentage of *Bacillus* isolates following each profile
hblC	*hblD*	*hblA*	
-	-	-	46.70
+	+	+	50.96
-	+	+	1.94
+	-	-	0.40

Table 3.8 Profiles of occurrence of genes in the *nhe* operon

Occurrence of gene			Percentage of *Bacillus* isolates following each profile
nheA	*nheB*	*nheC*	
-	-	-	21.01
+	+	+	44.36
+	+	-	4.28
+	-	+	4.28
-	+	+	10.90
-	-	+	3.11
-	+	-	11.67
+	-	-	0.39

It can be seen from Table 3.7 and 3.8 that the patterns of occurrence of genes in the *nhe* operon have a greater variation than those in the *hbl* operon. The nonconservative patterns of co-occurrence of the *nhe* genes were also previously noted by other researchers (Hansen and Hendriksen, 2001), but at a less detailed study. These, in combination with low amount of product obtained (as discussed in 3.2.1), suggest a possibility of some degree of variation of nucleotides within the gene sequences. Further studies are required to clarify this.

3.3 Development of a multiplex PCR for detection of 8 *B. cereus*-enterotoxin genes in *Bacillus* species

3.3.1 Optimization of primers

In developing a multiplex PCR for detection of *B. cereus*-enterotoxin genes, it is necessary to know the patterns of distribution of the enterotoxin genes among *Bacillus* isolates as a basis of development. As there are genes arranged in operons, a possibility of using the best representatives from each operon was considered originally. However, the results from section 3.1 have revealed that there is not one acceptable representative of genes from these operons as nonconservative occurrence of the genes was observed. Therefore, in developing a multiplex PCR for *B. cereus*-enterotoxin genes, all of the eight genes were taken into account. So a multiplex PCR was performed using primers applied in single PCR of individual genes (section 3.2). However, it appeared that some sets of the primers (primers specific for *hblC* (hblC-F1, hblC-R1) and *nheB* (nheB-F1, nheB-R1)) could not be used in the multiplex reaction. The primer pair nheB-F1 and nheB-R1 could not be used because the size of the amplified fragment was indistinguishable from that of *cytK* after agarose gel electrophoresis (Fig. 3.3). The primers FcytK and RcytK were not changed because *cytK* appeared to be problematic to amplify in previous reactions in which 3 other different sets of primers were used. The primer pair hblC-F1 and hblC-R1 could not be used since it seemed to require different conditions from the primers specific to *cytK* when used in a multiplex PCR. Therefore, new primers were designed or selected for amplification of the fragment of the *hblC* genes (HBLC-N and HBLC-C) and *nheB* (NHEB-N and NHEB-C).

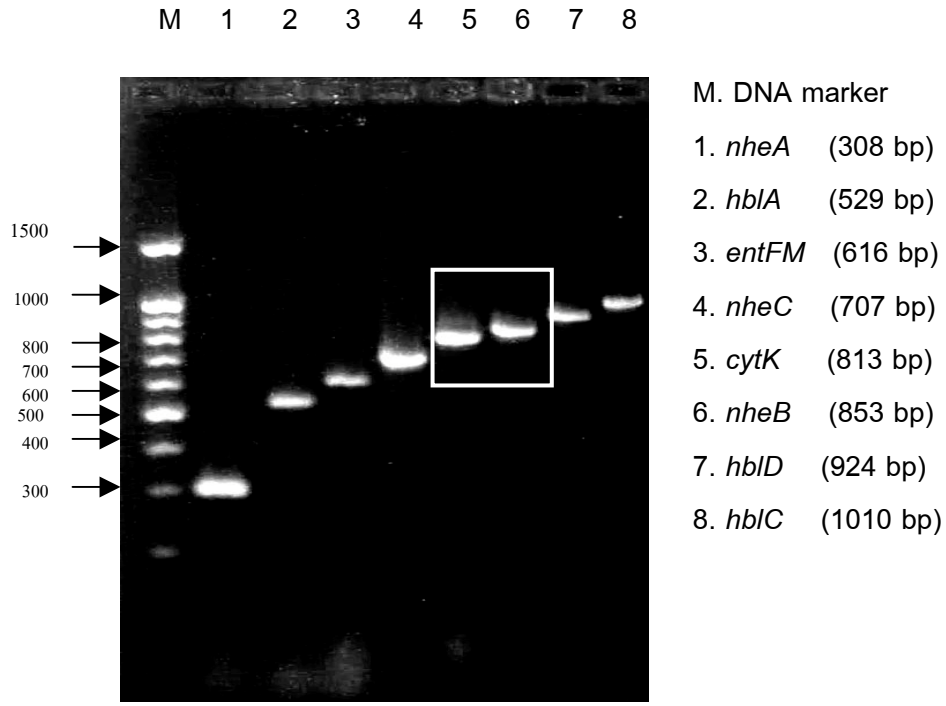


Fig. 3.3 Amplified products obtained with primers used in amplification of individual genes in single PCRs. The amplified *nheB* fragment (with primers nheB-F1 and nheB-R1) would be indistinguishable from that of *cytK* (indicated by frame) and were reserved from application in a multiplex reaction.

3.3.2 Optimization of annealing temperature

Eight pairs of selected primers (see 3.3.1 and Table 2.2) were subject to multiplex PCR. Originally, annealing temperature of 59 $^{\circ}$C, at which all individual genes were successfully amplified, was used. However, *hblC* was not amplified with the newly selected corresponding primers (3.3.1). Therefore, the multiplex PCR was tested at different annealing temperatures in order to identify the most optimal annealing temperature for the multiplex reaction. The varied annealing temperatures were set using a temperature gradient function (program C, annealing temperatures programmed to range from 40-60 $^{\circ}$ C) of the thermal cycler (2.7). This was carried out using reference chromosomal DNA from *B. cereus* strains NVH 1230-88 (reference for all enterotoxin genes) and NVH 0391-98 (strain that *cytK* was originally isolated) as DNA templates. A reaction mixture, an

aliquot of 50 µl from one master mixture preparation, contained 2.0 mM $MgCl_2$, 0.22 mM dNTPs, 0.2 pM forward and reverse primer, approx. 15-40 ng chromosomal DNA templates (from strains NVH 1230-88 and NVH 0391-98, the latter was used since it was an original strain from which *cytK* had been identified), and 4 U DNA polymerase. The fragments of 8 enterotoxin genes amplified in multiplex PCR at different annealing temperatures are shown in Fig. 3.4.

