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Full Length Research Paper

Bacteriophage specific to nisin producing-*Lactococcus lactis* subsp. *lactis* TFF221, a starter culture inThai fermented food

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A lytic bacteriophage ϕTFF221, specific for nisin-producing *Lactococcus lactis* subsp. *lactis* TFF221, was first isolated from kung-jom, a Thai traditional fermented shrimp paste. The plaques were clear and round-shaped on the lawn of bacteria, indicating its lytic activity. Restriction analysis indicated that genome of phage ϕTFF221 was double-stranded DNA. Transmission electron microscopy revealed that it had an icosahedral head with a contractile tail and a baseplate, and might be tentatively classified as a member of the *Myoviridae* family. Structural protein profile of phage ϕTFF221 by SDS-PAGE showed five bands with molecular masses estimated at, 45.3, 39.8, 26.8, 16.5, and 8.9 kDa. One-step growth experiment of its lytic activity appeared latent, and burst periods of 30 and 120 min, respectively, with a burst size of about 60 PFU/infected cell. The phage was highly specific to its host. None of the tested LAB strains was sensitive to phage ϕTFF221. Phage was stable over a wide pH range of 4 to 10 at 30°C for 1 h, and survived after heating at 70°C for at least 3 min. Phage had more survivals in 1 to 5% of NaCl than in 10% of NaCl. Kinetic monitoring of *L. lactis* subsp. *lactis* TFF221-GFP$^+$ starter, which was containing pN014-GFP and had a fluorescent ability, and phage ϕTFF221 in food model, demonstrated the considerable decrease in the number of the starter in the presence of phage. Knowledge of these basic properties of this phage might be important for the development of using starter fermentation.

Key words: Bacteriophage, fermented food, Lactococcus lactis.

INTRODUCTION

Lactic acid bacteria (LAB) are commonly used as starter cultures in the production of a great multitude of fermented foods (Caplice and Fitzgerald, 1999). These organisms are able to produce a wide variety of antimicrobial compounds, including proteinaceous substances called bacteriocins (Leroy and de Vuyst, 2004). The bacteriocin nisin, produced by some strains of *Lactococcus lactis*, has received particular attention because of its large inhibitory effect against wide variety of food-spoilage microorganisms and food-borne pathogens (Klaenhammer, 1993). Nisin is the only bacteriocin generally regarded as safe (GRAS), and has approved its use as a food additive (Delves-Broughton, 1990). Therefore, the development of nisin-producing lactic acid bacteria to be used as starter culture in food fermentation has been widely studied (Noonpakdee et al., 2003; Rattanachaikunsopon and Phumkhachorn, 2008).

In our previous study, *L. lactis* subsp. *lactis* TFF221, which is nisin-producing, has been isolated from kung-jom, a Thai traditional fermented shrimp paste (Rattanachaikunsopon and Phumkhachorn, 2008). It has also been developed for use as a functional starter culture in order to obtain products with high quality, consistency and safety from some serious food pathogens. However, one of the most persistent and stubborn problems in using starter culture fermentation is bacteriophage (phage) infection which prevents normal ripening that causes fermentation delay or failure, and results in financial losses (Moineau et al., 1996). The problem has stimulated researchers and manufacturers to continually devise innovative and effective procedures

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to alleviate the devastating consequences of the attacking phage. In order to effectively control the phage infection, it is imperative to thoroughly understand the important properties of the trouble phage.

Phages, which are viruses that infect bacteria, require bacterial host cells for growth and replication. At the initial step of phage infection, the phage attaches itself to the outermost part of the bacterial host cell and then, injects its genetic material into the cell. Inside the cell, the genetic material encodes phage protein coats, for the progeny phages which are rapidly filled with newly synthesized phage genome. Finally, the bacterial cell lyses and eventually dies as lots of progeny phages are released. The released phages attack new bacteria nearby (Sandeep, 2006). This process continues until all the bacteria are entirely eliminated from the ecosystem. Therefore, if there is any contaminated phage which is specific to the added starter bacterial strain in the fermentation system, it can rapidly bring fermentation to a halt.

Phage infection of the starter culture is a common occurrence in various kinds of fermented foods (Jarvis, 1989). Among the phages that infect starters in fermented foods, those isolated from dairy foods and vegetable fermentations have been studied most extensively (Lu et al., 2003; Capra et al., 2006; Yoon et al., 2007). Taxonomic classification of lactococcal phages continues to be an area of debate. All *L. lactis* phages documented so far are double-stranded DNA phages, and the vast majority has been classified in *Siphoviridae* family (Kotsonis et al., 2008). However, knowledge about lactococcal phage specific to starter culture in Thai fermented food is quite scarce.

