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Characteristics and Distribution of Actinophages Isolated from Thai Soil Samples

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ABSTRACT: Indigenous streptomycetes isolated from soil samples collected from several parts of Thailand were used as hosts for isolation of phages. Twenty-four phages were obtained by an enrichment procedure. The phages were plaque purified and morphologically examined under a transmission electron microscope. It was observed that all phages had hexagonal heads and long non-contractile tails. Thus, they belonged to group B of Bradley's classification and type B1 in the Family Siphoviridae. The host range of the phages was determined against 163 streptomycete strains and was found to vary from narrow to broad. Ten phages were characterized based on their morphology, physiology, genome, proteins, antigenic relationship and biology. Phages No.1 and No.15, which had broad host ranges, showed dissimilar patterns in the one step growth experiment. The majority of phages gave maximum replication at neutral pH although phage No.15 preferred basic conditions. Phages No.30 and No.27 were closely related serologically. The number and size of fragments obtained after digestion with restriction enzymes showed that all phages were unique. The results showed that they were distinguished from previously reported phages.

KEYWORDS: characteristics, distribution, streptomycetes, actinophages, Thai soil samples.

INTRODUCTION

The term actinophage was used to designate the phages of actinomycetes when they are first described in 1936 by Dmitrieff and Souteeff[1,2]. All actinophages possess tails and contain double-strand DNA. They comprise six morphological types[3,4] and belong to three families of Myoviridae, Siphoviridae and Podoviridae[5].

Soil possesses a wide variety of actinophages with highly polyvalent characteristics[6]. Streptomycete phages are popular and well-studied amomg actinophages, for example, phage ϕC31[7]. Many streptomycete phages have been isolated from soil and molecular biology of some of their genes have been characterized[7,8,9,10,11,12,13,14,15,16,17,18]. Also, the stability and ecology of phages in soil have been investigated[19]. So far, the study of the distribution and diversity of streptomycete phages has been rather discontinuous. Therefore, the objective of this study was to isolate and characterise the phages isolated against streptomycete species from Thai soil samples and determined their activity spectra.

MATERIAL AND METHODS

Soil Sampling and Isolation of *Streptomyces* sp.

Soil samples were taken from several provinces in Thailand. The pH of the soil was measured[20]. Isolation of *Streptomyces* sp. from soil was carried out on humic acid vitamin agar medium or HV-agar[21]. Soils were dried at 40-50°C for 2 h to reduce the bacterial population[22]. Then, 1 g of the dried soil was added to 9 ml of distilled water and serially diluted and enumerated on HV-agar by incubation at 30°C for 7-14 days. The spore suspensions of actinomycetes obtained were stored in 20 % (V/V) glycerol at –20°C.

Isolation of Novel Streptomycetes Phages

Isolation of phages was carried out following Dowding's method[23]. Spores (10^7 cfu/ml) of streptomycete hosts and twenty-five grams of soil sample were inoculated into a 250 ml Erlenmeyer flask containing 50 ml of nutrient broth and incubated at 200 rpm on a rotary shaker for 18 h at 30°C. The suspension was filter-sterilized through a sterile 0.45 mm cellulose acetate filter and the presence of phages was determined by the soft-agar overlay or double layer method using nutrient agar and soft nutrient agar as the media, pH 7.0[24]. The plates were incubated at 30°C until a confluent lawn was produced. A plaque forming plate was prepared for purification of single plaque. A single plaque was picked up by using a sterile needle and immersed into 2 ml of nutrient broth. Subsequently, 10 ml of the original host spore

suspensions was added, and incubated overnight. Plaque formation was assayed and the purification step was repeated at least 5 times with the original host in order to test their ability in reinfecting their hosts. Subsequently, high stock of phage lysate was prepared by pouring 5 ml of nutrient broth onto each plate containing plaques. This was then allowed to stand at room temperature for 5 h. Phage lysate was filter-sterilized through a 0.45 µm cellulose acetate filter, titred and stored at 4°C.