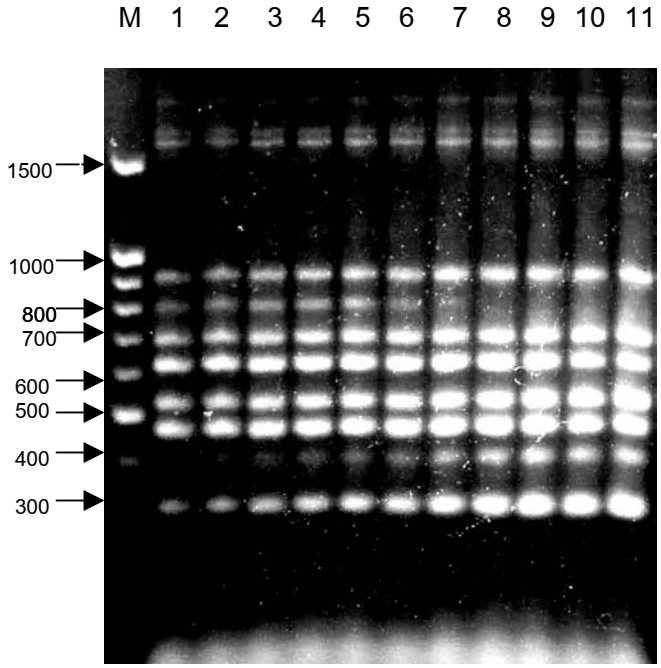


Fig. 3.4 Amplified results from 8 pairs of primers used in multiplex PCR, performed at different gradient temperatures of: **lane (1)** 40.1 °C, **lane (2)** 40.8 °C, **lane (3)** 42.2 °C, **lane (4)** 44.2 °C, **lane (5)** 46.7 °C, **lane (6)** 49.4 °C, **lane (7)** 52.1 °C, **lane (8)** 54.7 °C, **lane (9)** 57.1 °C, **lane (10)** 59.0 °C, **lane (11)** 60.2 °C; **(M)** DNA marker.

From Fig. 3.4, it can be seen that all genes except *cytK* and *hblC* were amplified best at different annealing temperature ranges. The gene *cytK* was amplified reasonably at annealing temperatures between 40.0-49.4 °C, with the maximum yield obtained at 46.7 °C, while *hblC* was amplified at annealing temperatures between 42.2-60.2°C, with the maximum yield obtained between 57.7-60.2 °C. At annealing temperatures of 42.2-49.4 °

C, all 8 enterotoxin genes were amplified. The annealing temperature that seemed to be the best compromise for *cytK* and *hblC* is 46.7 °C. Therefore, this temperature was chosen for further optimization of other factors involved in PCR, with an attempt to improve the yield of both *cytK* and *hblC*.

3.3.3 Optimization of reagent factors involved in multiplex PCR

The main reagent factors involved in PCR that could have an effect to the yield and specificity of amplified products are magnesium concentration, dNTP concentration, primer concentration, amount of DNA polymerase, and amount of DNA template present in the reaction mixture. In this case, the yield of *cytK* and *hblC* fragments was to be improved, in order to be applicable with the test isolates. Experiments were carried out to optimize all of these factors. Magnesium concentration was varied between 2.0-3.0 mM. Concentration of dNTPs in the reaction was varied between 0.2-0.5 mM. The amount of DNA polymerase was fixed at 0.5 units per gene per reaction (total 4 units per 8 genes), as it was found that increasing amount of DNA polymerase was not necessary. Finally, an optimum condition of reagents was obtained, giving an improved yield of amplified *cytK* and *hblC* fragments, which can be clearly observed on agarose gel. The optimized reagent factors include 2.0 mM $MgCl_2$, 0.35 dNTPs, 0.2 pM each primer, approx. 15-40 ng DNA templates (from strains NVH 1230-88 and NVH 0391-98), and 4 U DNA polymerase (0.5 U/gene/reaction) in 50 μl reaction volume. It is clearly seen that increasing dNTP concentration contributes greatly to improvement of the yield of the problematically amplified genes.

3.3.4 Optimization of primer annealing condition

As mentioned in 3.3.2, one possible annealing temperature for operating the multiplex PCR is 46.7 °C. Another approach for maximizing the yield of the amplified fragments of *cytK* and *hblC* genes were to use two-step PCR, in which there are two annealing

temperatures set in one program. The two-step PCR was designed for this special case in which two fragments were amplified with maximum yield at different annealing temperatures. There were 2 programs of two-step PCR: “two-step-down PCR” and “two-step-up PCR”. The “two-step-down PCR” operated at a higher annealing temperature for 20 cycles, then at a lower annealing temperature for another 20 cycles, whereas the “two-step-up PCR” operated at a lower annealing temperature for 20 cycles, then at a higher annealing temperature for another 20 cycles (see 2.7, program G and I, respectively). This experiment was set with an attempt to maximize the yield after the reagent factors were optimized. Operation of the multiplex PCR for 8 enterotoxin genes using the three programs (H, G, I) were carried out using the optimized reagent condition (3.3.3). The results from the 3 programs are shown in Fig. 3.5.

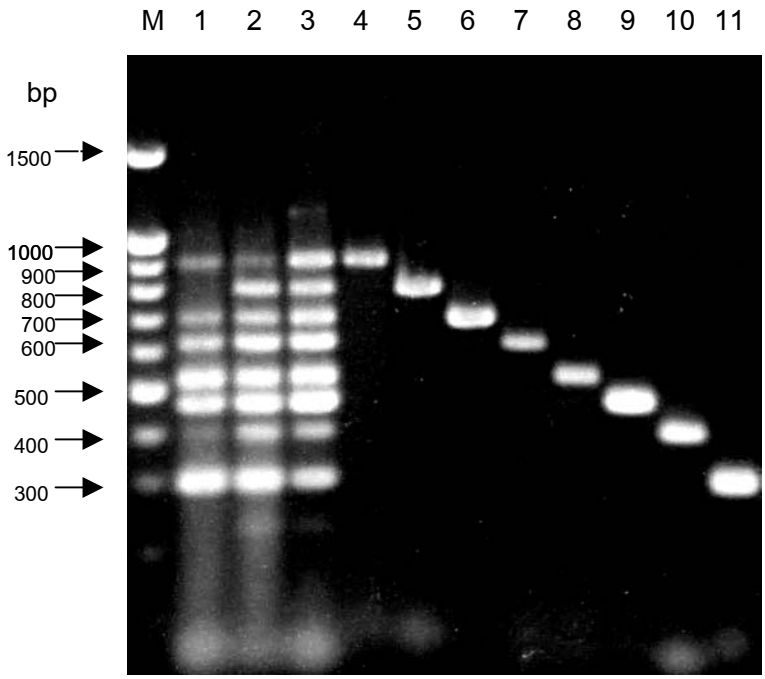


Fig. 3.5 Results from multiplex PCR performed with different annealing conditions set by 3 different programs: **lane (1)** “2-step-down” with annealing temperatures 60 $^{\circ}$C for 20 cycles followed by 45 $^{\circ}$C for 20 cycles, **lane (2)** “2-step-up” with annealing temperatures 45 $^{\circ}$C for 20 cycles followed by 60 $^{\circ}$C for 20 cycles, **lane (3)** annealing temperature set at 46.7 $^{\circ}$C, **lane (4) to (11)** reference amplified fragments of genes *hblD, cytK, nheC, entFM, hblA, nheB, hblC, nheA*, respectively.

