Transduction Efficiency and morphology of Thai *Salmonella* phages

ประสิทธิภาพในการถ่ายโอนยีนแบบทรานสดักชั่นและสัณฐานวิทยาของซาลโมเนลลาเฟจที่พบในประเทศไทย

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ABSTRACT

Phage is a virus that uses bacterium as its host. Phages have two life cycles, lytic and lysogenic, and these cycles allow host genetic transfer by phages called transduction. The abilities of phages to transduce under various environmental conditions such as varying temperature, pH, and concentrations of NaCl and heavy metals were tested. PCR technique was used to confirm the resistance gene transfer from *Salmonella* donor to recipient via transduction process. The results showed that alkaline pH and high salinity affected transduction efficiencies. However, these conditions did not affect transduction during host adsorption step. In addition, transmission electron microscopy was used to analyze 24 Thai *Salmonella* phages for their morphological characteristics. Information from both transduction efficiency and phage morphology allow us to better understand environmen tal conditions that affect bacterial host gene transfer and phage structure involved in the process.

บทคัดย่อ

เฟจ (Phage) เป็นไวรัสในแบคทีเรีย การดำรงชีวิตของเฟจมีสองวงจรคือ แบบ lytic และแบบ lysogenic วงจรชีวิตของเฟจทำให้เฟจเป็นพาหะในการถ่ายทอดยีนระหว่างแบคทีเรีย หรือที่เรียกว่า ทรานสดักชั่น ในการศึกษาประสิทธิภาพในถ่ายโอนยีนแบบทรานสดักชั่นนั้น ได้ทำการศึกษาภายใต้สภาพแวดล้อมต่างๆ เช่น การเปลี่ยนแปลงของอุณหภูมิ ค่า pH ความเข้มข้นของโซเดียมคลอไรด์และความเข้มข้นของโลหะ และใช้เทคนิค PCR ในการยืนยันการถ่ายโอนยีนดื้อยาปฏิชีวนะระหว่างเชื้อซาลโมเนลลาโดยกระบวนการทรานสดักชั่น ผลการทดลองแสดงว่า ความเป็นด่างและความเป็นเกลือส่งผลกระทบต่อทรานสดักชั่นแต่ไม่ใช่ในขั้นตอนการเกาะติด (adsorption) ของเฟจกับเชื้อซาลโมเนลลา นอกจากนี้ยังทำการศึกษาลักษณะทั่วไปของซาลโมเนลลาเฟจ 24 ชนิด ที่พบในประเทศไทยโดยใช้กล้องจุลทรรศน์อิเล็กตรอนแบบ ทรานสมิสชันวิเคราะห์ลักษณะทางสัณฐานวิทยา ผลการวิจัยจากการศึกษาครั้งนี้ช่วยให้เราเข้าใจสภาวะที่กระทบต่อ ทรานสดักชั่นของซาลโมเนลลาเฟจและโครงสร้างของเฟจที่อาจเกี่ยวข้องกับกระบวนการนี้

Key Words: *Salmonella*, Phages, Transduction

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Introduction

Salmonella enterica is a gram-negative, rod-shaped bacterium in the family *Enterobacteriaceae*. *Salmonella* can grow at moderate temperatures about 8-45°C; however, some strains can grow at less than 5°C (D'Aoust, 1989) or at as high as 49.5°C (ICMSF, 1996). *Salmonella* grows well in the pH between 7-7.5. In some strains that are resistant to acidic or alkaline conditions, *Salmonella* can grow even at pH 3.8 and pH 9.5 (ICMSF, 1996). At optimal growth conditions, *S. enterica* can grow and cause a foodborne disease called salmonellosis from contaminated raw meat, eggs, raw milk, or their products (e.g., butter or ice cream) as well as some vegetables (Gomez-Aldapa et al., 2012). *S. enterica* continues to be a major foodborne pathogen in animals and humans (Hendriksen et al., 2004). Patients with salmonellosis have symptoms including diarrhea, abdominal pain, fever, and vomiting (Burnett et al., 2001). In Thailand, from the year 2006 to 2008, a study of *S. enterica* contamination in meat products by Chiangthian *et al.* (2009) showed high levels of contamination at 46.39%, 46.62% and 50.85%, respectively. Ministry of Public Health (Thailand) enforces absence of *S. enterica* in 25 g of food products (such as eggs, egg products, meat, meat products, milk, or other dairy products).

