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RESEARCH ARTICLE

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Bacteriophage ΦLPN014 infecting *Lactobacillus plantarum* N014, A potential starter culture for NHAM fermentation

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ABSTRACT

In the production of fermented foods if a starter culture is infected by a bacteriophage, fermentation will be failed and undesirable products will be obtained. Characterization of a bacteriophage infecting a starter culture will provide information useful for finding a strategy to prevent the starter culture from bacteriophage infection. In this study, a bacteriophage, designated ΦLPN014, was isolated from nham. It was able to infect Lactobacillus plantarum N014, a potential starter culture in nham fermentation. Characterization of ΦLPN014 showed that the bacteriophage was highly specific to L. plantarum N014 and was completely inactivated by heat at 80°C and 90°C for 30 min. Ninety four percent of ΦLPN014 particles adsorbed on L. plantarum N014 after adding the bacteriophage in the host cell culture for 20 min. According to the one-step growth curve of ΦLPN014, the latent period, burst period and burst size were about 30 min, 120 min and 135 PFU/infected cell, respectively. The bacteriophage has an isometric head and a long non-contractile tail. Its nucleic acid was sensitive to restriction enzyme BglII but resistant to Ribonuclease A and Nuclease S_1. Based on its nucleic acid and morphology, ΦLPN014 was classified in the family Siphoviridae.

INTRODUCTION

Nham is an indigeneous Thai fermented pork. It is one of the most popular Thai fermented meat products. It is usually consumed uncooked. The fermentation of nham generally relies on natural contamination by environmental flora. Potential contamination of the product by pathogenic microorganisms such as *Salmonella*, *Staphylococcus aureus* and *Listeria monocytogenes* has been reported [1] and poses a safety risk for consumption of the product. Improved starter cultures and monitoring programs and good manufacturing practice could contribute to improving the overall consistency, quality and safety of nham.

Many research groups have been trying to isolate and identify the predominant lactic acid bacteria strain involved in nham fermentation for use as a starter or co-culture microorganisms [2, 3]. The use of bacteriocin-producing strain as starter cultures is one of the possible ways to control the fermentation process and to improve products' safety and quality. In our previous study, *Lactobacillus plantarum* N014 which is an interesting functional starter culture has been isolated from nham. The strain is highly competitive and is able to lead the fermentation process. It produces at least one bacteriocin that is active towards a wide range of bacteria and that has a strong antilisterial activity [3]. One problem for starter culture dependent fermentations is the infection of starter cultures by bacteriophages. The presence of bacteriophages is troublesome for many kinds of fermentation and if left unchecked they can cause slow acid production or total failure of the fermentation resulting in unexpected and undesired food products.

To develop an effective approach to prevent fermentation failure caused by a bacteriophage that has ability to infect the starter culture, characteristics of the bacteriophage are crucial information needed to be elucidated. The objective of this study was to isolate and morphological characterize phages specific for *L. plantarum* N014 which has been being developed for use as a starter culture for nham fermentation. To our knowledge, this is the first report of

investigation the presence of bacteriophage attacking *L. plantaturm* in nham fermentation. The results may be used as basic information for the development of an approach to control the bacteriophage in nham fermentation process or for developing a bacteriophage resistance strain of *L. plantaturm* N014 for being used as a starter culture in nham fermentation.

MATERIALS AND METHODS

Bacterial strains and culture conditions

L. plantarum N014 was used as the indicator strain for the isolation, propagation, and characterization of the bacteriophage named ΦLPN014. Strains of lactic acid bacteria used for bacteriophage host range study were *Lactobacillus brevis* ATCC 14869, *Lactobacillus curvatus* ATCC 25601, *Lactobacillus lactis* ATCC 12315, *Lactobacillus pentosaceus* ATCC 8041, *Lactobacillus plantarum* ATCC 14917, *Lactococcus lactic* ATCC 11454, *Leuconostoc mesenteroides* TISTR 473, *Pediococcus dextrinicus* ATCC 33087 and *Streptococcus faecalis* ATCC 8043.

