Research Article

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Wallapat Phongtang, Ekachai Chukeatirote*

Incidence and characterisation of *Bacillus cereus* bacteriophages from Thua Nao, a Thai fermented soybean product

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Abstract: *Bacillus cereus* is considered to be an important food poisoning agent causing diarrhea and vomiting. In this study, the occurrence of *B. cereus* bacteriophages in Thai fermented soybean products (Thua Nao) was studied using five *B. cereus sensu lato* indicator strains (four *B. cereus* strains and one *B. thuringiensis* strain). In a total of 26 Thua Nao samples, there were only two bacteriophages namely BaceFT01 and BaceCM02 exhibiting lytic activity against *B. cereus*. Morphological analysis revealed that these two bacteriophages belonged to the *Myoviridae*. Both phages were specific to *B. cereus* and not able to lyse other tested bacteria including *B. licheniformis* and *B. subtilis*. The two phages were able to survive in a pH range between 5 and 12. However, both phages were inactive either by treatment of 50°C for 2 h or exposure of UV for 2 h. It should be noted that both phages were chloroform-insensitive, however. This is the first report describing the presence of bacteriophages in Thua Nao products. The characterization of these two phages is expected to be useful in the food industry for an alternative strategy including the potential use of the phages as a biocontrol candidate against foodborne pathogenic bacteria.

Keywords: *Bacillus cereus*; bacteriophages; fermented soybean; stability.

Introduction

Bacillus cereus, a member of *B. cereus sensu lato* group, is a Gram-positive, rod-shaped, spore-forming bacterium which is widely distributed in nature [1–3]. *B. cereus* is

also a well-known food poisoning bacterial pathogen; the species can be isolated from various kinds of food products including cereals, dairy products, dried foods, eggs, meat, rice, spices, and vegetables [4]. It has been recognized as one of the major important foodborne pathogens although its occurrence and outbreak is believed to be underestimated [5]. For example, *B. cereus* has now been described as the second most frequently encountered causative agent of confirmed and suspected food-borne outbreaks in France after *Staphylococcus aureus* [6]. In addition, during the past decade, numerous reports related to foodborne illness outbreaks have shown that these incidences are due to *B. cereus* contamination which includes the outbreaks in Spain [7], Australia [8], Argentina [9], England [10], and Indonesia [11]. *B. cereus* causes two types of gastrointestinal diseases: diarrheal syndrome (intestinal infection) characterized by diarrhea and abdominal pain associated with enterotoxins produced during bacterial growth in the intestines; and emetic syndrome (food poisoning) characterized by nausea and vomiting related to cereulide synthesized by *B. cereus* cells in food products [12]. The diarrheal type is related to enterotoxins, produced during the growth of *B. cereus* in the digestive tracts, while the emetic type is produced by the growing *B. cereus* cells in the food. Nevertheless, surviving spores are found to be the source of both intestinal infection and food poisoning. Although *B. cereus* is not a competitive microbe, it grows well after cooking and cooling. Moreover, after heat treatment, its spores germinate and produce toxins associated with these illnesses [13].

At present, several technologies have been employed and developed to control the foodborne pathogenic microbes. These include physical, chemical, and biological approaches with a major aim to inactivate food pathogens [14]. However, the effectiveness of treatments depends on many factors (e.g., food type, nutrient quality, consumer's acceptability). For example, the treatments available for fresh food products that have to be eaten raw

*Corresponding author: Ekachai Chukeatirote, School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand, E-mail: ekachai@mfu.ac.th

Wallapat Phongtang, School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand

appear to be limited. Therefore, there remains a search for new techniques that can be used to guarantee food quality and safety. Bacteriophages (or phages) are natural killers of bacteria. They are omnipresent and, according to a number of studies, they are probably the most abundant entities on Earth [15, 16]. Since their discovery, phages have been used and applied in various themes (e.g., phage therapy and phage display). In the food industry, phages are also significant, as they can kill the bacterial starter culture, leading to a delay or disruption of a fermentation process. In contrast, phage biocontrol is introduced and involves the application of phages aiming to reduce the bacterial population. When used in clinical trials, this strategy is known as phage therapy. Potential advantages of phages include their ability to destroy their host specifically. Unlike antibiotics, the use of phages is expected to be less disruptive to commensal microbial communities. Besides, the development of phage-resistant bacteria is less (although possible) when comparing with the antibiotic treatment. Several successful examples of phage biocontrol in food products have been described [17, 18]. For *B. cereus*, only a few studies have shown that the phages could be isolated and had a potential in controlling *B. cereus* [19-21]. This leaves room for further research and development of phage infecting *B. cereus*.

