The Natural History Journal of Chulalongkorn University 3(2): 1-6, October 2003

Antibody Against Actinophages Isolated from Soil in Thailand

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ABSTRACT.–Streptomycetes and phages were isolated from soil in different locations of Thailand. The isolated phages, distinguished by host range determination, plaque morphology and phage morphology under transmission electron microscope, were partially purified by single plaque isolation. From host range determination in a variety of phages, phage Roi-1 was selected to serve as an antigen for immunization in rabbits. High titers of phage suspension were prepared by removal of host cells passing through 0.45 μm membrane filter. Five milliliters of the pure phage suspension was injected to three rabbits twice a week for 1 month. Sera from rabbits were subsequently collected and tested for cross reactivation against another 8 phages. The results revealed that phage Nsaw-30 gave a high degree of relatedness to phage Roi-1.

KEY WORDS: actinophage; streptomycetes; soil; antibody

INTRODUCTION

Bacteriophage or phage, was discovered in 1915 and 1917 by Twort and d'Herelle. Like all viruses, phage is the obligate intracellular parasite. In general, phage consists of nucleic acid called core surrounded by protein coat which is known as capsid (Lwoff et al., 1959). Phage can be easily determined by a technique called "plaque assay". The plaque is resulted from phage destroyed bacteria cell at a localized area in agar and giving a clear, turbid circular on the lawn of bacteria. The number of phages by plaque assay was referred as the approximately phage particles in the samples. The morphology of plaque can be used as a distinction between virutlent (lytic) phages and temperate phages. For a lytic cycle, the virulent phage causes the bacterial cells lysis and released large amounts of phage progeny in each generation cycle whereas temperate phages undergo to either lytic or lysogenic cycle. The lysogenic cycle is starting with phage DNA integrates to the bacterial chromosome. Phage DNA multiplies along with bacterial replication without phage progeny. Hence, the temperate phage generates a turbid plaque resulting from the lysogen which carried phage DNA grows within the plaque.

Enrichment procedures are used for detection of phage in the samples, including the incuba-tion of broths containing a soil sample and a high concentration of the potential host cells. The period of incubation depends on the growth cycle of the host in the growth medium.

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Streptomyces phages are isolated from soil by using enrichment procedure (Goyal et al., 1987). They are useful to employ in numerous subjects, especially in study for host genetics and provide as tools for studies or exploitation of their hosts (Kieser et al., 2000).

Phage are discriminated by several characteristics, principally, the biological characteristics such as host range relatedness, morphological characters and antigenic relationships. Generally, the phages themselves are non-toxic and non-pathogenic to animals and can be injected with large amounts without damaging the host cells. Rabbits are the most suitable and practical subject used for phage immunization (Adams, 1959).

Antibody of phages was employed in numerous virus researches, for example, serologic classification of viruses into groups in which the antigenic relationships are correlated with morphologic and biologic resemblances (Delbruck, 1946). To approach the relatedness of isolated phages; therefore, the purpose of this study was to study phages from several sources relevant to antibody neutralization test. The relationship among phages will understand in distribution of phages in Thailand. The phages obtained will be of valuable in further work of molecular biology.

MATERIALS AND METHODS

Isolation of hosts

Streptomycetes and phages were isolated from soil samples collected from several provinces of Thailand. Streptomycetes were isolated by using humic acid vitamin agar medium (HV-agar: Humic acid, 0.1%; Na_2HPO_4, 0.05%; KCl, 0.17 %; $MgSO_4.7H_2O$, 0.005 %; $FeSO_4.7H_2O$, 0.001 %; $CaCO_3$, 0.002 %; Cycloheximide, 0.0005 %; Vitamin B; Agar, 1.8 % [pH 7.2]) (Hayakawa and Nonomura, 1987). One gram of soil was added to 9 ml of distilled water and made serially 10-fold dilutions. Each dilution was spreaded on HV-agar and incubated at 30^{0}C for 7-14 days. Colonies from each plate were subcultured on Mannitol mungbean agar until spore formed and then observed under light microscope. Preparation of streptomycetes spore suspension was prepared by adding five milliliters of distilled water to spores of streptomycete on Mannitol mungbean agar (Diced mungbean, 1%; Mannitol, 1%; Agar, 1.5%; Tap water, 50%; Distilled water, 50%[pH 7.0]), then scraped the surface of the culture and filtered the suspension through sterile cotton wool in a filter tube. The filtered suspension was centrifuged at 1,200 xg for 10 min to pellet the spores, poured of supernatant, and added 20 % (V/V) glycerol. The suspension was transferred to a screw cap for freezing at –20 ^{0}C until used.

