การกระจายและความหลากหลายของแอคติโนฟาจในดินที่มีสเตรปโตมัยซิทิส

นางสาวอรอนงค์ พริ้งศุลกะ

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DISTRIBUTION AND DIVERSITY OF ACTINOPHAGES IN SOILS POPULATED WITH STREPTOMYCETES

Miss Onanong Pringsulaka

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ได้ทำการศึกษาการกระจายและความหลากหลายของแอคติโนฟาจจากแหล่งดินกระจายตามภาคต่าง ๆของประเทศ โดยเริ่มจากการแยกสเตรปโตมัยซิทิสซึ่งเป็นโฮสท์ (ตัวให้อาศัย) จากนั้นทำการแยกฟาจโดยวิธีส่งเสริมการเจริญและใช้สเตรปโตมัยซิทิสจากดินแหล่งเดียวกันเป็นโฮสท์ พบว่าสามารถแยกฟาจได้ทั้งหมด 24 ชนิด และได้ศึกษาการสร้างสารปฏิชีวนะของโฮสท์ นำฟาจที่ได้มาทำให้บริสุทธิ์บางส่วนด้วยวิธีการแยกพลัคเดี่ยว แยกความแตกต่างของฟาจโดยดูจากรูปร่างลักษณะของพลัคที่เกิดกับโฮสท์และรูปร่างลักษณะของฟาจภายใต้กล้องจุลทรรศน์อิเลคตรอนแบบส่องผ่าน พบว่าฟาจที่ได้มีขนาดและรูปร่างแตกต่างกัน แต่มีลักษณะที่คล้ายกันคือมีส่วนหัวเป็นหกเหลี่ยม ส่วนหางยาวไม่สามารถหดได้ ด้วยลักษณะดังกล่าวจึงจัดฟาจเหล่านี้อยู่ในกลุ่มบี ตามการจัดจำแนกตามลักษณะรูปร่างของ Bradley และเป็นชนิด B1 ใน Family Siphoviridae ตามการจัดจำแนกของ Ackermann and Eisenstark's taxonomy จากการศึกษาโฮสท์เรนจ์หรือความสามารถในการติดเชื้อในสเตรปโตมัยซิทิสสายพันธุ์ต่าง ๆ ทั้งหมด 164 สายพันธุ์ พบว่าฟาจทั้งหมดให้รูปแบบโฮสท์เรนจ์แคบ-กว้างต่างกัน โดยฟาจ Roi-1 และ Yok-15 ให้โฮสท์เรนจ์กว้างที่สุด จากนั้นนำฟาจจำนวน 9 ชนิด ได้แก่ Roi-1, Ray-7, Surat-11, Yok-15, Sin-27, Nsaw-28, Lam-29, Nsaw-30 และ Ac- 7 ที่มีโฮสท์เรนจ์กว้างมาศึกษาขั้นต่อไป จากการทดลอง one step growth พบว่าให้รูปแบบแตกต่างกัน จากผลของพีเอชต่อการเพิ่มจำนวนของฟาจพบว่าฟาจส่วนใหญ่สามารถเพิ่มจำนวนได้มากที่พีเอชเป็นกลางยกเว้นฟาจ Yok-15 ที่เพิ่มจำนวนได้ดีที่พีเอชเป็นด่าง ผลของแคทไอออนทั้ง 2 ชนิดได้แก่แคลเซียมและแมกนีเซียม พบว่าฟาจบางชนิดไม่ต้องการ ส่วนชนิดที่ต้องการแคทไอออนทั้งสองก็ต้องการในปริมาณต่ำยกเว้นฟาจ 4 ชนิดคือ Yok-15, Sin-27, Nsaw-28 และ Nsaw-30 ที่ต้องการแคลเซียมที่ความเข้มข้นสูง ผลของการยับยั้งฤทธิ์ของแอนติบอดีที่ได้จากฟาจ Roi-1 ต่อฟาจชนิดที่ทำการทดสอบ พบว่าให้ผลการยับยั้งต่อฟาจ 2 ชนิด คือ Nsaw-30, Sin-27 ได้ดีที่สุด ส่วนการศึกษาโปรตีนของฟาจโดยวิธี SDS-PAGE พบว่าฟาจที่ใช้มีขนาดและความเข้มของแถบโปรตีนต่างกัน การศึกษาจีโนมของฟาจโดยการตัดดีเอ็นเอด้วยเอนไซม์ตัดจำเพาะหลาย ๆชนิด พบว่าดีเอ็นเอของฟาจหลังจากตัดด้วยเอนไซม์ให้รูปแบบของแถบและน้ำหนักโมเลกุลต่างกัน นอกจากนี้เมื่อศึกษาความคล้ายคลึงกันของดีเอ็นเอของฟาจ โดยวิธี plaque hybridization พบว่าเมื่อใช้ดีเอ็นเอของฟาจ Surat-11, Ray-7, Yok-15 และ Roi-1 เป็นตัวติดตาม (probe) พบความคล้ายคลึงกัน (homology) ของดีเอ็นเอของฟาจเป็นจำนวน 5, 4, 4 และ 0 ชนิด ตามลำดับ

อนึ่งจากการศึกษาความสัมพันธ์ระหว่างฟาจ Yok-15 (ซึ่งให้ผลของการติดเชื้อในสเตรปโตมัยซิทิสสายพันธุ์อ้างอิงได้มากที่สุดคือ 39 สายพันธุ์ จาก 46 สายพันธุ์) กับโฮสท์ 4 สายพันธุ์คือ *Streptomyces* sp. 15, *S. coelicolor*, *S. griseus* และ *S.viridochromogenes* ในดินไทย 2 ชนิดและดินญี่ปุ่น 1 ชนิด เมื่อบ่มฟาจ และ Streptomyces sp. 15 ในดินไทยทั้ง 2 ชนิด พบว่าฟาจสามารถเพิ่มจำนวนได้จนถึงปริมาณสูงสุด หลังจากนั้นปริมาณลดลงอย่างรวดเร็ว อย่างไรก็ตามเมื่อนำฟาจ Yok-15 มาบ่มในดินไทยกับโฮสท์อื่นอีก 3 สายพันธุ์ พบว่าไม่สามารถเพิ่มจำนวนได้ อีกทั้งไม่มีการเพิ่มจำนวนเลยในโฮสท์ทั้ง 4 สายพันธุ์เมื่อบ่มในดินญี่ปุ่น นอกจากนี้ยังพบว่าเมื่อบ่มฟาจ Yok-15 กับสเตรปโตมัยซิสทั้ง 4 สายพันธุ์ ปริมาณฟาจที่ได้มีจำนวนน้อยกว่าปริมาณของฟาจอิสระ (ที่ไม่ได้บ่มกับโฮสท์) ในดินทั้ง 3 ชนิด จากผลดังกล่าวชี้ให้เห็นว่าภาวะของดินและโฮสท์ที่ใช้มีผลต่อการรอดชีวิตและการเพิ่มจำนวนของฟาจในดิน ในทางกลับกันภาวะของดินมีผลต่อการการรอดชีวิตของสเตรปโตมัยซิทิสมากกว่าการติดเชื้อด้วยฟาจ

เมื่อทำการจัดจำแนกสปีชีส์ของ *Streptomyces* sp. 15 โดยใช้ส่วนของลำดับเบสที่ประมวลรหัสของ 16S rDNA พบว่ามีความคล้ายคลึงกับ Streptomyces sp. NRRL5183 แต่เนื่องจากไม่มีรายงานถึงเชื้อนี้โดยละเอียด จึงได้นำ *Streptomyces* sp. 15 เข้าไปจดทะเบียนใน Genbank เป็นเชื้อใหม่ (หมายเลข AY128706) สำหรับฟาจ Yok-15 พบว่าไม่มีความเสถียรต่อคลอโรฟอร์ม มีความเสถียรที่อุณหภูมิ 4 องศาเซลเซียส ที่พีเอช 9 เป็นเวลา 5 ชั่วโมง และลดปริมาณอย่างรวดเร็วในที่มีโซเดียมไพโรฟอสเฟต และ EDTA นอกจากนี้ฟาจสามารถเพิ่มจำนวนได้ในโฮสท์ที่เลี้ยงในอาหารเหลว โดยพบว่าฟาจสามารถรอดชีวิตในโฮสท์ที่เลี้ยงในอาหารเหลวที่พีเอช 5 และเพิ่มจำนวนได้ดีที่สุดที่พีเอช 9 ความเร็วรอบของเครื่องเหวี่ยง 200 รอบต่อนาที ปริมาณของโฮสท์และฟาจที่เหมาะสมคือ 1.41×10^8 cfu/ml และ 10^6 pfu/ml ตามลำดับ

ภาควิชา.....-..... ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา....2545..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORDS: ACTINOPHAGES/ STREPTOMYCETES/ SOIL/ DISTRIBUTION/ DIVERSITY

ONANONG PRINGSULAKA: DISTRIBUTION AND DIVERSITY OF ACTINOPHAGES IN SOILS POPULATED WITH STREPTOMYCETES. THESIS ADVISOR: ASSOC. PROF. SURINA CHAVANICH, Ph.D., THESIS CO-ADVISOR: PROF. SEIYA OGATA, Ph.D., 170 pp. ISBN 974-17-1297-9

Distribution and diversity of actinophages from soil samples collected from several parts of Thailand were investigated. At first, streptomycetes were isolated and used as hosts for isolation of phages. By enrichment procedure, 24 phages were obtained and their hosts were further studied for antimicrobial producing activity. The phages were partially purified by single plaque isolation and then distinguished by plaque morphology and morphology examination under transmission electron microscope. All phages gave differences in size and shape but they all had a hexagonal head and long non-contractile tails. Thus, they were belonged to group B of Bradley's classification and type B1, in Family Siphoviridae of Ackermann and Eisenstark's taxonomy. Determination of host range of phage with 164 streptomycetes strains, it revealed that all phages displayed the different host range pattern by forming plaques on *Streptomyces* strains and varied from narrow to broad host range. Phage Roi-1 and Yok-15 each gave very broad host range. The phages giving broad host range were brought to further studied. They showed dissimilar patterns in one step growth experiment. Most of them were able to give maximum replication at neutral pH except phage Yok-15, which was at basidic pH. Requirement of cations, Ca^{2+} and Mg^{2+}, they were also different. Some phages did not require at all, some required small amounts except Yok-15, Sin-27, Nsaw-28 and Nsaw-30 that required high amounts of Ca^{2+}. Antigenic relationship between the 9 phages and anti-phage Roi-1 serum revealed that Nsaw-30 and Sing-27 were highly inactivated, thus they were closely serological relatedness. SDS-PAGE showed that the phages displayed a different size of protein bands. The number and size of fragments of all phage DNA, which obtained after digestion with restriction enzymes, showed a unique and different molecular weights. The homology of phage DNA was also employed by plaque hybridization. When using DNA of Surat-11, Ray-7, Yok-15 and Roi-1 DNA as probes, it resulted that the homology of phage DNA toward these probes were 5, 4, 4 and 0, respectively.

The fate of actinophage Yok-15, and its host bacteria, *Streptomyces* sp. 15 (isolated from Thai soil as well as phage Yok-15*)*, *S. coelicolor*, *S. griseus* and *S. viridochromogenes* in two kinds of Thai and Japanese sterile soils using batch microcosms, was also investigated. Phage Yok-15 had a very wide host range, and could infect 39 important strains of *Streptomyces* species from 48 strains tested. In two kinds of Thai soils, phage Yok-15 multiplied in host strain 15 at a maximal titer level, then decreased rapidly in titer level during incubation. However, no multiplication of phage YoK-15 was observed during the incubation with other three streptomycetes in Thai soils, and also with all used streptomycetes in Japanese soil. During incubation with these streptomycetes, the phages decreased in their titer more rapidly than free phages in both Thai and Japanese soils. These results indicated that natures or conditions of soil and strains of host streptomycetes may affect on the survival and multiplication of phages in soil. On the other hand, the survival of host streptomycetes seemed to be strongly affected by the conditions of soil rather than phage infection.

Streptomyces sp. 15 was identified by partial 16S rDNA sequences covering the variable region. It resulted that *Streptomyces* sp. 15 was closely resembled to *Streptomyces* sp. NRRL 5183 (accession number AJ391815) which has published in year 2000 and has no any report supported since then. Thus, this strain was designated as a new strain (accession number AY128706). To further characterization of phages Yok-15, the phage was sensitive to chloroform, it stabilized at temperature of 4 ^{0}C, at pH 9 for 5 h, and rapidly inactivated by detergents: sodium pyrophosphate and EDTA. In addition, it could multiply in liquid culture with maximum yields at 200 rpm, with initial host and phage concentration at 1.41×10^{8} cfu/ml and 10^{6} pfu/ml, respectively, and could survive at pH 5.

Department .....-..... Student's signature.....
Field of study.....Biotechnology..... Advisor's signature.....
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สถาบันวิทยบริการ
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CHAPTER I

INTRODUCTION

Bacteriophages (phages) are obligate intracellular parasites that multiply inside bacteria by using host biosynthetic machinery, therefore, phage metabolites are inert in their extracellular surroundings. The first bacteriophage, *Bacteriophagum intestinale* was described by Felix D'Herelle (D'Herelle, 1918). The replication of bacteriophages is very different than that of animal viruses. With animal viruses, the whole virus is often taken into the cell and the nucleic acid uncoated therein (Luria *et al.*, 1978). While, bacterial viruses, the separation of nucleic acid and protein always occurs at the cell surface and only nucleic acid was injected. Currently, over 5000 negatively stained phages and defective phages, observed by electron microscopy since 1960, could be attributed to 13 virus families (Ackermann, 2001). Defective phages include shadowed phages, prophages, mutants and phages without hosts (e.g., phages observed in water or rumen and not propagated)(Van Regenmortel *et al.*, 2000). Phages infecting most of the major groups of eubacteria and archaebacteria have been identified and observed in the natural habitats of their hosts. Quantification of phage densities is complicated by the fact that plaque counts enumerate only those phages plating on a specific host and are generally lower than electron microscopic counts which may include many inactive particles (Campbell, 1994).

1.1 Basic structure of bacteriophages

The main structure of all viruses consists of a nucleic acid core surrounded by a protein coat. The nucleic acid forms a long filamentous molecule and usually consists of a single nucleic acid molecule, which can be either single-stranded or double-stranded, linear or circular DNA, or single-stranded linear RNA. In 1959, Lwoff and his collaborators described these parts. The nucleic acid was called the core and the protein coat, protecting nucleic acid from nucleases and harmful substances, was called the capsid. The capsid is made up

of morphological subunits as seen under the electron microscope, called capsomeres. The capsomeres consist of a number of protein subunits or molecules called protomers (Pelczar *et al*., 1988). In both icosahedral tailess and tailed phages, the nucleic acid is contained in a hollow region formed by the capsid and is highly compact. In a filamentous phage the nucleic acid is embedded in the capsid and is present in an extended helical form. The complete infectious virus particle is known as the virion (Bradley, 1967).

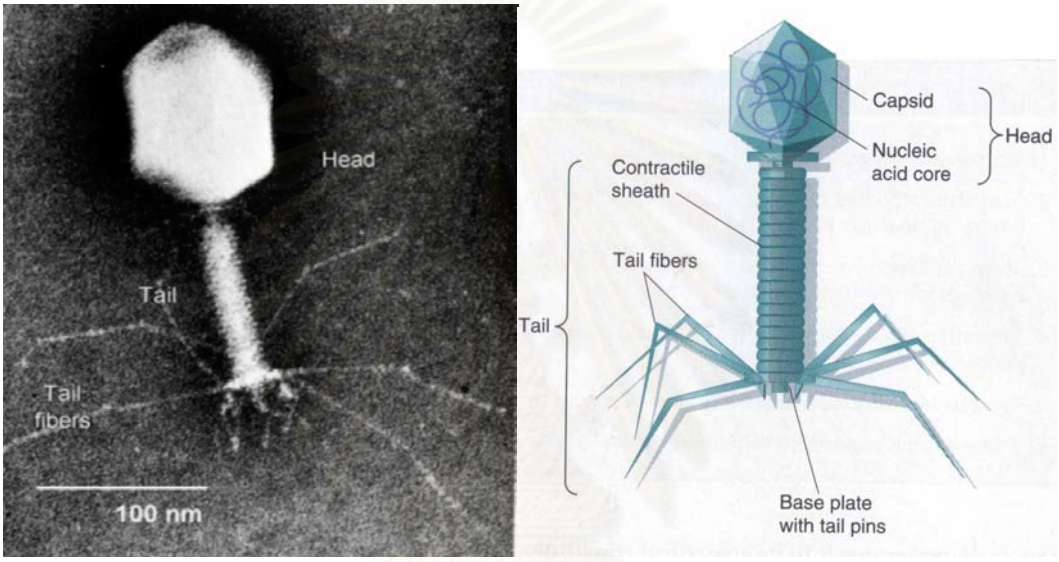

Fig. 1.1 Basic structure of bacteriophages
(www.mansfield.ohio-state.edu; McKane and Kandel, 1996)

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1.2 Basic Morphological Types of Bacteriophages

According to Bradley (1967), phages can be divided into 7 types. (Fig. 1.2) The first type (A) has a hexagonal head, which may or may not be elongated and a tail with a contractile sheath attached to it. This is usually rigid and may have various appendages such as fibers or terminal structures. The second group (B) also has a six-sided head and a tail, however, the tail is relatively flexible. The tail may or may not have terminal appendages and longer than the head diameter without contractile apparatus. The third type (C) also has a tail, a six-sided head and a non-contractile apparatus, but the tail is shorter than the head diameter and it may also have appendages attached to it. The fourth group (D) has no tail but is still six-sided in outline. Some posses knob or large capsomere on each apex of the hexagon. In the fifth group (E), the large capsomere are absent and the virion presents a simple hexagonal outline. The sixth group (F) is dissimilar from the others, the virion is in the form of a long flexible filament. Phages of the final group (G) have a lipid envelope and are pleomorphic (Pelczar *et al.*, 1988).

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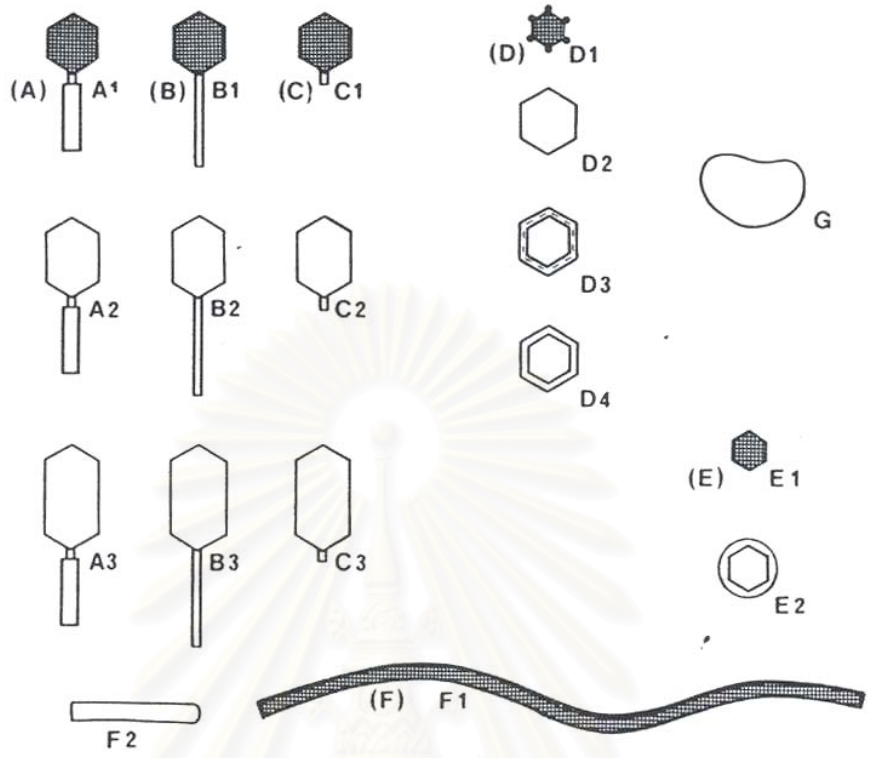

Fig. 1.2 Basic morphological types of bacteriophages (Coetzee, 1987)

1.3 Bacteriophage taxonomy and classification

Procedures for viral taxonomy and nomenclature were organized in 1966 with the establishment of the International Committee for Taxonomy of Viruses (ICTV) (Maniloff *et al.*, 1994). The ICTV developed the nomenclature and taxonomy for virus agreement. In 2001, primarily based on the 7th ICTV report and subsequent taxonomic decisions approved during the ICTV meeting at the ICTV Congress in Sydney 1999, the taxonomic lists of viruses consisted of families and genera of phages as shown in Table 1.1 and 1.2. The properties used for phage classification are listed in Table 1.3.

Table 1.1 Families and genera of phages (Van Regenmortel *et al*., 2000)

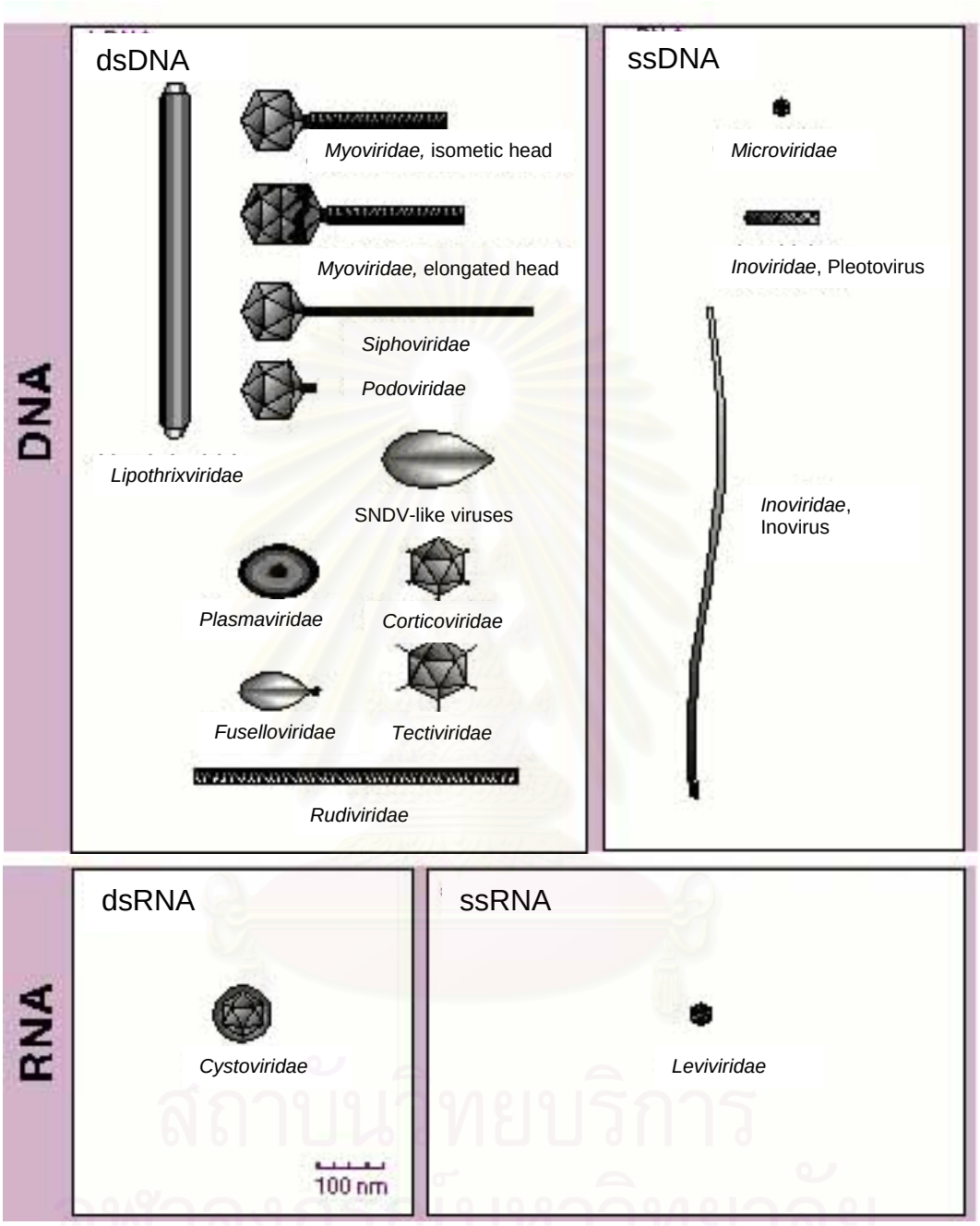

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Table 1.2 Descriptive of orders and families of phages

(Van Regenmortel *et al.*, 2000)

Order	Family [Subfamily]	Genus	Type Species
	Fuselloviridae	Fusellovirus	Sulfolobus virus SSV1
	Lipothrixviridae	Lipothrixvirus	Thermoproteus virus 1
Caudovirales	Myoviridae	ϕH-like viruses	Halobacterium virus ϕH
	Rudiviridae	Rudivirus	Sulfolobus virus SIRV1
Caudovirales	Siphoviridae	ψM1-like viruses	Methanobacterium ψM1
		Sulfolobus SNDV-like viruses	Sulfolobus virus SNDV
Caudovirales	Myoviridae	T4-like viruses	Enterobacteria phage T4
		P1-like viruses	Enterobacteria phage P1
		P2-like viruses	Enterobacteria phage P2
		Mu-like viruses	Enterobacteria phage Mu
		SPO1-like viruses	Bacillus phage SPO1
	Podoviridae	T7-like viruses	Enterobacteria phage T7
		ϕ29-like viruses	Bacillus phage ϕ29
		P22-like viruses	Enterobacteria phage P22
	Siphoviridae	λ-like viruses	Enterobacteria phage λ
		T1-like viruses	Enterobacteria phage T1
		T5-like viruses	Enterobacteria phage T5
		c2-like viruses	Lactococcus phage c2
		L5-like viruses	Mycobacterium phage L5
	Corticoviridae	Corticovirus	Alteromonas phage PM2
	Cystoviridae	Cystovirus	Pseudomonas phage ψ6
	Inoviridae	Inovirus	Enterobacteria phage M13
		Plectrovirus	Acholeplasma phage MV-L51
	Leviviridae	Levivirus	Enterobacteria phage MS2
		Allolevivirus	Enterobacteria phage Qβ

Table 1.2 (continue)

Order	Family [Subfamily]	Genus	Type Species
	Microviridae	Microvirus	Enterobacteria phage ϕX174
		Spiromicrovirus	Spiroplasma phage 4
		Bdellomicrovirus	Bdellovibrio phage MAC1
		Chlamydiamicrovirus	Chlamydia phage 1
	Plasmaviridae	Plasmavirus	Acholeplasma phage L2
	Tectiviridae	Tectivirus	Enterobacteria phage PRD1

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Table 1.3 Properties for phage classification (Van Regenmortel *et al.*, 2000)

I. Virion properties

A. Morphology properties of virions

1 Virion size

2 Virion shape

3. Presence or absence of an envelope and peplomers

4. Capsomeric symmetry and structure

B. Physical properties of virons

1. Molecular mass of virions

2. Buoyant density of virions

3. Sedimentation coefficient

4. pH stability

5. Thermal stability

6. Cation (Mg++, Mn++) stability

7. Solvent stability

8. Detergent stability

9. Radiation stability

C. Properties of genome

1 Type of nucleic acid - DNA or RNA

2. Strandedness - single stranded or double stranded

3. Linear or circular

4. Sense - positive, negative, or ambisense

5. Number of segments

6. Size of genome or genome segments

7. Presence or absence and type of 5′-terminal cap

8. Presence or absence of 5′-terminal covalently linked polypeptide

9. Presence or absence of 3′-terminal poly(A) tract (or other specific tract)

10. Nucleotide sequence comparisons

Table 1.3 (continue)

D. Properties of proteins

1. Number of proteins
2. Size of proteins
3. Functional activities of proteins
4. Amino acid sequence comparisons

E. Lipids

1. Presence or absence of lipids
2. Nature of lipids

F. Carbohydrates

1. Presence or absence of carbohydrates
2. Nature of carbohydrates

II. Genome organization and replication

1. Genome organization
2. Strategy of replication of nucleic acid
3. Characteristics of transcription
4. Characteristics of translation and post-translational processing
5. Site of accumulation of virion proteins, site of assembly, site of maturation and release
6. Cytopathology, inclusion body formation

III. Antigenic properties

1. Serological relationships
2. Mapping epitopes

IV. Biological properties

1. Host range, natural and experimental
2. Pathogenicity, association with disease
3. Tissue tropisms, pathology, histopathology
4. Mode of transmission in nature
5. Vector relationships
6. Geographic distribution

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1.4 Stages in the phage life cycle (Maloy *et al.*, 1994)

The phage life cycle is divided into two distinct categories: the lytic and lysogenic cycles. A phage in the lytic cycle converts an infected cell into a phage factory, and many phage progeny are produced. A phage capable only of lytic growth is called virulent. In the lysogenic cycle, which has been observed only with phages containing double-stranded DNA, no progeny particles are produced. Instead, the phage DNA integrates into bacterial chromosome. A phage capable of undergo such life cycle is called temperate phage. Most temperate phages also undergo a lytic cycle under certain circumstances.

When a virulent phage forms a plaque on a lawn of growing bacteria, the plaque form a halo zone since all bacteria in the center of the plaque are killed and lysed. However, temperate phages, such as λ, form a plaque with a turbid center. The turbidity is caused by the growth of phage-immune lysogenic cells in the plaque.

1.4.1 Lytic cycle

The typical lytic cycle of phages containing double - stranded DNA is described below (Fig. 1.3).

1. Adsorption of the phage to specific receptors on the bacterial surface is taken place. Many different types of phage receptors are existing, typically, phage receptors are proteins or carbohydrates present on the surface of the bacteria that normally serve for other purposes other than phage adsorption. It is observed that specific strain of bacteriophages can only adsorb to specific strain of host bacteria which known as viral specificity.

2. The DNA from the phage is then passed through the bacterial cell wall in a process known as penetration.

3. The infected bacterium is converted into a phage, producing cell. After phage infection, bacteria often lose the ability to either replicate or to transcribe their own DNA.

4. Production of phage nucleic acid and proteins then takes place. This stage occurs either through synthesis of phage-specific DNA and RNA

polymerases or the specificity modification of bacterial polymerases by phage proteins.

5. The assembly of phage particles known as morphogenesis. Two types of proteins are needed for the assembly process: structural proteins, which are present in the phage particle, and catalytic proteins, which participate in the assembly process but do not be form parts of the phage particle.

6. The release of newly synthesized phage then occurs, from which 50 to 200 phages may be produced per infected bacterium.

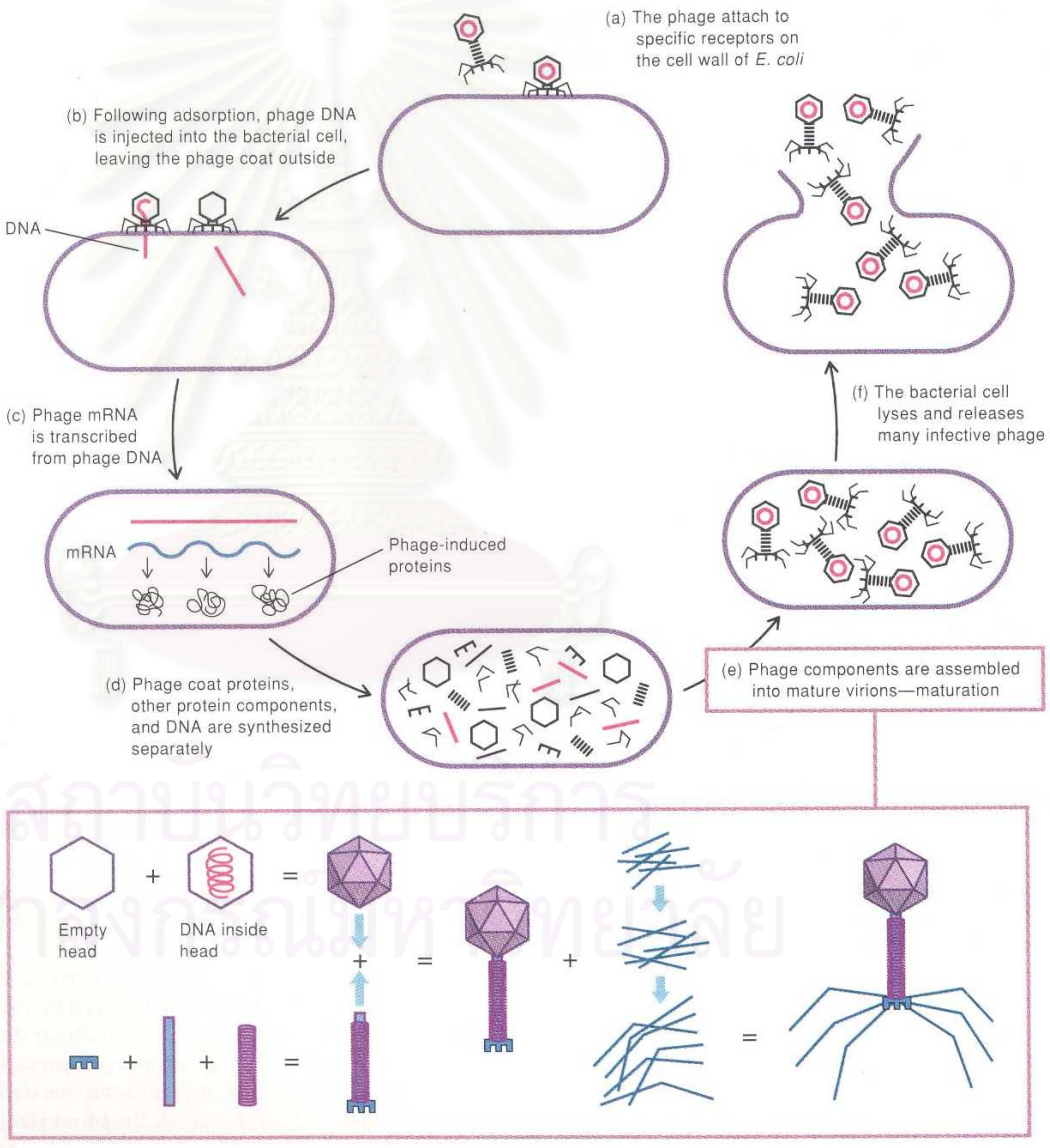

Fig. 1.3 (a-f) Steps in the replication of T-even phages during the infection of *Escherichia coli* (Nester *et al.*, 1995)

1.4.2 Lysogenic cycle

There are two types of lysogenic cycles. In Fig. 1.4, the most common lysogenic pathway: the linear phage DNA molecule is injected into a bacterium. A brief period of mRNA synthesis is needed to synthesize a repressor protein which inhibits the synthesis of the mRNA species that encode the lytic functions and a site-specific recombination enzyme. Phage mRNA synthesis is then turned off by the repressor. Recombination of the phage DNA molecule and the DNA of the bacterium inserts the phage DNA into the bacterial chromosome. The bacterium continues to grow and multiply, and the phage genes replicate as part of the bacterial chromosome.

The second type of lysogenic pathway, which is less common, differs from the preceding one in that there is no DNA-insertion system, and the phage DNA becomes a plasmid (an independently replicating circular DNA molecule) rather than a segment of the host chromosome. *E. coli* phage P1 is an example of this type of lysogenic pathway (Maloy *et al*., 1994).

A phage capable of entering both lytic and lysogenic life cycle is called a temperate phage. A bacterium containing a complete set of phage genes is called a lysogen and the phage DNA in lysogens is called a prophage.

Lysogens have two important properties. They are resistant to reinfection by a same type of phage that previously lysogenized the cell. This resistance to superinfection is called immunity. Secondly, a lysogen can initiate a lytic cycle even after many cell generations in this process called induction, by which the phage genes are excised as a single segment of DNA.

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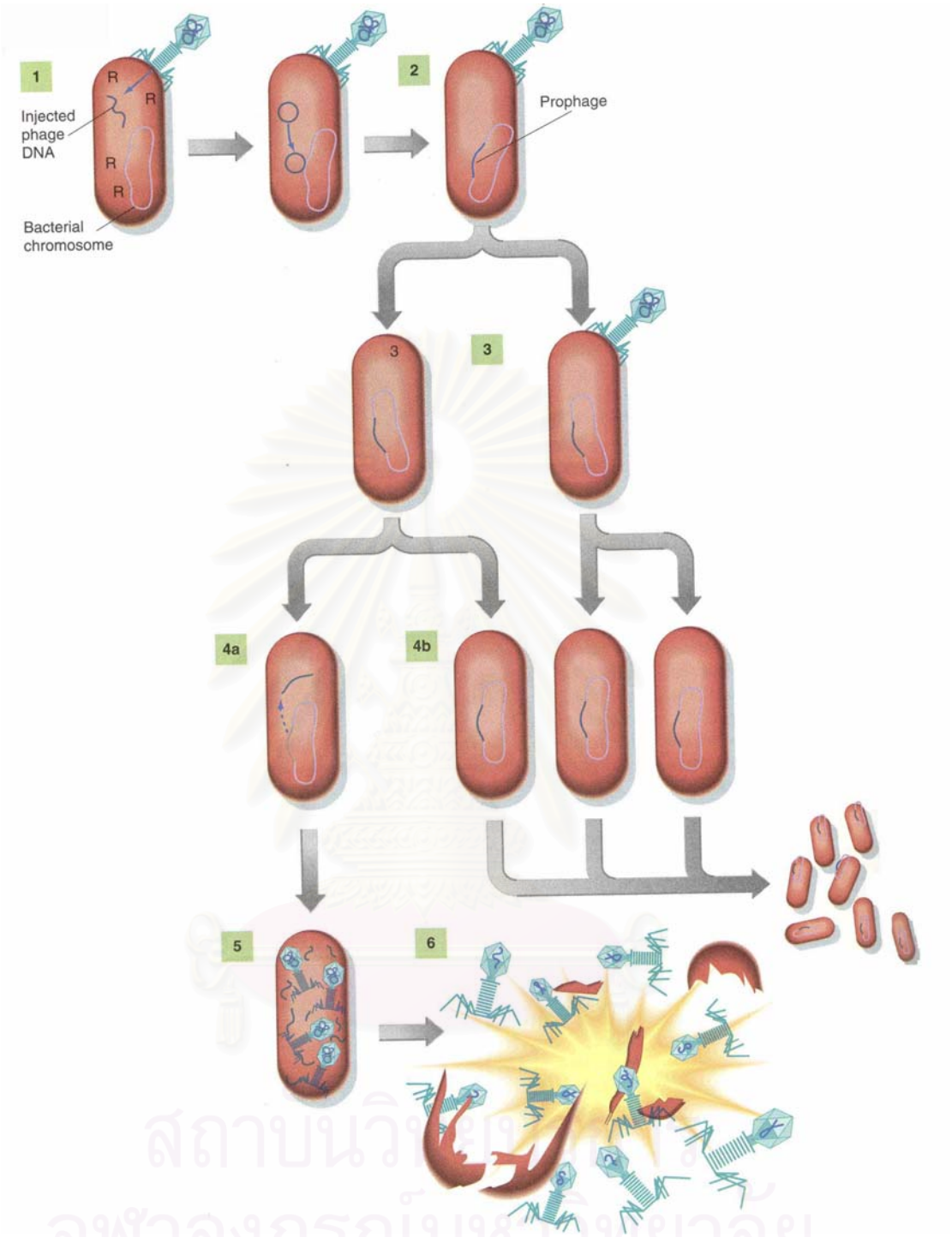

Fig. 1.4 Lysogenic cycle (McKane and Kandel, 1996)

(1) Temperate phage infected the cell and represses its own replication (R= repressor molecules). (2) Phage DNA integrates into host chromosome. (3) Prophage is replicated with host chromosome. (4b) Most of cells continue to divide, showing no evidence of viral infection. (4a) Spontaneous induction: In a small percentage of lysogens, prophage DNA splits away from the bacterial chromosome. (5) Following excision, phage DNA replicates and progeny temperate phages assemble. (6) The induced cell lyses and infectious temperate phages are released.