Thua Nao is a Thai fermented soybean product which is widely consumed in northern Thailand [22]. Similar products include Japanese Natto and Indian Kinema. At present, the most promising phage research focuses on other fermented foods; these include fermented cucumber, fermented milk products, kimchi, and sauerkraut [23-25]. The study of phages in fermented soybean product is also limited to Natto and Cheonggukjang [26]. Because of these reasons, this study was proposed focusing on Thua Nao product and bacteriophages. The aims of the present study were i) to evaluate the incidence of *Bacillus*-infecting phages especially for those specific to *B. cereus*; and ii) to characterize the phages infecting *B. cereus* for possible biocontrol application.

Materials and Methods

Samples collection

Twenty-six Thua Nao samples (nine fresh and 17 dried samples) were purchased from different local markets in Chiang Rai (19 samples), Chiang Mai (five samples), Lampang (one sample), and Mae Hong Son (one sample). In general, approximately 100 g of each sample was

collected in sampling bags, transferred to the laboratory, and stored at 4°C until required.

Isolation of phages

Bacterial strains used for bacteriophage isolation were three *B. cereus* reference strains (ATCC 11778, TISTR 687, and TISTR 1527), *B. cereus* TN69, and *B. thuringiensis* S2-3. These bacteria were grown in either tryptic soy broth (TSB) or agar (Difco Laboratory, Detroit, MI, USA) at 37°C overnight. To isolate the bacteriophages, 20 g of the sample was mixed with 80 ml of TSB and 1 ml of each overnight-grown bacterial host culture. The mixture was incubated at 37°C with shaking (180 rpm) for 72 h. Then, the culture was centrifuged at 8000 x g, 10 min, and the supernatant was filtered through a 0.2 µm membrane filter (Whatman, Little Chalfont, UK). The filtrate was used in a plaque assay in order to detect bacteriophages, that were then propagated and purified following the method previously described by Sambrook and Russel [27].

Host range determination

Isolated bacteriophages were tested for host range against *B. cereus* strains (ATCC 11778, TISTR 687, TISTR 1527, and TN69); *B. subtilis* strains (ATCC 6633, ASA, S1-13, S4-5, TN3, and TN51); *B. thuringiensis* S2-3; *B. licheniformis* TISTR 1109; and five non-*Bacillus* species (*Enterobacter aerogenes* TISTR 1540, *Escherichia coli* TISTR 527, *Micrococcus luteus* TISTR 884, *Pseudomonas aeruginosa* TISTR 781, and *Staphylococcus aureus* TISTR 746). The spot test was then performed to determine the host range of the isolated phages. For this, the soft agar overlayer containing the indicator strain was prepared, on which 20 µl of the phage stock (10^4 – 10^6 PFU/ml) was spotted and incubated overnight for lysis zone formation.

Determination of multiplicity of infection (MOI)

MOI was typically defined as the ratio of virus particles to potential host cells. In order to determine the optimal MOI, the early exponential phase bacterial host strain cultured in TSB broth at 37°C were inoculated with dilutions of the phage samples to give the MOI of 0.1, 1, 10 and 100. Bacteria-free suspensions and phage-free suspensions were also prepared as the controls for all experiments. After incubation at 37°C for 2-3 h, the samples in triplicate

were taken from each MOI experiment, and the viable bacterial cell counts were determined by plating samples on TSB agar and then incubating overnight at 37°C. To evaluate the optimal MOI, a reduction rate ($\log N_t/N_c$) was calculated by comparing the number of the host cells in the control (N_c) with the number of the host cells treated with the bacteriophage (N_t) after 2-3 h incubation.

Phage morphology examination

A drop of purified phage stock (approximately 10^8 PFU/ml) was spotted onto a carbon-coated copper grid and left at room temperature for 6 min. After removal of excess fluid, negative staining with 2% (w/v) uranyl acetate for 10 min was then applied on the sample. Excess fluid was removed by absorbent paper. After drying, the phage morphology was observed with a JEOL transmission electron microscope (JEM-2010, Japan) at an accelerating voltage of 160kV. The phages were classified based on the information of the International Committee on Taxonomy of Viruses [28]. The phage size was determined from the average of at least three independent measurements.

Phage stability under different conditions

The stability of the isolated phages was then evaluated under different conditions, including pH, temperature, UV, and chloroform. Phage samples were incubated in TSB broths with different pH values in the range of 3 - 13 at 37°C for 2 h. Similarly, phage stocks were incubated at 4, 37, 50, 55 and 60°C for 2 h. For UV stability assay, the phage samples were placed in sterile Petri dishes and irradiated for 0, 15, 30, 45, 60 and 120 min with a UV lamp ($\lambda = 254$ nm) in a laminar flow cabinet. Resistance to chloroform was also determined by adding 5% chloroform to the phage samples. In addition, to investigate the presence of lipid in the viral capsid, an equal volume of the phage sample was mixed with chloroform and incubated for 30 min at room temperature [29]. After all these experiments, the bacteriophage titre was determined using the double-layer agar technique.