From Fig. 3.5, it can be seen that the multiplex PCR performed using program H with annealing temperature of 46.7 $^{\circ}$C gave the best result. The program “2-step-up PCR” (program I) gave a better result than that yielded by program “2-step down PCR” (program G). When these 2-step programs were designed, it was hoped that the higher annealing temperature would allow *hblC*, while the lower would allow *cytK* to be amplified maximally. It is noteworthy that the order of operation of two (high and low) annealing temperatures had a significant influence on the amplification. It seems that when there is a problem with the yield of amplification for problematic fragments which are amplified at different conditions, a compromise annealing temperature or a 2-step-up program starting with a lower temperature could be a solution. Program 2-step-down, with the provided conditions, cannot be used for operation of this multiplex PCR since it did not amplify all genes. This “2-step-up PCR” may be used for amplification of multi-enterotoxin genes of *B. cereus* as an alternative to the basic program (H), if require. However, in this experiment, the result obtained from program H is the most satisfactory and therefore this program proceeded to evaluation of the method with the test isolates (3.4).

3.4 Efficiency of the multiplex PCR with the test isolates

From section 3.3, the best optimized condition for multiplex PCR amplifying 8 *B. cereus*-enterotoxin genes was developed. This condition was then applied for PCR with 60 test isolates (randomly chosen from different gene profiles) in order to evaluate the accuracy of this multiplex PCR. The presence of the 8 enterotoxin genes of these 60 test isolates had been examined earlier in single PCRs (3.2.1). Amplified results of different gene profiles are demonstrated in Fig. 3.6. Alignment of results obtained from the multiplex PCR compared with those obtained from single PCRs is shown in Table 3.9, and the evaluation of the multiplex PCR is shown in Table 3.10.

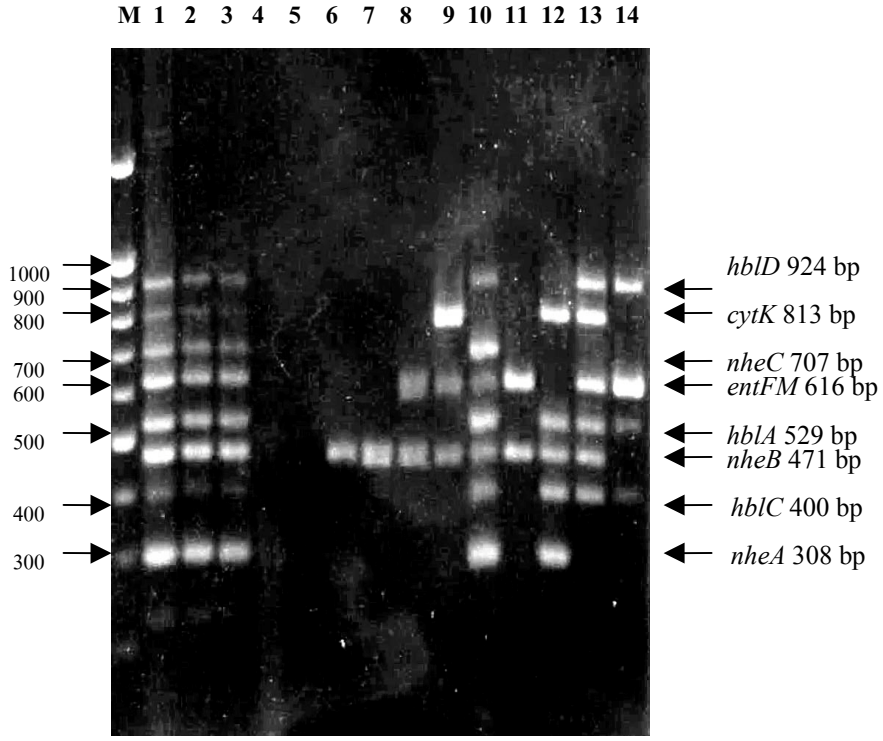


Fig. 3.6 Profiles of multiplex PCR of *B. cereus* enterotoxin genes in test isolates of *Bacillus* spp. **(M)** DNA marker; **lane (1)** reference strains (8/8, meaning 8 genes detected by single PCR and 8 genes detected by multiplex PCR); **lane (2)** *B. cereus* BC89 (8/8); **lane (3)** *B. cereus* BC15 (7/8); **lane (4)** *B. cereus* BC111 (0/0); **lane (5)** *B. licheniformis* M15 (1/0, *nheB* not detected); **lane (6)** *B. mycoides* CD28 (1/1); **lane (7)** *B. cereus* BC26 (2/1, *nheA* not detected); **lane (8)** *B. cereus* CD12 (2/2); **lane (9)** *B. cereus* M4 (3/3); **lane (10)** *B. licheniformis* BC93 (8/7, *cytK* not detected); **lane (11)** *B. licheniformis* BC2 (3/2, *nheA* not detected); **lane (12)** *B. cereus* BC5 (6/5, *hblD* not detected); **lane (13)** *B. cereus* BC51 (6/6); **lane (14)** *B. mycoides* BC104 (4/4)

Table 3.9 Alignment of results from single PCR and multiplex PCR of *Bacillus* species

isolate	species	ocuurrence of gene obtained from single PCR								ocuurrence of gene obtained from multiplex PCR							
		entFM	*cytK*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*entFM*	*cytK*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*
BC89	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M28	*B. cereus*	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+
BC90	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC67	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CD20	*B. licheniformis*	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-
AB40	*B. cereus*	-	+	+	+ (f)	+	+	+	+	+	+ (f)	+	+ (f)	+ (f)	+	+	+ (f)
BC60	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC111	*B. cereus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD68	*B. thuringiensis*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC124	*B. cereus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BC32	*B. mycoides*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AB48	*B. cereus*	+	+ (f)	+	+	+	+	+ (f)	+	+	+ (f)	+ (f)	+	+	+	+ (f)	+
CD14	*B. licheniformis*	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-
BC68	*B. cereus*	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
BC5	*B. cereus*	-	+	+	+	+	+	+	-	-	+	+	+ (f)	-	+	+	-
M4	*B. cereus*	+	+	-	-	-	-	+	-	+	+	-	-	-	-	+	-

Table 3.9 Alignment of results from single PCR and multiplex PCR of *Bacillus* species (cond.)

isolate	species	ocuurrence of gene obtained from single PCR								ocuurrence of gene obtained from multiplex PCR							
		entFM	*cytK*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*entFM*	*cytK*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*
M7	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC78	*B. licheniformis*	+	+	+	+	+	+	+	+	+	+ (f)	+	+	+ (f)	+	+	+ (f)
BC93	*B. licheniformis*	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AB46	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC15	*B. cereus*	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M25	*B. cereus*	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
DE26	*B. licheniformis*	+	+	+	+ (f)	+	+	+	+	+	+	+	+	+	+	+	+
BC16	*B. cereus*	+	+	+	+ (f)	+	+	+	+	+	+	+	+	+	+	+	+
M26	*B. cereus*	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+
M12	*B. cereus*	+	+	-	-	-	-	+	-	+	+	-	-	-	-	+	-
AB43	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M32	*B. cereus*	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-
CD71	*B. thuringiensis*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC79	*B. mycoides*	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
AB16	*B. cereus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD2	*B. cereus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD28	*B. mycoides*	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-
M2	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.9 Alignment of results from single PCR and multiplex PCR of *Bacillus* species (cond.)