Transmission Electron Microscopy

Phage lysate (0.2 ml) was dropped onto the surface of a carbon-coated grid and left standing for 1 min, then remaining suspension was removed. The specimen was stained with 1% uranyl acetate for 1 min and examined with a JCM-200 CZ transmission electron microscope, at the magnifications of 72,000×and 100,000×[23,25,26].

Host Range Determination

The host ranges of phages were determined against 45 type strains of streptomycetes (kindly supplied by the National Science and Technology Development Agency (NSTDA) and by Prof. Dr. Seiya Ogata) and 118 isolated strains. Spore suspensions (10^7 spores/ml) were infected with phage at a multiplicity of infection (m.o.i.) of 0.1-0.01. The presence of plaques was examined after 18 h of incubation. In this study, thirty phages were characterized using the criteria described by van Regenmortel, *et al* (2000) [27].

One-step Growth Curve Experiment

The one-step growth experiment that was used followed the method of Dowding (1973) [23].

Effect of pH on Survival of Bacteriophage Isolates

Nutrient broth with various pH values was used for phage cultivation. The culture with phage infected was incubated at 30°C for 1 h. The number of phages was examined by the double agar method with nutrient agar and soft nutrient agar as the medium.

The optimal pH for phage multiplication was determined. Nutrient agar plates and soft agar overlays were adjusted to a specified pH in the pH range of 4.0 to 9.0. The number of phages was examined by the double agar method.

Preparation of Antisera

Antisera against phages were prepared according to the method of Adams (1959) [28]. The phage stocks used for immunization had at least 10^{10} pfu/ml, at which concentration they provided an adequate immunological response and showed no toxicity and pathogenicity to the animal. The host cells which might have been present were eliminated by membrane filtration through a 0.22 µm cellulose acetate filter. The purified phage particles (10^{10} pfu/ml; 5 ml) were injected into 2.5 kg, 6–month-old New Zealand white rabbits via a subcutaneous route twice a week for 3 weeks. Analysis of the reaction between phage and corresponded antibody was carried out via the equation $K = 2.3\ D/t \times \log p_0 / p$, where p_0 = phages examined at 0 min, p = phages examined at time t min, D = final dilution of serum in the phage-serum mixture and K = velocity constant.

Preparation of Streptomycetes Phage DNA

High titered phage lysates were prepared by the double layer technique from 100 plates of nutrient agar. Phage suspension was harvested from lysate plates after 24 h, then soaked with 5 ml nutrient broth for 5 h and then centrifuged at 1,200 xg at 4°C for 10 min to remove host debris. Phage DNA was extracted as described by Sambrook (1989)[31]. Quality of the extracted DNA was examined by agarose gel electrophoresis.

Plaque Hybridization

A lysate plate after incubated at 30°C overnight, was left standing at 4°C for 2 h to harden the top agar. Then, a dried nitrocellulose membrane was placed over the agar surface for 10 min. Prehybridization was carried out according to Benton and Davies (1977)[30]. Plaque hybridization was detected using a digoxigenin (DIG) high prime DNA labeling and detection starter kit I (company, city, state/country) according to the manufacturer's instructions.

Results and Discussion

Soil samples, designated as no. 1-31, were taken from several provinces of Thailand and pH values of the soil particles were determined. The average pH was in the range of 5.4 -8.0.

Under a transmission electron microscope, all phages had hexagonal heads and long non-contractile tails, similar to previously characterized *Streptomyces* phages (Fig. 1). Therefore, they belonged to group B of Bradley's classification[31], type B1. According to Ackermann and Eisenstark's taxonomy, all of them were in the family Siphoviridae[3]. Average size of heads ranged from 40 to 67 nm, except that of phage No.22 which was 80±5 nm. Tail length of all phages was in the range of 133 to 280 nm.