Isolation of phages

Phages from soil samples were isolated by enrichment procedure using the potential host isolates (Dowding, 1973). Twenty-five grams of soil sample was inoculated into 250 ml Erlenmeyer flask containing 50 ml of nutrient broth (Beef extract, 0.3%; Bacto-peptone, 0.5%[pH 7.0]) and 4 mM $CaCl_2$ and added spores of the determined streptomycetes host strain to give a final concentration of 10^7 cfu/ml. The mixture was incubated overnight at 200 rpm on a rotary shaker. To examine the presence of phages, Soft-agar overlay plate method or double layer method (Gratia, 1936) was generally used for phage detection, 0.1 ml of the filtrate was diluted to 10^{-1} and 10^{-2} in nutrient broth and plated 0.1 ml of undiluted and diluted into nutrient agar agar (Nutrient broth + agar 1.5%), overlaid with 0.1 ml top agar (Nutrient broth + agar 0.5%) containing spore suspension, left agar to solidify for 5 min and incubated at 30 ^{0}C for overnight. Plaques were then purified by single plaque isolation prior to make a high titer stocks. The presence of bacteriophages in the sample (titering) was enumerated. Only one dilution should be used to determine the titer of pfu/ml.

Host range of phages

Host ranges of phages were determined by the presence of plaques against 44 reference streptomycetes (kindly supplied from National

Science and Technology Development Agency (NSTDA) and from Prof. Dr. Seiya Ogata) and 88 streptomycetes strains isolated from Thai soil.

Morphology of phages

Phages morphology were observed under transmission electron microscope. Carbon-coated grid was placed on a center of plaque and left stand for 1 min, stained with 1% uranyl acetate for 1 min then morphology of phage particles were examined with a JCM-200 CZ transmission electron microscope, at magnifications of x72,000 and x100,000 (William and Fisher, 1970; Dowding, 1973; Kuhn et al., 1987).

Antisera preparation procedure

Antisera were prepared according to Adams (1959). Five millilitres of 10^{10} pfu/ml of phage titer was inoculated to 2.5 kgs weight and 6-month-old New Zealand White rabbits via subcutaneous route twice a week for 3 weeks. Three Rabbits was used for triplicate. Animals were bled by slitting marginal ear vein and about 5 ml of blood was collected. Antibodies were separated by low speed centrifugation and stored in sterile screw-capped vials in the refrigerator.

To determine relationship among selected

TABLE 1. Localities and number of host isolates.

No.	Province	District	pH	No. of host isolates	No. of phage isolates
1	Roi-ed	Muang	7.2	2	3
2	Nonthaburi	Muang	6.6	1	2
3.	Phetchaburi	Muang	6.6	3	3
4.	Prachuap Khiri Khan	Huahin	6.2	1	-
5.	Bangkok	Bangphlat	6.6	2	3
6.	Angthong	Muang	6.3	3	2
7.	Rayong	Muang	6.2	4	2
8.	Samut Sakhon	Mahachai	6.9	2	-
9.	Nakhon Pathom	Muang	7.1	1	2
10.	Kanchanaburi	Muang	6.9	9	2
11.	Surat Thani	Punpin	5.8	5	2
12.	Nakhon Ratchasima	Pakthongchai	7.3	12	3
13.	Khon Kaen	Baanphai	7.6	3	3
14.	Maha Sarakham	Muang	5.4	11	2
15.	Nakhonnayok	Parkplee	7.2	3	3
16.	Prachin Buri	Muang	7.7	2	2
17.	Chachoengsao	Muang	8.0	2	2
18.	Pathum Thani	Thanyaburi	3.8	2	2
19.	Chon Buri	Muang	6.9	7	1
20.	Khon Kaen	Muang	7.0	7	2
21.	Loei	Poo Kra Doong	7.3	5	-
22.	Pathumthani	Muang	7.6	6	2
23.	Chon Buri	Pattaya	7.1	7	2
24.	Chantaburi	Muang	6.9	8	2
25.	Chiengmai	Chieng Dao	6.7	7	2
26.	Chiengmai	Phang	7.3	6	2
27.	Singburi	Promburi	6.7	6	2
28.	Nakhonsawan	Muang	7.2	2	2
29.	Lampoon	Pa-sang	6.7	2	3
30.	Nakhonsawan	Taklee	6.8	3	1