isolate	species	ocuurrence of gene obtained from single PCR								ocuurrence of gene obtained from multiplex PCR							
		entFM	*cytK*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*entFM*	*cytK*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*
BC51	*B. cereus*	+	+	+	+	+	-	+	-	+	+	+	+	+	-	+	-
CD3	*B. thuringiensis*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC26	*B. cereus*	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-
BC110	*B. cereus*	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DE22	*B. cereus*	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
CD8	*B. mycoides*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC93	*B. licheniformis*	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
CD12	*B. cereus*	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-
CD18	*B. cereus*	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-
M3	*B. mycoides*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC17	*B. cereus*	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
M12	*B. cereus*	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-
M29	*B. licheniformis*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CD24	*B. cereus*	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-
BC104	*B. mycoides*	+	-	+	+	+	-	-	-	+ (f)	-	+	+	+	-	-	-
BC58	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC105	*B. mycoides*	+	-	+	+	+	+	+	+	+ (f)	-	+	+	+	+	+	+
CD15	*B. mycoides*	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-

Table 3.9 Alignment of results from single PCR and multiplex PCR of *Bacillus* species (cond.)

isolate	species	ocuurrence of gene obtained from single PCR								ocuurrence of gene obtained from multiplex PCR							
		entFM	*cytK*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*entFM*	*cytK*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*
M15	*B. licheniformis*	-	-	-	-	-	-	+ (f)	-	-	-	-	-	-	-	-	-
BC2	*B. licheniformis*	+	-	-	-	-	+	+	-	+	-	-	-	-	-	+	-
BC13	*B. licheniformis*	+	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-
BC30	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC40	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BC46	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M26	*B. cereus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CD48	*B. mycoides*	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-

note: +: PCR product observed; +(f): faint signal, indicating low yield of PCR product observed; -: no PCR product observed.

Table 3.10 Efficiency of multiplex PCR for amplification of *B. cereus*-enterotoxin genes in *Bacillus* species in comparison with single PCR

species[1]	no. of genes detected by[2]		no. of isolates in each case			gene for which results from 2 methods are not identical
	S-PCR	M-PCR	MP-PCR identical to S-PCR	M-PCR more sensitive[3] than S-PCR	M-PCR less sensitive[3] than S-PCR	
Bc, Bm, Bt, Bl	8	8	23			
Bc, Bm, Bl	7	7	8			
Bc, Bl	7	8		4		*entFM, cytK*
Bc	6	6	1			
Bc	6	5			1	*hblD*
Bm, Bl	4	4	3			
Bc	3	3	2			
Bl	3	2			1	*nheA*
Bc, Bm, Bl	2	2	9			
Bc	2	1			1	*nheA*
Bm	1	1	1			
Bl	1	0			1[4]	*nheB*
Bc, Bl	0	0	5			
no. from total 60 isolates (%)			52 (86.66 %)	4 (6.67 %)	4 (6.67 %)	

Note: [1]Bc: *B. cereus*, Bm: *B. mycoides*, Bt: *B. thuringiensis*, Bl: *B. licheniformis*.

[2]S-PCR: single PCR, M-PCR:multiplex PCR.

[3]less sensitive: fewer genes were detected; more sensitive: more genes were detected.

[4]only this case gives false negative result.

From Table 3.9 and 3.10, it can be seen that the results from the two methods appeared to be well correlated. Multiplex PCR gave 86.66 % identical results to single PCR, 6.67 % more sensitive, and 6.67 % less sensitive than single PCR. Most of the less sensitive cases (fewer genes detected by multiplex PCR) occurred with *nhe* or *hbl* genes (Table 3.10). False negative result (none of the genes detected by multiplex PCR while at least 1 gene detected by single PCR) given by multiplex PCR was observed in only one isolate (1.67 %) as shown in Table 3.10. Considering the fact that 97.28 % of *Bacillus* isolates harbor at least one enterotoxin gene, the chance of having false negative is very low. To our knowledge, this is the first described multiplex PCR for *B. cereus*-enterotoxin genes that covers different gene identities. Operation of multiplex PCR reduces time and reagent spent in single PCR significantly. This method is now available as an in-house rapid-and-efficient alternative for food laboratories to enumerate or detect not only *B. cereus* or some limited types of toxins but all potentially toxigenic *Bacillus* species.

4. Conclusions

From the study, it can be concluded that:

(1) As many as 97.28 % of *Bacillus* isolates tested harbor at least one of the enterotoxin genes and the genes are found distributed among *B. cereus, B. thuriengiensis, B. mycoides,* and *B. licheniformis* at different percentages for different genes and in different species, with no exception for the genes in *hbl* and *nhe* operon in which non conservative occurrence of the three genes were observed with the method used.

(2) A multiplex PCR for detection of 8 *B. cereus*-enterotoxin genes has been successfully developed. Having satisfactory performance with test *Bacillus* isolates and good correlation with single PCR, the method is now available as a more advantageous alternative to the existing methods, allowing rapid and efficient detection of potentially toxigenic *Bacillus* species.

5. Suggestions for future study

From this study, some new information and development of method, relating to entrotoxigenic factors of *B. cereus* and other species, were achieved. These are presently beneficial to the food industry and organizations associating with epidemiology or disease control and prevention. They have also provided basis for further study and development, in which some suggestions are given below:

(1) The fact that all enterotoxin genes are broadly distributed among *Bacillus* species, not only in *B. cereus* but also in *B. thuringiensis, B. mycoides,* and *B. licheniformis*, which are not generally recognized as food pathogens, this suggests a possibility that these species are potentially enterotoxigenic. The ability of these species to produce enterotoxins and their pathogenicity should be further investigated. Meanwhile, we suggest inclusion of the toxigenic isolates of these species in preventive measures of food industry and food protection agency.

(2) As it was found in this study, there is inconsistency in the occurrence profiles of the genes in *nhe* and *hbl* operons which suggests possible variation of nucleotide sequence within these operons. This should be confirmed by other methods such as Southern blotting and hybridization which, with a well-designed probe, was reported to be more sensitive than PCR in some cases (Guinebretière *et al.*, 2002). Another approach to confirm the heterogeneity in these operons is through DNA sequencing. Such data will provide a basis for further characterization of the genes and the role of the individual genes in the operons in pathogenicity.

(3) In this study, a condition of a multiplex PCR for detection of *B-cereus* enterotoxin genes has been optimized. Further development of this multiplex PCR in terms of increasing convenience for operation and transport should be carried out for wider application in food laboratories.