Bacterial stock cultures were stored at -80°C in MRS (Man Rogosa and Sharpe) broth (Difco Laboratories, Detroit, MI, USA) with 15% glycerol. Before use in experiments, frozen cultures were propagated twice in MRS broth at 37°C for 24 h. The bacteriophage was propagated on its host cultured in MRS medium supplemented with 10 mM $CaCl_2$. Soft agar was prepared with MRS broth supplemented with 0.5% agar.

Bacteriophage isolation

Nham samples were purchased at local markets. Ten g portion of each sample were blended in 90 ml of phosphate buffer (pH 7.2) with a Lab Blender 400 Stomacher (Seward, West Sussex, UK) for 2 min. To remove food ingredient particles and bacterial cells, the homogenate was centrifuged at 5,000 xg for 10 min. The supernatant was filtrated through 0.45-µm-pore-size membrane filter (Pall Corporation, Ann Arbor, MI, USA). For bacteriophage enrichment, the filtrate was added to equal amount of MRS broth supplemented with 10 mM $CaCl_2$ (MRS-Ca^{2+}) and inoculated with a log phase of *L. plantarum* N014 (10^6 cfu/ml) at 37°C for 24 h. After incubation, the culture was centrifuged at 5000 xg for 10 min. The supernatant obtained was membrane filtered for sterilization. The resulting filtrate was labeled as bacteriophage suspension and stored at 4°C for later testing the presence of bacteriophage active against *L. plantarum* N014.

Bacteriophage detection

The presence of a bacteriophage specific to *L. plantarum* N014 was examined by the spot test method as described by Chang et al. [4]. *L. plantarum* N014 was grown at 37°C for 24 h in MRS broth. One hundred µl of the culture was mixed well with 3 ml of MRS-Ca^{2+} soft agar and then overlaid on the surface of the solidified basal MRS agar (1.5%). Ten µl of the bacteriophage suspension was spotted onto the plate, which was then incubated at 37°C for 24 h. Bacterial sensitivity to the bacteriophage was established by a clear zone at the spot where the bacteriophage suspension was deposited.

Bacteriophage lysate preparation

Bacteriophage suspension was further purified by three rounds of plaque assay as described by Paterson et al. [5] with some modifications. Bacteriophage lysate was prepared from plaques formed on the last overlay plate. Single plaque was picked and mixed with 100 µl of a log phase culture of *L. plantarum* N014 (10^6 cfu/ml). The mixture was added to 5 ml of MRS-Ca^{2+} broth, and incubated at 37°C for 24 h. The bacteriophage lysate was centrifuged at 5000 xg for 10 min. The supernatant was filtered through a 0.45-µm-pore-size membrane filter. The resulting filtrate called bacteriophage lysate was stored at 4°C for later use.

Plaque assay

Bacteriophage titer was determined by plaque assay as described by Paterson et al. [5] with some modifications. Bacteriohage sample was serial diluted with phosphate buffer saline (pH 7.2). One hundred µl of each dilution and one hundred µl of a log phase culture of *L. plantarum* N014 (10^6 cfu/ml) were added to a tube containing 3 ml of MRS-Ca^{2+} soft agar. The mixture was overlaid onto the surface of a MRS agar plate and incubated at 37°C for 24 h. Plaques was counted and recorded as plaque forming units per milliliter (PFU/ml).

Bacteriophage host range examination

Bacteriophage host range was studied by using the spot test method [4] as mentioned earlier. The lactic acid bacteria used as hosts in this experiment are shown in Table 1.

Thermal inactivation test

A 1.5 ml microcentrifuge tube containing 900 µl of sterile, deionized water was preheated to a desirable temperature, ranging from 50 to 90°C. One hundred µl of bacteriophage lysate was added to the tube to obtain the final bacteriophage concentration of about at 10^6 PFU/ml (initial titer). After heating for 30 min, the tube was placed in an ice-water bath. The remaining bacteriophage titer (final titer) was determined by plaque assay. The percentage of bacteriophage survival was calculated as follow: [(initial titer-final titer)/initial titer] x 100%.