The objective of this study is to isolate and characterize phage specific to nisin-producing *L. lactis* subsp. *lactis* TFF221. To our knowledge, this is one of the very first works concerning the investigation of the presence of phage attacking starter culture in Thai fermented foods. Our study also examines the inoculated phage and its host proliferation in food matrixes. The results of this experiment emphasize the importance of this phage as a distinct devastator in starter fermentations. Results presented in this study could be used as basic information to develop defense strategies and systems for curtailing the phage in fermentation process, or for developing a phage-resistance strain of nisin-producing *L. lactis* subsp. *lactis* TFF221 to be used as a suitable starter in commercial practice.

MATERIALS AND METHODS

Bacteria, phage, culture conditions and phage titration

Nisin-producing *L. lactis* subsp. *lactis* TFF221 strain was used as a host bacterium for phage isolation and propagation. The strain was grown in deMan Rogosa Sharpe (MRS) medium at 30°C. To test the host range of the phage, 17 strains of LAB were used (Table 1). All of LAB used in this study was grown in MRS medium at 30°C.

Table 1. Host range specificity study of phage ϕTFF221.

Bacterial strains[a]	Spot test[b]
Lactococcus lactis ATCC11454	–
Lactococcus lactis subsp. *lactis* TFF221	+
Lactococcus lactis subsp. *lactis* ATCC11007	–
Lactobacullus acidophilus ATCC4356	–
Lactobacillus brevis ATCC14869	–
Lactobacillus brevis UBUB001	–
Lactobacillus casei ATCC334	–
Lactobacillus curvatus ATCC25601	–
Lactobacillus delbrueckii subsp. *lactis* ATCC12315	–
Lactobacillus pentosus ATCC8041	–
Lactobacillus plantarum ATCC8014	–
Leuconostoc cremoris ATCC19254	–
Leuconostoc fallax ATCC700006	–
Leuconostoc mesenteroides TISTR473	–
Pediococcus pentosaceus ATCC25745	–
Pediococcus pentosaceus TISTR374	–
Enterococcus faecalis ATCC29212	–
Enterococcus faecalis TISTR927	–

a; American type culture collection (ATCC); Thailand Institute of Scientific and Technological Research (TISTR); TFF and UBUB, Culture Collection of Biological Science Department, Faculty of Science, Ubon Ratchathani University. b+, clear zone; – no clear zone.

The bacterial cultures were stored as stock cultures in MRS broth supplemented with 20% (vol/vol) glycerol at -70°C.

The phage was purified by single plaque isolation (Lu et al., 2003). A single plaque was picked from the lawn of the bacterial host, and propagated in 10 ml of an early log phase *L. lactis* subsp. *lactis* TFF221 culture (10^6 CFU/ml) in MRS broth, supplemented with 10 mM $CaCl_2$ (MRS-Ca). After incubating at 30°C overnight, phage lysate was centrifuged at 4500 × *g* for 10 min. The supernatant was filtered through 0.45 µm membrane filter (Sartorius, Goettingen, Germany). Phage stock was stored at 4°C, and an aliquot was frozen at -70°C.

Phage titer was enumerated as plaque forming unit (PFU/ml) by using the double-layer agar plaque method. Briefly, 100 µl of diluted phage solution, 100 µl of the bacterial overnight culture, and 5 ml of MRS-Ca soft agar (0.7% agar) were mixed in a glass tube and poured onto a MRS agar containing Petri dish. Plates were incubated for 18 h after which, plaque forming unit were counted.

Food samples preparation and phage isolation

Thirty of traditional fermented food samples were randomly purchased at local markets in Ubon Ratchathani province, Thailand (Table 2). To prepare the food samples for phage isolation, 25 ml of brine samples of fermented vegetable samples (“pak-dong”) were collected and centrifuged at 4500 × *g* for 10 min and supernatants were used for phage isolation. Five grams of fermented meat samples (“nham”, “sai-krog”, “mom”, “kung- jom”, “pla-som” and “kem-buk-nud”) were mildly blended in 50 ml of phosphate buffered saline (PBS) with stomacher apparatus and the homogenates were centrifuged at 4500 × *g* for 10 min and the supernatants were collected for phage isolation.

Table 2. Types of fermented food for phage isolation.