Bacteriophage or phage is a virus that infects a bacterium and uses bacterial host machineries to propagate. Phages are the natural killers of bacteria. Recently, there is much interest in the use of phages as agents of foodborne pathogens (Goodridge et al., 2011). Phage has two life cycles. Virulent phage enters lytic life cycle and destroys the bacteria in order to increase the number of particles while temperate phage enters lysogenic life cycle that is able to insert genetic material into the bacterial host DNA (Khakhum et al., 2010). Transduction is a DNA transfer from one bacterium to other bacteria via virus (Hartl *et al.,* 1988). Transduction can occur through either the lytic cycle or lysogenic cycle. Transduction process can be divided into two types, generalized and specialized transductions. In generalized transduction, DNA fragments are transferred from one bacterium to another via lytic phage (Fineran et al., 2009). Two important types of phages capable of generalized transduction are P22 phage in *Salmonella* and P1 phage in *E. coli.* The other type of transduction is specialized transduction which is the process that the genes are transferred to another bacterium by temperate phage during the lysogenic life cycle. Transduction is; therefore, one of the keys that can lead to bacterial genetic diversity.

In the study herein, *S. enterica* and its phages are used as models to understand phage transduction, phage physiology, and genetics. Multiple experiments were performed to test the abilities of phage transduction and adsorption under various environmental conditions and to characterize phage morphology and genetics. Using *Salmonella* as a model, the hypothesis was that changing conditions such as temperature, pH, NaCl and heavy metals would alter transduction of antibiotic resistance gene from host to recipient bacteria. Transmission electron microscopy was used to study morphologies of selected *Salmonella* phages isolated from dairy farms in Thailand.

Objective of the study

Based on the transduction studies in *Vibrio cholerae* (Faruque et al., 2000; Lipp et al., 2002) which demonstrated the effects of climate change on transduction, varying temperatures, pH values, and heavy metal concentrations on the gene transfer by vibrio phage transduction. Therefore this study aims to better understand environmental conditions that affect *Salmonella* gene transfer and phage structure that may involve in such process.

Methodology

Bacterial strains

Two *Salmonella* strains were used for transduction experiment. Donor strain (R8-3980) was a *Salmonella* Typhimurium containing chloramphenicol resistant gene (*cat*) and *lacZ*. Recipient strain (R8-3981) was a wild type *Salmonella* Typhimurium strain. Two *Salmonella* strains were used for phage propagation. *Salmonella* Dublin (S5-368) was used for propagations of phages SPT7, SPT10 and SPT19 while *Salmonella* Typhimurium (S5-370) was used for propagation of phage SPT30.

Phage strains

Salmonella phage P22 was used to study transduction ability under various environmental conditions. Selected Thai phages (Wongsuntornpoj et al., 2014), namely SPT7, SPT10, SPT19 and SPT30 were used in transduction ability. Phages were selected under the following criteria (i) differences in genome sizes, (ii) differences in host range profiles, and (iii) differences in morphology. Selected Thai phages (Wongsuntornpoj et al., 2014), namely SPT1, SPT5, SPT7, SPT8, SPT9, SPT710, SPT712, SPT13, SPT16, SPT19, SPT20, SPT22, SPT25, SPT26, SPT30, SPT31, SPT36, SPT37, SPT71, SPT43, SPT746, SPT48, SPT50, and SPT51 were used to study morphologies.

Media and chemicals

For maintaining bacterial culture, tryptic soy broth (TSB) and tryptic soy agar (TSA) media were used. TSA (0.7 % agar) was used for phage propagation. For isolation of the transduced bacteria (containing chloramphenicol resistance gene), TSA plates containing 1.25 µg/ml chloramphenicol and 10 mM EGTA (ethylene glycol tetraacetic acid) were used. SM Buffer used for washing and harvesting phages phage were composed of 0.05 M Tris-HCl at pH 7.5, 0.1 M NaCl, and 0.01 M $MgSO_4$.

