

CHARACTERIZATION OF BACTERIOPHAGE Φ T25 AND PHAGE-RESISTANT
DERIVATIVES ISOLATED FROM FERMENTED MILK INDUSTRY IN THAILAND

A THESIS
BY
SIRINTHORN SUNTHORNTHUMMAS

Presented in Partial Fulfillment of the Requirements for
the Doctor of Philosophy Degree in Biotechnology
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AN ABSRACT

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Bacteriophage infection is a serious problem in industrial milk fermentation. The consequences of a phage infection are a delay in fermentation process, an alteration of the product quality, and in severe cases involve the losses of the products that cause economic loss. This study was the first report of isolation and characterization of lactic acid bacteria phage from abnormal fermented milk in Thailand. Bacteriophage Φ T25 is a novel virulent phage which has shown a strong lytic activity only against *Lactobacillus paracasei* LPC (host strain). Transmission electron microscopy revealed that phage Φ T25 belonged to the member of *Siphoviridae* family, with a hexagonal head (55 ± 4.6 nm in diameter) and a long noncontractile tail (184 ± 7.8 nm in length). The one-step growth curve of the phage indicated that the latent period and the rise period were both 55 min and the average burst size was approximately 38 phage particles per infected cell. The presence of calcium ions had an influence on phage adsorption and improved plaque formation. Genome analysis indicated that the phage Φ T25 genome was a linear, double-stranded DNA of 38,421 base pairs with an overall G+C content of 44.5 mol%. A total of 50 possible open reading frames (ORFs) were identified and as a consequence 31 of these were assigned predicted functions. The phage Φ T25 genome was organized into five functional modules, including DNA packaging, replication, lysogenic, lysis and morphogenesis. This was highlighted by the genome organization which differed slightly to the consensus pattern of genomic organization usually found in *Siphoviridae* phages. Analyses of restriction profile revealed no differences between banding profiles with and without prior annealing, suggesting the absence of cohesive ends in the DNA molecule of the phage Φ T25. Therefore, phage T25 was predicted to be *pac*-type phage using a head-full DNA packaging strategy. The identification of genes involved in the establishment of lysogeny indicated that the phage Φ T25 may have originated as a temperate phage. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles showed that the phage Φ T25 contained four structural proteins (12.7, 22, 42.4 and 71.5 kDa). Furthermore, spontaneous phage-resistant mutants were isolated from *Lb. paracasei* LPC, using the phage Φ T25 with secondary culture and agar plate methods. Phenotypic characteristics related to their phage-resistance capacities, as well as their technological properties, were also determined. The results in the present study revealed that the agar plate method was most useful for obtaining true phage-resistant mutants. Most of the mutants were completely or partially unable to adsorb phage particles. Three mutants (AP1-2, AP3-17 and AP3-31) were selected on the basis of their stability and high level of phage resistance. The comparison of random amplified of polymorphic DNA (RAPD) profiles revealed the highest similarity coefficient (87%) among parent strains and the three selected mutants confirming that each mutant has been derived from their parent strains. A good performance during milk fermentation and the subsequent refrigerated storage in the presence of phages was demonstrated when these mutant were employed. These selected phage-resistant derivatives could be used as improved strains or industrial rotation programs when commercial strains become sensitive to phages present in industrial environments.

การศึกษาลักษณะของ $\Phi T25$ และสายพันธุ์กลายที่ดื้อต่อการติดเชื้อด้วย
แบคทีเรียโอเฟจที่แยกได้จากโรงงานผลิตนมเปรี้ยวในประเทศไทย



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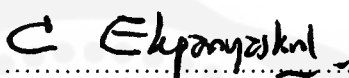
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การปนเปื้อนแบคทีเรียโอเฟจในโรงงานอุตสาหกรรมนมหมักนั้น ถือเป็นปัญหาสำคัญ โดยเฟจจะส่งผลทำให้ประสิทธิภาพในการผลิตกรดของหัวเชื้อช้าลง ทำให้คุณภาพของผลิตภัณฑ์ต่ำลง และในกรณีร้ายแรงคืออาจสูญเสียผลิตภัณฑ์นั้น ซึ่งส่งผลให้สูญเสียมูลค่าทางเศรษฐกิจ งานวิจัยนี้เป็นงานวิจัยแรกๆ ที่ทำการคัดแยกและศึกษาลักษณะของเชื้อแบคทีเรียแลคติกจากตัวอย่างนมหมักที่ล้มเหลวในประเทศไทย โดยพบว่าสามารถแยกเฟจใหม่และให้ชื่อว่า Φ T25 ซึ่งเป็นเฟจที่ทำให้เกิดการแตกสลายและมีความจำเพาะสูง กล่าวคือสามารถทำให้เกิดการติดเชื้อ *Lb. paracasei* LPC (host strain) ได้เพียงสายพันธุ์เดียว เมื่อทำการศึกษารูปร่างภายใต้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน พบว่า เฟจ Φ T25 จัดอยู่ในแฟมิลี *Siphoviridae* ประกอบด้วย ส่วนหัวมีลักษณะเป็นหกเหลี่ยมขนาด 55 ± 4.6 นาโนเมตร และมีส่วนของหางยาว 184 ± 7.8 นาโนเมตร จากกราฟการเจริญของเฟจ บ่งชี้ว่า Φ T25 มี latent period เท่ากับ rise period คือ 55 นาที และมีค่าเฉลี่ยของ burst size เท่ากับ 38 phage particles/infected cell จากการศึกษาผลของการเติมเซลล์เชื้อในอาหาร พบว่าเซลล์เชื้อมีผลต่อการเข้าเกาะติดของเฟจและการเกิดพลาค เมื่อศึกษาจีโนมของเฟจ Φ T25 พบว่า เป็น DNA สายคู่ ที่เป็นเส้นตรงขนาด 38,421 คู่เบส มีปริมาณ G+C เท่ากับ 44.5 โมลเปอร์เซ็นต์ และประกอบด้วย open reading frame (ORF) จำนวน 50 ORF และในจำนวนนี้มีเพียง 31 ORF ที่สามารถทำนายหน้าที่ของยีนได้ จากการศึกษาแบบการตัดจีโนมของเฟจด้วยเอนไซม์ตัดจำเพาะ พบว่าไม่มีความแตกต่างของแถบดีเอ็นเอที่จับกันก่อนและหลังให้ความร้อน ซึ่งให้เห็นว่า ดีเอ็นเอของเฟจ Φ T25 ไม่มี cohesive end ดังนั้นเฟจ Φ T25 อาจจัดเป็น *pac*-type phage ซึ่งอาศัย head-full packaging system เพื่อบรรจุสารพันธุกรรมเข้าสู่แคปซิด นอกจากนี้จีโนมของเฟจ Φ T25 สามารถจัดเรียงตามหน้าที่ได้ 5 โมดูลคือ การประกอบตัวของดีเอ็นเอ การจำลองสารพันธุกรรม ความเกี่ยวข้องกับการเกิดไลโซเจนิค การทำให้เกิดการแตกสลาย และการประกอบตัวเพื่อพัฒนาเป็นเฟจใหม่ จากการจัดเรียงตัวของจีโนมพบว่า มีความแตกต่างกับรูปแบบการจัดเรียงตัวของเฟจในแฟมิลี *Siphoviridae* เล็กน้อย จากข้อมูลจีโนมยังพบว่า เฟจ Φ T25 มีลำดับเบสที่กำหนดการแสดงออกของยีนที่สัมพันธ์กับการเป็น lysogeny ซึ่งให้เห็นว่า เฟจ Φ T25 มีต้นกำเนิดมาจาก temperate phage เมื่อทำการศึกษารูปแบบของโปรตีนของเฟจ Φ T25 โดยวิธี sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) พบว่าเฟจประกอบด้วยโปรตีนโครงสร้าง 4 แถบโปรตีน (12.7, 22, 42.4 และ 71.5 กิโลดาลตัน) นอกจากนี้งานวิจัยนี้ได้ทำการคัดแยกสายพันธุ์กลายที่ดื้อต่อการติดเชื้อด้วยเฟจจากสายพันธุ์ *Lb. paracasei* LPC โดยอาศัยเฟจ Φ T25 พร้อมด้วยวิธี secondary culture และ agar plate รวมทั้งศึกษาลักษณะทางฟิโนไทป์ที่เกี่ยวข้องกับความสามารถในการติดเชื้อ และการนำไปพัฒนาทางเทคโนโลยีในการหมักนม ผลการศึกษาพบว่า วิธี agar plate มีประสิทธิภาพในการคัดแยกสายพันธุ์กลายที่ดื้อต่อการติดเชื้อด้วยเฟจอย่างแท้จริง และมีสายพันธุ์กลายส่วนใหญ่สามารถป้องกันไม่ให้เฟจเกาะติดเชื้ออย่างสมบูรณ์ หรือเพียงบางส่วนได้ ทำการคัดเลือกสายพันธุ์กลายจำนวน 3 สายพันธุ์จากความเสถียรและความทนทานต่อการติดเชื้อด้วยเฟจ มาทำการศึกษารูปแบบความคล้ายคลึงกันของดีเอ็นเอ ด้วยวิธี random amplified polymorphic DNA (RAPD) ซึ่งให้เห็นว่ามีความคล้ายคลึงกันสูงมาก (87%) ระหว่างสายพันธุ์ตั้งต้นและสายพันธุ์กลาย ซึ่งยืนยันได้ว่าสายพันธุ์กลายมีการพัฒนาจากสายพันธุ์ตั้งต้น และเมื่อทำการศึกษาประสิทธิภาพที่ดีในการผลิตกรดระหว่างการหมักนมและการเก็บรักษาที่อุณหภูมิตู้เย็นของสายพันธุ์กลายทั้ง 3 สายพันธุ์ ผลการศึกษาพบว่า สายพันธุ์กลายที่คัดเลือกสามารถนำไปใช้ในการทดแทนหรือหมუნเวียนกับสายพันธุ์ตั้งต้น เมื่อสายพันธุ์ตั้งต้นที่ใช้ในการค้าไวต่อเฟจที่พบในสิ่งแวดล้อมที่ใช้ในอุตสาหกรรมนมหมักได้

The thesis titled
"Characterization of bacteriophage Φ T25 and phage-resistant derivatives isolated from
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by
Sirinthorn Sunthornthummas

has been approved by the Graduate School as partial fulfillment of the the requirements for the
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.....Dean of Graduate School

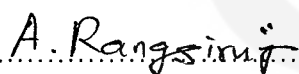
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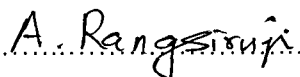
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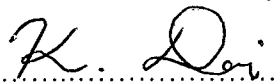
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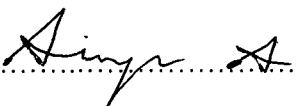
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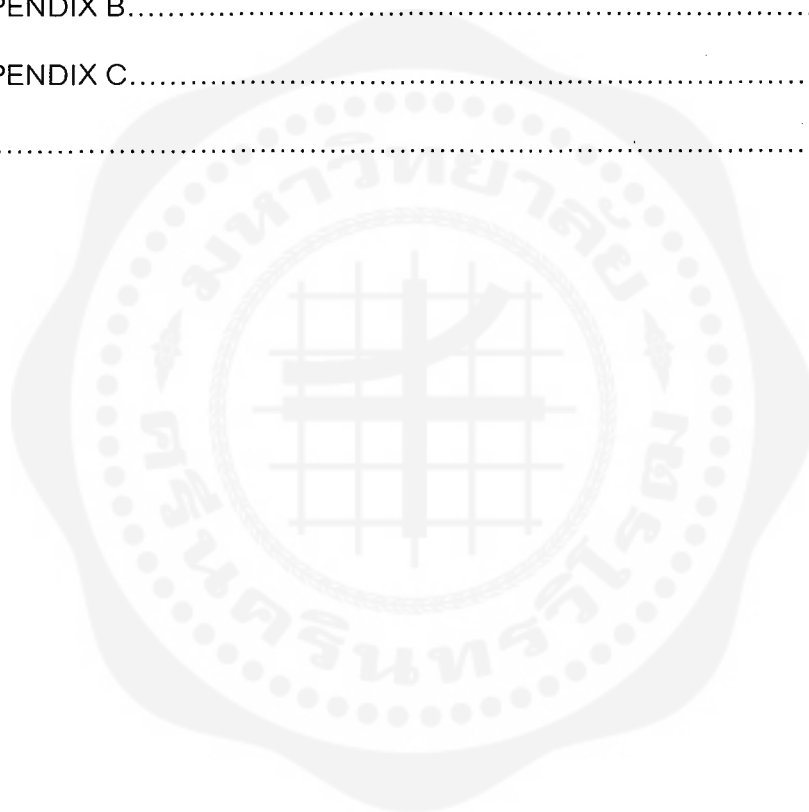
Sirinthorn Sunthornthummas

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CHAPTER 1

INTRODUCTION

Background

In recent years, increasing interest has been shown in the use of lactobacilli as probiotic adjuncts and some species are used worldwide as industrial starters for the manufacture of fermented dairy products. Lactobacilli starter culture strains contribute to improve in texture, flavor and nutritional value of dairy products. Among lactobacilli, members of *Lactobacillus casei* group (*Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus* and *Lb. zeae*) are most commonly used as starter culture in the dairy fermentation processes. However, the expanding use of these strains as starter cultures and probiotics can lead to bacteriophage (phage) infection in dairy processing plant environment.⁽¹⁾

Phage infection of dairy starter cultures has been recognized as a significant and persistent problem for the dairy industry. Its infection of lactic acid bacteria (LAB) starter culture can lead to a partial or total delay in the acidifying activity of starter, reducing the quality of the final products and resulting in significant economic losses.⁽²⁾ The presence of phages is unavoidable in the dairy plant environments, phage control strategies are usually designed to control rather than eradicate them. Many of the strategies employed for minimizing bacteriophage impact on dairy fermentation include the use of phage-resistant starter culture, direct vat inoculation of starters, optimized sanitation and culture rotation.⁽³⁾

Since each commercial lactobacilli starter culture brings its own special characteristics, including body, flavor and texture of the final fermented milk products, it is very difficult to find strains with similar technological properties differing in resistance to phages.⁽⁴⁾ The isolation of spontaneous phage-resistant mutants is the first approach used to obtain phage-resistant derivatives from phage-sensitive strains. This technique is simple and does not involve genetic manipulation, therefore the resistant strains recovered can be used to replace the sensitive strains in food industry. Although many spontaneous phage-

resistant strains have been identified,⁷ the mechanisms for phage insensitivity of *Lb. paracasei* are still underexplored. Investigation into the mechanisms of phage resistance can provide information that will aid in the selection of the phage-resistant strains. Moreover, the spontaneous phage-resistant strains usually have a high frequency of phenotype reversion and physiological bacterial modifications, thus, they should be tested for their technological performance, including phage resistance stability, acidification ability, favorable organoleptic properties and suitability for large-scale production.⁽⁴⁾

Due to the economic impact of phage infection on the dairy industry, the morphology, physiology and genomics of *Lactobacillus* bacteriophages have been extensively studied. In addition, since the phages are known to be the main vectors of gene exchange phenomena, they are considered the most important in driving evolution in prokaryotes.^(5,6) To date, more than 40 *Lactobacillus* bacteriophages genome sequences are available in public databases.⁽⁷⁾ The availability of these data allows for comparison of viral genomes in order to understand the genetic relationships among different phages and the function of putative genes. Although knowledge on phages and genomes derived from lactic acid bacteria of the dairy environment is increasing, only few reports are available for *Lb. paracasei* phages.

The aims of this study were to isolate *Lb. paracasei* phage and the spontaneous phage-resistant derivatives from *Lb. paracasei* commercial starter culture of fermented milk. The isolated bacteriophage will be characterized in various aspects, including morphological and physiological characteristics and complete sequence analysis. The phage-resistant derivatives will be characterized by means of phage resistance phenotype, their genetic diversity, the mechanisms of phage resistance and technological characteristics for industrial uses. The data obtained will be used for developing the phage-resistant starter culture and the rotation program to control phage attack in the dairy industry. The genome sequence of *Lb. paracasei* bacteriophage may provide useful information to further understand the phage defense systems that may confer higher levels of resistance. This will be the first study of phage infecting dairy starter cultures in Thailand.

Objectives of the study

1. To isolate *Lb. paracasei* bacteriophage from unsuccessful milk fermentation in dairy industry in Thailand.
2. To characterize *Lb. paracasei* bacteriophage in various aspects, including its morphology and physiology.
3. To analyze whole genome of *Lb. paracasei* bacteriophage.
4. To isolate spontaneous phage-resistant derivatives from *Lb. paracasei*.
5. To characterize spontaneous phage-resistant derivatives by means of phage resistance phenotype, their genetic diversity, the mechanisms of phage resistance and technological characteristics.

Significance of the study

The data obtained from this study can be used for developing the phage-resistant starter culture and the rotation program to control phage attack in the dairy industry. The genome sequence of *Lb. paracasei* bacteriophage may provide useful information to further understand the phage defense systems that confer higher levels of resistance and/or broader phage specificity.

Scope of the study

The unsuccessful milk fermentation sample from dairy plant was used to isolate and characterize bacteriophage by starter culture host. The isolated phage was characterized in various aspects, including morphology, physiology and whole-genome sequencing. In addition, this study aims to investigate the possibility of isolating spontaneous phage-resistant mutants from commercial strains by agar plate method and secondary culture method. Spontaneous phage-resistant mutants were characterized by means of phage resistance phenotype, their genetic diversity, the mechanisms of phage resistance and adequate technological characteristics for industrial uses.

CHAPTER 2

REVIEW OF LITERATURE

1. Lactic acid bacteria

1.1 General introduction

The term lactic acid bacteria (LAB) has long been referred to "milk-souring bacteria". The first pure culture of LAB was *Bacterium lactis* (probably *Lactococcus lactis*) that was acquired by J. Lister in 1873. The first monograph of lactic acid bacteria was written by Orla-Jensen (1919) and classification was based on morphology (cocci or rod, tetrad formation), mode of glucose fermentation (homo- or heterofermentation), growth at different temperatures (e.g. 10°C and 45°C) and range of sugar utilization. These characteristics are still important in the classification of LAB.⁽⁸⁾

The typical LAB grown under standard conditions (nonlimiting glucose concentration, growth factors and oxygen limitation) are gram-positive, non-spore-forming, rod or cocci-shaped, catalase-negative, devoid of cytochromes, fastidious nutrition requirement, acid-tolerant and strictly fermentative, with lactic acid as the major end product during sugar fermentation. The G+C composition of LAB genome is below 55 mol%.⁽⁹⁾ The inability to synthesize cytochromes and porphyrin groups (e.g. heme) is a key feature that must be emphasized of physiological characteristics of LAB. When grown in laboratory media which lacking heme or related compounds, LAB will not produce true-catalase and cytochromes. However, some LAB may produce a non-heme catalase or "pseudocatalase" when grown on media with low carbohydrate content.⁽⁸⁾ Thus, LAB can generate ATP only through fermentation. LAB are commonly found in various nutrition environments, including milk and dairy products, vegetables, cereals, meat and meat products. LAB species are also found among the resident microbiota of the gastrointestinal tract and genitourinary tract of humans and animals where they fulfill variety of health-

promoting function and protection of tissue against pathogens that are designated as probiotic.⁽¹⁰⁾

Two main sugar fermentation pathways can be distinguished among LAB. First, glycolysis (Embden-Meyerhof-Parnas pathway) results in lactic acid as major end product under standard conditions and is referred to as a homolactic fermentation. The other, 6-phosphogluconate/phosphoketolase pathway results in significant amounts of other end products such as ethanol and/or acetate and CO₂ in addition to lactic acid and is referred to as a heterolactic fermentation.⁽⁸⁾

1.2 Metabolism of LAB

Lactic acid bacteria utilize sugars (e.g. glucose) to form lactic acid by either the homofermentative or heterofermentative pathway. Homofermentative LAB utilize hexoses by the glycolytic pathway and the heterofermentative LAB, by the pentose phosphoketolase pathway. The latter requires phosphoketolase enzyme which is absent in the obligate homofermentative pathway but present in the facultative and obligately heterofermentative LAB.

1.3 Classification of LAB

The classification of LAB genera has been mainly based on Orla-Jensen's work although a number of new genera and species are increasing. The phenotypic characteristics such as morphology still remain the basic feature of characterizing LAB. The differentiation of the LAB genera with classical phenotypic tests was shown in Table 1. LAB can be separated into rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera). With the exception of the genus *Weissella* that encompasses both cocci and rods.⁽¹¹⁾ Coccus-shaped LAB can be differentiated by cell arrangement as tetrad-forming genera which are *Aerococcus*, *Pediococcus* and *Tetragenococcus*. In addition, other coccus-shaped LAB can be identified according to growth at certain temperatures (10°C and 45°C) such as *Enterococcus* can grow at both 10°C and 45°C, *Lactococcus* and *Vagococcus* grow only at 10°C and some species of *Streptococcus* grow at 45°C. These genera can also be distinguished by salt tolerance (6.5% NaCl). Furthermore, tolerance to acid and/or alkaline conditions can be used to differentiate aerococci, carnobacteria, enterococci, tetragenococci and vagococci.⁽¹²⁾

For preliminary classification of LAB at the species level, the phenotypic and biochemical methods are used as mentioned above. Other characteristics such as range of carbohydrates fermented, arginine hydrolysis, acetoin formation (Voges-Proskauer test), bile tolerance, type of hemolysis, production of extracellular polysaccharides, growth factor requirements, presence of certain enzymes (e.g. β -galactosidase and β -glucuronidase), growth characteristics in milk and serological typing are also useful in the species-level classification. Moreover, molecular and chemotaxonomic approaches, including type of diamino acid in the peptidoglycan, presence and type of teichoic acid, presence and type of menaquinones, guanine+cytosine (G+C) ratio of the DNA, fatty acid composition and electrophoretic mobility of the lactate dehydrogenase (LDH), are necessary for characterizing species of LAB.⁽⁸⁾

Table 1 Differential characteristics of lactic acid bacteria⁽⁸⁾

Character	Rods				Cocci					
	<i>Carnob.</i>	<i>Lactob.</i>	<i>Aeroc.</i>	<i>Enteroc.</i>	<i>Lactac.</i> <i>Vagoc.</i>	<i>Leucon.</i> <i>Oenoc.</i>	<i>Pedioc.</i>	<i>Streptoc.</i>	<i>Tetragenoc.</i>	<i>Weissella</i> ^a
Tetrad formation	—	—	+	—	—	—	+	—	+	—
CO ₂ from glucose ^b	— ^c	±	—	—	—	+	—	—	—	+
Growth at 10°C	+	±	+	+	+	+	±	—	+	+
Growth at 45°C	—	±	—	+	—	—	±	±	—	—
Growth in 6.5% NaCl	ND ^d	±	+	+	—	±	±	—	+	±
Growth in 18% NaCl	—	—	—	—	—	—	—	—	+	—
Growth at pH 4.4	ND	±	—	+	±	±	+	—	—	±
Growth at pH 9.6	—	—	+	+	—	—	—	—	+	—
Lactic acid ^e	L.	D, L, DL ^f	L.	L.	L.	D	L, DL ^f	L.	L.	D, DL ^f

+, positive; —, negative; ±, response varies between species; ND, not determined.

^a*Weissella* strains may also be rod-shaped.

^bTest for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.

^cSmall amounts of CO₂ can be produced, depending on media.

^dNo growth in 8% NaCl has been reported.

^eConfiguration of lactic acid produced from glucose.

^fProduction of D-, L-, or DL-lactic acid varies between species.

Source: Salminen S, Wright AVE, Ouwehand A, editors. Lactic acid bacteria microbiological and functional aspects. 3rd ed. New York: Marcel Dekker, Inc.; 2004.

1.3.1 *Lactobacillus* (Lb.)

Lactobacillus is the largest group of LAB and to date it contains 215 species.⁽¹³⁾ Lactobacilli are rod-shaped bacteria. It is heterogeneous, encompassing species with a variety of phenotypic, biochemical and physiological properties. The G+C content of lactobacilli is 33-55 mol%.⁽¹⁴⁾ Orla-Jensen (1919) divided the genus into three groups based on carbohydrate fermentation pathways (Table 2): Group I, obligately homofermentative lactobacilli which ferment hexoses almost entirely to lactic acid, while pentoses or gluconate is not fermented; Group II facultatively heterofermentative lactobacilli which ferment hexoses either almost entirely to lactic acid or, under glucose-limiting conditions, to lactic acid, acetic acid, ethanol and formic acid, and pentoses to lactic acid and acetic acid; and Group III obligately heterofermentative lactobacilli which ferment hexoses to lactic acid, CO₂, acetic acid and/or ethanol and pentoses to lactic acid and acetic acid.^(8,15)

Table 2 Differentiation of *Lactobacillus* based on carbohydrate fermentation pathway⁽⁸⁾

Characteristic	Group I, Obligately homofermentative	Group II, Facultatively heterofermentative	Group III, Obligately heterofermentative
Pentose fermentation	—	+	—
CO ₂ from glucose	—	—	+
CO ₂ from gluconate	—	+ ^a	+ ^a
FDP aldolase present	+	+	—
Phosphoketolase present	—	+ ^b	+
	<i>Lb. acidophilus</i>	<i>Lb. casei</i>	<i>Lb. brevis</i>
	<i>Lb. delbrückii</i>	<i>Lb. curvatus</i>	<i>Lb. buchneri</i>
	<i>Lb. helveticus</i>	<i>Lb. plantarum</i>	<i>Lb. fermentum</i>
	<i>Lb. salivarius</i>	<i>Lb. sakei</i>	<i>Lb. reuteri</i>

^aWhen fermented.

^bInducible by pentoses.

Source: Salminen S, Wright AVE, Ouwehand A, editors. Lactic acid bacteria microbiological and functional aspects. 3rd ed. New York: Marcel Dekker, Inc.; 2004.

The sequences of 16S rRNA gene and DNA-DNA homology have led to the reclassification of a number of lactobacilli. Detailed grouping of the species was updated by Felis and Dellaglio (2007) which resulted in separating lactobacilli into 12 species groups: (1) *Lb. delbrueckii* (delb), (2) *Lb. salivarius* (sal), (3) *Lb. reuteri* (reu), (4) *Lb. buchneri* (buch), (5) *Lb. alimentarius*–*Lb. farciminis* (al-far), (6) *Lb. casei* (casei), (7) *Lb. sakei* (sakei), (8) *Lb. fructivorans* (fru), (9) *Lb. coryniformis* (cor), (10) *Lb. plantarum* (plan), (11) *Lb. perolens* (per) and (12) *Lb. brevis* (bre).⁽¹⁶⁾

1.3.1.1 *Lactobacillus casei* group

The members of *Lb. casei* group are nonmotile and the G+C ratios of their DNA are between 45 and 47 mol%. The cell wall peptidoglycan is the Lys-D-Asp type and no teichoic acid is detected. They are facultatively heterofermentative lactobacilli that produce lactic acid as the major end product of fermentation and do not produce gas from glucose. They also ferment pentose by phosphoketolase, producing lactic acid and acetic acid. Generally, only L(+) isomer of lactic acid is produced, some strains of *Lb. paracasei* can form an equal amount of both L(+)- and D(-)- isomers.⁽¹⁷⁾ Historically, the members of this group were classified as *Lb. casei* with five subspecies (*Lb. casei* subspecies *alactosus*, *casei*, *pseudoplantarum*, *rhamnosus* and *tolerans*) that are based on phenotypic properties and particularly carbohydrate fermentation patterns as shown in Table 3. Subsequently, the application of molecular techniques, 16S rRNA sequencing (V1 region) and DNA-DNA homology, has led to reclassification of *Lb. casei* group into three species.^(18,19)

1. *Lb. casei*

Lb. casei can be differentiated from other members by its inability to ferment lactose. It can grow at 10°C but not at 45°C; the characteristic which can be used to distinguish *Lb. casei* from *Lb. rhamnosus*. The type strain is strain ATCC 393^T.

2. *Lb. paracasei*

Lb. paracasei can be divided into two subspecies: *Lb. paracasei* subspecies *paracasei* (type strain: ATCC 25302^T) and *Lb. paracasei* subspecies *tolerans* (type strain: ATCC 25599^T). The ability to survive at 72°C is the main feature to distinguish between subspecies of *Lb. paracasei*. *Lb. paracasei* subsp. *paracasei* is able to ferment

fewer carbohydrates than the other subspecies and gives a very different fermentation profile in most identification kits.

3. *Lb. rhamnosus*

Lb. rhamnosus can be distinguished from the other members of the *Lb. casei* group by its ability to produce acid from rhamnose and its ability to grow at 45°C. The type strain is strain ATCC 7469^T.



Table 3 Carbohydrate fermentation by species of the *Lb. casei* group⁽¹⁴⁾

Acid produced from	<i>Lb. casei</i>	<i>Lb. paracasei</i>	<i>Lb. paracasei</i>	<i>Lb. rhamnosus</i>
		<i>subsp. paracasei</i>	<i>subsp. tolerans</i>	
<i>N</i> -acetyl-glucosamine	+	+	+	+
Adonitol	-	d	-	-
Amygdalin	+	+	-	+
<i>D</i> -Arabinose	-	d	-	d
<i>L</i> -Arabinose	-	-	-	-
<i>D</i> -Arabitol	-	-	-	-
<i>L</i> -Arabitol	-	-	-	-
Arbutin	+	+	-	+
Cellobiose	+	+	-	+
Dulcitol	-	d	-	d
Erythritol	-	-	-	-
Esculin	+	+	-	+
<i>D</i> -Fructose	+	+	+	+
<i>D</i> -Fucose	-	d	-	-
<i>L</i> -Fucose	D	-	-	d
Galactose	+	+	+	+
<i>b</i> -Gentibiose	-	+	-	+
Gluconate	+	+	+	+
<i>D</i> -Glucose	+	+	+	+
Glycerol	-	d	-	D
Glycogen	-	-	-	-
Inositol	-	d	-	+
Inulin	-	d	-	-
2-Keto-gluconate	-	-	-	-
5-Keto-gluconate	-	-	-	-
Lactose	+	D	+	+
<i>D</i> -Lyxose	-	d	-	D
Maltose	D	+	-	+
Mannitol	+	+	-	+
<i>D</i> -Mannose	+	+	-	+
Melezitose	+	+	-	+
Melibiose	-	-	-	-
<i>n</i> -Methyl glucoside	-	D	-	+
<i>n</i> -Methyl mannoside	-	-	-	-
β -Methyl xyloside	-	-	-	-
Raffinose	-	-	-	-
Rhamnose	+	-	-	+
Ribose	-	+	-	+
Salicin	+	+	-	+
Sorbitol	-	D	-	+
<i>L</i> -Sorbitose	-	d	-	D
Starch	-	d	-	-
Sucrose	-	+	-	+
<i>D</i> -Tagatose	+	+	+	+
Trehalose	+	+	+	+
<i>D</i> -Turanose	-	+	-	+
Xylitol	-	-	-	-
<i>D</i> -Xylose	-	-	-	-
<i>L</i> -Xylose	-	-	-	-

d, 10: 49% of strains; D, 50: 90% of strains produce acid.

Source: Collins MD, Rodrigues U, Ash C, Aguirre M, Farrow JAE, Martinez-Murcia A, et al. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA gene. FEMS Microbiol Lett 1991;77:5-12.

1.4 Applications of LAB

1.4.1 Production of antimicrobial metabolites

LAB produce a number of substances such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins which possess antimicrobial properties. Bacteriocins are generally defined as proteinaceous macromolecules produced by bacteria that are active against closely related bacteria. Bacteriocins produced by LAB have received more attention because of their "GRAS" (Generally Recognized As Safe) status and their potential use as food additives for biopreservation.⁽²⁰⁾ For example, nisin produced by *Lc. lactis* is used predominantly in thermally processed foods and dairy products due to its effectiveness to spoilage, pathogenic bacteria and heat-resistant spore-forming bacteria, including *Bacillus*, *Clostridium*.⁽²¹⁾

1.4.2 Probiotics

Probiotics are described as "live microorganisms which when administered in adequate numbers confer a health benefit on the host". Most probiotic microorganisms belong to LAB such as *Lactobacillus* species, *S. thermophilus* and *E. faecium*.

1.4.2.1 Mechanisms of action of probiotics

The probiotics must be viable at high concentration (at least 10^7 CFU/mL or /g) and can survive in the acidic, gastric environment. Epithelial cells lining the mouth, intestinal tract and vagina, are host to millions of receptor sites for various microbes. Some probiotic bacteria have high binding affinities to colonize the receptor sites on epithelial cells so they can prevent colonization by pathogens. The probiotics can improve bioavailability of nutrients, proteins and lipids, by breaking down in the digestive tract, thus providing benefits, especially for infants and patients.