Fermented food	Number of samples	Number of isolated phage
pak-dong	7	0
nham	4	0
sai-krog	4	0
mom	3	0
kung-jom	4	1
pla-som	4	0
kem-buk-nud	4	0

Phages were isolated from the samples by using enrichment protocol (Lu et al., 2003). The obtained supernatant of a food sample was filtered through 0.45 µm pore size membrane filter. The filtrate was added to an equal volume of double strength MRS-Ca broth and inoculated with an early log phase of host culture. After overnight incubation at 30°C, the medium was centrifuged at 4500 × *g* for 10 min. The obtained supernatant was passed through 0.45 µm membrane filter for ascertainable bacterial sterilization and then the filtrate was tested for the presence of phages.

Phage detection and host range determination

Phage detection was performed, using a spot test method (Lu et al., 2003). The test was used for the presence of phage by observing lytic activity of phages. Soft agar (0.7% agar) in 5 ml MRS-Ca broth was seeded with 0.1 ml of early log phase host culture, mixed thoroughly, and poured onto an MRS agar plate. After solidification, 10 µl of phage filtrate was spotted onto the top agar layer. After drying, the plate was incubated at 30°C overnight. A clear zone in the plate, resulting from the lysis of host cells, indicated the presence of phage. Spot test was also used for host range studies with LAB listed in the Table 1.

Phage preparation for electron microscopy

Phage preparation for direct visualization by electron microscopy was carried out as described by Watanabe et al. (1970) with some modifications. Briefly outlined, 500 ml of MRS-Ca medium was inoculated with a host, *L. lactis* subsp. *lactis* TFF221, and grown to an optical density at 600 nm of 0.5. The bacterial host was then infected with 5 ml of a phage suspension (10^5 PFU/ml) and incubated overnight at 30°C. After centrifugation at 4500 × *g* for 20 min, the supernatant was collected, and was centrifuged at 4°C with a 70.1Ti rotor at 28500 × *g* for 1 h in a Beckman L-80 ultracentrifuge (Beckman, CA, USA). The resulting pellets were re-suspended in 5 ml of phage buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$). A purified phage was recovered after centrifugation (4500 × *g* for 20 min) and the supernatant was passed through a 0.45 µm membrane filter. The purified phage was stored at 4°C for electron microscopy.

Electron microscopy

A drop of the purified phage suspension was applied to a carbon-coated grid for 5 min, then removed with a pipette and immediately replaced with a solution of 2% (wt/vol) uranyl acetate. After 1 min, the excess liquid was removed with a filter paper. The grid was allowed to air dry for 10 min and examined in a transmission electron microscope (JEOL, JEM-1230, Japan).

One-step growth curve of phage

For one-step growth experiments, a method of Capra et al. (2006) was used. A mid-exponential phase culture of *L. lactis* subsp. *lactis* TFF221 (optical density at 600 nm = 0.5) was harvested and suspended in one-fifth of the initial volume of fresh MRS-Ca broth. Phages were added at a multiplicity of infection (MOI) of 0.5, and allowed to adsorb for 30 min at 30°C. Cells were harvested by centrifugation (10000 × *g* for 5 min), and re-suspended in MRS-Ca broth. Decimal dilutions were made, incubated at 30°C, and at intervals, aliquots from each dilution were collected for phage counts. Experiment was performed at three different occasions and values depict the Mean of three observations ± standard deviation (SD). Latent period, burst time and burst size were calculated from the one-step growth curve.

Digestion of phage genome by restriction endonucleases

Phage genome was isolated according to the protocol provided with a commercial kit (PureLink™ Viral RNA/DNA Mini Kit, Invitrogen, Carlsbad, CA, USA). Purified phage genome was digested with either RNase, *Afl*II or *Hpa*I restriction endonucleases under the conditions prescribed by the manufacturer (Promega, Madison, WI, USA). After digestion, the samples were heated for 10 min at 70°C to avoid possible cohesive end ligation. Electrophoresis of digested genome was carried out on 1% agarose gel in 1× TAE (0.04 mM Tris-acetate, 0.001 M EDTA) and band patterns were visualized by a Dark Reader transilluminator (Clare Chemical Research) after staining with GelStar (Lonza Bioscience, Rockland, ME, USA). A lambda DNA-*Hin*dIII digest (Promega) and a pGEM® DNA marker (Promega) were used as reference DNA.

SDS-PAGE analysis of structural phage proteins

Purified phage suspension was precipitated with 4 volumes of ice-cold acetone. After centrifugation (10000 × *g*, 10 min, 4°C), the pellet was air-dried and re-suspended in PBS buffer. Phage structural proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Briefly, the sample was mixed with 2× sample loading buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue) and then heated in a boiling water bath for 3 min, followed by separating the proteins in the gel (12%). Protein bands were visualized by staining the gel with Coomassie brilliant blue.