Phage preparation

Phage propagation

First, *Salmonella* host was prepared by streaking out a frozen stock on TSA plate to isolate for the single colony. One host colony was inoculated into 5 ml of TSB and cultured overnight (12-18 hr) at 37 °C. Then, host culture was diluted 10-folds (500 µl of bacteria: 4.5 ml of TSB). Next, phage was prepared by making a 10-fold dilution (20 µl of phage: 180 µl of NaCl buffer), a 100-fold and 1000-fold dilutions from phage stock. The prepared phage (150 µl) was added into 300 µl of *Salmonella* host cell in each test tube and incubated for 10-15 min at room temperature. Next, 4 ml of 0.7% TSA (pre-warmed at 55°C) was added into this tube and poured onto TSA (1.5% agar) plate. The overlay was incubated overnight at 37°C. SM buffer was then poured onto each plate and incubated at 37°C for 2-3 hr with periodic shaking. After that, SM buffer was pipetted and the surface of the 0.7% TSA was scraped. Chloroform was added to kill bacterial host cell. The

mixed solution was centrifuged at 10,000 rpm for 10-12 min (4°C). Supernatant was collected and 1 drop of chloroform was added to eliminate the bacterial cells. Supernatant was filtered using 0.2 µm pore with a syringe. The filtrate was stored at 4°C for next the experiments (Moreno, 2008).

Phage titer

Before each experiment, phage titers were enumerated. Host was prepared as described previously. Overnight culture of host cell was diluted 10-fold (500 µl of bacteria: 4.5 ml of TSB). An overlay of 300 µl host suspension and 0.7% TSA were prepared. Phage was prepared by performing the 10-fold serial dilutions (10^{-1}-10^{-10}). An aliquot of 10 µl of each phage dilution was spotted on TSA plate with host bacteria spread on the surface. The plate was incubated at 37°C overnight. The titer of the phage was determined and calculated from the clear zones (Moreno, 2008).

Transduction

Phage preparation

Salmonella donor strain was grown in 5 ml of TSB overnight at 37°C and 200 µl of overnight culture was added into 1 ml of P22 phage broth ($5x10^6$ plaque forming unit (pfu)/ml). P22 was used as a positive control of this experiment. For selected *Salmonella* phage, 1 ml of TSB and phage ($5x10^6$pfu/ml) was added into 200 µl of overnight culture of bacteria donor. For negative control, phage was not added and contained only 200 µl of overnight bacteria donor and 1 ml of TSB. The mixtures were incubated overnight at 37°C. A 4-5 drop of chloroform was added and incubated at 37°C for 20 min. Bacteria were killed and then centrifuged at 12,000 rpm for 2 min. The supernatant was saved and a drop of chloroform was added to ensure that the bacteria were eliminated. A 10 µl of supernatant was streaked onto TSA plate containing chloramphenicol and incubated at 37°C overnight to ensure that none of bacterial donor strain survived (Moreno, 2008).

Infection of recipient strain

Recipient strain was grown in 5 ml TSB overnight at 37°C and a 0.1 ml of phage prepared previously was added into 5 ml of TSB. The mixture was incubated at 37°C for 1.5-3 hr (to mid-log phase). An aliquot of 10 µl of P22 was added to 0.5 ml of recipient strain, an aliquot of 10 µl of negative control was added to 0.5 ml of recipient strain. For selected phages, 0.1 µl, 1 µl, 10 µl and 100 µl were added to 0.5 ml of recipient strain and incubated at 37°C for 30 min, without shaking. Different volumes of tested phages were used in order to determine the optimal multiplicity of infection (MOI). Then, 0.5 ml of 20 mM EGTA was added to chelate the calcium needed for phage adsorption and avoid secondary infection. The mixture was further incubated at 37°C for 1 hr, with shaking. The mixture was centrifuged at 8,000 rpm for 5 min and then supernatant was discarded. Pellet was resuspended with 100 µl of TSB and then spread on antibiotic plate with chloramphenicol and 10 mM EGTA. The plate was incubated overnight at 37°C, after that the number of colonies were counted and calculated for transduction frequencies i.e., the number of transductants/pfu used for transduction (Moreno Switt et al., 2013).