Bacteriophage adsorption assay

The adsorption experiment was carried out as described by Lu et al. [6] with some modifications. A log phase culture of *L. plantarum* N014 (10^6 CFU/ml) in MRS-Ca^{2+} broth was infected with the bacteriophage lysate to give a multiplicity of infection (MOI) of 0.01 and incubated at 37°C. Aliquots of 0.5 ml were taken at 5-min intervals (up to 30 min) after infection and immediately filtered through a 0.45-µm-pore-size membrane filter. Filtrates were subjected to plaque assay to determine titers of unadsorbed bacteriophage particles (residual titers). The MRS-Ca^{2+} broth containing only bacteriophage (without the host) was used as a control. The percentage of bacteriophage adsorption was calculated as follow: [(control titer-residual titer)/control titer] × 100%.

One-step growth experiment

One-step growth experiment was performed as described by Caso et al. [7] with some modifications. Ten ml of log phase culture of *L. plantarum* N014 was harvested by centrifugation at 5,000 xg for 10 min and resuspended in 5 ml of fresh MRS broth in order to obtain a final concentration of 10^8 cfu/ml. To this suspension, 5 ml of bacteriophage lysate was added in order to have a MOI of 0.01 and the bacteriophage was allowed to adsorb for 20 min at 37°C. The mixture was centrifuged at 5,000 xg for 10 min, and the pellet containing infected bacterial cells was resuspended in prewarmed (37°C) BHI broth, followed by incubation at 37°C. Samples were taken at 10-min intervals (upto 3 h) and immediately diluted, and then titers were determined by plaque assay.

Examination of bacteriophage morphology

The morphology of bacteriophage was examined by transmission electron microscopy (TEM). Bacteriophage particles were fixed by mixing 25 µl of the bacteriophage lysate with 25 µl of 50% glutaraldehyde in 4% paraformaldeyde. A 5 µl aliquote of this mixture was placed on a carbon a Formvar-coated copper grid (Proscitech, Brisbane, Queenland, Australia) and allowed to adsorb for 5 min at room temperature. The bacteriophage was negatively stained with 0.5% uranyl acetate for 2 min and then inspected with a Philips EM 300 electron microscope (Philips, Eindhoven, the Netherlands) operated at 60 kV. The bacteriophage size was determined from the average of five independent measurements.

Analysis of phage nucleic acids

Bacteriophage nucleic acid was extracted by using PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. The purified nucleic acid was tested for sensitivity to Ribonuclease A, Nuclease S_1 and restriction enzyme *Bgl*II (all from Sigma-Aldrich, St. Louis, MO, USA) according to the supplier's recommendations. The results were analyzed by 0.8% agarose gel electrophoresis.

RESULTS

A total of twenty nham samples purchased from different local markets were screened for a bacteriophage specific to *L. plantarum* N014 by spot test method. Only one sample was positive for bacteriophage isolation. The bacteriophage produced a clear zone on the lawn of its host where the bacteriophage lysate was spotted. The bacteriophage was designated ΦLPN014.

Specifictity of ΦLPN014 to 10 different strains of several lactic acid bacteria was examined by spot test method. Only *L. plantarum* N014 was susceptible to the bacteriophage while the rest of the tested bacteria were not sensitive to the bacteriophage. According to the data presented in Table 1, ΦLPN014 was highly specific to the bacterial strain level because the bacteriophage was lytic to *L. plantarum* N014 but not to *L. plantarum* ATCC 14917.

Thermal stability of ΦLPN014 was investigated by treating the bacteriophage with heat at 50, 60, 70, 80 and 90°C for 30 min. The reduction of bacteriophage titer was temperature dependent. The complete inactivation of ΦLPN014 was observed when it was incubated at 80 and 90°C for 30 min (Table 2).

The adsorption rate of ΦLPN014 on cell surface of *L. plantarum* N014 is shown in Figure 1. Most of bacteriophage particles were adsorbed on host cells within the first 10 min of observation peroid. During that time the percentage of adsorption raised from 0 to 82%. In the next 10 min of observation peroid, the percentage of adsorption slowly increased from 82% to 94%. In the last 10 min of observation peroid, the adsorption rate was stable at 94%.