The probiotics have been proposed for the prevention of diarrhea. For instance, *Lb. rhamnosus* 35 constitutes the active substance of pharmaceutical products that have been successfully used in the treatment and prevention of diarrhea.⁽¹⁴⁾ Several studies have been reported how probiotics counteract diarrhea such as competing with pathogens for nutrients and receptors, inducing hydrolysis of toxins and receptors and inducing production of antimicrobial substances, including peptides of the innate immune system.⁽²²⁾

1.4.2.2 Safety of probiotics

Although most of LAB fall into the category of organisms classified as “GRAS” status, other bacterial genera such as *Enterococcus*, *Bacillus* and streptococci which are not “GRAS”, have also been used as probiotics. Many factors must be addressed in the evaluation of safety of probiotics, including pathogenicity, infectivity and virulence factors, metabolic activity and the intrinsic properties of the microbes.⁽²³⁾

1.4.3 Starter culture

Dairy starter cultures are actively growing cultures of LAB to drive the fermentation process. They have a multifunctional role in manufacturing. The capability of LAB to produce lactic acid from lactose is the major role of dairy starter cultures because acid affects a number of aspects of the manufacturing process, including the composition, quality and texture of the final product. The decrease of pH indirectly affects flavor by controlling the proteolytic activity of both natural milk proteinases and by influencing the biochemical reactions involved in the formation of other flavor compounds. Moreover, other important advantages such as inhibition of undesirable organisms, as well as contribution to health benefits are recognized.⁽²⁴⁾ Dairy products and the respective LAB starters are gathered in Table 4.

Table 4 Various dairy products and LAB species applied in their production.⁽³⁾

Product	LAB species
Yoghurt	<i>Streptococcus thermophilus</i> , <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>
Cottage cheese, Cheddar, Pasta Filata	<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> <i>Streptococcus thermophilus</i> *
Tvarog, blue cheese	<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> <i>Lactococcus lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i> <i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>
Butter milk, fermented cream, butter	<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> <i>Lactococcus lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i>
Ryazanka	<i>Streptococcus thermophilus</i> **
Cheddar, Feta	<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i>
Mozzarella, Pizza cheese	<i>Streptococcus thermophilus</i> , <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>
Masdamer, Gouda, Edam, Tilsitter, soft mould ripened cheese, quark, fermented milk beverages	<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> <i>Lactococcus lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i> <i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>
Mozzarella, Swiss, stabilized soft mould ripened cheese	<i>Streptococcus thermophilus</i>
Swiss, Grana	<i>Lactobacillus helveticus</i> , <i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>
Fermented cream, fermented milk beverages	<i>Lactobacillus acidophilus</i> , <i>Streptococcus thermophilus</i>
Actimel®-like products	<i>Lactobacillus casei</i> , <i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> <i>Lactobacillus rhamnosus</i> , <i>Lactobacillus acidophilus</i>
Swiss, Italian	<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> <i>Lactococcus lactis</i> subsp. <i>lactis</i> var. <i>diacetylactis</i> <i>Lactobacillus helveticus</i> , <i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>

* seldom applied in cottage cheese, ** texturizing strains

Source: Marcelino K. Lactic acid bacteria – R&D for food, health and livestock purposes. Texas, USA: In Tech; 2013.

2. Bacteriophage

2.1 General introduction

Bacteriophages or phages, like all viruses, are obligate intracellular parasites but they can infect only bacteria. Phages are the most ubiquitous organisms on earth, found in all aquatic and terrestrial environment or wherever their host thrives. Typically, one phage can infect only one species or subgroup of a species. Each phage particle contains its genome that is protected by protein coat, or capsid. Phage genome can be either single- or double- stranded DNA or RNA which encode the structural proteins of capsid and other viral proteins essential for virus replication. Phages are not capable of generating energy and have no ribosomes for making proteins.⁽²⁵⁻²⁶⁾

2.2 Basic morphological type of bacteriophages

According to Bradley (1967), phages are divided into six basic types (A to F) based on nucleic acid type and gross morphology (Figure 1). 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 type (B) has a six-sided head and a tail. The tail is relatively flexible which may or may not have terminal appendages. Moreover, the tail is longer than the head diameter and has no contractile apparatus. The third type (C) also has a tail and a six-sided head, but the tail is shorter than the head diameter and may also have appendages attached to it. The fourth type (D) has no tail but is still six-sided in outline though of symmetrical appearance. Each apex of the hexagon has a knob or large capsomere on it. In the fifth type (E), the large capsomeres are absent and the virion presents a simple regular hexagonal outline. The sixth type (F) is quite unlike the others, the virion being in the form of a long flexible filament. Later, the last type of phages (G) was reported. The virion's shaped is pleomorphic with a lipid envelope.⁽²⁷⁻²⁸⁾

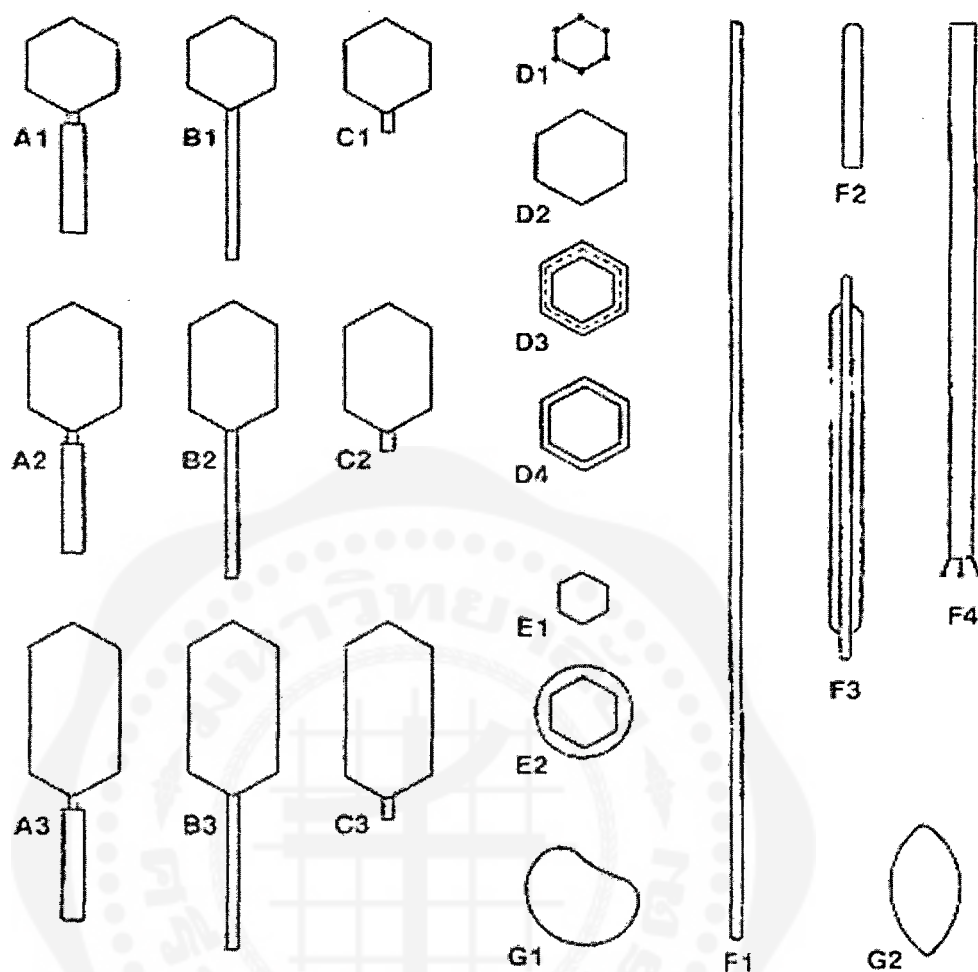


Figure 1 Basic morphological types of bacteriophages.⁽²⁹⁾

Source: Goyal SM, Gerbra CP, Bitton G. Phage ecology. USA: A Wiley-Interscience; 1987.

2.4 Bacteriophage taxonomy and classification

In order to develop a single, universal taxonomic scheme for all viruses, the International Committee on Taxonomy of Viruses (ICTV) was established at an international conference on microbiology in Moscow in 1966.⁽³⁰⁾ Based on the 7th ICTV report and subsequent taxonomic decision approved during the ICTV meeting at the ICTV Congress in Sydney in 1999, the taxonomic lists of virus consisted of families and genera of phages as

shown in Figure 2 and Table 5. The properties used for phage classification are listed in Figure 3.

The most recent development is the recognition of tailed phages to the order *Caudovirales* and of 15 tailed phage genera. In the present ICTV phage classification, the phages are classified into 1 order, 14 families and 37 genera.⁽³¹⁾ The names of the order, families and genera are typically derived from Latin or Greek roots and end in *-virales*, *-viridae* and *-virus*, respectively.⁽²⁵⁾



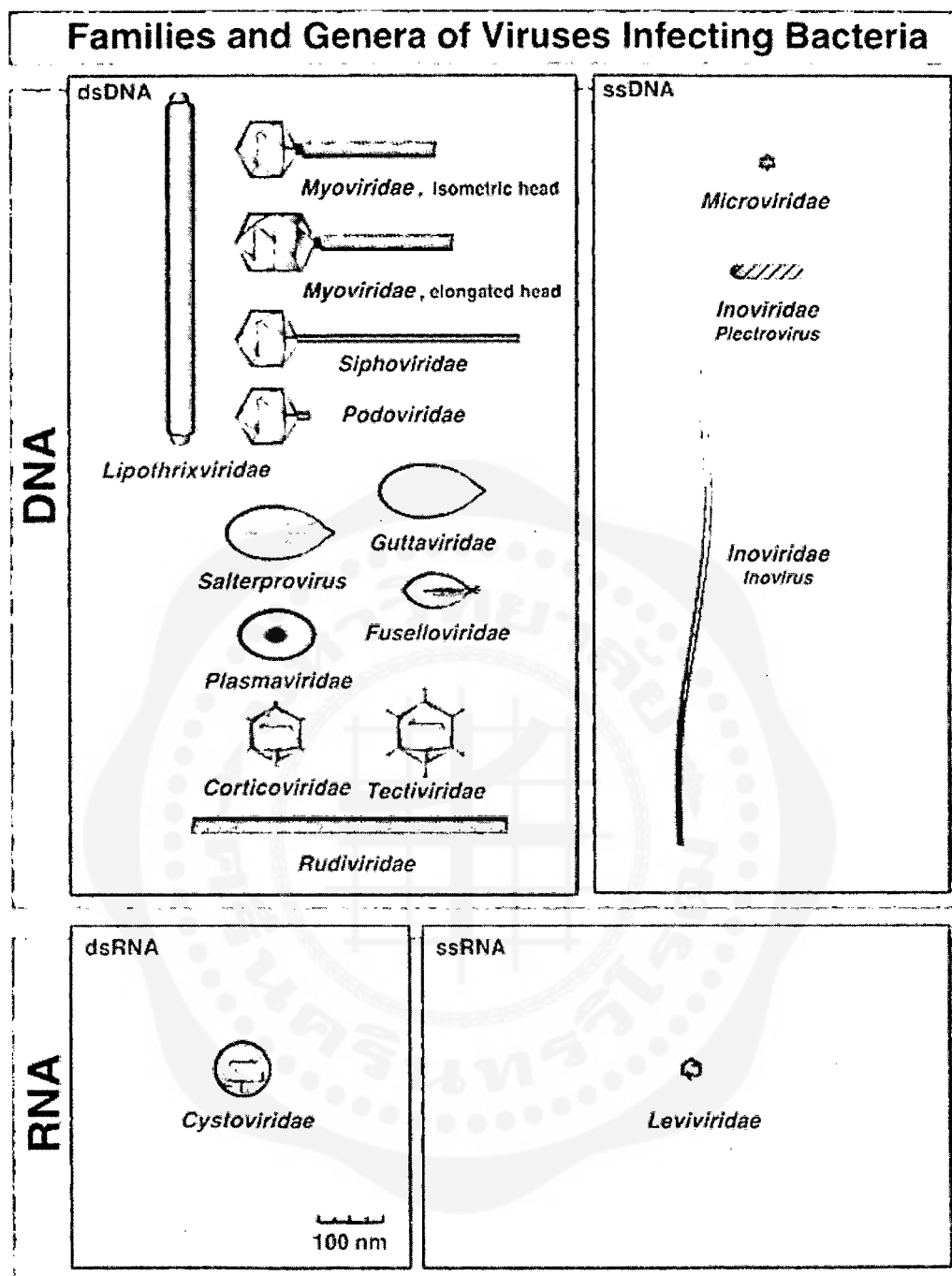


Figure 2 Families and genera of phages.⁽³⁰⁾

Source: Goyal SM, Gerbra CP, Bitton G. Phage ecology. USA: A Wiley-Interscience; 1987.

Table 5 Description of order and families of phages.⁽³⁰⁾

Family	Nucleic acid	Shape	Genus	Type species
Myoviridae	Linear dsDNA	Tailed	T4-like viruses P1-like viruses P2-like viruses Mu-like viruses SP1-like viruses Φ H-like viruses	Enterobacteria phage T4 Enterobacteria phage P1 Enterobacteria phage P2 Enterobacteria phage Mu Bacillus phage SP1 Halobacterium phage Φ H
Siphoviridae	Linear dsDNA	Tailed	λ -like viruses T1-like viruses T5-like viruses L5-like viruses c2-like viruses ψ M1-like viruses Φ C31-like viruses N15-like viruses	Enterobacteria phage λ Enterobacteria phage T1 Enterobacteria phage T5 Mycobacterium phage L5 Lactococcus phage c2 Methanobacterium phage ψ M1 Streptomyces phage Φ C31 Enterobacteria phage N15
Podoviridae	Linear dsDNA	Tailed	T7-like viruses P2-like viruses Φ 29-like viruses N4-like viruses	Enterobacteria phage T7 Enterobacteria phage P2 Bacillus phage Φ 29 Enterobacteria phage N4
Tectiviridae	Linear dsDNA	Polyhedral	Tectivirus	Enterobacteria phage PRD1
Corticoviridae	Circular dsDNA	Polyhedral	Corticovirus	Pseudoalteromonas phage PM2
Plasmaviridae	Circular dsDNA	Pleomorphic	Plasmavirus	Acholeplasma phage L2
Lipothrixviridae	Linear dsDNA	Filamentous	Alphalipothrixvirus Betalipothrixvirus Gammalipothrixvirus	Thermoproteus tenax virus 1 Sulfolobus islandicus filamentous virus Acidianus filamentous virus 1

Table 5 (continued).

Family	Nucleic acid	Shape	Genus	Type species
Rudiviridae	Linear dsDNA	Filamentous	Rudivirus	Sulfolobus islandicus rod-shaped virus 2
Fuselloviridae	Circular dsDNA	Pleomorphic	Fusellovirus	Sulfolobus spindle-shaped virus 1
			Salterprovirus	His1 virus
Guttaviridae	Circular dsDNA	Pleomorphic	Guttavirus	Sulfolobus newzealandicus droplet-shaped virus
Inoviridae	Circular ssDNA	Filamentous	Inovirus	Enterobacteria phage M13
			Plectrovirus	Acholeplasma phage L51
Microviridae	Circular ssDNA	Polyhedral	Microvirus	Enterobacteria phage Φ X174
			Chlamydia microvirus	Chlamydia phage 1
			Bdellovibrionavirus	Bdellovibrio phage MAC1
			Spiromicrovirus	Spiroplasma phage 4
Cystoviridae	Linear dsRNA	Polyhedral	Cystovirus	Pseudomonas phage ψ 6
Leviviridae	Linear ssRNA	Polyhedral	Levivirus	Enterobacteria phage MS2
			Allolevivirus	Enterobacteria phage Q β

*C, circular; L, linear; M, multipartite; S, supercoiled.

Source: Van Regenmortel MHV, Fauquet CM, Bishop DH. Virus taxonomy: Seventh report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press; 2000.

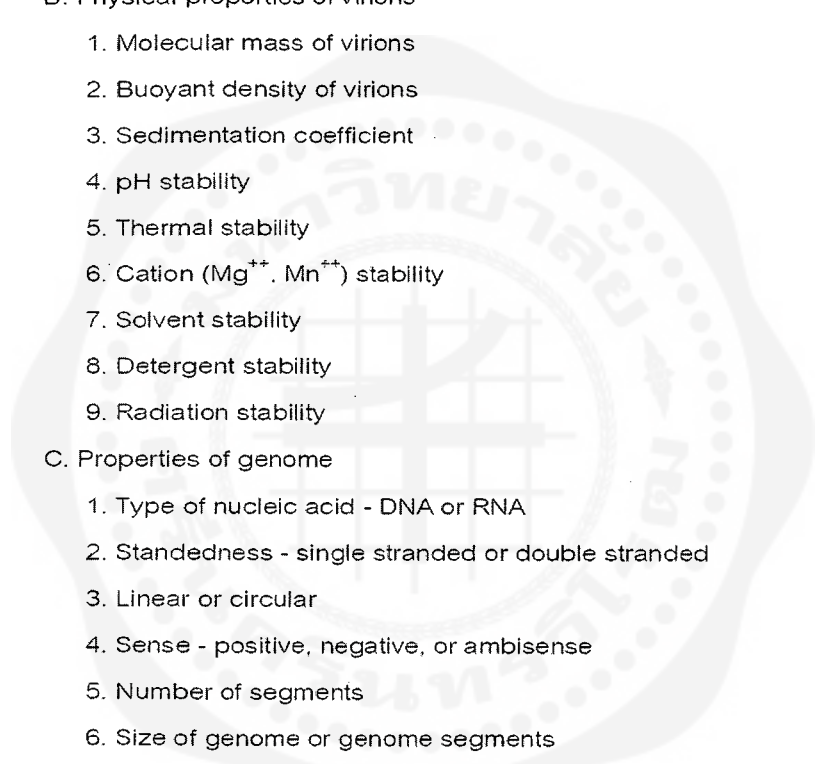
- 
- I. Virion properties
 - A. Morphology properties of virions
 - 1. Virion size
 - 2. Virion shape
 - 3. Presence or absence of an envelope and peplomers
 - 4. Capsomeric symmetry and structure
 - B. Physical properties of virions
 - 1. Molecular mass of virions
 - 2. Buoyant density of virions
 - 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. Standedness - 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
 - D. Properties of proteins
 - 1. Number of proteins
 - 2. Size of proteins
 - 3. Functional activities of proteins
 - 4. Amino acid sequence comparisons

Figure 3 Properties for phage classification.⁽³⁰⁾

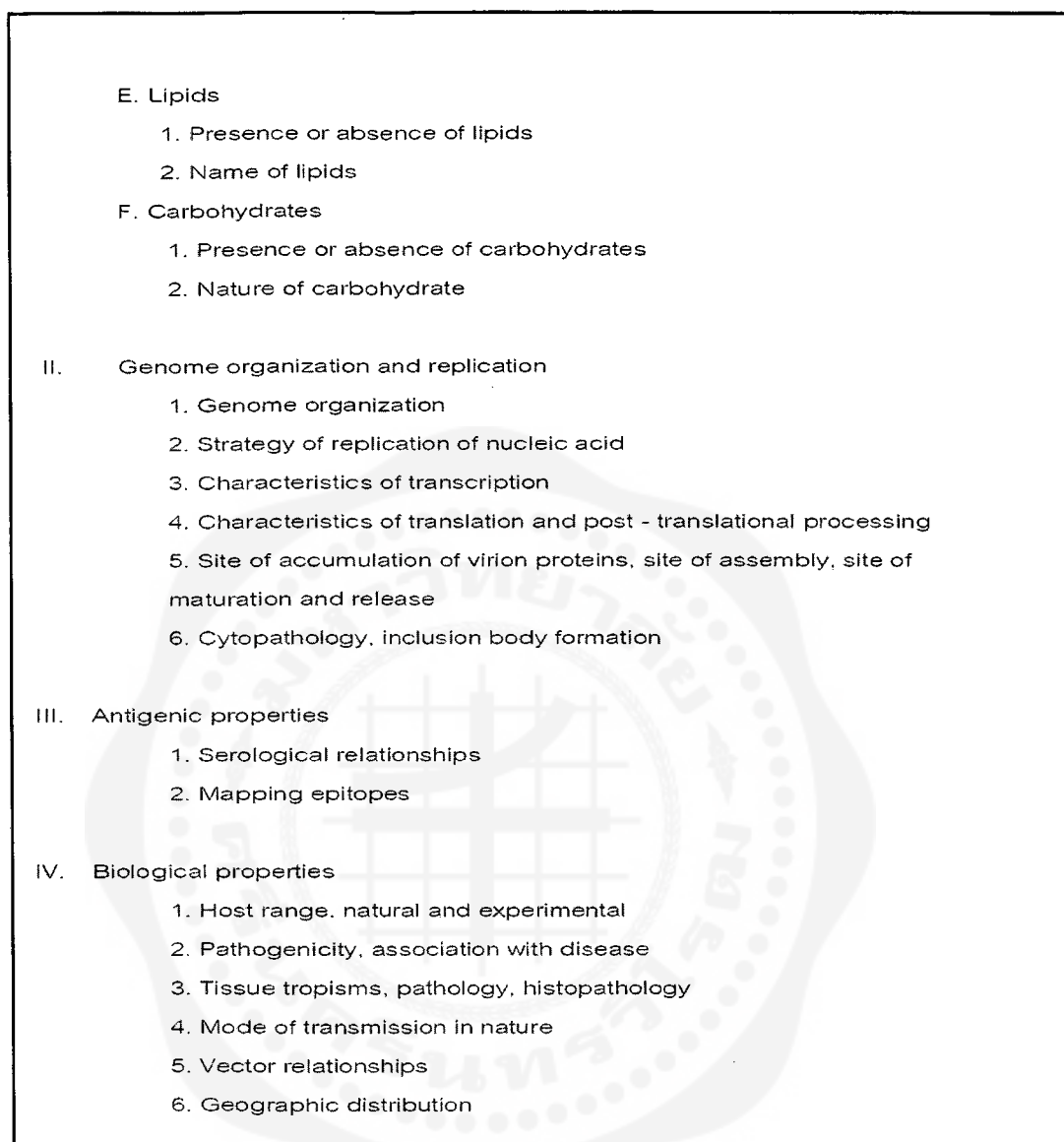


Figure 3 (continued).

Source: Van Regenmortel MHV, Fauquet CM, Bishop DH. Virus taxonomy: Seventh report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press; 2000.

2.4.1 Tailed phages (Order *Caudovirales*)

Tailed phages constitute the largest group of bacterial virus and infect both eubacteria and archaea. Virions consist of a head with cubic symmetry and a helical tail. Tailed phages are extremely varied in size, DNA content, genome structure and physiology.⁽³²⁾

2.4.1.1 Structure of tailed phages

Virions have no envelope and consist typically of protein and DNA. Their DNA compositions generally resemble that of their hosts, but some DNAs contain unusual bases such as 5-hydroxymethylcytosine or 5-hydroxymethyluracil. Their heads are icosahedral either regular or prolate. Capsid proteins are organized into capsomers. Tails are true helical or consist of stacked disks and terminal adsorption structures such as base plates, spikes, or fibers as shown in Figure 4. Head, tail and tail fibers are synthesized by separate pathways and assembled later.⁽³³⁾

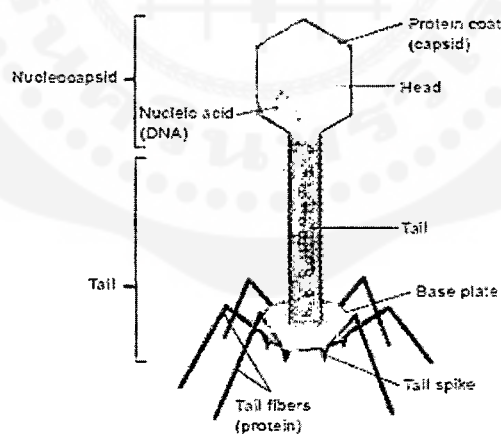


Figure 4 Typical structure of tailed phage (T4).⁽³³⁾

Source: Nester EW, Robert CB, Nester MT. Microbiology: a human perspective. USA: William C Brown; 1995.

2.4.1.2 Classification of tailed phages

Tailed phages are divided into three families.

1. *Myoviridae* (25% of tailed phages)

Myoviridae has contractile tails consisting of a sheath and a central tube. The sheath is separated from the head by a neck. The examples of phage in this family are coliphage T4 and coliphage P1.

2. *Siphoviridae* (61% of tailed phages)

Siphoviridae has long and non-contractile tails. The examples of phage in this family are coliphages λ and T5.

3. *Podoviridae* (14% of tailed phages)

Podoviridae has short tails. The examples of phage in this family are *Salmonella* Typhimurium bacteriophage P22 and coliphage T7.

2.5 Virulent-temperate phages

Phages can be divided into two groups based on life cycle. The first group is virulent phages that can only multiply by means of lytic cycle. In the lytic cycle, phage virion adsorbs to the surface of host cell and injects its genome which induces switching of host metabolism and sets up molecular machinery to produce phage progeny. The host cell then lyses, liberating many new phages. The other group is temperate phages which are able to initiating a lytic cycle like their virulent counterparts, but they are also capable of initiating a lysogenic cycle. In the lysogenic cycle, phage DNA is integrated into host genome and assumes a quiescent state called a prophage. It remains in this condition indefinitely, being replicated as its host cell and new bacteria will inherit the viral DNA. When the bacterium contains a silent phage DNA, these cells are said to be lysogenized or lysogenic because one of these prophages occasionally comes out of its quiescent condition and enters the lytic cycle.⁽²⁵⁾

2.5.1 Virulent phages

The virulent phages generally encode many host-lethal proteins which result in disruption of host replication, transcription, or translation and may also degrade the host genome, destroy or redirect certain host enzymes, or alter the bacterial membrane.⁽²⁵⁾

2.5.2 Temperate phages

In 1950, Lwoff and Gutmann discovered the individual cells of *Bacillus megaterium* in microdrops which could continue to divide in phage-free medium with no sign of phage production, but that an occasional cell would lyse spontaneously and liberate phages. Lwoff named the hypothetical intracellular state of the phage genome as a prophage, and showed later that by treating lysogenic cells with inducing agents such as ultraviolet light, the prophage could be uniformly induced to come out of its quiescent state and initiate lytic growth.⁽²⁵⁾

Lwoff (1953)⁽³⁴⁾ listed the characteristic signs of lysogeny as follows:

1. In a lysogenic culture, lysogenesis is a property of every cell and every spore.
2. Bacteria of a lysogenic culture generally can adsorb the mature phage produced by the culture, but are not damaged by it.
3. Lysis of lysogenic bacteria by enzymes, by other phages, or by mechanical means does not liberate mature phage particles. The intracellular phage in lysogenic bacteria is noninfectious.
4. Infection of a susceptible bacterial culture by a temperate phage may result in the conversion of a considerable proportion of the bacterial cells to the lysogenic condition, potentially capable of liberating the same kind of phage that was used to infect them.
5. Lysogenic bacteria can multiply without liberating mature phage and can undergo many cell divisions in the absence of external phage without losing the lysogenic propensity.
6. Lysis of single lysogenic bacterial cells occurs spontaneously or after a characteristic latent period following induction is accompanied by the release of many mature phage particles.

Temperate phages can carry their host genes from one bacterial cell to another which is also called transduction. Most of the temperate phages have their own specific sites of integration and when their prophages are induced during the transition to lytic growth, the prophage DNA is sometimes excised mistakenly along with a piece of host

DNA. This produces a transducing phage which can affect a specialized transduction by carrying this particular piece of host DNA into other cells, thus changing their genomes through recombination with the transduced segment. However, some temperate phages integrate randomly into the genome and then always carry some host DNA with them, so they are able to affect a generalized transduction. Both temperate and virulent phages may also produce generalized transduction by mistakenly producing particles filled with host rather than phage DNA; those are generally rare and cannot reproduce, but may still contribute substantially to bacterial genetic exchange in nature.

2.6 The infection process

In 1939, Ellis and Delbrück studied details of phage infective process by using one-step growth curve experiments (Figure 5). This type of experiments is performed by mixing the phages and appropriate host cell at a low multiplicity of infection and diluting the infected cells after a few minutes for phage adsorption.⁽³³⁾ The samples are withdrawn at time intervals to determine the minimum latent period of intracellular virus growth and the infective centers. The latent period is defined as the minimum time between the adsorption of phage to host cell and the lysis of host cell for the liberation of phage progeny. The plaque count generally remains constant until the end of the latent period. It then rises sharply until all infected cells have lysed and finally remains constant at the new higher level. The ratio between the plaque count obtained before and after lysis gives the average burst size. The infective center is either a single phage particle or an infected cell that bursts on the plate to produce a single plaque.^(25,34)

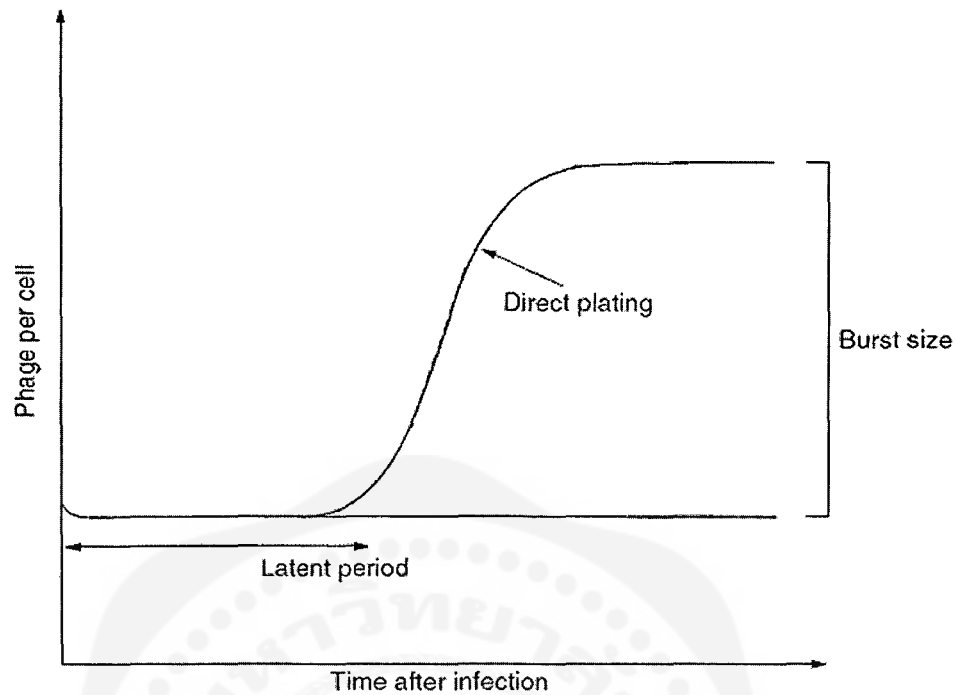


Figure 5 Classical one-step growth of bacteriophage.⁽⁸⁾

Source: Salminen S, Wright AVE, Ouwehand A, editors. Lactic acid bacteria microbiological and functional aspects. 3rd ed. New York: Marcel Dekker, Inc.; 2004.

Both the burst size and the latent period are characteristics of each phage and host strain and are influenced by other physical factors such as type of medium and temperature.⁽¹⁹⁾ Moreover, the eclipse period can be defined as the time period taken for the appearance of first intracellular phages as determined by artificial lysis and during the period only naked phage DNA is present in the cell. The typical lytic cycle of phages is shown in Figure 6.