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7. Outputs from this research project

From this research project, manuscript of research papers and proposal for patenting are being prepared, as followed:

7.1 Manuscript of research paper entitled: Occurrence Profiles and Distribution of *Bacillus cereus*-enterotoxin genes among food isolates of *Bacillus cereus* and other *Bacillus* species (in final revision before submission to Applied and Environmental Microbiology - see appendix 1)

7.2 Manuscript of research paper entitled: Development of a multiplex PCR system for detection of potentially enterotoxigenic *Bacillus* species (in preparation)

7.3 Proposal for patenting: Multiplex PCR system for detection of potentially enterotoxigenic *Bacillus* species (in process)

Appendix 1

Manuscript of a research paper entitled “Occurrence Profiles and Distribution of *Bacillus cereus*-enterotoxin genes among food isolates of *Bacillus cereus* and other *Bacillus* species”

Occurrence Profiles and Distribution of *Bacillus cereus*-Enterotoxin Genes among Food Isolates of *Bacillus cereus* and Other *Bacillus* Species

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Bacillus cereus is known to produce enterotoxins of different molecular entities, such as Hemolysin BL (HBL), Non-Hemolytic Enterotoxin (NHE), Cytotoxin K, and Enterotoxin FM. This study aimed to investigate occurrence and distribution of genes associated with these enterotoxins in *B. cereus* and other *Bacillus* species isolated from foods through Polymyxin Egg-yolk Mannitol Bromothymol blue agar (PEMBA), a commonly used *B. cereus* selective medium. Occurrence of the genes *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *entFM*, and *cytK* was investigated among 257 food-isolates of *Bacillus cereus* and other *Bacillus* species through polymerase chain reaction. Gene *hblA, hblC, hblD, nheA, nheB, nheC, entFM,* and *cytK* were found to distribute among isolates of *B. cereus* with a frequency of 64.0, 61.6, 64.0, 59.2, 70.4, 68.8, 60.0, and 70.4 %, respectively. These enterotoxin genes were also found present in isolates of *B. thuringiensis*, *B. mycoides*, and *B. licheniformis* at different percentages for different genes and in different species, with no exception for the genes in *hbl* and *nhe* operon in which non conservative occurrence of the three genes were observed with the method used. All genes, except *entFM* in all cases and *nheB* in some cases, distributed broader among isolates of *B. thuringiensis* and *B. mycoides*, species that are closely related to *B. cereus*, than among those of *B. licheniformis*. As many as 97.28 % of *Bacillus* isolates tested harbor at least one of the enterotoxin genes. These data indicate potential toxigenicity of these *Bacillus* species which are normally recovered from foods. The potential of these species to produce entertoxins and cause illnesses should be noted in the food industry and those involved with disease control and prevention.

Bacillus cereus, a well recognized food enteropathogen, is known to produce two types of enterotoxins, emetic and diarrheagenic. Among the diarrheagenic type of toxins, there are many enterotoxin involved, which have different molecular entities. To date, at least 4 entities have been identified with sufficient evidences to have enterotxic activity. These include Hemolysin BL (HBL) [3, 4], Non Hemolytic Enterotoxin (NHE) [8, 9], Enterotoxin FM [2], and Cytotoxin K [16]. A protein called Enterotoxin T was also identified [1], however, the enterotoxic and molecular properties of this protein are proposed to be reevaluated [12]. Hemolysin BL comprises of three components, one binding component (designated "B") and two lytic components (designated L_1 and L_2), according to their cooperative mechanism for hemolysis. The B, L_1 and L_2 component are encoded by the genes *hblA*, *hblD*, and *hblC*, respectively, which are adjacent to each other [14, 25]. Part of a structural gene following the *hblA* gene, named *hblB*, was also found but the function of this gene has not yet been clarified. Non Hemolytic Enterotoxin is another enterotoxin composed of 3 components designated "NheA", "NheB", and "NheC", which are encoded by the genes *nheA*, *nheB*, and *nheC*, respectively [9]. Parts of the NHE complex are cytotoxic to CaCo-2 cells [17]. Enterotoxin FM was a protein previously reported to show cytotoxic activity [27] and eventually was found to be encoded by the gene *entFM* [2]. Cytotoxin K, a protein isolated from a strain that caused a serious gastrointestinal illness and was later reported to be cytotoxic to CaCo-2 cells, is encoded by the gene *cytK* [13].

There have been reports about the occurrence of some of these enterotoxin genes in other *Bacillus* species. [11,18,23]. Moreover, there are evidences that some other species beside *B. cereus* were involved in foodborne gastrointestinal diseases [26]. These species have normally been found in foods [18, 24]. However, since these species

have not been widely recognized as food pathogens, they are normally discarded during confirmation stage through biochemical tests. This study investigated occurrence and distribution of *B. cereus*-enterotoxin encoding genes among isolates of these *Bacillus* species obtained from food through Polymyxin Egg-yolk Mannitol Bromothymol blue agar (PEMBA), a commonly used *B. cereus* selective plating medium, in order to evaluate potential toxigenicity of *Bacillus* species obtained from foods.

MATERIALS AND METHODS

Isolation and identification of *Bacillus* species from foods. Food sample (25 g) were homogenated in 225 ml of buffered peptone water, homogenized, and serial ten-fold dilution was made in 9 ml of buffered peptone water to 10^{-3}. From the homogenate of each dilution, 0.1 ml was drawn and plated on Polymyxin Egg-yolk Mannitol Bromothymol blue (PEMB) agar (*Bacillus cereus* selective agar base (Oxoid) with addition of egg-yolk and separately sterilized polymyxin. The plates were incubated at 37 °C for 24 h. All presumptive *Bacillus* colonies (colonies with diameter 3-5 mm) were drawn and subject to physiological and biochemical tests for identification of species. Gram reaction, cell morphology, spore production, swollenness of sporangia, motility, reduction of nitrate, tyrosine decomposition, resistance to lysozyme, anaerobic utilization of glucose, VP reaction, Rhizoid growth, hemolysis, and crystal toxin productions, as described in Bacteriological Analytical Manual [22], were included. Identification of species were based on Bergey's manual of systematic bacteriology [5].

Bacterial strains and isolates. A strain of *B. cereus* (NVH 1230-88, harbors *hbl, nhe, entFM, cytK*; provided by P. E. Granum, The Norwegian School of Veterinary

Science, Oslo, Norway) was used as a reference. The 257 *Bacillus* isolates used in this study were obtained from foods, consisted of 125, 15, 28, and 89 isolates of *B. cereus, B. thuringiensis, B. mycoides,* and *B. licheniformis*, respectively.

Isolation of chromosomal DNA. To isolate chromosomal DNA from a *Bacillus* culture, the isolate was grown in Tryptone soy yeast extract (TSYE) broth at 37 °C for 24 h, and the cell pellet from a 3 ml (1.5 ml × 2 times) culture was collected by centrifugation at 6000 × g for 30 s. The cell pellet was washed twice in 500 µl phosphate buffer saline (PBS, pH 7.2) and resuspended in 120 µl of 2 M sodium acetate (pH 5.2), followed by 160 µl of 10 % sodium dodecyl sulfate. The lysed cells were added with 700 µl of phenol:chloroform:isoamylalcohol (25:24:1) and mixed by converting the tube for 5 min. The mixture was then centrifuged at 12,000 × g for 5 min and the aqueous phase was transferred to a fresh tube, leaving white interphase behind. To the aqueous, 1 ml of isopropanol and 120 µl of 3 M sodium acetate (pH 5.2) was added. The mixture was placed at -70 °C for 30 min before being centrifuged at 12,000 × g for 20 min. The DNA pellet was washed with 500 µl of 70 % (v/v) ethanol, centrifuged (12,000 g, 10 min), drained, and left to dry at room temperature for 30 min or until dried. The pellet was resuspended in 50 µl of TE buffer and incubated at 37 °C to dissolve the DNA. The DNA was stored at 4°C. Throughout the preparation, filtered pipette tips were used. This preparation was routinely sufficient for direct application in PCR.