One hundred and eighteen strains of the isolated

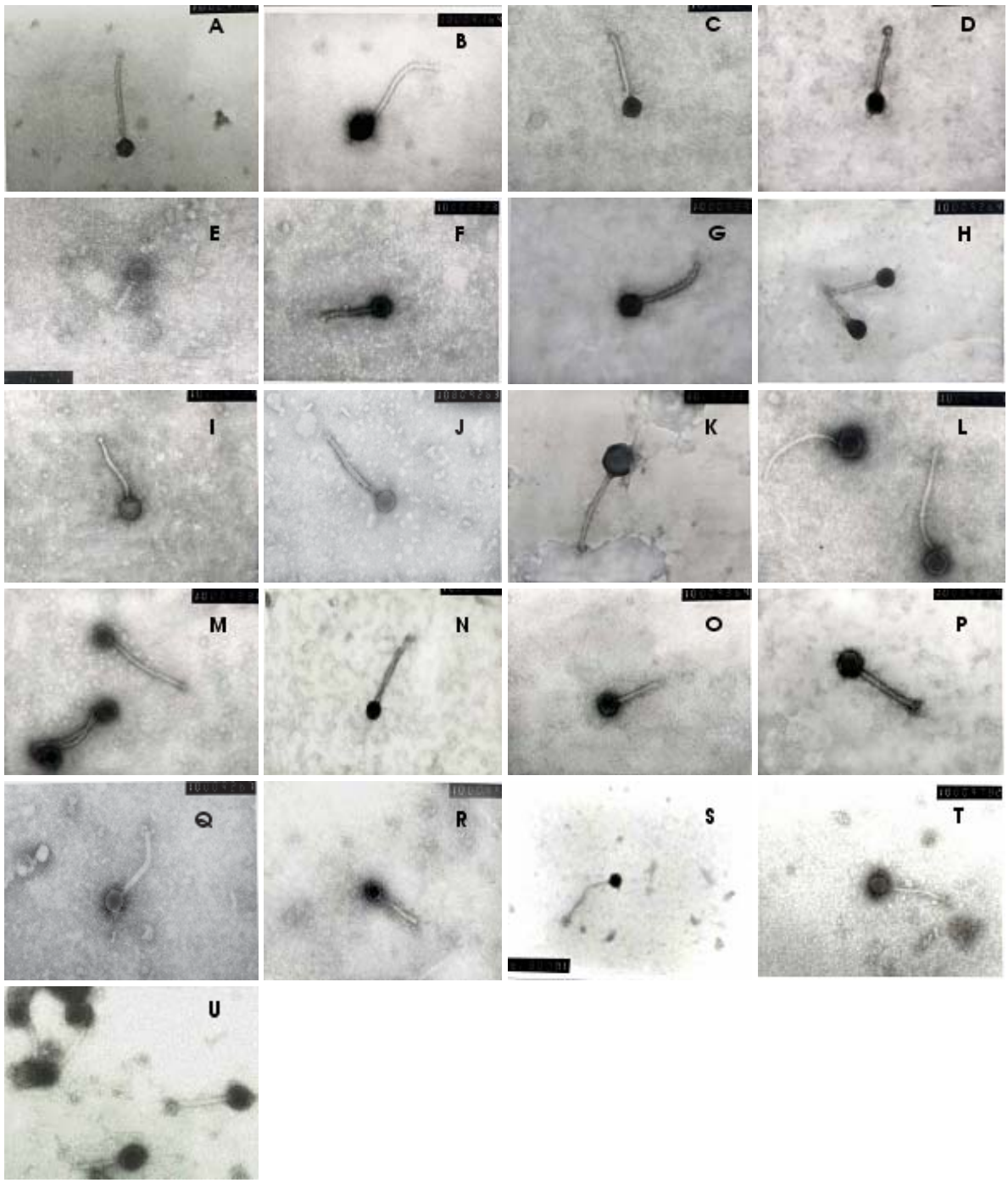

Fig 1. Phage morphology. (A) No.1, (B) No.2, (C) No.3, (D) No.6, (E) No.7, (F) No.9, (G) No.11, (H) No.12, (I) No.13, (J) No.15, (K) No.16, (L) No.17, (M) No.18, (N) No.19, (O) No.20, (P) No.22, (Q) No.27, (R) No.28, (S) No.29, (T) No.30, (U) No.31 (bar, 100 nm).

streptomycetes and 45 reference strains were used for host range determination. Among the isolated phages, phage No. 1 showed the widest range of hosts, 109 infected strains among 163 strains used, including *S. lividans* TK 21, *S. coelicolor* Muller (Table 1). However, it did not infect *S. thermovulgaris* JCM 4520 (thermophilic *Streptomyces*), while phages No.12, No.18, and No.29 did. None of phages could infect *Kitasatospora griseola* JCM 3339. All phages displayed different host range patterns that were narrow (No.2, No.3, No.5, No.22 and No.24) or broad (No.1, No.7, No.11, No.12, No.15, No.27, No.28, No.29, No.30 and

Table 1. Host range of phages.