1.5 Enumeration of bacteriophage particles (Adams, 1959)

The three principal assay methods that have been used are as follows:

1.5.1 Plaque counts

This method was first described by D'Herelle in 1917. An appropriate dilution of a phage suspension is inoculated to susceptible bacteria then an aliquot of the mixture is sampling and plated onto an agar plate. After incubation, the host bacteria have been lysed and the small areas on the agar surface or plaques are presented. Eventually, the circular zone of lysis may become large enough in area to be readily visible to the naked eye. These areas were called "taches vierges" (virgin spot) or plaques. Each plaque arises from a single viral particle in the original sample. The number of plaques was proportional to the original number of phage in the inoculum.

A modification of D'Herelle's plaque counting method was called agar layer method. This method was invented by Gratia (1936). About 2 ml of melted 0.6 % agar is cooled to 45^{0}C and inoculated with a drop of a concentrated suspension of the host bacterium. A measured volume of phage suspension is then added and poured over the surface of nutrient agar. After the upper agar layer has hardened, the plate is incubated. The bacteria grow and form an opaque background against plaques.

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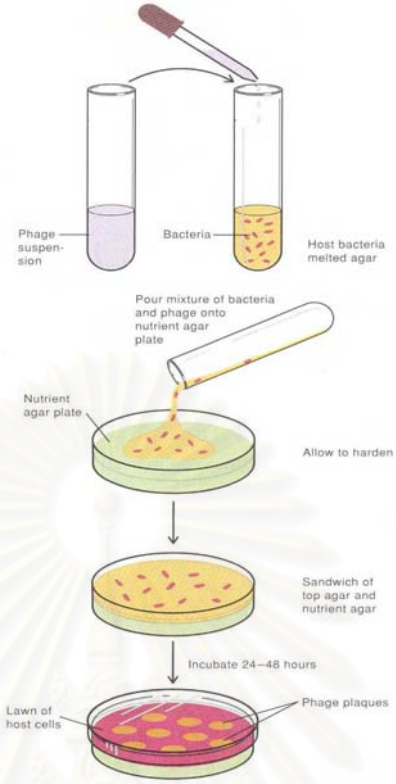

Fig. 1.5 Double layer method (Nester *et al*., 1995)

1.5.2 Dilution end-points

This method uses the lysis of fluid bacterial cultures as an indicator for the presence of phage and performed by multiplying the number of tubes tested at the limiting dilution. The phage preparation is diluted to the point where each sample should contain about one phage particle.

1.5.3 Measurements of the length of time required for lysis of a standard fluid bacterial culture

This method was developed by Krueger (1930). The procedure requires that one add the appropriate dilutions of phage to tubes containing suspended host cells. The length of time required for lysis is determined by the reduction of bacterial turbidity and is inversely proportional to the logarithm of the initial phage concentration over a considerable range.

1.6 General properties of phages

1.6.1 Antigenic properties of bacteriophage(Adams, 1959)

Antibodies against viruses are useful in viral research. Generally, the phages themselves are non-toxic and non- pathogenic for animals and can be usually injected in large amounts without damage to the recipient. In the case of some toxic substances that are produced by their hosts, they should be removed by fractionation and inactivated. Rabbits are the most suitable and practical animals to use for phage immunization.

1.6.2 The one-step growth curve

The replication of bacteriophage from the time of attachment until the release of virions from the infected bacterium can be conveniently studied by means of the one-step growth experiment of Ellis and Delbruck (1939). This method gives the minimum latent period of intracellular virus growth and the average burst size. The latent period is defined as the minimum time between the adsorption of the phage to the host cell and the lysis of the host cell with release of phage progeny. The burst size is the mean yield of phage particles per infected bacterium.

In this experiment, a culture is infected with a m.o.i. (multiplicity of infection: the average number of adsorbed phage per bacterium) of about 0.1 (to ensure that no cell is infected with more than one phage). Antibody to the phage is then added to inactivate any unadsorbed phage. The infected cells are diluted about 1000-fold into a fresh warm medium (to prevent inactivation of progeny phage by the antibody), and at various times aliquots of the supernatant are taken and plated on a lawn of sensitive bacteria. At first the number of plaques is constant (Fig. 1.6) because plaques are formed only by phage released later by infected unlysed cells. This is called the latent period. Some time after infection (the length of time is characteristic for each phage), the number of plaques increase. During this short time interval (the rise period), the infected cells are lysing. When all infected cells have lysed, the phage concentration remains constant. The ratio of phage produced to the initial number of infective

centers is the burst size, and the time (minutes) prior to the increase in plaque number is their lysis time.

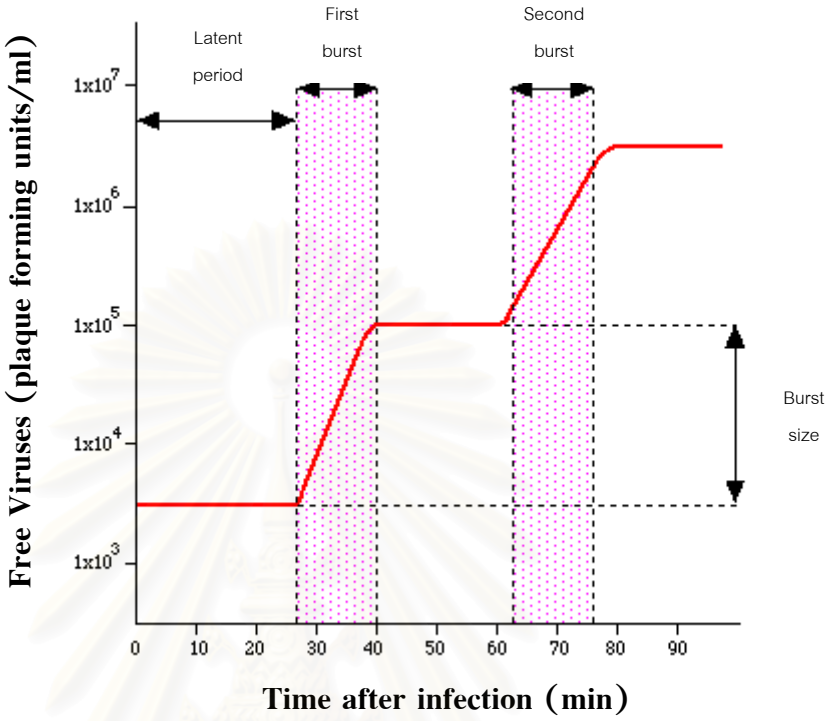

Fig. 1.6 The one-step growth curve (Nester *et al.*, 1995)

1.7 The use of bacteriophages

1.7.1. Bacterial identification (DuBow, 1994)

Bacteriophages have been used in bacterial identification and classification since 1925. The specificity of phage adsorption and lytic propagation on bacterial species known as phage typing, is still an important use of bacteriophages. Phage typing proves advantageous because it has a relatively easy procedure and lacks the need for sophisticated equipment. Phages can be used when bacterial strains causing a particular industrial or epidemiological problem differ from other strains in only a limited number of characteristics. Such characteristics include the presence of a particular surface antigen which the phage may use as an adsorption site, a particular restriction and modification system of the host, or even the presence of a particular temperate phage. These host characteristics may play a role in the complete lytic development of the

phage after infection, and their absence or presence can be quickly detected by the growth of a particular set of typing phages.

Most phage typing schemes are for bacterial of medical importance. The discovery in 1934 of the Vi antigen on the surface of the typhoid bacillus *Salmonella typhi* led to the isolation of Vi phages capable of lysing the typhoid bacillus, but only if it expressed the Vi antigen. These phages made possible the differentiation and typing of this medically important bacterial species. In addition, phages can be used as indicators of contamination by their hosts, for example, coliphages are used to detect fecal contamination of water. In practical, lytic phages, forming clear plaques due to the complete lysis of the host, are easier to use than temperate phages.

1.7.2 Biochemical and molecular biological research http://www.mun.ca/biochem/courses/3107/Lectures/Topics/bacteriophage.html)

1.7.2.1 T series of DNA bacteriophages

The T series of bacteriophages have a central role in the development of molecular biology. The study of the T bacteriophage has contributed a great deal to our understanding of molecular biology and genetic regulation. The genome of all of phages consists of a single linear molecule of dsDNA. However, circular forms and/or circular permutations can exist. The T-even phages, T2, T4 and T6, are all related serologically and all have large genomes. The T-odd phages fall into three serological groups; as T3 and T7 are related to each other but not to T1 or to T5 which are unrelated. Bacteriophage T7 has been used for the study of DNA replication because of its linear chromosome and the fact that it encodes its own DNA polymerase.

1.7.2.2 Temperate bacteriophages

Bacteriophage lambda, which infects *E. coli*, is a well-known example of a temperate bacteriophage. It has a linear dsDNA genome of 48,502 bp, which circularizes after infection. Bacteriophage lambda has been

used in the field of molecular biology and also played an important role as a cloning vector.

1.7.2.3 Small DNA bacteriophages

- The spherical phages (PhiX174, G4, S13) are broadly similar to the filamentous phage. The capsid is icosahedral not helical and is not enveloped (these phage lyse the host cell). Their genome consists of a circular ssDNA molecule.

- Filamentous phages (M13, fd, f1) only infect *E. coli* cells carrying the F plasmid, since the phage must adsorb to the F pilus to gain entry to the cells. Their life-cycle involves a dsDNA intermediate replicative form within the cell, which is converted to a ssDNA molecule prior to encapsidation. They are utilized in molecular biological laboratory since they provide an easy means to prepare ssDNA for DNA sequencing.

1.7.2.4 RNA bacteriophages (MS2, R17 and f2 form one family; Q beta forms another)

The ssRNA bacteriophages are the simplest viruses known. All have linear ssRNA genomes. Because of this, they have served as useful sources of RNA for studies of translation and protein synthesis.

1.8 Bacteriophage in soil

The role of phage in bacterial ecology was determined by Anderson (1957) who pointed out that phages may influence a naturally occuring bacterial population in the following ways: (a) virulent phages may eliminate sensitive organisms and cause the replacement of a population by phage-resistant mutants; (b) temperate phages may destroy a fraction of the population and lysogenize the remainder, and lysogenization may cause specific character changes associated with the prophage; (c) both temperate and virulent phages may bring about transduction of genetic properties. Therefore, the interaction and state in soil has been thoroughly considered and is interesting.

1.8.1 Detection of phages in soil

1.8.1.1 Enrichment procedures

Enrichment procedures include the incubation 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.

1.8.1.2 Direct counts

Direct counts aim to determine the number of phages present in sample in vivo. Soil samples of known weight are suspended, diluted and filtered to remove bacteria. The filtrates are then plated against one or more putative host isolates. The number of plaques indicates how many of phage particles which are active against the host used are in the soil samples.

1.8.2 The stability of free phages in soil

1.8.2.1 pH

Soil pH is an important factor influencing phage stability. Most studies have been conducted using typical neutrophilic host, active in a range from about pH 5.0 to pH 8.0. It has proved difficult to isolate phages from acidic soils (<pH 5.0).

1.8.2.2 Temperature

Phages are generally more stable at lower temperatures in laboratory conditions. When streptomycete phages were added to soil and incubated at temperatures ranging from 5 to 40^{0}C, results showed that phages could survived at low temperatures, but were totally inactive at 40^{0}C (Williams and Lanning , 1984).

1.8.2.3 Moisture

Soil moisture has a major influence on bacterial growth, primarily through its effect on aeration.

1.8.2.4 Soil colloids

Many phages can be adsorbed to colloidal particles in their natural habitat (Reanney and Marsh, 1973). This may result in their protection or inactivation (Duboise *et al*., 1979). Also, many streptomycete phage, when isolated from soil, were adsorbed by unsubstituted or sodium treated kaolin. Fewer were adsorbed by sodium and calcium montmorillonite or by calcium- and aluminum-treated kaolin (Sykes and Williams, 1978).

1.8.2.5 Other Factors

Water content was found to have little effect on a streptomycete phage except for when there is a decrease at high moisture holding capacities (Williams and Lanning, 1984). The addition of herbicides exerted no significant effect on two phages for *Streptomyces chrysomallus* (Roslycky, 1982).

1.9 Actinomycetes

Actinomycetes, a form of fungi-like bacteria that form long, thread-like branched filaments and high G+C organisms, are gram positive. They are numerous and widely distributed not only in soil but in a variety of other habitats including composts, river muds, and lake bottoms. To isolate actinomycetes, selective media are used, preferable supplemented with antimicrobial compounds (Ottow, 1972). Chitin is also supplemented because almost actinomycetes strains can use this polysaccharide at a higher percentage than bacteria and fungi (Kuznetsov and Iangulova, 1970). The period of incubation must be longer than that of bacteria.

Environmental factors are influential to actinomycetes. Some of these include organic matter, pH, moisture, and temperature. They are abundant in the presence of available carbon but rarely found in extreme conditions such as in a low pH, high moisture content or high temperature. Most actinomycetes are not tolerant to low pH and fail to multiply below pH 5.0. They have an optimum temperature range of about 25-30 ^{0}C (Alexander, 1977).

Actinomycetes are the primary decomposers of tough plant materials like bark and woody stems. They produce many extracellular enzymes and play an important role of biodegradation in soil by decomposing complex mixtures in dead plants, animal and fungal materials (McCarthy, 1987; Crawford, 1988; Wang *et al.*, 1989) and by recycling nutrients (McCarthy and Williams, 1992).

The genus *Streptomyces* are numerically dominant and found up to 70 to 90% of the actinomycetes colonies on most agar media. *Nocardia* spp. and *Micromonospora* spp. are the second and third most abundant (Alexander, 1977). Soil is the most popular habitat for isolation, they produce well-developed branched and rarely fragmented substrate mycelia. Screening of new *Streptomyces* provided a great variety of new compounds that have been used as human and animal medicines, as well as agricultural chemicals (Kudo, 1997).

1.10 Actinophages

Actinophage was used to designate the phage of actinomycetes. Dmitrieff (1934), Dmitrieff and Souteeff (1936) were the first to observe the lysis of an actinomycete culture. They recognized the significance of this reaction in the life cycle of the organism. A culture of an organism called *Actinomyces boris* by the authors, and evidently belonging to the genus *Streptomyces*.

At present, all classified actinophages posses tails and contained double-strand DNA. They comprised of six morphological types (Ackermann and Eisenstark, 1974) and belong to three families of Myoviridae, Siphoviridae and Podoviridae (Matthews, 1982).

They are numerous reviews on actinophage, especially streptomycetes phages since they are widely distributed in soil including compost. In addition,

they produce a lot of extracellular enzymes and the important antibiotics. Most actinophages infecting streptomycetes have been isolated from soil by enrichment techniques including both virulent and temperate phages.

Besides of streptomycetes phages, there were several reports on phage infecting *Nocardia* spp.(Bradley and Anderson, 1958; Brownell and Adams, 1967; Riverin *et al*., 1970; Brownell and Adams, 1976; Brownell, 1976; Pulverer *et al*., 1975; Brownell and Clark, 1974). Actinophage of *Micromonospora purpurea*, an industrial strain producing aminoglycoside and macrolide antibiotics, were also characterized (Caso *et al*., 1990; Tilley *et al*., 1990; Kikuchi and Perlman, 1978).

1.11 Streptomycetes phages

Streptomycetes phages have been studied for a variety of reasons. They caused problems in the fermentation industry, their use in the typing of streptomycetes, host genetics, phage biology and diversity. They are useful tools for studies or exploitation of their hosts, especially in restriction and modification systems (Chater, 1980) while some of them were also developed for cloning vectors.

1.11.1 Isolation and storage of streptomycetes phages

The natural habitat of most streptomycetes is soil. Their phages are readily isolated from soil, provided the pH is above 5.0 (Kieser *et al*., 2000). Most phages isolated from soil have a wide host-range except for those with a high specificity for *S. venezuelae* (Stuttard, 1983). Besides, streptomycetes themselves provide a second source of phage isolation from their ability of harbouring prophages. However, it is rare to obtain phages in this way, because the endogenous phages often have a narrow host-range and need a suitable indicator strain. Moreover, few streptomycetes prophages are UV-inducible and only two (Sat-1 and R4) are induced by mitomycin C (Ogata *et al*,. 1985; Kieser *et al*., 2000).

The unusually virulent phages have been found to contaminate industrial fermenter cultures. However, this has not always taken place in well-understood streptomycetes phages.

In general, phages are stored at 4 ^{0}C in the dark. Filtration is necessary to minimize the loss of phage titre by adsorption to host debris. For long-term preservation, it is advisable to store phages as DNA (usually at 4^{0}; in order to recover the phages by transfection) and/or as lyophils (using filter-paper strips dipped into a high-titer suspension). To recover phages from lyophils, one simply places the filter paper strip on a fresh conventional overlay plate containing spores of the host strain in the top layer, and incubates the filter paper strip overnight.

1.11.2 Properties and characteristics of some streptomycetes phages

There are several reports on phages as followings;

Cox and Baltz (1984) determined the general biological relevance of restriction endonuclease in *Streptomyces* species which presented the barrier to restrict phage DNA.

Godany *et al.* (1996) isolated a *Xho* I isoshizomer (*Sau*LPI) in the resistant colonies of *S. aureofaciens* after actinophage μ1/6 and B1 infection.

Zhou, *et al*. (1994) reported the characterization of ϕ HAU3. This phage is a temperate phage with cohesive ends and a broad host range that includes *S. hygroscopicus* 10-22, a producer of antifungal compounds.

Many studies isolated deletion mutants of actinophage. For example, Anne *et al.* (1984; 1990) isolated temperate actinophage VWB infected *S. venezualae* ETH14630. This phage has a genome size of 47.3 kb with cohesive ends and is able to package at least 4 kb of additional DNA. Consequently, it can be developed into a phage-based cloning vector as well as a Sat-1 (Ogata *et al*., 1985), SH10 (Klaus *et al*., 1981; Walter *et al*., 1981),

Pa16 (Rosner, 1980), ϕ C31 (Sladkova *et al*., 1981; Suarez *et al*., 1984), R4 (Isogai *et al*., 1981), Bα (Ishihara *et al*., 1982), and Sat 28 (Suenaga *et al*., 1994). The isolation of deletion mutants and the presence of unique sites has facilitated the establishment of phage vectors in *Streptomyces* spp.

Soils contained a wide diversity of actinophages with highly polyvalent characteristics (Robinson and Corke, 1959). As of present, the study on distribution and diversity of streptomycetes was rather discontinuous. Therefore, the present study is aimed for isolation of *Streptomyces* spp. and actinophages from soil, characterize and observe their morphologies, determine relationship between phage and streptomycetes, construct phage DNA's restriction map and elucidate the interaction of phage and host in soil. The results of this study will give us some understanding of the distribution and diversity of phage in soil populated with streptomycetes. The report will provide useful information concerning the distribution of actinophages from different parts of Thailand. The isolated phages and information obtained will be of valuable in further work of molecular biology.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Agarose gel electrophoresis equipment, Mupid, Japan.

Controlled environment incubator shaker: model R-88, New Brunswick Scientific Co.Ltd., U.S.A.

Electronic top loader, A200S, Sartorius, Germany.

Electrophoresis unit: Model Mini-PROTEAN II Electrophoresis Cell, Bio-Rad, U.S.A.

Freezer -20 ^{0}C, MDF-U332, Sanyo, Japan.

Gene amp PCR system 2400, Perkin Elmer, U.S.A.

Haemacytometer, 1/10 nm bright line deep, Boeco, West Germany.

Incubator D-601: model 500, Memert, U.S.A.

Laminar flow, International Scientific Supply Co. Ltd., Thailand.

Microcentrifuge, KM-15200, Kubota, Japan.

Microscope, CHS, Olympus Optical Co. Ltd, Japan.

pH-meter, Cyberscan 1000, Eutech Cyberscan, Singapore.

Polycarbonate syringe filter holder 25 mm, Sartorius, Germany.

Power supply, BIO-RAD Laboratories, U.S.A.

Refrigerated centrifuge: model HERTZ, Kubota Corporation, Japan.

Stereo microscope, SZH-10, Olympus Optical Co. Ltd, Japan.

Swinnex filter unit, Millipore Corporation, U.S.A.

Syringe 10 ml, Nipro, Thailand.

Transmission electron microscope, model JCM-200 CZ, JEOL Japan.

UV transiluminator, model 3-3602, Fotodyne, U.S.A.

Vortex mixer, vortex-2 Genei, model G-560E, Scientific Industries, U.S.A.

Water bath, model aquatherm G-86, New Brunswick Scientific Co.Ltd. U.S.A.

Water bath, shaker, Memmert, Germany.

2.2 Chemicals

Absolute ethanol, Merck, U.S.A.

Acrylamide, Sigma Chemical, U.S.A.

Agarose, FMC Bioproducts, U.S.A.

Ammonium persulfate, Merck, U.S.A.

Bacto peptone, Difco, U.S.A.

Beef extract, Difco, U.S.A.

Biotin, Sigma Chemical, U.S.A.

Boric acid, Merck, U.S.A.

Calcium carbonate, Fluka, Switzerland.

Calcium chloride, Merck, U.S.A.

Chloroform, Merck, U.S.A.

Coomassie brilliant blue R-250, Sigma Chemical, U.S.A.

CTAB(cetyltrimethylammonium bromide), Sigma Chemical, U.S.A.

D-mannitol, Difco, U.S.A.

Ethidium bromide, Fluka, Switzerland.

Ethylenediaminetetra-acetic acid(EDTA), Bio-Rad Laboratories., U.S.A.

Glacial acetic acid, Merck, U.S.A.

Glucose anhydrous, Fluka, Switzerland.

Glycerol, Merck, U.S.A.

Glycine, Merck, U.S.A.

Humic acid, Sigma Chemical, U.S.A.

Hydrochloric acid, BDH Laboratory Supplies., England.

Isoamyl alcohol, Sigma, U.S.A.

Magnesium sulfate, Merck, U.S.A.

Maleic acid, Fluka, Switzerland.

Mannitol, Difco Laboratories, U.S.A.

N,N' -methylene - bis - acrylamide , Sigma, U.S.A.

Niacin, Sigma Chemical, U.S.A.

N-Z amine, Humko Sheffield, U.S.A.

p- aminobenzoic acid, Sigma Chemical, U.S.A.

Phenol crystal, Merck, U.S.A.

Polyethylene glycol 6000, Sigma Chemical, U.S.A.

Polyethylene glycol 8000, Fluka, Switzerland.

Pyridoxin-HCl, Sigma Chemical, U.S.A.

Riboflavin, Merck, Germany.

Sodium acetate, APS Ajax Chemicals, Australia.

Sodium chloride, Merck, U.S.A.

Sodium dodecyl sulfate, BDH Laboratory Supplies, England.

TEMED, Sigma Chemical, U.S.A.

Thiamine- HCl, Sigma Chemical, U.S.A.

Trichloroacetic acid, Sigma Chemical, U.S.A.

Trisma base, USB, U.S.A.

Tween 20, Promega, U.S.A.

Yeast extract, Difco, U.S.A.

2.3 Supplies

Nitrocellulose transfer membrane, Schleicher and Schuell Inc. U.S.A.

Polaroid film, Polaroid, U.S.A.

Cellulose acetate filter, pore size 0.45 μm, Sartorius, Germany.

2.4 Kit

DIG high prime DNA labelling and detection starter kit, Roche, Germany.

2.5 Enzymes and restriction enzymes

100 bp DNA ladder, Pacific Science Ltd., Thailand.

Ampli Taq DNA polymerase, Promega, U.S.A.

*Apa*I, Takara Shuzo, Japan.

*Bam*HI, Promega, U.S.A.

*Bgl*II, Promega, U.S.A.

Deoxyribonuclease, Sigma Chemical, U.S.A.

λ DNA/*Hind* III marker, Promega, U.S.A.

*EcoR*I, Promega, U.S.A.

*EcoR*V, Promega, U.S.A.

*Hind*III, Promega, U.S.A.

*Kpn*I, Takara Shuzo, Japan.

Lysozyme, Sigma, U.S.A.

*Mlu*I, Promega, U.S.A.

*Mun*I, Takara Shuzo, Japan.

*Not*I, Takara Shuzo, Japan.

PCR buffer, Promega, U.S.A.

Proteinase K, Life Technology, U.S.A.

*Pst*I, Takara Shuzo, Japan.

Ribonuclease I, Sigma Chemical, U.S.A.

*Sac*I, Takara Shuzo, Japan.

*Sal*I, Takara Shuzo, Japan.

*Spe*I, Takara Shuzo, Japan.

*Sph*I, Takara Shuzo, Japan.

*Xba*I, Takara Shuzo, Japan.

*Xho*I, Takara Shuzo, Japan.

2.6 Actinomycetes strains and tested organisms

Kitasatospora griseola JCM 3339***

Streptomyces alboniger ATCC 12461**

S. albovinaceus ATCC 15823**

S. albus ATCC 14510***

S. ambofaciens ATCC 23877***

S. aminophilus ATCC 14961**

S. aureus ATCC 3309***

S. azureus PK 100C***

S. badius JCM 4350***

S. bikiniensis JCM 4011***

S. coelicolor ATCC 23899***

S. coelicolor M 145***

S. coeruleofuscus ATCC 23618/ATCC 19742***

S. coerulescens ISP 5146***

S. coralus JCM 4313*

S. cyaneus ATCC 14923***

S. echinatus ATCC 19748***

S. endus ATCC 23904***

S. griseus KA-1198*

S. griseus ATCC 2926***

S. humifer ATCC 13748***

S. hygroscopicus subsp. *hygroscopicus* ATCC 27438*

S. hygroscopicus subsp. *hygroscopicus* BCC 3299*

S. kanamyceticus KCCS-0433***

S. kanamyceticus KCCS-0775***

S. laurentii PO***

S. lavendulae subsp. *grosserius* ATCC 25457***

S. lavendulae subsp. *lavendulae* ATCC 8664***

S. lincolnensis JCM 4287***

S. lividans ATCC 19844***

S. lividans TK 24***

S. luteofluorescens ATCC 25469***

S. luteogriseus ATCC 15072*

S. melanosporofaciens ATCC 25473*

S. nivous ATCC 19793***

S. nodosus JCM 4297***

S. pervullus BCC 688*

S. perciperlis BCC 840*

S. pilosus IFO 12807***

S. puniceus ATCC 1980***1

S. sparsogenes IFO 13086***

S. tauricus IFO 13456***

S. thermovulgaris JCM 4520***

S. *violaceochromogenes* ATCC 19932***

S. viridochromogenes IFO 12337***

Tested organisms

Aspergillus niger ATCC6275**

Bacillus subtilis ATCC 6633**

Candida albicans ATCC 70014**

Escherichia coli ATCC 25922**

Micrococcus luteus ATCC 9341**

*Pseudomonas fluoresce*ns K. Komagata**

Saccharomyces cerevisiae TISTR 5169**

Staphylococcus aureus ATCC 25923**

* kindly supplied from National Science and Technology Development Agency (NSTDA).

**purchased from Bangkok-MIRCENS (Thailand Institute of Scientific and Technological Research; TISTR)

***Gift from Prof. Dr. Seiya Ogata.

2.7 Storage of microorganisms

Streptomycetes spores or mycelia were cultured at 30 ^{0}C for 7-14 days on various kinds of medium: Rye-flake agar medium (Appendix I, No. 4), Mannitol-mungbean agar medium (Appendix I, No. 11), Oat-meal agar medium (Appendix I, No. 8). The kind of medium used, depending on the strains, was for obtaining the maximal yields of spore. Five milliliters of distilled water was added to the culture, followed by scraping the surface of the culture and filtering the suspension through sterile cotton wool in a filter tube. The filtered suspension was centrifuged at 1,200 xg for 10 min to precipitate the spores. The supernatant was discarded, followed by the addition of 10 % (V/V)

glycerol then vortexed. The suspension was transferred to a screw cap tube for freezing at −20 ^{0}C until used (Wellinton and Williams, 1978).

Phage lysate was stored at 4 ^{0} C in nutrient broth (Appendix I, No. 1) with appropriate cations of $CaCl_2$ or $MgSO_4$ and kept in dark bottle.

Test organisms were cultured separately as followings:

A. niger was cultured on Potato dextrose agar (Appendix I, No.7) at 30 ^{0}C for 3-4 days

B. subtilis, *E. coli*, *M. luteus*, *Ps. fluorescens*, *S. aureus* were cultured on Nutrient agar (Appendix I, No. 2) at 37 ^{0}C for 24 h.

S. cerevisiae, *C. albicans* were cultured on yeast extract-malt extract agar (Appendix I, No. 9) at 30 ^{0}C for 24 h.

2.8 Soil sampling

Soil samples were taken from several provinces of Thailand (see Fig 3.1 in Chapter III). pH of the soil was measured at first. Soil pH was taken in a 1: 1 (w: v) suspension of air dried soil in distilled water. For this determination, weigh out 10 g of soil, add 10 ml of distilled water, stirred vigorously and allowed to stand for 10 min, inserted the glass electrode assembly and read the pH (Thomas, 1996).

2.9 Isolation of *Streptomyces* sp.

Isolation of *Streptomyces* sp. from soil was carried out by humic acid vitamin agar medium or HV-agar (Appendix I, No. 5). Soils were dried at 40-50 ^{0}C for 2 h. Then, 1 g 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.

2.10 Antibiotic tests

Isolated *Streptomyces* spp. were streaked in the center of plates and incubated for 3 days. Streak the organism to be tested in vertical line across the *Streptomyces* streak and incubated for 24 h at appropriate temperature. Positive test was observed by the appearance of clear zone between the crossing line.

2.11 Preparation of streptomycetes spore suspension

Five milliliters of distilled water were added to spores of streptomycete on Mannitol mungbean agar 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, vortex mixed and added 20 % (V/V) glycerol. The suspension was transferred to a screw cap for freezing at −20 ^{0}C until used.

2.12 Detection of phage

Soft-agar overlay plate method or double layer method (Gratia, 1936) was generally used for phage detection. The spore suspension of host strain was mixed with the phage suspension at a multiplicity of infection (m.o.i.) of approximately 0.1 in top agar, mixed the mixture gently and poured onto nutrient agar. The top agar was allowed to solidify, inverted and incubated at 30^{0}C overnight.

2.13 Isolation of new streptomycetes phages (Dowding, 1973)

Twenty-five grams of soil sample was inoculated into 250 ml Erlenmeyer flask containing 50 ml of nutrient broth 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. Then, the particulate matter was removed by centrifuging at 3,000 rpm for 10 min and carefully transferred supernatant to a fresh 20 ml screw cap bottle and filtered through a sterile 0.45 μm cellulose acetate filter. To examine the presence of phages, double layer method was used, 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, overlaid with 0.1 ml top agar (Appendix I, No. 1) containing spore suspension of the indicated strain, swirled immediately and briefly, 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.

2.14 Enumeration of bacteriophages

The presence of bacteriophages in the sample (titering) was enumerated. Only one dilution should be used to determine the titer of pfu/ml.

A serial dilution of the phage suspension was made in nutrient broth. Then, double layer method was employed exactly as described in section 2.12. The number of plaques was counted then calculated the titer using this formula:

$$n = y/(v).(x)$$

where:

n is the titer (pfu/ml) of the original sample,

y is the average number of plaques counted,

v is the volume plated, and x is the dilution plated.

2.15 Single plaque purification of phages

A plaque forming plate was prepared. The next day, a single plaque was picked up by using a sterile toothpick or inoculated needle and immersed the plaque into 2 ml of nutrient broth. 10 µl of host spore suspensions was added, and incubated overnight. Then, plaque formation was tested as described in section 2.12, repeated at least 3 times and phage lysate was prepared as following.

2.16 Preparation of phage lysates

2.16.1. Double layer method

After plaque forming, the next day, poured 5 ml of nutrient broth to plates, allowed plates to set at room temperature for 5 h with occasional shaking. The suspension in each plate was pooled and centrifuged at 1,200 g for 10 min to remove unlysed host cells. Phage lysate was filter-sterilized by passing through a 0.45 μm pore size cellulose acetate membrane filter. Titer of this lysate was measured and stored at 4 ^{0}C.

2.16.2. Liquid method

One milliliter of spore suspension ($1x10^{8}$ spores /ml) was added into 50 ml nutrient broth in 250 ml Erlenmeyer flask and incubated for 5 h until spore germinated. After that, the phage suspension was added at a multiplicity of infection (m.o.i.) of approximately 0.1 in a flask and shake continuously overnight. The next day, the mixture was centrifuged at 1,200 xg for 10 min, then filter-sterilized and stored at 4 ^{0}C.

2.17 Observation of phage particle under transmission electron microscope

Phage lysates were prepared in large amounts as describe in section 2.16. Then 0.5 M NaCl and 10 % polyethylene glycol MW. 8000 were added, mixed the solution throughoutly and allowed to stay overnight at 4 ^{0}C. The next day, phage particle was precipitated by centrifugation at 14,000 rpm for 20 min, phage pellets were dissolved in 10 mM ammonium acetate buffer containing 1 mM $MgCl_2$, pH 7.0 (Appendix II, No. 17). The suspended phage was dropped on carbon-coated grid 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).

2.18 Host range determination

Host range of phages was determined against 45 type strains of reference streptomycetes and 118 strains isolated from this laboratory. Spore suspension (10^7 spores/ml), prepared in section 2.11 was infected with phage m.o.i. at 0.1-0.01. After 18 h of incubation, the presence of plaques was examined and compared with a control of a non-infected host strain.

2.19 One step growth curve experiment (Dowding, 1973)

A susceptible spore suspension of initial concentration approximately 10^7 spores /ml was inoculated in nutrient broth and shaken for 5 h to let the spores germinate. An approximately titer of phage was added to give m.o.i. 0.1-0.01, then continued shaking and incubated for another 20 min. A 10 ml sample of the mixture was removed and membrane-filtered. Unadsorbed phages were removed from the filter membrane by washing the filter with nutrient broth several times. The filter membrane was transferred to the first flask containing 50 ml of nutrient broth at 30^0C and agitated, then diluted to 1/50 to another second flask. The two flasks were reincubated at 30 ^{0}C. About 0.1 ml of each sample was removed from the two flasks. Of the first flask was removed at the initial, of the second flask was removed after 35 min and plated immediately.

2.20 Effect of pH

2.20.1 pH stability of phage

Sterilized nutrient broth adjusted to desired pH values with HCl and NaOH was determined for phage stability over a range of pH 4 to 9. Phage suspension in each dilution broth was incubated at 30 ^{0}C for 1 h. The number of phages was assayed by double agar method and incubated plates for 18-24 h.

2.20.2 Optimal pH for phage multiplication

Nutrient agar plates and soft agar overlay were adjusted pH over a range of pH 4 to 9. The number of phages was assayed by double agar method and incubated plates for 18-24 h.

2.21 Role of divalent cations

The effect of divalent cations concentration, for examples, $MgSO_4$ and $CaCl_2$, was determined. The test was done by plating the proper dilutions of the host and phage mixture into soft agar overlays on nutrient agar plates to which either $MgSO_4$ or $CaCl_2$ is supplemented at concentration range from 0-50 mM. The plates were incubated overnight then counted for the number of visible plaques.

2.22 Preparation of antisera (Adams, 1959)

Three rabbits were used for immunization of phage. Phage infection was carried out via subcutaneous route. The purified phage particles (10^{10} pfu/ml) of 5 ml were inoculated twice per week for 3 weeks. Animals were bled by slitting marginal ear vein and about 5 ml of blood was collected. After centrifugation, serum was decanted, recentrifuged again to remove residual red blood cells, and stored in sterile screw-capped vials in the refrigerator.