Oligonucleotide primers. The oligonucletide primers were designed using the "WebPrimer" (Stanford University, available online at http:genome-www2.stanford.edu/cgi-binlSGDweb-primer), according to the published sequence of

the gene *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *entFM*, and *cytK*. Primers were supplied by Bio Basic. The sequence and other details of oligonucleotide primers are shown in Table 1.

DNA amplification. PCR reactions were performed in a total volume of 10 µl containing 1 × reaction buffer (10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, DynaZyme), 2.0 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dATP, dTTP, dGTP, and dCTP), 1 pM forward and reverse primer, approx. 5 ng chromosomal DNA template, and 0.5 U DNA polymerase (DynaZyme) in 10 µl total reaction volume. The amplification was carried out in a thermal cycler (Master Cycler, Appendorf) operated with an initial denaturation step at 94 °C for 5 min, 35 amplification cycles (94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min 30 s), followed by final extension at 72 °C for 10 min. Filtered pipette tips were used in all steps involved in preparation of reagents.

Detection of PCR products. A 5-µl aliquot of the PCR product was analyzed on 1.5 % (w/v) TAE agarose gel. DNA marker (100 bp-1.5 kb) (SibEnzyme) was used as molecular size marker. Agarose gel after electrophoresis was stained with ethidium bromide solution (~500 µg/l in TAE buffer). Nucleic acid was visualized on a UV transilluminator (Ultra LUM) and photographed by a digital camera attached to the chamber of the UV transilluminator which links to a gel documentation system (DigiDoc, Biorad).

RESULTS

Occurrence of *Bacillus* species in foods. Colonies grown on PEMBA were initially examined on morphological basis for rod-shaped Gram positive cultures with spores. Some of these colonies were first restreaked on PEMB agar, and some white colonies from the isolation step appeared blue on a restreaked plate, more obviously where density of culture was high. The cultures were then subject to physiological and biochemical tests (listed above). They were identified as *B. cereus, B. thuringiensis, B. mycoides,* and *B. licheniformis*. Two *B. anthracis* isolates were also recovered, however, the isolates were not used further in this study. The percentages of samples containing each *Bacillus* spp. and range of numbers of viable *Bacillus* in each type of food are given in Table 2.

Occurrence of *B. cereus*-enterotoxin encoding genes in food isolates of *Bacillus* spp. Occurrence of genes *hblA, hblC, hblD, nheA, nheB, nheC, entFM,* and *cytK* were examined in 257 *Bacillus* isolates, including 125 *B. cereus*, 15 *B. thuringiensis*, 28 *B. mycoides*, and 89 *B. licheniformis* isolates through PCR with primers listed in Table 1. Positive control (PCR with HVH 1230-88 DNA template) and negative control (PCR with no DNA template) were included in every batch of amplification. Inconsistency in co-occurrence of the genes in the *hbl* and *nhe* operons was observed in some isolates, in which amplification was repeated. Occurrence of these 8 enterotoxin genes in all test isolates was sorted into profiles. In total, 39 profiles are observed, as presented in Table 3.

Distribution of *B. cereus*-enterotoxin genes in *B. cereus* and other *Bacillus* spp. Detection of 8 enterotoxin genes in 257 *Bacillus* isolates by PCR revealed that up to 97.28 % of the isolates harbor at least one of the enterotoxin genes, only 2.72 %

harbor none of the genes. Gene *hblA, hblC, hblD, nheA, nheB, nheC, entFM,* and *cytK* were found to present in isolates of *B. cereus* with a frequency of 64.0, 61.6, 64.0, 59.2, 70.4, 68.8, 60.0, and 70.4 %, respectively. The genes were also found present in other species. Frequency of distribution of the genes is found to be of different percentages for different genes within one species and in different species. Frequency of distribution of these enterotoxin genes among isolates of different *Bacillus* species are illustrated in Fig. 1.

DISCUSSION

Among members of genus *Bacillus*, *B. cereus* is the only species that is recognized as food enteropathogen. In many selective plating media, including PEMBA, other *Bacillus* spp. are normally isolated together with *B. cereus* [24]. We found that the species belonging to *B. cereus* group are always isolated together, as they share a common characteristic of egg-yolk lecithinase production. *B. licheniformis* was a main species outside *B. cereus* group that was isolated from food in this study, however, this species was egg-yolk lecithinase negative and is normally screened out during selective plating procedure. In this study, *B. cereus*, *B. thuringiensis, B. mycoides,* and *B. licheniformis* are the main species present in pasteurized milk and products from rice and wheat. The food like pasteurized milk is potentially hazardous especially when storage temperature is abused. Processed food like noodles, rice meal, or pasta could also be potentially hazardous when prepared into dishes and left to stand at room temperature. Although numbers of *B. cereus* occur in these foods are not high enough to cause illnesses (Table 2), the numbers could increase to an infective level, provided that factors affecting growth and enterotoxin production are suitable. Considering the fact that as high as 97.28 % of the isolates harbor at least one of the

enterotoxin genes (as presented above), this means that most of *Bacillus* spp. presented in food would be potentially enterotoxigenic, and could contribute to food toxicoinfection when intrinsic and extrinsic factors permit.

Recent researches have revealed that there are many genes involved in *B. cereus* pathogenicity, as reviewed above. In this study, we observed that each enterotoxin gene occurred in more than half of the *B. cereus* isolates tested. Occurrence of these traditionally recognized *B. cereus*-enterotoxin genes in other species beside *B. cereus* has previously been reported [21], and is confirmed by the results from this study. In general, isolates belonging to *B. cereus* group (including *B. cereus, B. thuringiensis, B. mycoides*) are observed to have all genes (except *entFM*, and *nheB* in some cases) distributed among them more frequent than among *B. licheniformis* isolates.

Interestingly, all enterotoxin genes were found present in 15 *B. thuringiensis* isolates tested in this study. These isolates were not necessarily obtained from the same food source. Hansen and Hendriksen (2001) examined enterotoxin genes (*hblA, hblC, hblD, nheA, nheB,* and *nheC*) in *B. thuringiensis* and found high distribution of these genes among the isolates. The possible potential of *B. thuringiensis*, *B. mycoides*, and some other species to be enterotoxigenic was raised by other researchers [11, 15, 20]. Some *B. thuringiensis* strains had been proved to cause fluid accumulation in intestine, a traditional test indicating enterotoxic property [28]. *EntFM* was another gene that had been found present in other species belonging to *B. cereus* group [2, 15]. Our study reveals that *entFM* was found in *B. licheniformis*, a species outside *B. cereus* group as well, at a high percentage of 91.01 %. Since various enterotoxin-encoding genes were found in *B. licheniformis*, they could have been associated with toxicity of food-poisoning strains of this species [26] in some cases. As for *cytK*, the gene encoding

Cytotoxin K, a highly virulent entertoxin, it was found present in food-borne *B. cereus* isolates with a higher frequency (70.4 %) than what has been reported (37.2 %) in a previous study [10].