Conditions affecting transduction efficiency

In this study, the effects of changing environmental conditions such as

temperature, pH, and heavy metal concentration, on transduction efficiencies were tested (Table 1). Selected conditions included varying (i) temperatures from 4 to 45°C, (ii) pH values from 4 to 9, (iii) concentrations of NaCl 0%, 1%, 2%, 3%, 4% and 5%, and (iv) concentrations of the heavy metals such as iron, copper, cadmium and zinc. Temperature and pH ranges were chosen based on conditions that allow growth of *Salmonella*. The selected conditions, specifically heavy metal concentrations, were based on minimum and mean concentrations found in industry areas in Thailand (Simasuwannarong et al., 2012). Effects of these conditions on transduction efficiency were tested during the transfer of resistance gene to the recipient bacteria.

Table 1: Conditions used in this study

Environmental factors	Specific conditions	Reference
(i) Temperature	4, 15, 25, 37 and 45°C	
(ii) pH	4, 7, 9 and 10	
(iii) NaCl concentration	0%,1%, 2%, 3%, 4% and 5%	
(iv) Heavy metal concentration	1.5 and 30 g/l	Simasuwannarong et al., 2011
Fe (iron)	4.5 and 40 mg/l	
Cu (copper)	0.03 and 3.5 mg/l	
Cd (cadmium)		
Zn (zinc)	3.5 and 35 mg/l	

PCR confirmation

Polymerase Chain Reaction (PCR) technique was used to confirm the resistance gene transfer from *Salmonella* donor to recipient via transduction process. The PCR mixtures were prepared as follows: 17.875 µl of water, 2.5 µl of 10x Mg-free buffer (500 mM KCl, 100 mM Tris-HCl and 1% Tritron X-100), 2 µl of dNTP, 1.5 µl of 1.5 mM $MgCl_2$, 0.5 µl of primer (primers including: c1 [5'-TTATACGCAAGGCGACAAGG-3'] and c2 [5'-GATCTTCCGTCACAGG-TAGG-3'] for *cat* gene), 0.125 µl Tag-polymerase and 0.5 µl of recipient DNA template. PCR was performed in the thermal cycler with 95°C for 10 min (denaturation), 55°C for 45 sec (anneal), and 72°C for 1 min (polymerization). The expected product size (1.1 kb), if transduction occurred, was determined by 1% agarose gel electrophoresis. Gel was stained with SYBR gold and visualized through transilluminator.

Statistical analysis of transduction data

For analysis of transduction data, one-way analysis of variance (ANOVA) of SPSS software program version 18.0 was used with the significance level at $P \leq 0.05$.

Adsorption

Adsorption experiment was performed according to De Lappe et al. (2009). *Salmonella* was grown in 5 ml of TSB until *Salmonella* reached exponential-phase (about 10^9cell/ml). Phage at approximate concentration of 10^6 pfu/ml was added and incubated for 0 and 15 min at 37°C (or conditions allowing increased transduction efficiency as determined above). The mixture was centrifuged for 4 min at 12,000 rpm and 10µl of phage dilution from supernatant was spotted on bacterial host cell lawn and incubated overnight at 37°C. Titer was determined in supernatant containing free phage and in resuspended pellet containing adsorbed phages.

Transmission electron microscopy (TEM)

The morphological characteristics Thai phages were analyzed using TEM. First, phage was fixed by adding 100 µl of phage suspension to 10 µl

of fixative (10% formaldehyde and 5% glutaraldehyde). Forceps were used to pinch a grid and 5 µl of phage suspension was dropped onto the shiny side of grid. Suspension was let to dry for 1-3 min and phage was adsorbed to the grid. Then, filter paper was used to pull out the rest of the liquid. The grid was placed into grid box, air-dried overnight at room temperature, and finally observed under TEM. Photograph was taken at around 5000x to observe capsid diameter and tail length of each phage. Phage was identified to the Family level.

Results

Transduction

The ability of phage transduction under various environmental conditions was tested to identify phage capability in generalized transduction and environmental conditions that affect such process. The varying conditions in temperature, pH, concentration of NaCl, and concentration of heavy metals were used. In this study, P22 phage and *Salmonella* phages were used as models. The statistical analysis of transduction data using one-way ANOVA with $P \leq 0.05$ as a cut-off for significance level revealed that only pH 10 and 5% NaCl affected transduction efficiencies. pH 10 (Figure 1) and 5% NaCl (Figure 2) significantly decreased the numbers of transductants suggesting that these conditions affected the ability to transfer *cat* gene from *Salmonella* donor strain to the recipient strain and, therefore, decreased transduction abilities.

