# Contaminated *Bacillus cereus* in Lao and Thai fermented soybean "Tua Nao"

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## Abstract

Fermented soybeans without salt ("Tua Nao") are commonly produced and consumed in northern areas of Lao PDR and Thailand. Contamination of pathogenic *Bacillus* (*B.*) *cereus* during its production may cause toxic food poisoning. To evaluate the practical risk, we purchased 10 Lao and 23 Thai Tua Nao products from local markets in the northern areas of both countries, and then confirmed *B. cereus* contamination. Nine (90%) of the 10 Lao samples and 18 (78%) of the 23 Thai samples contained *B. cereus*. Two of the Thai isolates produced diarrheal enterotoxin confirmed by a reversed passive latex agglutination test (an immunological method). Emetic toxin coding gene (*crs*) was detected from two Lao isolates by the PCR method. These five (potential) toxin-producing *B. cereus* strains exhibited different random amplified polymorphic DNA (RAPD)-PCR patterns. Improvement of general hygiene control and the use of starter culture are considered necessary to prevent foodborne illnesses caused by *B. cereus* in fermented soybean produced in these countries.

## Introduction

Alkaline fermented soybeans mainly fermented by *Bacillus* (*B.*) *subtilis* without salt are commonly prepared and eaten in East Asia, Southeast Asia, and West Africa as seasonings or side dishes (Wang and Fung 1996, Doyle et al. 2001, Inatsu et al. 2002). "Tua Nao" is one such traditional food produced in Northern Thailand (Leejeerajumnean et al. 2001). Conventionally for Tua Nao production, boiled and smashed soybeans are subjected to fermentation in banana leaves for 2-3 days at ambient temperature. Alternatively, the soybean products are allowed to ferment outdoors exposed to sunlight. The sun-dried Tua Nao can be stored for several months at room temperature (Chantawannakul et al. 2002). Similar sun-dried fermented soybean foods are also produced in Nepal, Yunnan province of China, and the northern areas of Laos and Myanmar (Nikkuni et al. 1995, Inatsu et al. 2002).

*Bacillus* (*B.*) *cereus* is a Gram-positive spore forming bacterium. Some strains produce an emetic (vomit-inducing) toxin and/or diarrheal toxins (enterotoxins) in foods, with acute symptoms appearing 8 to 16 hrs. after the ingestion of polluted foods (Drobniewski 1993, McKillip 2000, Notermans & Batt 1998). A spontaneous remission is observed after 24 hrs. in most cases, and one fatal case caused by polluted pasta salad has been described (Dierick et al. 2005).

According to Mikami et al. (1995), 66% and 3.1% of 524 *B. cereus* strains isolated from the environment and foods produced diarrheal and emetic toxins (cereulide), respectively. A higher (12%) ratio of emetic toxin positive strains was isolated from starch foods made from cereal powders (Kashiwagi et al. 2005). *B. cereus* grows well at room temperature in starch foods to produce heat-resistant emetic toxin (Agata 1997, 2002). For these reasons, many *B. cereus* food poisoning incidents are caused by the intake of cooked rice, noodles or sticky rice cakes (Shinagawa 1990). In December 2001, 346 people who ate sweet red bean paste covered with sticky rice cake (*An-iri-mochi*) in Kumamoto prefecture in Japan suffered food poisoning caused by cereulide (Huruse et al. 2004, Matsuoka et al. 2003).

Fermentation during Tua Nao production occurs due

to naturally contaminated *B. subtilis* strains in ambivalent conditions. Some *B. cereus* strains, a genetically close space with *B. subtilis*, produce either diarrheal or emetic toxins, or both. *B. cereus* may have chances to contaminate during Tua Nao production. However, the risk of contamination is unclear. The objective of this study is to confirm the possibility of *B. cereus* contamination in Tua Nao and evaluate the genetic diversity of toxin-producing strains by using randomly amplified polymorphic DNA (RAPD) fingerprinting analysis.

