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Characterization of Phage Behaviors Against Antibiotic-Resistant *Salmonella* Typhimurium

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ABSTRACT - This study was designed to investigate the dynamic behaviors of phages against *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 19585 (ST^{WT}), *S.* Typhimurium KCCM 40253 (ST^{KCCM}), ciprofloxacin-induced *S.* Typhimurium ATCC 19585 strains (ST^{CIP}), and *S.* Typhimurium CCARM 8009 (ST^{CCARM}). Phages, including PBST-10, PBST-13, PBST-32, PBST-35, P-22, and P-22 B1 had narrow host ranges. The adsorption rates of all phages ranged from 47 to 85%, 58 to 95%, and 61 to 93%, respectively, against ST^{WT}, ST^{KCCM}, and ST^{CIP}, while the lowest adsorption rates ranged from 14 to 36% against ST^{CCARM}. The phage burst sizes were from 43 to 350, 37 to 530, 66 to 500, and 24 to 500 plaque-forming units (PFUs) per infected ST^{WT}, ST^{KCCM}, ST^{CIP}, and ST^{CCARM}, respectively. The ST^{CIP} strain was effectively inhibited by all phages at the early of incubation period. These results provide useful information for better understanding the phage behaviors against antibiotic-resistant and antibiotic-sensitive pathogens.

Key words: Antibiotic resistance, *Salmonella*, Phage adsorption, Burst size, Lytic activity

Antibiotic, particularly penicillin, was greatly appreciated at the time of World War II. Over many long years, the overuse of antibiotics has led to the rapid emergence of resistance in bacteria[1]. In particular, antibiotic-resistant *Salmonella* spp. can cause severe life-threatening illness and become serious health problems[2]. The prevalence of antibiotic resistance can arise due to the activation in efflux mechanism, alteration of cell membrane permeability, and enzymatic inactivation of antibiotics[3,4]. Hence, new therapeutic methods are required to control the antibiotic resistance. Recently, bacteriophages (phages) have considered as an alternative therapeutic option over current antibiotics[5].

Virulent lytic phages can be used for the control and detection of bacterial pathogens due to the high specificity of phage-bacterium interactions[6,7]. Phage specificity predominantly depends on the phage-host binding efficacy, which is directly associated with the lytic activity of phages[8,9]. The bacterial surface receptors and phage tail fiber affect the host ranges of phages. Phage-binding receptors on the surface of bacteria include outer membrane proteins, flagella, and lipopolysaccharides[10]. There is still a challenging question whether phages are applicable to control antibiotic-resistant bacteria. However, relatively few studies have characterized the phage behavior in antibiotic-resistant pathogens. Therefore, the aim of this study was to evaluate the possibility of using phages to control antibiotic-resistant *S.* Typhimurium.

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Materials and Methods

Bacterial strains and culture conditions

Strains of *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 19585 (ST^{WT}), *S.* Typhimurium KCCM 40253 (ST^{KCCM}) and *S.* Typhimurium CCARM 8009 (ST^{CCARM}) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), Korean Culture Center of Microorganism (KCCM, Seoul, Korea), and Culture Collection of Antibiotic Resistant Microbes (CCARM, Seoul, Korea), respectively. Ciprofloxacin-induced *S.* Typhimurium ATCC 19585 strains (ST^{CIP}) was obtained by a serial passage method[11] from 0 to 2 μg/mL of ciprofloxacin in trypticase soy broth (TSB; Difco, Becton, Dickinson and Co., Sparks, MD, USA). All *S.* Typhimurium strains were cultured in TSB with aerobic condition at 37°C for 20 h and harvested by a centrifugation at 3,000 × g for 20 min at 4°C. The collected cells were washed twice with phosphate-buffered saline (PBS, pH 7.2).

Bacteriophage propagation

Salmonella phages, P-22, P-22 B1, PBST-10, PBST-13, PBST-32, and PBST-35, were purchased from ATCC and Bacteriophage Bank (PB) at Hankuk University of Foreign Studies (Yongin, Gyeonggi, Korea). All phages were propagated in TSB with the host strain ST^{KCCM} at 37°C for 20 h. The proliferated phages were harvested by centrifugation at 5,000 × *g* for 10 min then filtered using 0.2 μm pore-size disposable syringe filters. The phage stock solution was determined using the agar overlay assay and stored at 4°C. In brief, the serially diluted phages (1:10) were gently mixed to avoid air bubble with the host strain (10^8 CFU/ml) in TSB[12]. The mixtures were poured onto the pre-heated TSA plates and incubated at 37°C for 20 h to count the phage plaques which were estimated as plaque-forming unit (PFU).