2.23 Assay of phage antisera

To assay, serum was diluted to 1:100 and 1:1,000 in the nutrient broth, the same medium as of the phage dilution. The phage stock 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 indicated above 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 reaction between phage and its antibody was calculated by the equation as:

$$K = 2.3\ D/t \times \log p_0 / p \text{ (integral)}$$

$$p/p_0 = e^{-Kt/D} \text{ (exponential)}$$

In which p_0 = phage assay at zero time,

p = phage assay at time t min,

D = final dilution of serum in the phage-serum mixture,

K = velocity constant

2.24 Preparation of streptomycetes phage DNA (Sambrook, 1989)

High titer phage lysates was prepared by double layer technique from 100 plates on solid nutrient agar or either from 1,000 ml of liquid culture as described in section 2.16. In the following day, each of the plaque in the plates or plate lysates was soaked with 5 ml nutrient broth for each plates, allowed to sit at room temperature for 5 h. Then, phage suspension was harvested and centrifuged at 1,200 xg at 4^0C for 10 min to remove host debris. The supernatant was transferred to a fresh flask and then added DNaseI and RNaseI to final concentration of 1 µg/ml and 10 µg/ml, respectively and incubated at 37^oC for 30 min, after that, added NaCl and PEG MW. 8000 to final concentration of 1 M and 10 %, respectively, mixed thoroughly until the salt and PEG were completely dissolved. The flask may be left at 4 ^{0}C for overnight. The phage-PEG complex was pelleted by centrifugation at 10,000 rpm for 15 min at 4^0C, discarded the supernatant, added 500 µl TE pH 8.0 (Appendix II, No. 8) and gradually resuspended the pellet by repeated pipetting, following by added 0.5 M NaCl and 10 % SDS (Appendix II, No.16). The tube was incubated at 68 ^{0}C for 10 min. The phage was extracted twice with 1 volume of chloroform to remove some of the PEG. To burst phage and remove phage proteins, an equal volume of phenol was added, mixed by inversion and spinned in eppendorf tube about 1-3 min until upper aqueous phase becomes clear. The aqueous phase was transferred to a fresh eppendorf tube, added a 1: 1 mixture of phenol: chloroform and repeated once again using a 24: 1 mixture of chloroform: isoamyl alcohol. The phage DNA was precipitated by adding 1/10 volume of 3 M sodium acetate, then 1 ml of ice cold absolute ethanol. The DNA pellet was precipitated by centrifuging at 15,000 rpm for 15 min in a microfuge. The pellet was washed with 200 µl of 70% ethanol, vortex briefly, then centrifuge briefly. Then, the pellet was dried by leaving on the bench top, or by placing the sample

in the vacuum chamber for 5-10 min. The last step, 50-100 µl TE buffer was added and allowed the pellet to resuspend slowly. The quality of the DNA was checked by electrophoresis using a sample size of about 1 µl.

2.25 Agarose gel electrophoresis of DNA fragment

An appropriate amount of phage DNA was digested with restriction endonuclease in a suitable restriction buffer and incubated for 1-3 h in the conditions according to supplier recommendation. After digestion, phage DNA was loaded onto the 0.6 % agarose gel and also loaded a *Hind*III digested phage λ DNA. Electrophoresis was operated in 1x TAE buffer (Appendix II, No. 17) at 50 V. After electrophoresis was complete, visualized the separated DNA bands under UV illumination and photographed.

2.26 Plaque hybridization / transfer of phage DNA from plaques to nylon membrane or nitrocellulose (Benton and Davies, 1977)

The overnight lysate plate was kept at 4 ^{0}C for 2 h to harden the top agar. Then, a dry nitrocellulose membrane was placed over the plaques for 10 min, air bubbles between the agar and the membrane should be avoided, marked asymmetrically two positions. The membrane was removed and placed it with plaque side up between two sheets of 3 MM Whatman paper soaked in denaturation solution (Appendix II, No. 10). After 10 min, the filter was removed and transferred onto another two sheets of Whatman paper soaked in neutralization buffer for 10 min (Appendix II, No. 11). The membrane was transferred again onto Whatman papers soaked in 2x SSC (Appendix II, No. 12) for 10 min, sandwiched and dried between Whatman papers at room temperature for 30 min and baked at 80 ^{0}C for 1.5-2 h in a vacuum oven. The membrane was stored at room temperature in a plastic bag until needed for hybridization.

2.27 Southern blotting procedure (Southern, 1975)

The procedure was carried out after visualized the phage DNA bands by agarose gel electrophoresis. The gel was rinsed in distilled water and then placed in 1 N HCl for 10 min with gentle agitation, HCl was discarded, gel was rinsed in water. For transfer to nitrocellulose or nylon membranes, the gel was placed in denaturation buffer for 15 min and repeated again with fresh denaturation solution. The denaturation buffer was discarded, rinsed the gel in distilled water and placed in neutralization buffer for 20 min with gentle shaking and repeated again with fresh neutralization solution. The capillary transfer was set up as illustrated in Fig. 2.1, wetted two pieces of 3 MM Whatman paper with 10x SSC solution and placed across the blot blocks which dip into 10x SSC solution. Gel was carefully placed onto the wetted filter paper while avoid of trapped air bubbles, wetted the nylon membranes and transferred to a tray containing 10x SSC and soaked for 20 min. Nitrocellulose or nylon membrane was placed with the same size of gel on to the gel and removed air bubbles. Then, another two pieces of 3 MM Whatman paper which cut to the same size of the gel was wetted in 10x SSC and placed it on the membrane, removed air bubbles, placed a set of paper towels (5 inches high) on top and allowed it to stand overnight. The next day, paper towels was discarded and transferred the membranes to a tray containing 500 ml 2x SSC, washed membrane at room temperature in 2x SSC twice, 15 min each wash. The membrane was air-dried, baked the blots (between Whatman papers) at 80 ^{0}C in the vacuum oven for 1-2 h.

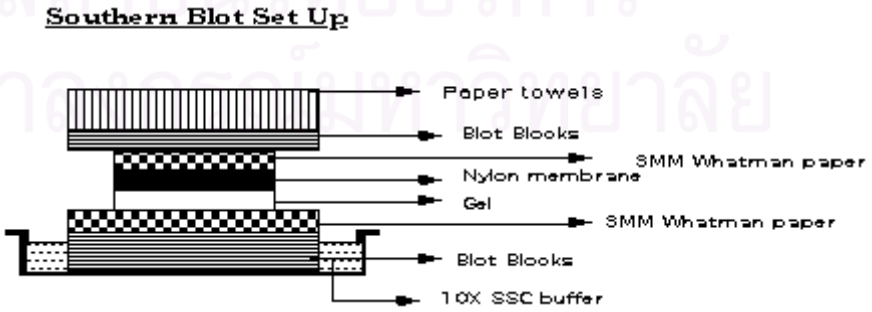

Fig. 2.1 Southern blot set up
(http://hdklab.wustl.edu/lab_manual/southern/southern1.html)

2.28 Detection of plaque hybridization and southern blot via chemiluminescence (Boehringer mannheim manual, version 2.0, catalogue number 101 023.)

2.28.1. Prehybridization

Pre-heat the filter (from plaque hybridization or southern blot) with an appropriate volume of digoxigenin (DIG) easy hybridization (10 ml/100 cm^2) to hybridization temperature (37-42 ^{0}C).

2.28.2. Hybridization

Add DIG labeled probe to fresh prehybridization solution, and incubated at 37 ^{0}C, 2-15 h, with gentle agitation, poured off and saved hybridization solution. This can be reused for additional blots (the preparation of labeled probe was shown in Appendix III, No. 2, the determination of labeling efficiency of probe was shown in Appendix III, No. 3).

2.28.3. Detection

The membrane was washed in 2x SSC, 0.1% SDS (Appendix II, No. 13), twice, 5 min each, at room temperature and then washed blot in 0.5 x SSC, 0.1% SDS (Appendix II, No. 14) at 65 ^{0}C, twice, 15 min each. Blot was equilibrated for 1 min in washing buffer (Appendix II, No. 5), at room temperature followed by soaking the membrane in blocking solution at room temperature for 30 min and poured off blocking solution (Appendix II, No. 2). After preparing anti-DIG antibody-alkaline phosphatase conjugate (Appendix II, No. 3) in antibody solution, this solution was replaced and incubated 30 min at room temperature. The next step, anti-DIG antibody-alkaline phosphatase conjugate was poured off and washed blot in washing buffer twice, 15 min each at room temperature. The blot was equilibrated for 2 min in detection buffer (Appendix II, No. 7). After poured off the detection buffer, the blot was quickly placed in the plastic sheets and dropped 0.5 to 1.0 ml of NBT/BCIP solution (Appendix II, No. 4) onto membrane (DNA side up) and incubated for 2-24 h until the color appeared.

2.29 Structural phage protein analysis by SDS/ polyacrylamide gel electrophoresis (Laemmli 1970)

2.29.1 Preparation of phage protein

The purified phage suspensions were treated with 12 % (w/v) trichloroacetic acid to precipitate phage protein and allowed to set on the ice bath for 30 min. Then the phage proteins were precipitated by centrifuge at 14,000 rpm for 15 min and washed with distilled water twice. SDS-PAGE of phage proteins was performed by the methods as described by Laemmli (1970).

2.29.2 Preparation of running gel solution

The separating gel was set up by preparing separating gel monomer (Appendix III, No. 4) and added TEMED just prior to pouring gel. The gel was allowed to polymerize before adding stacking gel by overlaying gently with water or n-butanol. After the separating gel had polymerized, decanted the overlay, prepared the stacking monomer (Appendix III, No. 4), added the TEMED, and poured. At last, the comb was inserted and allowed to polymerize completely before running. Gel was run at constant current, 200 V. SDS-PAGE running buffer was diluted from 5x running buffer (Appendix II, No. 17) to 1x before use. Samples were diluted at least 1:4 with sample buffer (Appendix II, No. 18), heated at 95^{0}C for 4 min prior to loading. For protein marker, brought the desired amount of the protein marker (7 µl for mini-gels) (Appendix IV) over to a separate tube and heated to 95 ^{0}C for 3-5 min. After a quick microcentrifugation, the samples were loaded directly onto a gel.

2.29.3 Running conditions

The constant voltage was being set at 200 volts.

2.29.4. Staining

Bands were stained with coomassie brilliant blue R250 in fixative (40 % methanol, 10 % acetic acid) and destained with several changes

of 40 % methanol and 10 % acetic acid until clear background was obtained. Molecular marker of proteins was used to estimate size of protein.

2.30 Investigation of some characteristics of *Streptomyces* sp. 15

2.30.1 Isolation of genomic DNA of *Streptomyces* sp. 15

(Murray and Thompson, 1980; Ausubel *et al*., 1994)

Streptomyces spore was inoculated in tryptic soy broth (Appendix I, No. 6) for 2 days. Mycelium was harvested by centrifuge at 6,000 rpm for 10 min. The pellet was resuspended in 5 ml of TE25S buffer (Appendix II, No. 20). One hundred microliters of lysozyme (100 mg/ml in water) was added and incubated at 30-60 min at 37 ^{0}C. Five hundred microliters of proteinase K (20 mg/ml in water) was added, mixed and then added 300 µl of 10 % SDS, mixed by inversion, incubated at 55 ^{0}C for 1 h. After that, 1 ml of 5 M NaCl was added and mixed, added 0.65 ml of CTAB/NaCl (10% CTAB in 0.7 M NaCl) and incubated at 55 ^{0}C for 10 min, cooled to 37 ^{0}C, added 5 ml chloroform: isoamyl alcohol (24: 1), and mixed by inversion for 30 min. Upper supernatant was separated by centrifugation at 13,500 xg for 15 min, at 20 ^{0}C, transferred to a new tube, added 0.6 vol of isopropanol and mixed by inversion. After 3 min, DNA was spooled, rinsed DNA with 5 ml of 70 % ethanol, dried and dissolved in 0.5-1 ml of TE buffer at 55^{0}C.

2.30.2 Identification of *Streptomyces* sp. 15 by using partial 16S rDNA

After extraction from section 2.30.1, DNA was used for amplify by PCR reaction. Primer was designed to cover the variable region of 16 S rDNA. PCR reaction was performed in 50 µl reaction mixture (5 µl, 10x PCR buffer; 5 µl, dNTP; 1 µl, primer 1 (sense primer for PCR: 5'-TCACGGAGAGTTTGATCCTG-3'); 1 µl, primer 2 (anti-sense primer for PCR : 5'-GCGGCTGCTGGCACGTGGGCAATCTG-3'); 3 µl, $MgCl_2$; 5 µl, Template; 1 µl, Taq polymerase and for 35 cycles of denaturation (for 30 s at 97 ^{0}C), annealing (for 1 min at 50 ^{0}C), and extension (for 1 min at 72 ^{0}C). Whole sample was fractionated by agarose gel electrophoresis, and PCR products

of 500 bps were recovered using a Gene Clean II kit (Kataoka *et al.*, 1997). DNA sequencing was determined by using the Bioservice Unit, NSTDA. Data was analyzed by DNAsis program and compared the similarity of sequences with BLAST program from website: http://www.ncbi.nlm.nih.gov/BLAST.

2.31 Experimentation in soil

2.31.1 Materials

Organisms: Three organisms were used namely: *Streptomyces* sp. 15, *Streptomyces coelicolor* ATCC 23899, *Streptomyces griseus* ATCC 2926 and *Streptomyces viridochromogenes* IFO12337. Many other *Streptomyces* strains were also used for examination of the host range of phages. To prepare spore suspension, these strains were cultured on a rye-flake agar medium at 30 ^{0}C for 7-14 days until obtaining a large amount of spores. The spores gathered were suspended in 20 % (V/V) glycerol and then stored at -20 ^{0}C until use.

Phage: Phage Yok-15, employed in this study, was isolated from Nakhonnayok Province using strain 15.

Soil: Three soil samples were used for this study. The first Thai soil, which was collected from Nakhonnayok Province at 7-10 centimeters depth and used for the screening of *Streptomyces* strains and phages, was pH 7.4. The second sample was collected from Bangkok with operating pH of 6.8. The third was Japanese garden soil from Fukuoka Prefecture in Kyushu with pH of 6.6. Soil samples were sterilized by autoclaving twice. After autoclaving, 0.6 % sterile glucose solution was added to the soils to give moisture content of 15% (w/w) which allowed the germination and growth of streptomycetes.

2.31.2 Assay

Colony assay: colony assay for streptomycetes was done by spreading appropriate dilution of spores and mycelia onto a rye-flake agar medium described above. After 7 days of incubation at 28 ^{0}C, colony forming units per ml or per g (cfu/ml or cfu/g) were calculated.

Phage assay: phage assay was done by double layer method. After 1 or 2 days of incubation, plaque forming units per ml or per g (pfu/ml or pfu/g) were calculated.

Assay of phage resistant mutants: the presence and occurrence of phage-resistant mutants in a population of phage-susceptible streptomycetes was detected by plating the population with an excess of phage (Eisenstark, 1967). The colonies formed were calculated as above.

Experimental methods in simple batch microcosms: A 50 g of sterile Thai or Japanese soil in 250 ml Erlenmeyer flask with cotton plug was inoculated with spores of the host, *Streptomyces* sp. strain 15, *S. coelicolor*, *S. griseus*, or *S. viridochromogenes*, at concentration of 1.0×10^5 to 5×10^6 cfu/g. These prepared microcosms were preincubated for spore germination and early growth of streptomycetes for 12 h at 28 ^{0}C then infected with phage Yok-15 at multiplicity of infection (m.o.i.) of 0.01 or 0.1. The outline of the experiments was shown on Table 2.1. Three microcosms were employed for each experiment. Aliquot size of 1 g was periodically taken and assayed for phages and streptomycetes.

The data shown in each figures are the average of three measurements in two independent experiments.

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Table 2.1 The experimental designs

Microcosm	Experiments	Soil types
Streptomyces sp. 15 with/ without phage Yok-15 (A, B, C1, D1)	A: host 10^5 cfu/g and phage 10^3pfu/g B: host 10^5 cfu/g and phage 10^4 pfu/g	Thai soil (I) (Nakhonnayok Province)
S. coelicolor, *S. griseus*, *S. viridochromogenes* with/without phage Yok-15 (C2, D2)	C: host 10^6 cfu/g and phage 10^4pfu /g D: host 10^6 cfu /g and phage 10^5 pfu /g for 8 day's incubation	
Streptomyces sp. 15 with/ without phage Yok-15 (E, F)	E: host 10^6 cfu /g and phage 10^4 pfu /g F: host 10^6 cfu /g and phage 10^5 pfu /g for 8 day's incubation	Thai soil (II) (Bangkok)
Streptomyces sp. 15 with/ without phage Yok-15 (G1, H1, I1, J1)	G: host 10^5 cfu/g and phage 10^3pfu/g H: host 10^5 cfu/g and phage 10^4 pfu/g	Japanese soil (Fukuoka Prefecture)
S. coelicolor, *S. viridochromogenes* with/without phage Yok-15 (G2, H2, I2, J2)	I: host 10^6 cfu/g and phage 10^4 pfu/g J: host 10^6 cfu/g and phage 10^5 pfu/g for 4 day's incubation	

2.31.3 Efficiency of plating (Ellis and Delbruck, 1939)

Phage was diluted in broth that there is somewhat less than 1 infectious particle/ml and then distributed in tubes to give 50 samples of 1 ml each. Each sample was inoculated with a 0.1 ml of host and incubated overnight. At the end of this time, 0.1 ml sample from each tube is plated in the usual way to see whether phage is present or not. The proportion of samples containing no phage is determined and the average number of phage particles per sample is calculated from the Poisson formula:

$$P(0) = e^{-n}$$

In which

P(0) = the fraction of samples containing no phage,

n = the average number of phage particles per sample,

e = the base of natural logarithms

In addition, efficiency of plating was calculated from the number of plaque on indicator strain divided to number of plaque on previous host. An appropriate dilution of phage suspension was mixed with each spore suspension of certain hosts in overlaid agar and plated on nutrient agar. The pfu/ml was counted

2.31.4 Statistical Analysis

Significant differences and standard errors were determined by analysis of variance via student t -test, using the STATVIEW Software.

2.32 Characteristics of phage Yok-15

2.32.1 Sensitivity to chloroform

Five milliliters of a sterile phage suspension was transferred to a sterile tube and 0.1 ml of 1 % chloroform was added (Crosse and Hingorani, 1958). The tube was shaken on a rotary shaker at 200 rpm for 5 min and

allowed to stand at 4 ^{0}C for 2 min. The aqueous suspension was assayed for pfu/ml by double layer method (Lanning and Williams, 1982).

2.32.2 Temperature stability

Phage Yok-15 with titer 10^{2} pfu/ml was incubated at 4 ^{0}C and at 30 ^{0}C for 5 and 7 h and the titer of phage was determined.

2.32.3 Sensitivity to EDTA and pyrophosphate

EDTA and sodium pyrophosphate in various concentrations were used for this purpose. After adding these reagents, the samples were taken at interval time. Double agar layer method was done as in section 2.12 in order to determine the titer of phage.

3.32.4 Multiplication of phage Yok-15 in liquid culture

pH of nutrient broth was adjusted over the range of 5 to 11. *Streptomyces* sp. 15 and *S.viridochromogenes* were used as host for phage Yok-15 multiplication. Double agar layer method was done as in section 2.12. The titer of phage was determined.

2.32.5 Optimal conditions for obtaining the high titers of phage

2.32.5.1 Agitation speed

The rotation speed to cultivate phage Yok-15 was determined at 150, 200, and 250 rpm. Double agar layer method was done as in section 2.12. The pfu/ ml of phage was calculated.

2.32.5.2 Initial host and phage concentrations

Various dilution of host and phage concentration was independently tested to obtain the high titer of phages. Double agar layer method was done as in section 2.12 and the pfu/ ml of phage was calculated.

CHAPTER III

RESULTS

3.1 Soil Sampling

Soil samples, designated number 1-30, were taken from several provinces of Thailand (Fig 3.1). Collected date, pH and characteristic of soil particles were recorded as showed in Table 3.1.

Table 3.1 Localities and characteristics of soil samples

No.	Province	District	Collected Date	pH	Soil texture
1	Roi-ed	Muang	1999/1/2	7.2	Loam, dark brown
2.	Nonthaburi	Muang	1998/6/28	6.6	Loam, black
3.	Phetchaburi	Muang	1998/6/28	6.6	Clay, brown
4.	Prachuap Khiri Khan	Huahin	1998/6/28	6.2	Sand, brown
5.	Bangkok	Bangphlat	1998/6/28	6.6	Clay, black
6.	Angthong	Muang	1998/7/1	6.3	Loam, black
7.	Rayong	Muang	1998/7/1	6.2	Sand, black
8.	Samut Sakhon	Mahachai	1998/7/3	6.9	Clay, black
9.	Nakhon Pathom	Muang	1998/7/10	7.1	Loam, black
10.	Kanchanaburi	Muang	1998/7/10	6.9	Loam, brown
11.	Surat Thani	Punpin	1998/7/18	5.8	Loam,black
12.	Nakhon Ratchasima	Pakthongchai	1998/7/29	7.3	Sand, dark brown
13.	Khon Kaen	Baanphai	1998/7/29	7.6	Loam, brown
14.	Maha Sarakham	Muang	1998/7/29	5.4	Sand, brown
15.	Nakhonnayok	Parkplee	1998/8/8	7.2	Loam, brown

Table 3.1 (continue)

No.	Province	District	Collected Date	pH	Soil texture
16.	Prachin Buri	Muang	1998/8/8	7.7	Loam ,dark brown
17.	Chachoengsao	Muang	1998/8/8	8.0	Sand, reddish brown
18.	Pathum Thani	Thanyaburi	1998/8/8	3.8	Clay, dark brown
19.	Chon Buri	Muang	1998/8/17	6.9	Loam, black
20.	Khon Kaen	Muang	1998/8/17	7.0	Sand, reddish brown
21.	Loei	Poo Kra Doong	1998/8/17	7.3	Loam, black
22.	Pathumthani	Muang	1998/8/17	7. 6	Loam ,dark brown
23.	Chon Buri	Pattaya	1998/9/15	7.1	Loam, black
24.	Chantaburi	Muang	1998/10/12	6.9	Loam, dark brown
25.	Chiengmai	Chieng Dao	1999/2/22	6.7	Loam, reddish brown
26.	Chiengmai	Phang	1999/2/24	7.3	Loam, black
27.	Singburi	Promburi	2000/1/15	6.7	Clay, dark brown
28.	Nakhonsawan	Muang	2001/5/3	7.2	Loam, black
29.	Lampoon	Pa-sang	2001/5/5	6.7	Loam, brown
30.	Nakhonsawan	Taklee	2001/5/3	6.8	Loam, dark brown

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Fig. 3.1 Map of Thailand showing sampling localities of soil

For number refer to Table 3.1

3.2 Isolation of host cells

Streptomyces host cells were isolated by using humic acid vitamin agar medium. Normally each streptomycete and its phage were isolated from the same soil sample. The cultural characteristics of selected streptomycetes strains were observed on inorganic salt starch agar (Appendix I, No. 10) (Williams *et al.*, 1989). Other characteristics observed were; antibiotic producing activity to which test was carried via sensitivity test for their inhibition zone area surrounding tested organisms. The results were shown in Table 3.2.

Table 3.2 Cultural characteristics on plate and antibiotic activity tests of the streptomycetes

Streptomycetes Isolates	Tested organisms						
	C. albican	*E.coli*	*S. cerevisiae*	*B. subtilis*	*M. luteus*	*S. aureus*	*A. niger*
Streptomyces sp. 1	–	+	–	+	–	+	–
Streptomyces sp. 2	–	–	–	–	–	–	–
Streptomyces sp. 3	–	–	–	–	+	–	–
Streptomyces sp. 5	–	–	–	+	–	+	–
Streptomyces sp. 6	–	–	–	–	–	–	–
Streptomyces sp. 7	–	–	–	–	+	–	–
Streptomyces sp. 9	+	+	+	+	+	–	+
Streptomyces sp. 10	–	–	–	–	+	–	–
Streptomyces sp. 11	–	–	–	–	–	–	–
Streptomyces sp. 12	–	–	–	+	–	–	+
Streptomyces sp. 14	–	–	–	–	–	–	–
Streptomyces sp. 15	–	+	–	+	+	–	+
Streptomyces sp. 16	–	–	–	–	+	–	–
Streptomyces sp. 17	–	–	–	–	+	–	+

Table 3.2 (continue)

Streptomycetes Isolates	Tested organisms						
	C. albican	*E.coli*	*S. cerevisiae*	*B. subtilis*	*M. luteus*	*S. aureus*	*A. niger*
Streptomyces sp. 18	–	–	–	–	–	–	+
Streptomyces sp. 19	–	–	–	–	–	–	–
Streptomyces sp. 20	–	+	–	+	+	+	+
Streptomyces sp. 22	–	–	–	–	–	–	–
Streptomyces sp. 24	–	–	–	–	–	–	–
Streptomyces sp. 27	–	–	–	–	+	–	–

Symbol: +, positive; –, negative

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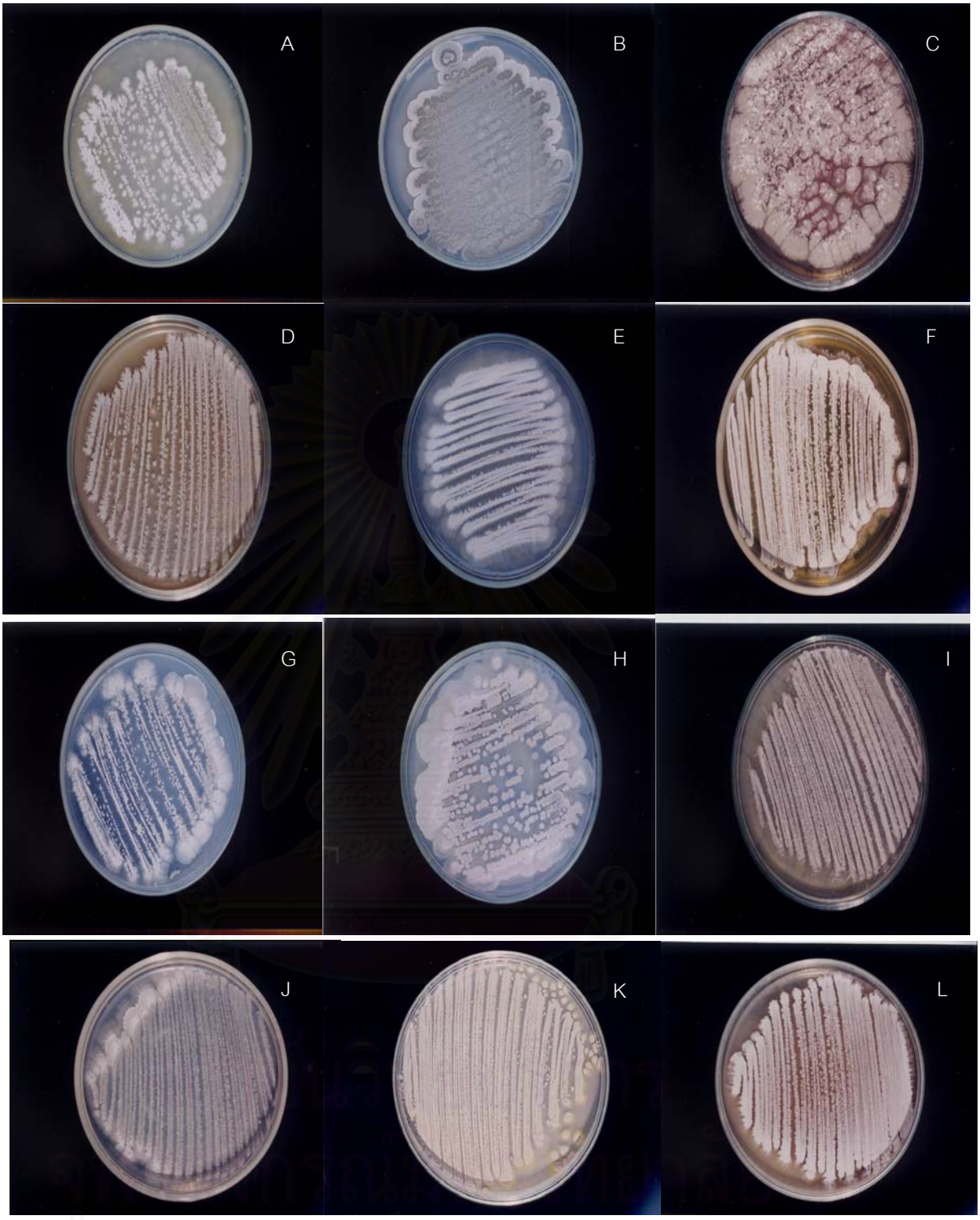

Fig. 3.2 Cultural characteristics of streptomycetes:

A) *Streptomyces* sp. 1, B) *Streptomyces* sp. 2, C) *Streptomyces* sp. 3,
D) *Streptomyces* sp. 5, E) *Streptomyces* sp. 6, F) *Streptomyces* sp. 7,
G) *Streptomyces* sp. 9, H) *Streptomyces* sp. 10, I) *Streptomyces* sp. 11,
J) *Streptomyces* sp. 12, K) *Streptomyces* sp. 13, L) *Streptomyces* sp. 14

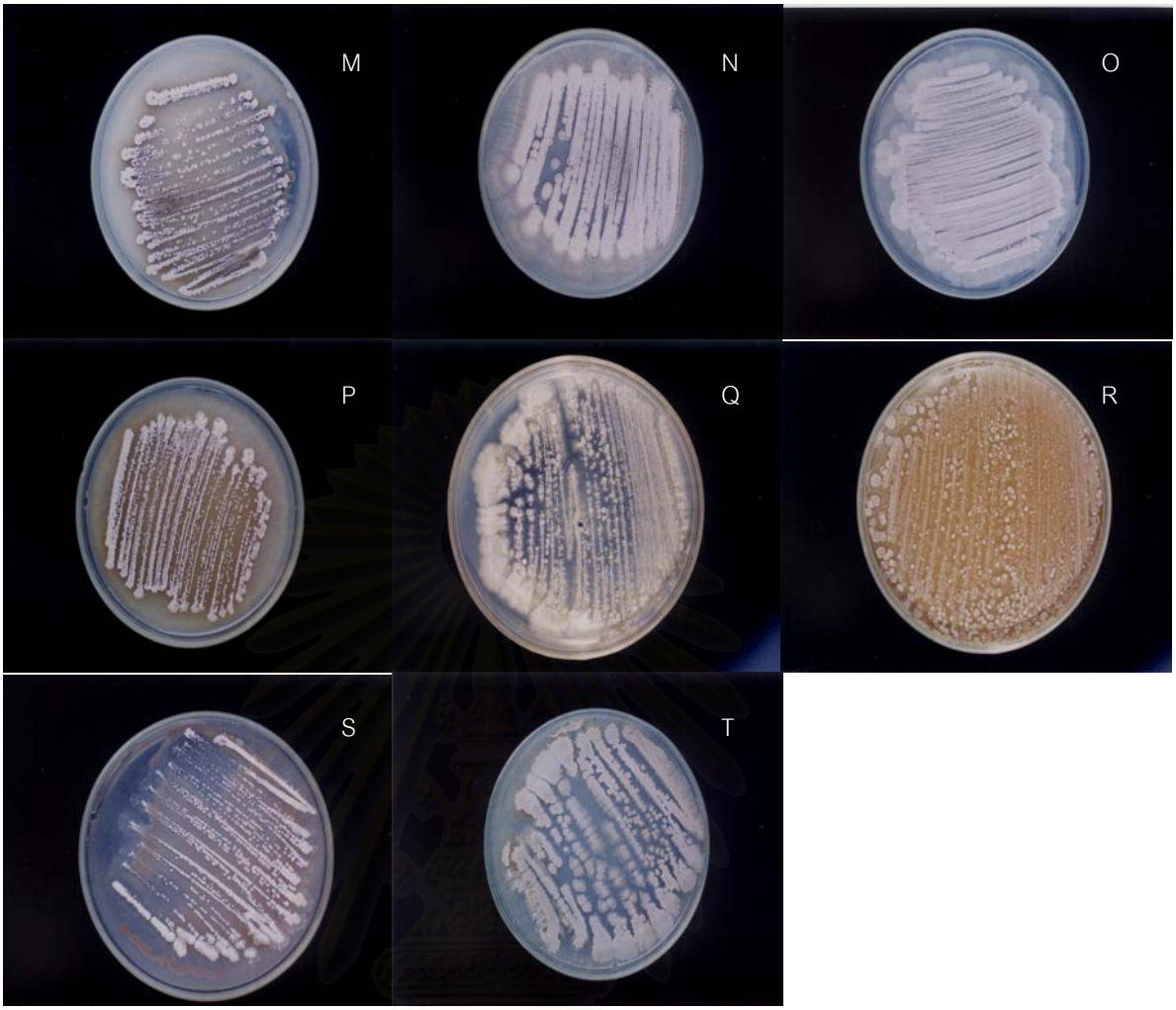

Fig. 3.2 Cultural characteristics of streptomycetes (continue):
M) *Streptomyces* sp. 15, N) *Streptomyces* sp. 16, O) *Streptomyces* sp. 17, P) *Streptomyces* sp. 19, Q) *Streptomyces* sp. 20, R) *Streptomyces* sp. 22, S) *Streptomyces* sp. 24, T) *Streptomyces* sp. 27

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3.3 Morphological characteristic of plaques

Phages were detected by plaque appearance in the lawn of *Streptomyces* after overnight incubation at 30 ^{0}C. Initial phage counts from soil samples were determined. Phages were distinguished by their respective morphology; size and turbidity. Morphological characteristics of plaques were shown in Table 3.3.