Streptomycetes	Phage No.																	
	1	2	3	5	7	9	11	12	15	18	20	22	24	27	28	29	30	31
Kitasatospora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. alboniger	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. albovinaceous	-	-	-	-		+	+	+	-	+	-	-	-	+	-	-	-	-
S. albus	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	-	-	-
S. ambofaciens	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+
S. aminophilus	-	+	-	-	+	+	-	-	+	+	-	-	-	+	-	-	-	+
S. aureus	+	-	-	-	+	-	-	-	+	+	-	-	-	+	-	-	-	-
S. azureus	+	+	-	-	+	+	+	+	+	+	-	-	-	+	-	-	+	-
S. badius	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. bikiniensis	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
S. coelicolor M 145	+	+	-	-	+	-	-	-	+	+	-	-	-	+	-	-	+	-
S. coelicolor Muller	+	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+	+	T
S. coeruleofuscus	+	+	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-
S. coerulescens	-	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
S. coralus	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	-	+	+
S. cyaneus	+	+	-	-	+	-	+	+	+	-	+	-	-	-	-	-	-	-
S. echinatus	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+
S. endus	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	T
S. eurythermus	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
S. griseus ATCC 2926	+	+	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-
S. griseus KA-1198	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	+	+	-
S. humifer	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-
*S. hygroscopicus*4772	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-
*S. hygroscopicus*NOV-1	+	+	-	-	-	+	+	-	+	-	-	-	-	-	+	+	+	-
S. kanamyceticus KCCS-0775	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. kanamyceticus KCCS-0433	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. laurentii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. lavendualae subsp. *lavendulae*	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
S. lavendulae subsp. *japonicus*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. lavendulae subsp. *grasserius*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. lincoensis	+	-	-	-	+	-		-	+	-	-	-	-	+	+	+	-	-
S. lividans TK24	+	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+	+	-
S. luteogriseus	+	+	-	-	+	+	+	+	+	+	+	-	-	+	+	-	+	T
S. melanosporofaciens	+	+	-	-	+	-	+	-	+	-	-	-	-	-	+	+	+	-
S. niveous	+	+	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	+
S. nodosus	+	-	-	-	+	-	+	T	+	-	-	-	-	-	+	+	-	-
S. parvullus	+	+	-	-	+	-	+	+	+	+	-	-	-	-	+	+	+	+
S. perciperlis	+	+	-	-	+	-	-	+	+	+	-	+	-	-	+	-	+	-
S. pilosus	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. puniceus	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. sparsogenes	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
S. tauricus	+	-	-	-	+	-	-	+	+	-	-	-	-	+	+	+	+	-
S. thermovulgaris	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-
S. violaceochromogenes	+	-	-	-	+	-	-	+	+	-	-	+	-	-	-	-	-	-
S. violaceusniger	+	+	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-
S. viridochromogenes	+	-	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-
Subtotal 1(referenced streptomycetes)	32	15	0	0	22	8	16	18	39	16	5	3	0	10	14	12	14	9
Subtotal 2(isolated streptomycetes) (data not shown)	77	1	0	3	6	10	6	30	68	2	6	0	0	9	68	72	76	30
Total (1+2)	109	16	0	3	29	18	22	48	107	18	11	3	0	19	82	84	90	39

Symbol: +, infected; -, non-infected; T, turbid

Table 2. One-step growth experiments on phages.

