Associations between Bacteriophage Resistance and Antibiotic Resistance Phenotypes in Laboratory and Clinical strains of Salmonella Typhimurium

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Research article

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Abstract

Background Bacteriophages have received great attention as alternative over antibiotics due to the host specificity. Therefore, this study was designed to evaluate the associations between bacteriophage-insensitive (BI) and antibiotic-resistant mutants of *Salmonella* Typhimurium strains. Bacteriophage-sensitive *Salmonella* Typhimurium ATCC 19585 (BSSTWT), ciprofloxacin-induced *S. Typhimurium* ATCC 19585 (BSSTCIP), *S. Typhimurium* KCCM 40253 (BSSTLAB), and clinically isolated multidrug-resistant *S. Typhimurium* CCARM 8009 (BSSTMDR) were used to induce the bacteriophage-insensitive mutants (BISTWT, BISTCIP, BISTLAB, and BISTMDR) against bacteriophage P22. **Results** The numbers of BSSTWT, BSSTCIP, and BSSTLAB were reduced by P22 (>3 log), while the least lytic activity was observed for BSSTMDR. BSSTWT treated with P22 showed the large variation in the cell state (CV $>40\%$) and highest mutant frequency (62%), followed by 25% for STCIP. The least similarities between BSSTWT and BISTWT were observed at P22 and PBST-13 ($<12\%$). The antibiotic susceptibilities were not significantly changed or slightly increased against BISTWT, BISTCIP, BISTLAB, and BISTMDR. The relative expression levels of bacteriophage-binding receptor-related genes (*btuB*, *fhuA*, *fliK*, *fljB*, *ompC*, *ompF*, *rfaL*, and *tolC*) were decreased in BISTCIP and BSSTMDR. **Conclusion** The results could pave the way for the application of bacteriophages as an alternative to control antibiotic-resistant bacteria.

Background

Over the last few decades, the emergence of antibiotic-resistant pathogens has become a serious threat to public health due to the risk of antibiotic treatment failure [1]. The misuse and overuse of antibiotics have accelerated the development of multidrug-resistant (MDR) pathogens [2]. MDR *Salmonella* strains are resistant to ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, and tetracycline, leading to high morbidity and mortality rates [2-5]. MDR *Salmonella* strains are one of the most common causes of infectious diseases with 90 million cases of gastroenteritis and approximately 155,000 deaths worldwide each year [1]. The current chemotherapeutic treatments become further complicated with MDR *Salmonella* infections. Therefore, the development of effective alternatives over antibiotics has been a major endeavor towards treatment of MDR *Salmonella* infections.

Recently, bacteriophages have received growing attention due to their host specificity [6, 7]. The host-bacteriophage interaction depends on the ability of bacteriophages to bind the host cell receptors, including appendages, glycocalyx, and cell wall components [8, 9]. However, there still remains a challenging question with respect to bacteriophage resistance. The alterations in the host cell receptors may confer the resistance to bacteriophages [10]. The bacterial host can evolve several bacteriophage resistance mechanisms, including inhibition of bacteriophage adsorption, prevention of bacteriophage DNA entry (superinfection exclusion system), degradation of bacteriophage DNA (restriction-modification system), development of abortive infection system, and CRISPR/Cas system [11-14]. The antibiotic resistance can result in the conformational change in bacteriophage-binding receptors on the host cell surface [10, 15]. However, there is relatively few information on the relationship between bacteriophage

resistance and antibiotic resistance in *Salmonella* Typhimurium. Therefore, this purpose of this study was to characterize the bacteriophage mutants of *Salmonella* Typhimurium in association with bacteriophage adsorption, antibiotic susceptibility, and gene expression.

Methods

Bacterial Strains and Culture Conditions

Strains of *Salmonella* Typhimurium ATCC 19585, *S. Typhimurium* KCCM 40253, and *S. Typhimurium* CCARM 8009 were obtained from 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. The bacterial cells were cultured in trypticase soy broth (TSB; BD, Becton, Dickinson and Co., Sparks, MD, USA) at 37°C for 20 h, centrifuged at $7000 \times g$ for 10 min at 4°C, and washed with phosphate-buffered saline (PBS; pH 7.2). The harvested cells were diluted to 10^8 CFU/mL for further assays.