Table 3.3 Plaque morphological characteristics of different phages on double layer agar (bar, 5 mm)

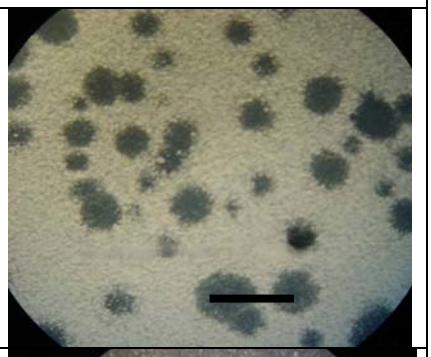
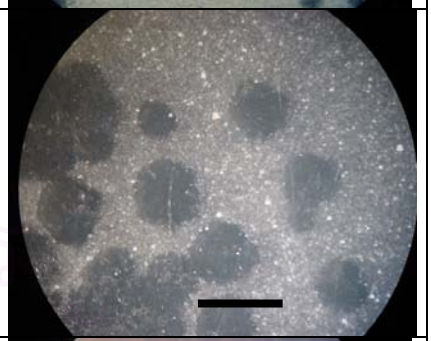
Phage	Initial plaque counts from soil (pfu/ml)	Characteristic of plaque			Morphological characteristics
		Size (mm)	Transparency	Edge	
Roi-1	6 x 10 ^{5}	0.4-4.0	Clear	Sharp	
Non-2	3	1.8-5.0	Clear	Sharp	
Phet-3	49 x 10^{2}	1.0-2.0	Turbid	Diffuse	

Table 3.3 (continue) (bar, 5 mm)

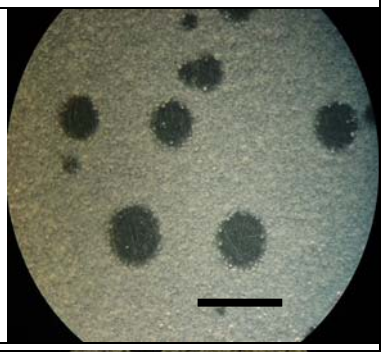
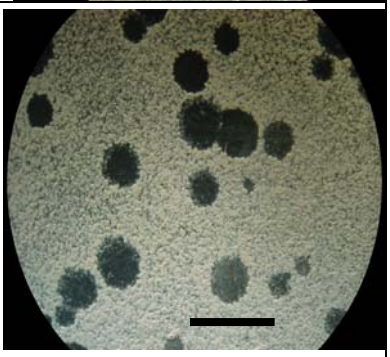
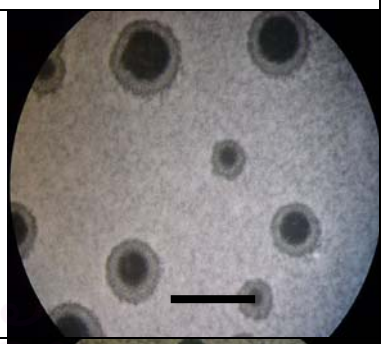
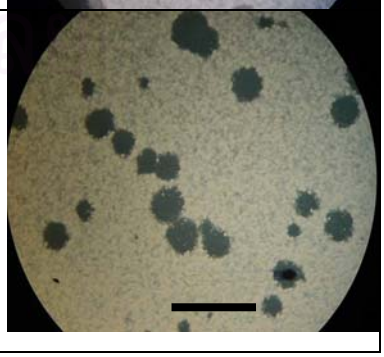
Phage	Initial plaque counts from soil (pfu/ml)	Plaque morphology			Morphological characteristics
		Size (mm)	Transparency	Edge	
Ban-5	2.50×10^3	1.0-5.0	Clear	Diffuse	
Ang-6	22	0.4-3.8	Turbid	Sharp	
Ray-7	50	1.8-4.9	Clear	Sharp and ring - shaped	
Pathom-9	1.60×10^7	0.4-2.8	Clear	Diffuse	

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Table 3.3 (continue) (bar, 5 mm)

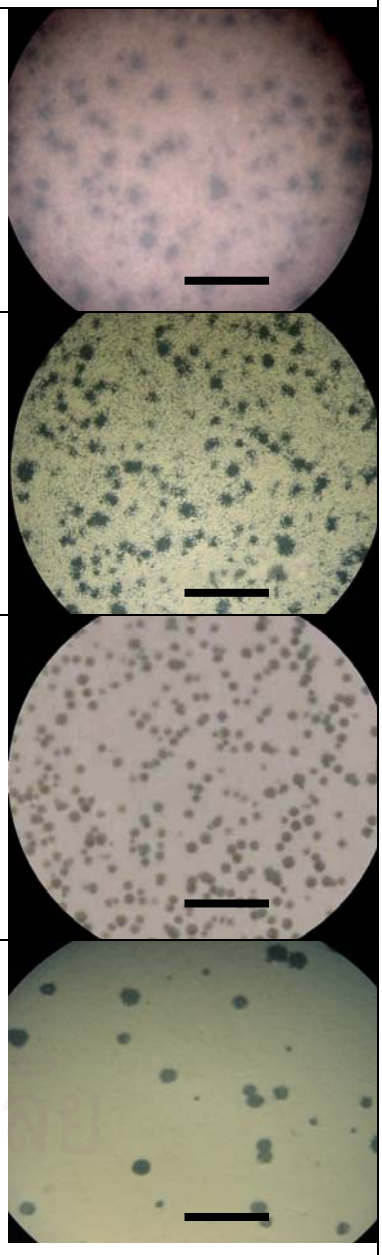
Phage	Initial plaque counts from soil (pfu/ml)	Plaque morphology			Morphological characteristics
		Size (mm)	Transparency	Edge	
Kan-10	1.65×10^3	0.5-1.8	Turbid	Sharp	
Surat -11	2.29×10^5	0.1-1.9	Clear	Diffuse	
Korat -12	6.20×10^4	0.1-1.0	Clear	Sharp	
KK-13	1.50×10^2	0.2-1.9	Clear	Sharp	

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Table 3.3 (continue) (bar, 5 mm)

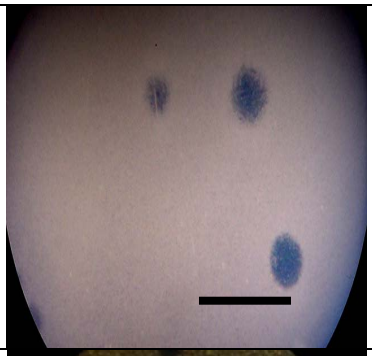
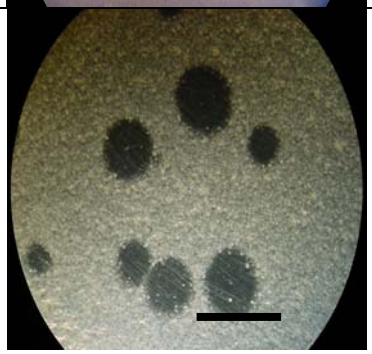
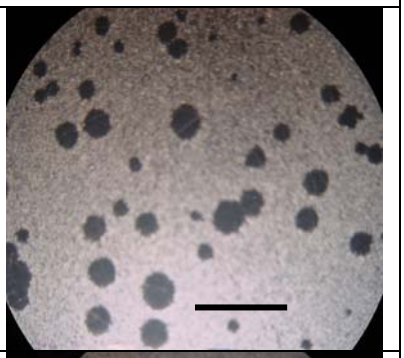
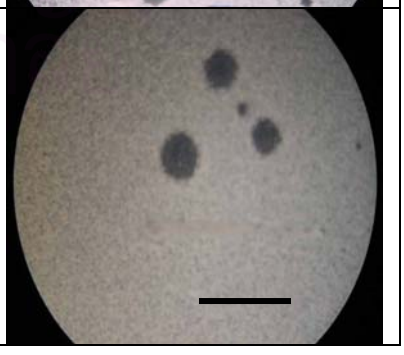
Phage	Initial plaque counts from soil (pfu/ml)	Plaque morphology			Morphological characteristics
		Size (mm)	Transparency	Edge	
Sarakham-14	1.95×10^3	0.2-3.0	Clear	Diffuse	
Yok-15	7.70×10^4	1.1-4.2	Clear	Sharp	
Prachin-16	4.10×10^3	0.4-3.4	Clear	Sharp	
Cha-17	2.33×10^5	1.2-3.1	Clear	Sharp	

Table 3.3 (continue) (bar, 5 mm)

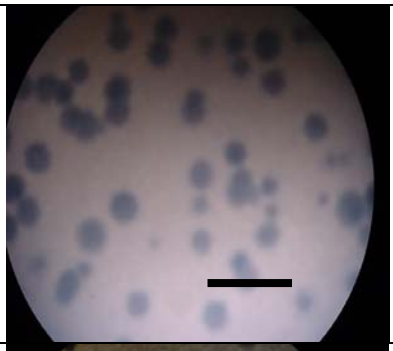
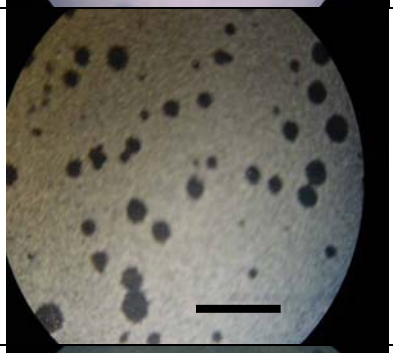
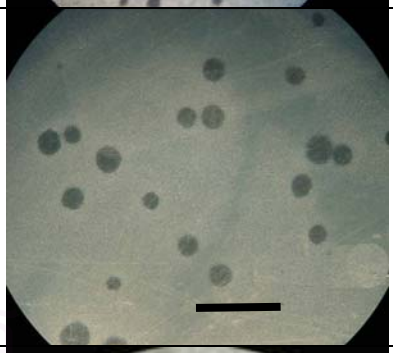
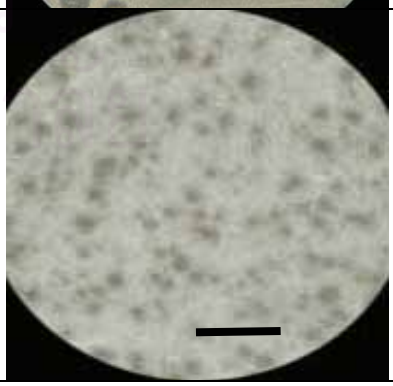
Phage	Initial plaque counts from soil (pfu/ml)	Plaque morphology			Morphological characteristics
		Size (mm)	Transparency	Edge	
Pathum -18	10	0.5-2.5	Clear	Sharp	
Chon-19	48	0.4-3.1	Clear	Sharp	
KK-20	2.25×10^3	0.2-1.5	Clear	Sharp	
Pathum -22	7.25×10^4	0.2-1.0	Turbid	Diffuse	

Table 3.3 (continue) (bar, 5 mm)

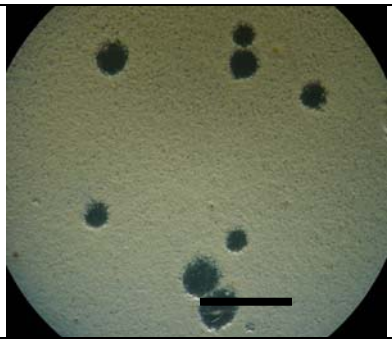
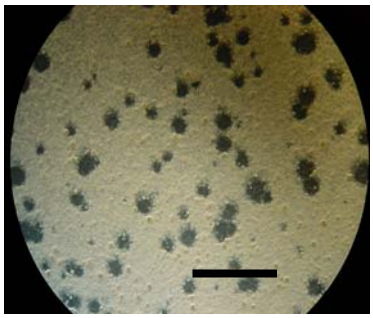
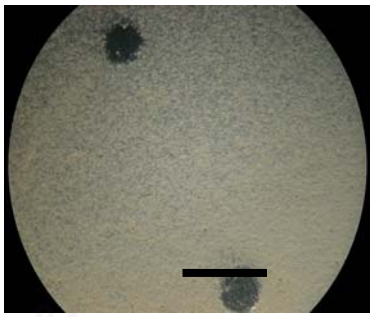
Phage	Initial plaque counts from soil (pfu/ml)	Plaque morphology			Morphological characteristics
		Size (mm)	Transparency	Edge	
Chan-24	125	0.2-3.3	Turbid	Sharp	
Sin-27	3.10×10^4	0.4-1.0	Clear	Diffuse	
Nsaw-28*	61	2.0-2.3	Clear	Sharp	

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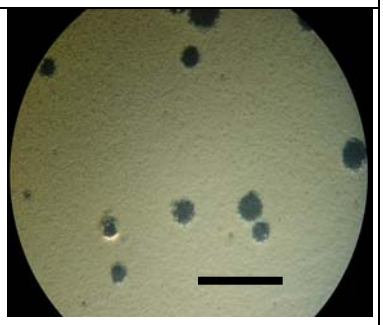
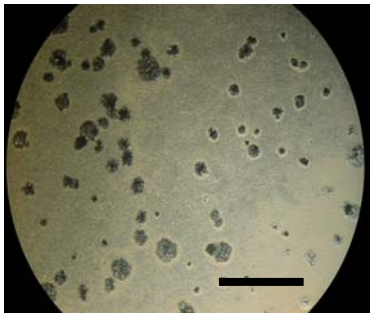
Table 3.3 (continue) (bar, 5 mm)

Phage	Initial plaque counts from soil (pfu/ml)	Plaque morphology			Morphological characteristics
		Size (mm)	Transparency	Edge	
Lam-29*	16	0.9-1.4	Clear	Sharp	
Nsaw-30*	82	0.2-0.9	Turbid	Diffuse	

* isolated by using *S. viridochromogenes* IFO 12337 as a host.

3.4 Morphology of phages

Phages were distinguished by plaque morphology and their appearance under electron microscope. The latter showed that all phages had a six-sided head and a long non-contractile tail which belonged to Bradley's group B (Bradley, 1959) or Siphoviridae family according to International Committee on Taxonomy of viruses (Matthews, 1982).

Table 3.4 Phage morphology (bar, 100 nm)

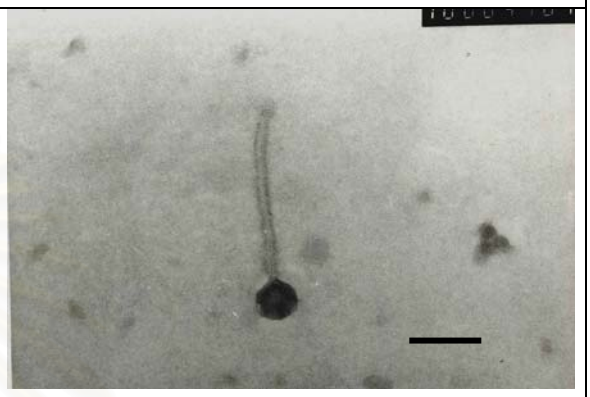
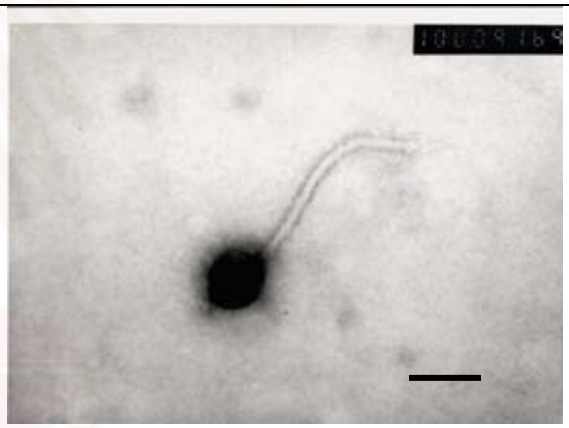
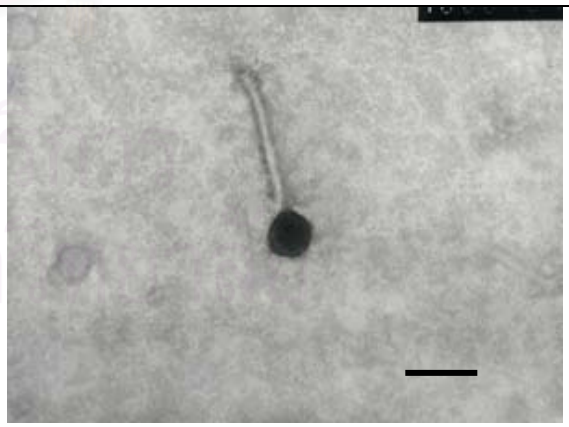
Phage	Phage dimension (nm)			Phage morphology
	Head	Tail		
		Width	Length	
Roi-1	40±7	10±2	213±47	
Non-2	67±60	12±4	273±7	
Phet-3	53±1	6.7±4	153±40	

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Table 3.4 (continue) (bar, 100 nm)

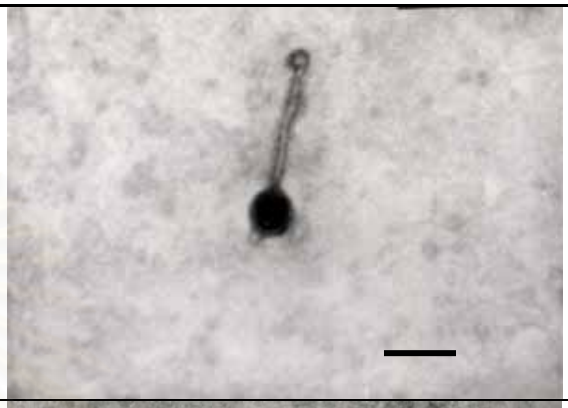
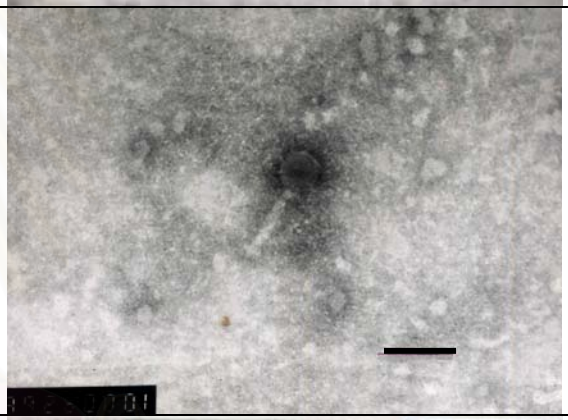
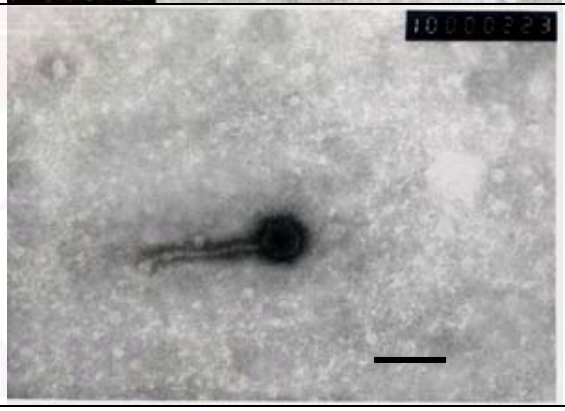
Phage	Phage dimension (nm)			Phage morphology
	Head	Tail		
		Width	Length	
Ang-6	47±5	13.3±7	187±50	
Ray-7	47±20	13.3±7	100±50	
Pathom-9	43±10	6.7±6	143±5	

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Table 3.4 (continue) (bar, 100 nm)

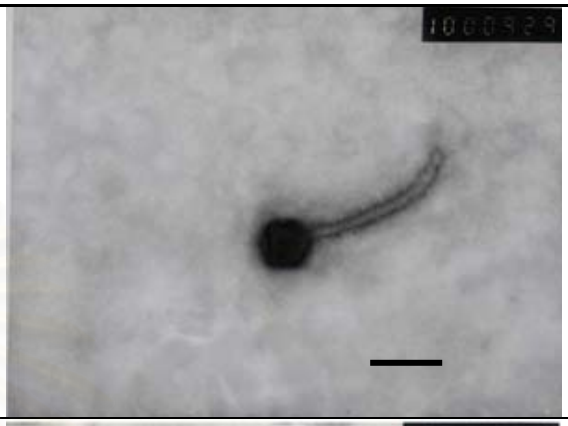
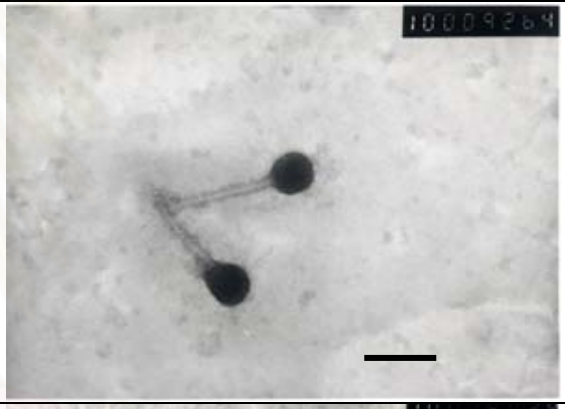
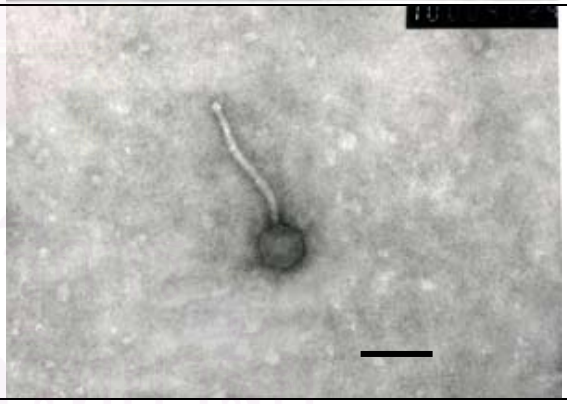
Phage	Phage dimension (nm)			Phage morphology
	Head	Tail		
		Width	Length	
Surat -11	60±1	8±2	213±50	
Korat -12	53±7	7±4	147±20	
KK-13	53±20	6.7±10	213±20	

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Table 3.4 (continue) (bar, 100 nm)

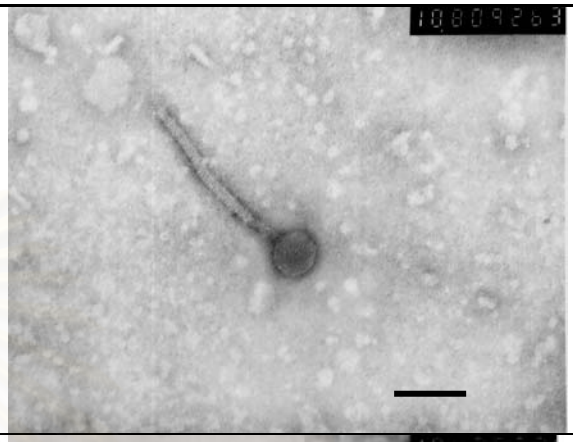
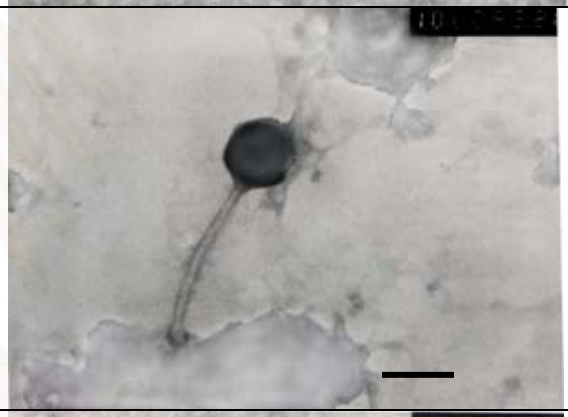
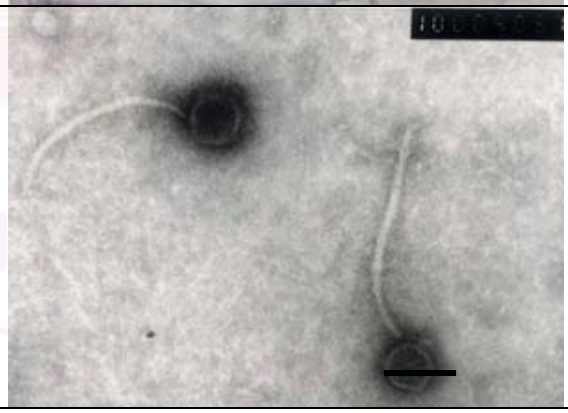
Phage	Phage dimension (nm)			Phage morphology
	Head	Tail		
		Width	Length	
Yok-15	50±4	6.7±10	210±4	
Prachin-16	60±13	10±5	280±23	
Cha-17	60±11	10±10	260±20	

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Table 3.4 (continue) (bar, 100 nm)

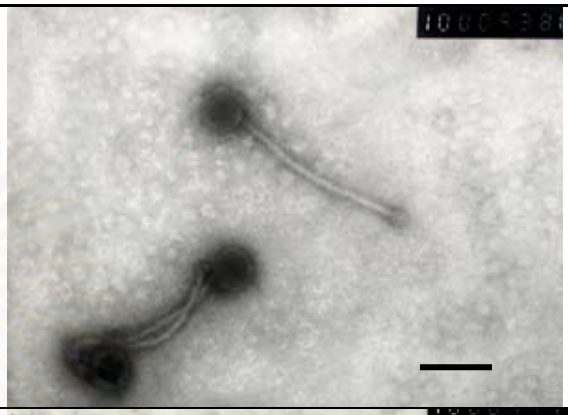
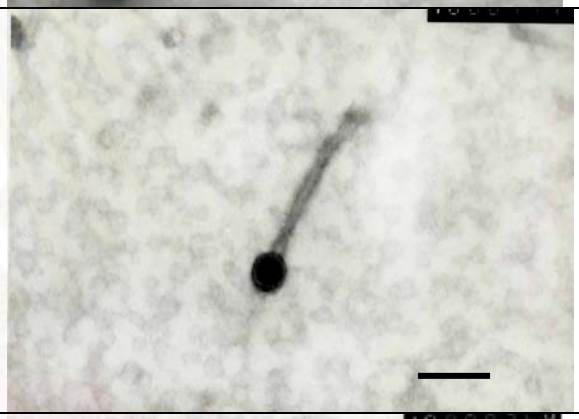
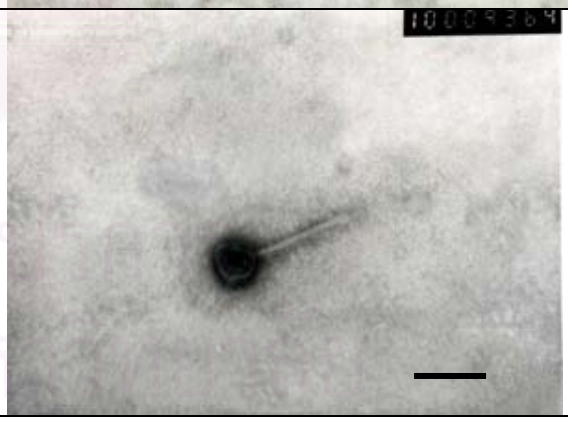
Phage	Phage dimension (nm)			Phage morphology
	Head	Tail		
		Width	Length	
Pathum-18	53±7	10±5	253±20	
Chon-19	46±5	10±5	220±20	
KK-20	53±5	12±5	133±30	

Table 3.4 (continue) (bar, 100 nm)

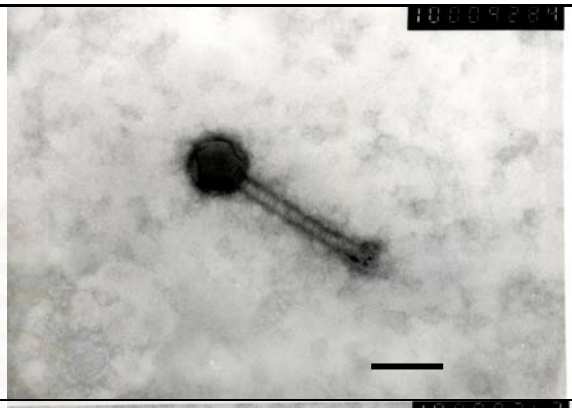
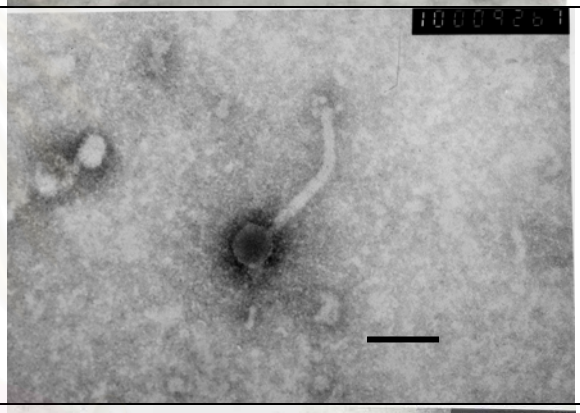
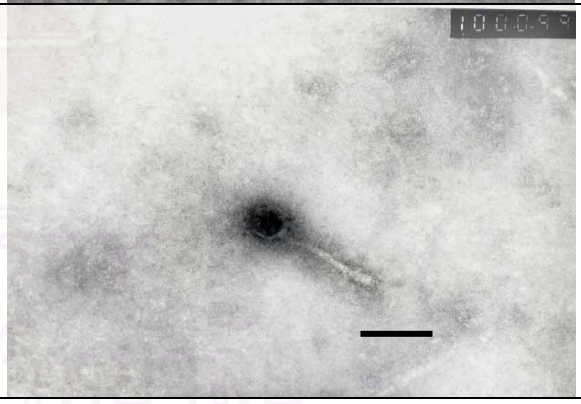
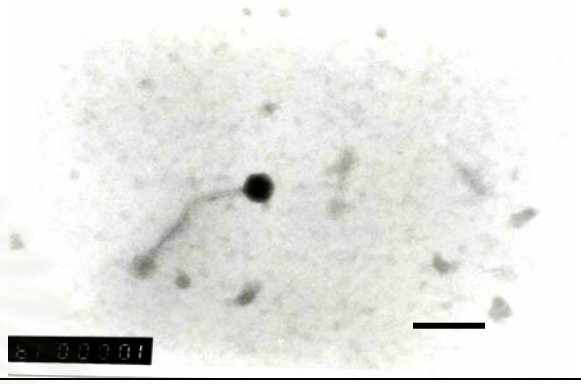
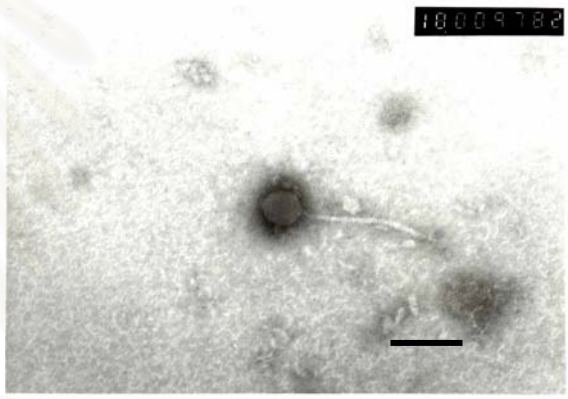
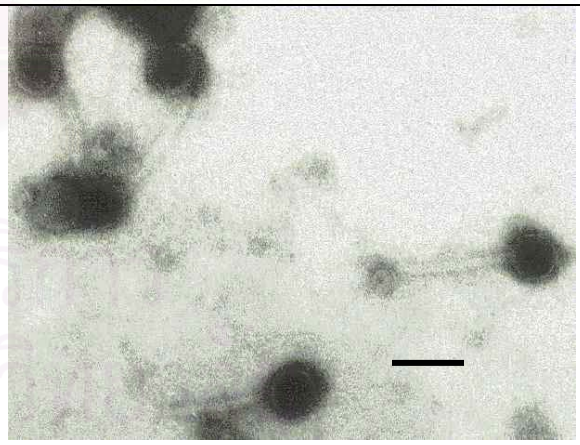
Phage	Phage dimension (nm)			Phage morphology
	Head	Tail		
		Width	Length	
Pathum-22	80±5	20±5	240±30	
Sin-27	53±7	20±10	200±50	
Nsaw-28	40±5	7.33±10	133±30	

Table 3.4 (continue) (bar, 100 nm)

Phage	Phage dimension (nm)			Phage morphology
	Head	Tail		
		Width	Length	
Lam-29	40±10	4 ±1	200±15	
Nsaw-30	36±6	6±2	140±10	
Ac 7*	53.3	13.3	133.3	

Symbol: *, Janejira Deatraksa, 2000.

3.5 Host range determination

From 118 strains of isolated streptomycetes and 45 strains of streptomycetes reference strains were used for host range determination. Among these, phage Roi-1 showed the widest host range followed by strains Roi-1, Yok-15, Nsaw-30, Lam-29, Nsaw-28, Korat-12, Ac-7, Ray-7, Surat-11, Sin-27, Pathum-18, Pathom-9, Non-2, KK-20, Ban-5, Pathum-22 could infect 109, 107, 90, 84, 82, 48, 39, 29, 22, 19, 18, 18, 16, 11, 5, and 3, respectively. 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 and the other closely relatedness strains (based on 16SrDNA) in family Streptomycetaceae, for instance: *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 (Table 3.5).

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Table 3.5 Host range determination

Streptomycetes	phage																		
	Roi-1	Non-2	Phet-3	Ban-5	Ray-7	Pathom-9	Surat-11	Korat-12	Yok-15	Pathum-18	KK-20	Pathum-22	Chan-24	Sing-27	Nsaw-28	Lam-29	Nsaw-30	Ac 7
Kitasatospora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. alboniger	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. albovinaceous	-	-	-	-		+	+	+	-	+	-	-	-	+	-	-	-	-
S. albus	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	-	-	-
S. amboflaciens	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-
S. aminophilus	-	+	-	-	+	+	-	-	+	+	-	-	-	+	-	-	-	+
S. aureus	+	-	-	-	+	-	-	-	+	+	-	-	-	+	-	-	-	-
S. azureus	+	+	-	-	+	+	+	+	+	+	-	-	-	+	-	-	-	-
S. badius	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. bikiniensis	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
S. coelicolor M 145	+	+	-	-	+	-	-	-	+	+	-	-	-	+	-	-	-	-
S. coelicolor Muller	+	-	-	-	-	-	-	-	+	+	-	+	-	-	+	-	-	+
S. coeruleofuscus	+	+	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-
S. coerulescens	-	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
S. coralus	+	+	-	-	+	+	+	-	+	+	+	-	-	-	+	-	+	-
S. cyaneus	+	+	-	-	+	-	+	+	+	-	+	-	-	-	-	-	-	-
S. echinatus	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
S. endus	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+
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. lavendulae subsp. *lavendulae*	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
S. lavendulae subsp. *japonicus*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. lavendulae subsp. *grasserius*	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. lincoensis	+	-	-	-	+	-		-	+	-	-	-	-	+	-	+	-	-
S. lividans TK24	+	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	-	-
S. luteogriseus	+	+	-	-	+	+	+	+	+	+	+	-	-	+	+	-	-	+
S. melanosporofaciens	+	+	-	-	+	-	+	-	+	-	-	-	-	-	+	+	-	-
S. niveous	+	+	-	-	-	-	-	+	+	-	-	-	-	-	+	+	-	-
S. nodosus	+	-	-	-	+	-	+	+	+	-	-	-	-	-	+	+	-	-
S. parvullus	+	+	-	-	+	-	+	+	+	+	-	-	-	-	+	+	-	-
S. periperlis	+	+	-	-	+	-	-	+	+	+	-	+	-	-	+	-	-	-
S. pilosus	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. puniceus	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S. sparsogenes	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. tauricus	+	-	-	-	+	-	-	+	+	-	-	-	-	+	+	-	-	-
S. thermovulgaris	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-
S. violaceochromogenes	+	-	-	-	+	-	-	+	+	-	-	+	-	-	-	-	-	-
S. violaceusniger	+	+	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-
S. viridochromogenes	+	-	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-
Subtotal 1 (referenced streptomycetes)	32	15	0	0	23	8	16	18	39	16	5	3	0	10	14	12	14	9

Table 3.5 (continue)

Streptomycetes	phage																	
	Roi-1	Non-2	Phet-3	Ban-5	Ray-7	Pathom-9	Surat-11	Korat-12	Yok-15	Pathum-18	KK-20	Pathum-22	Chan-24	Sing-27	Nsaw-28	Lam-29	Nsaw-30	Ac 7
S1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
S2.2	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
S3.1	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
S3.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+
S3.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
S5.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
S5.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S6.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
S6.2	-	-	-	+	-	-	-	+	+	-	-	-	-	-	+	+	-	-
S6.3	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-
S7.1	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-
S7.2	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	+	-
S7.4	+	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	+	-
S8.1	+	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-
S8.2	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-
S10.1	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-
S10.2	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+
S10.3	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-
S10.4	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-
S10.5	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+		-
S10.6	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-		-
S10.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
S11.2	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-
S11.1	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
S11.3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S11.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S11.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S12.1	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
S12.2	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
S12.3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
S12.4	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
S12.5	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+		-
S12.6	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	+		-
S12.7	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-

Table 3.5 (continue)

Streptomycetes	phage																	
	Roi-1	Non-2	Phet-3	Ban-5	Ray-7	Pathom-9	Surat-11	Korat-12	Yok-15	Pathum-18	KK-20	Pathum-22	Chan-24	Sing-27	Nsaw-28	Lam-29	Nsaw-30	Ac 7
S12.8	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-		-
S12.9	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
S12.10	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+		-	-
S12.11	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S12.12	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S13.1	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-
S13.2	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-
S13.2	-	-	-	-	-	+	-	+	+	-	-	-	-	-	+	+	-	-
S14.1	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
S14.11	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
S14.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14.3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14.4	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
S14.5	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
S14.9	-	-	-	-	-	+	-	+	+	-	+	-	-	-	+	-	-	+
S15.1	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-
S15.2	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-
S15.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S16.1	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
S16.1	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S16.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-
S16.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S18.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
S18.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
S19.1	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
S19.2	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
S19.3	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-
S19.4	+	-	-	-	+	-	-	+	+	-	-	-	-	-	+	-	-	-
S19.5	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+			
S19.6	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
S19.7	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-			
S20.1	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	
S20.2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-
S20.3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
S20.4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		

Table 3.5 (continue)

Streptomycetes	phage																	
	Roi-1	Non-2	Phet-3	Ban-5	Ray-7	Pathom-9	Surat-11	Korat-12	Yok-15	Pathum-18	KK-20	Pathum-22	Chan-24	Sing-27	Nsaw-28	Lam-29	Nsaw-30	Ac 7
S20.5	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S20.6	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
S20.7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
S21.1	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-		-
S21.2	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-
S21.3	+	-	-	-	-	+	-	-	+	-	-	-	-	-	+	+	-	-
S21.4	+	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	-
S21.5		-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
S22.1	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-		-
S22.2	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+	-
S22.3	+	-	-	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-
S22.4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
S22.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
S22.6	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S23.1	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S23.2	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
S23.3	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-
S23.4	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-
S23.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S23.6	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
S23.7	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
S24.1	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-
S24.2	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+	-
S24.3	+	-	-	-	-	-	+	+	+	-	-	-	-	-	+	+	-	-
S24.4	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-
S24.5	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	+	-	-
S24.6	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+		+	-
S24.7	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-
S24.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+			-
S25.1	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-
S25.2	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
S25.3	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S25.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S25.5	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-
S25.6	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-

Table 3.5 (continue)

Streptomycetes	phage																	
	Roi-1	Non-2	Phet-3	Ban-5	Ray-7	Pathom-9	Surat-11	Korat-12	Yok-15	Pathum-18	KK-20	Pathum-22	Chan-24	Sing-27	Nsaw-28	Lam-29	Nsaw-30	Ac 7
S25.7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
S26.1	+	-	-	-	+	-	-	+	+	-	-	-	-	-	+	-	-	-
S26.2	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
S26.3	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
S26.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S26.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S26.6	+	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-
S27.1	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
S27.2	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-
S27.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
S27.4	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
S27.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S27.6	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Subtotal (2)	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

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3.6 One step growth experiments

Production of phages were determined in one step growth experiments at 30 ^{0}C as shown in Table 3.6 and Fig. 3.3. However, this experiment on phage Sin-27 and Ac 7 was not accomplished since two phages could not infect their hosts in liquid culture.

Table 3.6 One step growth experiments of phages

Phages	Latent period (min)	Rise period (min)	Burst size
Roi-1	10	20	30-35
Ray-7	20	40	95-100
Surat-11	20	20	110-115
Yok-15	30	30	30-35
Nsaw-28	20	40	40-45
Lam-29	20	60	30-35
Nsaw-30	25	35	10-20

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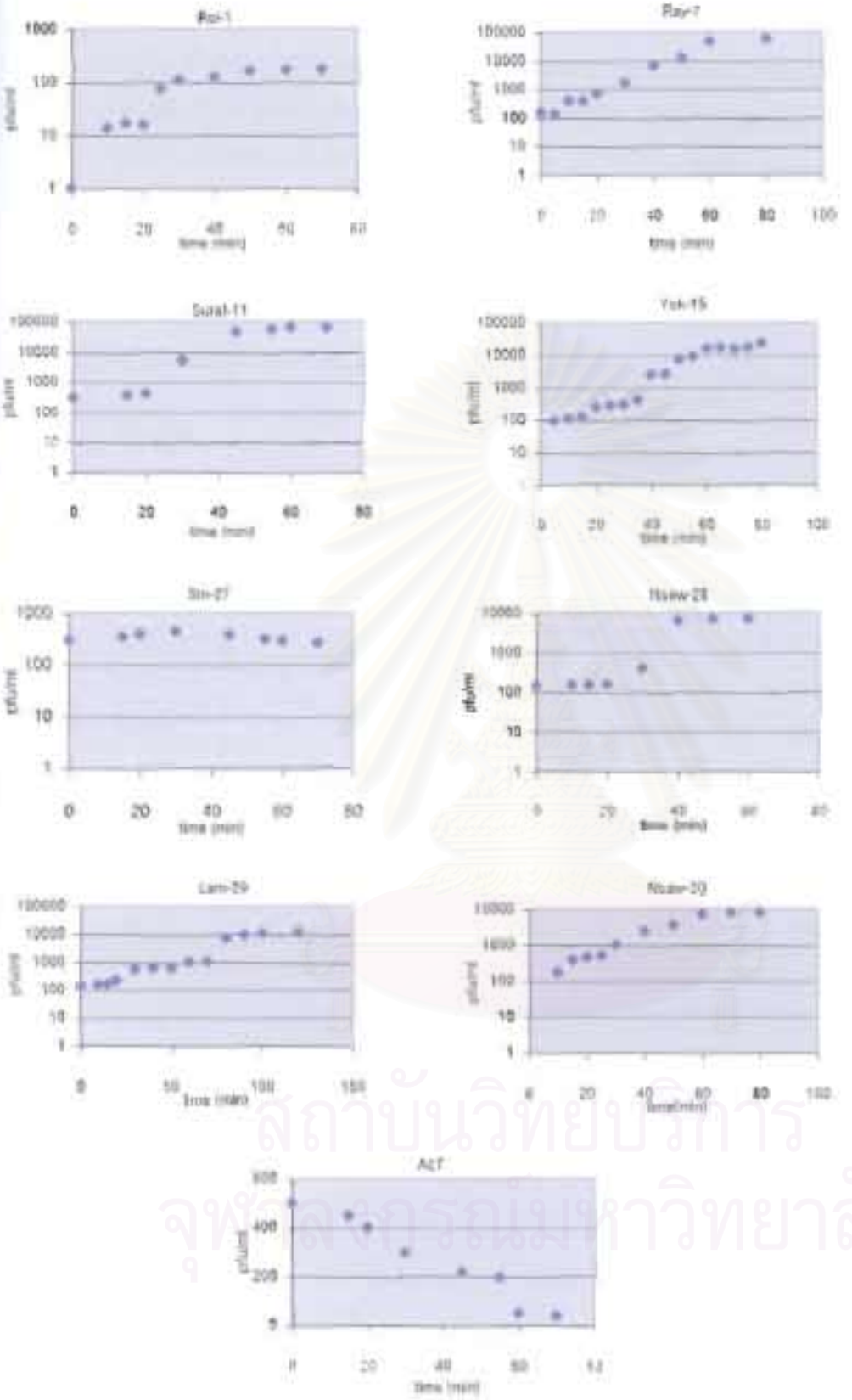

Fig. 3.3 One step growth experiments

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3.7 Effect of pH

Effect of the pH on phage showed that most phages were stable at the tested pH (4-9) while gave maximal yields at neutral pH. The exception to this was phage Yok-15 that its optimal and stable pH was 9. Six out of nine tested phages were able to survive at pH 4 (Table 3.7, 3.8; Fig 3.4, 3.5).