## Methods

### 1. Isolation and identification of *B. cereus* strains

Four and six Tua Nao samples were purchased from local markets in the northern area of Lao PDR (Oudomxai, Louang Namta, and Muang Sing) in September 2009 and September 2013, respectively. In July 2017, 23 Tua Nao samples were purchased from local markets in the northern area of Thailand (Chiang Rai and Mae Hong Son). Each of the 33 collected sun-dried samples was put into a separate bag and stocked at a cool and dry (25 to 10°C) location until use.

One typical *B. cereus* strain was isolated from each of the collected samples by the method described below. Several small pieces (3 to 5 g) of a sample were put into 25 mL of buffered peptone water (Merck Co. Ltd., Germany). After the sample was mashed by a sterile glass rod, pre-enrichment cultivation was performed for 18 to 24 hrs. at 30°C. One loop of the culture was streaked on an NGKG agar plate (Nissui Co. Ltd., Tokyo) and incubated for 18 to 24 hrs. at 30°C. A typical morphology (including positive egg yolk reaction and positive acid production from mannitol) showing colony was picked up and streaked on an X-BC agar plate (Nissui Co. Ltd., Tokyo). A typical blue colony was picked up after 18 to 24 hrs. of incubation at 30°C and then streaked on a 1% glucose containing Luria-Bertani (LB) agar plate (BD Difco, Co. Ltd., USA). The contents on the NGKG and X-BC agar plates were developed and used commonly to isolate *Bacillus cereus* effectively from food samples. The morphology of cells on the plates after 18 to 24 hrs. of incubation at 30°C was observed by a phase contrast microscope. The strains having several lighting holes in vegetative cells and extrasellar spores were assigned as *B. cereus* (Knaysi 1961). For further identification of the strains, biochemical tests were performed by using the API 50 CHB test kit (Biomerieux Co. Ltd., France) (Ballows et al. 1996, Aruwa & Olatope 2015). This test kit can identify 50 kinds of biochemical characteristics (enzyme production and carbohydrate fermentation) of *Bacillus* strains. Amylase production on a 1% starch

containing LB agar plate was confirmed by the $I_2$-KI method (Inatsu 2002). This method is based on the blue color change of iodine-potassium iodide solution with starch.

### 2. Confirming the toxin production (related gene) of isolated strains

Isolated *B. cereus* strains were incubated for 18 hrs. at 30°C. Bacterial cells and supernatants were separated by centrifuging (8,000 G, 10 min. at 4°C).

The supernatants were used for the assay of diarrheal toxin production after sequential dilution by phosphate buffered saline. A test kit ("CRET-RPLA", Denka Seiken Co. Ltd., Tokyo) based on a reversed passive latex agglutination test was used for testing. This immunological technique enables soluble antigens such as bacterial toxins to be detected in an agglutination assay.

Total DNA in each of the *B. cereus* strains was extracted from the harvested bacterial pellet after centrifuging. "PrepMan Ultra sample preparation reagent" (Applied Biosystems Co. Ltd., California) was used for DNA extraction. The emetic toxin coding gene (*crs* gene) was detected by the PCR method. The *Bacillus cereus* (CRS gene) PCR Detection Kit (Takara Bio Co. Ltd., Kyoto) was used for the PCR reaction. The hemolysin BL gene (*hbl* C) that codes a diarrhea toxin was also assayed by the PCR method (Roman et al. 2003). The obtained PCR products were subjected to 2% agarose gel electrophoresis. DNA fragments in the gel were detected under UV lighting after staining by ethidium bromide.

### 3. Molecular diversity analysis of (potential) toxin-producing strains

Random amplified polymorphic DNA (RAPD) fingerprinting by the PCR reaction of the isolated strains was performed according to Sarkar et al. (2002). The extraction and purification of the chromosomal DNA were done as described previously (Inatu et al. 2002). One of the eight 12-mer synthetic DNA primers listed in Table 1 and Premix Taq (Takara Co. Ltd., Tokyo) were used for each PCR reaction. The 45 cycles of the PCR reaction program were set as "94°C / 1 min. of denature, 40°C / 1 min. of annealing, and 72°C / 1 min. of extension" according to Kuwana et al. (2012). The obtained PCR fragments were electrophoresed on 1.5% agarose gel and visualized with Cyber Green under UV exposure. A 100-bp ladder DNA (Nippon Gene Co. Ltd., Tokyo) was used for the molecular weight marker.