Phage stability

Thermal inactivation of phage was examined at 60, 70, 80, and

90°C. A tube containing sterile distilled water was preheated to a desirable temperature. Phage solution was added into the tube at final concentration of 10^8 PFU/ml. After heating for 3 min, phage samples were taken and immediately placed on ice and enumerated to determine surviving PFU. Phage stability was examined at pH values ranging from 2 to 10. Phages were added into each pH value at final concentration of 10^8 PFU/ml and incubation at 30°C for 1 h. After incubation, the pH was brought to neutrality and the surviving phages were enumerated. Effect of salt concentration on stability of phage was also investigated. Sodium chloride was added into tubes containing sterile distilled water to obtain the concentration of 1, 2, 5, and 10% (wt/vol). Phages were added to each tube at final concentration of 10^8 PFU/ml and the tubes were kept at 30°C. After incubation for 7 days, phage samples of each tube were removed and the phage titers were enumerated. All experiments on phage stability were performed in triplicate and values were the Mean of the 3 determinations.

Transformation of bacterial host

The plasmid pN014-GFP, an *E.coli/Lactococcus* shuttle vector, containing a green fluorescent protein (gfp*)* gene and an erythromycin resistance marker, was obtained from our previous study (Phumkhachorn et al., 2007). *L. lactis* subsp. *lactis* TFF221 was transformed with a CelljecT PRO (Hybaid, Ashford, UK) by electroporation procedure as described by Holo and Nes (1989). The MRS plates containing 5 μg/ml of erythromycin were used for selection of erythromycin-resistant transformants. Transformants were selected after 3 days of incubation at 30°C. The selected transformants were tested for the presence of pN014-GFP by plasmid isolation according to Anderson and McKay (1983), and analyzed by agarose gel electrophoresis. The fluorescent mutant, GFP-expressing *L. lactis* subsp. *lactis* TFF221 (or *L. lactis* subsp. *lactis* TFF221-GFP$^+$), was grown on MRS medium containing erythromycin, and its colonies were examined by eye, using a Hoefer MacroVue UVis-20 translilluminator (Amersham Biosciences) after 48 h of incubation at 30°C. The fluorescent mutant strain was ascertained for phage susceptibility and subjected to be used as a starter culture in fermented food model.

Monitoring of bacterial host and phage counts in fermented food model

Kung-jom was used as a fermented food model for studying dynamic populations of phage and its host. Kung-jom was prepared by combining a mixture of dry small shrimp, salt, garlic and ground rice. Two separate sets of kung-jom fermentation were simultaneously prepared. The experimental set was added both the starter culture, *L. lactis* subsp. *lactis* TFF221-GFP$^+$ (10^4 CFU/g of food) and phage ϕTFF221 (10^3 PFU/g of food), but the control set was added only the starter culture. The ingredient mixture was thoroughly mixed and tightly packed in containers and incubated for 5 days at room temperature. During the incubation period, the samples of kung-jom were randomly collected at start of ripening process (time 0), daily. The collected samples were subjected to enumerate the number of both bacteria and phages. The number of bacteria in the samples was estimated by spread plate technique on MRS medium containing erythromycin. Starter bacterial colonies were distinctly identified and easily counted by fluorescent ability on the agar medium under the UV transilluminator. The number of phages presenting in the samples was determined by double-layer agar plaque method. Bacterial and phage counts were performed three times independently. All measurements were reported as Mean ± SD.

RESULTS

In this study, 30 Thai fermented food samples were examined for phages against nisin-producing *L. lactis* subsp. *lactis* TFF221 strain (Table 2). These samples were screened for the presence of phages by spot test using *L. lactis* subsp. *lactis* TFF221 as an indicator host cell. One sample from kung-jom was found to produce a clear zone indicating the lytic nature of the phage (Table 2). The isolated phage, designated as phage ϕTFF221, was purified by three consecutive single plaque isolations and propagated for investigation in more details.