Table 3.7 Stability of phage propagation at various pH

Phages	Number of pfu/ml					
	pH					
	4	5	6	7	8	9
Roi-1	7	1.62×10^3	1.40×10^3	6.40×10^3	2.73×10^3	2.50×10^3
Ray-7	100	6.00×10^4	1.13×10^5	1.61×10^5	1.88×10^5	1.55×10^4
Surat-11	0	203	2.50×10^4	5.20×10^4	4.90×10^3	2.50×10^3
Yok-15	0	521	4.00×10^3	5.80×10^3	8.30×10^3	1.10×10^4
Sin-27	52	291	358	449	250	200
Nsaw-28	41	59	1.80×10^3	1.90×10^3	1.20×10^3	200
Lam-29	62	2.20×10^3	6.10×10^3	952	53	40
Nsaw-30	0	51	146	156	176	145
Ac 7	46	162	231	267	293	226

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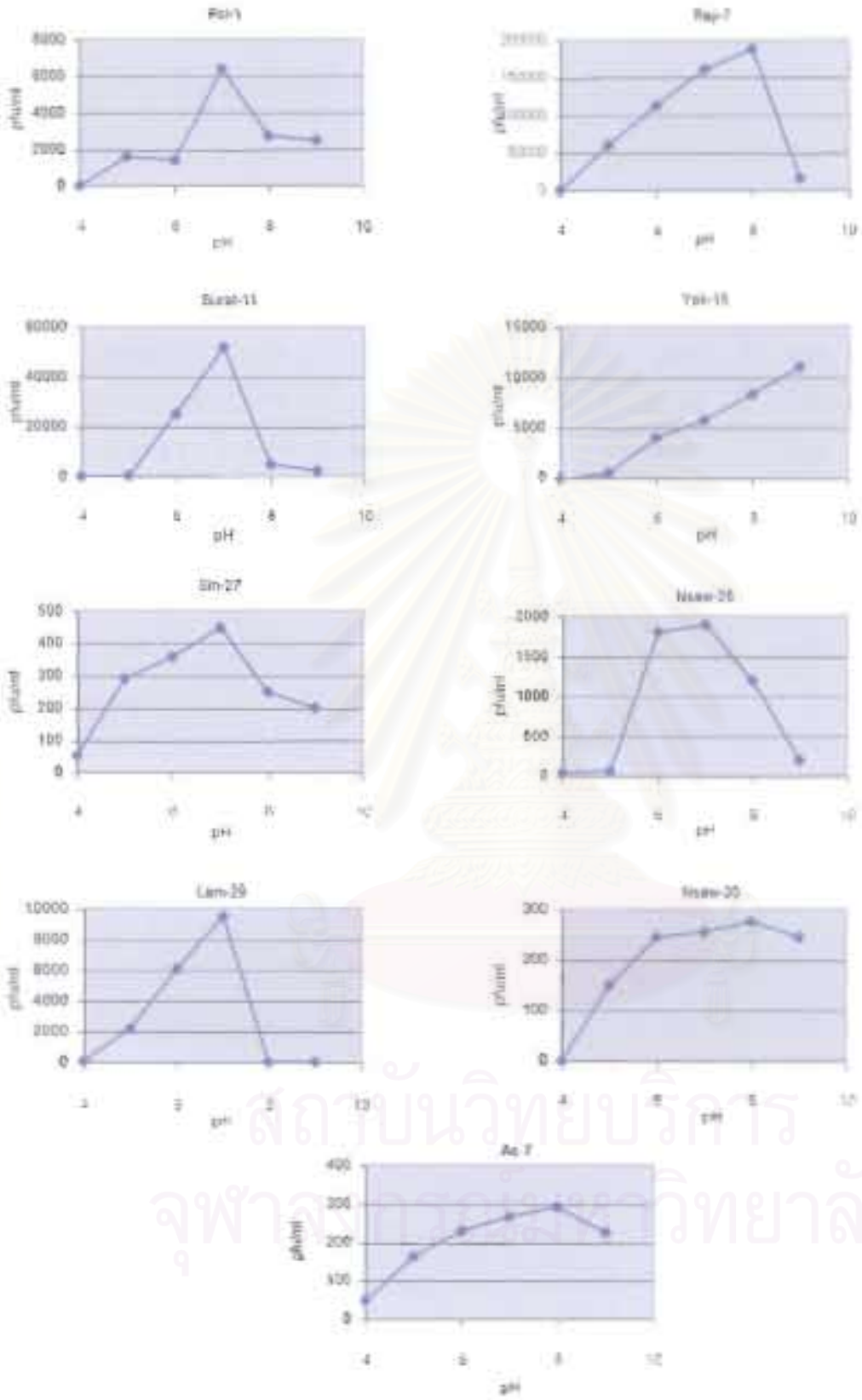

Fig. 3.4 Stability of phage propagation at various pH

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Table 3.8 Optimal pH for phage propagation

Phages	Number of pfu/ml					
	pH					
	4	5	6	7	8	9
Roi-1	10	50	320	260	920	470
Ray-7	10	170	840	790	110	30
Surat-11	0	144	730	990	490	370
Yok-15	0	2,890	4,880	5,280	5,290	5,520
Sin-27	29	404	788	968	724	796
Nsaw-28	80	2,310	5,160	4,160	1,820	1,350
Lam-29	32	478	1,024	1,200	836	696
Nsaw-10	0	270	360	455	200	120
Ac 7	75	920	4,560	5,760	400	392

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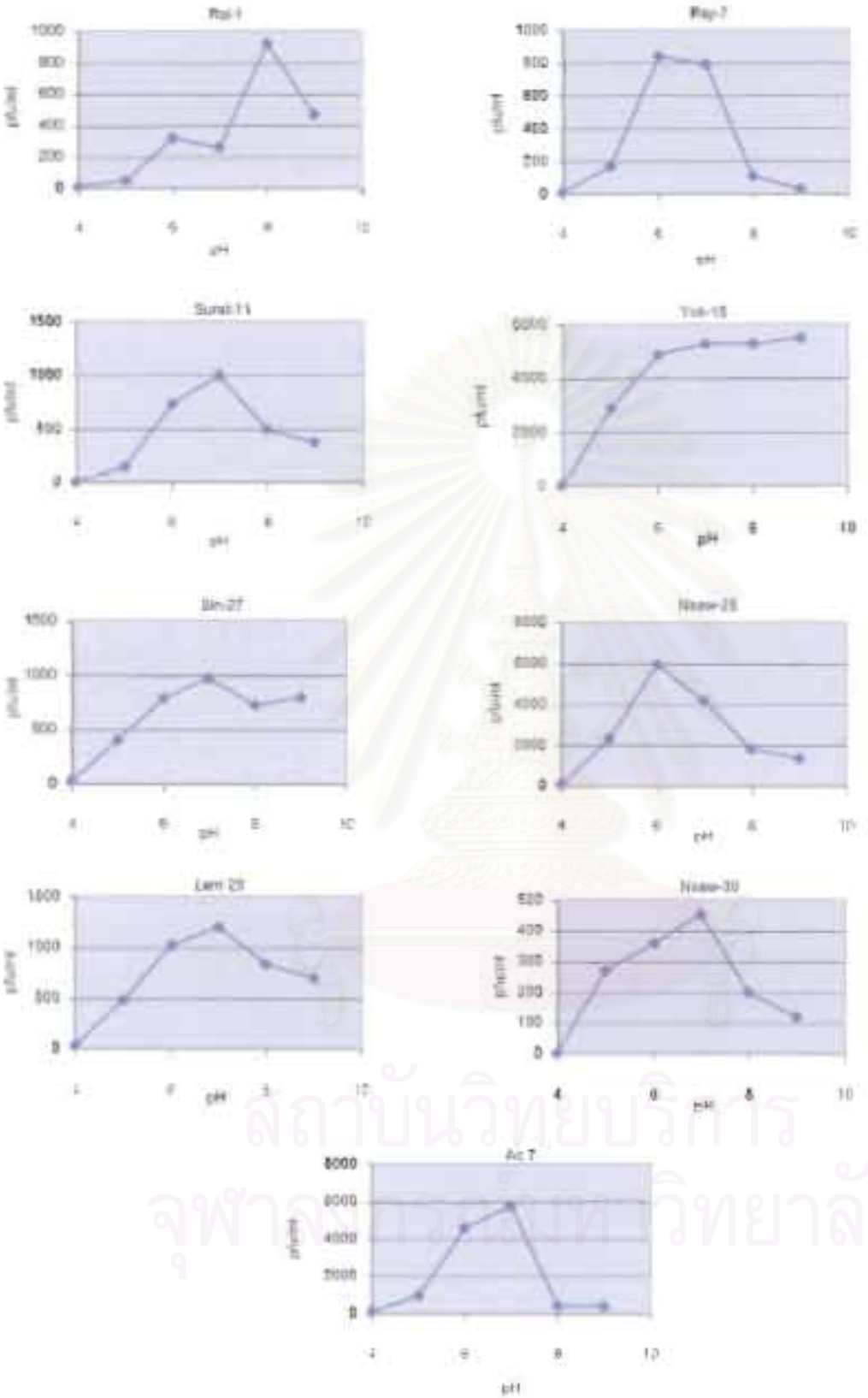

Fig. 3.5 Optimal pH for phage propagation

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3.8 Cation requirement for phage multiplication

Most phages required Mg^{2+} and/or Ca^{2+} for their multiplication, but some did not. To determine the effect of divalent cation on number of phage yields, 9 phages were selected based on result of host range determination and high titers phage obtained. Data of these were shown in Table 3.9; Fig. 3.6 for Mg^{2+} and Table 3.10; Fig. 3.7 for Ca^{2+} requirement.

Table 3.9 Effect of $MgSO_4$ on phage propagation

phages	Number of pfu/ml					
	$MgSO_4$ concentration (mM)					
	0	10	20	30	40	50
Roi-1	3.80×10^4	2.90×10^4	5.00×10^3	2.00×10^3	0	0
Ray-7	2.60×10^4	1.00×10^4	0	0	0	0
Surat-11	5.00×10^3	2.50×10^4	2.10×10^4	1.20×10^4	2.00×10^3	2.00×10^3
Yok-15	4.60×10^4	9.70×10^4	6.20×10^4	5.60×10^4	3.70×10^4	4.00×10^4
Sin-27	7.90×10^4	9.60×10^4	9.00×10^4	8.40×10^4	5.40×10^4	1.00×10^4
Nsaw-28	6.20×10^4	1.86×10^5	1.73×10^5	1.47×10^5	9.30×10^4	6.30×10^4
Lam-29	3.20×10^4	1.40×10^4	0	0	0	0
Nsaw-30	7.00×10^4	7.60×10^4	7.70×10^4	1.20×10^4	9.00×10^3	1.00×10^3
Ac-7	1.53×10^5	4.00×10^3	0	0	0	0

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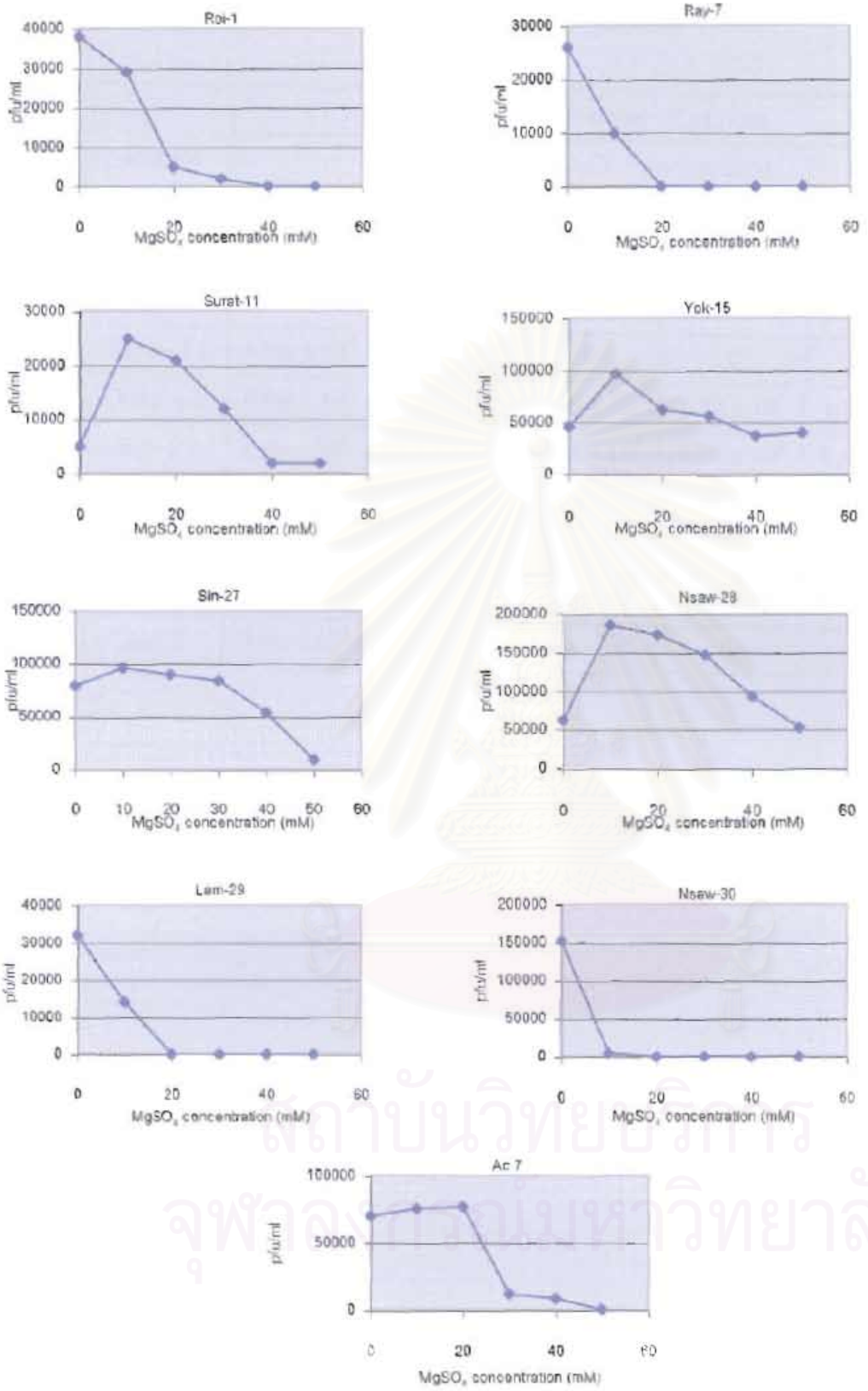

Fig. 3.6 $MgSO_4$ concentration for phage propagation

Table 3.10 Effect of $CaCl_2$ on phage propagation

Phages	Number of pfu/ml					
	$CaCl_2$ concentration					
	0	10	20	30	40	50
Roi-1	4.00×10^3	8.00×10^4	7.10×10^4	6.20×10^4	4.60×10^4	5.10×10^4
Ray-7	9.10×10^4	1.86×10^5	1.51×10^5	1.44×10^5	1.18×10^5	1.07×10^5
Surat-11	5.00×10^3	0	0	0	0	0
Yok-15	8.60×10^4	1.36×10^5	1.86×10^5	2.23×10^5	1.53×10^5	1.08×10^5
Sin-27	3.30×10^4	9.10×10^4	9.30×10^4	9.50×10^4	9.40×10^4	1.00×10^5
Nsaw-28	5.10×10^4	8.60×10^4	1.22×10^5	1.89×10^5	1.98×10^5	1.33×10^5
Lam-29	2.90×10^4	0	0	0	0	0
Nsaw-30	4.70×10^4	1.24×10^5	1.19×10^5	7.70×10^4	8.10×10^4	7.70×10^5
Ac-7	5.50×10^4	1.60×10^4	1.00×10^4	0	0	0

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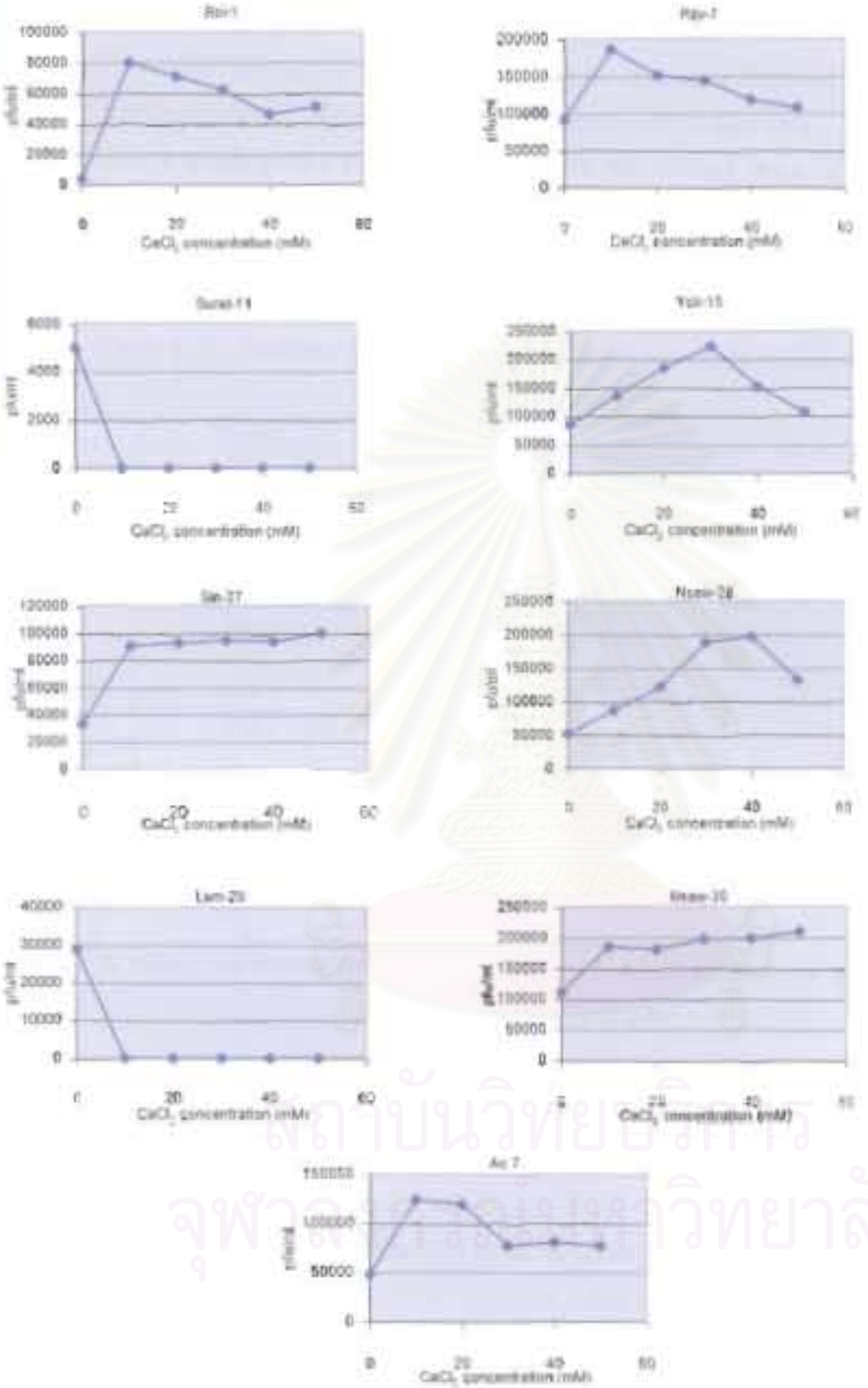


Fig. 3.7 $CaCl_2$ concentration for phage propagation

3.9 Cross reactivity of phages

An attempt to study the serological relevance between selected phages and antiserum against phage Roi-1, which possess widest host range, was conducted. Anti-Roi-1 serum gave a cross-reactivity against the selected strain of phages. Some phages showed closely related group pattern (Table 3.11). The closest serological relatedness to that of phage Roi-1 was found in phage Nsaw-10.

Table 3.11 Neutralizing activity of phage Roi-1 antiserum against selected phages

Phage	K value*	% of inactivation**
Roi-1	185.90	100
Ray-7	9.295	5
Surat-11	81.80	44
Yok-15	14.87	8
Sin-27	109.68	59
Nsaw -28	81.80	44
Lam-29	14.87	8
Nsaw-30	141.28	76
Ac7	79.94	43

*K values: the amount of phage antibodies, was calculated from the equation,

$$K = 2.3\ D/t \times \log (p_0/p)$$

where p_0= number of phage at zero time,

p = number of phage at time t min,

D= final dilution of serum in the phage-serum mixture (Adams, 1959)

** % of inactivation was calculated from $\frac{\text{K value of tested phage}}{\text{K value of phage Roi-1}} \times 100$

3.10 Restriction Analysis

After extraction of phage DNA as described in the section 2.24, a small amount of DNA was allowed to check their quality by electrophoresis using a sample size of about 1 µl (Fig 3.8). Then, an appropriate amount of phage DNA was digested with restriction endonuclease in a suitable restriction buffer and incubated for 1-3 h in the conditions according to supplier recommendation. The results were shown in Fig. 3.10-3.21.

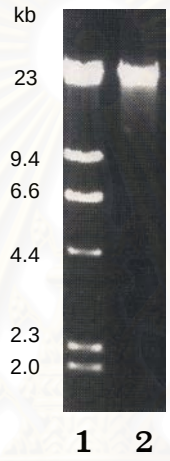


Fig 3.8 Agarose gel electrophoresis of phage DNA
(lane 1 : λ-*Hind*III markers, lane 2: phage Yok-15 DNA)

One way of making a restriction map is to compare single-enzyme digests with double digests. Two restriction enzymes are applied in separate digestions, and then the two enzymes are used together. After cutting, the fragment sizes are determined by electrophoresis. The double digest shows whether a fragment produced by one enzyme contains sites for the other; if so, the fragment disappears and is replaced by two or smaller fragments. Comparison of the sizes of fragments produced in the different digestions allows an approximate localization of the restriction target sites. The procedure was illustrated in Fig. 3.9 and the restriction map of some phage DNA was constructed as followings.

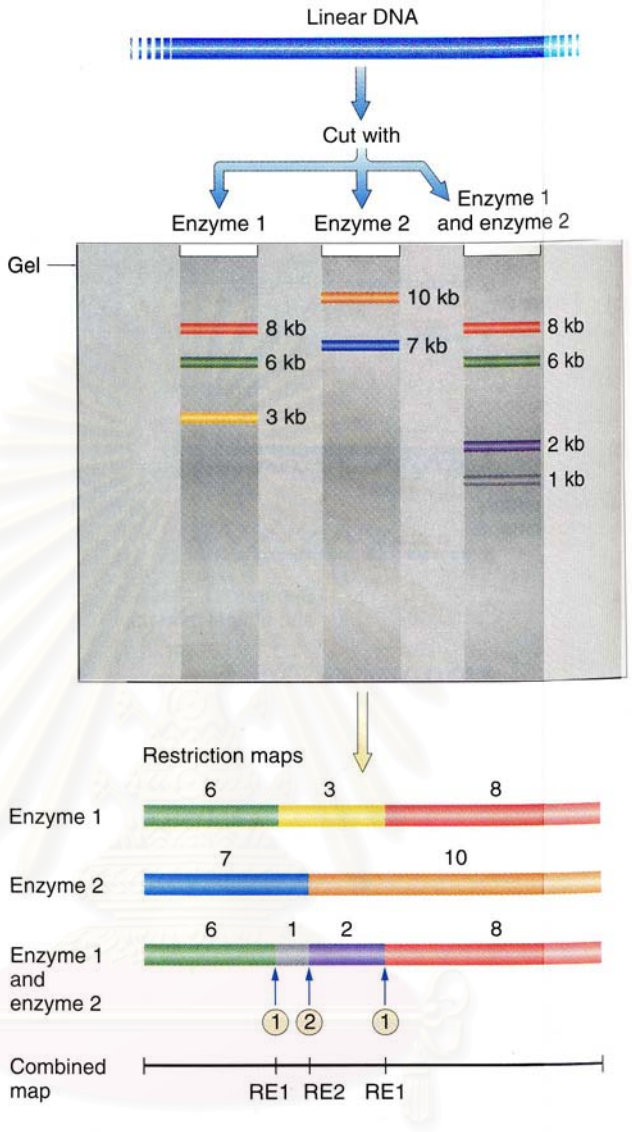


Fig. 3.9 Restriction mapping by comparing electrophoretic separations of single and multiple digests. In this simplified example, digestion with enzyme 1 shows that there are two restriction sites for this enzyme but does not reveal whether the 3-kb segment is in the middle or on the end of the digested sequence, which is 17 kb long. Combined digestion by both enzyme 1 and enzyme 2 leaves the 6- and 8- kb segments intact but cleaves the 3-kb fragment, showing that enzyme 2 cuts at a site within this enzyme 1 fragment. If the 3-kb section were on the outside of the sequence being studied, digestion by enzyme 2 alone would yield a 1- or 2-kb fragments produced by enzyme 1, the 3-kb fragment must lie in the middle. That the RE2 site lies closer to the 6-kb section than the 8-kb section can be inferred from the 7- and 10-kb lengths of the enzyme 2 digestion. (Griffiths *et al.*, 2000)

3.10.1 Restriction analysis of phage Roi-1

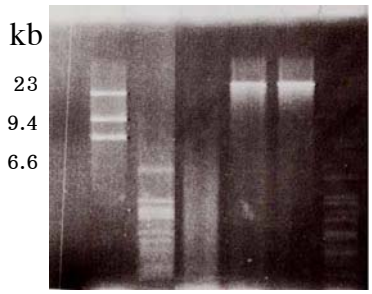


Fig 3.10 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Roi-1 generated with restriction enzymes, respectively, from lane 1 to lane 6: *Bgl*II, *Sal* I, *Ban* II, *Not*I, *Apa* I, *Sac* I.

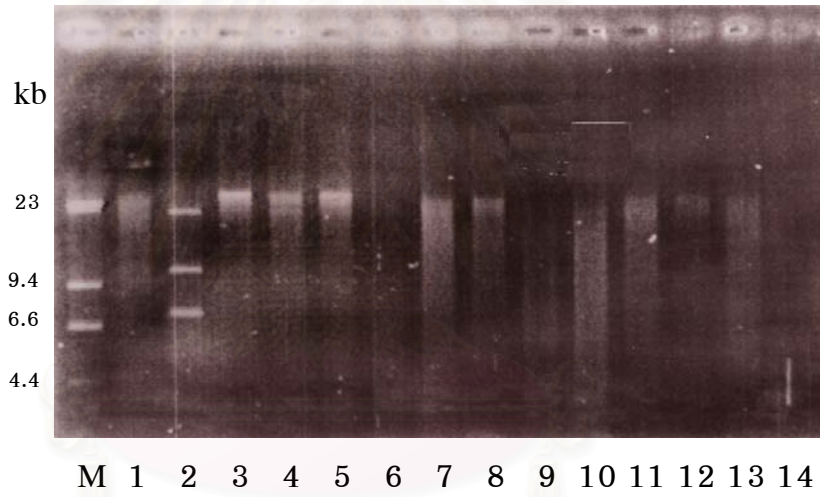


Fig 3.11 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Roi-1 generated with restriction enzymes, respectively, from lane 1 to lane 14: *EcoR*I, *Bgl* II, *Mlu*I, *Pst*I, *Xho* I, *Sal* I, *Sph*I, *EcoR*V, *Ban* II, *Bam* HI, *Spe* I, *Hind* III, *Apa*I, *Sac* I.

3.10.2 Restriction analysis of phage Ray-7

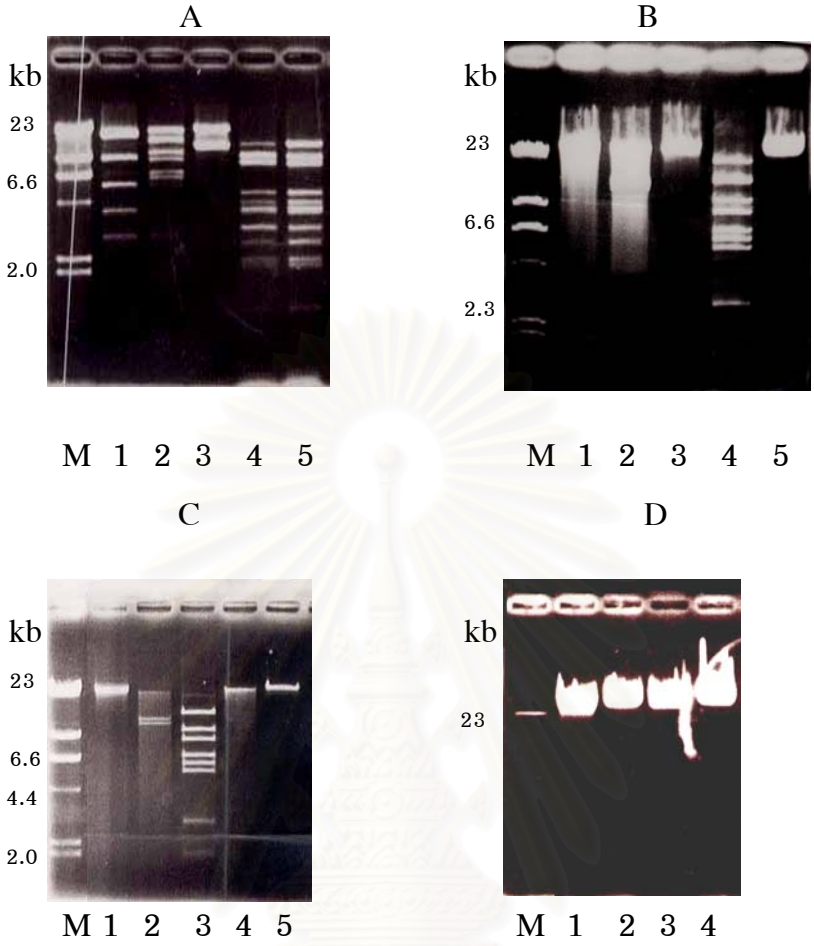


Fig 3.12 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M)and DNA fragment of phage Ray-7 generated with restriction enzymes, as follow:

(A) from lane 1 to lane 5: *Sph*I, *EcoR*I, *Mun* I, *Mun* I plus *Bam*HI, *Bam*HI, respectively,

(B) from lane 1 to lane 5: *Apa*I, *Mun* I, *Xba* I, *EcoR*I, *Xho* I, respectively,

(C) from lane 1 to lane 5: *Bgl*II, *Mlu* I, *Kpn* I, *EcoR*I, *EcoR*V, respectively,

(D) from lane 1 to lane 4: *Ban*II, *Hind* III, *Bgl* II, *Dra*I , respectively.

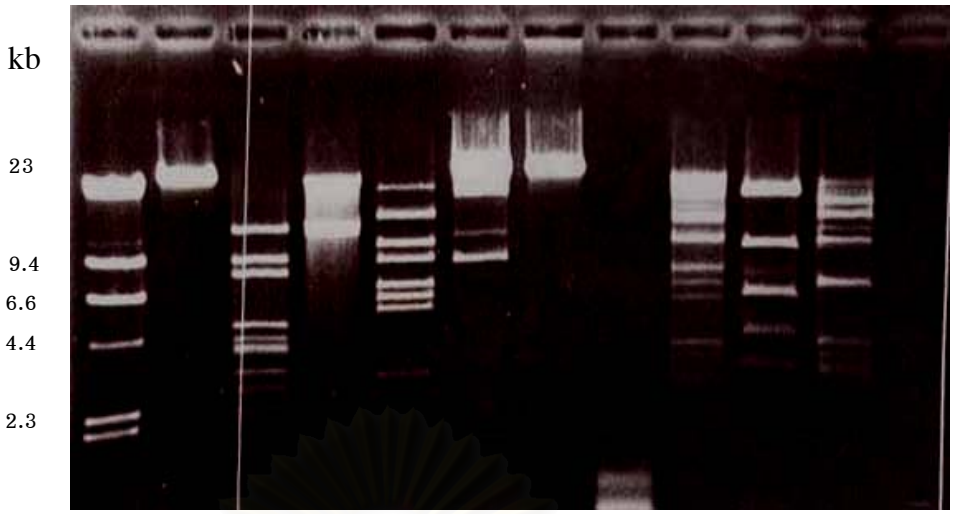
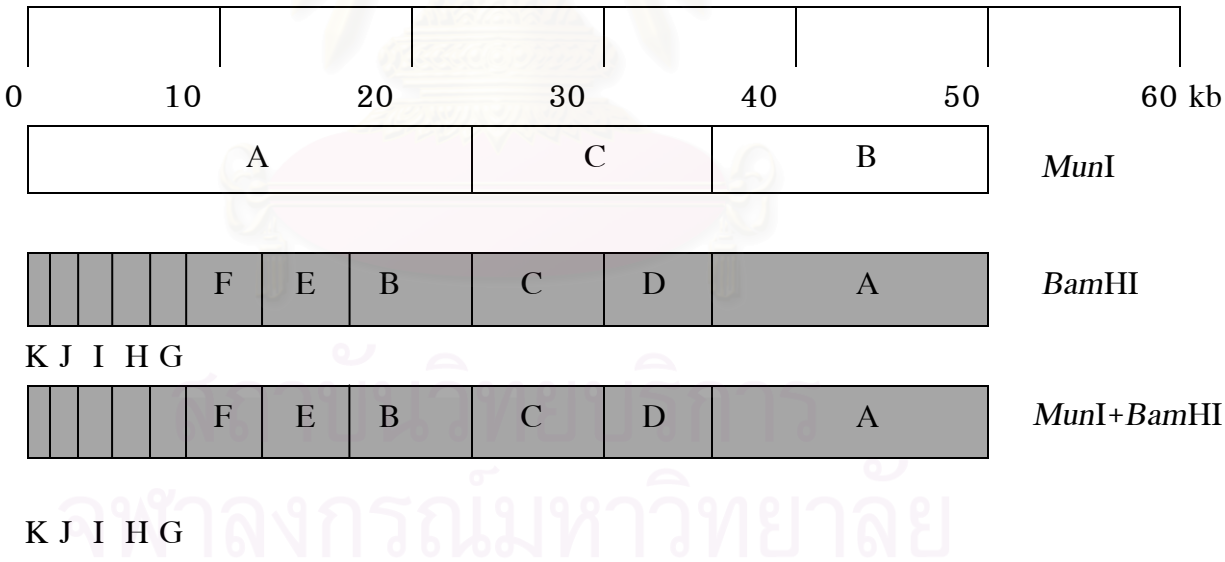


Fig 3.13 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Ray-7 generated with restriction enzymes, respectively, from lane 1 to lane 10: *Nhe*I, *Bam*HI, *Mun*I, *EcoR*I, *Nde* I, *Not* I, *Alu* I, *Kpn*I, *Sph* I, *Mlu* I.

A brief restriction map of phage Ray-7 (derived from Fig. 3.12 A, lane 3-5)



3.10.3 Restriction analysis of phage Surat-11

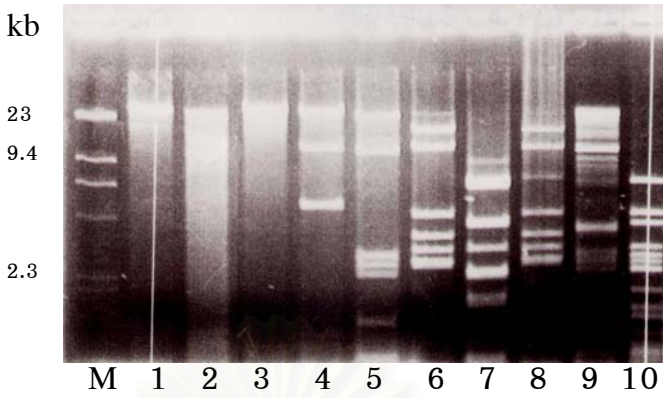


Fig. 3.14 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Surat-11 generated with restriction enzymes, respectively, from lane 1 to lane 10: *Sph*I, *Not*I, *Ban*II, *Nde*I, *Eco*RI, *Bgl*II, *Eco*RV, *Eco*RI plus *Bgl* II, *Eco*RI plus *Eco*RV, *Bgl* II plus *Eco*RV.