All experiments were conducted three times to confirm the reproducibility of the results.

**Table 1. DNA sequences of RAPD-PCR primers used in this study**

| Name | Sequence |
|------|----------|
| Primer-A | ATCAGCGCACCA |
| Primer-B | GCCAGCTGTACG |
| Primer-C | TGCCTCGCACCA |
| Primer-D | GCCCCGTTAGCA |
| Primer-E | CCGCAGTTAGAT |
| Primer-F | ACTGGCCGAGGG |
| Primer-G | TTCGGACGAATA |
| Primer-H | AGAATTGGACGA |

Eight DNA primers (12-mer) were used for preliminary experiments, and four were used for the main experiments (see Fig. 4).

## Result and discussion

*B. cereus* strains were isolated from 9 of 10 Lao (90%) and 18 of 23 (78%) Thai Tua Nao samples, respectively. Because three Lao *B. cereus* strains (N11, N41, and O21) harbored the cereulide coding (*crs*) gene (Fig 1 (a)), there is a possibility that some or all of the strains produce a vomit-inducing (emetic) toxin. (The PCR method is commonly used because directly detecting a vomit-inducing toxin is not easy.) Two Thai *B. cereus* strains (B71 and B91) harbored the hemolysin BL coding gene (*hbl* C) (Fig 1 (b)). The diarrhea toxin activity of the culture supernatants of the B71 or B91 strains was detected after $2^6$ or $2^1$ times dilution, respectively (No other isolated strains exhibited this activity).

In this work, we found five toxin-producing *B. cereus* strains from 27 isolated *B. cereus* strains. This means that 19% of the collected Tua Nao samples were contaminated with toxin-producing *B. cereus* strains. Precisely estimating this proportion only from the results obtained in this work is difficult, however, due to the low numbers of assayed samples. (Lower and upper 95% confidence intervals (CI) of this value were calculated as 6% and 38%, respectively.) In cases where a more precise risk assessment of *B. cereus* in Tua Nao is required, at least 10 times the number of samples should be assayed (The 95% CI will theoretically change from 14 to 24% under the same average value).

Figure 2 shows the cell morphology and colony morphology of these five (potent) toxin-producing strains. Though there were two different shapes of colonies on the NGKG agar plates, we could not find a clear relationship between colony morphology and the production of each toxin. Based on the assay of Japanese *B. cereus* strains isolated from the foods causing diarrheal food poisoning or in the victims thereof, Shinagawa (1990) found a positive relationship between the starch hydrolysis and enterotoxin production of *B. cereus* strains (In this study, 96% of 113 *B. cereus* strains isolated from wholesome foods hydrolyzed starch and produced enterotoxin). Ogawa et al. (2014) also obtained a similar result (All nine *B. cereus* strains isolated from the victims of food poisoning and 95% of the 21 *B. cereus* strains
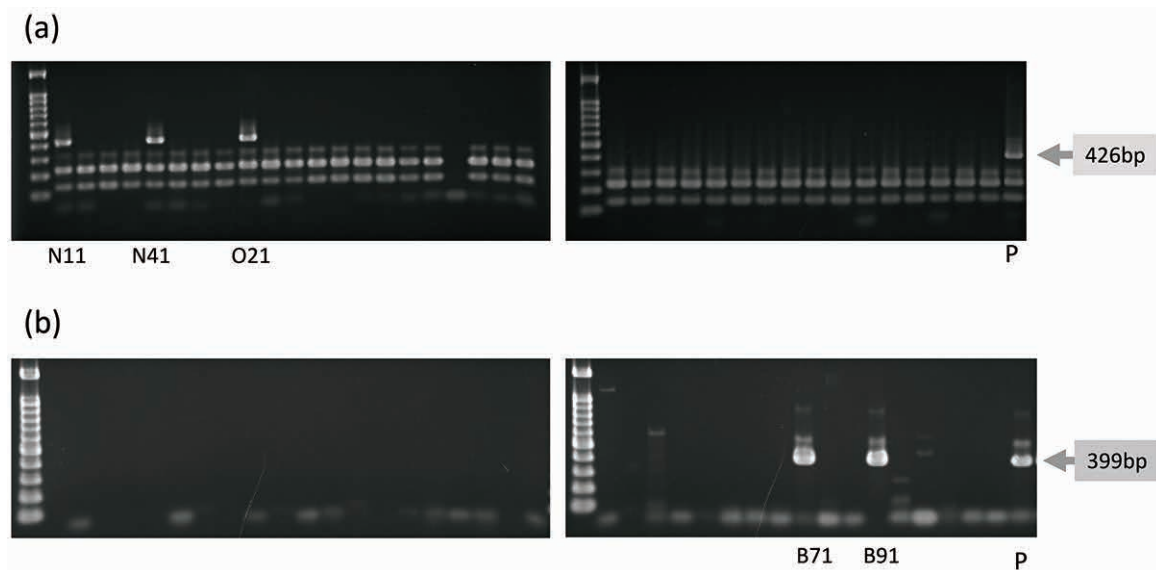