Multiplication parameters of the lytic cycle of ΦLPN014 including latent period, burst period and burst size were determined from the dynamic change in the number of phage during one replicative cycle (Figure 2). It was found that ΦLPN014 had latent and burst periods of about 30 and 120 min, respectively. The burst size estimated from the one-step growth curve was about 150 PFU/infected cell.

Morphology of ΦLPN014 was examined by TEM (Figure 3). The dimension of the bacteriophage was estimated by determining the mean values of 5 bacteriophage particles. The head appeared to be isometric with a diameter of about 33 ± 1.2 nm. The long non-contractile tail was about157 ± 6.3 nm in length and 8 ± 0.7 nm in width. No collar or tail appendages were observed.

The nucleic acid extracted from ΦLPN014 was examined for its sensitivity to different nucleic acid digesting enzymes. It was found that the nucleic acid could not digested by Ribonuclease A and Nuclease S_1. However, it was sensitive to the restriction enzyme *Bgl*II (Figure 4).

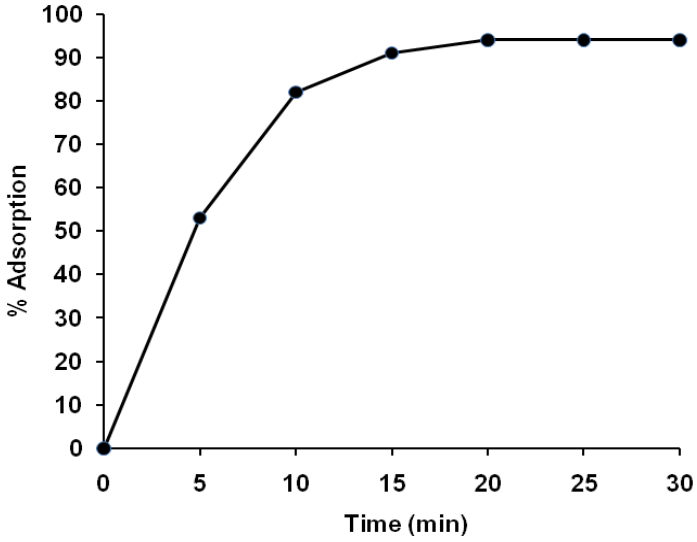

Figure 1: Adsorption rate of ΦLPN014 on *L. plantarum* N014

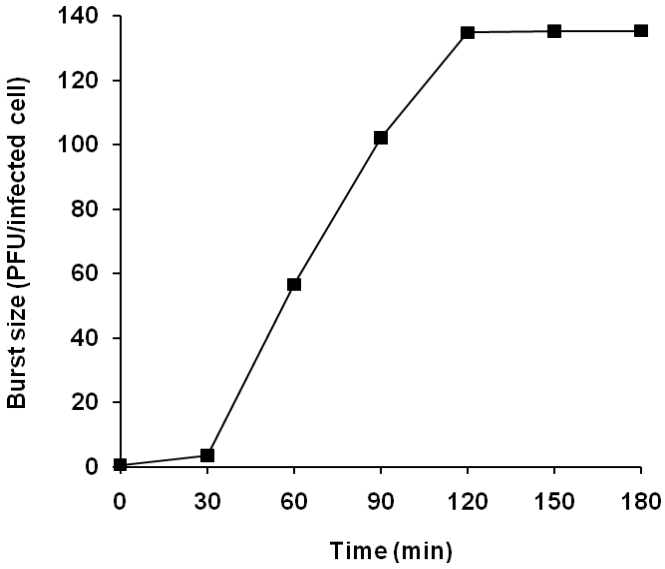

Figure 2: One-step growth of ΦLPN014

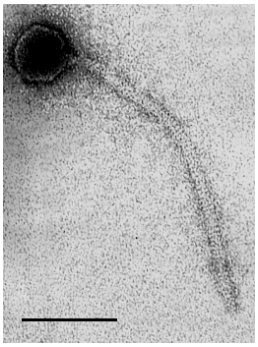

Figure 3: Morphology of ΦLPN014 as examined by TEM. Bar = 50 nm

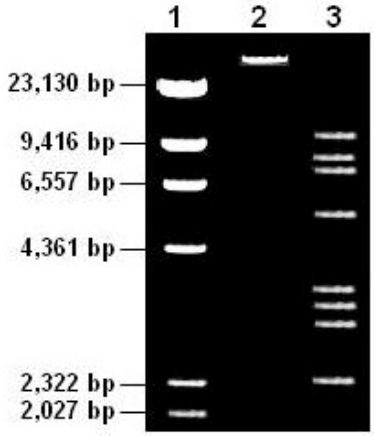

Figure 4: Agarose gel electrophoresis of ΦLPN014 nucleic acid
Lane 1, lambda DNA digested with HindIII marker,
Lane 2 uncut ΦLPN014 nucleic acid,
Lane 3, ΦLPN014 nucleic acid cut with BglII

Table 1: Host range of ΦLPN014

Bacterial strain	Lysis[a]
Lactobacillus plantarum N014	+
Lactobacillus brevis ATCC 14869	-
Lactobacillus curvatus ATCC 25601	-
Lactobacillus lactis ATCC 12315	-
Lactobacillus pentosaceus ATCC 8041	-
Lactobacillus plantarum ATCC 14917	-
Lactococcus lactic ATCC 11454	-
Leuconostoc mesenteroides TISTR 473	-
Pediococcus dextrinicus ATCC 33087	-
Streptococcus faecalis ATCC 8043	-

[a] *+ = clear zone form, - = no clear zone formed*

Table 2: Thermal stability of ΦLPN014

Heat Treatment	% Survival
50 ℃	96.53
60 ℃	74.82
70 ℃	56.95
80 ℃	0
90 ℃	0

DISCUSSION