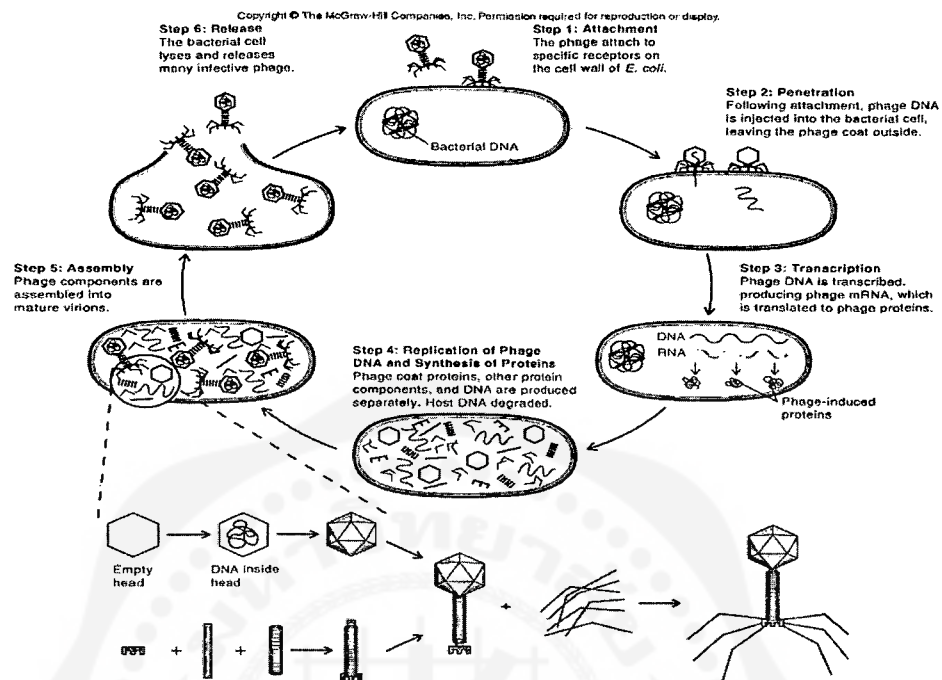


Figure 6 Steps in the replication of T4 phages in *E. coli*.⁽³³⁾

Source: Nester EW, Robert CB, Nester MT. Microbiology: a human perspective. USA: William C Brown; 1995.

2.6.1 Adsorption

Adsorption is a key to determine the specificity of the phage-bacterium relationship. Tailed phages attach to specific surface molecules or capsules on their specific host cells by adsorption structures, such as fibers or spikes. A single mutation on the part of specific surface molecules of a host cell can result in loss of ability to adsorb a particular bacteriophage, without loss of susceptibility to other closely related phages.⁽³⁴⁾ Efficiency of adsorption is important parameter that may vary for a given phage-host system depending on external factor and physiological state of host cell.⁽²⁵⁾ The ionic environment

is a crucial factor in adsorption process. Many phages require adsorption cofactors, such as Ca^{2+} , Mg^{2+} , or some amino acids. For example, coliphage T4 requires tryptophan for binding to their host cell. Conversely, unfavorable salt concentration can prevent phage adsorption by coating the bacterial receptor sites. The physiological state of host cell can change the concentration of particular cell-surface molecules and thus alterations in the efficiency of infection by certain phages.

Some phages have two stages and two different receptors of the adsorption, such as T4-like phages that bind at least three of their six long tail fibers to primary receptor molecules to trigger rearrangements of baseplate components and then irreversibly bind to a second receptor. In this family, members may use a variety of primary receptors, but they seem to use the heptose residue of the LPS inner core for the secondary receptor.⁽²⁵⁾

2.6.2 Penetration

Following the irreversible attachment, the phage DNA is injected into the host cell through the tail. Generally, tail tip has an enzymatic mechanism for penetrating the peptidoglycan layer and the inner membrane to release the DNA directly into the host cell. Many phages circularize their DNA rapidly by means of sticky ends or terminal redundancies, or have the linear ends protected because their DNA are potentially susceptible to host exonucleases and restriction enzymes.⁽²⁵⁾

2.6.3 Transition from host to phage-directed metabolism

Once inside the cell, the strong phage promoters are recognized by the host RNA polymerase resulting in transcription of immediate early genes. The products of these early genes have functions to protect the phage genome and restructure the host appropriately for phage replication, for instance, they inactivate host proteases and restriction enzymes, directly terminate various host macromolecular biosyntheses, or destroy some host proteins. The set of middle- and late- genes are often then transcribed, producing products that synthesize the new phage DNA and the components of the phage particle, respectively.

2.6.4 Morphogenesis

The phage DNA is packaged into preassembled icosahedral protein shells known as procapsids. Morphogenesis step usually involves complex interactions between

the major head structural proteins and specific scaffolding proteins, the latter plays a role in determining the size and shape of the phage head. Phage genome enters the procapsid through the portal which is embedded, replacing one of the 12 pentamers of the icosahedron to form a channel through the capsid wall.

2.6.5 Cell lysis

The final step, lysis of the host cell requires two phage-encoded protein factors, an endolysin or lysozyme and a second protein called a holin. The endolysin is a soluble enzyme that accumulates within the host cytoplasm during the late stages of infection, with the capacity to cleavage the bond holding the host peptidoglycan. Holin proteins form holes in the inner membrane at the appropriate time to allow the lysin to reach the peptidoglycan layer, causing rapid cell lysis and releasing the phage progeny from the cell.

3. Bacteriophages of lactic acid bacteria and their impacts on dairy fermentation

3.1 Bacteriophages of lactic acid bacteria

The infection of lactic acid bacteria by phages is particularly problematic in the dairy fermentation process which repeatedly use the same cultures or mixed cultures that are sensitive to phage infection. The dairy industry has been dealing with the phage infection and has relied on a variety of practical strategies to control phages which include adapted factory design, improved sanitation, process changes, adequate ventilation, improved starter medium, culture rotation and the use of phage-intensitive mutants. Nevertheless, phage infection of starter culture still occurs nowadays leading to fermentation failure in the dairy industry.⁽⁴⁹⁻⁵⁰⁾

All known phages of lactic acid bacteria or LAB phages belong to the order *Caudovirales* and most of them are classified in the family *Siphoviridae* that possess icosahedral capsids and long non-contractile tails. LAB phages contain double-stranded DNA with genome sizes range from 14 to 141 kb. Their G+C contents are similar to those of their hosts, ranging from 33 mol% in *Lb. delbrueckii* subsp. *lactis* phages to 48 mol% in *Lb.*

casei phages. LAB phages have been isolated from a variety of natural sources such as raw milk, yogurt, cheese, sauerkraut, coffee, sewage, saliva, human vagina and upon induction of lysogens. Most of them showed very restricted host ranges, limited to one or a few starter strains of the same species. Temperate phages generally exhibit rather narrow host ranges which are caused by homoimmunity against superinfection by other related phages or restriction modification systems.⁽³⁴⁾

3.1.1 *Lactobacillus* phages

Lactobacillus phages are of substantial research interest since their host bacteria are involved in numerous fermented food products. The variety of food fermentation lactobacilli-based processes can become disrupted by phage contamination. For example, the coculture of *Lb. delbrueckii* and *S. thermophilus* used in the production of yogurt, phage attack leads to failed or slow fermentation. *Lactobacillus* phages have been isolated from numerous other spontaneous fermentation processes of coffee, pickled cucumbers, cereals, wine and meat.⁽³⁵⁻³⁶⁾

Many virulent phages of lactobacilli have been isolated from milk fermentation processes. These include phages for *Lb. delbrueckii* subsp. *lactis* and *bulgaricus*, *Lb. helveticus*, *Lb. acidophilus*, *Lb. gasseri*, *Lb. casei* and *Lb. rhamnosus*. They mainly belong to *Siphoviridae* with a few in the family *Myoviridae*.⁽³⁷⁻³⁸⁾ Lysogeny is widespread throughout the family Lactobacillaceae such as *Lb. casei* S-1 strain (ATCC 27139) harbors the temperate phage Φ FSW which was not inducible with mitomycin C or ultraviolet light, although spontaneous induction was observed.⁽³⁹⁾ In addition, a virulent form, Φ FSV, that has been originated from Φ FSW was isolated from abnormal fermentations with S-1 strain. This represented the first formal evidence that the temperate bacteriophages can be a source of virulent phage outbreaks in dairy fermentations. The presence of the temperate phages may be a constant source of virulent phages and leads to problems in fermentation processes.^(34,40)

3.2 Sources of phage contamination

In dairy plants, phages can originate from various sources. It is important to identify the potential sources of phage contamination and to limit the entry to the manufacture processes.

3.2.1 Raw milk

The most abundant source of virulent phages is raw milk which is an ecological niche for some LAB. LAB phages occur naturally in raw milk at low titers (between 10^1 - 10^3 PFU/mL) and higher in number in dairy processing.⁽⁴¹⁻⁴²⁾ Phage concentrations in raw milk also depend on conditions of collecting, handling and storing of milk by the supplier (farm), transporting to the plant and finally, handling of the milk in the plant itself. Phage biodiversity is increased after adding milk collected from different farms and the number can be even higher in processed milk. Madera et al. (2004) reported that almost 10% of raw milk samples collected from different dairy farms in Spain contained infectious lactococcal phages.⁽⁴²⁻⁴³⁾ Using a multiplex PCR method, lactococcal and streptococcal phages have been detected in more than one-third of milk samples used for yogurt production in Spain.⁽⁴⁴⁾

3.2.2 Milk powder and whey protein concentrates

The dairy industry, particularly cheese manufacturing, recycles whey protein concentrates (WPC) to improve the taste or texture, to increase its nutrient value and to standardize milk before the fermentation process or to increase the yield of the final product.⁽⁴⁵⁻⁴⁷⁾ Upon whey or milk protein concentration, phages may remain in the whey protein concentrate (liquid or dried) and contaminate the products to which it is added.⁽⁴⁸⁾ When using membranes to separate whey components, it is highly possible that phages will be retained by ultrafiltration and/or microfiltration.⁽⁴⁹⁾ Depending on whether the retentate or the permeate is used, phages might still be present and cause problems in subsequent manufacturing processes.

Ideally, milk by-products should either be treated to inactivate the phages or be used in a type of fermentation that is driven by different starter cultures. For example, if the whey was collected from a cheddar fermentation made using mesophilic starter cultures, by-products of this whey could safely be used in yogurt manufacture or in a cheese process requiring thermophilic cultures. In addition, the use of concentrated milk products from another dairy plant (which may use different starter cultures) can offer additional protection. Although the latter will also most likely increase the phage biodiversity within the factory.⁽⁴⁵⁾

3.2.3 Factory environment

The dairy plant environment is one of the most abundant sources of virulent phages and several dispersion pathways may be occurred in the plant. Aerosolization is currently recognized as an important route of dispersion.⁽⁵⁰⁾ Personnel movements or transport of equipment and/or raw materials might cause the dispersion of phage particles as an aerosol. The consequences of this aerosolization are even worse if dispersion is unrestricted between contaminated and uncontaminated zones. The most common sources of phage contaminations are valves, crevices and “dead ends” (difficult cleaning and disinfection places) of production lines. Also, the formation of biofilms on dairy equipment can lead to serious phage problems. Recently, Verreault et al. (2011) showed that qPCR can detect lactococcal phages at high levels on various equipments and objects found in cheese plants such as walls, pipes, door handles, floors, office tables and even on cleaning materials.⁽⁵¹⁾ Raw milk handling, cheese milk processed in open vats and whey handling can lead to spreading of phages in the air. Consequently, virulent phages can circulate through the air far away from their aerosolization source due to the ability to bind to small particles ($<2.1 \mu\text{m}$), since bioaerosols can remain in the air for long periods.⁽⁵⁰⁻⁵²⁾ Even though qPCR cannot distinguish infectious from non-infectious viral particles, this data demonstrated the importance of good manufacturing and cleaning practices as well as personal training to avoid contamination of the raw or treated products and dissemination of phages.⁽⁴⁵⁾

3.2.4 Starter cultures

Data derived from genome sequencing projects have shown that many LAB strains contain prophages.⁽⁵³⁾ When bacteria carry a prophage, the cell is called a lysogen. The use of lysogenic starter can be a source of virulent phages because prophage can either start the lytic cycle or its genome can integrate into the bacterial chromosome and follow bacterial multiplication. Under stress conditions, such as heat, salt, antimicrobials, starvation or UV can induce prophages and leading to lytic cycle. Moreover, the induction can occur naturally with a frequency of even up to 9%.⁽⁵⁴⁾ Thus, the use of lysogenic strains in a starter culture may lead to cell lysis and failure fermentation.

Capra et al. (2010) reported the isolation of two lytic phages from a pure culture of *Lb. paracasei* and indicated that both phages could most probably have evolved from a lysogenic state.⁽⁵⁵⁾ The most recent analysis revealed that 25 out of 30 commercial collection or dairy isolated *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* were found to carry inducible prophages.⁽⁵⁶⁾ Thus, it should be mentioned that most starter culture suppliers should test their strains for the presence of prophages and their natural induction rate. Lysogenic strains with easily inducible prophages should be carefully investigated when selecting strains and designing cultures for specific industrial fermentation processes.⁽⁴⁶⁾

3.3 Phage control strategy

Since the presence of phages is unavoidable in the dairy plant environments, phage control strategies are designed to manage rather than eradicate them.⁽²⁵⁾ The procedures applied to minimize phage in dairy environment are plant design, optimized sanitation, ingredients treatment, phage inhibitory media and design of starters.

3.3.1 Plant design

One of the critical factors for preventing phage infection is the layout of a dairy factory. Contact between raw materials and waste (whey or water) should be avoided. For example, the milk reception should be separated from other plant areas, as phage-containing aerosols can be generated during tanker emptying and raw milk spoilage. The whey tanks and separators should be placed in a separate area situated as far as possible from the starter room and the cheese manufacturing vats.⁽⁵⁷⁾ The starter preparation room should be sealed off from the production areas and maintained under a positive pressure of filtered air. The air in the starter preparation room must be filtered to remove small particles which may bind phages and the air inlet for the filters should be located as far as possible from the milk silos and whey tanks. The efficiency of the filters should be checked routinely.⁽⁵⁸⁻⁵⁹⁾

Additionally, stainless steel with a high grade of polish is the ideal material for all the equipment used in the fermentation processes, as it should be subjected to an efficient cleaning and sanitizing before and after use. The fermentation tanks must be closed, sterilized by heat or sanitizers, with positive pressure filtered air in the headspace. The tanks should be monitored periodically for cracks.⁽⁵⁹⁾

3.3.2 Sanitation

Effectiveness of sanitation procedure is a critical factor in avoiding the contamination of bacteria and it is also the most efficient way of maintaining phage levels as low as possible within the dairy. Following factors are considered in the selection of a sanitizer: a fast antimicrobial activity, ease of application, low cost, lack of negative impact on the final product and degradation into harmless final compounds. Recently, the effectiveness on phage inactivation is a criterion taken into consideration which is reflected by an increasing number of studies on their viral effectiveness.⁽⁴³⁾

3.3.3 Ingredient treatment

As LAB phages are commonly present in milk and milk-derived ingredients, they should be treated to reduce the viral titers. Although heat treatment and pasteurization are commonly used for the pre-treatment of raw milk, high thermal resistance has been reported for some phages infecting *Lc. lactis*, *S. thermophilus*, *Lb. casei* and *Lb. paracasei*.⁽⁶⁰⁾ Thermo-resistant phages have also been found in whey, brine and cream at high titers (10^9 PFU/mL). Thus, the recycling of these by-products should be avoided since the return of even small quantities of phages can lead to constant propagation and heat treatment of by-products, prior to recycling, is recommended to prevent recontamination.⁽⁴⁹⁻⁵⁰⁾

3.3.4 Phage inhibitory media

Phage inhibitory media (PIM) for bulk starter media are usually employed to inhibit or delay phage propagation. For example, culture media that contain chelating agents, such as phosphates or citrates are capable of binding divalent cations, thereby preventing the phages to complete their lytic cycle.⁽⁶¹⁾ The use of sodium tripolyphosphate-high solubility (TAS) at low concentrations (0.3–0.5%) in milk was effective at inhibiting the lytic cycle of LAB phages.⁽⁶²⁻⁶³⁾ However, some bacterial strains showed a delayed growth and acidification activity due to the buffer ability of the added phosphates.

3.3.5 Design of starters

In the past, the production of traditional dairy products, particularly cheese, commonly use natural starters which are composed of an undefined mixture of different strains and/or species. The undefined starters might have consisted of phage-resistant strains because they are grown in the presence of phages. Due to the limited

reproducibility of their technological performance, modern industrial fermentation replaces undefined starters by well-defined, direct vat inoculated (DVI), high concentrated ($>10^{10}$ CFU/g) and product-optimized starters, containing from two to five phage-unrelated strains.⁽⁶⁴⁻⁶⁵⁾ The defined starter cultures are widely used in large-scale production facilities because of the significant degree of control over fermentation processes and complementary fermentation properties, such as rapid acidification, gas formation, texturization and development of flavor and aroma compounds. Defined starter cultures usually consisted of two or three phage-unrelated strains which can consistently enable producers to obtain high quality standard products.⁽³⁾ In addition, the rotation of defined phage-unrelated cultures is an efficient phage control system to avoid recontamination of the same phage.

3.4 Phage defense mechanisms of lactic acid bacteria

In order for the lactic acid bacteria to survive in an environment full of LAB phages, they have evolved defense mechanisms against the phages. These defense mechanisms have been divided into five groups, each one targeting a different step of phage multiplication cycle: (i) inhibition of phage adsorption, (ii) blocking of phage DNA injection, (iii) restriction modification systems, (iv) phage abortive infection systems and finally, (v) CRISPR/cas systems.

3.4.1 Inhibition of phage adsorption

Inhibition of phage adsorption to bacterial cell is a simple method that can often be used to induce the generation of bacteriophage-insensitive mutants (BIMs). The mechanisms are associated either with physical masking of the receptor, or with changes in its structure, or even with its absence in the cell envelopes.⁽⁶⁴⁾ Lack of a functional receptor might be due to spontaneous mutations in the genetic material, leading in turn to bacteriophage-insensitive mutants.

The inhibition of phage adsorption mechanisms are not only mediated by the bacterial chromosome, but also by acquired plasmids. The best documented plasmid-encoded mechanisms of inhibition of phage adsorption rely on either direct synthesis of cell surface antigens or the production of extracellular carbohydrates. The former reveals phage specificity, whereas the latter seems to restrict access to the bacterial cell for various

harmful factors.⁽⁶⁶⁾ The production of extracellular carbohydrates, called exopolysaccharides (EPSs) is important in the protection of bacterial cell not only against harsh environments, but also against the phages. There is some evidence that EPSs contain sugar residues which are similar or even identical to initial phage receptors. For example, phage-insensitive strains that carry EPS-encoding plasmids may cause phage immobilization by binding to EPS.⁽⁶⁷⁾ However, EPS-producing phage-resistant strains might be limited to a narrow range of dairy products because the polysaccharides have effects on the final product properties such as texture, viscosity and smoothness of mouthfeel.⁽³⁾

3.4.2 Blocking of phage DNA injection

Following successful adsorption, phage DNA is introduced into the bacterial cell and leading to the transcription of phage DNA and liberation of phage progeny. Watanabe et al. (1984) investigated on the interaction between phage PL-1 and *Lb. casei*.⁽⁶⁸⁾ In this study, an electron microscopy image showed the adsorption and injection of phage PL-1 to its host cell after empty capsids were observed on the surface of the sensitive strain. However, it was shown that no bacterial lysis occurred, despite phage adsorption to cell envelopes. These results suggested that phage DNA injection might be interrupted. Intensive attempts to elucidate the injection blocking phenomenon have led to important identification of different Sie (superinfection exclusion) or Sie-like systems which are membrane-associated proteins that prevent the injection of DNA into the host cell and are often found encoded on prophage regions of the bacterial chromosome.⁽⁶⁹⁾

3.4.3 Restriction modification systems

After the phages inject their DNA into the host cell, phage infection might be completed or hindered by the presence of restriction modification (RM) systems. The RM systems are widely distributed in LAB, particularly in lactococci. These systems recognize a specific DNA sequence and involve two enzymes: endonuclease (for restriction) and methyltransferase (for modification). The endonuclease is responsible for degradation of unmethylated foreign DNA, including phage DNA which lacks a unique methylation pattern, while the methyltransferase protects the host DNA against degradation by transferring of a methyl group from S-adenosyl-L-methionine to either on adenine or cytosine located within

the recognition sequence.⁽⁸⁾ Thus, this RM system is an anti-phage mechanism that limits phage proliferation and causing no harm to host cell.

Generally, the RM systems have a broad range of efficiency and can inactivate many different kinds of phages as they are not dependent on phage species or morphology. It only requires that the recognition sequence specific for the RM system is present in the phage DNA and this sequence has not been modified by methylation. The DNA of progeny phages that has escaped restriction by an RM system will be methylated and therefore, these phages can circumvent that specific RM system and reinfect the bacteria with a high efficiency. Thus, the RM systems alone are not very powerful, unless they are combined with other kinds of defense mechanisms. The efficiency with which they are able to restrict the phage DNA depends on the number of recognition sites. As the number of recognition sites in viral DNA increases, the efficiency of plaquing (EOP; the plaque count obtained under a certain set of conditions relative to the plaque count obtained under standard conditions) decreases exponentially.⁽⁷⁰⁾

3.4.4 Phage abortive infection systems

When phages injecting their DNA into the host cell without being digested by the activity of the RM systems, the abortive infection mechanisms (Abi) are able to interrupt phage development (genome replication, transcription, translation, packaging, or assembly of phage particles). These mechanisms can lead to a release of few or no phage progeny and to the death of the individual infected cell. Thus, it also resulted in a complete absence of phage-derived plaques or a severe reduction in plaque size, decreased EOP and burst size.⁽⁸⁾

3.4.5 CRISPR/cas systems

CRISPR/cas systems have recently been described in the naturally-occurring distinct phage defense system of prokaryotes. To date, CRISPR loci are found in the genome of many LAB species, namely, *S. thermophilus*, *Lb. acidophilus*, *Lb. brevis*, *Lb. casei*, *Lb. crispatus*, *Lb. delbrueckii*, *Lb. fermentum*, *Lb. helveticus*, *Lb. rhamnosus* and *Lb. salivarius*.⁽⁴⁵⁾ CRISPR/cas systems are composed of two specific determinants: (i) clustered regularly interspaced short palindromic regions (CRISPR array) and (ii) regions encoding CRISPR associated (Cas) proteins. The CRISPR arrays consist of non-coding sequences

composed of unique phage-derived spacers (21-72 bp) separated by short direct repeated sequences (21-48 bp) of bacterial origin. On the other hand, *cas* genes encode a polymorphic family of proteins constitute a heterologous group of proteins, which contain various functional domains, for example those typical for nucleases, helicases and nucleic acid binding proteins.⁽⁷¹⁾ The specific role of individual Cas proteins vary as they were shown to be engaged at various stages of CRISPR-conferred resistance. CRISPRs, together with *cas* genes form the CRISPR/*cas* immune systems that provide adaptive immunity against phage and invasive genetic elements.

3.5 Selection of phage-resistant strains

3.5.1 Classical methods

The method used to isolate phage-resistant strains is called secondary culture technique in which the sensitive strains undergo selective pressure of their specific phages. After liquid cultures exhibit complete lysis of the sensitive strains, they are further incubated 1-2 days for the secondary growth. This secondary growth culture is streaked on adequate solid medium. The grown colonies are consecutively cultured in liquid medium with the same selected phage for at least three rounds. Resultant isolates that are able to grow normally in the presence of the specific phage are considered as true phage-resistant mutants.^(4,72)

3.5.2 Bacteriophage insensitive mutant (BIM) system

The development of BIMs is a general way to obtain phage-resistant mutant strain without genetic manipulations. After the long-term susceptible bacteria expose to the phages, some of them survive. This may be because some bacterial cells have lost the capacity to adsorb phage due to a spontaneous mutation in the gene encoding phage receptor. The lack of a polysaccharide receptor for 936- and P335-type phages is associated with mutations in genes involved in its synthesis or transport. It is well documented that phage insensitivity of *Lc. lactis* strains is correlated with loss of the galactose-associated receptor in the cell wall.⁽³⁾ This disturbs the synthesis of wall components and, as a consequence, insensitive strains often lose their industrial properties, such as the ability to produce acids and reveal poor growth in comparison to wild type strains. Moreover, BIMs may have substantial disadvantages such as a high frequency of

phenotype reversion and changes in bacterial physiology (e.g. a reduced growth rate). Nonetheless, many BIMs have been isolated and are being used successfully under industrial conditions. Thus, the potential of BIMs needs to be evaluated because it may be unable to withstand phage attacks in industrial settings.

3.5.3 Plasmid concept

Among the acknowledged and widely applied methods of obtaining starter strains resistant to phage infections is conjugational transfer of plasmids conferring phage resistance determinants.⁽⁷³⁻⁷⁴⁾ In lactococci, most of the bacteriophage defense systems occur naturally on plasmids (natural, plasmid-encoded phage-resistance systems) such as restriction/modification (R/M) or abortive infection (heat-sensitive phage resistant (*Hsp*+) or other abortive infection (*Abi*+)). In this species, many various conjugal plasmids conferring phage resistance have been identified. For example, plasmid pTR2030 was isolated from strain ME2 that encoded heat-sensitive phage resistance (*Hsp*), restriction-modification (*LlaIR/M*) as well as conjugal transfer (*Tra*+) genes.⁽⁷⁵⁾ Its introduction via conjugation into other lactococcal strains, including *Lc. lactis* subsp. *cremoris*, resulted in phage-resistance phenotypes.⁽⁷⁶⁾

CHAPTER 3

MATERIALS AND METHODS

3.1 Equipment

Agarose gel electrophoresis equipment (Mupid, Japan)
Amicon Ultra-50 centrifugal filters (Merck, USA)
Autoclave (Tomy, Japan)
Freezer -20°C (Sanden Intercool, Thailand)
Geldoc XR⁺ (Bio-Rad, USA)
HPLC (Shimadzu, Japan)
Hot-air sterilizing oven (Thermo Fisher Scientific, USA)
Incubator (Gallenkamp, UK)
Laminar flow (Science Tech, Canada)
Microcentrifuge (Witeg Labortechnik GmbH, Germany)
Microscope (Olympus Optical Co. Ltd, Japan)
pH meter model 215 (Denver Instrument, USA)
Refrigerated centrifuge (Thermo Fisher Scientific, USA)
Shaker (Chemoscience, Thailand)
Syringe 10 mL (Nipro, Thailand)
Thermal cycler (Bioer, China)
Transmission electron microscope model JCM-200 CZ (Jeol, Japan)
Ultracentrifuge (Beckman coulter, USA)
Vortex mixer (Scientific Industries, Inc., USA)
Waterbath (Witeg Labortechnik GmbH, Germany)

3.2 Chemicals and culture media

Acrylamide (Serva, Germany)

Agarose (Invitrogen, New Zealand)

Ammonium persulfate (Serva, Germany)

Beef extract (Himedia, India)

Bromophenol blue (Thermo Fisher Scientific, USA)

Calcium carbonate (Thermo Fisher Scientific, USA)

Calcium chloride (Thermo Fisher Scientific, USA)

Caesium chloride (Serva, Germany)

Chloroform (RCI Labscan Limited, Thailand)

Coomassie brilliant blue R-250 (Bio Basic Inc., Canada)

D(+)-glucose (Thermo Fisher Scientific, USA)

Dipotassium hydrogen phosphate (Sigma Chemical, USA)

Ethanol (Merck, USA)

Ethidium bromide (Sigma Chemical, USA)

Ethylenediaminetetra-acetic acid (EDTA) (Thermo Fisher Scientific, USA)

Glacial acetic acid (Merck, USA)

Glycerol (Merck, USA)

Isopropanol (Merck, USA)

Manganese sulfate tetrahydrate (Thermo Fisher Scientific, USA)

Magnesium sulfate heptahydrate (Thermo Fisher Scientific, USA)

Mitomycin-C (Kyowa Hakko Kogyo Co. Ltd, Japan)

MRS broth (Himedia, India)

N,N' -methylene-bis-acrylamide (Sigma Chemical, USA)

Peptone (Himedia, India)

Phenol (Amresco, USA)

Phenolphthalein (Thermo Fisher Scientific, USA)

Polyethylene glycol 8000 (Sigma Chemical, USA)

Skim milk (Titan Biotech, India)

Sodium acetate trihydrate (Merck, USA)

Sodium chloride (Thermo Fisher Scientific, USA)

Sodium dodecyl sulfate (Vivantis, USA)

TE buffer (Bio Basic Inc., Canada)
TEMED (Sigma Chemical, USA)
Triammonium citrate (Thermo Fisher Scientific, USA)
Tris base (Serva, Germany)
Tween 80 (QRecTM, New Zealand)
Yeast extract (Himedia, India)

3.3 Supplies

Cellulose acetate filter, pore size 0.45 µm (Sartorius, Germany)
Polycarbonate syringe filter holder 25 mm (Sartorius, Germany)

3.4 Kits

API 50 CHL kit (Biomerieux, France)
MinElute Gel Extraction kit (Qiagen, USA)

3.5 Enzymes and restriction enzymes

2-Log DNA Ladder (Thermo Fisher Scientific, USA)
AseI (Thermo Fisher Scientific, USA)
BclI (Thermo Fisher Scientific, USA)
BglII (Taraka Shuzo, Japan)
BstE (Thermo Fisher Scientific, USA)
DNase (Amresco, USA)
Eco91I (Thermo Fisher Scientific, USA)
EcoRI (Thermo Fisher Scientific, USA)
EcoRV (Thermo Fisher Scientific, USA)
FbaI (Taraka Shuzo, Japan)
HindIII (Thermo Fisher Scientific, USA)
MluI (Thermo Fisher Scientific, USA)
MunI (Taraka Shuzo, Japan)
NcoI (Thermo Fisher Scientific, USA)

NheI (Thermo Fisher Scientific, USA)

Perfect *Taq* Plus Master mix (5 PRIME, USA)

PvuII (Thermo Fisher Scientific, USA)

RNase (Amresco, USA)

3.6 Molecular markers

100 bp Ladder (Thermo Fisher Scientific, USA)

Generuler™ DNA ladder mix

Lambda DNA marker/*HindIII* (Thermo Fisher Scientific, USA)

Loading dye (Thermo Fisher Scientific, USA)

3.7 Reference strains

Bifidobacterium longum subsp. *infantis* JCM 1222^T

Lb. sp. NGRI 0130Q

Lb. camelliae JCM 13995

Lb. casei TISTR 389

Lb. casei TISTR 453

Lb. casei subsp. *ramnosus* TISTR 047

Lb. casei subsp. *ramnosus* TISTR 108

Lb. gasseri JCM 1131

Lb. johnsonii JCM 2012

Lb. kisonensis JCM 15041

Lb. otakiensis JCM 15040

Lb. pentosus NGRI 0506

Lb. plantarum NGRI 0320

Lb. plantarum NGRI 0529

Lb. rapi JCM 15042

Lb. rhamnosus NGRI 0110

Lb. sunkii JCM 15039

Lc. lactis subsp. *hordinae* JCM 1180

Lc. lactis subsp. *lactis* JCM 1158

Lc. raffinolactis JCM 5706

Leu. citreum JCM 9698

Leu. mesenteroides subsp. *mesenteroides* JCM 6124

P. acidilactici JCM 8797

W. cibaria N22

3.7 Methods

3.7.1 Bacterial strains, bacteriophage and culture conditions

Lb. paracasei strain LPC, a commercial starter obtained from one of fermented milk factories in Thailand, was used as host for isolation of phages. The other LAB reference strains used to determine host range in this study were grown on MRS plates or in MRS broth (Appendix A, No. 1) and incubated in the conditions as shown in Table 1. All strains of LAB stock cultures were stored at -20°C in MRS broth containing 20% (v/v) glycerol. Before use, frozen cultures were plated onto MRS agar supplemented with 20 mM CaCO₃ (Appendix A, No. 3).

Phage counts were expressed as plaque-forming units per milliliter (PFU/mL). Phage Φ T25 stock which was used throughout this study, was stored in the dark at 4°C in MRS broth supplemented with 20 mM CaCl₂ (MRS-Ca).

3.7.2 Identification of *Lb. paracasei* LPC

Lb. paracasei LPC was identified by morphological and biochemical characteristics, genetics analysed based on 16S rRNA gene as well as species-specific primers.

3.7.2.1 Morphological and biochemical test

Morphological and biochemical characteristics were initially examined by Gram staining and catalase production. Fermentation of different carbohydrates using the API 50 CHL system is intended for the correct identification of the genus *Lactobacillus* and related genera. The API 50 CHL strip consists of 50 microtubes used to study the carbohydrate family and its derivatives (Appendix C, No. 1).