Determination of phage host range

The host ranges of phages were analyzed using a phage spot test with minor modifications[13]. In brief, the bacterial strains used in this study were ST^{WT}, ST^{KCCM}, ST^{CCARM}, ST^{CIP}, *Klebsiella pneumoniae* ATCC 23357, *K. pneumoniae* KCCM 11257, *K. pneumoniae* CCARM 10237, ciprofloxacin-induced *K. pneumoniae* ATCC 23357, *Staphylococcus aureus* ATCC 15564, *S. aureus* KCCM 13236, *S. aureus* CCARM 3080, ciprofloxacin-induced *S. aureus* ATCC 15564, and oxacillin-induced *S. aureus* ATCC 15564. Soft-agar was overlaid with 200 μL of each strain on pre-solidified trypticase soy agar (TSA; Difco, Becton, Dickinson and Co.) plate and then 2 μL of phages (10^8 PFU/mL) were spotted and incubated at 37°C for 20 h. Proper hosts were determined by the formation of clear zone, showing the lytic capability of phages.

Efficacy of phage adsorption

The adsorption assay was determined to evaluate the ability of phage to bind the host cell surface[14]. Each bacterial strain (10^5 CFU/mL) was infected with phage (P-22, P-22 B1, PBST-10, PBST-13, PBST-32, or PBST-35,) at multiplicity of infection (MOI) of 1 and allowed to adsorb at 37°C for 20 min. After incubation, bacterial cells were centrifuged at 13,000 × *g* for 5 min and the titers of adsorbed phages were determined using a soft-agar overlay assay.

One-step growth curve analysis

The one-step growth curves were used to estimate the phage burst size against antibiotic-susceptible and antibiotic-resistant host strains. The phages infected to bacterial cells (10^4 CFU/mL) at MOI of 1 for 20 min were centrifuged at 13,000 × *g* for 5 min to remove unabsorbed free phages. The collected phage-absorbed cell pellets were resuspended in TSB and incubated at 37°C for 30 min. The phage titers were estimated at every 5 min for 30 min by using the agar-overlay assay. The phage burst size was estimated by the ratio of the total number of phages released to the initial number of infected bacterial cells[15].

Determination of lytic activity

To evaluate the lytic activity of phages[13], the bacterial cells (10^4 CFU/mL) were infected with phages at MOI of 1 and incubated at 37°C for 12 h. The bacterial cells were centrifuged at 3,000 × g for 20 min. The collected bacterial cells were serially diluted (1:10) with phosphate buffer saline (PBS, pH7.2) and plated on TSA plate using an Autoplate® Spiral Plating System (Spiral Biotech Inc., USA). After incubation at 37°C for 24 h, the viable cells were enumerated using a QCount® Colony Counter (Spiral Biotech Inc., USA).

Statistical analysis

All experiments were conducted with three replicates. Data were analyzed within the Statistical Analysis System software 9.4 (SAS, Institute Inc., Cary, NC, USA) using the general linear model (GLM) and least significant difference (LSD) procedures. The significant mean differences between treatments were determined at $P<0.05$.

Results and Discussion

This study describes the interactions between phage and antibiotic-resistant *S.* Typhimurium in association with adsorption rate, one-step growth curve, and lytic activity. It is worth understanding the phage-host interactions in order to develop effective control and detection systems for antibiotic-resistant pathogens.

Host range and adsorption rate of *Salmonella* phages

The host ranges of *Salmonella* phages (PBST-10, PBST-13, PBST-32, PBST-35, P-22, and P-22 B1) were determined by using phage spot assay against ST^{WT}, ST^{KCCM}, ST^{CCARM}, ST^{CIP}, KP^{WT}, KP^{KCCM}, KP^{CCARM}, KP^{CIP}, SA^{WT}, SA^{KCCM}, SA^{CCARM}, SA^{CIP}, and SA^{OXA}. The lytic spectrum of phages varied against *S.* Typhimurium strains (data not shown). All phages have a narrow host range, which only lysed all *Salmonella* strains excepting ST^{CCARM}. The result suggests that the phage-binding receptors were altered in multidrug-resistant ST^{CCARM}. In addition, no intraspecies and interspecies infections were observed for *Salmonella* phages against *K. pneumoniae* and *S. aureus* strains. In general, phages can infect bacterial pathogens at close range[16]. The adsorption of phages (PBST-10, PBST-13, PBST-32, PBST-35, P-22, and P-22 B1) to the surfaces of host cells (ST^{WT}, ST^{KCCM}, ST^{CCARM}, and ST^{CIP}) were determined after 20 min