In vitro Stepwise Selection Assay

To induce antibiotic-resistant *S. Typhimurium* ATCC 19585 was exposed with serially increasing ciprofloxacin concentrations according to the serial passage procedure [16]. *Salmonella* Typhimurium ATCC 19585 was repeatedly cultured in TSB and trypticase soy agar (TSA) containing ciprofloxacin concentrations from 0.0078 to 1 µg/mL. The ciprofloxacin-induced resistant *S. Typhimurium* ATCC 19585 was stable for more than ten passages in antibiotic-free TSB at 37°C for 20 h prior to use.

Bacteriophage Propagation

Salmonella bacteriophages, P22, P22-B1, PBST-10, PBST-13, PBST-32, and PBST-35, were obtained from ATCC and Bacteriophage Bank at Hankuk University of Foreign Studies (Yongin, Gyeonggi, Korea). All bacteriophages were propagated at 37°C for 20 h in TSB containing *S. Typhimurium* KCCM 40253. The propagated bacteriophages were collected by centrifuging at $6000 \times g$ for 10 min, filtered through by a 0.2-µm filter to remove bacterial lysates, and further purified using polyethylene glycol (PEG) precipitation assay [17]. The titers of bacteriophage were determined by using a soft-agar overlay method [18]. In brief, the selected bacteriophages were serially (1:10) diluted with PBS and gently mixed with the host cells (10^7 CFU/mL) in TSB containing 0.5% agar. The mixture was poured onto the pre-warmed base agar and solidified at room temperature, and then incubated at 37°C for 20 h to enumerate the bacteriophages expressed as a plaque-forming unit (PFU).

Lytic Activity of Bacteriophage

Salmonella bacteriophages (P22, P22-B1, PBST-10, PBST-13, PBST-32, and PBST-35) were used to evaluate the lytic activity against ST^{WT} , ST^{CIP} , ST^{LAB} , and ST^{MDR} . The selected strains (10^8 CFU/mL each) were mixed with bacteriophage (10^{10} PFU/mL each) and incubated at 37°C for 10 min. The incubated cultures were centrifuged at $6,000 \times g$ for 5 min, serially diluted (1:10) with PBS, and plated on TSA using an Autoplate® Spiral Plating System (Spiral Biotech Inc.). The plates were incubated at 37°C for 24-48 h. The lytic activity was expressed as $\log N/N_0$; N and N_0 denote the counts of bacterial cells treated with and without bacteriophages, respectively.

Fluctuation Assay

The fluctuation assay was used to determine mutant distribution whether ST^{WT} , ST^{CIP} , ST^{LAB} , and ST^{MDR} mutants were spontaneous or inducible in the presence of bacteriophages [19]. In brief, ST^{WT} , ST^{CIP} , ST^{LAB} , or ST^{MDR} cells (10^3 CFU/mL) were distributed into 10 test tubes (0.2 mL; Group A) and one test tube (2 mL; Group B). The tubes were incubated at 37°C for 3 h. Group A (1 replicate/tube) and Group B (10 replicates/tube) were plated on TSA with P22 and incubated for 37°C for 24-48 h to enumerate viable cells.

Induction of Bacteriophage-insensitive *Salmonella* Typhimurium

Bacteriophage P22-insensitive mutant *S. Typhimurium* ATCC 19585 (ST^{WT}), ciprofloxacin-induced *S. Typhimurium* ATCC 19585 (ST^{CIP}), *S. Typhimurium* KCCM 40253 (ST^{LAB}), and clinically isolated multidrug-resistant *S. Typhimurium* CCARM 8009 (ST^{MDR}) were isolated using the spot plate assay [20]. P22 (2×10^6 PFU/5 μ l) were spotted on 0.5 % soft-agar containing $BSST^{WT}$, $BSST^{CIP}$, $BSST^{LAB}$, and $BSST^{MDR}$ (10^7 CFU/mL each) and incubated at 37°C until recovery of host cells within the clear zone, which were assigned as $BIST^{WT}$, $BIST^{CIP}$, $BIST^{LAB}$, and $BIST^{MDR}$, respectively. The isolated colonies were subcultured in TSB at 37°C for 20 h. The BIST cells were suspended gently in 0.5 % soft-agar plates and poured onto the pre-warmed base agar. The 10-fold diluted P22 (5 μ l each) was spotted onto the plates. After 20 h incubation at 37°C, the bacteriophage-insensitive mutants ($BIST^{WT}$, $BIST^{CIP}$, $BIST^{LAB}$, and $BIST^{MDR}$) were tested for the multiple-resistance to P22, P22-B1, PBST-10, PBST-13, PBST-32, and PBST-35.