Bacteriophage infection of starter cultures poses a major problem in manufacture of fermented food products and can result in significant monetary losses. Bacteriophages retard or, in the case of severe infection, eliminate the activity of the starter cultures resulting in low and unacceptable food quality. Screening and characterizing bacteriophages attacking starter cultures is a first important step towards the understanding of properties of the bacteriophages that is valuable for developing bacteriophage control strategies for achieving high and consistent quality of fermented products**.**

In the present study, 20 of nham samples were screened for bacteriophages active against *L. plantarum* N014, which is a potential starter in nham fermentation. Only one bacteriophage, named ΦLPN014, was isolated from the samples. Although *L. plantarum* bacteriophages have been isolated from various fermented food products [6-8], *L. plantarum* bacteriophage from nham has not been reported. Thus, this is the first report of *L. plantarum* bacteriophage isolated from nham.

Several lactic acid bacteria other than *L. plantarum* N014 were subjected to test for the host range of ΦLPN014. None of these bacteria were susceptible to ΦLPN014, indicating a narrow host range of the bacteriophage. Furthermore, the result also showed that the infectivity of the bacteriophage was strain specific because it infected *L. plantarum* N014 but not *L. plantarum* ATCC 14917. Some lactic acid bacteria specific bacteriophages with a narrow host range or infecting only one host have been isolated from fermented food products. These included bacteriophages Y4 and Y20 from sauerkraut [9] and bacteriophage PL-1 from Yakult [10]. However, there were some evidences on lactic acid bacteria specific bacteriophages with a broad host range including ΦJL-1 from cucumber fermentation [6], bacteriophage SC921 from kimchi [8], bacteriophages Y5, Y10, Y11, Y12, R1, R2 and R3 from sauerkraut [9], Φ1, Φ2, Φ3, Φ4, Φ5, Φ6, Φ7, Φ8, Φ9, Φ10, and Φ11 from yogurt [11], bacteriophages jw30, jw31 and jw32 from cheddar cheese [12] and bacteriophage DT1 from mozzarella whey [13].

From the thermal inactivation study, it was shown that the heat sensitivity of ΦLPN014 was temperature dependent. The higher temperature treatment was applied the higher loss of bacteriophage titer was observed. The same behavior was also previously observed for a *L. plantarum* bacteriophage ΦJL-1 isolated from cucumber fermentation [6]. Information about heat sensitivity of ΦLPN014 may be exploited in designing heat processes to reduce the possibility of bacteriophage contamination in appropriate steps of nham fermentation prior to starter culture inoculation.