phages and others, serum was diluted to 1:100 and 1:1,000 in the nutrient broth, the same medium as of the phage dilution. To assay, The phage suspension was diluted to a titer of 10^7 pfu/ml and 0.1 ml of phage was added to 0.9 ml of diluted serum and incubated at 30 ^{0}C. For each 5 min intervals, 0.1 ml of the phage-serum mixture were added to 9.9 ml of nutrient broth to stop antibody reaction and 0.1 ml samples of this dilution were plated by double layer technique. If no activation of phage occurring, about 1,000 plaques will be appeared after incubation of the plates. The amount of phage antibodies, K values, were calculated from the equation, $K=2.3\ D/t \times \log (p_0/p)$, where $p_0=$ phage assay at zero time, $p=$ phage assay at time t min and $D=$ final dilution of serum in the phage-serum mixture (Adams, 1959). Antiserum cross-reactivity was calculated from dividing K values of different phages by K value of tested phage.

RESULTS AND DISCUSSION

Soil samples, designated number 1-30, were taken from several provinces of Thailand. The number of host isolates for phage isolation and pH were shown in Table 1.

Isolation of phages

Streptomyces spp. isolated from the same soil was then used as the hosts. The results showed that phages were isolated from most soil samples except the soil No. 4, 8 and 21. After steps of single plaque purification and observed under transmission electron microscope, phage in each soil samples which was seen in electron microscope were collected and used for characterization of phage.

Host range determination of phages

Phage host range is a useful marker for epidermiological and genetically studies of phages. From 88 strains of isolated streptom ycetes from our labolatory and 44 strains of streptomycetes reference strains were used for host range determination. Among these, phage

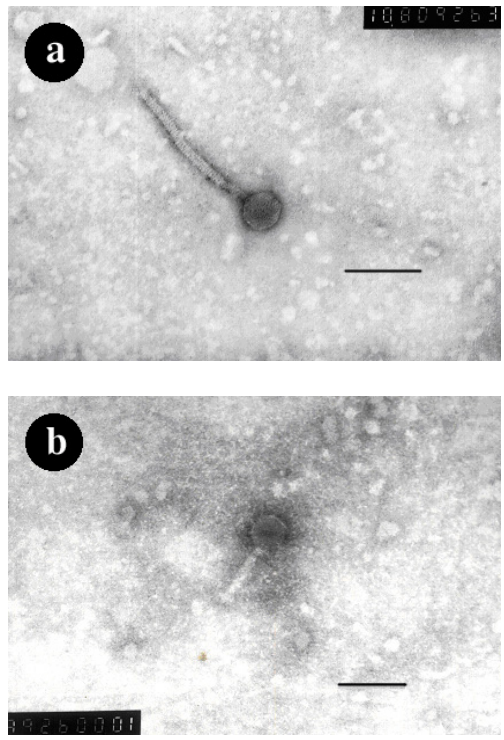

FIGURE 1. The morphology of phages (a) Yok-15 (b) Ray-7 (bar = 100 nm)