3.7.2.2 16S ribosomal RNA gene analyses

The amplification of 16S rRNA gene was performed by colony PCR (Appendix C, No. 2). Universal oligonucleotides used were; 27f, 519r, 530f, 1110r, 1114f, 1492r and 1525r (Table 6).⁽⁷⁷⁾ PCR reactions were carried out in a volume of 50 µL containing 25.0 µL of 1X PerfectTaq Plus PCR buffer, deoxynucleoside triphosphates (200 µM each), 2 primers (0.2 µM each), 2 mM MgCl₂, 1.25 U of PerfectTaq Plus DNA polymerase (5 PRIME) and 1.0 µL of DNA template (50 ng/µL). The reaction mixture was subjected to 1 cycle of 95°C preheating for 2 min and 30 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 1 min) steps followed by a final extension at 72°C for 10 min. PCR amplifications were performed in a prepared by thermal cycler. The amplified DNA products were analyzed by electrophoresis in 0.6% (w/v) agarose gels at 50 V for 3 h in 1X Tris-acetate EDTA (TAE) buffer (Appendix B, No. 1) and visualized via ethidium bromide staining (Appendix B, No. 2). The 16S rRNA gene fragments were purified with a MinElute Gel Extraction kit, sequenced and compared to a database in GenBank using BLASTn (Basic Local Alignment Search Tool).

Table 6 The universal forward and reverse primers for 16S ribosomal RNA gene.⁽⁷⁷⁾

Primer (direction)	Nucleotide sequence (5' to 3')
27f (forward)	AGAGTTTGATC(A/C)TGGCTCAG
519r (reverse)	GWATTACCGCGGCKGCTG
530f (forward)	GTGCCAGCMGCCGCGG
1110r (reverse)	GGGTTGCGCTCGTTG
1114f (forward)	GCAACGAGCGCAACCC
1492r (reverse)	GGTTACCTTGTTACGACTT
1525r (reverse)	AAGGAGGTGWTCCARCC

3.7.2.3 Species-specific PCR assay

Identification of *Lb. paracasei* LPC at species level was carried out using a species-specific PCR assay.⁽⁷⁸⁾ Primers used were listed in Table 7. Amplifications were carried out in a Bioer thermal cycler, in a total volume of 50 μ L containing 25.0 μ L of 1X PerfectTaq Plus PCR buffer, deoxynucleoside triphosphates (200 μ M each), 2 primers (0.2 μ M each), 2 mM MgCl₂, 1.25 U of PerfectTaq Plus DNA polymerase and 1.0 μ L of DNA template (50 ng/ μ L). The cycling program consisted of an initial denaturation step at 94°C for 180 s and then 30 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 60 s. The final elongation was completed at 72°C for 5 min. PCR products were analyzed by electrophoresis using 1.2% (w/v) agarose gels at 50 V for 3 h in 1X TAE buffer and visualized via ethidium bromide staining. The PCR fragments were purified with the MinElute Gel Extraction kit, sequenced and compared in a BLAST search using the GenBank database.

Table 7 Combinations of primers used to assign strains to species of *Lactobacillus casei* group in a species-specific PCR assay.⁽⁷⁸⁾

Primer (direction)	Nucleotide sequence (5' to 3')	<i>Lb.</i> <i>casei</i>	<i>Lb.</i> <i>paracasei</i>	<i>Lb.</i> <i>rhamnosus</i>
W1 (forward)	TGCACTGAGATTGACTTAA	X		
W2 (forward)	CACCGAGATTCAACATGG		X	
W3 (forward)	TGCATCTTGATTTAATTTTG			X
Y2 (reverse)	CCCACTGCTGCCTCCCGTAGGAGT	X	X	X

3.7.3 Isolation and characterization *Lb. paracasei* LPC phage (Φ T25)

3.7.3.1 Isolation of *Lb. paracasei* LPC phage

The phage Φ T25 was isolated from an abnormal fermented milk sample in the industrial tanks inoculated with a *Lb. paracasei* LPC starter culture. An overnight culture of *Lb. paracasei* LPC was propagated in 25 ml of double strength MRS broth in a 250-mL Erlenmeyer flask for 4-6 h. Then, 25 mL of fermented milk sample was added and further incubated at 37°C for 24 h. The culture broth was centrifuged at 4,000 $\times g$ for 10 min and the supernatant was filtered using a 0.45 μ m pore size syringe filter. To examine the presence of phages, the double layer plaque titration method was used with some modifications as follows. The 0.1 mL of decimal dilutions of filtrates and 0.2 mL of a log phase culture of the sensitive strain were added into 4 mL of warm MRS-soft agar (0.6%, w/v) supplemented with 20 mM CaCl₂ (Appendix A, No. 6), mixed gently and overlaid onto a base layer of MRS agar (1.5%, w/v) supplemented with 20 mM CaCl₂ (Appendix A, No. 5). Incubation was carried out at 37°C for 18 h under microaerophilic conditions. Plaques were then purified by single plaque isolation prior to making a high titer of phage stocks.

3.7.3.2 Single plaque purification

Single plaques were isolated and purified by picking up plaques into 300 μ L of MRS broth containing early-exponential-phase *Lb. paracasei* LPC and incubated at 37°C overnight under microaerophilic conditions. Then, plaque formation was tested by double

layer plaque titration method and this step was repeated three times to ensure isolation of a pure phage.

3.7.3.3 Preparation of phage lysates

Plaque forming plates were prepared by double layer plaque titration method. After overnight of incubation, the top agar layer was scraped into 25 mL of MRS broth in a 250-mL Erlenmeyer flask and further incubated for 4-6 h. Then, the suspension was centrifuged at 8,000 $\times g$ for 10 min to remove agar and host cells. Phage lysate was filter-sterilized by passing through a 0.45 μm pore size cellulose acetate membrane filter. Titer of the phage lysate was measured by double layer titration method and the lysate was stored at 4°C. Long-term storage can be achieved by adding autoclaved 80% (v/v) glycerol to the phage lysate and kept at -80°C.

3.7.3.4 Transmission electron microscopy (TEM)

Phage morphology was determined by transmission electron microscopy (TEM). The carbon coated grids were gently placed on the fresh overnight plaques and negatively stained with 1% (w/v) uranyl acetate (pH 4.5). Phage morphology was examined with a TEM - 1010 transmission electron microscope (JEOL, Japan) operated at 120 kV and at a magnification of $\times 120,000$.

3.7.3.5 Host-range determination

Host-range of the phage Φ T25 was determined against 24 reference strains of lactic acid bacteria (Table 8) by the agar spot test method.⁽⁷⁹⁾ Briefly, each of the reference strain (with a concentration of 10^9 CFU/mL) was added to 4 mL liquefied MRS soft agar (MRS broth with 0.6% agar), mixed gently and poured over the MRS agar plate. After the agar was hardened, single drops (about 10 μL of phage suspension) were spotted on the lawn of bacteria and incubated overnight at 37°C under microaerophilic conditions. Bacterial susceptibility to a phage was determined by lysis formed at the spot where the phage suspension drop was deposited.

Table 8 LAB strains and culture conditions.

Bacterial strain	Culture condition
<i>Bifidobacterium</i> (B.)	
<i>B. longum</i> subsp. <i>infantis</i> JCM ^a 1222 ^T	<i>Bifidobacterium</i> medium, 37°C, Anaerobic
<i>Lactobacillus</i> (Lb.)	
<i>Lactobacillus</i> sp. NGRI ^b 0130Q	MRS, 37°C
<i>Lb. pentosus</i> NGRI 0506	MRS, 37°C
<i>Lb. camelliae</i> JCM 13995 ^T	MRS, 30°C
<i>Lb. casei</i> TISTR ^c 389	MRS, 37°C
<i>Lb. casei</i> TISTR 453	MRS, 37°C
<i>Lb. casei</i> subsp. <i>ramnosus</i> TISTR 047	MRS, 37°C
<i>Lb. casei</i> subsp. <i>ramnosus</i> TISTR 108	MRS, 37°C
<i>Lb. gasseri</i> JCM 1131	MRS, 37°C
<i>Lb. johnsonii</i> JCM 2012	MRS, 37°C
<i>Lb. kisonensis</i> JCM 15041	MRS, 30°C
<i>Lb. otakiensis</i> JCM 15040	MRS, 30°C
<i>Lb. plantarum</i> NGRI 0320	MRS, 37°C
<i>Lb. plantarum</i> NGRI 0529	MRS, 37°C
<i>Lb. rapi</i> JCM 15042	MRS, 30°C
<i>Lb. rhamnosus</i> NGRI 0110	MRS, 37°C
<i>Lb. sunkii</i> JCM 15039	MRS, 30°C
<i>Lactococcus</i> (Lc.)	
<i>Lc. lactis</i> subsp. <i>hordinae</i> JCM 1180 ^T	MRS, 30°C
<i>Lc. lactis</i> subsp. <i>lactis</i> JCM 1158	MRS, 37°C
<i>Lc. raffinolactis</i> JCM 5706 ^T	MRS, 30°C

Table 8 (continued).

Bacterial strains	Culture conditions
<i>Leuconostoc</i> (<i>Leu.</i>)	
<i>Leu. citreum</i> JCM 9698 ^T	MRS, 30°C
<i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> JCM 6124	MRS, 30°C
<i>Pediococcus</i> (<i>P.</i>)	
<i>P. acidilactici</i> JCM 8797	MRS, 30°C
<i>Weissella</i> (<i>W.</i>)	
<i>W. cibaria</i> N22	MRS, 37°C

^a JCM: Japan Collection of Microorganisms, Japan.

^b NGRI: National Grassland Research Institute, Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan.

^c TISTR: Thailand Institute of Scientific and Technological Research, Thailand.

T: Type strain

3.7.3.6 Determination of optimal multiplicity of infection (MOI)

Multiplicity of infection (MOI) is defined as the ratio of infectious virus particles to host cells.⁽⁸⁰⁾ To determine the optimal MOI, an early log phase of *Lb. paracasei* LPC (OD_{600nm} = 0.6) was grown in MRS broth at 37°C and then infected with the phage Φ T25 at four different ratios (0.0001, 0.001, 0.01, 0.1, 1 and 10 PFU/CFU). After 3.5 h of incubation at 37°C, the mixtures were centrifuged at 9000 ×g for 3 min. The supernatant was filtered by passing through a 0.45 µm pore size cellulose acetate membrane filter and used to determine the phage titer.⁽³⁵⁾

3.7.3.7 One-step growth curve experiments

Lb. paracasei LPC culture (10⁸ CFU/mL) was resuspended in 1 mL of MRS broth and incubated with equal volume of phage (10⁷ PFU/mL) to generate an MOI of 0.1. After 10 min of adsorption at 37°C, the suspension was filtered through 0.45 µm pore size

syringe filter. Unadsorbed phages were removed from the filter membrane by washing the filter with MRS broth several times. Infected cells were harvested and resuspended in 20 mL of MRS broth. An aliquot of each dilution was withdrawn at intervals for phage counts.⁽⁸¹⁾ Latent period, rise period and burst size were calculated from the one-step growth curve.⁽⁴⁸⁾

3.7.3.8 Calcium effect on phage adsorption, propagation and plaque

Calcium effect on phage adsorption was determined as described by Lu et al. (2003).⁽³⁵⁾ Ten milliliters of early exponential-phase host culture ($OD_{600nm} = 0.6$) was inoculated into MRS broth supplemented with 0, 10, 20, or 30 mM $CaCl_2$ (Appendix A, No. 2). The final volume was adjusted with MRS broth and infected with the phage $\Phi T25$ at an MOI of 0.001. All tubes were incubated at 37°C. A sample was withdrawn at time intervals and filtered through a 0.45 μm pore size syringe filter. The titers of unadsorbed free phages in the supernatant were determined by double layer plaque titration method and the results were calculated as:⁽⁸²⁾

$$\frac{\text{Control titer} - \text{Residual titer}}{\text{Control titer}} \times 100$$

Influence of calcium on phage propagation was investigated by incubation (37°C) of infected (MOI of 0.001) *Lb. paracasei* LPC cultures in MRS broth, with $CaCl_2$ in a final concentration of 0, 10, 20, or 30 mM. Plaque formation was investigated using the double layer plaque titration method.⁽³⁵⁾

The effects of calcium ions on the plaque size were determined by supplementing calcium into MRS soft overlay agar and/or MRS agar plates to achieve final concentrations of 0, 10, 20, or 30 mM. Plaque formation was investigated using the double layer plaque titration method.⁽³⁵⁾

3.7.3.9 Bacteriophage DNA extraction and restriction enzyme analyses

Bacteriophage DNA was extracted by phenol-chloroform method⁽⁸³⁾. High titer phage lysate was prepared by double layer plaque titration method from 100 plates on MRS agar. After incubated overnight at 37°C, each five top layer of plates was scraped into 20 mL MRS broth and shaking at 100 rpm at room temperature for 4 h. Then, phage

suspension was harvested by centrifugation at 8,000 $\times g$ at 10 min to remove host debris. The supernatant was transferred to a fresh flask and then DNase I and RNase I were added to final concentrations of 1 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$, respectively and further incubated at 37°C for 30 min. After that NaCl and PEG 8000 were added to final concentrations of 1 M and 10%, respectively, mixed thoroughly until the salt and PEG were completely dissolved. The flask was incubated at 4°C for overnight to allow phage particles to form precipitates. The phage-PEG complex was pelleted by centrifugation at 10,000 $\times g$ for 15 min at 4°C, the supernatant was discarded and the pellet was resuspended in 500 μL TE pH 8.0 (Appendix B, No. 3). Then, 0.5 M NaCl and 10% (w/v) SDS were added to the pellet suspension and incubated at 65°C for 10 min. Equal volume of chloroform was added to the pellet suspension and centrifuged at 3,000 $\times g$ for 15 min to remove some of PEG. To burst phage and remove phage proteins, an equal volume of phenol was added, mixed by inversion and spinned in a microcentrifuge tube about 1-3 min until an aqueous phase became clear. The aqueous phase was transferred to a fresh microcentrifuge tube, then an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v) was added and centrifuged at 10,000 $\times g$ at 4°C for 10 min. The aqueous phase was transferred to a fresh microcentrifuge tube and repeated the phenol: chloroform: isoamyl alcohol step was repeated until no protein was visible at the interface of the organic phase and aqueous phase. The equal volume of chloroform: isoamyl alcohol (24: 1, v/v) was added to the nucleic suspension and centrifuged at 10,000 $\times g$ at 4°C for 10 min. The phage DNA was precipitated with 0.1 volume of 3 M sodium acetate (Appendix B, No. 4) and 1 mL of ice cold absolute ethanol before incubation at 4°C overnight. The phage DNA pellet was collected by centrifugation at 14,000 $\times g$ at 4°C for 15 min, washed once with cold 70% ethanol, vortexed, then centrifuged briefly. The pellet was dried by leaving on the bench top and resuspended in TE buffer, pH 8.0. The quality of the DNA was checked by electrophoresis.

Each restriction enzyme digestion contained approximately 1 μg of phage genomic DNA and the digestion buffer. Incubation time, temperature and termination of reaction were performed as recommended by the manufacturer (Appendix C, No. 3). After digestion, phage DNA was loaded onto the 0.6% agarose gel with a *HindIII* digested phage

λ marker. Electrophoresis was operated in 1X TAE buffer at 50 V. After electrophoresis was complete, DNA bands were visualized under UV illumination and photographed.

3.7.3.10 Caesium chloride (CsCl) purification

Phage particles can be separated from contaminants according to their buoyant densities by sedimentation in CsCl gradients. Different CsCl solutions were prepared by dissolving the salt in the phage buffer (Appendix B, No. 5). Step gradients were prepared in Beckman Ultra-Clear centrifuge tubes by floating the lighter gradient concentrations up by adding increasing density solutions to the tube bottom using a hypodermic syringe with a long needle. Then, five milliliters of phage suspensions from PEG precipitation was layered on the top of a step gradient and centrifuged at a rotor speed of 30,000 rpm for 3 h, in Beckman SW41. The phage formed a whitish band and it was removed using a syringe. After that the CsCl was removed from the phage suspensions (Appendix B, No. 5) by dialysis at 4°C, two or three times for 30 min against ca. 500 volumes of phage buffer and stored the dialyzed phage suspension at 4°C with a few drops of chloroform to avoid microbial contamination.

3.7.3.11 Cohesive end DNA analysis

Purified phage DNA was digested by restriction enzymes that resulted in the display of the two end fragments. After that the reactions were heated at 75°C for 15 min and divided into two equal portions. One portion was cooled rapidly by placing in wet ice and another portion was cooled slowly at room temperature. The resulting DNA bands were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.⁽⁸⁴⁾

3.7.3.12 Structural phage protein analysis by SDS/ polyacrylamide gel electrophoresis⁽⁸⁵⁾

The purified phage suspensions obtained from 3.7.3.10 were concentrated with Amicon Ultra-15 filter. SDS-PAGE of phage proteins was performed using methods described by Laemmli (1970).⁽⁸⁶⁾

Two gel layers were prepared with resolving gel at the bottom layer and stacking gel on the top layer. A 12.5% running gel topped with 4% stacking gel was prepared (Appendix C, No. 4). Electrophoresis was performed in a vertical direction from cathode to anode until the bromophenol blue band migrated to the end of the glass slab.

Gels were submersed in 1X SDS-PAGE running buffer (Appendix B, No.13) at 80 V for 100 min. Proteins were visualized by staining with Coomassie Brilliant Blue R250 (Appendix B, No. 14) in a fixative (40% methanol and 10% acetic acid) and destained several times with 40% methanol and 10% acetic acid until clear background was obtained. A protein weight molecular marker was used to estimate sizes of proteins obtained.

3.7.4 Isolation and characterization of phage-resistant mutants

3.7.4.1 Isolation of phage-resistant mutants

Two methodologies were used; the agar plate method (AP)⁽⁸⁷⁾ and the secondary culture method (SC).⁽⁸⁴⁾ The AP was modified as follows. The phage Φ T25 suspension of 0.1 mL was mixed with 0.2 mL of a log phase culture of *Lb. paracasei* LPC and 4 mL of warm MRS-soft agar (0.6%, w/v) supplemented with 20 mM CaCl_2 (top layer). Then the suspension was overlaid onto a base layer of MRS agar (1.5%, w/v) supplemented with 20 mM CaCl_2 . After incubation at 37°C for 18 h under microaerophilic conditions, the double layer plates which exhibited plaques on the bacterial lawn were further incubated at 37°C for 7 days. Phage-resistant mutant colonies which appeared within the plaques were selected and streaked on the MRS agar plates. For the SC method, overnight cultures of *Lb. paracasei* LPC were infected, at different infection ratios (multiplicity of infection, MOI of 1, 0.1 and 0.01), with the suspensions of phage Φ T25. After incubation at 37°C for 6-8 h, cultures exhibiting complete lysis and secondary growth after further incubation for up to 48 h at 37°C were selected and streaked on the MRS agar plates. Presumptive phage-resistant variants (A) obtained from both methods were confirmed their resistance by challenging each mutant under the pressure of phage Φ T25 lytic phage. Three consecutive subcultures with corresponding phage were performed and isolates that were able to grow normally under these conditions, were retained as true phage-resistant mutants (B).⁽⁹⁴⁾ The efficiency of each isolation method was calculated as:

$$\frac{\text{Number of true phage-resistant mutants (A)}}{\text{Number of presumptive phage-resistant mutants (B)}} \times 100$$

3.7.4.2 Characterization of phage-resistance phenotype

1. Phage resistance stability

Phage resistance stability was determined by seven sequential subcultures of the mutants obtained from 3.7.4.1 in MRS broth with independent infection with phages at each subculture.⁽⁸⁵⁾ The loss of phage resistance was determined by the culture lysis in comparison with a control a mutant strain without adding phage. The subculture at which lysis occurred for each mutant was recorded.

2. Level of resistance

The level of resistance of phage-resistant variants was investigated by using these variants as sensitive strains in double layer plaque titration method. The ratio PFU/mL (obtained on each mutant) / PFU/mL (obtained on the parent strain, *Lactobacillus paracasei* LPC) was defined as efficiency of plaquing or EOP.⁽⁸⁵⁾

3. Adsorption rates

To determine adsorption rates for parent and mutant strains, exponential phase culture of each parent and mutant strain were mixed with the phage Φ T25, separately, at an MOI of 0.02 and then incubated for 30 min at 37°C for adsorption. The phage-bacterial cell mixtures were centrifuged at 12,000 $\times g$ for 4 min and the presence of phage particles in the supernatants (unadsorbed phages) was determined by the double layer plaque titration method. To evaluate the degree of phage adsorption, the results were compared with the titer of a control without cells.⁽⁸⁸⁾

4. Lysogeny

The resistance phenotype is associated with lysogeny, leading to a possible phage resistance mechanism. To examine mutants for lysogeny, an overnight MRS broth culture of each phage-resistant mutant was centrifuged at 12,000 $\times g$ for 4 min and the supernatant was mixed with the corresponding sensitive strain in MRS soft agar and then overlaid on a plate of MRS-Ca agar (Appendix A, No. 5)⁽⁸⁵⁾, to reveal phage particles released spontaneously by mutants that were able to infect sensitive strains.

5. Prophage induction

All resistant strains were determined for the presence of prophage by using the inducing agent mitomycin C. An overnight culture of phage-resistant mutant cells

were inoculated in 5 mL of fresh MRS medium. Prior to the addition of mitomycin C, the cells were measured by determining absorbance at 600 nm and then left in a waterbath at 37°C for 30 min. Mitomycin C was added to a final concentration of 0.1-0.5 µg/mL and the absorbance was measured at 600 nm every hour for 6-8 h (or until a decrease of the optical density was observed). Lysates from mitomycin C induction were centrifuged at 3000 ×g for 12 min at 4°C and filtered through a 0.45 µm pore size syringe filter. The sterile supernatant was stored at 4°C. The presence of mitomycin C-inducible prophages was determined by double layer plaque titration agar method.⁽⁸⁹⁾

6. Plasmid curing

An overnight culture of phage-resistant mutants was inoculated in 10 mL MRS broth containing ethidium bromide or sodium dodecyl sulphate at concentrations ranging from 1.0 to 10 µg/mL and 0.1 to 1.0 mg/mL, respectively. After incubation overnight at 37°C, phage-resistant mutants were checked for the presence of plasmids after curing by electrophoresis and also checked for their resistance to the phage ΦT25.

3.7.4.3 Characterization of phage-resistant mutants

Cells were examined microscopically under 1,000X magnification and colony morphologies on MRS agar were determined. Acidification kinetics of the phage-resistant mutants were evaluated by their growth in 2% (v/v) skim milk-yeast extract medium (Appendix A, No. 4) with an overnight culture of each mutant and incubated for 24 h at 37°C. The acidity developed was measured with a pH meter and plotted against time, and further determined using high-performance liquid chromatography (HPLC). Overnight cultures were harvested by centrifugation at 5,000 ×g for 5 min, washed twice with 0.85% NaCl and resuspended in the fresh MRS broth to an optical density at 600 nm of 1.00, corresponding to approximately 10⁹ CFU/mL. Lactic acid in the supernatant was quantified by HPLC using an InertSustain C18 column with a mobile phase of 10 mM NH₄H₂PO₄ (pH 2.6), flow rate of 1.0 mL/min and temperature of 40°C.

Technological performance was evaluated by the manufacture of fermented milk. In one trial, the sensitive strain *Lb. paracasei* LPC was used as a starter (control fermented milk) and, in the other, five selected phage-resistant mutants isolated from *Lb. paracasei* LPC were used (experimental fermented milk). To assay the performance of the

resistant variants in the presence of phages, two additional trials were carried out by infecting both the sensitive strain (control infected fermented milk) and the selected phage-resistant mutant (experimental infected fermented milk) with the phage Φ T25. Skim milk-yeast extract medium was inoculated at 2% (v/v) with an overnight culture of each strain (about 10^6 CFU/mL) and a mixture of the phage Φ T25 (about 10^4 PFU/mL; MOI of 0.01) was also added in the infected trials. The acidification proceeded at 37°C in a waterbath until reaching pH 4.4 in the uninfected cultures. Fermented milks were stored under refrigerated conditions (12°C) for three weeks. Changes in pH were monitored by using the pH meter, during the manufactures and in the final products after 1, 2 and 3 weeks of refrigerated storage. Bacterial cell counts (MRS agar, 37°C, 48 h) and phage enumeration (if applicable) were also carried out during and after manufacture.

3.7.4.4 Genetic analyses

Random amplified polymorphic DNA (RAPD) was applied to the phage-sensitive strains and their phage-resistant variants, to determine their genetic similarity. Oligonucleotide primers M13 (5'-GAGGGTGGCGGTCT-3')⁽⁹⁰⁻⁹¹⁾, 1254 (5'-CCGCAGCCAA-3')⁽⁹²⁾, B06 (5'-TGCTCTGCCC-3')⁽⁹³⁾ and B10 (5'-CTGCTGGGAC-3')⁽⁹³⁾ were used in separate amplification assays. PCR reactions were carried out in a total volume of 50 μ L containing 25.0 μ L of 1X PerfectTaq PlusPCR buffer, deoxynucleoside triphosphates (200 μ M each), 2 primers (0.2 μ M each), 2 mM MgCl₂, 1.25 U of PerfectTaq Plus DNA polymerase and 1.0 μ L of DNA template. PCR programs differed according to the primers used; for primer M13: 1 initial cycle of 3 min at 94°C followed by 40 cycles of 1 min at 94°C, 20 s at 45°C and 2 min at 72°C; for primer 1254: 4 initial cycles of 5 min at 94°C, 5 min at 36°C and 5 min at 72°C followed by 30 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C; for primers B06 and B10: 1 initial cycle of 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 2 min at 36°C and 2 min at 72°C. All programs ended with an extension step at 72°C for 7 min (M13 and 1254) or 10 min (B06 and B10). Amplified DNA fragments were analyzed by electrophoresis on 1% (w/v) agarose gels at 50 V for 1 h in 1X TAE buffer and visualized through ethidium bromide staining.

3.7.5 Phage genomic DNA sequencing

The caesium chloride purified phages from the section 3.7.3.10 were used to extract genomic DNA by phenol-chloroform method.⁽³⁵⁾ The phage Φ T25 genome was sequenced using MiSeq (Illumina). Open reading frames (ORFs) were predicted using a combination of MiGAP and GenMark programs. Sequence similarity analyses were performed with the gapped blastx and blastp algorithms against the nonredundant database provided by NCBI.⁽⁹⁴⁾



CHAPTER 4

RESULTS

4.1 Identification of fermented milk starter culture *Lactobacilli* strain LPC

A starter culture *Lactobacilli* strain LPC was initially identified by Gram staining and its ability to produce lactic acid by the presence of clear halo surrounding the colonies on MRS agar plates supplemented with CaCO_3 . The results showed that the strain LPC is Gram positive bacilli and exhibited the clear zone around the colony (Figure 7). The biochemical characteristics including a catalase test and a carbohydrate fermentation profile using API 50 CHL revealed that the strain LPC was catalase-negative and belonged to *Lb. paracasei* subsp. *paracasei* with 99.7% identity (Figure 8 and Table 9,10). The results from the 16S rRNA gene sequence revealed that the strain LPC was identified as either *Lb. casei* or *Lb. paracasei* with 99% identity (Figure 9). To discriminate between the two species, the species-specific PCR primers that targeted the conserve sequence from the V1 region of 16S rRNA gene were used to carry out PCR reaction. The forward primer Y2 which was designed from the conserved 16S rRNA gene was used along with either reverse primers, *casei* W1 or *para* W2 which were designed from the conserved V1 region of the 16S rRNA gene of *Lb. casei* or *Lb. paracasei*, respectively. In this study, PCR products of approximately 295 bp were obtained from all tested strains of *Lb. casei* group with both primer pairs *casei* W1/Y2 and *para* W2/Y2 as shown in Figure 10. By using *Lb. paracasei* LPC template, PCR fragment obtained from the *para* W2/Y2 primer pair was further sequenced and aligned. The results showed that the nucleotide sequence of the V1 region of the strain LPC shared 100% identity with the V1 region sequence of the type strain *Lb. paracasei* ATCC 334 (Figure 11). Therefore, the strain LPC was determined as *Lb. paracasei*.⁽⁹⁵⁾



Figure 7 Morphological characteristics of *Lb. paracasei* LPC showing gram positive bacilli (x1000) (A); and clear halo surrounding the colonies on MRS agar plate supplemented with CaCO₃ (B).

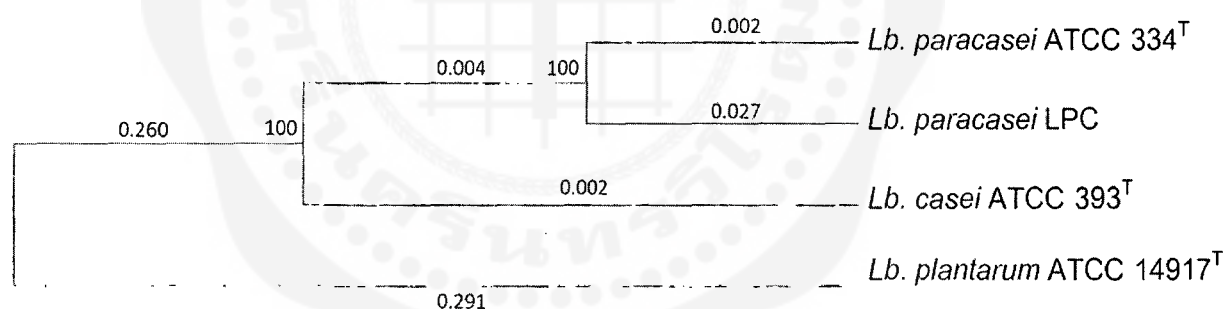


Figure 9 Neighbor-joining tree of 16S rRNA gene sequence of *Lb. paracasei* LPC. Bootstrap values (100%) of 1,000 replicates are represented on the branches. Decimal numbers indicated genetic distances.

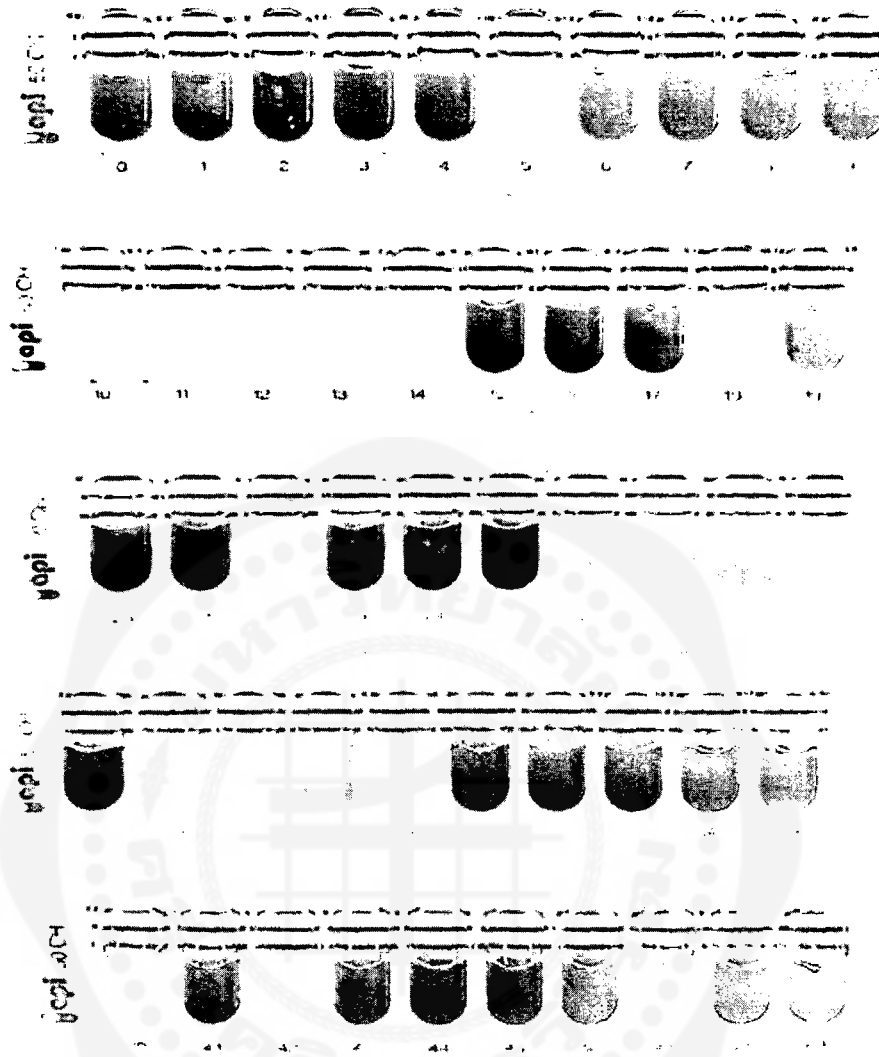


Figure 8 *Lb. paracasei* LPC carbohydrate fermentation profile on API 50 CHL system

Note: A positive test corresponded to acidification revealed a color change to yellow by the phenol red indicator contained in the medium. For the esculin test (tube No. 25), a change in color from red to black was observed.