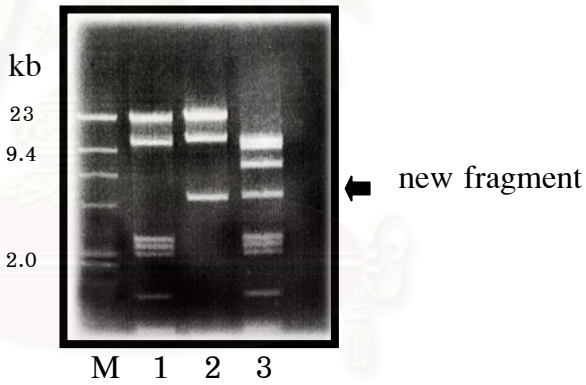


Fig. 3.15 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Surat-11 generated with restriction enzymes, respectively, from lane 1 to lane 3: *Eco*RI, *Nde*I, *Eco*RI plus *Nde*I.

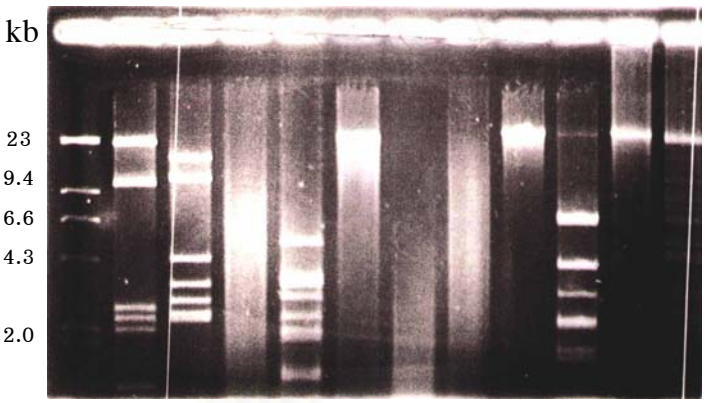
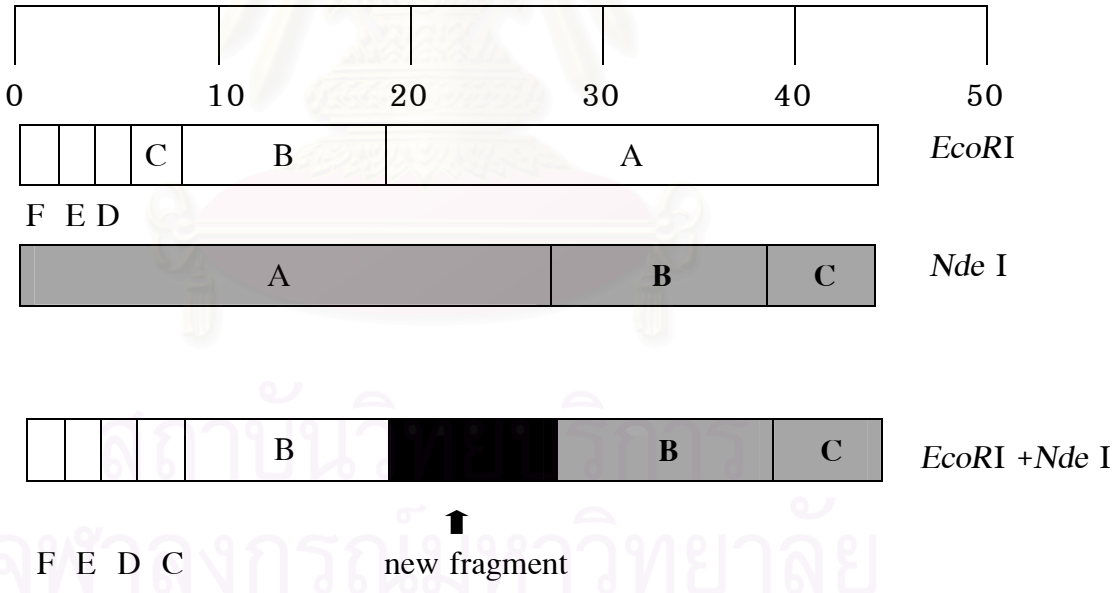


Fig. 3.16 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Surat-11 generated with restriction enzymes, respectively, from lane 1 to lane 11: *Eco*RI, *Bgl*II, *Spe*I, *Mlu*I, *Ban*II, *Apa*I, *Sac*I, *Xho*I, *Eco*RV, *Pst*I, *Nhe*I.

A brief restriction map of phage Surat -11 (derived from Fig. 3.15)



3.10.4 Restriction analysis of phage Korat-12

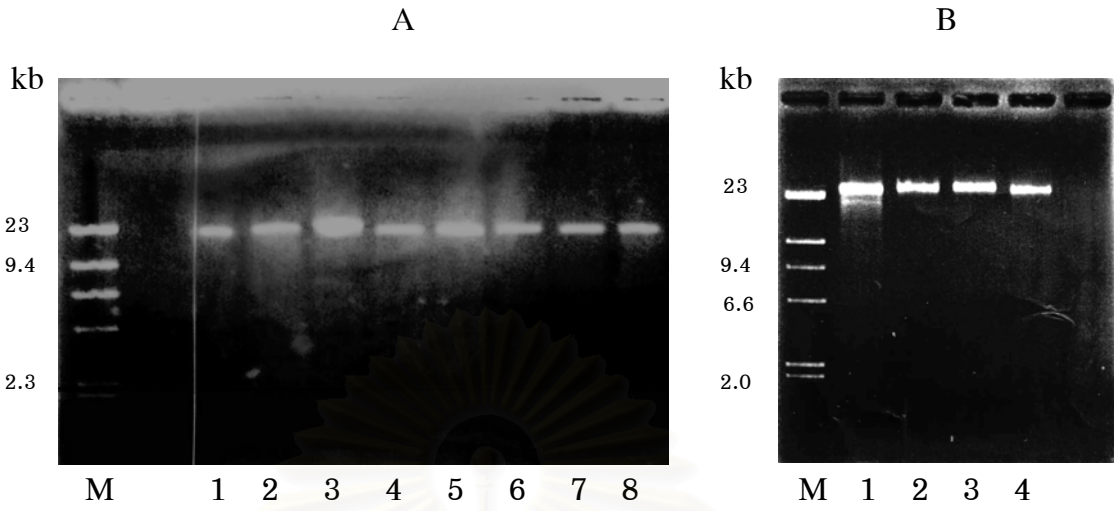


Fig. 3.17 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Korat-12 generated with restriction enzymes, respectively, from lane 1 to lane 8: (A) *Bam*HI , *Ban*II, *Hind*III, *Bgl*II , *Spe*I, *Sph*I , *Sal*I, *Nde*I, (B) from lane 1 to lane 4: *Eco*RI, *Eco*RV, *Mlu*I, *Pst*I.

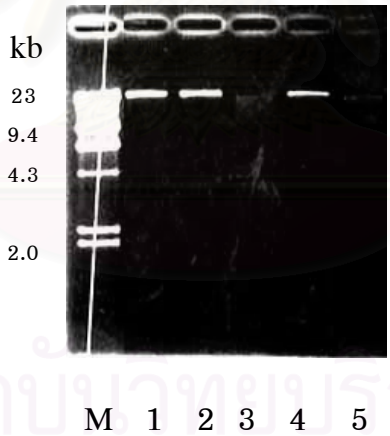


Fig. 3.18 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Korat-12 generated with restriction enzymes, respectively, from lane 1 to lane 5: *Eco*RI, *Mlu*I, *Kpn*I, *Pst*I, *Sac*I.

3.10.5 Restriction analysis of phage Yok-15

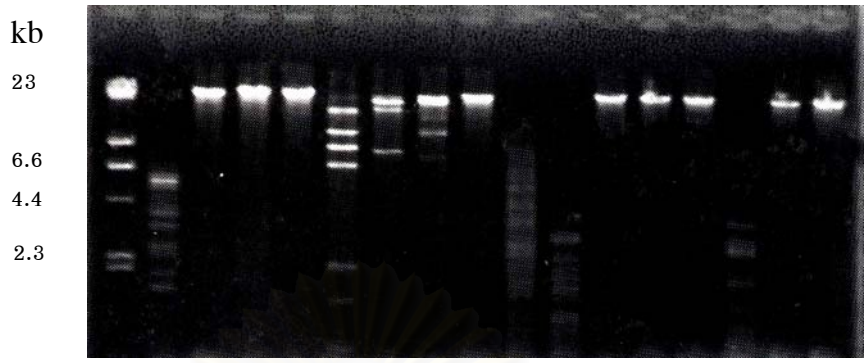


Fig. 3.19 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Yok-15 generated with restriction enzymes, respectively, from lane 1 to lane 16: *Apa*I, *Bam*HI, *Ban*II, *Hind*III, *Bgl*II, *EcoR*I, *Mlu*I, *Spe*I, *Sac*I, *Sal*I, *EcoR*V, *Pst*I, *Afl*II, *Bgl*I, *Mun*I, *Nhe*I.

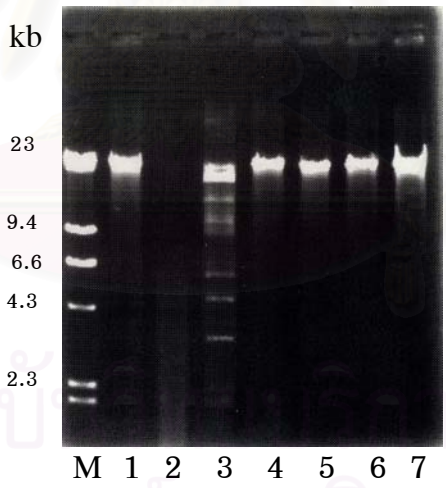


Fig. 3.20 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Yok-15 generated with restriction enzymes, respectively, from lane 1 to lane 7: *Dra*I, *Alu*I, *Sph*I, *Xho*I, *Xba*I, *Not*I, *Nde*I.

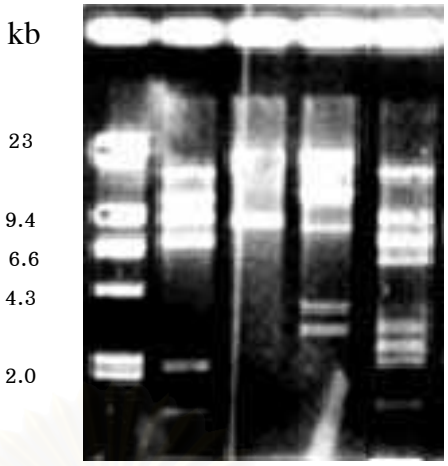


Fig. 3.21 Agarose gel electrophoresis of *Hind*III/lambda DNA marker (M) and DNA fragment of phage Yok-15 generated with restriction enzymes, respectively, from lane 1 to lane 4: *Bgl*II, *EcoR*I, *Mlu*I, *Bgl*II plus *Mlu*I.

A brief restriction map of phage Yok-15 (derived from Fig. 3.21)

(symbol, ▨ = new fragment)

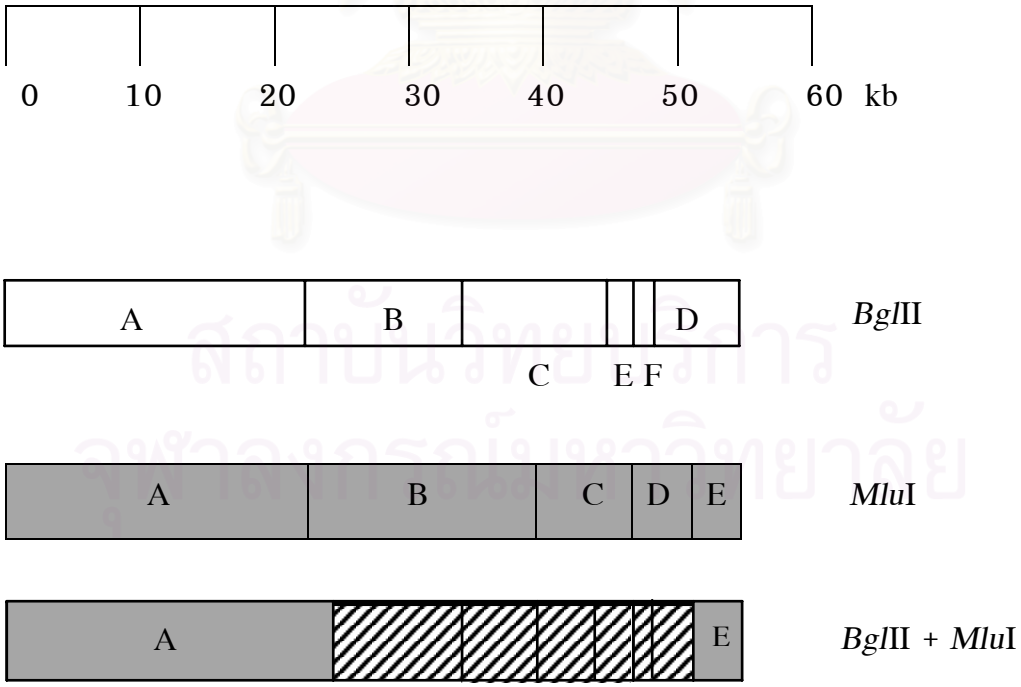


Table 3.12 Restriction analysis of phages

phages / enzymes	Restriction fragments				
	Roi-1	Ray-7	Surat-11	Korat-12	Yok-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	-
MW of genome	40,500	50,000	44,000	ND	55,000

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

3.11 Plaque hybridization

Plaque hybridization was used for examine the relatedness among phage DNA. Nutrient agar plates were overlaid with *S. viridochromogenes*, *Streptomyces* sp. 27. Then, 5 μl of various phages with amount of 10^4 pfu/ml was dropped on plates with space apart for an appropriated plaque appearance. The plates were kept still or not allowed to move until the agar was set up and the dropped phages were totally adsorbed. The plates were incubated at 30^0C for overnight. In following day, hybridization was prepared as of section 2.26. Results obtained were shown in Fig. 3.22-3.24.

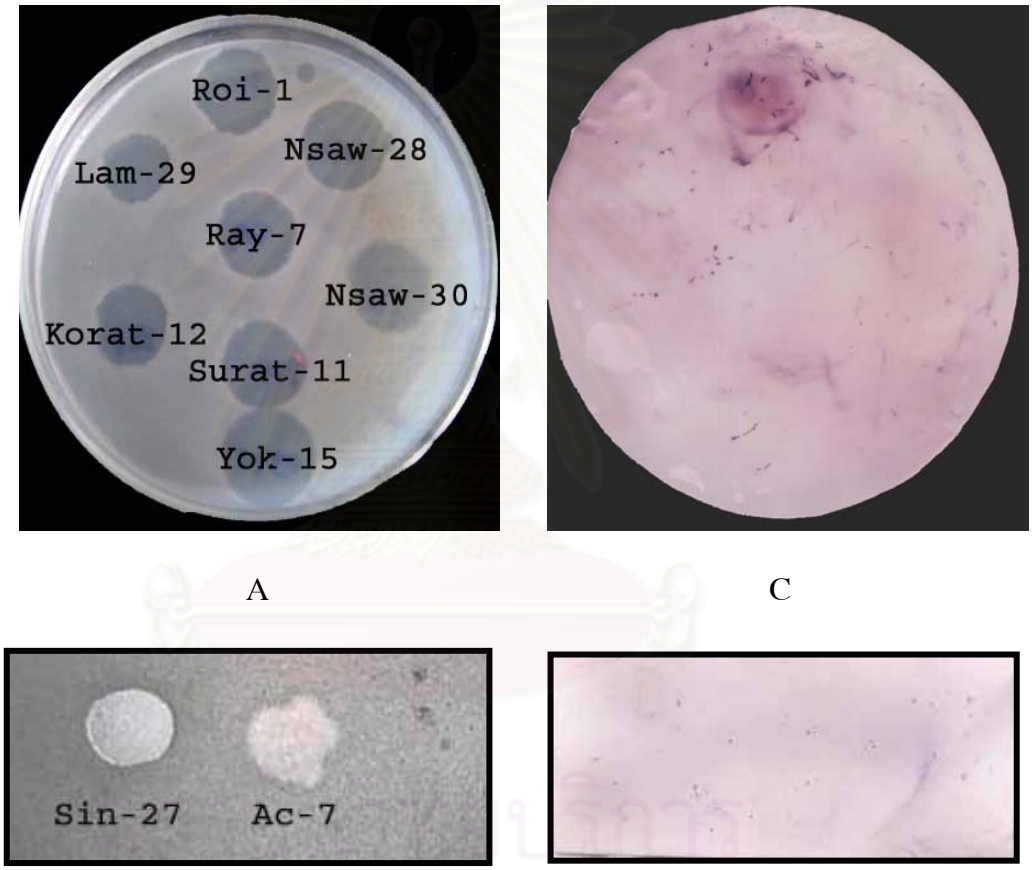


Fig. 3.22 Plaque hybridization using phage Roi-1 DNA as a probe. (A, B) plaque spots on double layer agar, (C, D) detection of plaque hybridization by chemiluminescence

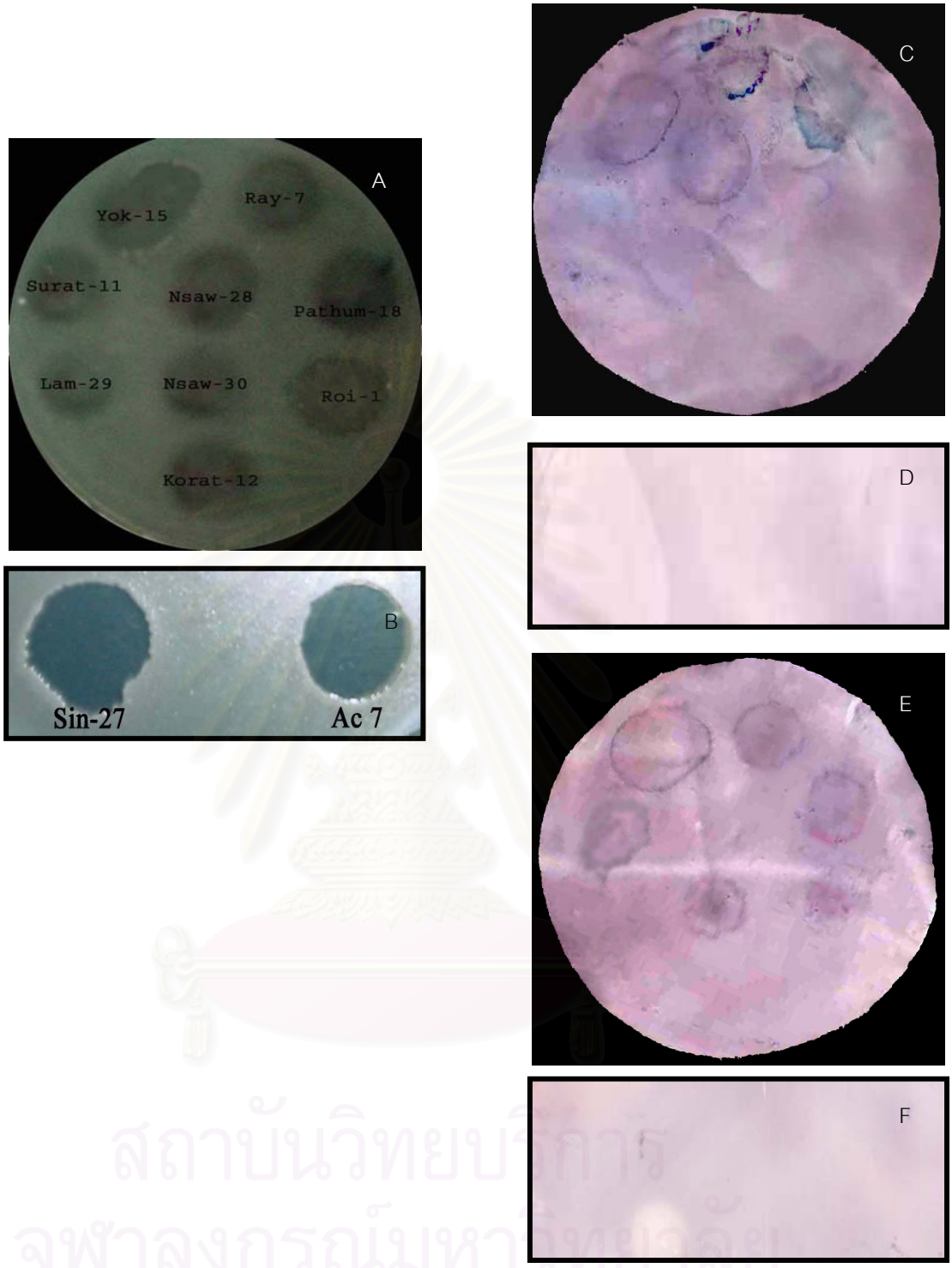


Fig. 3.23 Plaque hybridization. (A, B) plaque spots on double layer agar, (C, D) detection of plaque hybridization by chemiluminescence by using phage Ray-7 DNA and (E, F) using phage Surat -11 DNA as a probe

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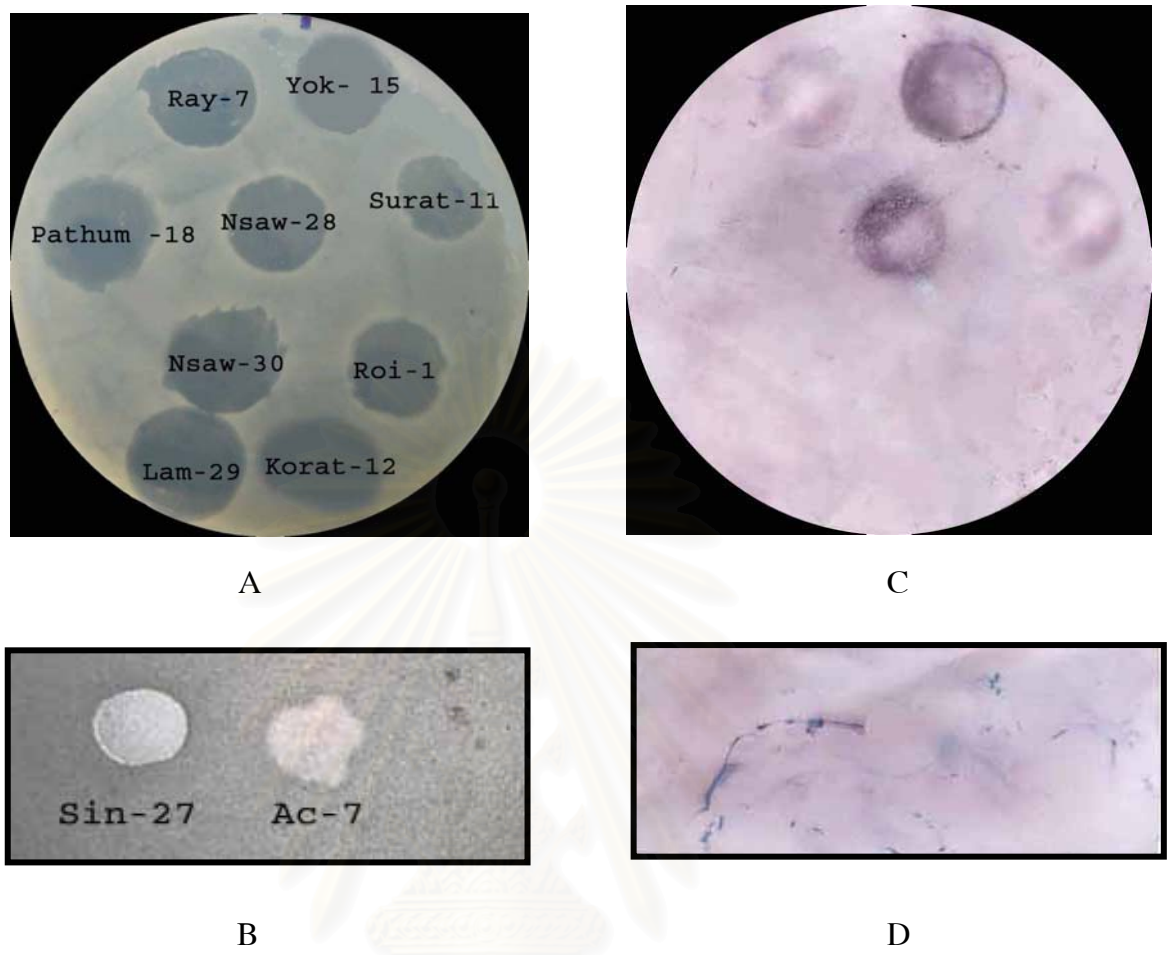

A C

B D

Fig. 3.24 Plaque hybridization using phage Yok-15 DNA as a probe. (A, B) plaque spots on double layer agar, (C, D) detection of plaque hybridization via chemiluminescence

Table 3.13 Plaque hybridization of phages

Probe	Homology										
	Roi -1	Ray- 7	Surat- 11	Korat- 12	Yok- 15	Pathum -18	Sin- 27	Nsaw- 28	Lam- 29	Nsaw -30	Ac- 7
Roi-1	+	–	–	–	–		–	–	–	–	–
Ray-7	–	+	–	–	+	–	–	+	–	+	–
Surat-11	–	+	+	–	+	+	–	–	–	+	–
Yok-15	–	+	+	–	+	–	–	+	–	–	–

Symbols: +, positive control; +, homology; –, non-homology

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3.13 Identification of *Streptomyces* sp. 15 by using partial 16S rDNA

Partial nucleotide sequences of the 16S rRNA gene of *Streptomyces* sp. 15 containing a variable α region used for *Streptomyces* species identification were amplified by PCR. A stretch approximately 500 bp in length was then sequenced and aligned. The sequences of the amplified products were analyzed by DNAsis program. The result was shown in Fig. 3.27.

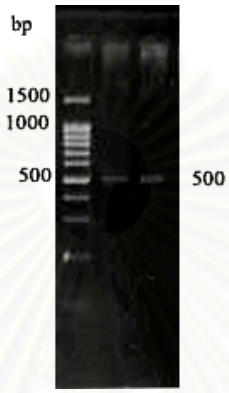


Fig. 3.26 Agarose gel electrophoresis of PCR products. Lane1: 100 bp DNA ladder, Lane 2: *Streptomyces coelicolor*, Lane 3: *Streptomyces* sp. 15

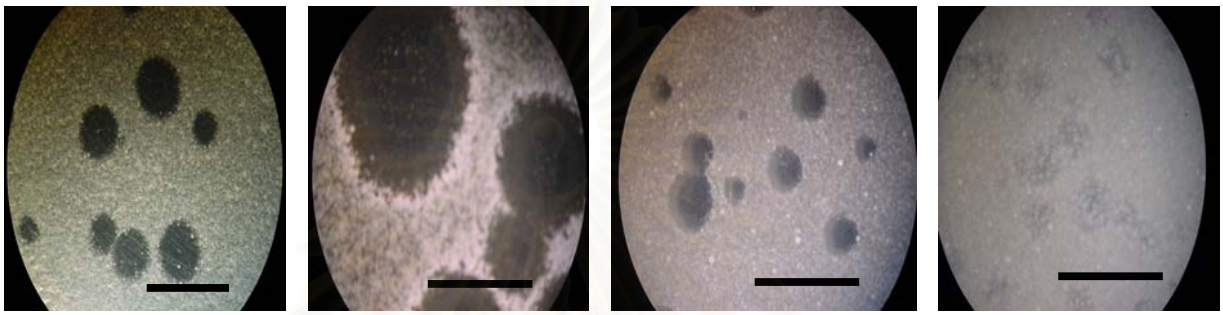
```
           10          20          30          40          50
5' GGTATAGAAG CGTTTTTGAC GTACAGTCAT TTCGCTTCTT CCTGCTGAAA
           60          70          80          90         100
   GAGGTTTACA ACCCGAAGGC CGTCATCCCT CACGCGGCGT CGCTGCATCA
          110         120         130         140         150
   GGCTTTCGCC CATTGTGCAA TATTCCCCAC TGCTGCCTCC CGTAGGAGTC
          160         170         180         190         200
   TGGGCCGTGT CTCAGTCCCA GTGTGGCCGG TCGCCCTCTC AGGCCGGCTA
          210         220         230         240         250
   CCCGTCGTCT GCCTTGGTGA GCCATTACCT CACCAACAAG CTGATAGGCC
          260         270         280         290         300
   GCGGGCTCAT CCTTCACCGC CGGAGCTTTT AACCTCCACC CACGAGAACA
          310         320         330         340         350
   GAAGTGTTAT CCGGTTTAG ACCCCGTTTC CAGGGCTTGT CCCAGAGTAA
          360         370         380         390         400
   AGGGCAGATT GCCCACGTGT TACTCACCCG TTCGCCACTA ATCCCCACCA
          410         420         430         440         450
   AAGTGGTTC ATCGTTCGAC TTGCATGTAT AAGCACGCCC CAGCGTTCGTC   3'
```

Fig. 3.27 Sequence of 16S rDNA of *Streptomyces* sp. 15.

Taxonomic characterization of strain 15 by physiological tests and 16S ribosomal DNA (rDNA) analysis (accession number AY128706) showed high similarity with that of *Streptomyces* sp. NRRL 5182 (accession number AJ391815).

3.14 Experimentations in soil

The fate of an actinophage Yok-15 having a wide host range and of its 4 streptomycete host strains in three soil samples as simple batch microcosms were monitored. Plaque morphological characteristics of phage in different hosts on double layer agar were shown in Fig. 3.28.



A B C D

Fig. 3.28 Plaque morphology of phage Yok-15 in individual hosts. (A) *Streptomyces* sp. 15, (B) *S. viridochromogenes*, (C) *S. coelicolor*, (D) *S. griseus* (bar, 5 mm)

3.14.1 Characteristics of phage Yok-15

From 30 strains of phages isolated from Thai soils, phage Yok-15 to which exhibited a polyvalent lytic phage, had the widest host range, and was able to infect important strains of streptomycetes. Among 45 strains of streptomycetes tested, Yok-15 could infect 39 strains, namely *S. ambofaciens*, *S. aminophilus*, *S. aureus*, *S. azureus*, *S. badius*, *S. bikiniensis*, *S. coelicolor*, *S. coeruleofuscus*, *S. coralus*, *S. cyaneus*, *S. echinatus*, *S. endus*, *S. griseus*, *S. hygroscopicus*, *S. kanamyceticus*, *S. lavendulae*, *S. lincolnensis*, *S. lividans*, *S. luteogriseus*, *S. melanosporofaciens*, *S. nivous*, *S. nodosus*, *S. pervullus*, *S. pilosus*, *S. puniceus*, *S. violaceochromogenes*, *S. viridochromogenes* and *Streptomyces* sp. 15. strain 15 was isolated from Thai soil upon which phage Yok-15 was isolated from. Indeed, phage Yok-15 showed a high multiplication toward these strains in both soft-agar overlaid plate and liquid cultures.

3.14.2 Efficiency of plating (EOP)

Relative EOP was calculated from equation in the section 2.31.1. Results of which were shown in Table 3.15.

Table 3.15 Relative efficiency of plating of phage Yok-15 on *Streptomyces* sp. 15 *S. viridochromogenes*, S. *coelicolor* and *S. griseus*

Host	P(0)*	n**	Efficiency of plating***	Efficiency of plating****
Streptomyces sp. 15	0.86	0.151	1.00	1.00
S. viridochromogenes	0.88	0.128	0.84	0.80
S. *coelicolor*	0.92	0.083	0.55	0.43
S. griseus	0.90	0.105	0.70	0.63

Symbols: * P(0) = the fraction of samples containing no phage

** n = the average number of phage particles per sample

*** Efficiency of plating, defined as number of plaques on indicator strain (n)/No. of plaques on previous host (*Streptomyces* sp. 15), was calculated from the Poisson formula as described in section 2.31.3

**** Efficiency of plating is defined as number of plaques on indicator strain by double agar method/ number of plaques on previous host (*Streptomyces* sp. 15)

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Table 3.16 The experimental designs

Microcosm	Experiments	Soil types	Data presented
Streptomyces sp. 15 with/ without phage Yok-15 (A, B, C1, D1)	A: host 10^5 cfu/g and phage 10^3 pfu/g B: host 10^5 cfu/g and phage 10^4 pfu/g	Thai soil (I) (Nakhon-nayok Province)	Fig. 3.29
S. coelicolor, *S. griseus*, *S. viridochromogenes* with/ without phage Yok-15 (C2, D2)	C: host 10^6 cfu/g and phage 10^4 pfu /g D: host 10^6 cfu /g and phage 10^5 pfu /g for 8 day's incubation		
Streptomyces sp. 15 with/ without phage Yok-15 (E, F)	E: host 10^6 cfu /g and phage 10^4 pfu /g F: host 10^6 cfu /g and phage 10^5 pfu /g for 8 day's incubation	Thai soil (II) (Bangkok)	Fig. 3.30
Streptomyces sp. 15 with/ without phage Yok-15 (G1, H1, I1, J1)	G: host 10^5 cfu/g and phage 10^3 pfu/g H: host 10^5 cfu/g and phage 10^4 pfu/g	Japanese soil (Fukuoka Prefecture)	Fig. 3.31
S. coelicolor, *S. viridochromogenes* with/ without phage Yok-15 (G2, H2, I2, J2)	I: host 10^6 cfu/g and phage 10^4 pfu/g J: host 10^6 cfu/g and phage 10^5 pfu/g for 4 day's incubation		

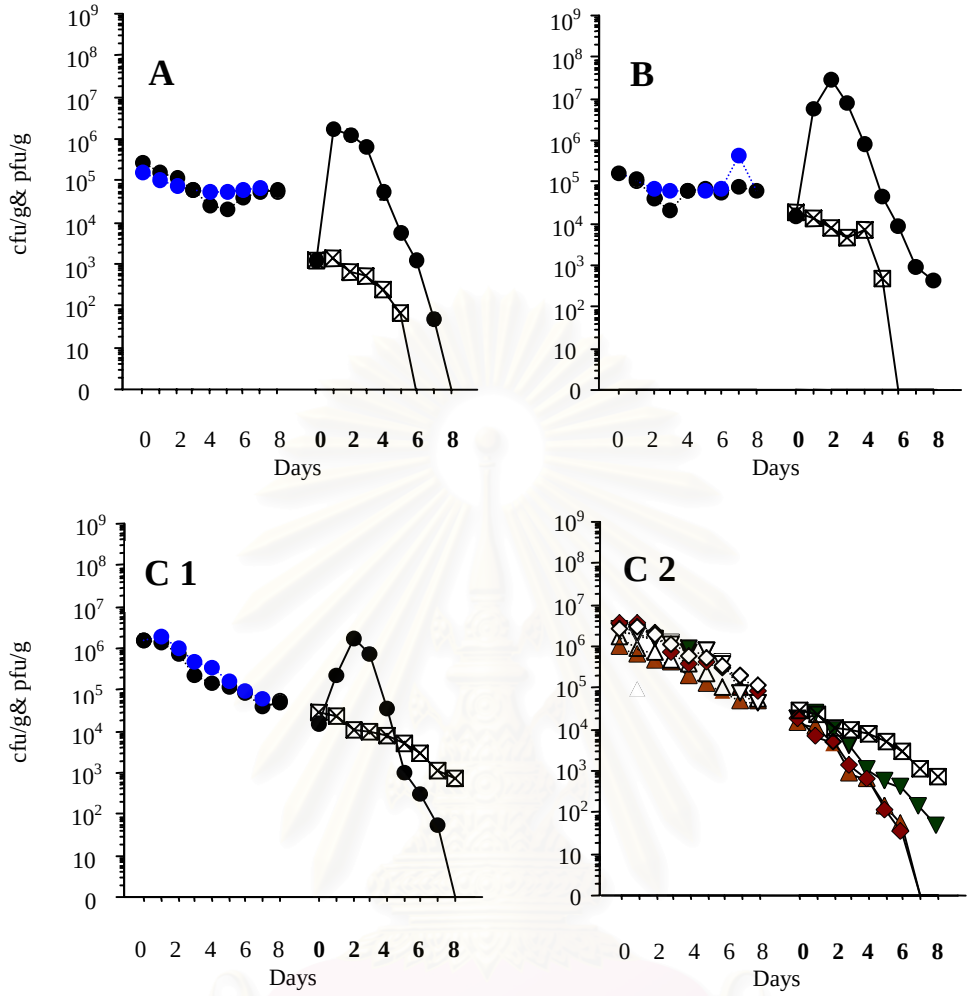

Fig. 3.29 Change in plaque forming units (pfu) of phage Yok-15 (solid line) and colony forming units (cfu) of streptomycetes (dotted line) in Thai soil (I). A, B, C1, C2, D1 and D2, See Table 3.16; ☒, Free phage Yok-15 ; ●,Strain 15 without phage Yok-15 ; ●, strain 15 with phage Yok-15; ∇, *S. coelicolor* without phage Yok-15; ▼, *S. coelicolor* with phage Yok-15; ◆, *S. griseus* with phage Yok-15; ◆, *S. griseus* with phage Yok-15; Δ, *S. viridochromogenes* without phage Yok-15; ▲, *S. viridochromogenes* with phage Yok-15