On the basis of spot test to check the host range, the phage was specifically lytic against its host strain. None of the 17 LAB strains showed susceptibility to this phage (Table 1). Multiplication parameters of the lytic cycle of phage ϕTFF221 were determined from the one-step growth curve in MRS-Ca broth at 30°C (Figure 1). The results showed that the latent period was about 30 min, the burst period was 120 min, and the burst size was estimated at 60 PFU per infected cell. Electron microscopy revealed that the phage ϕTFF221 had an icosahedral capsid of 89 × 100 nm in diameter connected to a long contractile tail of 17 × 139 nm. The distal end of the tail had a structural feature similar to the baseplate of 11× 39 nm (Figure 2). The virion morphology of this phage was classified as a member of the *Myoviridae* family according to Ackermann (2003). To examine the virion structural proteins, the phage ϕTFF221 were purified by ultracentrifugation and analysed by SDS-PAGE (Figure 3). At least, 5 bands could be clearly distinguished in the gel with molecular masses estimated at 45.3, 39.8, 26.8, 16.5, and 8.9 kDa. From the restriction enzyme digestion, the phage ϕTFF221 genome was cleaved by *Afl*II and *Hpa*I (Figure 4) but not by RNase (data not shown). The results of enzyme digestion confirmatory indicated that the phage was a double-stranded DNA virus.

Phage ϕTFF221 was able to survive thermal treatments at 60, 70, and 80°C for 3 min (Table 3). At 60°C, only a small reduction of the phage was observed. In contrast, the phage titers considerably decreased at 70 and 80°C. The higher temperature resulted in lesser phage titers. Heating at 90°C for 3 min completely inactivated the phage, so phage titer could not be detected in the sample. Phage ϕTFF221 maintained its infectivity when incubated at 30°C for 1 h in pH range between 4 and 10. In contrast, phage lost its infectivity completely at pH 3 or below. The highest stability of phage was observed at pH 7 and 8 (Table 3). Phage ϕTFF221 was found to be relatively stable at 1 to 5% NaCl. The phage titer at low-salt concentration (1, 2 and 5% NaCl) did not alter drastically. Reduction of phage titer was obviously seen at high-salt concentration (10% NaCl); nevertheless, the phage still survived within 7 days (Table 3).

The plasmid pN014-GFP containing the gfp gene expressed from a constitutive L-lactate dehydrogenase

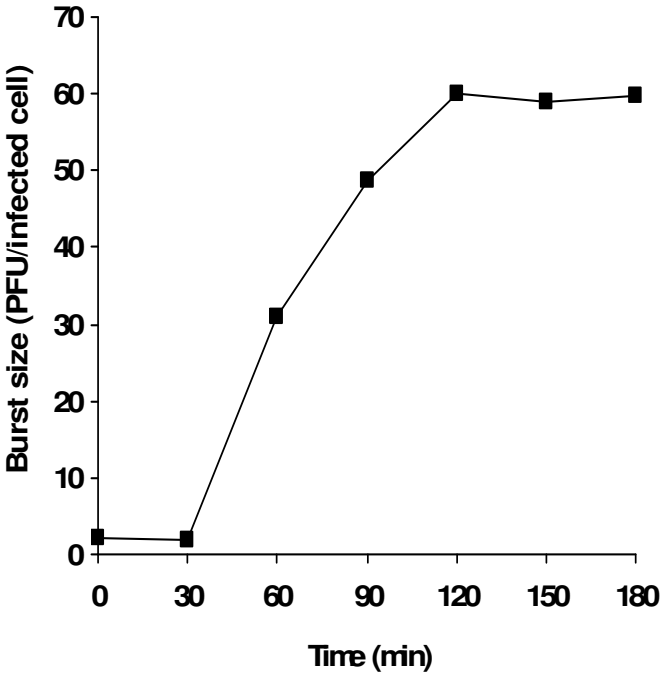

Figure 1. One-step growth curve of phage ϕTFF221 on *L. lactis* subsp. *lactis* TFF221.

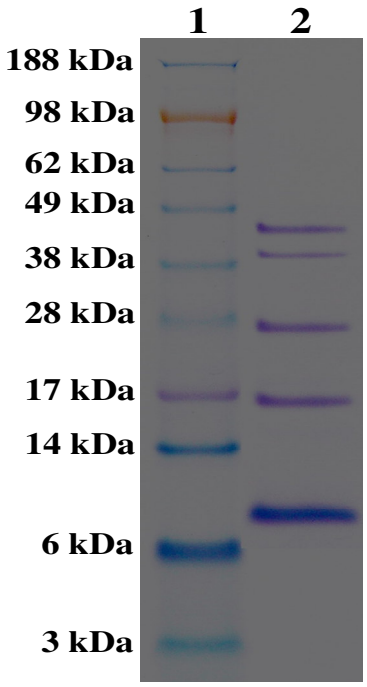

Figure 3. SDS-PAGE patterns of phage ϕTFF221 structural proteins. Lane 1, SeeBlue®Plus2 molecular mass markers; lane 2, phage ϕTFF221.