Thai phages SPT7, SPT10, SPT19 and SPT30 were used to study transduction abilities in the conditions that affected transduction ability (5% NaCl and pH 10). SPT7, SPT10 and SPT19 did not give the chloramphenicol-resistant transductant on the antibiotic plate. SPT30 gave one to two transductants on the antibiotic plates in the control condition (37°C, pH 7, 0.5% NaCl), not in high salinity (37°C, pH 7, NaCl 5.5%) and alkaline (37°C, pH 10, NaCl 0.5%) conditions.

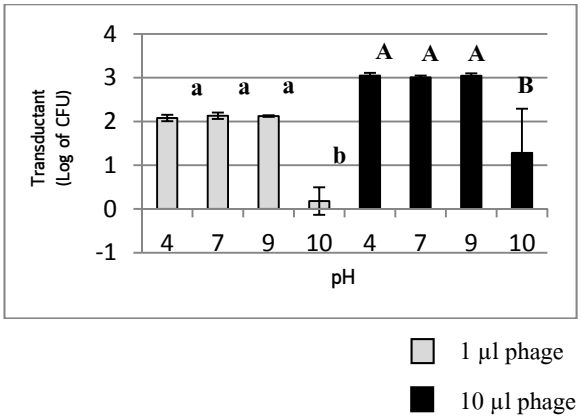

Figure 1 Phage transduction under different pH conditions. a, b, A, or B represents statistical group with significant difference ($P \leq 0.05$).

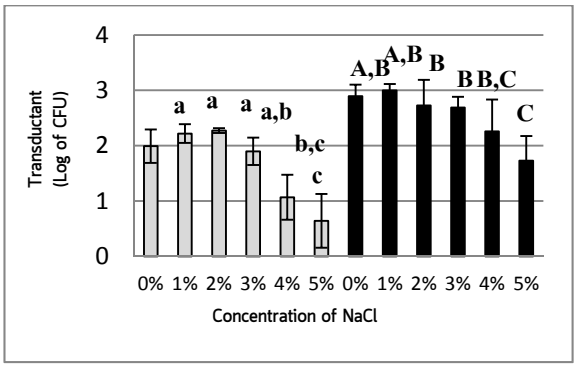

Figure 2 Phage transduction under varying concentrations of NaCl. a, b, c, A, B, or C represents statistical group with significant difference ($P \leq 0.05$).

PCR confirmation

PCR technique was used in confirmation of resistance gene transfer from *Salmonella* donor to recipient via transduction process. The PCR products size of designed primer was 1.1 kb. These primers could detect the *cat* gene in all transductants (data not shown).

Adsorption

Adsorption assay was performed to investigate whether high salinity (37°C, pH 7, NaCl 5.5%) and alkaline (37°C, pH 10, NaCl 0.5%) conditions affected transduction efficiencies during phage adsorption step. Phage at 10^6 pfu/ml was added and incubated for 0 and 15 min. Titer was determined in supernatant that contained free phage and in resuspended pellet that contained adsorbed phages. In both conditions, free phages were decreased by 2 logs pfu, while in the control condition (37°C, pH 7, NaCl 0.5%) free phages were decreased by 3 logs pfu. Statistical analysis of adsorption data using one-way ANOVA revealed no significance at $P \leq 0.05$. The results indicated that high salinity and alkaline pH do not affect the transduction efficiency during phage adsorption step.