The key step in the life cycle of most bacteriophages is the successful adsorption of bacteriophage particles with an appropriate host via a receptor on the cell surface. Among *Lactobacillus* bacteriophages, times required to obtain the maximum adsorption rates on their specific hosts vary depending on type of bacteriophage. For example, the maximum adsorption rates of ΦLPN014, ΦJL-1 [6] and ΦLP-1-A [7] was achieved within 20, 30 and 45 min after mixing the bacteriophages with their specific hosts, respectively. There are some different between receptors for bacteriophage adsorption on gram negative and gram positive bacteria. In gram negative bacteria the receptors have been identified as protein and lipopolysaccharide components of the outer membrane layer surrounding the peptidoglycan. A particular bacteriophage or group of bacteriophages adsorb to a specific site while different bacteriophages adsorb to different sites [14]. For gram positive bacteria the information on bacteriophage receptors is scarce. However, bacteriophages attacking *Lactococcus lactis* seem to bind initially to specific carbohydrate receptors exposed to the surface of the cell wall [15]. In this study, the receptor of ΦLPN014 on the host was not identified and it needs to be further investigated.

A particular *L. plantarum* bacteriohage has its own unique growth parameters. For examples, the burst size of ΦLPN014 was found in this study to be about 135 PFU/infected cell while that of ΦJL-1 [6], bacteriophage B2 [16] and bacteriophage fri [17] were about 22, 14 and 200 PFU/infected cell, respectively. Therefore, researchers cannot use previously reported growth parameters of other bacteriophages to estimate those of the bacteriophage being investigated.

One of the criteria for classification of bacteriophages to a particular group is their genetic and morphological characteristics. The nucleic acid of ΦLPN014 was double stranded DNA because it was resistant to Nuclease S_1, an single stranded DNA digesting enzyme, and Ribonuclease A, a RNA digesting enzyme, but sensitive to the restriction enzyme *Bgl*II, an double stranded DNA digesting enzyme. Transmission electron microscopy revealed that the bacteriophage was a tailed bacteriophage with an isometric head and a long non-contractile tail. Based on these characteristics, ΦLPN014 can be tentatively classified as a member of the family *Siphoviridae* according to the International Committee on Taxonomy of Viruses [18]. The order *Caudovirales* or the tailed bacteriophages can be divided into 3 families: *Myoviridae*, *Siphoviridae*, and *Podoviridae*. The bacteriophage investigated in this study was placed in to the family *Siphoviridae* family along with a number of *L. plantarum* bacteriophages including ΦJL-1 from cucumber fermentation [6], bacteriophage LP-1 from corn silage [7], bacteriophage ΦJL-2 from cheese whey [7], bacteriophage SC921 from kimchi [8], bacteriophage B1 from corn silage [19], and bacteriophage B2 from anaerobic sewage sludge [19]. However, *L. plantarum* bacteriophages in family *Myoviridae* have also been reported [9, 17, 20]. Thus, both *Siphoviridae* and *Myoviridae* family are so far the major groups of *L. plantarum* bacteriophages.

CONCLUSION

Food fermentations have been adversely affected by bacteriophage infections of starter cultures. These infections generally result in unexpected and undesired quality of the fermented food products. In order to prevent such infections in food fermentations, characteristics of the problematic bacteriophages are needed to be elucidated. In this study, we demonstrated that ΦLPN014 was able to infect *L. plantarum* N014, the potential starter culture for nham fermentation. The discovery of this bacteriophage provides valuable information that must be considered during conducting fermentation using *L. plantarum* N014 as a starter culture. Furthermore, characterization of ΦLPN014 performed in this study provides some basic information that may be useful for controlling the bacteriophage in fermentation process or for developing phage resistance strain of *L. plantaturm* N014 to be used as a suitable starter in nham manufacturing. However, to develop an efficient method for avoiding ΦLPN014 infection in nham fermentation, further research on its characteristics and its impact on nham fermentation is required.

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