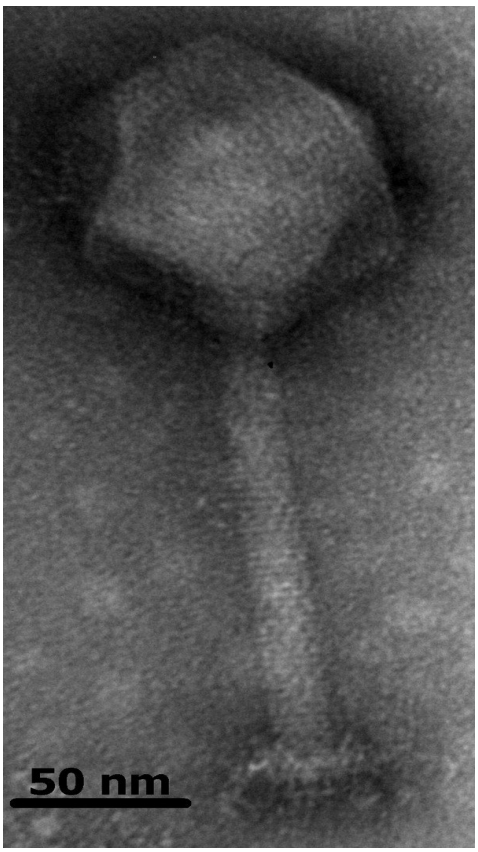

Figure 2. Electron micrograph of phage ϕTFF221 negatively stained with 2% uranyl acetate.

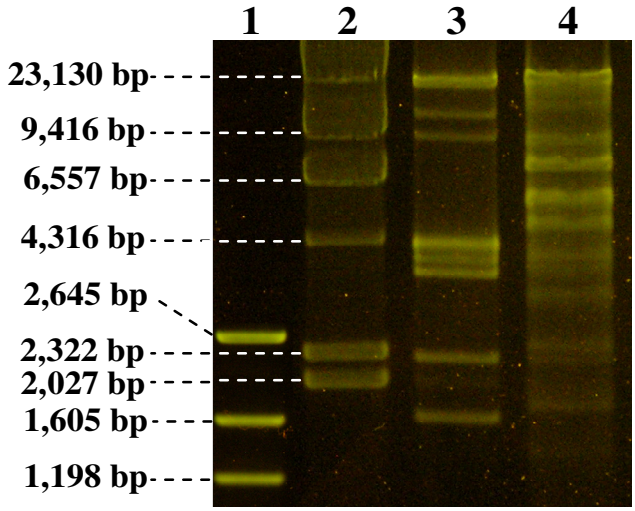

Figure 4. Analysis of phage ϕTFF221 genome digested with restriction endonucleases. Lane 1; pGEM DNA marker; lane 2; Lambda DNA cleaved with *Hin*dIII; lane 3; phage ϕTFF221 genome cut with *Afl*II; lane 3; phage ϕTFF221 genome cut wit *Hpa*I.

promoter, was available from our previous work (Phumkhachorn et al., 2007). Following transformation of *L. lactis* subsp. *lactis* TFF221 with the plasmid, it was

Table 3. Stability of phage ϕTFF221 to heat, pH and NaCl.

Treatment	Initial phage concentration (log PFU/ml)	Final phage concentration (log PFU/ml)
Heat (for 3 min) (°C)		
60	7.98	7.29
70	8.03	2.65
80	7.97	0.27
90	8.00	0
pH (for 1 h)		
2	7.99	0
3	7.97	0
4	8.02	5.28
5	7.96	5.53
6	7.99	5.72
7	8.00	6.81
8	7.98	6.72
9	8.01	5.56
10	7.96	2.99
NaCl (for 7 d) (%)		
1	8.00	7.45
2	7.98	7.74
5	7.96	7.02
10	8.02	3.81

found that the GFP protein in the transformant strain, *L. lactis* subsp. *lactis* TFF221-GFP$^+$, was active as revealed by the green fluorescent colonies of the transformants (data not shown). Transformation of *L. lactis* subsp. *lactis* TFF221 with the plasmid did not affect the phage susceptibility, in that, *L. lactis* subsp. *lactis* TFF221-GFP$^+$ is still sensitive to the phage ϕTFF221 by spot test and double-layer agar plaque assay. Therefore, *L. lactis* subsp. *lactis* TFF221-GFP$^+$ was used in a food model as the starter and its colony could be easily detected by placing the culture plate on the UV light.