of infection (Fig. 1). The phage adsorption to host cells is the initial step of phage infection process[8]. The highest adsorption rates of P-22 (85%), PBST-35 (80%), PBST-10 (75%), and PBST-32 (71%) were observed for ST^{WT}, while PBST-13 and P-22 B1 showed the lowest adsorption rates, respectively, 47% and 54%. The adsorption rates of PBST-10, P-22, PBST-35, PBST-32, and PBST-13 were 95%, 80%, 77%, 75%, and 73%, respectively, to ST^{KCCM}. In case of ST^{CIP}, the highest adsorption rate was more than 90% when infected with PBST-32 and PBST-35, followed by PBST-10 (80%), whereas the least adsorption rates of PBST-13, P-22, and P-22 B1 were 62%, 61%, and 59%, respectively, after 20 min of infection. The increased adsorption rates of PBST-32, PBST-35, and PBST-10 to ST^{CIP} (Fig. 1) are in a good agreement with previous report that ciprofloxacin enhanced the phage adsorption of Φ13 to *S. aureus*[17]. However, ST^{CCARM} showed the least adsorption rates when infected with all phages (<40%). ST^{CCARM}, a clinically isolate, was highly resistance to β-lactam antibiotics (ampicillin and penicillin G) and aminoglycoside antibiotics (streptomycin and kanamycin)[3]. Therefore, the alteration in the surface receptors of ST^{CCARM} resulted in the decrease in phage adsorption[18]. The phage adsorption is directly associated with bacterial surface receptors such as lipopolysaccharide, pili, flagella, and membrane porin[19]. Our data suggests that the alteration of bacterial membrane proteins and phage-binding receptors on the host cell surface were responsible for the reduction in the phage adsorption and binding specificity to ST^{CCARM} [9].

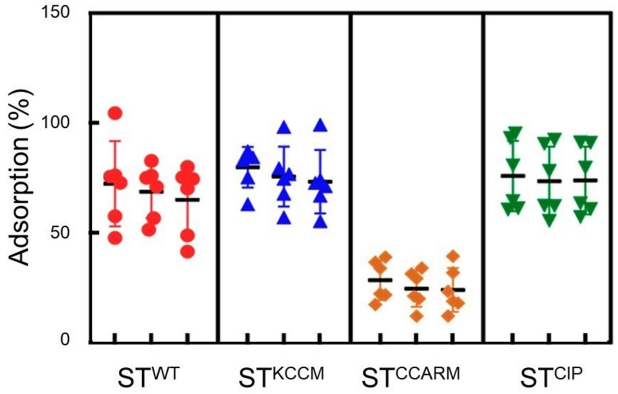

Fig. 1. Adsorption rates of PBST-10, PBST-13, PBST-32, PBST-35, P-22, and P-22 B1 against *Salmonella* Typhimurium ATCC 19585 (ST^{WT}; ●), *S.* Typhimurium KCCM 40253 (ST^{KCCM}; ▲), S. Typhimurium CCARM 8009 (ST^{CCARM}; ♦), and ciprofloxacin-induced *S.* Typhimurium ATCC 19585 strains (ST^{CIP}; ▼).

One-step growth curve and lytic activity of *Salmonella* phages

The one-step growth curves of PBST-10, PBST-13, PBST-32, PBST-35, P-22 and P-22 B1 were used to estimate the phage latent times and burst sizes against ST^{WT}, ST^{KCCM}, ST^{CCARM}, and ST^{CIP} (Fig. 2). The burst sizes varied from 24 to 1,156 PFU. The burst sizes of PBST-10, PBST-13, PBST-32, PBST-35, P-22 and P-22 B1 were 63, 63, 350, 267, 347,