Phage	Latent period (min)	Rise period (min)	Burst size(phage particles per infected cell)
No. 1	10	20	30-35
No.2	10	30	260-265
No.7	20	40	95-100
No.11	20	20	110-115
No.15	30	30	30-35
No.28	20	40	40-45
No.29	20	60	30-35
No.30	25	35	10-20

No.31) for forming plaques on *Streptomyces* strains. Host range determination revealed that most of the isolated phages acted as virulent phages and had a broad host range. Remarkably, thirty strains of the isolated phages could be distinguished to be closely related to family *Streptomyces* and *Kitasatospora* phages. Hahn *et al*. (1990)[32] suggested that the host range specificity of streptomycete phages which infected only *Streptomyces* sp could be applied to use as a tool for identification of streptomycete strains from soil. From the results, No.1 and No.15 had a very broad host range against the reference and isolated streptomycete strains. On the contrary, No.3, No.22, and No.24 displayed a narrow host range. No.3 and No.24 showed specific characteristics of being unable to form plaques with any other streptomycetes except the host from which they were originally isolated, which were *Streptomyces* sp. 3 and *Streptomyces* sp. 24, respectively. This characteristic might be profitably used to identify their host species, as previously reported in phages of *S. venezualae*[33].

One-Step Growth Experiments

Production of phages was determined in one-step growth experiments at 30°C, as shown in Table 2.

Effect of pH

Bacteriophages are usually stable over the pH range of 5.0 to 8.0[28] and the isolation of streptomycete phages from soil could be achieved above pH 5.0[34]. Results showed that all phages gave high titer at neutral pH, except phage No.15 (Table 3). Although stability of phages was found at pH 7.0 to 9.0, actinophages were rarely isolated when the pH of the eluting medium was below 7.0[35]. It has also been shown that the stability of actinophages is generally sensitive to pH values greater than 9.0[36]. Therefore, the optimum pH for the recovery of phages was preferably adjusted to a pH between 7.0-9.0. Phage No.15 was an exception, its optimum pH for isolation was 9.0 and it was stable at the same pH. Six out of nine phages were able to survive at pH 4.0 (data not shown).

Table 3. Optimum pH for phages.

Phages	pH optimum
No.1	8
No.7	6
No.11	7
No.15	9
No.27	7
No.28	6
No.29	7
No.30	7
No.31	7

Cross Reactivity of Phages

Hahn *et al.*, (1990)[32] studied the anti-FP-22 serum (very broad host specificity) and found that FP22 shared strong cross-immunity and antibody cross-reactivity with bacteriophage P23, but not with seven other streptomycete bacteriophages. An attempt to study the serological relevance between selected phages and antiserum against phage No.1, which posses the widest host range, was conducted. Anti-No.1 serum exhibited cross-reactivity against the selected strains of phages. Some phages showed closely related group patterns (Table 4). The closest serological relation to that of phage No.1 was found for phage No.10. Phages No.30 and No.27 showed moderate serological relevance to antiserum against phage No.1. Phages No.7, No.15 and No.29 were slightly related to phage No.1 with less than 10 % inactivation.

Plaque hybridization

The application of DNA probes to characterize heterogeneous phage populations may be used to simplify phylogenetic relationships among phages from similar habitats where they can be classified according to the level of DNA sequence similarities[37]. According to the DNA sequence similarity, it seemed that DNA of phage No.11 showed high similarity with that of 4 other

Table 4. Summary of phage properties.