**Fig. 1. Detection of an emetic toxin coding gene (a) or a diarrheal toxin coding gene (b) by the PCR method**
All 27 isolated *B. cereus* strains were assayed. Only the names of positive result samples are shown. A 426-bp fragment (a) or 399-bp fragment (b) corresponds to part of the *crs* gene or *hbl* C gene, respectively. The sample name "P" corresponds to a positive control strain. The lane in the most left position in each gel showed the bands of the 100-bp molecular weight marker (with the smallest band corresponding to 100 bp).

isolated from the foods causing said food poisoning hydrolyzed starch and produced enterotoxin). Based on these results, starch hydrolysis on an agar plate is commonly used for estimating the enterotoxin production of *B. cereus* strains at least in Japan. However, only the B91 strain produced both amylase and enterotoxin in this work (The B71 strain did not produce amylase). To clarify this different point, more toxin-producing *B. cereus* strains isolated from Tua Nao need to be assayed.

The genetical variety of isolated toxins producing *B. cereus* strains was confirmed by the RAPD-PCR method. To optimize the experimental conditions, one Thai and four Japanese toxin-producing strains isolated from

soybean curds were also used with the five toxin-producing strains isolated in this work (data not shown). Figure 3 shows the RAPD-PCR patterns of the five toxin-producing *B. cereus* strains isolated from the Tua Nao samples. Given the differences in fragment patterns among the five isolated toxin-producing strains, all the strains were thought to be different ones. Compared with primer B, the other three primers tended to give rather complicated fragments patterns. However, too many bands in a lane may cause confusion during analysis of the results. The use of two or more primers for the RAPD-PCR assay will be helpful toward increasing the resolution of the experimental results. This method will
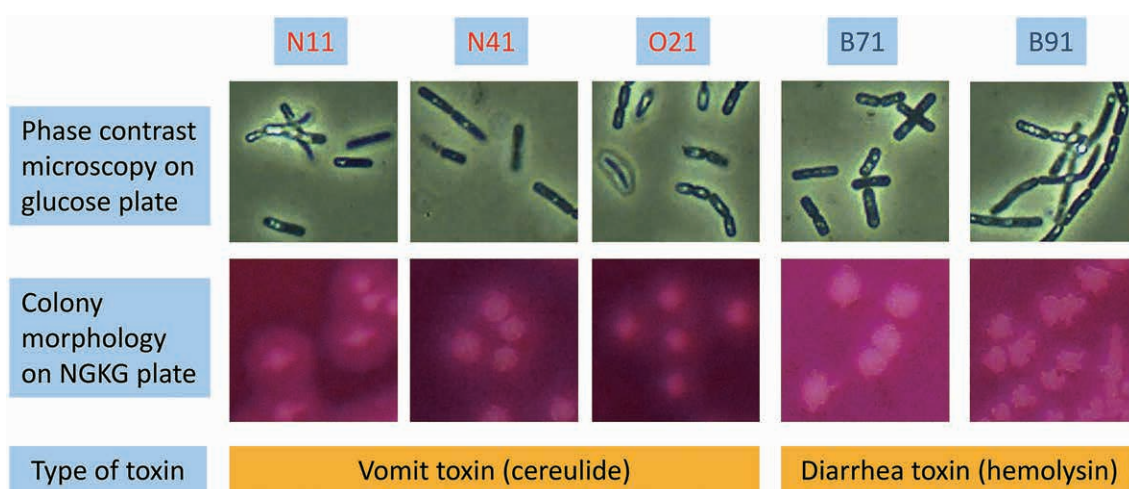