Ethical approval: The conducted research is not related to either human or animals use.

Results and Discussion

Incidence of *B. cereus* bacteriophages in Thua Nao

Twenty-six Thai fermented soybean samples (Thua Nao), including 17 dried samples and nine fresh samples, were used to screen and isolate *B. cereus* bacteriophages using five *B. cereus sensu lato* strains as indicators. Based on this experiment, only two Thua Nao samples were shown to contain bacteriophages (to form plaques), and two bacteriophages were thus isolated. These included BaceFT01 and BaceCM02 obtained from Chiang Rai and Chiang Mai sources, respectively. Both bacteriophages produced clear plaques with different sizes (Figure 1) on *B. cereus* TISTR687 bacterial lawn, and thus this bacterial strain was used for all subsequent experiments. For further characterization, the two phages were propagated and purified using the liquid culture technique. A high-titre stock was also prepared from the plate elution method.

The isolation rate of *B. cereus* bacteriophages obtained from our study was quite low (7.7%), when compared to the data reported by Shin et al. [20], Bandara et al. [30], and Oh et al. [21]. These previous works showed that the isolation rate of *B. cereus* phages from Cheonggukjang products (Korean fermented soybean samples) was high ranging from 37.8 to 100%. Thua Nao and Cheonggukjang are similar in terms of the manufacturing process and product type. Other similar fermented soybean products include Japanese Natto and Indian Kinema [22]. *B. cereus*, an opportunistic foodborne pathogenic bacterium, can be found in various food products including cereals, dairy products, and dried foods. The presence of *B. cereus* in the food products can be either from the original food substrates, production process, or environmental contamination of the food products. Interestingly, there might be a relationship between the *B. cereus* host and the *B. cereus* bacteriophages. Based on our study, *B. cereus* was not found in all Thua Nao samples when initially screened by mannitol egg yolk polymyxin (MYP) agar plates (data not shown). The low incidence (8.3%) of *B. cereus* in Thua Nao samples was also found as reported by Suriyanoi et al. [31]. It should be noted, however that the high incidence of *B. cereus* was also detected in Thua Nao (78%) as described by Inatsu et al. [32].

Figure 1: Plaque formation by double layer nutrient agar plates of *B. cereus* bacteriophages isolated from Thua Nao: BaceFT01 (A) and BaceCM02 (B).

Host range of *B. cereus* bacteriophages

The host range of the two bacteriophages BaceFT01 and BaceCM02 was determined against both *Bacillus* (twelve strains) and non-*Bacillus* bacterial species (five strains). Our data revealed that the BaceFT01 and BaceCM02 bacteriophages could infect only two *B. cereus* strains (ATCC 11778 and TISTR 687). Both bacteriophages did not infect other bacterial hosts used (including the remaining three *B. cereus sensu lato* strains and other *Bacillus* species). This finding was consistent with the previous work demonstrating that *B. cereus* bacteriophages isolated from the fermented soybean products were only specific to *B. cereus* bacterial host [20, 21, 30]. It is well known that most bacteriophages are specific for a bacterial species and many of them can only infect the specific strains within a species. There are advantages and disadvantages of this limited host range. For example, these two *B. cereus* bacteriophages may be of great importance if they could be used to prevent contamination of *B. cereus* during a soybean fermentation. Such a high specificity will not cause a harm to other microbes especially for the beneficial microbes (e.g., *B. subtilis*, a bacterial starter culture in the soybean fermentation process). In contrast, the bacteriophages with a broad host infectivity (i.e., to many *B. cereus* strains) would be of great significance for controlling the disease outbreaks.

Optimal multiplicity of infection (MOI)

The lytic activity of the two bacteriophages against *B. cereus* was then assessed at different MOIs ranging

from 0.1 to 100, and our data showed that the optimal MOI value of both bacteriophages was 100 (Figure 2). In general, the bacterial growth inhibition seemed to be MOI-dependent. This was evident especially for the bacterial growth inhibition derived from the treatment of the bacteriophage BaceFT01. Although the addition of the phage BaceCM02 with MOI values of 1 and 10 seemed not to affect the bacterial growth, it could be clearly seen that the phage BaceCM02 with an MOI of 100 showed the highest reduction in bacterial growth (more than 2 log reduction). The knowledge on the phage characteristics (e.g., growth and stability) is crucial considering from the application viewpoint. For example, the level of the bacteriophage may be a limiting factor, considering that during the initial infection process, the ratio between the phage and the host affects the attachment and adsorption, leading to specificity in the phage-host interactions [33]. In addition, the optimal MOI is suggested to be the recommended mixing ratio for large-scale proliferation and enrichment of the bacteriophage [34].