Heterogeneity in co-occurrence of the genes in *hbl* and *nhe* operons are observed in this study with the method used, especially with *nhe*. This has been noted previously by other researchers [8, 11, 29]. The occurrence of these genes in the isolates of question should be confirmed by other methods, and the effect of this heterogeneity (especially in *nhe*) on biological activity should be further investigated.

The role of these entertoxin genes associated with food toxicoinfection in the species other than *B. cereus* that are not recognized as food pathogens is still not conclusive and require further study. It should be noted that *B. thuringiensis* and *B. licheniformis* have historical evidences to have enterotoxic properties or to cause food-borne illnesses [26, 28], in which these currently known enterotoxins might have been involved. However, the occurrence of these genes in such species indicates the potential of these species to be enterotoxigenic and should be noted by those involved in pathogen diagnosis, epidemiology, disease control and prevention, and food quality assurance. We therefore strongly recommend that this problem be investigated, as all isolates used in this study were naturally present in food.

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TABLE 1. Primer details

primer name[a]	sequence 5′→3′	size (bp)	annealing site on gene[b]	PCR product size(bp)	gene (reference/ database)
entFM-F1	CAA GAA AAC GGT TGG TTC AA	20	280	616	*entFM*
entFM-R1	TTG TTG GCT TTT GTA CGT CT	20	895		(Asano *et al*., 1997)
FcytK	AAC AGA TAT CGG TCA AAA TGC	21	1858	813	*cytK*
RcytK	CCA ACC CAG TTA CCA GTT CC	20	2670		(Genbank/AJ277962)
hblA-F1	TGC GAG GTG AAA TTC AAC AA	20	470	529	*hblA*
hblA-R1	TTT TGA TCG GCT TTT TGA GC	20	998		(Genbank/L20441)
hblC-F1	ACG AAA ATT AGG TGC GCA AT	20	135	1010	*hblC*
hblC-R1	CTT GAT TTG CAA ACG TAT GC	20	1144		(Genbank/U63928)
hblD-F1	AGA AAG CAT TGG CTG AAA CA	20	152	924	*hblD*
hblD-R1	AAT TTG CGC CCA TTG TAT TC	20	1075		(Genbank/U63928)
nheA-F1	AAA AGT TCG CAA AGG CGA AT	20	256	308	*nheA*
nheA-R1	TGC TTC ATC GGC TTT AAT TG	20	563		(Genbank/Y19005)
nheB-F1	ATG CGG CTT TAA AAG GGA AA	20	287	853	*nheB*
nheB-R1	TTC CAG CTA TCT TTC GCA AT	20	1139		(Genbank/Y19005)
nheC-F1	TGG CAA CAA GTA ACG CAT TT	20	53	707	*nheC*
nheC-R1	TTT TTT CGC AAC CGC AAT CA	20	759		(Genbank/Y19005)

[a]primers with F or N included in the name are forward primers and those with R or C are reverse primers. FcytK and RcytK were resynthesized based on the sequences reported (Fagerlund *et al*, 2004). Other primers were designed in this study.

[b]according to the sequence retrieved from databases

TABLE 2. Occurrence of *Bacillus* spp. in foods.

food type	no. of sample	no. of food samples containing each species (range of *Bacillus* counts as CFU/g)				
		B. cereus	*B .mycoides*	*B .thuringiensis*	*B .anthracis*	*B .licheniformis*
pasteurized milk	18	15 (0.5×10^2-7.5×10^2)	6 (0.5×10^2-4.0×10^2)	0	0	15 (0.5×10^2-8.5×10^2)
rice, brown rice, glutinous rice	31	10 (0.5×10^2-7.0×10^2)	4 (0.5×10^2-1.5×10^2)	4 (0.5×10^2-1.0×10^2)	0	8 (0.5×10^2-8.5×10^2)
flour (rice, wheat, cassava, root)	9	5 (0.5×10^2-1.65×10^3)	2 (1.0×10^2-3.5×10^2)	1 (2.0×10^2)	1 (0.5×10^2)	3 (0.5×10^2-6.5×10^2)
noodles (rice, wheat)	14	2 (1.0×10^2-2.0×10^2)	0	0	0	1 (0.5×10^2)
semi-instant rice meal (chicken, pork, seafood flavor)	9	3 (0.5×10^2-1.0×10^2)	2 (0.5×10^2)	1 (0.5×10^2)	1 (0.5×10^2)	5 (0.5×10^2-1.0×10^2)
semi-instant macaroni soup	4	0	0	1 (2.0×10^2)	0	0

TABLE 3. Occurrence profiles of enterotoxin encoding genes

profile	isolates[a]	gene								no. of isolates (%)
		entFM	*cytK*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	
1	**Bc:** M7, M26, M28, AB43, AB46, AB48, BC16, BC30, BC40, BC58, BC60, BC67, BC84, BC89, BC91, BC94, BC100, BC102, BC103, BC125, BC126, CD5, CD6, CD52, DE23, AB11, BC46, BC115, BC119; **Bm:** M3, AB45, BC32, BC39, BC43; **Bt:** CD3, CD64, CD68, CD71, M24, AB1, AB2, AB3, AB4, AB10, AB13, AB14, AB49, CD69, CD70; **Bl:** M29, M30, AB22,BC48, BC59, BC63, BC72, BC76, BC78, BC82, BC85, BC116, CD10, DE23, DE26	+	+	+	+	+	+	+	+	64 (24.90)
2	**Bc:** M10, M18, M31, AB5, AB15, AB40, BC5, BC15, BC17,BC41, BC50, BC66, BC75, BC77, BC81, BC106, DE19, M20; **Bm:** BC79,BC101, BC127; **Bl:** BC61	-	+	+	+	+	+	+	+	22 (8.56)
3	**Bc:** BC42, BC47; **Bm:** BC36	+	+	+	+	+	+	-	+	3 (1.17)
4	**Bc:** M11, M13	+	+	+	-	+	+	+	+	2 (0.78)
5	**Bc:** BC52, BC56, BC57	+	+	+	+	+	-	+	+	3 (1.17)
6	**Bc:** BC90, BC110, DE22; **Bm:** M25, CD8; **Bl:** BC93, CD11	+	-	+	+	+	+	+	+	7 (2.72)
7	**Bc:** BC107, BC108; **Bm:** BC49	+	+	+	+	+	+	+	-	3 (1.17)
8	**Bc:** M6, M8, BC68; **Bl:** BC38	-	-	+	+	+	+	+	+	4 (1.56)
9	**Bc:** M21, M22, BC44	-	+	+	+	+	+	+	-	3 (1.17)
10	**Bc:** M19, M27, BC69, BC70	-	+	+	+	+	+	-	+	4 (1.56)
11	**Bc:** AB8	-	+	+	-	+	+	+	+	1 (0.39)
12	**Bc:** BC55	+	-	+	+	+	+	+	-	1 (0.39)
13	**Bm:** CD1, CD9	+	-	+	+	+	+	-	+	2 (0.78)
14	**Bc:** BC12, BC114	+	-	+	+	+	-	+	+	2 (0.78)
15	**Bc:** BC51	+	+	+	+	+	-	-	-	1 (0.39)
16	**Bl:** CD4	-	-	+	-	+	+	+	+	1 (0.39)
17	**Bl:** CD16	+	-	+	+	+	-	+	-	1 (0.39)
18	**Bc:** BC80	-	-	+	+	+	+	-	+	1 (0.39)
19	**Bc:** BC54, BC73; **Bl:** BC99	-	+	+	+	+	-	-	-	3 (1.17)
20	**Bc:** BC123; **Bm:** CD15; **Bl:** M14, M16, M17, AB21, AB25, BC1, BC2, BC3, BC18, BC29, BC31, BC33, BC34, BC35	+	-	-	-	-	+	+	+	16 (6.22)
21	**Bc:** CD57; **Bm:** BC104, BC105; **Bl:** BC13	+	-	+	+	+	-	-	-	4 (1.56)