Table 9 Carbohydrate fermentation profile of *Lb. paracasei* using the API 50 CHL system

No.	Carbohydrate	<i>Lb. paracasei</i> subsp. <i>paracasei</i>	<i>Lactobacillus</i> sp. LPC
1	Control	-	-
2	Glycerol	-	-
3	Erythritol	-	-
4	D-arabinose	-	-
5	L-arabinose	+	+
6	Ribose	-	-
7	D-xylose	-	-
8	L-xylose	-	-
9	Adonitol	-	-
10	β -methyl xyloside	+	+
11	Galactose	+	+
12	Glucose	+	+
13	Fructose	+	+
14	Mannose	-	+
15	Sorbose	-	-
16	Rhamnose	+	-
17	Ducitol	-	-
18	Inositol	+	+
19	Mannitol	+	?
20	Sorbitol	-	-
21	α -methyl mannoside	+	-
22	N-acetyl glucosamine	+	+
23	Amygdalin	+	-
24	Arbutin	+	-
25	Esculin	+	-
26	Salicin	+	+
27	Cellubiose	+	+
28	Maltose	+	?
29	Lactose	+	+

Table 9 (continued).

No.	Carbohydrates	<i>Lb. paracasei</i> subsp. <i>paracasei</i>	<i>Lactobacillus</i> sp. LPC
29	Lactose	+	+
30	Melibiose	-	-
31	Sucrose	+	+
32	Trehalose	+	+
33	Inulin	+	+
34	Melezitose	+	+
35	Raffinose	-	-
36	Starch	-	-
37	Glycogen	-	-
38	Xylitol	-	-
39	Gentobiose	V	?
40	D-turanose	+	+
41	D-Lyxose	-	-
42	D-tagatose	+	+
43	D-fucose	-	-
44	L-fucose	-	-
45	D-arabitol	-	-
46	L-arabitol	-	-
47	Gluconate	+	?
48	2-keto-gluconate	-	-
49	5-keto-gluconate	-	-

Symbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative;
d, 11-89% of strains are positive; ?, weak positive reaction

Table 10 Characteristics differentiating Group B lactobacilli (obligately heterofermentative) from Bergey's manual of systematic bacteriology.

Carbohydrates	<i>Lb. casei</i>	<i>Lb. paracasei</i>	<i>Lactobacillus sp.</i> LPC
Amygdalin	+	+	-
Arabinose	-	-	-
Cellobiose	+	+	+
Esculin	+	+	+
Gluconate	+	+	-
Mannitol	+	+	-
Melezitose	+	+	+
Melibiose	-	-	-
Raffinose	-	-	-
Ribose	+	+	+
Sorbitol	+	D	-
Sucrose	+	+	+
Xylose	-	-	-

Symbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative;

d; 11-89% of strains are positive

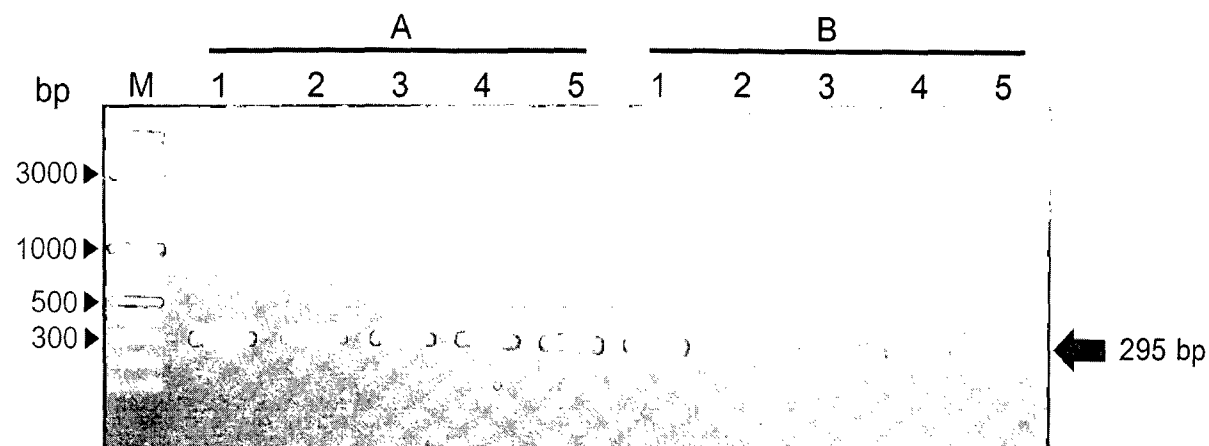


Figure 10 Agarose gel electrophoresis of PCR products obtained using different species-specific primer pairs; (A) *casei* W1/Y2; (B) *para* W2/Y2. Strains amplified from: 1, *Lb. casei* TISTR 389; 2, *Lb. casei* TISTR 390; 3, *Lb. casei* TISTR 1463; 4, *Lb. paracasei* ATCC BAA-52; 5, *Lb. paracasei* LPC. Lane M: DNA molecular size marker (100 bp ladder) (Generuler™ DNA ladder mix).

```

T_para      1 GAGTTCTCGTTGATGATCCGTGCTTGCACCGAGATTCAACATGGGAAACGAG      50
              |||
LPC         68 GAGTTCTCGTTGATGATCCGTGCTTGCACCGAGATTCAACATGGGAAACGAG      117
              |||

T_para      51 TGECGGACGG      60
              |||
LPC        118 TGECGGACGG      127

```

Figure 11 Alignment of nucleotide sequences of V1 region of strain LPC and V1 region of the type strain *Lb. paracasei* (ATCC 334). Sequence abbreviations: T_para, *Lb. paracasei* ATCC 334; LPC, *Lb. paracasei* LPC. The alignment was performed with the emboss matcher program.

4.2 Isolation and characterization of *Lb. paracasei* LPC phage from milk fermentation failure tank

4.2.1 Phage isolation and morphology

Lb. paracasei LPC was used as the host strain to isolate the phages from failed milk fermentation samples. Only one type of phage, designated as Φ T25 was isolated and purified by three successive single plaque isolations. The phage Φ T25 exhibited clear plaques with diameters ranging from 1.7 to 2.0 mm (Figure 12). Electron micrographs revealed that the phage Φ T25 has an icosahedral head 55 ± 4.6 nm in diameter and a non-contractile tail 184 ± 7.8 nm long (Figure 13). Therefore, this phage belonged to the *Siphoviridae* family or was Type B of Bradley's classification.^(27,96)

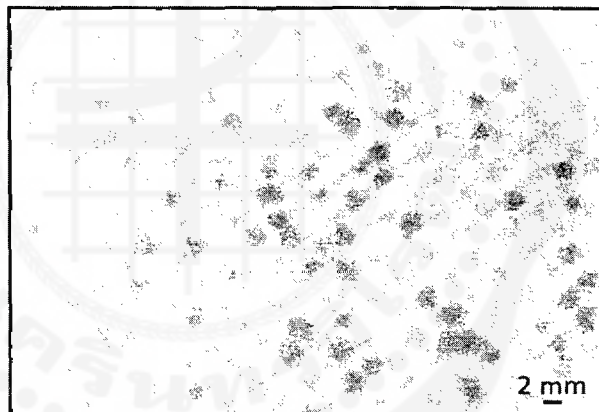


Figure 12 Plaque morphological characteristics of phage Φ T25 on MRS double layer agar.

Scale bar represents 2 mm.

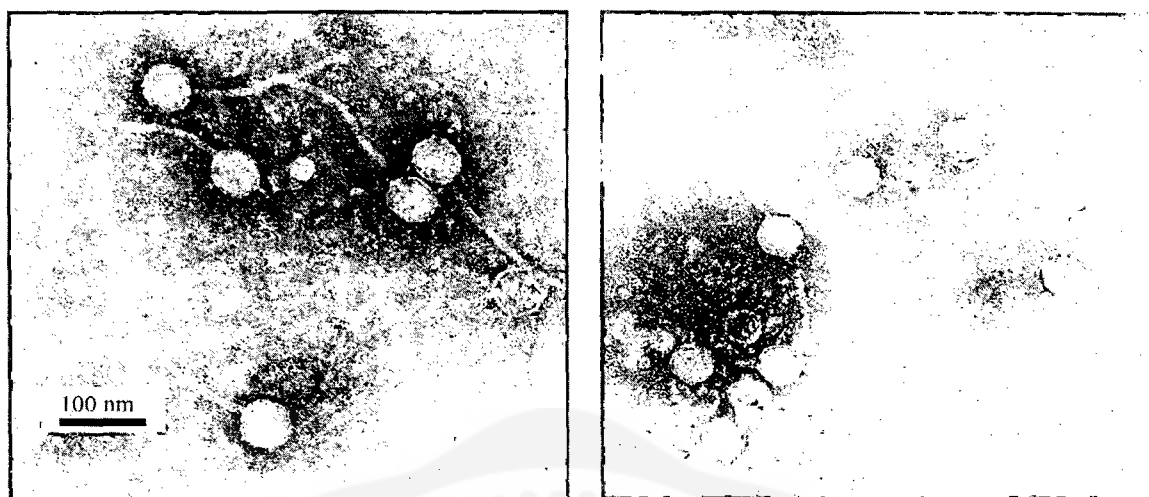


Figure 13 Electron micrograph of phage Φ T25 negatively stained with 2% uranyl acetate. Scale bar represents 100 nm.

4.2.2 Host-range determination

The host-range of the phage Φ T25 has been evaluated with 26 lactic acid bacteria reference strains using the agar spot test. The phage Φ T25 showed a specific inhibition only against *Lb. paracasei* LPC host strain. The tested strain *Lb. paracasei* ATCC BAA-52 and other closely related species of *Lb. casei* and *Lb. rhamnosus* were insensitive to this phage. Our results indicated that the phage Φ T25 is strain-specific, as reported elsewhere for all of the other *Lactobacillus* phages.⁽⁹⁵⁾

4.2.3 Determination of optimal multiplicity of infection (MOI)

The early log phase of *Lb. paracasei* LPC were infected with the phage Φ T25 at four different ratios of the phages and the host cells. The optimal MOI of the phage Φ T25 was determined to be 0.001 and the phage suspensions with high titer of 10^6 PFU/mL were obtained under these conditions (Table 11).

Table 11 Multiplicity of infection (MOI) of phage Φ T25. Comparison of phage titers at four ratios of MOI (0.001, 0.01, 0.01 and 1 PFU/CFU) in MRS medium.

Multiplicity of infection (MOI) (PFU/CFU)	Phage titers (log PFU/mL)
1	4.56±0.07
0.1	5.55±1.40
0.01	5.33±0.15
0.001	6.87±0.02

4.2.4 One-step growth curve of phage Φ T25

Multiplication parameters of the lytic cycle of the phage Φ T25 was determined by one-step growth curve. As it can be seen in Figure 14, the latent period was 55 min and followed by a 55 min rise period. The phage Φ T25 showed the highest burst size value approximately 38 PFU per infected cell.

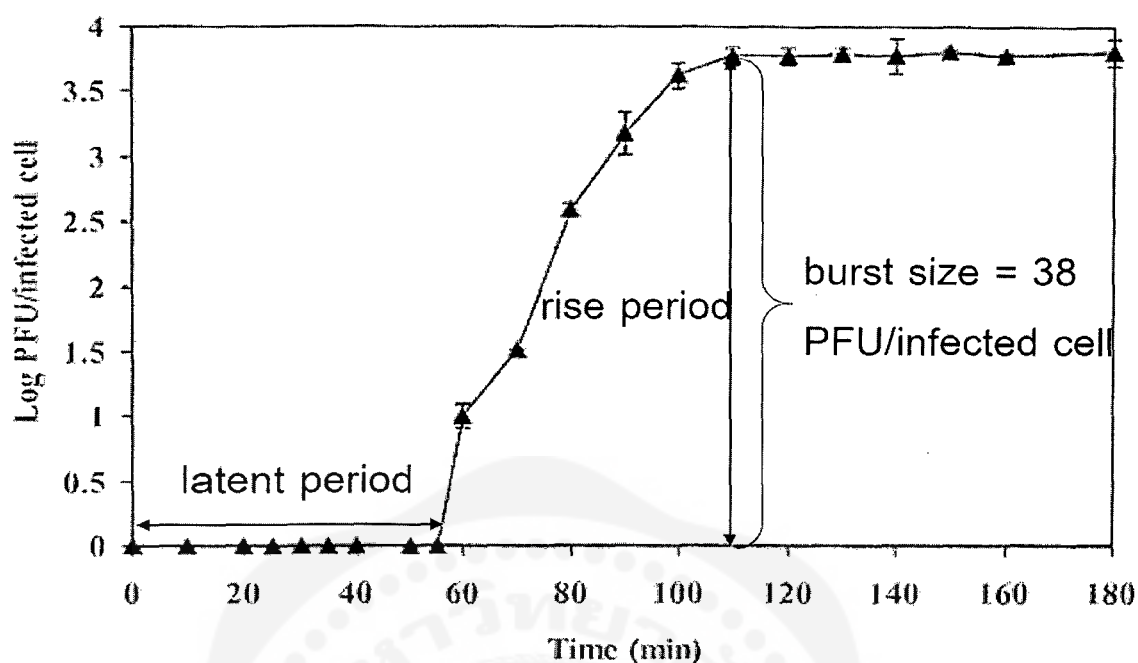


Figure 14 One-step growth curve of phage Φ T25 on the host strain *Lb. paracasei* LPC.

Mean

values used were obtained from three determinations.

4.2.5 Effects of calcium ions on phage adsorption, propagation and plaque

The effects of calcium ions on phage adsorption was shown in Figure 15. This result demonstrated that Ca^{2+} had a little influence on adsorption kinetics of this phage on the sensitive strain. In the presence of Ca^{2+} , the phage Φ T25 adsorbed slightly faster than in its absence. In all experiments, at least 50% of the phage particles were adsorbed after 15 min of incubation and increasing to more than 75% adsorption rates after 30 min of incubation. The maximum adsorption rate (91%) was achieved in the presence of 20 mM calcium ions after incubated for 30 min. By this time, 82% of phage particles were adsorbed in the absence of calcium ions.

Phage propagation rate was not influenced by calcium supplementation. Data presented in Figure 16 illustrated the indifference of phage proliferation either in the presence or absence of the calcium ions. Cell lysis was observed in MRS media containing

0, 10, 20, or 30 mM CaCl_2 within 6 hours. During this time, the final phage titer reached 10^8 PFU/mL in any concentration of the calcium ions.

The influence of Ca^{2+} on plaque formation by the phage ΦT25 was investigated by adding CaCl_2 to the MRS top agar and bottom agar layer. Addition of CaCl_2 to the bottom agar and the top agar contributed to enhance the plaque size and increased plaque productivity. Calcium supplementation in MRS media resulted in a large amount of plaques by 2-5 times when compared to the absence of calcium ions. Table 12 showed plaque counts from the various concentrations of CaCl_2 in the bottom agar and the top agar. Considerably higher number of plaques per plate were produced when using the concentrations of CaCl_2 at 10-20 mM in the bottom agar and 30mM in the top agar. In addition, the largest clear plaques were observed in the combination of 30 mM CaCl_2 MRS bottom agar layer and 20 mM CaCl_2 MRS top agar layer (Table 13). From these results, it was concluded that the Ca^{2+} was required for plaque formation.

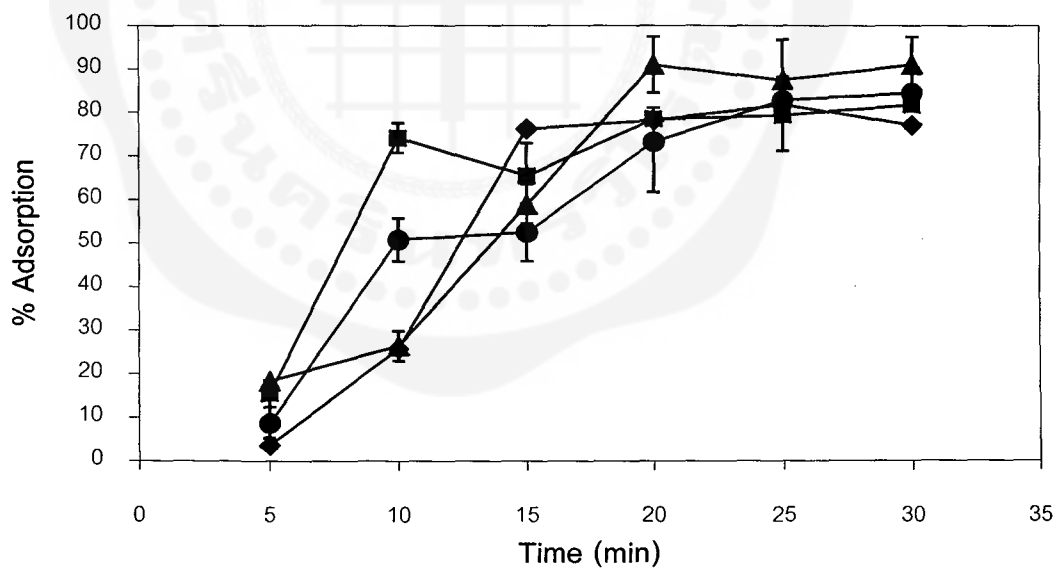


Figure 15 Influence of calcium ions on the adsorption of phage ΦT25 in MRS broth without calcium (●), with 10 mM calcium (◆), with 20 mM calcium (▲) and with 30 mM calcium (■). Mean values used were obtained from three determinations.

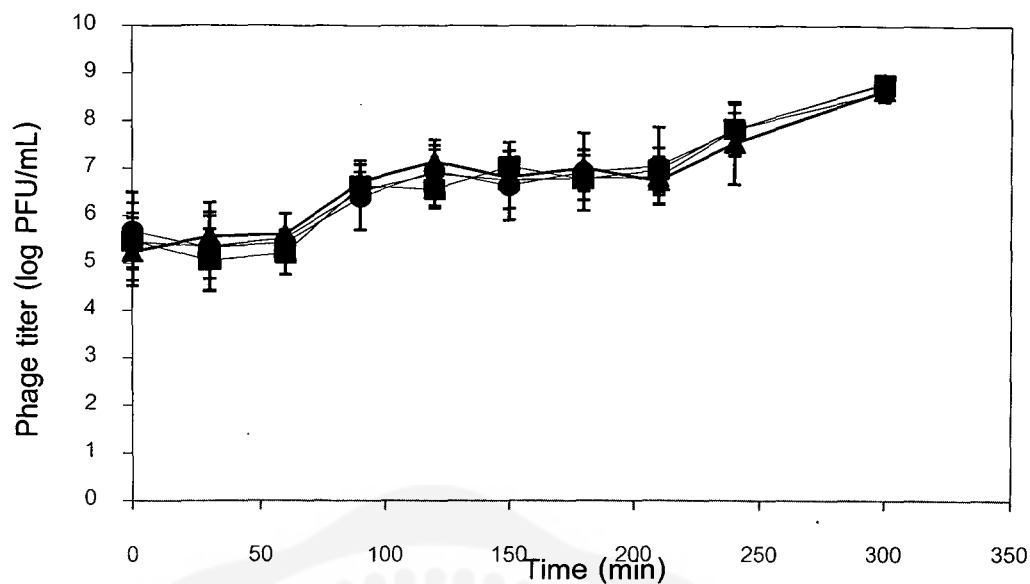


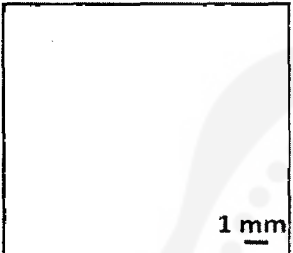
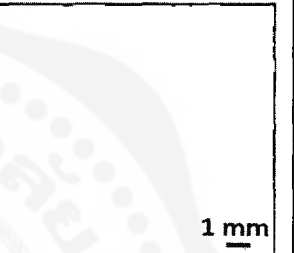
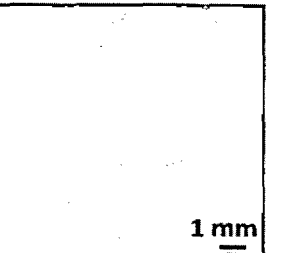
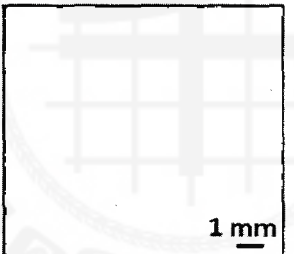
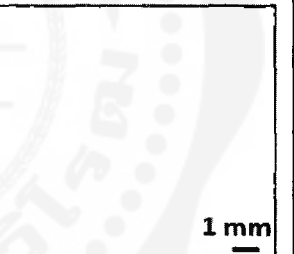
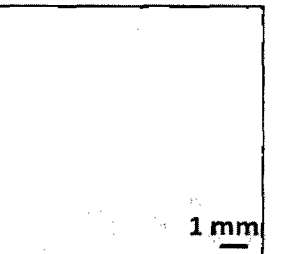
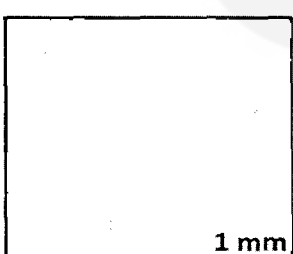
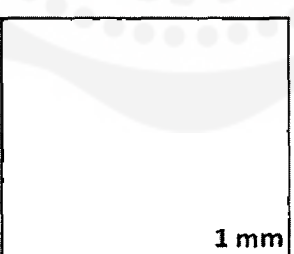
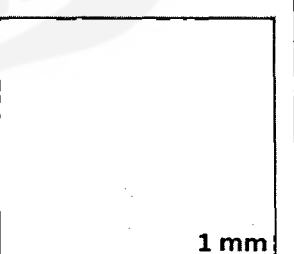
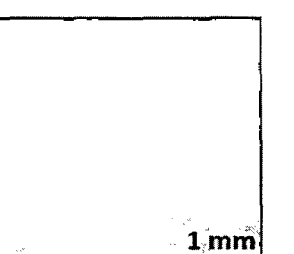
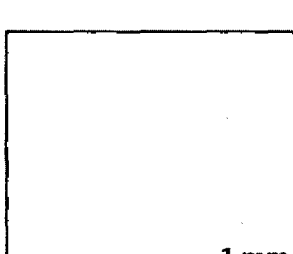
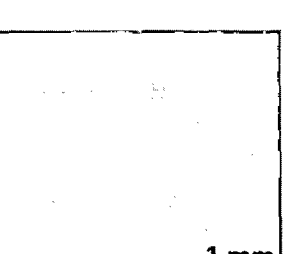
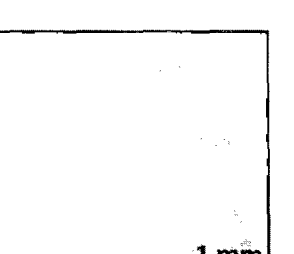
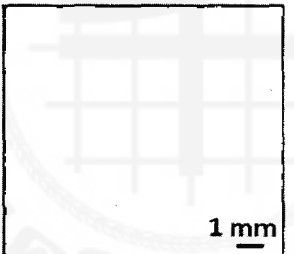
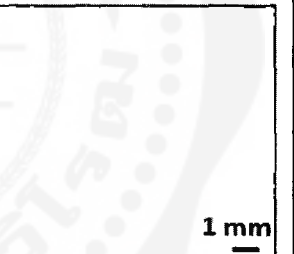
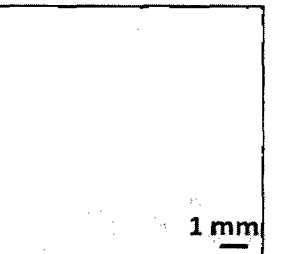
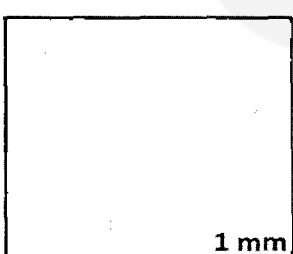
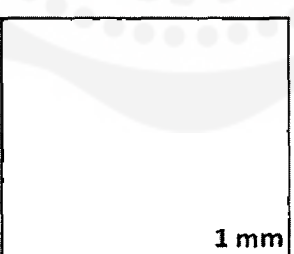
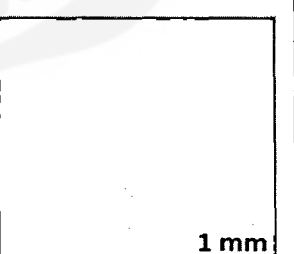
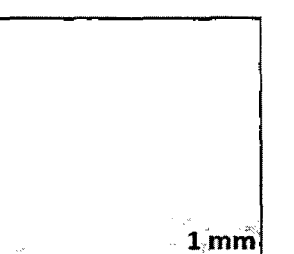
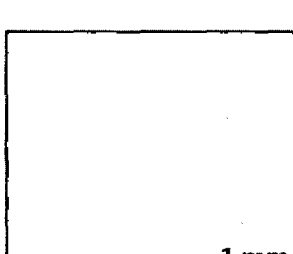
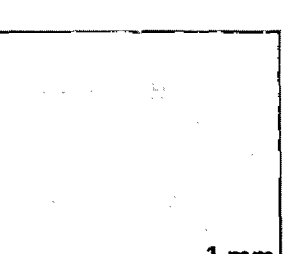
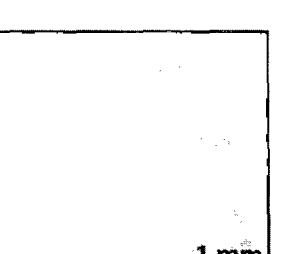
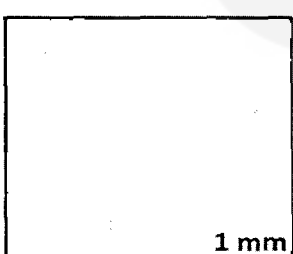
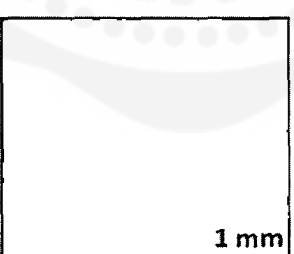
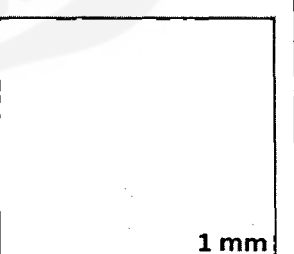
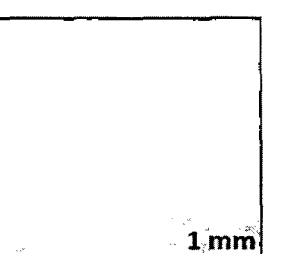
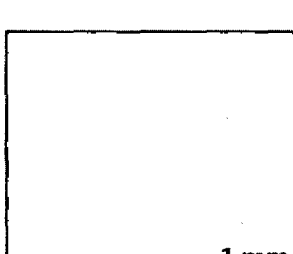
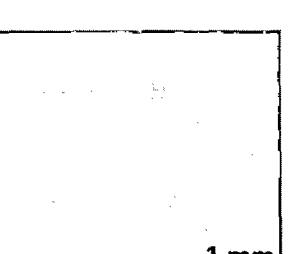
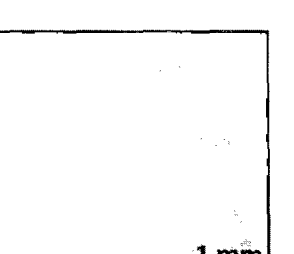
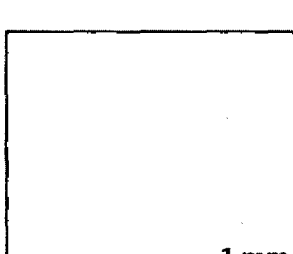
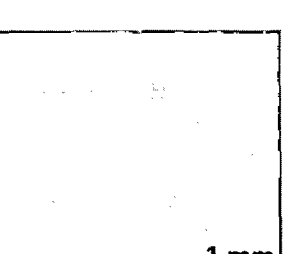
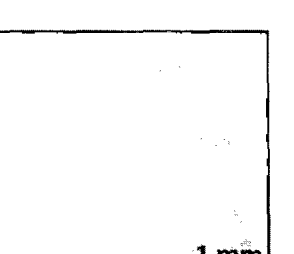
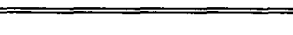
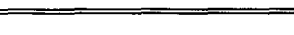
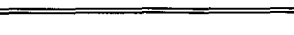
Figure 16 Influence of calcium ions on propagation of phage Φ T25 in MRS broth without calcium (●), with 10 mM calcium (◆), with 20 mM calcium (▲) and with 30 mM calcium (■). Mean values used were obtained from three determinations.

Table 12 The number of plaques per plate with different CaCl₂ concentrations in MRS bottom agar and top agar.

CaCl ₂ in bottom agar (mM)	CaCl ₂ in top agar (mM)			
	0	10	20	30
0	2.60±0.85	3.40±1.86	5.58±0.31	9.01±0.18
10	5.58±1.30	5.76±0.20	8.50±1.91	14.15±0.63
20	6.13±0.57	5.43±1.12	7.01±0.91	18.13±1.64
30	7.28±0.94	5.66±0.84	10.33±1.19	11.90±0.22

Note: Data presented means values (±SD) of at least three replicates.

Table 13 Plaque formation of phage Φ T25 on the lawn of *Lb. paracasei* LPC under the different concentrations of CaCl_2 in MRS bottom agar and top agar.

CaCl ₂ in bottom agar (mM)	CaCl ₂ in top agar (mM)			
	0	10	20	30
0				
				
				
				
10				
				
				
				
20				
				
				
				
30				
				
				
				

4.2.6 Analyses of phage genomes

Purified phage genomic DNA was digested with several restriction endonucleases, including *AseI*, *BglII*, *HindIII*, *EcoRI*, *EcoRV*, *MluI*, *MunI*, *NcoI*, *NheI* and *PvuII* and separated by agarose gel electrophoresis (Figure 17). The molecular weight of the phage Φ T25 DNA estimated by distinct restriction patterns was approximately 41 kb. The *cos* sites of the phage Φ T25 DNA were determined by comparing the restriction patterns generated by *BclI*, *BstEII*, *FbaI*, *MluI* and *NcoI* with and without heating the DNA templates for 10 min at 80°C prior to loading on an agarose gel. The results revealed the absence of an extra phage genomic fragment in the heated samples, suggesting that the phage Φ T25 DNA did not contain the cohesive ends (Figure 18). Instead, the phage Φ T25 may likely utilize the *pac* mechanism of DNA packaging.

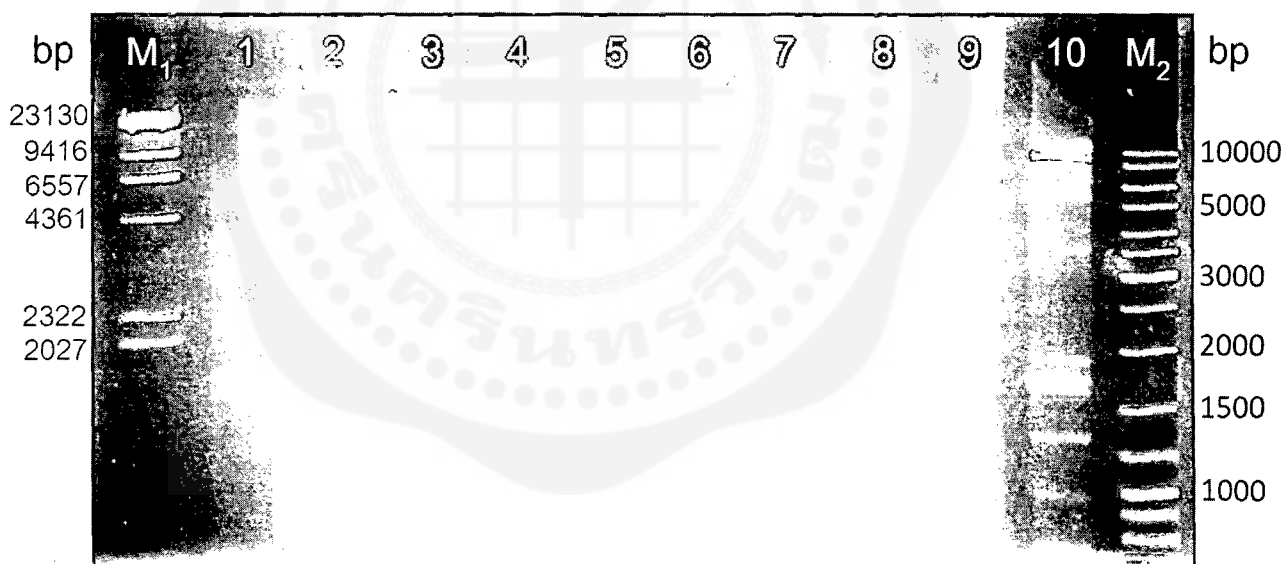


Figure 17 Restriction enzyme digests of phage Φ T25 DNA. Phage Φ T25 DNA templates were digested by *AseI* (lane 1), *BglII* (lane 2), *HindIII* (lane 3), *EcoRI* (lane 4), *EcoRV* (lane 5), *MluI* (lane 6), *MunI* (lane 7), *NcoI* (lane 8), *NheI* (lane 9), *PvuII* (lane 10). Lane M₁: Lambda DNA/*HindIII* marker; lane M₂ Generuler™ DNA ladder mix.