The results of kinetic monitoring of inoculated starter and phage in kung-jom are shown in Figure 5. *L. lactis* subsp. *lactis* TFF221-GFP$^+$ cells in the experimental set that inoculated with phage ϕTFF221 initially appeared in the food matrixes at a concentration of 3.84 log CFU/g and dropped to 2.16 log CFU/g at day 5 of fermentation process. Furthermore, the inoculated phage increased from an initial count of 2.84 log PFU/g to 4.88 log PFU/g at day 5. On the other hand, *L. lactis* subsp. *lactis* TFF221-GFP$^+$ cells in the control set were detected at a concentration of 3.88 log CFU/g at the beginning of the ripening process (time 0) and gradually increased to 5.83 log CFU/g at day 5. In this set, the titer of phage ϕTFF221 could not be detected throughout the experiment. These results demonstrated that the decrease in the number of *L. lactis* subsp. *lactis* TFF221-GFP$^+$ in the experimental

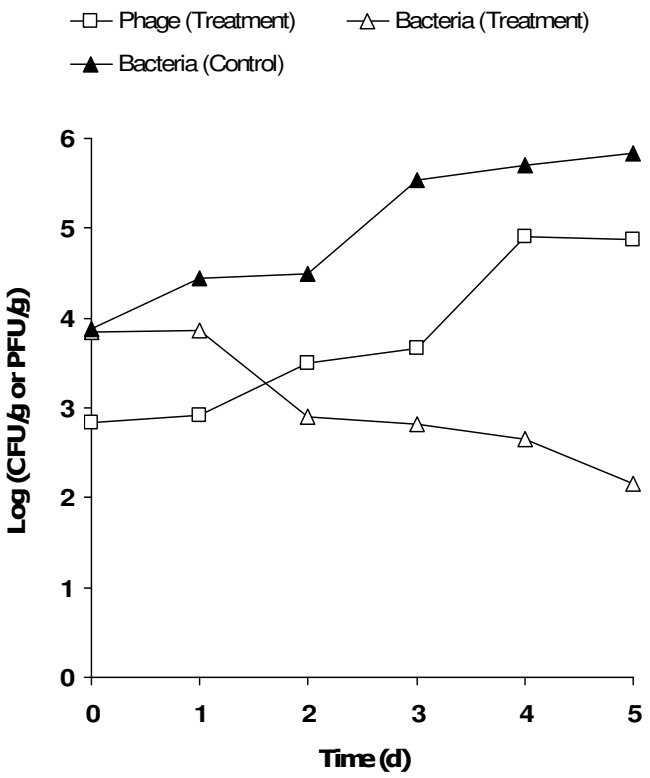

Figure 5. Kinetic monitoring of *L. lactis* subsp. *lactis* TFF221-GFP$^+$ and phage ϕTFF221 in food model.

set could have been due to the lytic activity of the phage through its multiplication. As compared to the experimental set, the gradual growth of *L. lactis* subsp. *lactis* TFF221-GFP$^+$ in the control set demonstrated that the presence of this phage could have adversely affected the number of added starter in kung-jom fermentation.

DISCUSSION

In this paper, we studied whether nisin-producing *L. lactis* subsp. *lactis* TFF221 which is a functional starter in kung-jom fermentation could be inhibited by phages. Among 30 Thai fermented food samples, one sample, from kung-jom, was found to have a virulent phage that could attack *L. lactis* subsp. *lactis* TFF221. This indicated that phage could be a factor which inhibits starter culture in Thai fermented foods. Finding of phages in the same kind of foods which the bacteria were isolated was not surprising, since phages could be usually found in environments where their hosts exist (Caso et al., 1995; Lu et al., 2003). Though, there have been many documents on phage in various fermented food, the published literature on phages in Thai fermented foods has not been reported. Thus, phage ϕTFF221 is the first *L. lactis* phage isolated from fermented product of Thailand.

The isolated phage, ϕTFF221, was further characterized. The results of host range studies showed that this novel lytic phage ϕTFF221 has a highly specific virulence towards nisin-producing *L. lactis* subsp. *lactis* TFF221. If this bacterial strain was used as a starter culture in kung-jom fermentation, the phage could adversely affect the quality of the food product by causing the fermentation delay or failure. Furthermore, the inactivation of the nisin-producing starter culture could allow the growth of some spoilage and pathogenic bacteria in the fermented product, hence affecting the safety of the food.