Roi-1 showed the widest host range followed by strains Yok-15, Nsaw-30, Lam-29, Nsaw-28, Ac-7, Ray-7, Sur-11, Sin-27, Pathum-18, Pathom-9, Non-2, KK-20, Ban-5, Pathum-22. In addition, Yok-15 and Roi-1 had given the wide host range against 39 and 32 reference strains, respectively. They both infected reference strain included *S. lividans* TK 21, *S. coelicolor* Muller, *S. bikiniensis* JCM4011, *S. lincolnensis* JCM 4287, *S. nodosus* JCM 4297, *S. badius* JCM4350. However, they did not infected *S. thermovulgaris* JCM 4520 (thermophilic *Streptomyces*) while the phages: Korat-12, Pathum-18, and Lam-29 did. None of phages could infect *Kitasatospora griseola* JCM 3339. Host range determination examined that mostly isolated phages acted as the virulent phage and gave a broad host range. Hahn et al. (1990) suggested the application of broad host range streptomycete phage. Since they limited to infect *Streptomyces* spp., it can be used to

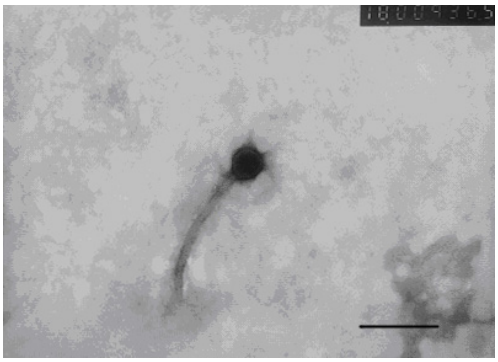

FIGURE 2. The morphology of phages Roi-1 (bar = 100 nm)

rapidly identify from new soil sources and their vectors might be used to shuttle DNA between wide range of academic strains, industrial strains and poorly characterized wild isolated from soils. Controversy, Phet-3, Ban-5, Pathum-22, and Chan-24 displayed a narrow host-range. This characteristic was profitably identified their host species and had been reported in phage of *S. venezualae* (Stuttard, 1989). Nevertheless, Roi-1 was selected to use as antigen for producing antiserum because it provided a widest host range. In addition, 8 phages; Nsaw–28, Nsaw 30, Lam-29, Ray-7, Sur-11, Yok-15, Sin-27, Ac-7, were selected for cross reactivation test according to a broad spectrum of host range.

Morphology of phages

After three repeated single plaque isolation, phage morphology was observed under Transmission electron microscope (Willium and Fisher, 1970) . They belonged to Siphoviridae (Van Rgeregenmortel, 2000) with icosahedral head and non- contractile tails. The morphology of phages (for instance) were shown in Fig. 1 and 2.

Cross reactivation of phages

An attempt to study selorogical relevance between phages and antiserum against phage Roi-1, having widest host range, was chosen for providing antiserum. Anti-Roi-1 gave a cross-reactivation against the selected strain

TABLE 2. Cross reactivation properties against antisera of phage Roi-1

Phage	% of inactivation
Nsaw–28	44
Nsaw-30	76
Lam-29	8
Ray-7	5
Sur-11	44
Yok-15	8
Sin-27	59
Ac-7	43

which examined by K value (Table 2). Antigenic properties of bacteriophage have been reviewed since 1937 by Burnet et al. In general, the rate of neutralization has been greater with homologous phage than with heterologous phages. For instance, the coliphages, T2, T4 and T6 form a closely related group and quite different when measured against T3 and T7 (Adams, 1959). According to percent of inactivation in Table 2, two phages: Nsaw-30, Sin-27, showed closely serological relevance between phages and antiserum against phage Roi-1. Phage Ray-7, Yok-15 and Lam-29 shared a slight relationship by which percent of inactivation below 10. Hahn et al., 1990 studied the anti-FP-22 serum (very broad host specificity). It was found that FP22 shared strong cross-immunity and antibody cross-reactivity with bacteriophage P23, but not with seven other streptomycete bacteriophages.

This report was firstly described on Streptomyces phages isolated in Thailand. We hope our isolated streptomycetes and phages will be carried for further study, applied for numerous usages and exploited these streptomycetes and phages to approach the ecological system in soil.

ACKNOWLEDGMENTS

This work was supported by Royal Golden Jubilee Program. We are grateful to National Science and Technology Development Agency for serving some typed strains of streptomycetes and Professor Dr. Keith Chater for 3 strains of streptomycetes.

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Received: 5 June 2002
Accepted: 21 March 2003