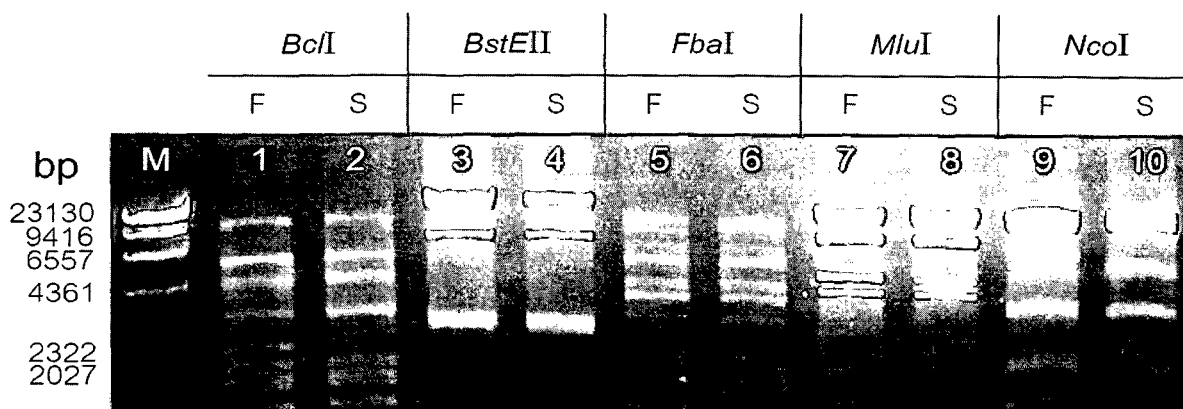


Figure 18 Restriction analysis of phage Φ T25 genomic DNA. The phage DNA templates were digested with *BclI*, *BstEII*, *FbaI*, *MluI*, *NcoI* and the products were separated by agarose gel electrophoresis. Lane M represents Lambda DNA/*HindIII* marker. The digests were heated to 80°C for 15 min and then allowed to cool down fast (F) or slowly (S) to room temperature prior to electrophoresis.

4.2.7 Analysis of the Φ T25 structural proteins

Phage stock was purified using Caesium chloride density gradient (Figure 19) and concentrated using an Amicon Ultra centrifugal filters to achieve phage titers of 10^{20} PFU/mL. The concentrated phage sample was further analyzed for structural proteins using 14% SDS-PAGE coupled with LC-MS/MS analysis. A total of 7 structural proteins were detected with molecular masses (MMs) estimated at 45 and 28 kDa for the major proteins and 80, 60, 55, 13 and 10 kDa for the minor proteins. (Figure 20).

The MMs of structural proteins estimated by SDS-PAGE obtained in this study agreement with the calculated masses from the corresponding gene sequences with the exception of the bands at 50 and 35 kDa. The major phage protein with a molecular mass of 22.1 kDa was identified as a major capsid protein (ORF 49). The minor protein with a molecular mass of 71.5 kDa was designated as a tail fiber protein (ORF 40). The two other minor proteins with molecular masses of 42.4 and 12.7 kDa were recognized as a portal protein (ORF 3) and a head-tail joining protein (ORF 47), respectively.

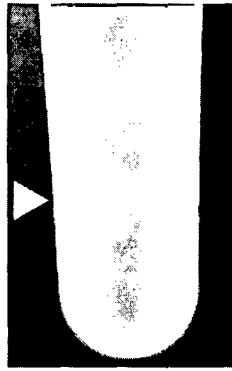


Figure 19 Caesium chloride density gradient from phage Φ T25 lysates. The white band (white arrow) indicated the purified virus fraction that was subsequently used for Φ T25 structural protein analyses.

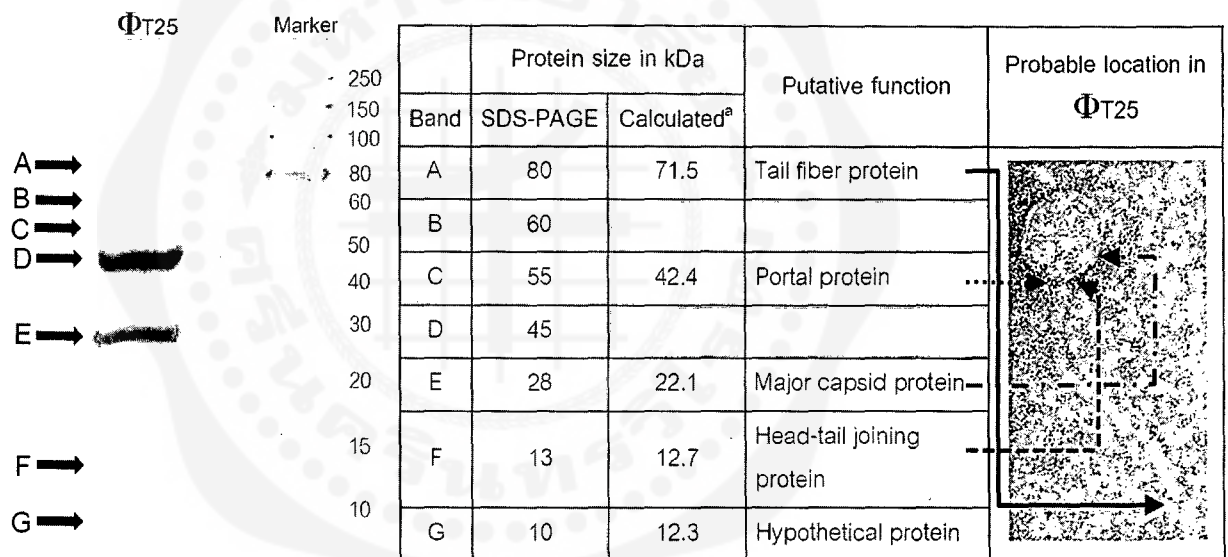


Figure 20 Analysis of phage Φ T25 structural proteins. (Left) SDS-polyacrylamide gel stained with Coomassie blue showed 7 virion protein bands (A-G). The sizes (in kDa) of the proteins in the broad-range molecular mass standard are indicated on the right side (Apro Science, Japan). (Right) Identification of phage Φ T25 proteins from corresponding bands shown in the left panel. Letter at the right corresponds to the letter indicated in the left panel.

^aCalculated values obtained from the Mascot program in combination with the gene sequences.

4.3 Whole genome analysis of phage Φ T25

The complete de novo double-stranded sequence of the phage Φ T25 revealed a genome of 38,421 bp with a G+C content of 44.5%. Bioinformatic analyses of the Φ T25 genome yielded 50 possible ORFs based on several criteria: (i) the ORF begins with either an ATG, GTG, or TTG and ended with either TGA, TAG, or TAA; (ii) the ORF contained at least 30 codons and (iii) the ORF that was preceded by an identifiable ribosomal binding site (RBS).⁽⁹⁷⁾ Of the 50 ORFs, 4 were initiated with the start codon TTG, 8 with GTG and 38 with ATG. A potential RBS, complementary with the 3' ends of 16S rRNA genes of various bacteria, could be identified upstream of most of the 50 ORFs. No evidence could be found for the presence of cohesive, protruding ends (*cos*) in the Φ T25 genome and ligation of its DNA did not alter the restriction patterns (as shown in Figure 18). This result confirmed that the phage Φ T25 likely utilized the *pac* mechanism of DNA packaging. The phage Φ T25 genome had clusters of genes involved in DNA packaging, replication, lysogeny, cell lysis and morphogenesis (Figure 21 and Table 14).

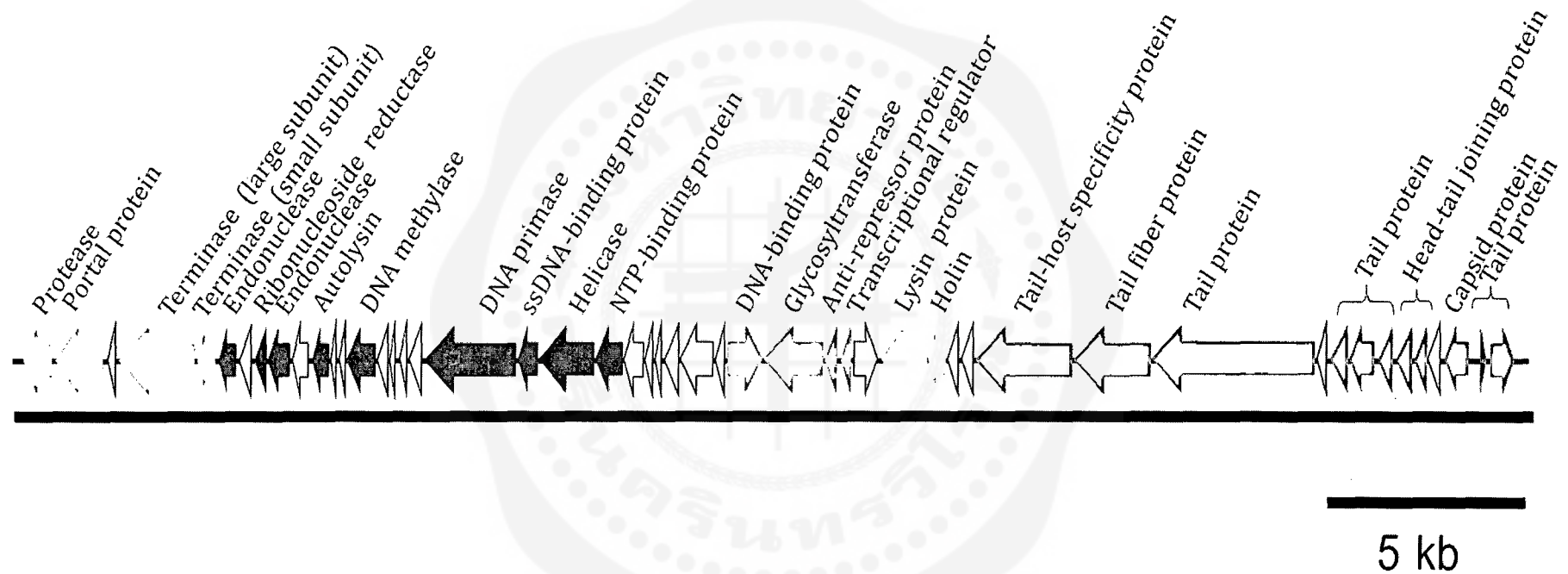


Figure 21 Genome map of phage Φ T25. Arrows indicate putative ORFs and their orientations. Modules are indicated as follows: DNA packaging (green); replication (red); lysogeny (pink); cell lysis (yellow); and morphogenesis (blue).

Table 14 Features of the ORFs of phage Φ T25, predicted functions of proteins and best matches with databases.

ORF	Strand	Position		Size (aa ⁴)	SD sequence	Best BLAST hit						Size (aa ⁴)	GenBank accession no.
		Start	Stop			Predicted function	Organism	Identity	E-value				
1	-	1195	1911	238	AAGGAGGTGATACGAATG	ATP-dependent caseinolytic protease	<i>Lb. paracasei</i> subsp. <i>paracasei</i> Lpp126	97%	2.00E-152	238	EPC67946.1		
2	-	1889	3076	384	AAGGAGGTGACTGATG	Phage portal protein	<i>Lb. rhamnosus</i>	97%	0	395	WP_032957347.1		
3	-	3076	3285	69	AGTTGGGTTTGAGGTGATG		<i>Lb. rhamnosus</i>	96%	2.00E-37	69	WP_080704920.1		
4	-	3272	5164	630	AGGAGGCGAIGTTAGGTG	Phage terminase-like protein, large subunit	<i>Lb. paracasei</i> subsp. <i>paracasei</i> Lpp126	99%	0	630	EPC67943.1		
5	-	5161	5634	157	ACTTGGCIGCCACAACGTTGTG	Phage terminase small subunit	<i>Lb. paracasei</i> subsp. <i>paracasei</i> Lpp126	99%	6.00E-112	157	EPC67942.1		
6	-	5769	6212	147	ACTTGGCIGCCACAACGTTGTG	HNH endonuclease	<i>Lb. paracasei</i>	95%	1.00E-100	162	WP_020967540.1		
7	-	6276	6584	102	GGAGGACTAGAATTG		<i>Lb. paracasei</i>	88%	2.00E-58	107	WP_016363344.1		
8	-	6587	6907	105	GGCGGGCGACCCCGATG	Phage-related ribonucleoside-diphosphate reductase	<i>Lb. paracasei</i>	88%	6.00E-64	106	WP_016365680.1		
9	-	6900	7469	174	AAGCATCTGTTGTATGCGTTCGTTTATG	Endonuclease	<i>Lb. casei</i>	86%	1.00E-105	177	WP_071798499.1		
10	-	7429	8646	405	GGAGGAAAGTAGATG		<i>Lactobacillus</i> phage Lc-Nu	98%	0.00E+00	405	YP_358805.1		
11	-	9016	9459	147	AAGGAGTGGACGCAATG	Autolysin regulatory protein	<i>Lactobacillus</i> phage iLp1308	96%	2.00E-102	147	YP_009208883.1		
12	-	9472	9585	34	GGAGGTGACTGACGATG		<i>Lb. casei</i>	91%	6.00E-04	130	WP_020751718.1		
13	-	9575	9712	45	GAGGTATGAATGTCGCAATATG		<i>Lb. sakei</i>	96%	2.00E-23	66	WP_035147693.1		
14	-	9748	10506	252	AAGAAAGTAGAGGATGAAAAATG	DNA methylase	<i>Lactobacillus</i> phage Lrm1	93%	2.00E-180	252	YP_002117711.1		
15	-	10503	10787	94	ACGAGGTGAAAAATG		<i>Lb. paracasei</i>	86%	4.00E-37	94	WP_050894227.1		

Table 14 (continued).

ORF	Strand	Position		Size (aa*)	SD sequence	Best BLAST hit				
		Start	Stop			Predicted function	Organism	Identity	E-value	Size (aa*) GenBank accession no.
16	-	10768	10908	45	<u>AAGAAAGT</u> AGAGGACGACCGATG		<i>Lb. casei</i> 32G	80%	2.00E-17	49 EKQ00900.1
17	-	10905	11090	61	<u>AGGAGG</u> CACACAGATG		<i>Lactobacillus</i> phage phiAT3	89%	2.00E-35	61 YP_025063.1
18	-	11181	11513	110	<u>AATCAGAAATA</u> ATG	VRR-NUC domain protein	<i>Lactobacillus</i> phage CL2	68%	1.00E-40	108 YP_009201841.1
19	-	11807	14107	766	GGAGGTAATGTGATG	DNA primase	<i>Lactobacillus</i> phage CL2	98%	0.00E+00	766 YP_009201840.1
20	-	14266	14784	172	<u>AGGAGGAA</u> ACTAATATG	Single-stranded DNA-binding protein	<i>Lactobacillus</i> phage CL2	95%	4.00E-123	172 YP_009201838.1
21	-	14908	16284	458	<u>AAGTTCGGAGG</u> IGACAGTG	Helicase	<i>Lactobacillus</i> phage CL2	90%	0.00E+00	458 YP_009201836.1
22	-	16247	16969	240	<u>GGGGG</u> IGCGGTAGATG	NTP-binding protein	<i>Lactobacillus</i> phage CL2	95%	1.00E-167	248 YP_009201835.1
23	-	16970	17455	161	AAGGAAAATATTATG		<i>Lactobacillus</i> phage Lrm1	98%	8.00E-113	161 YP_002117701.1
24	-	17474	17677	67	<u>AAGGGGATG</u> ACGTGATG		<i>Lactobacillus</i> phage J-1	88%	9.00E-38	67 YP_008767278.1
25	-	17682	17834	50	<u>AATTGGAGG</u> CAGIGGATTG		<i>Lactobacillus</i> phage J-1	94%	1.00E-26	50 YP_008767277.1
26	-	17919	18275	118	<u>AAGGAGGAAA</u> IGCCATG		<i>Lactobacillus</i> phage J-1	97%	4.00E-82	119 YP_008767276.1
27	-	18323	19135	267	AAGGAGGCATAGCAATG		<i>Lb. casei</i>	87%	2.00E-148	272 WP_003596701.1
28	-	19170	19409	79	<u>AAGCGAGG</u> IGACATCAAAATG		<i>Lb. rhamnosus</i>	100%	5.00E-51	79 WP_064613077.1
29	+	19537	20421	208	<u>AAGAGG</u> IGGACAAAATG	DNA-binding helix-turn-helix protein	<i>Lb. paracasei</i> subsp. <i>paracasei</i> ATCC 25302	90%	1.00E-132	277 EEI67788.1
30	-	20604	22094	496	<u>AGATG</u> ITCACGTCAAGAGAGGAATG	Glycosyltransferase	<i>Lactobacillus</i> phage Lb338-1	95%	0.00E+00	519 YP_002790859.1
31	-	22170	22337	46	<u>AAGGAGGAAA</u> ITTAATG	Phage repressor protein/antirepressor Ant	<i>Lb. paracasei</i>	91%	1.00E-21	252 WP_016373253.1
32	-	22338	22604	86	<u>AAGGAGG</u> GAAGGAATCATG	Transcriptional regulator	<i>Lb. harbinensis</i>	87%	5.00E-49	89 WP_035439868.1

Table 14 (continued).

ORF	Strand	Position		Size (aa*)	SD sequence	Best BLAST hit					
		Start	Stop			Predicted function	Organism	Identity	E-value	Size (aa*)	GenBank accession no.
33	+	22775	23353	192	<u>AGGAGGGAGCAATCATG</u>	Gp2 phage-like protein	<i>Lb. paracasei</i> subsp. <i>paracasei</i> Lpp70	77%	4.00E-103	194	EPD04849.1
34	-	23430	24596	388	<u>AAGGAGGAAACCATG</u>	Putative lysin protein	<i>Lb. casei</i> Lpc-37	99%	0.00E+00	388	EKQ29605.1
35	-	24607	25044	145	<u>GTAGGCATTAAAGGAGATAATG</u>	Phage holin, LL-H family	<i>Lb. paracasei</i> subsp. <i>paracasei</i> ATCC 25302	90%	3.00E-77	150	EEI67763.1
36	-	25031	25321	96	<u>AAGGAAGTGAGAAAGTG</u>		<i>Lactobacillus</i> phage Lrm1	91%	8.00E-59	96	YP_002117685.1
37	-	25475	25765	96	<u>GGTGGTGATTTAAATG</u>		<i>Lb. casei</i>	98%	1.00E-62	96	WP_003566386.1
38	-	25767	28187	806	<u>AAGGAGGCATGGCTGTAAATG</u>	Putative tail-host specificity protein	<i>Lactobacillus</i> phage phiAT3	86%	0.00E+00	806	YP_025042.1
39	-	28188	30110	640	<u>AAGGAGGAACCGITTAATTG</u>	Phage tail fiber protein	<i>Lb. rhamnosus</i>	93%	0.00E+00	640	WP_039141511.1
40	-	30111	34220	1369	<u>GGAAGCAATACCAAGAGAAAGGGTG</u>	Tail protein	<i>Lb. rhamnosus</i>	96%	0.00E+00	1369	WP_039141514.1
41	-	34217	34447	76	<u>AAGAAGCAGGATG</u>		<i>Lb. rhamnosus</i> Lc-Nu-like prophage	100%	3.00E-48	76	AAX07956.1
42	-	34425	34889	154	<u>ATAGCAACAACCTGATG</u>	Putative tail component	<i>Lactobacillus</i> phage phiAT3	96%	3.00E-76	116	YP_025037.1
43	-	34932	35564	198	<u>AGGAGGCATTCAAAATG</u>	Phage tail protein	<i>Lb. paracasei</i>	97%	9.00E-136	206	WP_085693993.1
44	-	35576	35947	123	<u>ATGGTGGTGGGGATGAATG</u>	Tail component	<i>Lb. casei</i>	97%	7.00E-82	126	WP_003579649.1
45	-	35944	36363	139	<u>AAGGGGGCATAACCGTG</u>	Putative head-tail joining protein	<i>Lactobacillus</i> phage Lc-Nu	92%	2.00E-88	139	YP_358767.1
46	-	36366	36710	114	<u>ACAGCGGTGATACTGATG</u>	Putative head-tail joining protein	<i>Lactobacillus</i> phage phiAT3	90%	1.00E-71	114	YP_025033.1
47	-	36700	37050	116	<u>AAGGTCGCCIATGAAAGAAACAGTTTG</u>		<i>Lb. paracasei</i> subsp. <i>paracasei</i> 8700:2	93%	3.00E-59	116	EEQ65370.1
48	-	37080	37694	204	<u>ATGCCGGTATTTCAACAATG</u>	Phage major capsid protein	<i>Lb. paracasei</i>	98%	7.00E-142	413	WP_003658489.1
49	+	37901	38074	57	<u>GATGTTCTCAAGATTGATGTG</u>	Putative tail component	<i>Lactobacillus</i> phage Lc-Nu	68%	1.00E-22	644	YP_358772.1
50	+	38075	38419	115	<u>AAGGAGGCCIGGCTATAAATG</u>	Phage tail protein	<i>Lb. rhamnosus</i>	100%	1.00E-72	811	WP_064658998.1

*aa, amino acid

4.4 Isolation of spontaneous phage-resistant derivatives

A total of 175 presumptive mutants were obtained from the AP and SC methods using *Lb. paracasei* LPC sensitive strains under pressure of the phage Φ T25 (Table 15). Among them, 98 isolates (56%) were true phage-resistant mutants since they maintained resistance up to the third subculture under the selective pressure. In this study, the SC method showed slightly more efficiency than the AP method for obtaining true phage-resistant variants.

Table 15 Phage-resistant mutants isolated from *Lb. paracasei* LPC using agar plate (AP) and secondary culture (SC) methods.

Isolation method	Number of presumptive phage-resistant mutants	Number of true phage-resistant mutants	Isolation efficiency of true phage-resistant mutants (%)*
Agar plate method (AP)	89	48	53.93
Secondary culture method (SC)	86	50	58.13
Total	175	98	

*Isolation efficiency of true phage-resistant mutants (%) was calculated by dividing number of true phage-resistant mutants by number of presumptive phage-resistant mutants.

4.5 Characterization of spontaneous phage-resistant derivatives

4.5.1 Phage resistance phenotype

4.5.1.1 Phage resistance stability

Most of the mutants lost the resistance phenotype at the second subculture. Only 58 true phage-resistant mutants maintained the phenotype up to the seventh subculture under the selective pressure phage Φ_{T25} . For the SC method, a high stability (%) of spontaneous phage-resistant mutants was observed, since 42% of them maintained resistance up to the seventh subculture as shown in Table 16.

When high titers of the phage Φ_{T25} were assayed on the previously selected mutants, diverse resistance levels expressing in terms of Efficiency of plaquing (EOP) were obtained (Table 17). All mutants exhibited EOP values ranging from 10^{-11} - 10^{-14} (phage-resistance level). Therefore, 31 out of 58 phage-resistant mutants were selected based on the low level of EOP for further studies.

Table 16 Stability of the phage resistance phenotype for *Lb. paracasei* LPC phage-resistant mutants (% of mutants able to successfully overcome each subculture).

Sensitive strain	Phage	Isolation method	Number of subculture						
			1	2	3	4	5	6	7
<i>Lb. paracasei</i> LPC	Φ_{T25}	AP ($n_r=89$)	100	57	53	52	49	43	42
		SC ($n_r=86$)	100	59	58	47	43	23	23

n_r , number of phage-resistant mutants, isolated from the respective parent strain.

Table 17 Efficiency of plaquing (EOP) of the phage resistance phenotype for *Lb. paracasei* LPC-derived phage-resistant mutants.

Sensitive strain	Phage	EOP* (number of mutant in each group)				
		10^{-1} - 10^{-3}	10^{-4} - 10^{-6}	10^{-7} - 10^{-10}	10^{-11} - 10^{-12}	10^{-13} - 10^{-14}
<i>Lb. paracasei</i> LPC	Φ T25	0	0	0	8	23

* Efficiency of plaquing (EOP) is calculated from the phage Φ T25 titer (in PFU/mL) on the test strain (phage-resistant) by the phage titer in PFU/mL (10^{14} PFU/mL) on the parent strain (*Lb. paracasei* LPC).

4.5.2 Phage resistance mechanism

In terms of the phage-resistance mechanisms, two corresponding mechanisms involved were demonstrated including the lower adsorption rate and the presence of prophage(s).

4.5.2.1 Adsorption rates

The phage-resistant mutants obtained from *Lb. paracasei* LPC were diverse with respect to adsorption, with rates ranging from low (0-30%), medium (31-70%) to high (71-100%). Only 6 mutants, AP3-1, AP3-13, AP3-16, AP3-31, AP3-42 and AP 3-46 exhibited low adsorption rates (Table 18). Most of the mutants (70%) were partially unable to bind the phage particles because they possessed the low and medium adsorption rates.

4.5.2.2 Lysogeny

Free phages were detected in the supernatant of broth cultures of 23 phage-resistant mutants. This result suggested that the resistance phenotype could be linked to an integration of the phage Φ T25 DNA in the genome of *Lb. paracasei* LPC, leading to a possible phage-resistance mechanism associated with the lysogenic immunity. To confirm whether the phage resistance was due to a superinfection immunity mechanism, an induction was attempted with sublethal concentrations of mitomycin C (0.6 μ g/mL). Detection of the prophage from the filtrates of mitomycin C-induction supernatants indicated

the presence of at least one prophage on 7 out of 31 strains analyzed. However, some mutants exhibiting the high adsorption rate may also harbor prophage(s). Among 31 strains, the strains AP3-13, AP3-15, AP3-16, AP3-34, SC4-39 and SC4-52 have shown to exist in true lysogenic association since prophage excisions can be induced by mitomycin C.

4.5.2.3 Plasmid curing

After curing of the plasmid with ethidium bromide, phage resistance stability of 5 selected mutants with no prophage detected including the strains AP1-2, AP1-16, AP3-17, AP3-31 and SC4-14 were examined. All variants still maintained their insensitivity to phage infection, suggesting that the phage resistance phenotype was not associated with the presence or absence of the plasmid.

Subsequently, 3 phage-resistant strains including AP1-2, AP3-17 and AP3-31 were selected based on their extreme resistance capacity to the phage Φ T25 (EOP < 10^{-10}) and their nonlysogenic property for further study.

Table 18 Phage resistance mechanisms present in phage-resistant mutants isolated from *Lb. paracasei* LPC.

No.	Mutant strain	Phage resistance mechanism			
		Adsorption rate ^a	Prophage ^b		Plasmid curing ^e
			Lysogeny ^c	Mitomycin C induction ^d	
1	AP1-2	High	-	-	S
2	AP1-16	Medium	-	-	S
3	AP2-25	High	+	-	
4	AP2-55	Medium	+	-	
5	AP3-1	Low	+	-	
6	AP3-6	Medium	+	+	
7	AP3-13	Low	+	+	
8	AP3-14	Medium	+	-	
9	AP3-15	Medium	+	+	
10	AP3-16	Low	+	+	
11	AP3-17	High	-	-	S
12	AP3-21	Medium	+	-	
13	AP3-25	High	+	-	
14	AP3-27	High	-	-	
15	AP3-28	Medium	+	-	

Table 18 (continued).

No.	Mutant strain	Phage resistance mechanism			
		Adsorption rate ^a	Prophage ^b		Plasmid curing ^e
			Lysogeny ^c	Mitomycin C induction ^d	
16	AP3-31	Low	-	-	S
17	AP3-33	Medium	-	-	
18	AP3-34	Medium	-	+	
19	AP3-42	Low	+	-	
20	AP3-44	Medium	+	-	
21	AP 3-46	Low	+	-	
22	AP 3-48	Medium	+	-	
23	AP 3-53	Medium	+	-	
24	SC4-14	High	-	-	S
25	SC4-39	Medium	+	+	
26	SC4-48	Medium	+	-	
27	SC4-52	High	+	+	
28	SC5-2	High	+	-	
29	SC5-8	Medium	+	-	
30	SC5-17	High	+	-	
31	SC5-29	Medium	+	-	

^aAdsorption rate of phage Φ T25 on each mutant strain in MRS broth after 30 min at 37°C.

^b -: absence; +: presence of prophage.

^cSpontaneous induction of free phages, detected in the supernatants of mutant cultures.

^dDetection of prophage from filtrates of mitomycin C-induction supernatants.

^ePhage resistance stability of 5 selected mutants after plasmid curing; S: stable resistance; U: unstable resistance.

4.5.2 Characterization of phage-resistant mutants

4.5.2.1 Cell and colony morphologies

Three phage-resistant strains, including AP1-2, AP3-17 and AP3-31 were identical to the corresponding parent strain *Lb. paracasei* LPC in cell and colony morphologies as shown in Figures 22 and 23.

4.5.2.2 Acidification kinetics

Lb. paracasei LPC and three selected strains were evaluated in relation to their acidification kinetics in the MRS and RSM media (Figure 24). All phage resistant variants developed acidity as fast as the parent strain and pH values in the MRS and RSM media after 24 h at 37°C ranged between 3.91-4.03 and 3.60-3.73, respectively. All mutants exhibited high acidifying activities, and to they were able to produce 12.09 ± 0.82 mg/mL lactic acid in the MRS medium. These amounts were indifferent to those exhibited by the parent strain (Table 19).

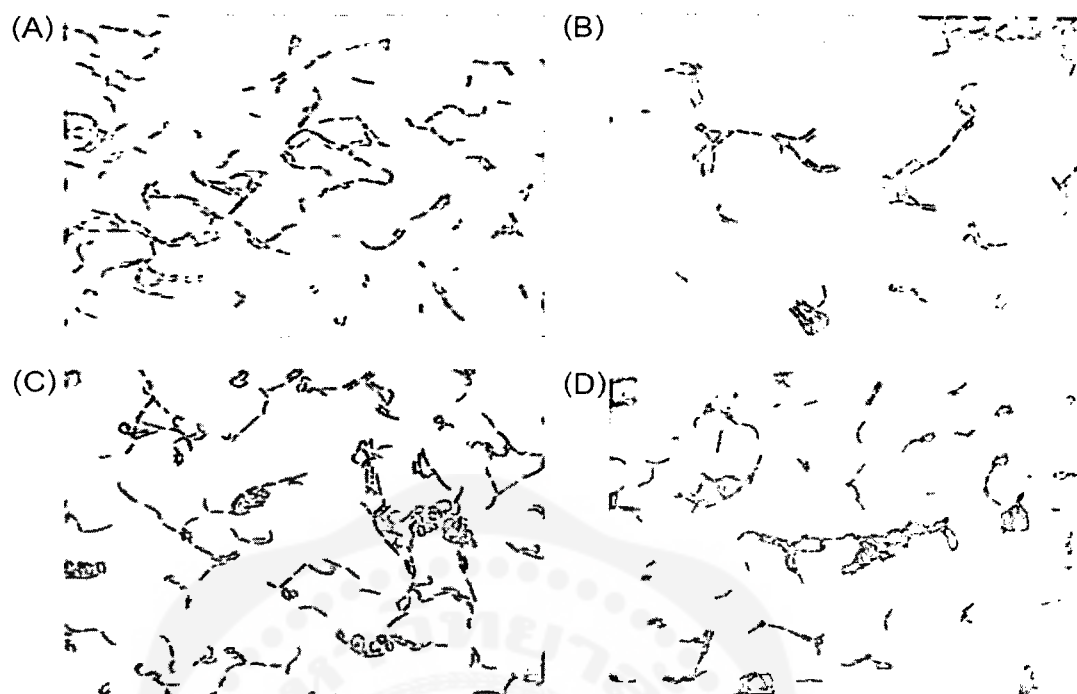


Figure 22 Gram stain of *Lb. paracasei* LPC (A) and their phage-resistant mutant strains, AP1-2 (B), AP3-17 (C) and AP3-31 (D).