Mutant Frequency Assay

The mutation rates of ST^{WT} , ST^{CIP} , ST^{LAB} , and ST^{MDR} cultured in the absence and presence of P22 for 48 h were estimated on the TSA containing bacteriophages (10^7 PFU/mL). The cultured cells were plated on TSA with and without bacteriophages and incubated at 37°C for 24-48 h. The mutant frequencies were estimated as the proportion of the numbers of surviving colonies on the TSA with and without bacteriophages.

Lysogenic Induction Assay

Lysogenic cells were induced by mitomycin C [18]. In brief, the cultured BSST^{WT}, BSST^{CIP}, BSST^{LAB}, BSST^{MDR}, BIST^{WT}, BIST^{CIP}, BIST^{LAB}, and BIST^{MDR} were treated with mitomycin C (0.5 µg/mL) at 37°C for 2 h. After incubation, the mixtures were centrifuged at $7000 \times g$ for 5 min and filtered through a 0.2-µm filter. The collected supernatants were spot tested to confirm the lytic bacteriophages against BSST^{LAB}.

Bacteriophage Adsorption Assay

Bacteriophage adsorption rates were estimated to evaluate the bacteriophage-binding receptors on the bacteriophage-sensitive and bacteriophage-insensitive ST^{WT} , ST^{CIP} , ST^{LAB} , and ST^{MDR} . In brief, BSST^{WT}, BSST^{CIP}, BSST^{LAB}, BSST^{MDR}, BIST^{WT}, BIST^{CIP}, BIST^{LAB}, and BIST^{MDR} (10^6 CFU/mL) were infected with the bacteriophages (P22, P22-B1, PBST-10, PBST-13, PBST-32, and PBST-35) at MOI of 0.1 and then allowed to adsorb at 37°C for 15 min. After incubation, the cultures were centrifuged at $16,000 \times g$ for 2 min at 4°C. The supernatants were serially diluted and plated to determine unabsorbed bacteriophage titers according to a soft-agar overlay assay. The adsorption percentage was estimated using the equation: Adsorption rate (%) = [(initial phage titer-phage titer in the supernatant) / (initial phage titer)] × 100.

Antibiotic Susceptibility Assay

The antibiotic susceptibilities of BSST^{WT}, BSST^{CIP}, BSST^{LAB}, and BSST^{MDR} were determined by using an agar disc diffusion assay to compare with those of BIST^{WT}, BIST^{CIP}, BIST^{LAB}, and BIST^{MDR}. The cultured cells (0.5 McFarlan) were spread on Mueller–Hinton agar plate and then allowed to dry for 5 min. The antibiotic discs (Becton, Dickinson and Company, NJ, USA), including ampicillin (10 µg), cephalothin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), imipenem (10 µg), streptomycin (10 µg), and tetracycline (30 µg) were placed on the surface on Mueller–Hinton agar and incubated at 37°C for 20 h. The diameter of the inhibition zone was measured by using a digital vernier caliper to evaluate the antibiotic susceptibility.