profile	isolates	gene								no. of isolates (%)
		entFM	*cytK*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	
22	**Bc:** AB9, AB34, BC7, BC10, BC19, BC118; **Bl:** CD30, CD41, CD59, DE27	+	+	-	-	-	-	+	+	10 (3.89)
23	**Bc:** M33, AB36, BC8, BC9, BC11, BC71	-	+	-	-	-	-	+	+	6 (2.33)
24	**Bc:** BC26; **Bl:** M15	-	-	-	-	-	+	+	+	2 (0.78)
25	**Bc:** BC53	+	-	-	-	-	+	-	+	1 (0.39)
26	**Bc:** BC95; **Bl:** AB47, BC4, CD67, DE9, DE15	+	+	-	-	-	-	+	-	6 (2.33)
27	**Bc:** CD13, CD27, **Bm:** CD31; **Bl:** CD21, CD46, CD34	+	-	-	-	-	-	+	+	6 (2.33)
28	**Bc:** CD62; **Bm:** BC20	+	+	-	-	-	-	-	+	2 (0.78)
29	**Bc:** BC14, BC124; **Bm:** BC97; **Bl:** BC92	-	-	+	+	+	-	-	-	4 (1.56)
30	**Bc:** BC112	-	-	+	-	+	-	+	-	1 (0.39)
31	**Bc:** M4, M12, BC88, BC122, M32; **Bl:** AB7, AB19, BC6	+	+	-	-	-	-	-	-	8 (3.11)
32	**Bc:** AB38; **Bm:** AB35, AB41	-	+	-	-	-	-	-	+	3 (1.17)
33	**Bc:** AB42	-	-	-	-	-	+	+	-	1 (0.39)
34	**Bc:** DE18	-	+	-	-	-	+	-	-	1 (0.39)
35	**Bc:** BC64; **Bl:** BC21, BC24	+	-	-	-	-	-	-	+	3 (1.17)
36	**Bc:** BC65	-	-	-	-	-	-	+	+	1 (0.39)
37	**Bc:** CD23; **Bl:** BC22, BC23,CD26, CD45, CD51, DE1, DE2, DE3, DE5, DE6, DE7, DE8 DE11, DE12, DE13, DE14, DE16, DE21, DE28	+	-	-	-	-	-	+	-	20 (7.77)
38	**Bc:** CD12, CD18, CD24, CD36, CD39, CD43, CD44, CD48, CD49, CD50; **Bm:** BC86, CD17, CD29, CD42, CD47; **Bl:** BC27, BC28, BC37, CD14, CD20, CD22, CD25, CD32, CD35, CD38, CD40, CD65	+	-	-	-	-	-	-	-	27 (10.50)
39	**Bc:** AB16, BC111, BC113, CD2, **Bm:** CD28; **Bl:** M2, CD66	-	-	-	-	-	-	-	-	7 (2.72)

[a]**Bc:** *B. cereus;* **Bm:** *B. mycoides;* **Bt:** *B. thuringiensis;* **Bl:** *B. licheniformis*

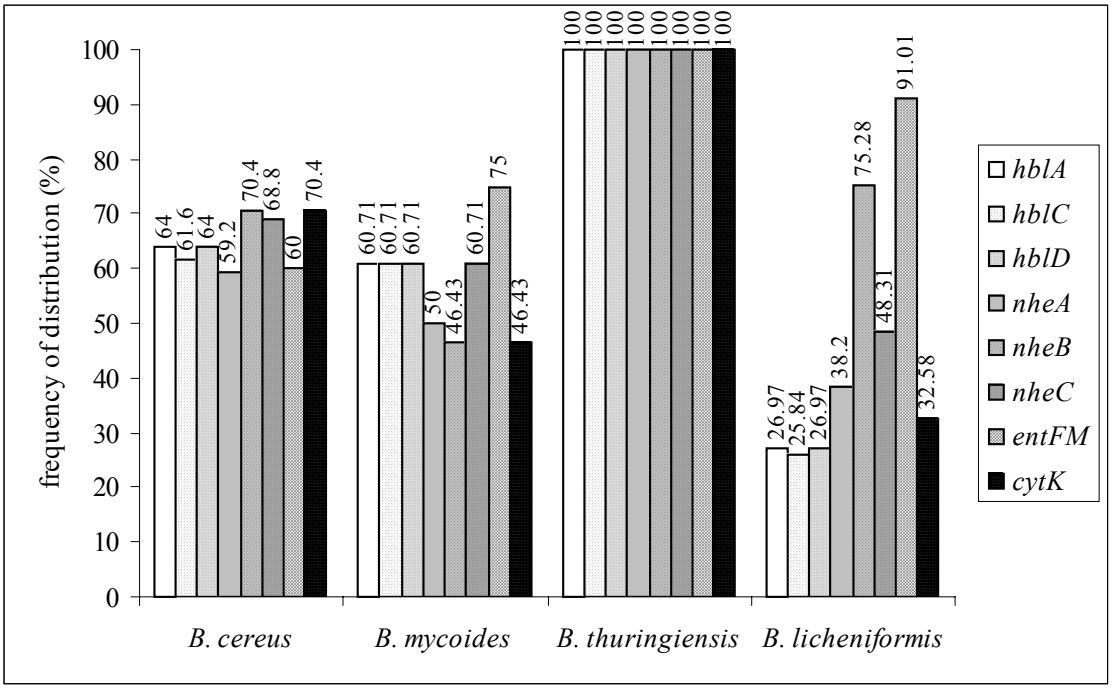

Fig 1. Frequency of distribution of *hblA*, *hblD*, *hblC*, *nheA*, *nheB*, *nheC*, *entFM*, and *cytK* amoung isolates of *Bacillus* species