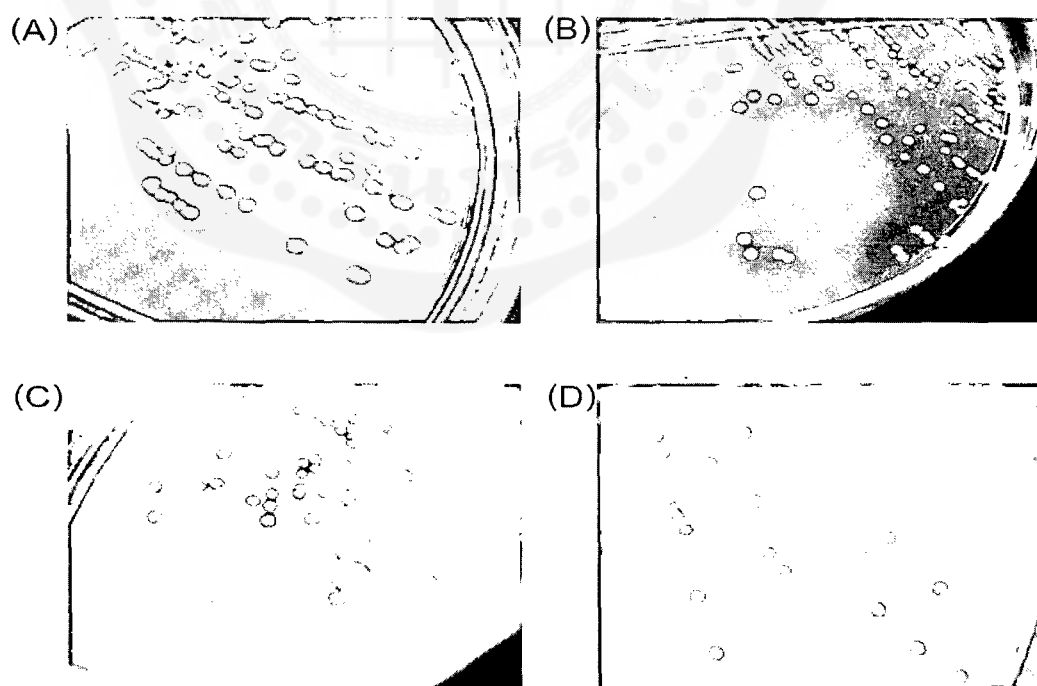


Figure 23 Colony morphology of *Lb. paracasei* LPC (A) and their phage-resistant mutant strains, AP1-2 (B), AP3-17 (C) and AP3-31 (D).

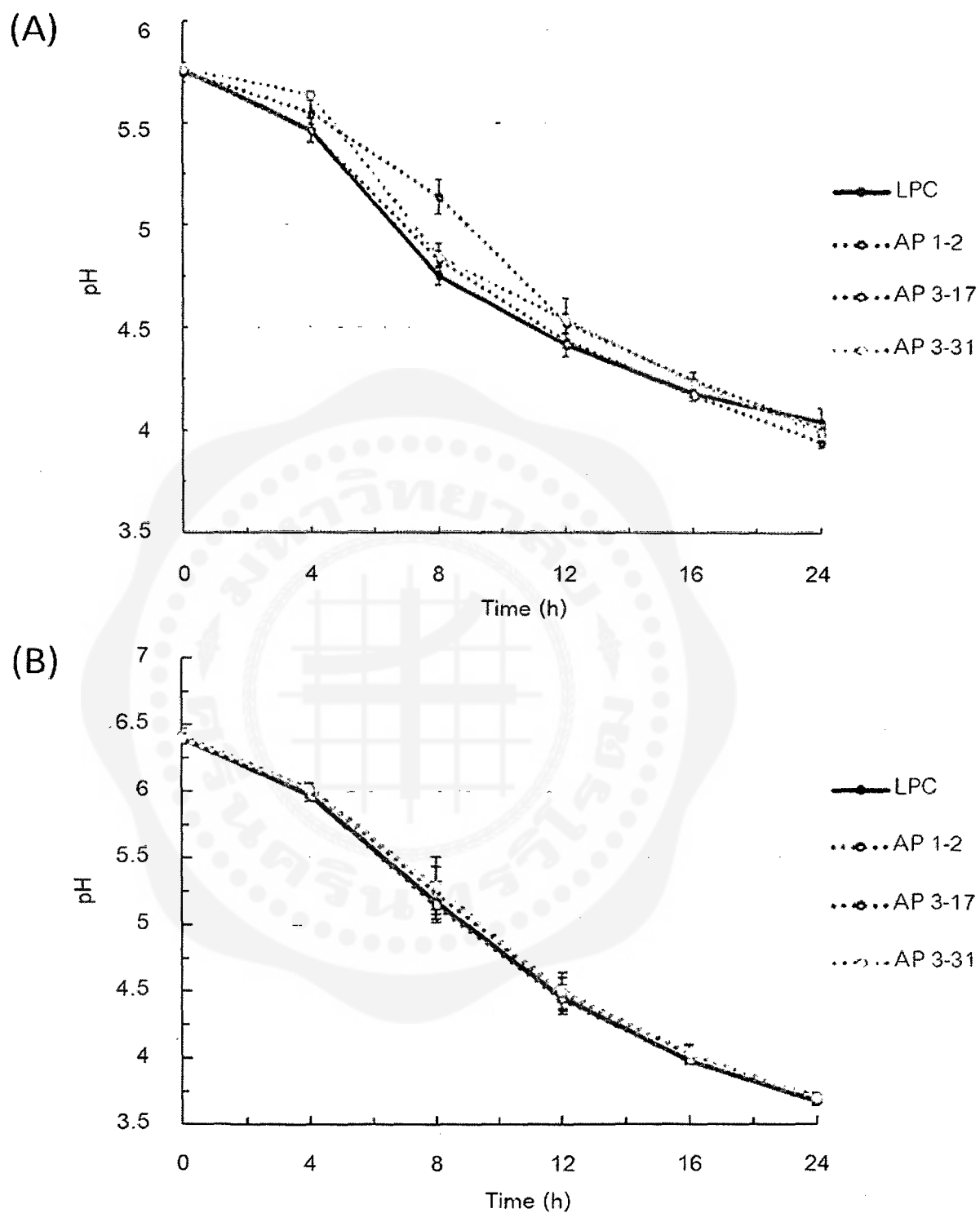


Figure 24 Change in pH values in MRS (A) and RSM (B) media of the sensitive strain *Lb. paracasei* LPC (—) and their phage-resistant mutants (- - -). Assays were performed in triplicate and data were reported as means $\pm$ standard deviations.

Table 19 Summary of acidification kinetics of *Lb. paracasei* LPC and their phage-resistant mutant strains, AP1-2, AP3-17 and AP3-31 in MRS and RSM media after incubation at 37°C for 24 h.

Strain	MRS medium	
	pH	Concentration of lactic acid ^a (mg/mL)
<i>Lb. paracasei</i> LPC	4.04±0.06	13.67±0.79
<i>Lb. paracasei</i> AP1-2	3.94±0.02	12.77±1.96
<i>Lb. paracasei</i> AP3-17	4.01±0.02	11.18±1.45
<i>Lb. paracasei</i> AP3-31	3.98±0.01	12.33±0.85

^a Lactic acid was determined by HPLC analysis.

4.5.2.3 Technological performance

To investigate the performance of the selected strains (AP1-2, AP3-17 and AP3-31) as starters and the stabilities of their phage resistance phenotypes, pilot milk fermentation assays were performed. Parallel fermentations were carried out with *Lb. paracasei* LPC which was used as a phage susceptible control. The changes in pH, lactobacilli count and phage titers throughout the manufacturing process were shown in Figures 25-27. The control fermentation (without phage inoculation), *Lb. paracasei* LPC and mutants grew up to 10^9 CFU/mL after 8 h of incubation and a pH reaching to 4.40 after 24 h. In the presence of Φ T25, viable cell counts of the sensitive strain LPC were drastically reduced to only 10^6 CFU/mL and the pH reaching to 4.78 after 24 h of incubation. As expected, the titer of Φ T25 increased during the same period, producing 10^6 PFU/mL. Conversely, milk inoculated with the combination of resistant variants of *Lb. paracasei* and the phages, all variants continued to grow with a bacterial count of 10^9 CFU/mL and the phage titer was ranged between 10^2 - 10^4 PFU/mL during 24 h of incubation. During refrigerated storage, both viability of the resistant variants (10^9 CFU/mL) and pH (4.40) remained constant until the end of the storage period (21 days), even in the presence of the phages (Figures 25D, 26D and 27D).

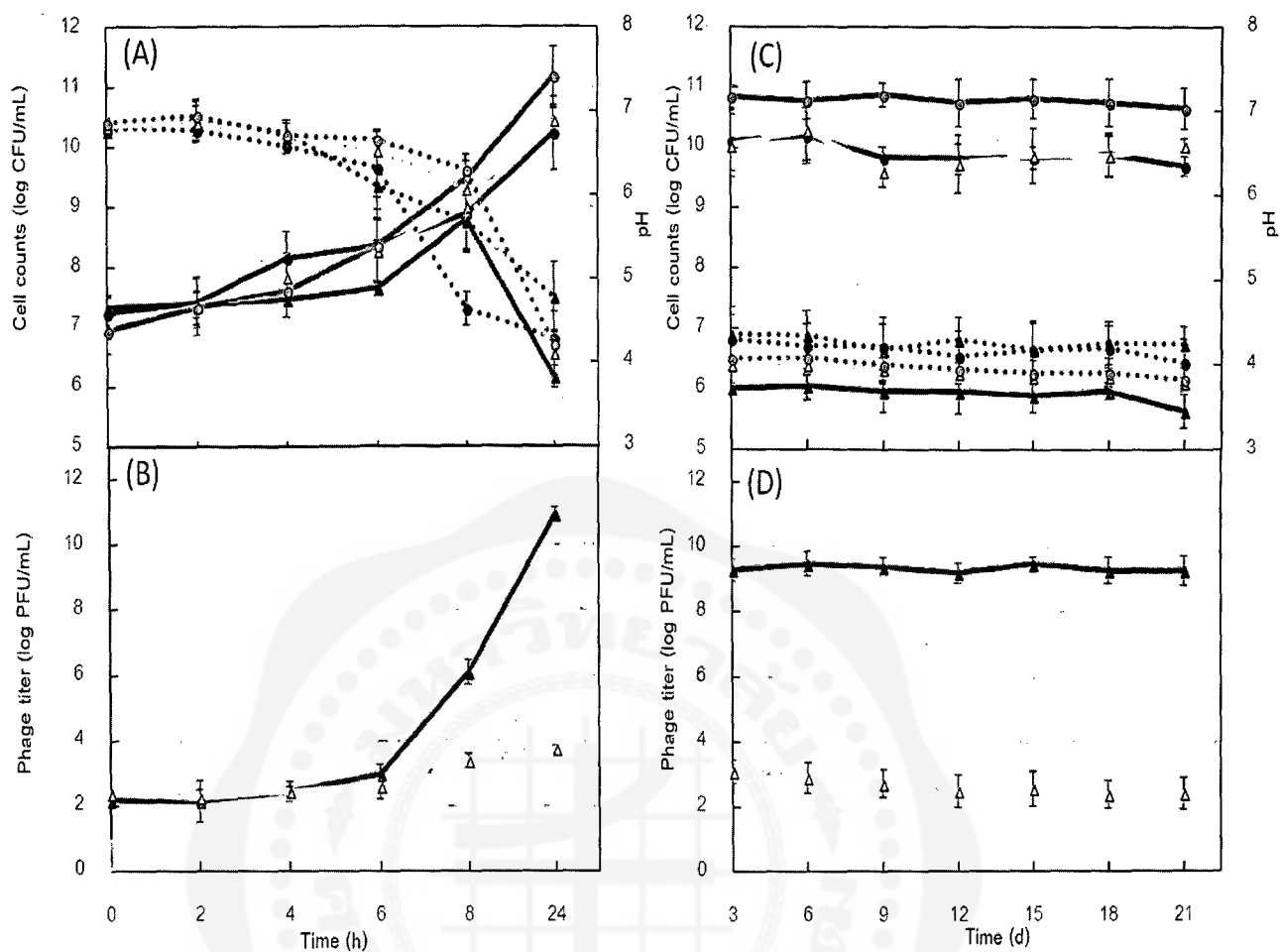


Figure 25 Changes in cell counts (—) and pH (---) values during fermentation (A) and subsequent refrigerated storage (C) of milk by using *Lb. paracasei* LPC ($\blacktriangle$, $\bullet$) or the mutant *Lb. paracasei* AP1-2 ($\triangle$, $\circ$), infected ($\blacktriangle$, $\triangle$) or not ($\bullet$, $\circ$) with phage Φ T25. Titers of phage Φ T25 during fermentation (B) and the subsequent refrigerated storage (D) of milk by using *Lb. paracasei* LPC ($\blacktriangle$) or the mutant AP1-2 ($\triangle$) were also shown. Error bars represented the standard deviation of three determinations.

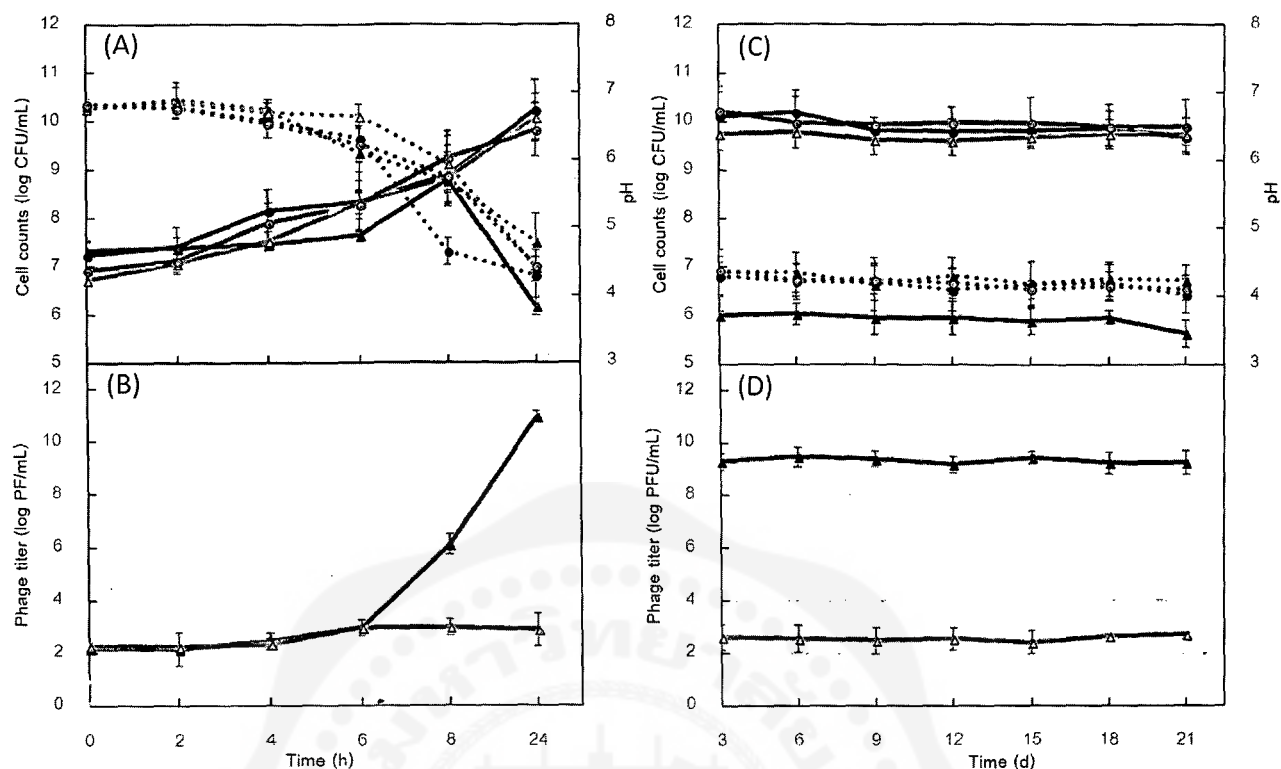


Figure 26 Changes in cell counts (—) and pH (---) values during fermentation (A) and subsequent refrigerated storage (C) milk by using *Lb. paracasei* LPC (▲,●) or the mutant *Lb. paracasei* AP3-17 (△,●), infected (▲,△) or not (●,●) with phage Φ T25. Titers of phage Φ T25 during fermentation (B) and the subsequent refrigerated storage (D) of milk by using *Lb. paracasei* LPC (▲) or the mutant AP3-17 (△) were also shown. Error bars represented the standard deviation of three determinations.

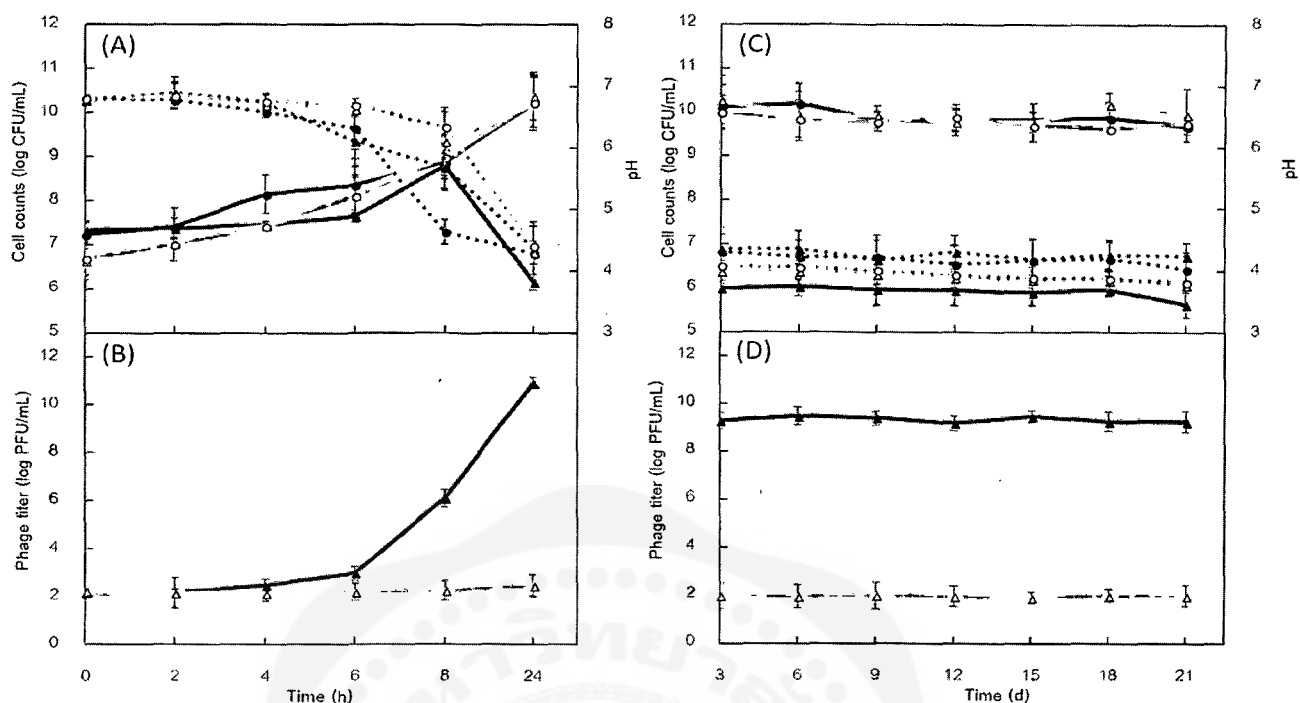


Figure 27 Changes in cell counts (—) and pH (---) values during fermentation (A) and subsequent refrigerated storage (C) of milk by using *Lb. paracasei* LPC ($\blacktriangle$, $\bullet$) or the mutant *Lb. paracasei* AP3-31 ($\triangle$, $\circ$), infected ($\blacktriangle$, $\triangle$) or not ($\bullet$, $\circ$) with phage Φ T25. Titers of phage Φ T25 during fermentation (B) and the subsequent refrigerated storage (D) of milk by using *Lb. paracasei* LPC ($\blacktriangle$) or the mutant AP3-31 ($\triangle$) were also shown. Error bars represented the standard deviation of three determinations.

4.5.2.4 Genetic analysis

All 3 variants that exhibited high stability of phage resistance phenotype and fast milk acidification activity as much as in the parent strain were subjected to RAPD fingerprinting analysis. The UPGMA dendrogram was obtained based on the RAPD profile of 4 primers, including B10, B06, M13 and 1254 (Figure 28). The similarity coefficients between *Lb. paracasei* LPC and their phage-resistant mutant strains were higher than 87%. The results revealed a strong genetic homology and confirmed that the selected phage-resistant mutants were certainly derived from the parent strain *Lb. paracasei* LPC.



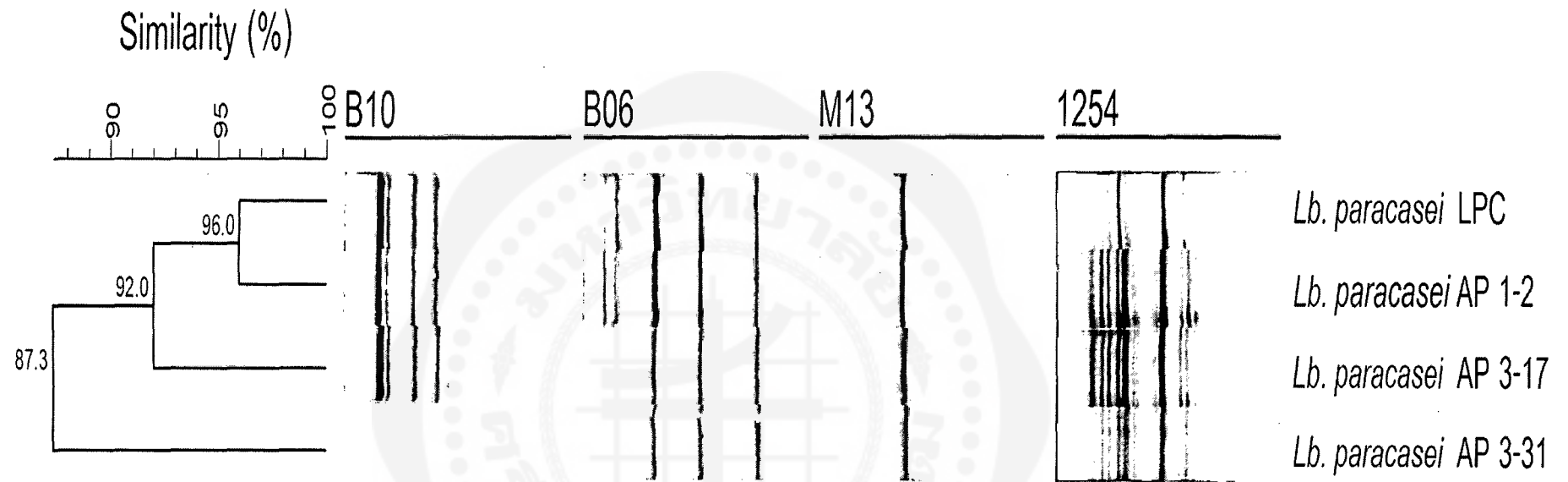


Figure 28 UPGMA dendrogram of RAPD profile from *Lb. paracasei* LPC and their spontaneous phage-resistant mutants, obtained with B10, B06, M13 and 1254 primers. Scale represents similarity coefficient values (%).

CHAPTER 5

CONCLUSIONS AND DISCUSSION

The manufacture of fermented milk products rely on actively growing lactic acid bacteria (LAB), known as starter cultures which contribute to high-quality final products. LAB produce not only lactic acid as a product of carbohydrate fermentation but also various flavor compounds which are desirable and characteristics of dairy products. Several species of LAB associated with probiotic potential are being used as the starter cultures such as *Lactococcus lactis*, *Streptococcus thermophilus* and *Lactobacillus* sp.⁽³¹⁾ In this study, *Lactobacillus* sp. starter culture was isolated from drinking yogurt samples and designated as strain LPC. *Lactobacillus* sp. LPC was identified by Gram-stain reaction, colony morphology, sugar-fermentation profiles using the API 50 CHL system and 16S rRNA gene sequence analysis. The results obtained by the API 50 CHL system of 99.7% indicated that the strain LPC could be *Lb. paracasei* subsp. *paracasei*, whereas the results from the 16S rRNA gene sequence analysis revealed that this strain could be either *Lb. casei* or *Lb. paracasei*. Confirmation of the identification of the strain LPC at species level was achieved by species-specific PCR, with the primers targeted on the V1 region of the 16S rRNA gene.⁽⁷⁸⁾ Sequencing of the V1 region of *Lactobacillus* sp. LPC showed 100% identity with V1 region sequence of *Lb. paracasei* ATCC 334 (type strain) but was different from the sequence of the V1 region of *Lb. casei* ATCC 393. Consequently, *Lactobacillus* sp. LPC was identified as *Lb. paracasei*. The species *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* are phylogenetically and phenotypically closely related and are regarded together as the *Lb. casei* group within the facultatively heterofermentative lactobacilli.⁽⁹⁸⁾ The classification and nomenclature of these species are controversial.⁽⁴⁰⁾ Within the *Lb. casei* group, the close genetic relationship as well as the similarity of biochemical properties among of the species may lead to misidentifications. Species-specific assays have been applied to differentiate the species of the *Lb. casei* group. Differences in the V1 region of the 16S rRNA gene were observed for three casei-group species.⁽⁹⁹⁾ Ward and Timmin (1999) have developed polymerase chain reaction primers which are specific for each of

these species based on differences in the V1 region of the 16S rRNA gene in conjunction with the primer Y2, enabled definitive and rapid species identification of the *Lb. casei* group.⁽¹⁰⁰⁾ Based on this study, the sequence differences in the V1 region of the 16S rRNA gene were sufficient to discriminate the species of the *Lb. casei* group. Recently, Wuyts et al. (2017) show that the *Lb. casei* group harbors three different taxonomic clades by using a core genome phylogenetic tree, GC content analysis, and pairwise genome distances. Reclassification of the *Lb. casei* group members led to the discovery of at least one catalase gene in all of the members of clade B, representing *Lb. casei*, making it the first described catalase-positive species in the whole *Lactobacillus* genus. Thus, if sequencing of the 16S rRNA gene leads to the identification of a member of the species *Lb. casei* or *Lb. paracasei*, then we propose the use of the heme-dependent catalase gene or the antioxidant superoxide dismutase -encoding gene as a marker gene for the correct identification of these species.⁽¹⁰¹⁾

After enrichment in the presence of *Lb. paracasei* LPC, only one type of phage was isolated from the fermented milk culture tank and designated as the phage Φ T25. According to its morphology, the phage Φ T25 was classified as a member of the *Siphoviridae* family of the *Caudovirales* order⁽⁴²⁾, as reported for all of the *Lactobacillus* phages with the exception of *Lb. paracasei* myophage Lb338-1.⁽¹⁰²⁾ The structural features of Φ T25 were similar to most of the other *Lactobacillus* phages (about 50 nm in diameter, 170-180 nm in length) as shown in Table 20. The phage Φ T25 was highly strain-specific to *Lb. paracasei* LPC; no lytic activity was observed on any lactobacilli strains except the closely related strains within the *Lb. casei* group. Similar results were reported for other the *Lb. paracasei* phages Lb338-1, iLp84 and iLp1308.⁽¹⁰²⁻¹⁰³⁾ The 55-min latent period estimated for the phage Φ T25 was higher than the average time reported for the *Lb. paracasei* phages MLC-A, C_L1, C_L2 and iLp1308, whereas the burst time of the phage Φ T25 was shorter than those reported by the other *Lb. paracasei* phages.^(55,103-104) The phage Φ T25 had an average burst size of 38 PFU/cell which was larger than that of the phage iLp1308 (28 PFU/cell), but smaller than that of the other *Lactobacillus* phages (42-160 PFU/cell). The multiplicity of infection (MOI) was defined as the ratio of virus particles to host cells. The high MOI strategy is used when the experiment requires that every cell in the culture is

infected and resulted in rapid cell death while low MOI is used when complete of multiplication cycle of phage is required. MOI resulting in the highest phage titer was considered to be optimal for the subsequent experiments.⁽³⁵⁾ In the present study, the optimal MOI 0.001 resulted in the highest phage titer of 6.87 log PFU/mL within 3.5 h. Determination of the optimal MOI is required to obtain the highest yields of phage. At low MOI, it is ensured that only one virus infecting each host cell and the infection process is initiated with the resulting production of phage progeny. For high MOI, on the other hand, a single host cell has been infected by too many phages, resulting in a lower yield of the phage progeny (lysis from without). The optimal MOI of the *Lb. plantarum* bacteriophage, Φ JL-1 was determined to be 0.01-0.03 while that for *Weissella cibaria* phage, Φ 22 was 0.01.^(35,105) Adsorption of the LAB phages is usually studied at low MOI (<0.02) to avoid variances due to the influence of the relative amounts of phage particles and host cells present.⁽¹⁰³⁾ Capra et al. (2006) studied the adsorption on the *Lb. paracasei* phages at MOI of up to 1.5, where a decrease in the adsorption was reasonably expected, due to a low number of available sites for phage attachment on the cell wall.⁽¹⁰⁴⁾

The initial step of phage infection is the adsorption of the phages to host cell surface. Phage adsorption is not only dependent on the presence of specific receptors on the cell surface⁽¹⁰⁵⁾, but may also depend on the presence of certain cations in the media. The phages usually require higher concentrations of divalent cations, such as calcium or magnesium, at some stage of their infection cycle than the concentration required for the growth of the host cells.⁽¹⁰⁶⁾ In addition, Sechaud et al. (1988) reported that the divalent cations play a crucial role in the phage life cycle including stabilization of the coiled DNA inside the phage capsid, improvement in adsorption rate and participation in the process of penetration of the phage genome into the host cytoplasm.⁽¹⁰⁷⁾ In this study, the addition of the calcium ions significantly increase the adsorption capabilities of the phage Φ T25. The maximum adsorption rates of the phage Φ T25 were achieved in the presence of 30 mM calcium ions after 20 min of incubation. In the presence of Ca^{2+} , the *Lb. paracasei* phages MLC-A, C_L1, C_L2, iLp1308 and iLp84 adsorbed faster than in its absence.^(55,103-104,108) Moreover, the presence of the calcium ions was efficient for cell lysis on agar plate, leading to higher titers and clearer plaques. The largest plaques with diffuse borders were obtained

on MRS agar with 30 mM CaCl_2 -MRS base layer. The bottom base layer normally provides nutrients, buffers against a reduction in pH and helps to maintain a soft and moist lawn.⁽¹⁰⁶⁾ Furthermore, the phage Φ T25 could normally in form plaques on MRS agar in the absence of the calcium ions which was similar to the results previously observed in the *Lactobacillus* phages FAKG1, FAKG2, J-1 and Ldb.^(109,110) In contrast, the calcium and magnesium ions were indispensable for forming the lysis plaques of other *Lactobacillus* phages such as BYM, Ib3, YAB and MLC-A.⁽¹⁰⁹⁾ The propagation rates of the phage Φ T25 in calcium-free MRS were almost the same as that in calcium-containing MRS, suggesting that the excess of calcium ions did not promote phage propagation and cell lysis in the liquid media. However, the requirements of the divalent cations vary in different types of the phages.⁽¹⁰⁸⁾ These results suggested that the levels of calcium and/or other cations in MRS media were sufficient for the initial infection steps but were not considered to be involved in the intracellular multiplication. In the case of the PL-1 phage of *Lb. casei* ATCC 27092, the calcium ions seemed to be necessary for a stage of penetration of the phage genome.⁽¹⁰⁶⁾

The restriction fragment analysis suggested that genome size of the phage Φ T25 was approximately 41 kb which was similar in range to the other *Lb. paracasei* phages known to date, including MLC-A; 37 kb, MLC-A2; 40 kb, MLC-A5; 40 kb, MLC-A8; 43 kb, MLC-A17; 45 kb and MLC-A19; 38 kb, FiLp84; 38 kb, FiLp1308; 34 kb, Lb338-1; 142 kb, CL_1; 39 kb, CL_2; 38 kb and iA2; 34 kb.^(7,55,102,103,104)

Table 20 General features of phages of *Lb. casei* group.