Properties	Phages
1. pH of soil samples:	
1.1 Neutral pH (6-8)	No.1, No.2, No.3, No.5, No.6, No.7, No.9, No.10, No.12, No.13, No.15, No.16, No.17, No.19, No.20, No.22, No.24, No.27, No.28, No.29, No.30
1.2 Acidic pH (<6)	No.11,No.18
2. Plaque transparency:	
2.1 Clear	No.1, No.2, No.5, No.7, No.9, No.11, No.12, No.13, No.15, No.16, No.17, No.18, No.19, No.20, No.27, No.28, No.29
2.2 Turbid	No.3, No.6, No.10, No.22, No.24, No.30, No.31
3. Phage Morphology:	
Six-sided head and a long non-contractile tail	All
4. Host range determination:	
4.1 Broad	No.1, No.2, No.6, No.7, No.9, No.11, No.12, No.13, No.15, No.16, No.17, No.18, No.19, No.20, No.27, No.28, No.29, No.30, No.31
4.2 Narrow	No.3 No.5, No.22, No.24
5. One-step growth experiment:	
5.1 Burst size 10-50	No.1, No.15, No.28, No.29, No.30
5.2 Burst size >50	No.7, No.11
6. Stability and optimal pH:	
6.1 Neutral pH (6-8)	No.1, No.7, No.11, No.27, No.28, No.29, No.30, No.31
6.2 Basic pH (>8)	No.15
7. Effect of cations:	
7.1 Mg^{2+} (0-20 mM)All	
7.2 Ca^{2+} (0-20 mM)	No.1, No.7, No.11, No.29, No.31
Ca^{2+} (>30 mM)	No.15 No.27, No.28, No.30
8. Serological relevance between phages and antiserum against phage No.1:	
8.1 Close	No.30, No.27
8.2 Moderate	No.11, No.28, No.31
8.3 Slight	No.7, No.15, No.29
9. DNA homology to:	
9.1 Phage No.1	-
9.2 Phage No.7	No.15, No.28, No.30
9.3 Phage No.11	No.7, No.15, No.18, No.30
9.4 Phage No.15	No.7, No.11, No.28

phages. Phages No.7 and No.15 each showed similarity to 3 phages, whereas No.1 did not resemble any other phage (Table 5).

Plaque hybridization was used to examine the relatedness among phage DNA. Nutrient agar plates were overlaid with *Streptomyces* sp. 27 and *S. viridochromogenes*. Then, 5 ml of various phages containing approximately 10^4 pfu/ml was dropped onto plates with adequate spacing for an appropriate plaque appearance.

Phage DNA

The DNA restriction pattern was used to distinguish phages. From this experiment, phage DNA, which were

Table 5. Plaque hybridization of phages.

Probe	Homology										
	No.1	No.7	No.11	No.12	No.15	No.18	No.27	No.28	No.29	No.30	No.31
No.1	+	-	-	-	-	-	-	-	-	-	-
No.7	-	+	-	-	+	-	-	+	-	+	-
No.11	-	+	+	-	+	+	-	-	-	+	-
No.15	-	+	+	-	+	-	-	+	-	-	-

Symbols: +, homology; -, non-homology

Table 6. Restriction analysis of phages.

Phage Enzyme	Restriction fragments				
	No.1	No.7	No.11	No.12	No.15
Afl II	ND	ND	ND	ND	-
Alu I	ND	> 10	ND	ND	> 10
Apa I	-	-	> 10	ND	> 10
Bam HI	> 10	9	ND	-	-
Ban II	> 10	-	-	-	-
Bgl I	ND	ND	ND	ND	> 10
Bgl II	3	-	7	-	8
Dra I	ND	-	ND	ND	-
EcoR I	-	9	6	-	3
EcoR V	-	ND	7/8	-	-
Hind III	-	-	ND	-	-
Kpn I	ND	11	ND	-	ND
Mlu I	-	10	8	-	5
Mun I	ND	3	ND	ND	-
Nde I	ND	4	3	-	-
Nhe I	ND	-	-	ND	-
Not I	ND	-	> 10	ND	-
Pst I	-	ND	-	-	-
Sac I	> 10	ND	> 10	-	> 10
Sal I	> 10	ND	ND	-	> 10
*Spe*I	-	ND	> 10	-	-
Sph I	-	5	-	-	9
Xba I	ND	ND	ND	ND	-
Xho I	-	ND	> 10	ND	-
Length of genome (bp)	40,500	50,000	44,000	ND	55,000

Symbols: -, no restriction fragments; ND, not determined

digested with restriction enzymes, showed unique patterns and were distinct from those of ϕC31[38], SH10[18], TG1[39] and any other phages that have previously been reported (Table 6).

It was found that properties of phages used in this study such as morphology, physiology, genome, antigenic relationship and biology were different from phages previously reported[7,8,9,10,11,12,13,14,15,16,17,18]. A summary of phage properties in this study is shown in Table 4.

This report was focused on *Streptomyces* phages isolated in Thailand and expected to generate some useful information concerning the distribution and diversity of phage in soil from different parts of Thailand, especially actinophages. The isolated phages and information obtained will be of value in further work on phage molecular biology.

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