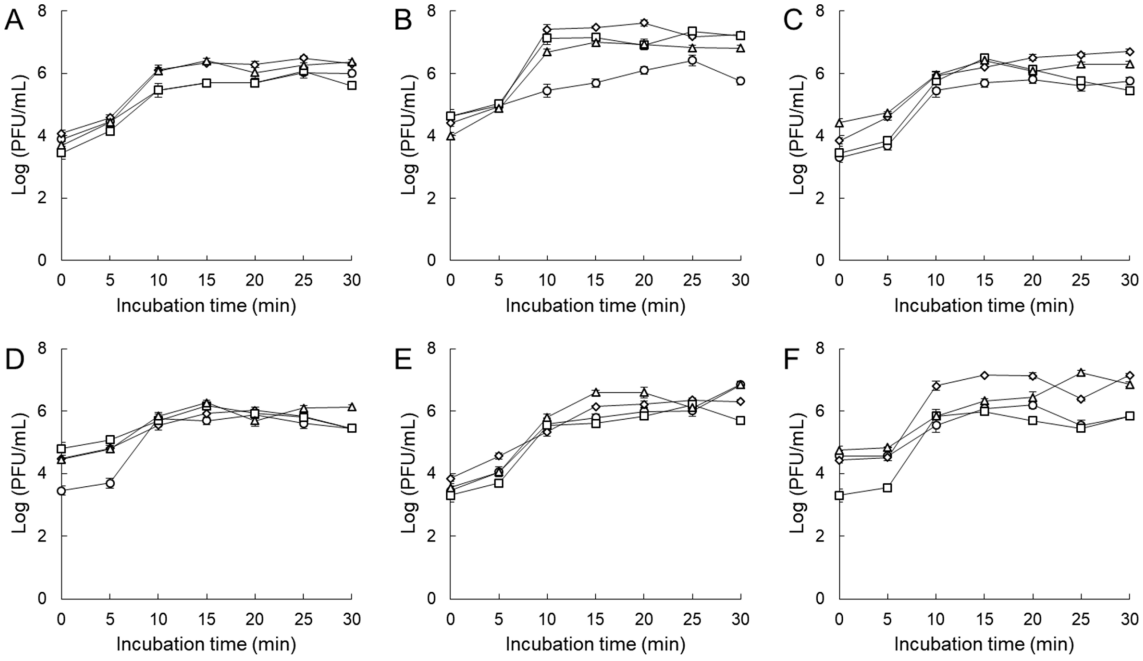

Fig. 2. One-step growth curves of PBST-10 (A), PBST-13 (B), PBST-32 (C), PBST-35 (D), P-22 (E), and P-22 B1 (F) against *Salmonella* Typhimurium ATCC 19585 (ST^{WT}; ○), *S.* Typhimurium KCCM 40253 (ST^{KCCM}; ◊), S. Typhimurium CCARM 8009 (ST^{CCARM}; □), and ciprofloxacin-induced *S.* Typhimurium ATCC 19585 strains (ST^{CIP}; Δ).

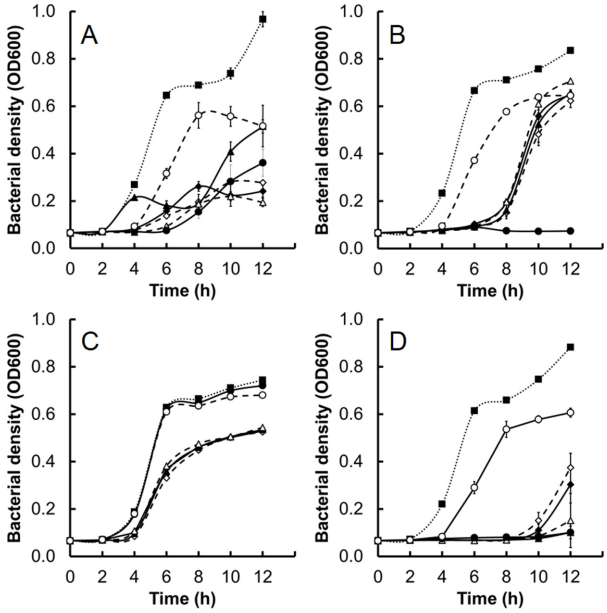

Fig. 3. Lytic activities of PBST-10 (♦), PBST-13 (◊), PBST-32 (▲), PBST-35 (Δ), P-22 (●), and P-22 B1 (○) against *Salmonella* Typhimurium ATCC 19585 (ST^{WT}; A), *S.* Typhimurium KCCM 40253 (ST^{KCCM}; B), *S. Typhimurium* CCARM 8009 (ST^{CCARM}; C), and ciprofloxacin-induced *S.* Typhimurium ATCC 19585 strains (ST^{CIP}; D). (■) No phage treatment was considered as a control.