Host	Phage	Life cycle	Head (nm)	Tail (nm)	Family	Latent period (min)	Rise period (min)	Burst size (PFU/cell)	Year	Reference
<i>Lb. casei</i> Shirota	J-1	Virulent	65	283	<i>Siphoviridae</i>				1965	111
<i>Lb. casei</i> Shirota	PL-1	Virulent	62	290	<i>Siphoviridae</i>	100	70	200	1970	111
<i>Lb. casei</i> S-1	FSW	Temperate	57	156	<i>Siphoviridae</i>				1983	39
<i>Lb. casei</i> ATCC 393	A2	Virulent	55	239	<i>Siphoviridae</i>	140	30	180-200	1994	112
<i>Lb. casei</i> ATCC 393	AT3	Temperate	53	200	<i>Siphoviridae</i>				2005	113
<i>Lb. paracasei</i> A	MLC-A	Virulent	57	156	<i>Siphoviridae</i>	30	135	69	2006	104
<i>Lb. paracasei</i> 338	Lb-338-1	Virulent	85	200	<i>Myoviridae</i>	90	105	42	2009	102
<i>Lb. paracasei</i> A	C _L 1	Temperate	54	152	<i>Siphoviridae</i>	30	120	160	2010	55
<i>Lb. paracasei</i> A	C _L 2	Temperate	54	152	<i>Siphoviridae</i>	30	120	90		
<i>Lb. paracasei</i> A	iA2	Temperate			<i>Siphoviridae</i>					

Table 20 (continued).

Host	Phage	Life cycle	Head (nm)	Tail (nm)	Family	Latent period (min)	Rise period (min)	Burst size (PFU/cell)	Year	Reference
<i>Lb. paracasei</i> 84	iLp84	Temperate	61	184	<i>Siphoviridae</i>	84	130	85	2015	113
<i>Lb. paracasei</i> CNRZ1308	iLp1308	Temperate	61	184	<i>Siphoviridae</i>	51	92	28		
<i>Lb. paracasei</i> LPC	ΦT25	Virulent	55	184	<i>Siphoviridae</i>	55	55	38	2016	106
<i>Lb. rhamnosus</i> Lc 1/3	Lc-Nu	Virulent	50	213	<i>Siphoviridae</i>				1993	114
<i>Lb. rhamnosus</i> M1	Lrm1	Temperate	55	275	<i>Siphoviridae</i>				2008	115

The phage Φ T25 genomic DNA was subjected to restriction digest with several endonucleases. The approximate size of the double-stranded DNA of the phage Φ T25 calculated from the sum of restriction fragments was 41 bp. This molecular weight was turned out to be overestimated when compared to the complete genome sequencing because of the incomplete digestion. The complete genome sequencing carried out using the MiSeq sequencing platform revealed a linear, double-stranded DNA genome with a size of 38,421 bp and a G+C content of 44.5%. The G+C content of the phage Φ T25 was in agreement with previous reports of the other *Lactobacillus* phages as shown in Table 21. No evidence could be found for the presence of cohesive ends in the phage Φ T25 genome and the ligation of its DNA did not alter the restriction patterns. Therefore, the phage Φ T25 likely utilized the *pac*-site for headful DNA packaging. Similarly, organized *pac*-site phages were identified in other *Lactobacillus casei* group species, including the phages C_L1, C_L2, iLp84, iLp1308 and Lb338-1. In total, fifty open reading frames (ORFs) were identified; the majority of genes were begun with ATG while twelve genes were started with unusual initiation codons (GTG or TTG). Based on DNA and amino acid sequence similarities, putative functions were assigned to 31 (62%) out of 50 predicted gene products. Only 21 ORFs showed homology to predicted phage proteins; the majority of the remaining ORFs showed sequence similarities to those of the lactobacilli genome. Although many of these ORFs were typical phage genes, it may be inferred that the matches in the bacterial genomes represented the prophages or remnants of the prophages.⁽¹¹⁶⁾ This result suggested that the phage Φ T25 itself has a prophage origin or a close evolutionary relationship to the temperate phages.

Table 21 Genome characteristics of *Lactobacillus casei* group-phage.

Host/phage	Life cycle	Genome size (kb)	Mol% G+C	Total number of ORFs (plus strand / minus strand)	Genome type	Year	Accession number
<i>Lb. casei</i>							
Lca1	Virulent	46.98	44.8	71 (56/15)	N.D.*	2006	DQ411856.1
A2	Temperate	43.41	44.9	61 (55/6)	cos	2007	NC_004112.1
phiAT3	Temperate	39.16	44.6	53 (50/3)	cos	2009	NC_005893
J-1	Virulent	40.93	44.8	63 (57/6)	cos	2014	NC_022756
PL-1	Virulent	38.88	44.9	59 (57/2)	cos	2014	NC_022757.1
<i>Lb. paracasei</i>							
Lb338-1	Virulent	142.11	37.4	199 (128/71)	pac	2009	NC_012530
C _L 1	Temperate	39.47	44.8	62 (48/14)	pac	2016	KR905066.1
C _L 2	Temperate	38.75	44.9	60 (59/1)	pac	2016	NC_028835.1
iA2	Temperate	34.15	45.6	50 (43/7)	cos	2016	NC_028830.1
iLp84	Temperate	39.39	45.1	60 (49/11)	pac	2016	NC_028783.1
iLp1308	Temperate	34.17	45.1	50 (42/8)	pac	2016	NC_028911.1
T25	Virulent	38.42	44.5	50 (5/45)	pac	2018	AP018361.1
<i>Lb. rhamnosus</i>							
Lc-Nu	Virulent	36.46	44.3	51 (50/1)	cos	2005	NC_007501.1
Lrm1	Temperate	39.98	45.5	54 (51/3)	cos	2008	EU246945.1

* N.D.: not determined.

The DNA packaging genes were located in the early gene cluster of the phage Φ T25 genome. The ORFs 1-6 encoded phage DNA packaging proteins, including protease, portal protein, terminase and HNH endonuclease. All of these proteins were homologous to the proteins found in the *Lb. casei* group, indicating a host species-specific manner. The derived protein products of the ORFs 4 and 5 were homologous to the large subunit and small subunit of phage terminase protein of *Lb. paracasei* subsp. *paracasei* Lpp126, respectively. In tailed phages, the small terminase subunit recognizes and binds specifically to its own DNA, while the large subunit provides the endonuclease and ATPase activities to cleave concatemeric phage DNA and allow prohead binding. As stated above, after the restriction digests of the phage Φ T25 DNA with the restriction enzymes, no alteration in the banding patterns were observed after heating the restricted DNA to 70°C, suggesting that the phage Φ T25 exploited the *pac* DNA packaging mechanism. Enterobacteria phage P22 is a well-known headful packaging or *pac*-type phage.⁽¹¹⁷⁾ In this case, the packaging initiation site (*pac*) is typically reserved for the site that terminase recognizes for beginning a headful packaging series. The *pac*-type phage requires a sensor that measures the amount of DNA head-filling and a nuclease that will cleave a non-specific DNA as soon the head is full. As the imprecision of the headful cut mechanism, the packaged DNA length in the headful packaging phages is typically between 102% and 110% the length of the genome sequence.⁽¹¹⁸⁾

The DNA replication module of the phage Φ T25 was covered from the ORF 8 to the ORF 28 which were located upstream of the lysis gene. This arrangement module differed from the other consensus *Siphoviridae* phage genomes by having the replication and morphogenesis modules in exchanged positions. This type of gene organization has also been reported in the *Pediococcus* phage cIP1.⁽¹¹⁹⁾ The four DNA replication-related genes which encoded primase (ORF 9), single-stranded DNA-binding (ORF 20), helicase (ORF 21) and NTP-binding protein (ORF 22), were highly similar to those of the *Lactobacillus* phage CL2. The results suggested that the phage Φ T25 and phage CL2 likely shared a common module of the replication genes. In the replication gene region, the origin of DNA replication (*ori*) of the phage is mainly characterized by noncoding region containing several inverted and direct repeats.⁽¹²⁰⁾ *Ori* regions are composed of genes that are expressed early after

infection. Special features of such *ori* regions include expression of helicase and primase, binding sites for specific initiator proteins and adjacent stretches of AT-rich regions. For the phage Φ T25, the *ori* site of this phage was located within a 350-bp non coding region between the ORFs 18 and 19. It consisted of an AT rich region (86% A+T within 65 bp) surrounding by direct and indirect repeats. As shown in Figure 29, the *ori* region of the phage Φ T25 included three copies of 9 bp repeats, four copies of 11 bp repeats and five copies of 13 bp repeats, each occurring in both orientations. This AT rich region also contained an inverted repeat that was capable of forming a stem-loop structure (nucleotides 73-81 and 120-128).

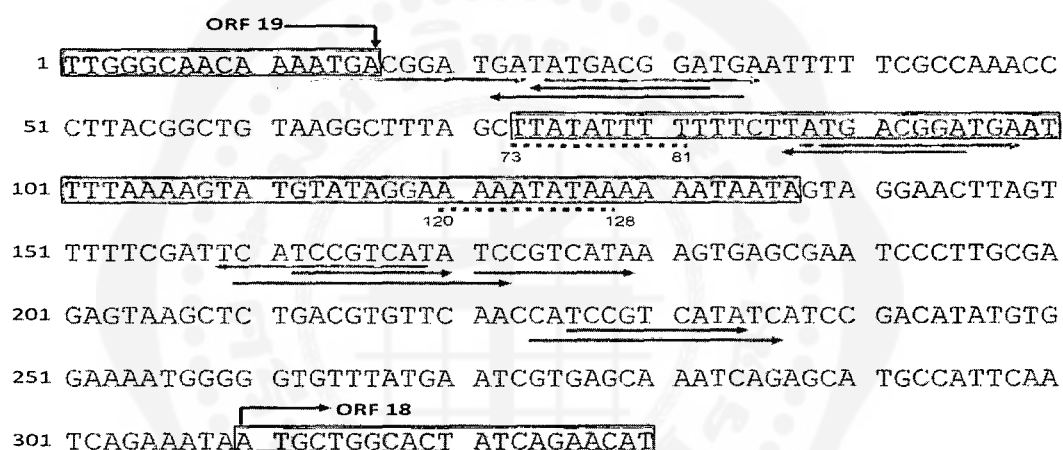


Figure 29 Nucleotide sequence of the possible origin of replication of phage Φ T25. the sequence corresponds to nucleotide sequence 26600-26929 of minus strand. The angled arrows mark the 3'- end of ORF 19 and the 5'- end of ORF 18. The three copies of 9 bp perfect repeats, four copies of a 11 bp perfect repeats and five copies of 13 bp perfect repeats occurring in both orientations are marked with green, yellow and blue arrows, respectively. A 65-nucleotide region displaying a content of 84% is shown in red boxes. A palindrome sequences that are capable to forming a stem-loop is marked with red dotted lines.

The lysogeny module of the phage Φ T25 was embedded between the lysis and replication cassettes, a typical organization found in the *Lactobacillus* phages. In the phage Φ T25, the ORFs 29, 31 and 32 encoded the predicted two phage anti-repressors

and Cro-like protein, respectively. However, the phage Φ T25 genome lacked an essential element for the commitment between lytic and lysogenic cycles such as integrase, excisionase and a CI repressor gene. The presence of genes involved in lysogeny indicated that the phage Φ T25 may be originated from the temperate phage. In tailed phages, the dual holin-lysins system is responsible for host cell lysis and progeny release. The lysis module including the putative lysin and putative holin of the phage Φ T25 was encoded by the ORFs 34 and 35, respectively. The ORF 34 contained a GH25 muramidase superfamily domain (cl10448) involving in cell wall degradation and a lysM superfamily domain (cd00118) involving in binding peptidoglycan. The product of the ORF 35 also encoded a conserved holin_6_1 superfamily domain (pfam09682) which was found in a number of phage and prophage regions of Gram-positive bacteria.⁽¹²¹⁾

The head-tail morphogenesis module of the phage Φ T25 was occupied by the ORFs 38 through 50. The proteins encoded by the ORFs 38, 42 and 48 shared high homologies to putative tail-host specificity protein, putative tail component and putative head-tail joining protein of the *Lactobacillus* phage phiAT3, respectively.⁽¹¹³⁾

Lactic acid bacteria phage research has progressed significantly over the past decade, but phage contamination is still nowadays recognized as one of the persistent problems of dairy industry. Several strategies have been invested to control phage infection of starter culture, such as uses of phage inhibitory media or phage-resistant culture, and optimisation of sanitation. Traditionally, these phage-resistant cultures have been obtained by recombinant DNA technology⁽¹²²⁾ or conjugal transfer of plasmids conferring phage resistance.⁽⁹¹⁾ The isolation of spontaneous phage-resistant mutants has been revalued recently because it is a simple and natural methodology to improve strains, since there are no regulatory restrictions for the use of resistant strains in industrial environments.^(15,123) Nevertheless, similar efforts regarding the use of the resistant strains of *Lb. paracasei* have been comparatively limited, despite its great technological importance in the yogurt industry. To date, only a few studies aimed to isolate the natural phage-resistant mutants in *Lb. paracasei*.⁽¹²⁴⁾

In an attempt to find a starter culture strain with an improved phage resistance, 175 spontaneous phage-resistant mutants were isolated from the *Lb. paracasei* LPC sensitive

strains under selective pressure of the specific phage Φ T25. In this study, the results indicated that the AP method proved to be more efficient than the SC method for the recovery of true phage-resistant variants from the *Lb. paracasei* LPC sensitive strains as previously reported in *Lb. plantarum*.⁽¹²⁵⁾ The variants obtained from the AP method showed a high stability of phage resistance phenotype up to seventh subculture with specific phage. The difference could be attributed to a rapid reversion of variants to the sensitive phenotype obtained from the SC method. The strains having a high phage resistance stability and effective phage defense mechanism are the basis of desired phenotypes for the defined starter cultures.⁽⁸⁷⁾ Moreover, all *Lb. paracasei* LPC phage-resistant mutants exhibited a high efficacy of natural phage defense mechanisms against the phage Φ T25, due to the extremely low EOP value (always lower than 10^{-11}). Moineau and Lévesque (2005) suggested that strong (EOPs in the range of 10^{-7} - 10^{-9}) and medium (EOPs of 10^{-4} - 10^{-6}) levels of phage resistance mechanism were sought by the dairy industry because weak level mechanisms (EOPs of 10^{-1} - 10^{-3}) may not provide sufficient protection during fermentation processes.⁽²⁴⁻²⁵⁾

There are several defense mechanisms of LAB to overcome phage infection including native defence strategies such as the prevention of adsorption and blocking of DNA injection, as well as resistance mechanisms such as restriction/modification (R/M), abortive infection (Abi) and the recently discovered CRISPR/Cas system.⁽¹²⁶⁾ However, a phage resistance mechanism linked to super-infection immunity or lysogeny may be present among the mutants which was due to the integration of the phage genome in the host chromosomes. Such phage-resistant strains containing the prophages should be discarded as starter strains. In the present study, free phages were detected in the supernatants of broth cultures of the most phage-resistant mutants (23 from 31 mutant strains), suggesting the presence of a phage resistance mechanism linked to lysogeny among these variants. Even though the prophages were detected in these variants, but not all these lysogenic strains could be induced by mitomycin-C. Similar results have been reported for the temperate phages C_L1 and C_L2, spontaneous releases from noninfected cultures of *Lb. paracasei* A, could not be obtained by mitomycin C treatment.⁽⁵⁵⁾ These results confirmed that lysogeny is widely distributed in lactococci and lactobacilli, including probiotic strains

of the *Lb. casei* group.⁽⁵⁶⁾ The understanding of lysogenic dairy starter culture is of considerable industrial importance since the strains harboring the prophage genome are considered as a major source of bacteriophage in the dairy plant and should be avoided. Shimizu-Kadota et al. (1983) reported that the *Lb. casei* virulent phage Φ FSV isolated from probiotic dairy fermentation was derived from the lysogenized phage Φ FSW.⁽³⁹⁾

Adsorption interference is the main phage resistance mechanism in lactic acid bacteria species. Among the variants of *Lb. paracasei* LPC, the adsorption rate was unevenly distributed, and the phage particles which were partially or completely unable to adsorb were observed. The failure of phage adsorption can result from a variety of mechanisms, including the change in carbohydrate composition, production of masking substance of the specific receptor, or alteration of specific phage protein receptors.⁽¹²⁶⁻¹²⁸⁾ Zago et al. (2017) showed that the S-layer protein of *Lb. helveticus* played a role in phage adsorption and that variation in one or more amino acids in the protein sequence can affect phage binding.⁽¹¹⁶⁾ The additional benefit of the adsorption inhibition for infection interference might be: (i) it is more efficient to obstructing successful phage infection than to stop it once it has started and (ii) there is not loss of cell viability, while other systems of phage resistance such as abortive infection usually induce death of the infected cells.⁽¹²⁸⁾

For the phage-resistant mutants which showed high adsorption rates without plaque formation, this could be caused by the interference during the injection of phage DNA. In the early steps of phage infection, phage can reversibly adsorb to a specific receptor site on the host cell surface, followed by the crucial step of irreversible interaction between phage and membrane protein which allowed phage DNA injection to proceed. The two-step of adsorption process can occur with the same receptor or, more frequently, different surface protein. In the reversible adsorption step, phage can be released from cells as infectious particles by dilution, whereas irreversibly bound phages are not recoverable as they become obligate to infection.⁽⁶⁸⁾ Watanabe et al. (1972) reported that the *Lb. casei* phage PL-1 normally adsorbed to the cell surface of a mutant strain but phage DNA penetration was often inhibited.⁽⁶⁸⁾ Electron microscopic examination revealed phage adsorption to cells, but no evidence of DNA injection such as empty phage heads was detected.⁽¹²⁹⁾ These results clearly indicated that the mechanism of resistance was not

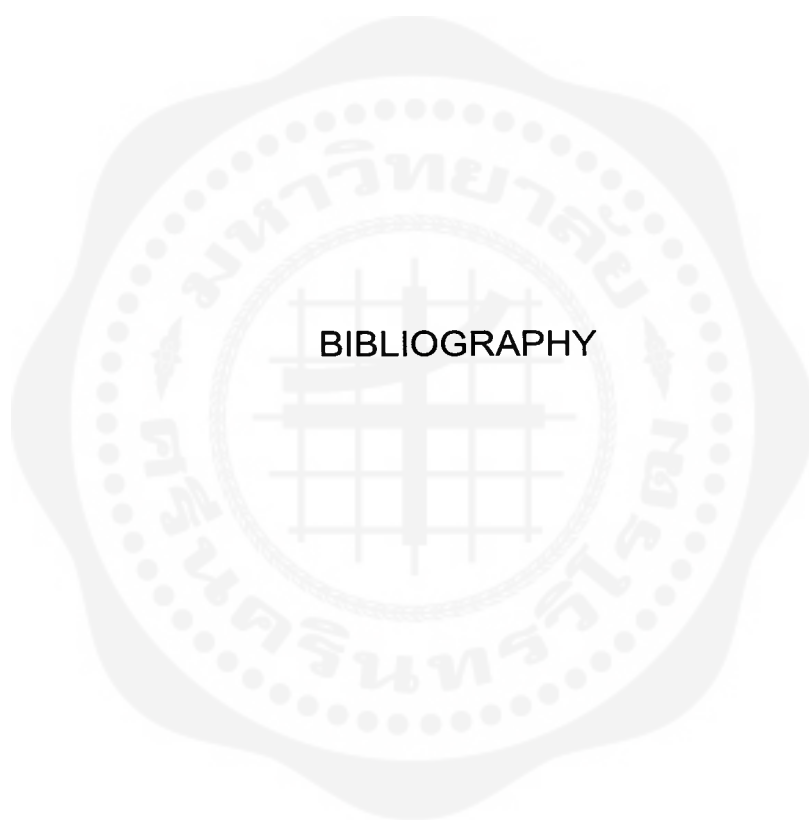
based on a failure to adsorb phage because of the absence of receptors, but due to a block in the ejection mechanism for phage DNA.

The use of RAPD technique enabled us to evaluate the diversity among strains belonging to a particular species.⁽¹³⁰⁾ In this study, the RAPD fingerprints obtained with four arbitrary primers were utilized to detect molecular diversity and relationship among the phage-resistant variants and the sensitive strain. The results showed a cluster structure with similarity coefficients higher than 87%. Therefore, the resistant mutants appeared to be almost identical to the parent strain. This suggested that the mutants were derived from its parent strain.

According to Moineau and Lévesque (2005), a good measure of the effectiveness of a phage-resistant mutant for use in industrial environments is its ability to withstand phage attacks during the fermentation process.⁽²⁴⁾ Moreover, the combination of two or three well characterized bacterial strains should be used to prevent the possible phage infection of the starter culture and subsequent fermentation or process failures.⁽²⁴⁾ The results of the present study demonstrated that the phage-resistant mutants isolated from *Lb. paracasei* LPC were identical to its parent strain in cell structure, colony morphologies and properties that were assayed. Particularly, the selected resistant mutants, AP1-2, AP3-17 and AP3-31, which had a strong phage resistance mechanism should be able to maintain or even improve the technological characteristics of the respective phage-sensitive strains.

In conclusion, to date, this is the first study on the isolation, morphological and genetic characterization of the novel virulent phage Φ T25 which lyses the drinking yogurt starter strain of *Lb. paracasei* LPC. This study has provided knowledge regarding the phage biology, the effect of the physical factors (temperature and pH) on phage viability that would help designing strategies to minimize the consequences of phage attacks in the fermented milk. Morphologically, this phage belonged to Bradley's group B or the *Siphoviridae* family by having hexagonal head and long, noncontractile tail which was found in most *lactobacillus* phage. This phage seemed to have a narrow host-range therefore, potential approaches such as the strain rotation or the use of phage-resistant starter cultures should be set up to control phage infection. Moreover, the spontaneous phage-resistant mutants were isolated from *Lb. paracasei* strain with good technological capacities. In particular,

the selected mutants (AP1-2, AP3-17 and AP3-31) were able to resist the high titers of phage during the manufacture process and the subsequent improvement in acidifying rates, acidifying and proteolytic powers. Consequently, these mutants should be considered as the alternative strains to be used in the fermented milk manufacture instead of the commercial phage-sensitive strains of *Lb. paracasei*, without any kind of regulatory restrictions. In addition, the complete genome sequence of the *Lb. paracasei* phage Φ T25 was generated. Analysis of the genomic DNA demonstrated that most of the identified genes of the phage Φ T25 showed remarkable similarities to those reported previously for the *Lactobacillus* phages. Many genes encoded by the phage Φ T25 genome showed homology to the genes derived from the *Lactobacillus* genome. This may be attributed to genetic exchange and recombination between phage and the related bacteria. The phage Φ T25 also possessed an incomplete lysogenic module that underlined its possible origin from a temperate phage. Ultimately, the results from this study may help improve the processes of lactic acid bacteria starter strain selection, thus minimizing the risk of phage proliferation in industrial environments and will lead to a better understanding of phage-host interactions.



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APPENDICES

APPENDIX A

MEDIA FOR BACTERIAL CULTURE

1. MRS broth/ agar

Peptone	10	g
Beef extract	8	g
Yeast extract	4	g
Dextrose	20	g
Sodium acetate	5	g
Tween 80	1	g
Dipotassium phosphate	2	g
Ammonium citrate	2	g
Magnesium sulfate	0.1	g
Manganese sulfate	0.05	g
Agar (for MRS agar)	15	g

These ingredients were dissolved and adjusted to a volume of 1 liter with distilled water. The medium was sterilized at 15 lbs pressure (121°C) for 15 minutes.

2. MRS broth supplemented with 10 mM calcium chloride

It consisted of MRS broth (1) with an addition of 1.5 grams of calcium chloride. These ingredients were dissolved and adjusted to a volume of 1 liter with distilled water. The medium was sterilized at 15 lbs pressure (121°C) for 15 minutes.

3. 1.5% MRS agar supplemented with 20 mM calcium carbonate

It consisted of MRS agar (1) with an addition of 3 grams of calcium carbonate. These ingredients were dissolved and adjusted to a volume of 1 liter with distilled water. The medium was sterilized at 15 lbs pressure (121°C) for 15 minutes. The medium was

mixed well and poured (18-20 mL) into sterile Petri plates. After the plates were cool, they were stored at 4°C for up to 2 months.

4. Skim milk-yeast extract medium

Skim milk	55	g
Yeast extract	10	g

These ingredients were dissolved and adjusted to a volume of 1 liter with distilled water. The medium was sterilized by autoclaving at 110°C for 5 min.

MEDIA FOR BACTERIOPHAGES

5. MRS with 1.5% agar supplemented with 20 mM calcium chloride

It consisted of MRS agar (1) with an addition of 3 grams of calcium chloride. These ingredients were dissolved and adjusted to a volume of 1 liter with distilled water. The medium was sterilized at 15 lbs pressure (121°C) for 15 minutes, mixed well and poured (18-20 mL) into sterile Petri plates. After the plates were cool, they were stored at 4°C for up to 2 months.

6. MRS agar with 0.6% supplemented with 20 mM calcium chloride

It consisted of MRS broth (1) with an addition of 6 grams of agar and 3 grams of calcium chloride. The medium was sterilized at 15 lbs pressure (121°C) for 15 minutes. When the medium was cooled to 60-70°C, 5 mL of the medium was dispensed to the top of each plate.

APPENDIX B

REAGENTS FOR DNA AND ELECTROPHORESIS

1. 50X TAE buffer

Tris base	24.2	g
Glacial acetic acid	5.7	mL
0.5 M EDTA (pH 8.0)	10	mL

These ingredients were dissolved and adjusted to 100 ml with distilled water. Then, the solution was sterilized at 15 lbs pressure (121°C) for 15 minutes and diluted to 1X with deionized water just before use in electrophoresis.

2. Ethidium bromide (10 mg/mL)

Ethidium bromide	1	g
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It was dissolved in distilled water, adjusted to 100 mL final volume with distilled water. Then, it was stored in a dark glass bottle at room temperature.

3. TE-buffer

Tris- HCl	0.1	M
EDTA	1	mM

It was adjusted to pH 8.0 and made up to a volume of 1 liter with distilled water and sterilized at 15 lbs pressure (121°C) for 15 minutes.

4. 3M Sodium acetate

Sodium acetate.3H ₂ O	408.3	g
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Sodium acetate.3H₂O was dissolved in 800 mL of distilled water and adjusted the pH to 7.0 with NaOH. It was made up to a volume to 1 liter with distilled water and sterilized at 15 lbs pressure (121°C) for 15 minutes.

5. Phage buffer

1 M Tris-HCl, pH 7.6	10	mL
Sodium chloride	0.4	g
Magnesium sulfate	10	mL

These ingredients were dissolved in 980 ml of distilled water and sterilized at 15 lbs pressure (121°C) for 15 minutes.

6. 12% Trichloroacetic acid (TCA)

Trichloroacetic acid	500	g
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It was dissolved in 227 mL of distilled water. The resulting solution contained 100% (w/v) TCA. It was diluted to 12% TCA by distilled water.

REAGENTS FOR PROTEIN AND SDS-PAGE**7. 1.5 M Tris-HCl pH 8.8**

Tris base	18.15	g
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It was dissolved in 800 mL of deionized water and adjusted to pH 8.8 with concentrated HCl and made up to 1 liter with deionized water. Then the solution was sterilized at 15 lbs pressure (121°C) for 15 minutes.

8. 1.5 M Tris-HCl pH 6.8

Tris base	12.1	g
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Tris base was dissolved in 800 mL of deionized water and adjusted to pH 6.8 with concentrated HCl and made up to 1 liter with deionized water. Then, the solution was sterilized at 15 lbs pressure (121°C) for 15 minutes.

9. 10% (w/v) Sodium dodecyl sulfate (SDS)

Sodium dodecyl sulfate	10	g
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Sodium dodecyl sulfate was dissolved in deionized water, and made up to a volume of 1 liter with distilled water. It was stored at room temperature.

10. 10% (w/v) Ammonium persulfate (APS)

Ammonium persulfate	1	g
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Ammonium persulfate was dissolved in 10 mL of distilled water and stored at 4°C.

11. Sample buffer (8 mL)

0.5 M Tris-HCl	1	g
10% SDS	1	g
Glycerol	1	g
Beta-mercaptoethanol	1	g
0.05% (w/v) Bromophenol blue	1	g
Distilled water	1	g

The solution was mixed well and stored at 4°C

12. 10X Glycine running buffer

Tris base	30.3	g
Glycine	143.75	g
SDS	10	g

The ingredients were dissolved and made up to 1 liter with deionized water. Then, the solution was diluted to 1X with deionized water just before use in electrophoresis.

13. Coomassie Brilliant Blue solution

Coomassie Brilliant Blue R-250	1.2	g
Absolute methanol	200	g
Phosphoric acid	100	g
Ammonium sulfate	100	g

The mixture of Coomassie brilliant blue R-250 and ammonium sulfate in absolute methanol were dissolved before adding phosphoric acid and 800 mL of distilled water. Then, the mixture was mixed and filtered through Whatman No. 1 membrane and stored in the dark at room temperature.

REAGENTS FOR CsCl PURIFICATION**14. CsCl solution**

Density (g/mL)	CsCl (g)	SM buffer (mL)
1.45	60	85
1.50	67	82
1.70	95	75

APPENDIX C

1. API 50 CHL® (BioMérieux manufacturer's manual)

The biochemical test was performed by API 50 CHL system for the correct identification of the genus *Lactobacillus* and related genera. The API 50 CHL strip consisted of 50 microtubes used for studies of the carbohydrate family and its derivatives. During incubation, fermentation was revealed by a color change in the tube, caused by the anaerobic production of acid and detected by the pH indicator present in the chosen medium. The first tube, which did not contain any active ingredient, was used as a negative control.

A suspension of *Lactobacillus paracasei* LPC and its mutant strains was prepared in the ampule of API 50 CHL medium with a turbidity equivalent to 2 McFarland. Then, the suspension was filled into the tubes containing API 50 CHL medium, and covered all the tubes with mineral oil. The API 50 CHL strip was incubated under microaerophilic conditions at 37°C for 48 h. A positive test corresponded to acidification was revealed by the bromocresol purple indicator contained in the medium changing to yellow. For the esculin test (tube No. 25), a change in color from purple to black was observed. The results were recorded on the result sheet and the biochemical profile was interpreted by using the apiweb™ identification software with database.

2. Colony PCR

Single colony was picked up by using a micropipette tip and resuspended 50 µL of sterile water. This suspension was heated at 75°C for 15 min, and used as DNA template in the PCR mixture.

3. List of enzymes used in the analysis of restriction enzyme digestion patterns.

Enzyme	Recognition sequence
<i>AseI</i>	5' A T ↓ T A A T 3' 3' T A A T ↑ T A 5'
<i>BglII</i>	5' A ↓ G A T C T 3' 3' T C T A G ↑ A 5'
<i>EcoRI</i>	5' G ↓ A A T T C 3' 3' C T T A A ↑ G 5'
<i>EcoRV</i>	5' G A T ↓ A T C 3' 3' C T A ↑ T A G 5'
<i>HindIII</i>	5' A ↓ A G C T T 3' 3' T T C G A ↑ A 5'
<i>FbaI</i>	5' T ↓ G A T C A 3' 3' A C T A G ↑ T 5'
<i>MluI</i>	5' A ↓ C G C G T 3' 3' T G C G C ↑ A 5'
<i>MunI</i>	5' C ↓ A A T T G 3' 3' G T T A A ↑ C 5'
<i>NcoI</i>	5' C ↓ C A T G G 3' 3' G G T A C ↑ C 5'
<i>NheI</i>	5' G ↓ C A C G 3' 3' C G T C ↑ G 5'
<i>PvuII</i>	5' C A G ↓ C T G 3' 3' G T C ↑ G A C 5'

4. Preparation of gels for SDS/ polyacrylamide gel electrophoresis

4.1 Separating gels

Solution	7%	10%	12%	15%
Distilled H ₂ O	5.1 mL	4.1 mL	3.4 mL	2.4 mL
1.5 M Tris-HCl, pH 8.8	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

4.2 4% Stacking gels

Solution	Volume
Distilled H ₂ O	3.075 mL
0.5 M Tris-HCl, pH 6.8	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 monomer	5.05 mL



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