Quantitative RT-PCR Assay

Total RNA was extracted from BSST^{WT}, BSST^{CIP}, BSST^{LAB}, BSST^{MDR}, BIST^{WT}, BIST^{CIP}, BIST^{LAB}, and BIST^{MDR} according to the protocol of RNeasy Protect Bacteria Mini kit protocol (Qiagen, Hilden, Germany). The pre-cultured cells were mixed with 1 mL of RNA protect Bacteria Reagent to stabilize RNA, and the mixture were centrifuged at 5,000 × g for 10 min. The collected cells were lysed with a lysozyme-containing buffer TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0). The lysate cells were mixed with 95% ethanol to extract RNA through an RNeasy mini column. According to the QuantiTect reverse transcription procedure (Qiagen), cDNA was synthesized. Briefly, the RNA extracts were rinsed with a Wipe buffer to remove genomic DNA and mixed with a master mixture containing reverse transcriptase, RT buffer, and RT primer mix. The mixture was incubated at 42°C for 15 min followed by 95°C for 3 min. For amplification, the PCR mixture (20 µl) containing 10 µl of 2× QuantiTect SYBR Green PCR Master, 2 µl of each primer, and 2 µl of cDNA, and 4 µl of RNase-free water was denatured at 95°C for 30 sec, followed by 45 cycles of 95°C for 5 sec, 55°C for 20 sec, and 72°C for 15 sec using an QuantStudio™ 3 Real-Time PCR System (Applied Biosystems™, USA). The synthesized oligonucleotide primers used in this study are listed in **Table 1**. The relative gene expression levels were determined using the comparative method [21].

Statistical Analysis

All analyses were performed in duplicate on three replicates. Data were analyzed using Statistical Analysis System (SAS). The general linear model (GLM) and least significant difference (LSD) procedures were used to determine significant mean differences among treatments at $P < 0.05$.

Results

Lytic activity of Bacteriophages against *Salmonella* Typhimurium

The lytic activities of bacteriophages (P22, P22-B1, PBST-10, PBST-13, PBST-32, and PBST-35) against *Salmonella* Typhimurium ATCC 19585 (ST^{WT}), ciprofloxacin-induced *S. Typhimurium* ATCC 19585 (ST^{CIP}), *S. Typhimurium* KCCM 40253 (ST^{LAB}), and clinically isolated multidrug-resistant *S. Typhimurium* CCARM 8009 (ST^{MDR}) were determined as shown in **Figure 1**. The highest lytic activities of P22 were observed against ST^{WT}, ST^{CIP}, and ST^{LAB}, showing more than 3 log reduction. Most bacteriophages had the least lytic activities against ST^{MDR} (< 1 log reduction).

Variability in Inherited and Induced Mutation in *Salmonella* Typhimurium

Fluctuation assay was used to evaluate the cell states of ST^{WT}, ST^{CIP}, ST^{LAB}, and ST^{MDR}, which can determine whether mutants were inherited (spontaneous) or induced in the presence of bacteriophages

[19]. ST^{WT}, ST^{LAB}, and ST^{MDR} showed large variation in group A (CV > 40%) and small variation (CV < 12%) from group B, while the small variations were observed in ST^{CIP} from both group A and group B (**Figure S1**). The bacteriophage-induced mutation in ST^{WT}, ST^{CIP}, ST^{LAB}, and ST^{MDR} was determined to evaluate the mutant frequency in the presence of bacteriophages (**Figure S2**). ST^{WT}, ST^{CIP}, ST^{LAB}, and ST^{MDR} varied in the resistance to bacteriophage. ST^{WT} treated with P22 had the highest mutant frequency of 62%, followed by 25% for ST^{CIP}.

Lytic Activity and Bacteriophage Specificity for P22-mutant *Salmonella* Typhimurium

The P22-induced BIST^{WT}, BIST^{LAB}, and BIST^{MDR} exhibited the resistance to P22 and P22-B1, while those were still susceptible to other bacteriophages (PBST-10, PBST-13, PBST-32, and PBST-35) (**Figure S3**). BIST^{CIP} was resistant to P22, P22-B1, PBST-32, and PBST-35. A dendrogram was depicted based on the adsorption rates of bacteriophages (P22, P22-B1, PBST-10, PBST-13, PBST-32, and PBST-35) to bacteriophage-sensitive (BS) and bacteriophage-insensitive (BI) ST^{WT}, ST^{CIP}, ST^{LAB}, and ST^{MDR}. The highest similarities between BSST^{WT} and BIST^{WT} were observed at PBST-35 (85%), followed by PBST-10 (78%), P22-B1 (76%), and PBST-32 (63%), while the least similarities were observed at P22 and PBST-13 (<12%) (**Figure 2**). The similarities between BSST^{CIP} and BIST^{CIP} were 68% at PBST-10 and 86% at PBST-13. BSST^{CIP} and BIST^{CIP} at P22, P22-B1, PBST-32, and PBST-35 had similarities of less than 20%. The highest similarity between BSST^{LAB} and BIST^{LAB} was 95% at PBST-13, while the least similarities were observed at P22, PBST-32, and PBST-35. BSST^{MDR} and BIST^{MDR} at PBST-13, and PBST-35 had similarities of 99% and 95%, respectively. No similarities were observed between BSST^{MDR} and BIST^{MDR} at PBST-10 and PBST-32.