Transmission electron microscopy (TEM)

Phages were analysed for their morphological characteristics using TEM. Photograph was taken at around 5000x magnification to observe phage capsid diameter and tail length. Selected Thai phages, namely SPT1, SPT5, SPT7, SPT8, SPT9, SPT710, SPT712, SPT13, SPT16, SPT19, SPT20, SPT22, SPT25, SPT26, SPT30, SPT31, SPT36, SPT37, SPT71, SPT43, SPT746, SPT48, SPT50 and SPT51 were used in this study. The result showed that Thai phages can be classified by contractile, long and noncontractile, or short tail into three families *Siphoviridae*, *Myoviridae*, and *Podoviridae.* Thai phages SPT1, SPT5, SPT8, SPT9, SPT16, SPT20, SPT22, SPT26, SPT30, SPT36, SPT41, SPT43, SPT46, SPT48, SPT50, and SPT51 have long and noncontractile tails as those in *Siphoviridae* Family. These phages have head widths of 45-200 nm, head lengths of 45-200 nm, and tail lengths of 75-400 nm. Phages SPT7, SPT10, SPT12, SPT13, and SPT 37 have long and contractile tails as those in *Myoviridae* Family. These phages have head widths of 50-180 nm, head lengths of 50-200 nm, and tail lengths of 125-200 nm. Phages SPT19, SPT25, and SPT31 have short and noncontractile tails and can be classified in *Podoviridae* Family. Phages in this group have heads with width range of 25-50 nm, head lengths of 100-200 nm, and tail lengths of 10-50 nm. Examples of Thai *Salmonella* phages are shown in Figure 3.

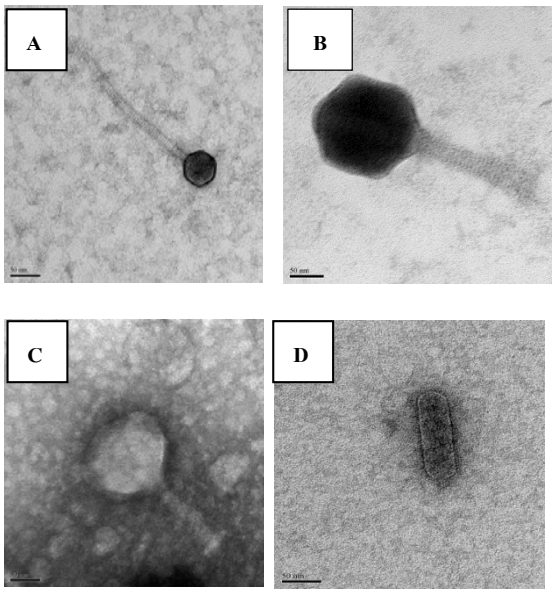

Figure 3 Transmission electron microscopy images of Thai phages. (**A**) SPT36 in *Siphoviridae* Family, (**B**) SPT5 in *Siphoviridae* Family, (**C**) SPT12 in *Myoviridae* Family, and (**D**) SPT31 in *Podoviridae* Family. Scale bar; 50 nm.

Discussion and Conclusions

Environmental conditions such as temperature, pH, and mineral and metal concentrations play roles in propagation of bacteria and their phages. This study has shown that high salinity (5% NaCl) and alkaline pH (pH 10) affected transduction by P22 phage. However, these conditions do not affect the adsorption of P22 phages to *Salmonella*. The lower transduction efficiencies observed under high salinity and alkaline pH must have occurred during phage invasion, propagation inside the host, or release. Thai phages SPT7, SPT10, SPT19 and SPT30 were also tested under high salinity and alkaline pH conditions for their transduction abilities. Under tested conditions, Thai phages were not able to transduce as well as P22 phage. This could be due to the fact that SPT7, SPT10, and SPT19 were originally isolated from *S.* Dublin; however, donor and recipient strains used in this study were *S.* Typhimurium. Serotypes of donor and recipient strains could also affect transduction efficiencies. SPT7, SPT10, SPT19 and SPT30 were further characterized for their morphologies along with 20 other Thai *Salmonella* phages using TEM. Thai *Salmonella* phages belong to three phage Families i.e., *Siphoviridae*, *Myoviridae* and *Podoviridae*. SPT7 and SPT10 are of *Myoviridae* Family with long and contractile tails. SPT30 belongs to *Siphoviridae* Family with long and noncontractile tail. SPT19 belongs to *Podoviridae* Family with short and noncontractile tail. Although SPT19 and P22 are of the same Family and have similar structures, the abilities to transduce genetic materials are significantly different. In conclusion, the efficiency of *Salmonella* phage transduction may depend on the bacterial host serotype, type of phage life cycle, and environmental conditions.

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