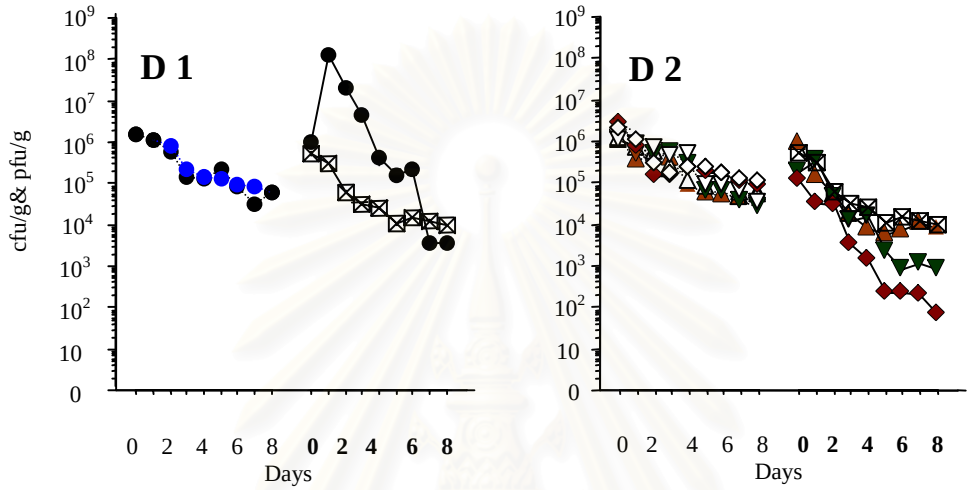

Fig. 3.29 (continue) Change in plaque forming units (pfu) of phage Yok-15 (solid line) and colony forming units (cfu) of streptomycetes (dotted line) in Thai soil (I). ☒, Free phage Yok-15 ; ●,Strain 15 without phage Yok-15 ; ●, strain 15 with phage Yok-15; ∇, *S. coelicolor* without phage Yok-15; ▼, *S. coelicolor* with phage Yok-15; ◆, *S. griseus* with phage Yok-15; ◆, *S. griseus* with phage Yok-15; Δ, *S. viridochromogenes* without phage Yok-15; ▲, *S. viridochromogenes* with phage Yok-15

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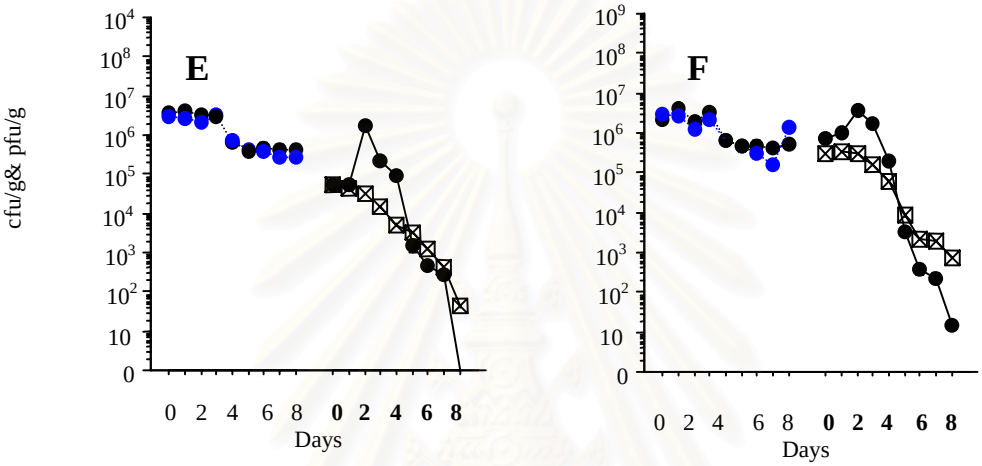

Fig. 3.30 Change in plaque forming units (pfu) of phage Yok-15 (solid line) and colony forming units (cfu) of streptomycetes (dotted line) in Thai soil (II). E and F, See Table 3.16; ☒, Free phage Yok-15 ; ●,Strain 15 without phage Yok-15; ●, strain 15 with phage Yok-15

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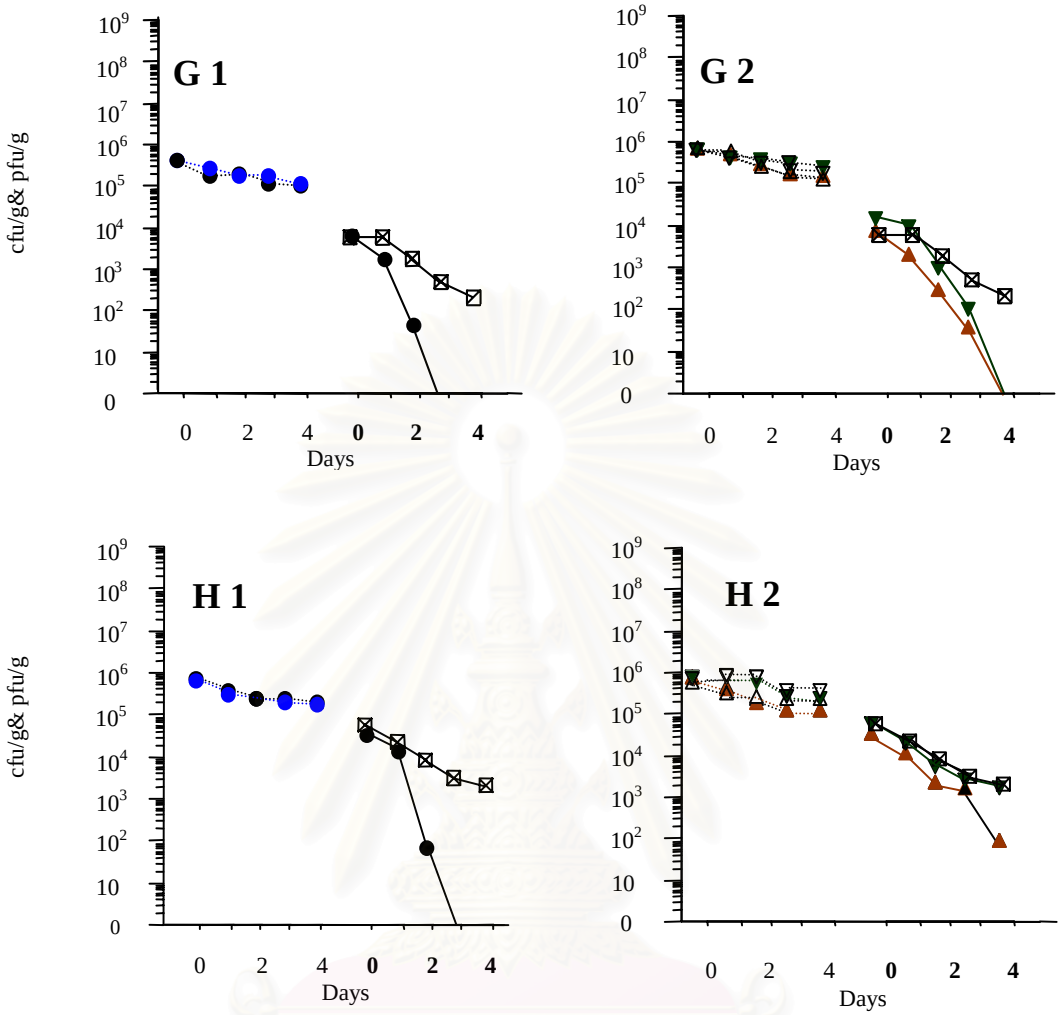
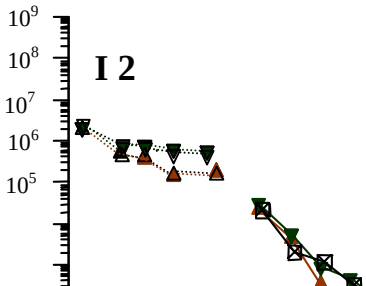


Fig. 3.31 Change in plaque forming units (pfu) of phage Yok-15 (solid line) and colony forming units (cfu) of streptomycetes (dotted line) in Japanese soil (I). G1, G2, H1, H2, I1, I2, J1 and J2, See Table 3.16; ☒, Free phage Yok-15 ; ●, Strain 15 without phage Yok-15 ; ●, strain 15 with phage Yok-15; ∇, *S. coelicolor* without phage Yok-15; ▼, *S. coelicolor* with phage Yok-15; Δ, *S. viridochromogenes* without phage Yok-15; ▲, *S. viridochromogenes* with phage Yok-15



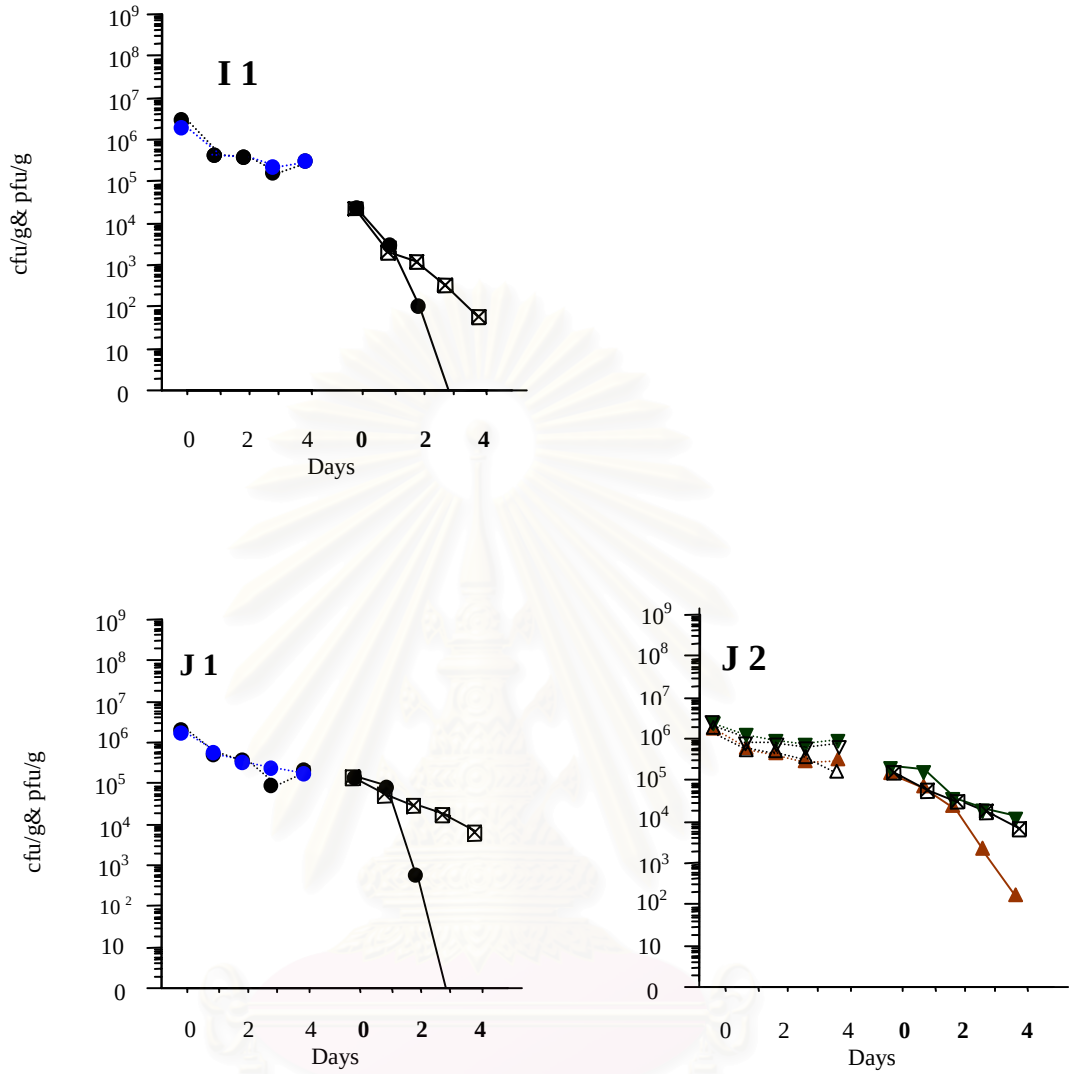


Fig. 3.31 (continue) Change in plaque forming units (pfu) of phage Yok-15 (solid line) and colony forming units (cfu) of streptomycetes (dotted line) in Japanese soil (I). I1, I2, J1 and J2, See Table 3.16; ☒, Free phage Yok-15 ; ●, Strain 15 without phage Yok-15 ; ●, strain 15 with phage Yok-15; ∇, *S. coelicolor* without phage Yok-15; ▼, *S. coelicolor* with phage Yok-15; Δ, *S. viridochromogenes* without phage Yok-15; ▲, *S. viridochromogenes* with phage Yok-15.

Table 3.17 Occurrence of phage resistant mutants

Strains	Day 0		Day 8	
	Number of phage resistants	Number of initial spores	Number of phage resistants	Number of survivals
Streptomyces sp. 15	2,424	8.6×10^6	10	5.5×10^4
S. coelicolor	1,952	6.8×10^6	143	3.0×10^4
S. griseus	1,520	2.0×10^6	3	7.0×10^4
S. viridochromogenes	2,542	9.1×10^6	6	4.0×10^4

Fate of phage in soils: Phage Yok-15 multiplied with a maximum number during incubation with strain 15 in all systems of both Thai soil samples, then decreased rapidly in its numbers (Fig. 3.29 and 3.30). Number of phages increased significantly between day 1 and day 2. The rate of decrease of phages incubated with strain 15 was faster than that of free phages. Furthermore, the phages incubated with *S. coelicolor*, *S. griseus* and *S. viridochromogenes* decreased more rapidly than free phages which showed no multiplication. These results indicated that strain 15 was suitable for phage multiplication in both Thai soil samples used.

On the other hand, in Japanese soil, phage Yok-15 could not multiply on strain 15 and other two strains used. The phage Yok-15 together with strain sp. 15 decreased significantly in their titer at early period ($p < 0.01$) and completely lost within 3 days. The decreasing rate of phages together with the streptomycetes hosts was also faster than that of free phages without them (Fig. 3.31): this pattern was similar to the pattern in

Thai soils. The existence of host streptomycetes seemed to be correlated with the decrease of phage titer.

Fate of streptomycetes hosts in soils: Number of strain 15 decreased gradually at early period of incubation, then abruptly decreased at the middle period then slow down and cease (or to recover) in late period. This trend seemed to be a general characteristic of strain 15 incubated with phages rather than those without phages. The decreasing rate of streptomycetes at the initial number of 10^6 cfu/g was faster than that at 10^5 cfu/g. *S. coelicolor*, *S. griseus* and *S. viridochromogenes* also decreased in the numbers as seen in strain 15. This difference of the decreasing rate may due to nutrient-poor condition of soil, however, we could not find any clear difference of their characteristics among these 4 strains in the present study. Furthermore, as shown in Table 3.17, the rate of occurrence of phage resistant mutants in these strains did not increase during 8 days of incubation in soil.

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3.15 Sensitivity to chloroform

Method for sterilize phage with 1 % chloroform was an alternative of filter sterilization. Phage Yok-15 was tested and comparison to other 2 phages. Results were shown in Table 3.18.

Table 3.18 Chloroform sensitivity of phages

Phages	Before treated (pfu/ml)	After treated (pfu/ml)
Yok-15	23×10^8	138
Non-2	28×10^7	172×10^5
Ray-7	57×10^7	63×10^5

3.16 Effect of temperature on the titer of phage Yok-15

In this study, known titer of phage Yok-15 was tested for their stability at 4 ^{0}C for 5 and 7 h in comparison as those at room temperature (RT). The recovery titer of phage was shown in Table 3.19.

Table 3.19 Effect of temperature on the titer of phage Yok-15

pH	Recovery of phage Yok-15 yields (pfu/ml) *			
	5 h		7 h	
	4 ^{0} C	RT	4 ^{0} C	RT
4	7.27	0.91	0	0
5	32.72	11.82	32.72	10
6	47.27	16.36	36.36	12.72
7	54.54	40.91	52.72	38.18
8	68.18	45.45	63.63	40.91
9	100	69.09	86.36	68.18

Symbol: * Percent of plaque-forming units recovered at 5 h, 4 ^{0}C, pH 9.

3.17 Sensitivity to EDTA and pyrophosphate

Phage Yok-15 was incubated along with EDTA or sodium pyrophosphate at different concentrations. Samples were taken at 5 and 10 min and determined of the phage titer.

3.17.1 Sodium pyrophosphate

Various concentrations of sodium pyrophosphate were tested with phage Yok-15 at 30 ^{0}C. The results of inactivation times were shown in Table 3.20 and Fig 3.32.

Table 3.20 Effect of sodium pyrophosphate on phage Yok-15 inactivation

Sodium pyrophosphate concentration (mM)	Phage titer (pfu/ml)	
	Time (min)	
	5	10
0	200	200
2	10	25
5	6	4
10	0	0
15	0	0

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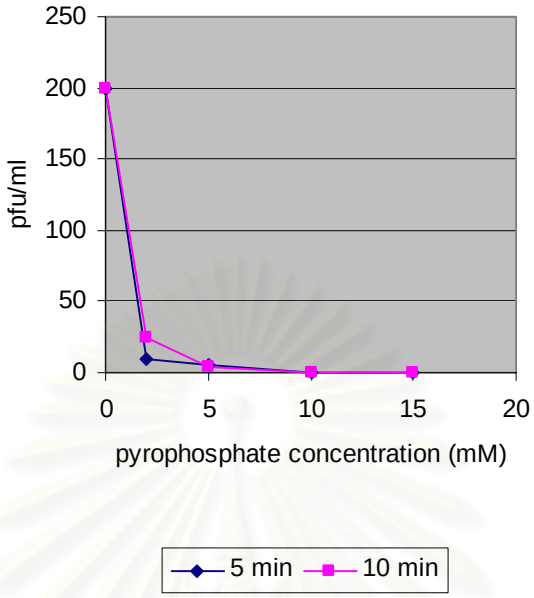

Fig. 3.32 Effect of sodium pyrophosphate on phage Yok-15 inactivation

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3.17.2 0.5 M EDTA (Ethylenediaminetetra-acetic acid)

The effect of EDTA on yields of phage Yok-15 was shown in Table 3.21 and Fig. 3.33.

Table 3.21 Effect of 0.5 M EDTA on phage Yok-15 yields

Time (min)	Phage titer (pfu/ml)
0	28×10^5
30	87×10^4
60	6×10^3
90	240
120	230
150	210
180	160

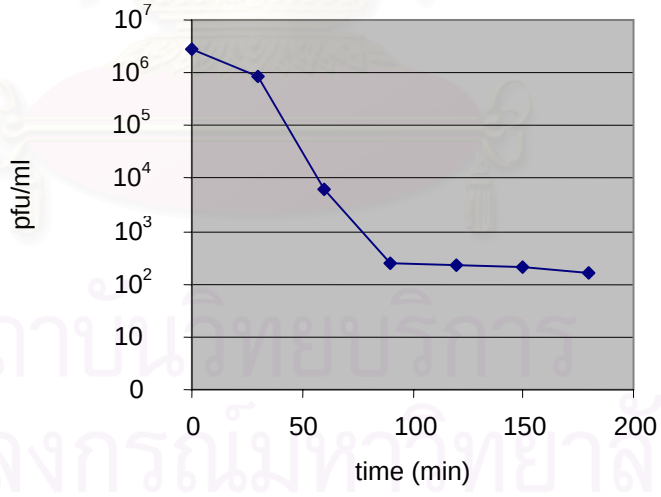


Fig. 3.33 Effect of 0.5 M EDTA on phage Yok-15

3.18 Multiplication of phage Yok-15 in liquid culture

Since the maximum titer of phage Yok-15 was found at pH 9 which was rather different from the other phages, therefore, the study on pH of this phage in liquid culture was considerably interesting. pH was extended over the range of 5 to 11. Normally, *Streptomyces* sp. 15 which used as host for phage Yok-15 multiplication, was capable in growing at pH more than 6.0. Therefore, at lower pH, *S. viridochromogenes* was used as substitution to *Streptomyces* sp. 15. The results were shown in Table 3.22, 3.23 and Fig. 3.34.

Table 3.22 Multiplication of phage Yok-15 in liquid culture at different pH from 4 to 6 using *S. viridochromogenes* as host

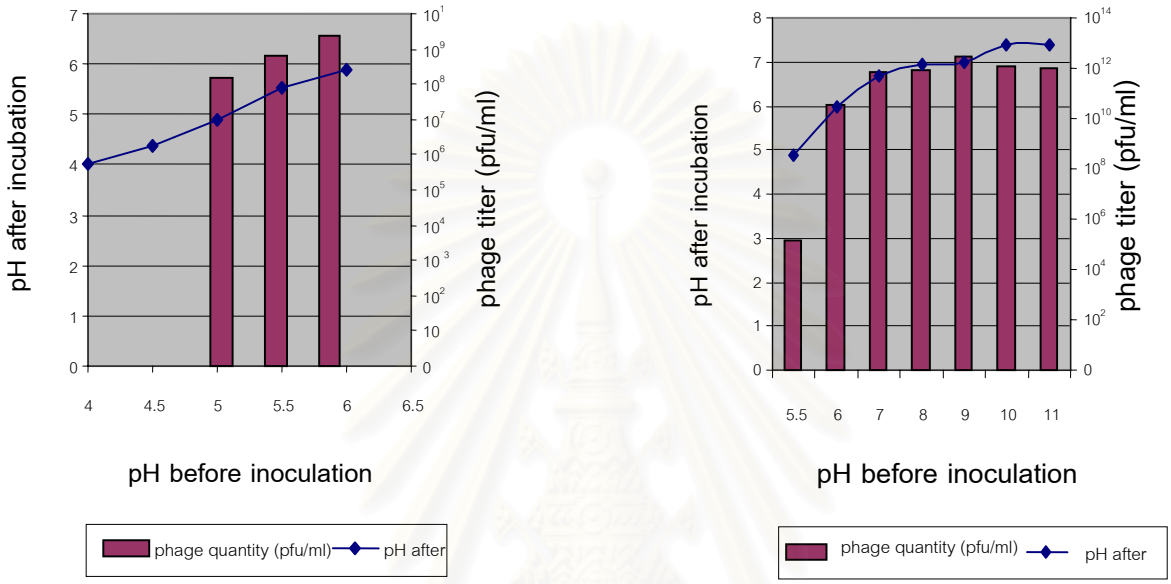
pH before	pH after	Phage titer (pfu/ml)
4	4.02	0
4.5	4.39	0
5	4.90	1.48×10^{6}
5.5	5.53	6.30×10^{7}
6	5.89	2.30×10^{9}

Note: Initial phage titer: 1×10^{6} pfu/ml, initial spore 1×10^{8} spores/ml

Table 3.23 Multiplication of phage Yok-15 in liquid culture at different pH from 5.5 to 11 using *Streptomyces* sp. 15 as host

pH before	pH after	Phage titer(pfu/ml)
5.5	4.86	1.40×10^{5}
6	6.00	3.30×10^{10}
7	6.66	7.00×10^{11}
8	6.96	8.00×10^{11}
9	7.01	2.99×10^{12}
10	7.40	1.14×10^{12}
11	7.39	1.08×10^{12}

Note: initial phage titer: 1.37×10^{7} pfu/ml, initial spore: 1×10^{8} spores/ml



(A) (B)

Fig. 3.34 Multiplication of phage Yok-15 in liquid culture at different pH. (A) host: *S. viridochromogenes*, at pH from 4 to 6, (B) host: *Streptomyces* sp. 15 at pH from 5.5 to 11

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3.19 Optimal conditions for obtaining high titer of phage Yok-15 in liquid culture

3.19.1. Agitation speed

Rotation speed to cultivate phage Yok-15 in host cell was operated at 150, 200 and 250 rpm. The results were shown in Table 3.24 and Fig. 3.35.

Table 3.24 Effect of rotation speed on phage Yok-15 yields

Rotation speed (rpm)	Phage titer (pfu/ml)
150	2.68×10^{10}
200	1.00×10^{12}
250	3.20×10^{11}

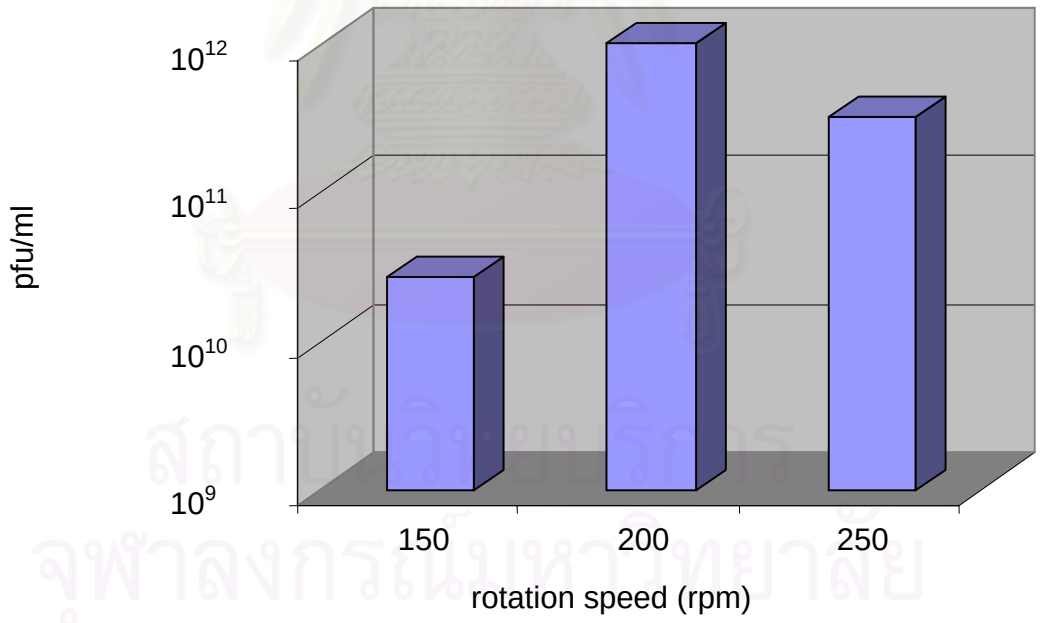

Fig. 3.35 Effect of rotation speed on phage Yok-15 yields

3.19.2. Initial host and phage concentrations

Various dilution of host and phage concentrations were independently tested in order to obtain high titers of phage. The results were shown in Table 3.25; Fig. 3.36 for varying initial host and Table 3.26; Fig. 3.37 for varying initial phage, respectively.

Table 3.25 Effect of initial number of spore on titer of phage Yok-15

Amount of spore(spores/ml)	phage titer (pfu/ml)
1.41×10^4	2.02×10^5
1.41×10^5	7.70×10^6
1.41×10^6	2.44×10^7
1.41×10^7	1.09×10^{12}
1.41×10^8	3.01×10^{12}

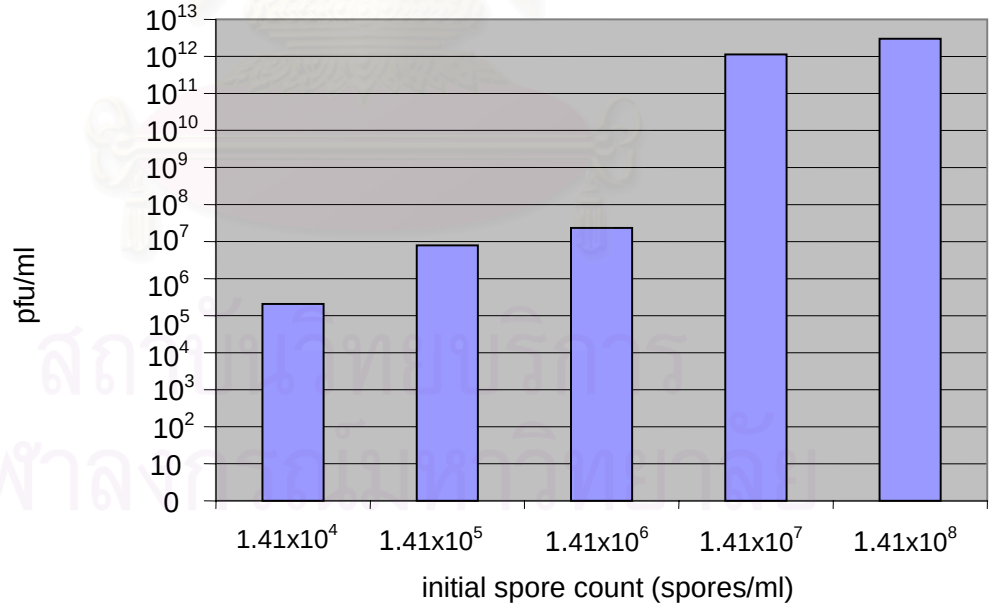

Fig. 3.36 Effect of initial number of spore on titer of phage Yok-15

Table 3.26 Effect of initial phage concentration on titer of phage Yok-15

Initial host (spore/ml)	initial phage titer (pfu/ml)	1 day after inoculation (pfu/ml)
10^8	10	1,100
	10^3	1.90×10^9
	10^5	1.00×10^9
	10^6	1.00×10^{10}

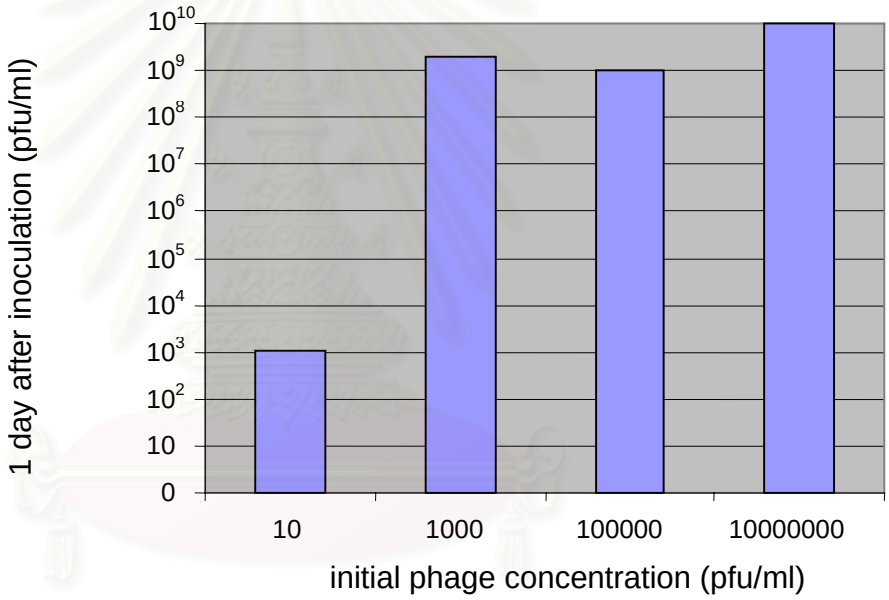

Fig. 3.37 Effect of initial phage concentration on titer of phage Yok-15

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CHAPTER IV
DISCUSSION

The objective of this study was to isolate *Streptomyces* spp. and actinophages from soils in Thailand with respect to their original habitats. Characterization, determination the relevance of phage and streptomycetes, construction phage DNA's restriction map, and investigation on the interaction of phage and host in soils were also examined.

Soil, fodder, and composts appear to be the primary reservoirs for streptomycetes (Williams *et al.*, 1989). In this report, *Streptomyces* spp. were isolated from soil using the humic acid vitamin agar medium (HV agar medium). This medium contained humic acid as the sole source of carbon and nitrogen and was found to be the most effective medium compared to other media used for the isolation of soil actinomycetes (Hayakawa and Nonomura, 1984; 1987). Practically, the soil samples were air dried at 45^0C for 2-16 h in order to reduce the proportion of some other bacteria in soil samples (Kutzner, 1981). The suitable dilution of soils was spread onto HV agar and then incubated for 7 days. As determined visually or under microscopic examination, the colonies of streptomycetes were obviously identified by their typical characteristics: the production of aerial mycelium, pigmentation as long as their incubation periods by which they produced spore chains on aerial mycelium after two weeks of incubation. On the other hand, colonies of *Micromonospora, Microbispora* and *Streptosporangium* required up to 3 to 4 weeks for sporulation. In this study, streptomycetes were isolated from 28 soil samples. After 7 days of incubation, their typical colonies were picked up and restreaked several times on mannitol mungbean agar. After 2 weeks of incubations, colonies of *Streptomyces* obtained were kept in form of spore in 10 % (v/v) glycerol at -20 ^{0}C to preserve their viability. All *Streptomyces* strains were used for phage selection and the uninfected strains were employed to determine their host range. It was observed

that some strains were able to produce antibiotic and antifungal agent. Results of which were shown in Table 4.1.

Table 4.1 Summary of inhibitory agents produced by isolated *Streptomyces* sp. against bacteria and fungi

Microorganisms	Yeast	Mold	Gram negative bacteria	Gram positive bacteria		
				B. subtilis	*M. luteus*	*S. aureus*
Streptomyces strain No.	9	9, 12, 15, 17, 18, and 20	1, 9, 15, and 20	1, 5, 9, 12, 15, and 20	3, 7, 9, 10, 15, 16, 17, 20, and 27	1, 5, 20, and 26

To isolate phage from soils, an enrichment procedure was carried out. This procedure was firstly devised by Gratia (1936) and described in details by Adams (1959). This method has proved to be both simple and effective, resulting in the isolation of phage specific to a variety of soil bacteria (Table 4.2). A positive result could be interpreted as host is present in the sample (Williams *et al*., 1994). *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. However, the initial number of phages was different. It is possible that the less amounts of phage isolated from some soil sources resulted from phage in soil samples that were not specific to *Streptomyces* isolates.

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Table 4.2 Examples of soil bacteria used for phage isolation by specific enrichment.

Propagation species	References
Arthrobacter globiformis	Casida and Liu (1974)
Bacillus spp.	Tan and Reanney (1976)
Nocardia spp.	Williams *et al.* (1980)
Pseudomonas aeruginosa	Bradley (1966)
Streptomyces spp.	Wellington and Williams (1981)
Streptoverticillium spp.	Wellington and Williams (1981)

Thirty actinophages were characterized and distinguished via the properties used in phage classification (Van Regenmortel *et al.*, 2000). The difference of plaque size and morphology were the initial screening criteria of characteristic features. These features may vary among different phages grown on the same bacterial host as well as same phage grown on different bacterial hosts. The results showed that plaque size were different in each experiment even in the same host and phage in accordance with report of St. Clair and McCoy (1958). When the two test systems were employed by the use of phage G with *Streptomyces olivaceus* and phage B with *S. griseus* 1947, the results showed that:

a) Concentration of agar has relatively little effect upon plaque size except at the extremes of 0.8 and 2.8 percent; higher concentrations producing slightly smaller plaques,

b) Culture of one day old on poured plates gave larger and more uniform plaques than older ones. Excessively dry plates cause erratic behavior, whereas freshly poured plates often contain enough surface moisture to produce irregularly shaped plaques,

c) Excess moisture introduced with the inoculum likewise causes irregularities and tendes to inhibit optimal sporulation of the background streptomycetes growth,

d) Thick agar gives larger plaques than thin agar, and

e) There was no appreciable difference in plaque form or size when 8 h germinated, rather than ungerminated spores were used as inoculum.

Preparation of phage stocks was necessary for routine study. High titer phage suspension was normally obtained by soak-outs from single plaques or confluent plates (double layer method). Thus, it is very important to purify each stock by several rounds of single plaque isolation. After retained the single plaque soak out, filter-sterilized was needed to minimize loss of phage titer by adsorption to host debris and contamination (Kieser *et al*., 2000). In this study, high titer stock of phages were recovered by soaking out with nutrient broth and stored at 4^0C in the dark bottle with their viability checked every month. In case of phage Yok-15, it could multiply by liquid culture method and gave titer similar with soak out method (as described in section 3.9, the initial titer of phage, 1.37×10^7 pfu/ml and after multiplication in liquid culture at pH 9, 2.99×10^{12} pfu/ml).

One of the well accepted procedures for characterizing taxonomy of phages is a morphological characterization of virions by electron microscopy. From taxonomic study, actinophages belong to three families: Myoviridae, Siphoviridae and Podoviridae (Bradley, 1967; Ackermann and Eisenstark, 1974; Ackermann *et al*., 1978) and most tailed actinophage, belong to the mophological type B1 (Ackermann and Eisenstark, 1974). Under observation of phage morphology by transmission electron microscope, all phages had hexagonal heads and long noncontractile tails like all previously characterized *Streptomyces* phages (Table 4.3). Therefore, they were belonged to group B of Bradley's classification, type B1 and fall into Family Siphoviridae according to Ackermann and Eisenstark's taxonomy (Ackermann and Eisenstark, 1974). The smallest phage, Ray-7, and the largest phage, Prachin-16, had a head of

47±20 and 60±13 nm and a tail 280±23 and 100±50 nm, respectively. The heads of the other phages ranged from 40 to 67 nm except the phage, Pathum-22 with big head of 80±5 nm. Length of the tail of all phages was varied in the range of 133 to 280 nm.

Table 4.3 Morphology of phages determined under electron microscopy

Actinophages	Average head diameter (nm)	Average tail width (nm)	Average tail length (nm)	References
Roi-1	40±7	10±2	213±47	This study
Non-2	67±60	12±4	273±7	This study
Phet-3	53±1	6.7±4	153±40	This study
Ang-6	47±5	13.3±7	187±50	This study
Ray-7	47±20	13.3±7	100±50	This study
Pathom-9	43±10	6.7±6	143±5	This study
Surat -11	60±1	8±2	213±50	This study
Korat -12	53±7	7±4	147±20	This study
KK-13	53±20	6.7±10	213±20	This study
Yok-15	50±4	6.7±10	210±4	This study
Prachin-16	60±13	10±5	280±23	This study
Cha-17	60±11	10±10	260±20	This study
Pathum-18	53±7	10±5	253±20	This study
Chon-19	46±5	10±5	220±20	This study
KK-20	53±5	12±5	133±30	This study
Pathum-22	80±5	20±5	240±30	This study
Sin-27	53±7	20±10	200±50	This study
Nsaw-28	40±5	7.33±10	133±30	This study
Lam-29	40±10	4 ±1	200±15	This study

Table 4.3 (continue)

Actinophages	Average head diameter (nm)	Average tail width (nm)	Average tail length (nm)	References
ϕHAU 3	60±3		200±7	Zhou *et al.* (1994)
Nsaw-30	36±2	6±2	140±10	This study
Ac-7	53.3	13.3	133.3	This study
Sat-1	60	6	115	Ogata *et al.* (1985)
R4	64	-	190	Chater *et al.* (1979)
ϕC 31	53	-	100	Lomovskaya *et al.* (1980)
FP43	50±3	-	223±30	Suarez *et al.* (1984)
SV 2	56	-	132 ± 14	Stuttard and Dwyer(1981)
VP II	55.0±1.5	-	220±9	Dowding (1973)
FP22	71	-	307	Hahn *et al.* (1990)

Phage host range is a useful marker for epidermiological and genetically studies of phages. A summary of these data was presented in Table 3.5. All of phage isolates displayed the different host range pattern by forming plaques on

Streptomyces strains and varied from narrow: Non-2, Phet-3, Ban-5, Pathum-22 and Chan-24, to broad: Roi-1, Yok-15, Nsaw-30, Lam-29, Nsaw-28, Korat-12, Ac 7, Ray-7, Surat-11and Sin-27. Phage Ac 7, firstly isolated by Janejira Deatraksa (2000) and proved to be temperate phage by Theerapat Wechaprasit (2001), was added in the study for further characterization. In addition, several phages formed turbid plaques with reference strains but after picking up the colony which is capable of growing in turbid plaques for testing whether they were lysogen. It was found that no lysogen obtained from these experiments. Host range determination examined that mostly isolated phages acted as the virulent phage and gave a broad host range. Remarkably, from the result, they were clearly discriminated the close relationship between *Streptomyces* and *Kitasatospora* by tested among 30 strains of phages. 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 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. From 30 phages used in the study, phage Yok-15 and Roi-113 gave a very broad host range against reference strains and isolated streptomycete strains. Cox and Blatz (1984) has been reported newly isolated phage, FP22 capable of forming plaques on 21 of the 29 species tested. So far as we know, this phage showed the widest host range of *Streptomyces* phage. However, we speculate that Yok-15 was exhibited the host range broader than FP22 against reference strain because it did infect 39 from 46 strains although their streptomycete strains used and the total numbers were different from this study. Controversy, Phet-3, Ban-5, Pathum-22, and Chan-24 displayed a narrow host-range especially Phet-3 and Chan-24 which were not capable of forming a plaque in any streptomycetes except on the host in which the phages were originally isolated: *Streptomyces* sp. 3 and *Streptomyces* sp. 24, respectively. This characteristic was profitably identified their host species and had been reported in phage of *S. venezualae* (Stuttard, 1989).