**Fig. 2. Colony morphology and cell morphology of five isolated (potential) toxin-producing *B. cereus* strains**
Cells grown on a 1% glucose containing agar plate at 30°C for 24 hrs. were observed directly by a phase contrast microscope (pace of expansion: 1,000 times). Colonies developed on the NGKG agar plate after 24 hrs. of incubation at 30°C are shown.
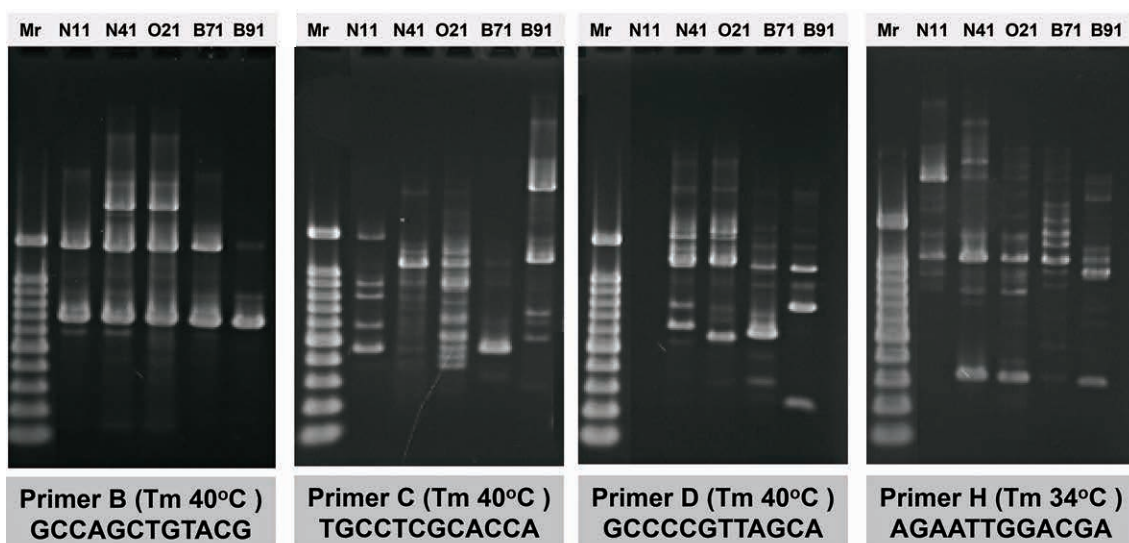


**Fig. 3. RAPD-PCR patterns of 5 isolated (potent) toxin-producing *B. cereus* strains**
Four kinds of 12-mer PCR primers were used for assaying each of the five strains. The lane "Mr" shows the bands of the 100-bp molecular weight marker (with the smallest band corresponding to 100 bp).

be applicable to fix the contamination routes of *B. cereus* into Tua Nao by comparing the fragments patterns of the strains isolated from the final product and suspected sources (The strains that show different patterns should be different strains).

    *B. cereus* and *B. subtilis* are ubiquitous in nature and frequently isolated from soil. As a common inhabitant of soil, *B. cereus* can easily be transmitted into vegetables or crops and hence into foods. *B. cereus* is genetically similar to *B. subtilis*, the main bacterium of Tua Nao fermentation. Because both bacteria exhibit similar growth characteristics under suitable conditions, the contamination of *B. cereus* during natural fermentation by *B. subtilis* may easily occur. Similar to *natto* production in Japan, the use of starter culture under hygienic conditions is one way to deal with this problem. Even today, some people in Lao PDR and Thailand eat homemade or raw Tua Nao produced by small businesses (Chukeatirote 2015). Because steamed or boiled soybeans used for Tua Nao production can be a good media for the growth of both *B. subtilis* (fermenting bacteria) and *B. cereus* (sometimes producing toxins), preventing the contamination caused by *B. cereus* requires the implementation of general hygienic management (i.e., keeping the workplace and cooking utensils clean).

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