Major criteria for phage classification are its morphology and type of nucleic acid. Electron microscopic examination of phage ϕTFF221 revealed that the phage particle had an icosahedral head with a contractile tail. Moreover, restriction digestion of the phage genome demonstrated that its genome was a molecule of double-stranded DNA with size of 65 kb. From these results, the phage ϕTFF221 was tentatively classified as a member of *Myoviridae* family. According to the International Committee on Taxonomy of Viruses, lactococcal phages are members of the *Caudovirales* order, an extremely large, morphologically and genetically diverse group that encompasses over 95% of all known phages (Ackermann, 2003). This order contains three families, namely, the *Myoviridae* (with long, contractile tail), the *Siphoviridae* (with long, non-contractile tail), and the *Podoviridae* (with short tail). Although lactococcal phages are mainly members of the *Siphoviridae* family with a few members in the *Podoviridae* family (Deveau et al., 2006), it does not mean that no lactococcal phage has been classified into the *Myoviridae* family. At least, two lactococcal myophages, phage RZh and c10III, have been evident (Deveau et al., 2006). Therefore, it is not an unusual case for our study to isolate a myophage infecting *L. lactis*.

Variations in nucleic acid sequence and numbers of genes and structural proteins of lactococcal phages have been reported (Deveau et al., 2006). In this study, five structural proteins of the phage ϕTFF221 with molecular masses ranging from 9 to 45 kDa were identified by SDS-PAGE analysis. However, their amino acid sequences and functions are still unknown and not included in this study. A detailed nucleic acid sequence analysis and identification of the open reading frames corresponding to the observed structural proteins will be the subject of future research. These data will be useful to unveil the relationship between, the novel phage and the known lactococcal phage.

Phage ϕTFF221 was quite stable. It was able to survive in the broad pH range, and at 60 to 70°C for at least 3 min. Thus, it is possible that the phage may not be completely inactivated by standard pasteurization. Results of pH and heat sensitivity may be exploited in reduction or prevention of this phage contamination in the fermentation processes. It is noteworthy that the survivals of phage in 1 to 5% of NaCl were higher than in 10% of NaCl. If this phage were contaminated in the process of fermentation, it would possibly provide more chances of starter infection in low-salt than in high-salt fermentation, and eventually causes starter culture failure.

During the fermentation process, it is important to know the interaction between the added starter culture and its specific phage. However, the starter culture always coexists in foods with several naturally occurring bacteria. This means the conventional tracing method must have many steps of microbiological and biochemical techniques to differentiate and identify the starter culture among those bacteria in the food environment. In this experiment, we used a green fluorescent protein gene (gfp) to trace starter bacteria in kung-jom environments. In pN014-GFP plasmid, the gfp gene was placed under the control of the constitutive promoter of L-lactate dehydrogenase (*ldh*L). Therefore, the expression of the gfp gene in *L. lactis* subsp. *lactis* TFF221-GFP$^+$ was reflected by the fluorescent ability of the bacteria. GFP has been successfully used as a marker in many LAB (Fernandez et al., 2000; Gory et al., 2001; Phumkhachorn et al., 2007). Marked bacterial cells are easily seen by placing the culture plate on UV transilluminator or seeing the living cell under fluorescence microscopy. Hence, the GFP-based tracing system provides a rapid tool for detecting the starter culture in the complex food environment.

L. lactis subsp. *lactis* TFF221-GFP$^+$ was used as the host for phage ϕTFF221 infection in the experimental

food model. To assess the stability and inactivation of the phage and its host, we monitored the infective phage (measured as log PFU/g), and the survival bacteria (measured as log CFU/g) in food during the course of the fermentation process. In comparison with the control set, the number of the starter culture considerably decreased in the presence of phage; moreover, the amount of phage was gradually increased in the experimental set. This study raised the possibility that the phage which might naturally occur in food fermentations could potentially affect the starter culture introduced, and it seemed to be stable in complex food matrixes.

In conclusion, to our knowledge, this is the first report with a detailed study of phage isolated from Thai fermented foods. The discovery of phage ϕTFF221 and its characteristics provide valuable information that should be considered when using the nisin-producing *L. lactis* subsp. *lactis* TFF221 strain in kung-jom fermentations. The study of phage stability and phage-host interaction in food environment could lead to a further understanding of this phage in the fermentation processes. Due to its potential to cause fermentation failure, the strategy for prevention of phage ϕTFF221 infections is needed and has been one of the main research subjects undertaken in our laboratory.

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