It may result from the factors adsorption specificity (Lomovskaya *et al*., 1977; 1980; Okanishi *et al*., 1968) and barely occurred from intracellular barrier, host restriction and modification system. Phage sensitivity patterns have provided some useful taxonomic information on streptomycetes and supporting evidence of the relationships between *Streptomyces* and other genera (Williams *et al*., 1989; Kurtboke *et al*., 1992).

One step growth experiment was the one of kinetics used to determine the lytic burst size and latent period, and rise period. This method was developed by Ellis and Delbruck (1939) and described by Adams (1959). In the one-step growth experiment, *Streptomyces* host in nutrient broth was mixed with phage to give a multiplicity of infection (m.o.i.) of 0.1 - 0.01. At this m.o.i. level, each host cell has no more than one phage adsorbed onto it. Therefore, each pfu scored was the result of one bacterium infected with one phage. Normally, after a suitable adsorption period, the mixture was diluted into anti phage antibody to stop adsorption and inactivate unadsorbed phage. However, the method used in this study is devised by Dowding (1973) as an alternative choice for avoiding laboratory animal injection. This procedure has shown that the washing procedure on filters was more than 99% effective in removing free phages since early samples from the first growth flask yielded very few free phage plaques. The overall data of one step growth experiment were shown in Table 3.6.

Bacteriophages are usually stable over the pH range of 5 to 8 (Adams, 1959) and streptomycetes phages isolated from soil provided the pH above c. 5.0 (Kieser *et al*., 2000). The results showed that all phages giving a high titer at neutral pH except phage Yok-15 (Table 4.4). In addition, stability of phages was found at pH 7 to 9. It was no doubt to clarify that the optimum pH for eluent for recovered phage preferable adjusted pH from 7.0-9.0 and actinophages were rarely isolated when pH of the eluting medium was <7.0 (Lanning and Williams, 1982). The stability of actinophages, however, is generally sensitive to pH values greater than 9.0 (Sykes *et al*., 1981).

To investigate the divalent cation affecting on phage propagation (Table 4.5), five actinophages required a small amounts of $MgSO_4$. In contrast, at least 4 actinophages required a high concentration of $CaCl_2$ for their multiplications. From this result, affects of Ca^{2+} and Mg^{2+} have different affects to phage multiplication depended on the phage strains. Carter (unpublished data) reviewed the optimized cation concentration of Ca^{2+} and Mg^{2+} on phage propagation (Kieser, *et al*., 2000), for example phage ϕC31 needed 10 mM of Mg^{2+} and 8 mM of Ca^{2+}, phage R4 needed 10 mM of Mg^{2+} and 25 mM of Ca^{2+}. In addition, Dowding (1973) determined the effect of cation concentration on VPII phage. It was found that Ca^{2+} required for adsorption and enhanced phage stability.

Table 4.4 Role of pH of phages

Phages	pH optimum	pH stability
Roi-1	8	7
Ray-7	6	8
Surat-11	7	7
Yok-15	9	9
Sin-27	7	7
Nsaw -28	6	7
Lam-29	7	7
Nsaw-30	7	8
Ac7	7	8

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Table 4.5 Role of cation for phage propagation

Phages	Final concentration of cation requirement (mM)	
	$MgSO_4$	$CaCl_2$
Roi-1	-	10
Ray-7	-	10
Surat-11	10	-
Yok-15	10	30
Sin-27	10	50
Nsaw -28	10	40
Lam-29	-	-
Nsaw-30	-	50
Ac7	20	10

Antigenic properties of bacteriophage have been reviewed since 1937 by Burnet *et al*. Antibody of phages was employed in many virus researches, for example, serologic classification of viruses into groups in which the antigenic relationships are correlated with morphologic and biologic resemblances (Delbruck, 1946), neutralization of free phage for other proposed study. 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 3.11, 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.

The profile of phage protein treated with 12% w/v trichloroacetic acid was performed in SDS-PAGE. This concentration was derived from Foschino *et al*. (2001). In this study, the presence of many protein bands for each of phages was also investigated. Most of them found the prominent band at molecular massed of 40-42 kDa, 23-26 kDa. Four of them: (Roi-1, Yok-15, Sin-27, and Nsaw-28) showed the band at 35-36.5 kDa and three of them (Ray-7, Surat-11 and Sin-27) showed the band at 10.2-10.4. Though the phage proteins were not clearly showed the distinguished but their profiles could display a similarity among phages. These results were in accordance with Anne *et al*.(1990) who revealed that phage VWB showed at least 17 protein bands and three prominent bands with molecular massed of 16.5 kDa, 27.2 kDa and 43 kDa. However, the prominent bands from this study were different from previous reports and seemed unclarify. It may result from the host proteins contamination and some protein components in cells existing. In addition, it was possible that the pattern of each phage is different depend upon the phage type, locality and other factors.

Characteristic of phage DNA pattern is used to distinguish phage. From these experiments, the number and size of fragments of all phage DNA, obtained after digestion with restriction enzymes, showed a uniqueness and distinct from ϕ C31(Harris *et al*., 1983), SH10 (Walter *et al*., 1981), TG1 (Foor *et al*., 1985) and other reported actinophages (Table 3.12). The restriction maps of phage DNA: Roi-1 and Korat-12 were not constructed because their DNA were rarely cut with restriction enzyme and/or cut with many sites (more than 10 sites). In case of Surat-11, restriction map was not completed since the restriction enzymes could not distinguished four small fragments apart (Fig. 3.13). From isolated phage DNA, three restriction enzymes: *Sac*I, *Sph*I, *Sal*I produced from *S. achromogenes*, *S. phaeochromogenes*, *S. albus* were also determined in some phages, especially *Sal*I restriction sites could relate with plaque formation on *S. albus* (see Table 3.5 host range determination). If plaque

formed, phage DNA were cleaved by restriction enzymes produced by the corresponding hosts.

The molecular mass of phages was calculated by analysis of the fragments by restriction enzymes. The samples of the genome length of streptomycetes phages were shown in Table 4.6.

Table 4.6 Phage genome in length

Phages	Genome length (kb)	References
Roi-1	40.5	This study
Ray-7	50	This study
Surat-11	44	This study
Yok-15	55	This study
phi SC623	57	Schneider (1990) and Kuhn (1987)
TG1	41	Foor *et al.*(1985)
VWB	47.3	Anne (1985)
FP22	131	Hahn *et al.* (1990)

The application of DNA probes to characterize heterogeneous phage populations may simplify phylogenetic investigation because different phages from similar habitats can be classified according to their level of DNA sequence similarity (Ogunseitan *et al.*, 1992). Therefore, this technique was employed to diverse phages. The results were shown in Table 3.13. The positive color showed the similarity of DNA among phages. It seemed that a phage Surat-11 DNA shared the most similarity of DNA to other 5 phages whereas Ray-7 and Yok-15 DNA gave a similarity of DNA to 4 phages and Roi-1 DNA was not resembling with any phage DNA.

From this study, properties of phages: morphology, physiology, genome, proteins, antigenic relationship and biology were distinguished from previously actinophages reported by Lomovskaya *et al*. (1980), Walter *et al*. (1981), Cox and Baltz (1984), Anne *et al*. (1984; 1985), Ogata *et al*. (1985), Greene and Goldberg (1985), Brzezinski *et al*. (1986), Coyne and Kirby (1986), Ishihara *et al*. (1982), and Hranueli *et al*. (1979). The summary of phage properties in this study was shown in Table 4.7.

Table 4.7 Summary of phage properties

Properties	Phages
1. pH of soil samples:	
1.1 Neutral pH (6-8)	Roi-1, Non-2, Phet-3, Ban-5, Ang-6, Ray-7, Pathom-9, Kan-10, Korat-12, KK-13, Yok-15, Prachin-16, Cha-17, Chon-19, KK-20, Pathum-22, Chan-24, Sin-27, Nsaw-28, Lam-29, Nsaw-30
1.2 Acidic pH (<6)	Surat-11,Pathum-18
2. Plaque transparency:	
2.1 Clear	Roi-1, Non-2, Ban-5, Ray-7, Pathom-9, Surat-11, Korat-12, KK-13, Yok-15, Prachin-16, Cha-17, Pathum-18, Chon-19, KK-20, Sin-27, Nsaw-28, Lam-29,
2.2 Turbid	Phet-3, Ang-6, Kan-10, Pathum-22, Chan-24, Nsaw-30, Ac 7
3. Phage Morphology: Six-sided head and a long non-contractile tail	All

Table 4.7 (continue)

Properties	Phages
4. Host range determination:	
4.1 Broad	Roi-1, Non-2, Ang-6, Ray-7, Pathom-9, Surat-11, Korat-12, KK-13, Yok-15, Prachin-16, Cha-17, Pathum-18, Chon-19, KK-20, Sin-27, Nsaw-28, Lam-29, Nsaw-30, Ac 7
4.2 Narrow	Phet-3, Ban-5, Pathum-22, Chan-24
5. One step growth experiment:	
5.1 Burst size 10-50	Roi-1, Yok-15, Nsaw-28, Lam-29, Nsaw-30
5.2 Burst size >50	Ray-7, Surat-11
6. Stability and optimal pH:	
6.1 Neutral pH (6-8)	Roi-1, Ray-7, Surat-11, Sin-27, Nsaw-28, Lam-29, Nsaw-30, Ac 7
6.2 Basidic pH (>8)	Yok-15
7. Effect of cation:	
7.1 Mg^{2+} (0-20 mM)	All
7.2 Ca^{2+} (0-20 mM)	Roi-1, Ray-7, Surat-11, Lam-29, Ac 7
Ca^{2+} (>30 mM)	Yok-15 Sin-27, Nsaw-28, Nsaw-30
8. Serological relevance between phages and antiserum against phage Roi-1:	
8.1 Close	Nsaw-30, Sin-27
8.2 Moderate	Surat-11, Nsaw-28, Ac 7
8.3 Slight	Ray-7, Yok-15, Lam-29

Table 4.7 (continue)

Properties	Phages
9. DNA homology to:	
9.1 Phage Roi-1	-
9.2 Phage Ray-7	Yok-15, Nsaw-28, Nsaw-30
9.3 Phage Surat-11	Ray-7, Yok-15, Pathum-18, Nsaw-30
9.4 Phage Yok-15	Ray-7, Surat-11, Nsaw-28
10.Protein prominent bands	
10.1 40-42 kDa	Ray-7, Surat-11, Yok-15, Sin-27, Nsaw-30
10.2 35-36.5 kDa	Roi-1, Yok-15, Sin-27, Nsaw-28, Ac 7
10.3 23-26 kDa	Ray-7, Surat-11, Yok-15, Sin-27, Nsaw-28, Lam-29, Ac 7
10.4 10.2-10.4 kDa	Ray-7, Surat-11, Sin-27

The ecology of phage in soil clearly merits more attention. They also provide a novel opportunity to apply and test the concepts of host-parasite interactions. At a more practical level, the presence of a phage in a sample that is active against the bacterial taxa of interest can be rapid means of screening large numbers of soils for the presence of selected bacteria (Williams *et al*., 1994).

From the results indicated that the identification of *Streptomyces* may be more advantage when studying the interaction in soil. In general, the method of 16S rRNA oligonucleotide cataloging has been applied to determine suprageneric relationships of actinomycetes and other bacteria (Stackebrandt and Woese, 1981; Stackebrandt *et al*., 1981; Stackebrandt and Schleifer, 1984) and found to exhibit conserved regions that can be used to study distantly related species,

regions of higher variability are useful for the analysis of closely related species (Woese, 1987; Simonet *et al.*, 1994). Hence, this method was carried out for identification of *Streptomyces* sp. 15. A database of partial subunits of 16S rDNA was examined by the method of Kataoka *et al.* (1997). From this report, partial 16SrDNA sequences covering the variable region in 89 strains of the genus *Streptomyces* were grouped and made the usefulness for rapid *Streptomyces* species identification. PCR product of 500 bp was amplified using specific primers, and sequenced the fragment in the last step, and the 120 bp bearing the hyper-variable region was sequenced. BLAST program (www.ncbi.nih.nlm.) was used for taxonomic characterization of streptomycetes. It was found that *Streptomyces* sp. 15 was closely resembled with *Streptomyces* sp. NRRL 5183 (accession number AJ391815), which has published in year 2000 and has no any report supported since then. The other resemblance strains also had no data support. Thus, this strain was still reported as a new strain (accession number AY128706).

The efficiency of plating (EOP) is defined as the fraction of phage particles that can form a plaque. In any case, we wait until lysis occured, only those samples which really contained no particle will show no plaques, quite independent of any inefficiency of plating. The value of EOP is 1 or nearly 1 for many phages but can be less than 1 (0.1 to 0.5) for phages that make very small plaques (Maloy *et al.*, 1994). Various hosts gave a different value of EOP and all 3 strains of host used in soil experiment did not provide a much different of EOP (0.84, 0.55 and 0.70 for *S. viridochromogenes*, S. *coelicolor* and *S. griseus*, respectively). The result indicated that *Streptomyces* sp. 15 was not restricted phage propagated in any alternative hosts. The EOP of *S. coelicolor* and *S. griseus* gave a lower EOP. This difference presumably resulted from the fact that they produced small plaques (Fig. 3.28).

The fate of phages in soil seems to be strongly affected by the conditions of soil and the natures of host streptomycetes, as reported elsewhere (Mayfield *et*

al., 1972; Burroughs *et al*., 2000). Growth of host cells should be useful for multiplication of phages. Strain 15 seemed to be more suitable to Thai soils, especially Thai soil (II), than other used strains for their growth. Thus, multiplication of phage Yok-15 should occur in strain 15.

There are many factors affecting the decrease in phages and their hosts displayed in soils. Williams and Lanning (1984) added known amounts of a streptomycete phage to soils to assess the effects of various environmental factors on its stability. However, influence of pH on phage Yok-15 and its host streptomycetes was not related to the factor because streptomycetes and actinophages are stable at pH between 5.5 and 9.0 in sterile soil (Sykes and Williams, 1978; Sykes *et al*., 1981). There are two factors influencing the titer of phage. First, components of soil are considered as a main factor. Many kinds of phages could be adsorbed to colloidal particles, especially clay in their natural habitat (Reanney and Marsh, 1973; Duboise *et al*., 1979). Several actinophages isolated from soil are adsorbed by kaolin (Sykes and Williams, 1978) as well as streptomycetes spores and mycelia adsorbed to kaolinite and other clays (Ruddick and Williams, 1972). Therefore, main decreasing in the titer of free phages in control experiments may be due to the adsorption to kaolin. Second, the phage titer in soil decreases by the adsorption to the mycelia of host streptomycetes. The phage adsorption correlated with the increase of mycelial density (Burroughs *et al*., 2000). Thus, phage Yok-15 incubated with the host streptomycetes decreased more rapidly than free phages without the host streptomycetes. Particularly, dying hyphae and their debris would strongly affect the decreasing rate of phages. Thus, phage Yok-15 was mainly decreased by the adsorption to host mycelia and soil.

From these results, we concluded that a multiplication of phage in soil occurring in phage compatible with its host streptomycetes that can grow in nutrient-poor condition of soil. However, enhancing of streptomycetes growth

and phage multiplication was hardly obtained, thus they lost their activities during incubation.

Phage – streptomycete interaction usually have low infection efficiencies (10-50 %) probably due to incomplete adsorption. Eventually, we hope this study will alternately clarify the phage-host microcosm and well increase understanding of their status in soil.

Phage suspension may be sterilized by treatment with chloroform and this does not inhibit growth of the streptomycete host (Lanning and Williams, 1982). However, many *Streptomyces* phages are inactivated rapidly by 1 % chloroform. Also, all of three phages, Yok-15, Non-2 and Ray-7, were inactivated. So it is inadvisable to use chloroform to sterilize suspensions without first testing the phage for chloroform resistant (Kieser *et al*., 2000).

The results in temperature sensitivity showed that the suitable temperature for storage phage Yok-15 was at 4^0C. Generally, phages are stored at 4^0C for most purposes with little long-term loss of infectivity, as long as they are protected from light (Kieser *et al*., 2000). Sodium pyrophosphate and EDTA rapidly inactivated phage Yok-15 and available for phage deletion mutant isolation (Chater *et al*., 1980). The inactivation process are resulted from EDTA may act by forming a complex with some cation bound to the phage particle(Adams, 1959) as well as the toxicity of pyrophosphate. Issinger and Falk (1976) found that sodium pyrophosphate treatment of T3 and T7 wild type particles led to morphologically aberrant forms which had partially or completely lost the hexagonal head structure and led to a release of the phage tails. In addition, optimal conditions for obtaining high titer yields of phage Yok-15 was found at 200 rpm with initial concentration of spore and phage at 10^6 pfu/ml and 1.41 x 10^8 spores/ml, respectively.

This report was principally described on *Streptomyces* phages isolated in Thailand. We hope our isolated streptomycetes and phages will be carry for further study and apply for numerous usages. For instance, antibiotic producing strains of streptomycetes will bring out the further applications especially uncharacterized species, *Streptomyces* sp. 15 as well as the characterized phages were employed for molecular study. In addition, the information on *Streptomyces* sp. 15 and phage Yok-15 was clarified their interaction in unsuitable conditions for *Streptomyces* growth. We expect the acquired phages being value to identify streptomycetes taxa by using the beneficial of their broad as well as narrow host range. We also tried to isolate novel streptomycetes strains which were easily attacked by phage in order to isolate phage in extreme conditions for example; in soil pH lower or higher than 4.0. Therefore, we hope to exploit these streptomycetes and phages to approach the ecological system in soil.

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APPENDICES

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APPENDIX I

Media

1. Nutrient broth

	Beef extract	3	g
	Bacto-peptone	5	g
For top layer;	Agar	5	g

Making up to a volume of 1 liter with distilled water and autoclave. To use, melt and then cool to 45°C.

For base layer;	Agar	15	g

Making up to a volume of 1 liter with distilled water and autoclave.

2. Nutrient agar

Beef extract	3	g
Bacto-peptone	5	g
Agar	15	g
Distilled water	1	l

3. Benette broth and agar

	Glucose	10	g
	Beef extract	1	g
	Yeast extract	1	g
	N-Z amine type A	2	g
For top layer;	Agar	5	g

Making up to a volume of 1 liter with distilled water and autoclave. To use, melt and then cool to 45°C.

For base layer;	Agar	15	g

Making up to a volume of 1 liter with distilled water and autoclave.

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4. Rye flakes agar

Rye flakes	10	g
Glucose	2	g
Yeast extract	1	g
Adjustto pH 7.2 and then added,		
$CaCO_3$	2	g
Agar	15	g

Making up to a volume of 1 liter with distilled water and autoclave.

5. Humic acid

*Humic acid	1	g
Na_2HPO_4	0.5	g
KCl	1.7	g
$MgSO_4.7H_2O$	0.05	g
$FeSO_4.\ 7H_2O$	0.01	g
$CaCO_3$	0.02	g
**Cycloheximide	50	mg
***Vitamin B		
Agar	18	g
Distilled water	1	l

Adjust to pH 7.2

* Dissolve humic acid in 10 ml of 0.2 N NaOH.

**Cycloheximide and vitamin B were filter sterilized by passing through a 0.45 μm pore size and added to a medium at 45^0C.

***Vitamin B

Thiamine-HCl	0.5	mg
Riboflavin	0.5	mg
Niacin	0.5	mg
Pyridoxin-HCl	0.5	mg
Inositol	0.5	mg

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p-aminobenzoic	0.5	mg
Biotin	0.25	mg

6. Tryptic soy broth (TSB)

Bacto-tryptone	17	g
Bacto-soytone	3	g
NaCl	5	g
$K_2 HPO_4$	2.5	g
Distilled water	1	l

Adjust to pH 7.3. Making up to a volume of 1 liter with distilled water and autoclave.

7. Potato dextrose agar

Diced potatoes	200	g
Dextrose	15	g
Agar	20	g
Distilled water	1	l

Boil potatoes for 1 h and pass the mixture through a fine sieve, add agar and boil until dissolved, add dextrose and stir. Autoclave at 121^{0}C for 20 min.

8. Oat meal agar medium

Oat meal	30	g
Agar	20	g
Distilled water	1	l

Boil Oat meal in water for 20 min and make up liquor to 1 liter with water. Add agar and dissolve by heating. Autoclave at 121 ^{0}C for 20 min.

9. Yeast extract-malt extract agar

Yeast extract	3	g
Malt extract	3	g

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Bacto-peptone	5	g
Dextrose	10	g
Agar	20	g
Distilled water	1	l

Adjust to pH 7.0. Making up to a volume of 1 liter with distilled water and autoclave.

10. Inorganic salt starch agar

Soluble starch	10	g
$CaCO_3$	2	g
$(NH_4)_2SO_4$	2	g
MgSO4	1	g
NaCl	1	g
Ferrous sulfate	1.0	mg
$MnCl_2.7H_2O$	1.0	mg
$ZnSO_4$	1.0	mg
Agar	20	g
Distilled water	1	l

Adjust to pH 7.2. Making up to a volume of 1 liter with distilled water and autoclave.

11. Mannitol mungbean agar

Diced mungbean	10	g
Mannitol	10	g
Agar	15	g
Tap water	500	ml
Distilled water	500	ml

Adjust to pH 7.0. Making up to a volume of 1 liter with distilled water and autoclave.

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APPENDIX II

Reagents

1. DIG high prime DNA labeling and detection starter kit I contents

Vial 1	: DIG- high prime 50 µl
Vial 2	: DIG-labeled control DNA 20 µl
Vial 3	: DNA dilution buffer 3 x 1 ml
Vial 4	: Anti-digoxigenin AP conjugate 100 µl
Vial 5	: NBT/BCIP 6 x 1 ml
Vial 6	: Blocking solution 4 x 100 ml
Vial 7	: DIG easy hyb granules

2. Blocking solution

Prepare a 1x working solution by diluting 10x blocking solution 1: 10 with maleic buffer (see below, No. 6).

3. Antibody solution

Centrifuge anti-digoxigenin-AP (vial 4) for 5 min at 10,000 rpm in the original vial prior to each use, and pipet the necessary amount carefully from the surface. Diluted anti-digoxigenin-AP 1: 5000 (150 mU/ml) in blocking solution.

4. Color substrate solution

Add 200 µl of NBT/BCIP stock solution (vial 5) to 10 ml of detection buffer.

5. Washing buffer

Maleic acid	0.1	M
NaCl	0.15	M
Tween 20	0.3 %	V/V

Adjust to pH 7.5. Making up to a volume of 1 liter with distilled water and autoclave.

6. Maleic acid buffer

Maleic acid	0.1	M
NaCl	0.15	M

Adjust to pH 7.5 with NaOH (solid). Making up to a volume of 1 liter with distilled water and autoclave.

7. Detection buffer

Tris-HCl	0.1	M
NaCl	0.1	M

Adjust to pH 9.5. Making up to a volume of 1 liter with distilled water and autoclave.

8. TE- buffer

Tris-HCl	0.1	M
EDTA	1	mM

Adjust to pH 8.0. Making up to a volume of 1 liter with distilled water and autoclave.

9. Blocking solution

Prepare a 1x working solution by diluting 10x Blocking solution 1: 10 with maleic buffer.

10. Denaturation buffer

NaOH	20	g
NaCl	87.66	g
Distilled water	1	l

11. Neutralization buffer

Tris-HCl	0.5	M
NaCl	3	M
Distilled water	1	l

Adjust to pH 7.0. Making up to a volume of 1 liter with distilled water and autoclave.

12. 2x SSC buffer

NaCl	17.532	g
Tri-sodium citrate dihydrate	8.823	g

Adjust to pH 7.0. Making up to a volume of 1 liter with distilled water and autoclave.

13. 2x SSC, 0.1 %SDS

20x SSC	10	ml
10 % SDS	1	ml

Making up to a volume of 1 liter with distilled water and autoclave.

14. 0.5x SSC, 0.1 % SDS

20x SSC	2.5	ml
10 % SDS	1	ml

Making up to a volume of 1 liter with distilled water and autoclave.

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15. 1.5 M Tris- HCl, pH 8.8

Tris base	27.23	g
Deionized water	80	ml

Adjust to pH 8.8 with 6 N HCl. Making up to a volume of 150 ml with deionized water and store at 4 ^{0}C.

16. 10% SDS

Sodium dodecyl sulfate (SDS)	100	g
Deionized water	500	ml

Making up to a volume of 1 liter with distilled water, autoclave and store at room temperature. Wear a face mask while weighing out SDS.

17. 5x Running Buffer, pH 8.3 (1 liter)

Tris base	15	g
Glycine	72	g
SDS	5	g

Making up to a volume of 1 liter with deionized water. Store at 4 ^{0}C. Warm to room temperature before use if precipitation occurs. Dilute 60 ml 5x stock with 240 ml deionized water for one electrophoretic run.

18. Sample Buffer (8 ml)

Distilled water	4.0	ml
0.5 M Tris-HCl	1.0	ml
Glycerol	0.8	ml
10% SDS	1.6	ml
Beta-mercaptoethanol	0.4	ml
0.05% (w/v) BromopHenol blue	0.2	ml

Dilute the sample at least 1: 4 with sample buffer, and heat at 95 ^{0}C for 4 minutes.

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19. 0.5 M Tris-HCl, pH 6.8

Tris base	6	g
Deionized water	60	ml

Adjust to pH 6.8 with 6 N HCl. Making up to a volume of 150 ml with deionized water and store at 4 ^{0}C.

20. TE25S buffer

Tris -HCl 25 mM pH 8, 25 mM EDTA pH 8, 0.3 M sucrose.

21. 0.1 M Ammonium acetate buffer

Ammonium acetate ($C_2H_7NO_2$)	0.77	g
Distilled water	1	l

Adjust to pH 7.0 with 1 N NaOH.

22. 50x TAE

Tris-base	242	g
Glacial acetic acid	57.1	g
0.5 M EDTA pH 8.0	1	l

For use: dilute to 1x by double distilled water.

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APPENDIX III

1. Moisture Content by Nancy Trautmann and Tom Richard

When deciding what proportions of various materials to mix together in making compost, the moisture of the resulting mixture is one of the critical factors to consider. The following steps outline how to design your initial mix so that it will have a suitable moisture level for optimal composting.

1.1 Calculate the % moisture for each of the materials you plan to compost.

a) Weigh a small container.

b) Weigh 10 g of the material into the container.

c) Dry the sample for 24 hours in a 105-110^{0}C oven.

d) Reweigh the sample, subtract the weight of the container, and determine the moisture content using the following equation:

$$Mn = ((Ww-Wd)/Ww) \times 100$$

in which:

Mn = moisture content (%) of material n

WW = wet weight of the sample, and

Wd = weight of the sample after drying.

Suppose, for example, that you weigh 10 g of grass clippings (Ww) into a 4 g container and that after drying the container plus clippings weighs 6.3 g. Subtracting out the 4-g. container weight leaves 2.3 g as the dry weight (Wd) of your sample. Percent moisture would be:

$$Mn = ((Ww-Wd)/Ww) \times 100$$

$$= ((10 - 2.3) / 10) \times 100$$

= 77% for the grass clippings

1.2 Choose a moisture goal for your compost mixture. Most literature recommends a moisture content of 50%-60% by weight for optimal composting conditions.

2. DIG-DNA Labeling (DIG High Prime DNA Labeling and Detection Starter Kit I, Instruction Manual, Version I)

2.1 Add 1 μg template DNA and autoclaved, double distilled water to a final volume of 16 μl to a reactor vial.

2.2 Denature the DNA by heating in a boiling water bath for 10 min and quickly chilling in an ice/water bath.

2.3 Mix DIG-High Prime (vial 1) thoroughly and add 4 μl to the denatured DNA, mix and centrifuge briefly. Incubate for 1 h or O/N at 37 ^{0}C

2.4 Stop the reaction by adding 2 μl 0.2 M EDTA (pH 8.0) and/or by heating to 65 ^{0}C for 10 min.

3. Determination of labeling efficiency(DIG High Prime DNA Labeling and Detection Starter Kit I, Instruction Manual, Version I)

3.1 A series of dilutions of DIG-labeled DNA is applied to a small strip of nitrocellulose membrane positively charged. Part of the nitrocellulose membrane is preloaded with defined dilution of DIG-labeled control DNA (vial2) which are used as standards.

3.2 The nitrocellulose membrane is subjected to immunological detection with anti-digoxigenin-AP conjugate (vial 4) and the premixed stock solution of NBT/BCIP(vial 5). The intensities of the dilution series of DIG-labeled DNA and control DNA are compared by exposure to Lumi-Imager.

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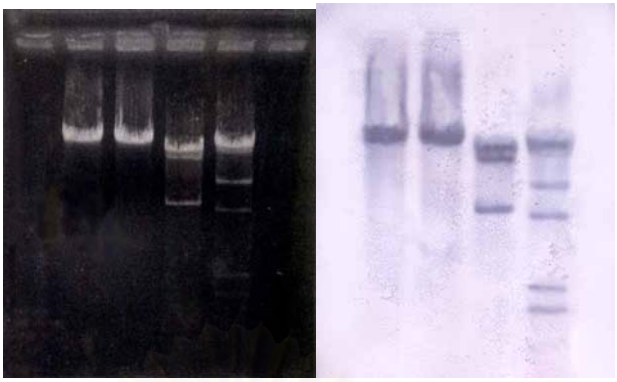

1 2 3 4 1 2 3 4

A B

Fig. 1 Determination of labeling efficiency. Agarose gel electrophoresis of phage Yok-15 DNA (A), southern blot hybridization (B). Lane 1, 2: Yok-15 DNA, lane 3: Yok-15 DNA cut with *Bgl*II, lane 4: Yok-15 DNA cut with *Mlu*I.

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4. Preparation of Gels for SDS/Polyacrylamide Gel Electrophoresis

Table 1 Separating Gels, in 0.375 M Tris, pH 8.8

	7%	10%	12%	15%
Distilled H_2O	5.1 ml	4.1 ml	3.4 ml	2.4 ml
1.5 M Tris-HCl, pH 8.8 (Appendix II, No. 15)	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% (w/v) SDS	0.05 ml	0.05 ml	0.05 ml	0.05 ml
Acrylamide/bis-acrylamide (30%/0.8% w/v)	2.3 ml	3.3 ml	4.0 ml	5.0 ml
10% (w/v) ammonium persulfate	0.05 ml	0.05 ml	0.05 ml	0.05 ml
TEMED	0.005 ml	0.005 ml	0.005 ml	0.005 ml
Total monomer	10.005 ml	10.005 ml	10.005 ml	10.005 ml

Table 2 Stacking Gels, 4.0% gel, 0.125 M Tris, pH 6.8

Distilled H_2O	3.075 ml
0.5 M Tris-HCl, pH 6.8 (Appendix II, No. 19)	1.25 ml
10% (w/v) SDS	0.025 ml
Acrylamide/bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate	0.025 ml
TEMED	0.005 ml
Total Stack monomer	5.05 ml

APPENDIX IV

Fig. 2 Molecular size of proteins in 10-20 % SDS PAGE

Table 3 Protein Marker (New England Biolabs Inc., Cat. No. 1-800-632-7799)

Protein	Source	Calculated MW (Da)
Myosin	Rabbit muscle	212,0001
MBT-β-galactosidase [2]	*E. coli*	158,194
β-galactosidase	*E. coli*	116,351
PHospHorylase *b*	Rabbit muscle	97,184
Serum albumin	Bovine	66,409
Glutamic dehydrogenase	Bovine liver	55,561
MBT2[*2]	*E. coli*	42,710
Lactate dehydrogenase M	Porcine muscle	36,487
TriosepHospHate isomerase	Rabbit muscle	26,625
Trypsin inhibitor[3]	Soybean	(20,040-20,167)
Lysozyme	Chicken egg white	14,313
Aprotinin [4]	Bovine lung	6,517
Insulin A, B chain [5]	Bovine pancreas	(2,340-3,400)

1 Hames, B.D. 1990.

2. MBP2* = maltose-binding protein.

3. Trypsin inhibitor (soybean) is a mixture of three isoforms: A-20,094 Da; B-20,040 Da ; C-20,167 Da.

4. Enzyme sequence from bovine pancreatic trypsin inhibitor.

5. Insulin chains are unresolved by SDS-PAGE (Tris-Glycine).

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APPENDIX V

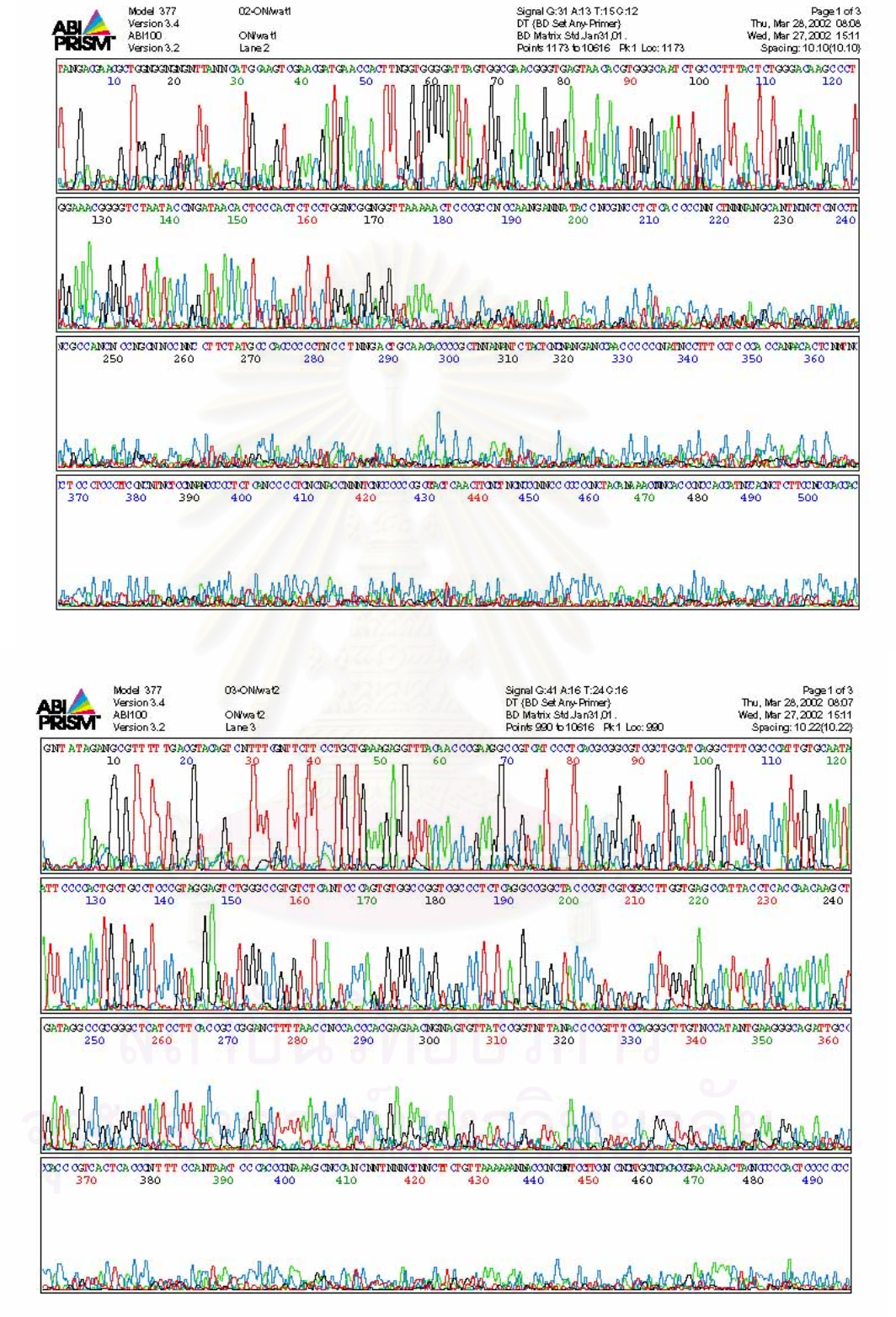

Fig. 3 16 S rDNA sequencing data analysis of *Streptomyces* sp. 15

BIOGRAPHY

Miss Onanong Pringsulaka was born on the 10 th of March 1974. She graduated with the Bachelor's Degree in Microbiology from Kasetsart University in 1994, Master's Degree in Industrial Microbiology from Chulalongkorn University in 1998 and further her Ph.D. study in Biotechnology Program.

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