Morphology of *B. cereus* bacteriophages

The morphological characteristics of *B. cereus* bacteriophages were determined using transmission electron microscopy (Figure 3). Both isolates consisted of an icosahedral head (90 and 62 nm in size for the phages BaceFT01 and BaceCM02, respectively), and a long contractile tail (166 and 188 nm, respectively) (Figure 3). Both bacteriophages were classified as *Myoviridae* phages based on their morphology. To date, most known bacteriophages (more than 96%) belong to

Figure 2: Log reduction of *B. cereus* TISTR 687 with different multiplicities of infection (MOIs) of bacteriophage BaceFT01 (Δ) and BaceCM02 (×).

Figure 3: Transmission electron micrographs of *B. cereus* bacteriophages: BaceFT01 (A) and BaceCM02 (B). The scale bars shown are 100 nm.

Figure 4: Influence of temperature on stability of the *B. cereus* bacteriophages BaceFT01 (Δ) and BaceCM02 (×).

the order *Caudovirales* (or tailed bacteriophage group) [35]. Based on this distinct tailed structure, the phages in the order *Caudovirales* can be classified into three families: *Myoviridae* (long contractile tails), *Siphoviridae* (long non-contractile tails), and *Podoviridae* (short non-contractile tails). At present, it has been reported that the bacteriophages infecting *B. cereus* are tailed bacteriophages being the members of the three families, although it should be noted that the two families of *Myoviridae* and *Siphoviridae* are the most abundant in the *B. cereus* group phage [36]. This finding coincided with previous studies, especially when considering that the *B. cereus* bacteriophages isolated from the fermented soybean products, were members of the *Myoviridae* and *Siphoviridae* families [26].

Phage stability

To evaluate the phage suitability for potential applications (e.g., as a biocontrol agent in the food industry), we undertook the phage stability assays under different conditions including pH, temperature, UV, and chloroform. We found that the activity of the two phages (BaceFT01 and BaceCM02) was similar as both phages were stable between 4 and 37°C (Figure 4). The temperature is one of the key factors for bacteriophage survival [37]. It is known that the temperature

involves the mechanism of attachment, penetration, multiplication, and the length of the latent period (in the case of lysogenic phages). For example, at lower than optimal temperatures, fewer phage genetic materials could penetrate into the bacterial hosts leading to a slow (or delayed) rate in the multiplication phase. Moreover, bacteriophages could potentially lose the ability to attach to the host cells or might simply disintegrate in the presence of high temperature [38]. For pH stability, both bacteriophages were stable (>60% of the phage titre remained) at pH values from 6 to 10 (Figure 5). The pH value is another important factor affecting the phage occurrence, stability, and survivability. Under an acidic condition, this also causes phage coagulation and precipitation [38]. The phage stability when treated with UV ($\lambda = 254$ nm) was also investigated. Figure 6 showed that the two phages (BaceFT01 and BaceCM02) remained active after 15 min of UV irradiation. Both phages were completely inactive after 2 h-UV treatment. The ultraviolet light is known to cause DNA damage, and besides, it can also cause a peptide bond alteration. The previous work has shown that UV is a major cause of phage mortality especially in marine habitats [39]. In the chloroform test, our results showed that the viability of both phages was unaffected (>90% of the phage titre remained) in the presence of 5% chloroform. Additionally, the treatment of chloroform (using an equal volume of chloroform and phage sample and incubating for 30 min) had no effect

Figure 5: Survival of the *B. cereus* bacteriophages BaceFT01 (Δ) and BaceCM02 (×) after 2 h at 37°C in TSB broths with different pH values in the range of 3 – 13.

Figure 6: Effect of ultraviolet irradiation on viability of the *B. cereus* bacteriophages BaceFT01 (Δ) and BaceCM02 (×).

on the activity of both phages, suggesting an absence of lipids in the phage capsid.

Conclusion

Based on the present study, we isolated two *Myoviridae* bacteriophages from Thua Nao, a Thai fermented soybean product. Both bacteriophages (BaceFT01 and BaceCM02)

were specific for *B. cereus*. Moreover, our data provided some fundamental knowledge as well as important characteristics of these two *B. cereus* bacteriophages. A promising use of bacteriophages as anti-*B. cereus* candidate in soybean fermentation may be developed for industrial purpose. This article also adds a growing body of research to the scientific community describing the potential role of phage as a biocontrol agent in foods.

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Conflict of interest: Authors state no conflict of interest

Data Availability Statement: The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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