and 43 PFU against ST^{WT}, 158, 1154, 400, 37, 271, and 530 PFU for ST^{KCCM}, 167, 335, 1067, 24, 350, and 500 PFU for ST^{CCARM}, and 500, 1000, 100, 66, 1000, and 309 PFU for ST^{CIP}, respectively, at MOI of 1 (Fig. 2). PBST-13, PBST-32, and P-22 showed the highest burst sizes against ST^{KCCM} (1153 PFU), and ST^{CCARM} (1067 PFU), and ST^{CIP} (1000 PFU), respectively (Fig. 2B, 2C, and 2E). The short latent time with high burst sizes is the best criterion for a potential phage therapeutic application[20]. The lytic activity of PBST-10, PBST-13, PBST-32, PBST-35, P-22, and P-22 B1 was evaluated against ST^{WT}, ST^{KCCM}, ST^{CCARM}, and ST^{CIP}, at the MOI of 1 (Fig. 3). The phage lytic patterns varied depending on bacteria stains. The highest lytic activity was observed for PBST-35 against ST^{WT}, followed by PBST-10 and PBST-13 (Fig. 3A). The P-22 showed the highest lytic activity against ST^{KCCM}, while other phages such as PBST-10, PBST-13, PBST-32, PBST-35, and P-22 B1 (23 ± 0.01%) showed the least lytic activities ($p<0.05$) (Fig. 3B). No significance in the lytic activity of P-22 and P-22 B1 was observed against ST^{CCARM} when compared to the control ($P>0.05$) (Fig. 3C). The growth of ST^{CIP} was well inhibited by all phages at the early of incubation period, excepting P-22 B1 (Fig. 3D). The phage lysis pattern and resistance range depend on the host type and phage species[21]. The low lytic efficacy of most phages against ST^{CCARM} may be attributed to the low binding affinity due to the alteration in phage-binding receptors[22]. Phage-host interaction is highly associated with the presence of phage-binding proteins and receptors[23]. Therefore, the phage resistance may occur as a result of host cell surface receptor modification that leads to reduce the lytic efficacy. The phage resistance of the host bacteria occurs due to the inhibition of phage adsorption and entry[24,25]. Further understanding the phage resistance mechanisms is essential to improve lytic activity of phages.

In conclusion, the most significant finding was that the phage behaviors of PBST-10, PBST-13, PBST-32, PBST-35, P-22, and P-22 B1 were varied depending on the degree of antibiotic resistance in *S.* Typhimurium, ST^{WT}, ST^{KCCM}, ST^{CIP}, and ST^{CCARM}. The highest adsorption rates were observed for all phages to ST^{WT}, ST^{KCCM}, and ST^{CIP}, except ST^{CCARM}. The phages burst sizes and lytic activities depended on the alteration of phage-binding surface receptors. This study provides useful information for designing phage control system.

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국문요약

본 연구는 다양한 항생제 내성을 갖는 *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 19585 (ST^{WT}), *S.* Typhimurium KCCM 40253 (ST^{KCCM}), ciprofloxacin-induced *S.* Typhimurium ATCC 19585 strains (ST^{CIP}), and S. Typhimurium CCARM 8009 (ST^{CCARM})에 대한 phage의 흡착 및 용균 특성을 평가하였다. PBST-10, PBST-13, PBST-32, PBST-35, P-22, P-22 B1 phages는 narrow host range를 보였다. 숙주인 ST^{WT}, ST^{KCCM}, ST^{CIP}에 대한 phage의 흡착률은 각각 47-85%, 58-95%, 61-93%였지만, ST^{CCARM}에 대한 phage의 흡착률은 14-36%의 낮은 수준을 보였다. ST^{WT}, ST^{KCCM}, ST^{CIP}, ST^{CCARM}에 대한 phage burst size는 각각 43-350, 37-530, 66-500, 24-500 plaque-forming unit (PFU)으로 다양하게 관찰되었다. P-22 B1을 제외한 모든 phage는 배양 초기에 ST^{CIP}숙주를 효과적으로 저해하였다. 이러한 결과는 항생제 내성균을 저해하기 위해 phage control system 개발에 유용한 정보로 활용될 것이다.

Conflict of Interest

There are no conflicts of interest to declare.

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