Lysogenic Conversion of Bacteriophage-insensitive *Salmonella* Typhimurium Mutants

The lysogen induction assay were performed to evaluate whether the bacteriophage resistance was owing to BIST^{WT}, BIST^{CIP}, BIST^{LAB}, and BIST^{MDR} lysogenic cells (**Figure S4**). BIST^{WT} and BIST^{LAB} treated with mitomycin C exhibited lytic growth, indicating that the P22-resistant BIST^{WT} and BIST^{LAB} strains have inducible prophages. However, BIST^{CIP} and BIST^{MDR} treated with mitomycin C did not show phage plaque, suggesting that the P22 was not lysogenized in the BIST^{CIP} and BIST^{MDR} (**Figure S4**).

Antibiotic Susceptibility of Bacteriophage-insensitive *Salmonella* Typhimurium Mutants

The antibiotic susceptibilities of BSST^{WT}, BSST^{CIP}, BSST^{LAB}, and BSST^{MDR} were evaluated using disk diffusion assay and compared with those of BIST^{WT}, BIST^{CIP}, BIST^{LAB}, and BSST^{MDR}, respectively (**Figure**

3). No noticeable changes in antibiotic susceptibilities were observed between bacteriophage-sensitive and bacteriophage-insensitive strains (**Figure 3A**). The susceptibilities of BIST^{CIP}, BIST^{LAB}, and BIST^{MDR} were significantly increased to ciprofloxacin (**Figure 3B**), ampicillin (**Figure 3C**), and tetracycline (**Figure 3D**), respectively, compared to BSST^{CIP}, BSST^{LAB}, and BSST^{MDR}.

Gene Expression in Bacteriophage-insensitive *Salmonella* Typhimurium Mutants

The relative gene expression of *btuB*, *fhuA*, *fliK*, *fljB*, *ompC*, *ompF*, *rfaL*, *seiA*, *stn*, and *tolC*

were observed in BIST^{WT}, BIST^{CIP}, BIST^{LAB}, and BIST^{MDR} compared to BSST^{WT}, BSST^{CIP}, BSST^{LAB}, and BSST^{MDR} (**Figure 4**). Most genes were slightly overexpressed in BIST^{WT} and BIST^{LAB}, while the relative expression levels of most genes were significantly decreased in BIST^{CIP} and BIST^{MDR}. The *seiA* was highly overexpressed in BIST^{WT} by 11-fold and BIST^{LAB} by 18-fold, while the relative expression levels of *stn* was decreased in the BIST^{CIP} by 7-fold and BIST^{MDR} by 4-fold.

Discussion

The application of bacteriophages for combatting antibiotic-resistant bacteria is often impeded by bacteriophage resistance [22]. However, bacteria under selection pressure can lead to a trade-off between bacteriophage-resistance and antibiotic resistance [23]. Therefore, this study describes the association between bacteriophage-resistance and antibiotic resistance in *S. Typhimurium* with different levels of antibiotic resistance, which needs to design effective bacteriophage-based therapy to control antibiotic-resistant bacteria.

P22 was able to most effectively lyse ST^{WT}, ST^{CIP}, and ST^{LAB} (**Figure 1**). The efficacy of lytic activity of bacteriophages depend on the specific recognition between bacterial cell surface receptors and receptor-binding proteins of bacteriophages [8]. As shown in **Figure 2**, the large variation from group A and small variation from group B imply that mutation was induced before bacteriophage infection [19]. The mutation occurred spontaneously before exposure to selection pressure. The alteration in bacteriophage-binding receptors on the host cells can lead bacteriophage-insensitive mutants [24]. The resistance of BIST^{LAB} to P22 was also due to the lysogenic conversion, which can lead superinfection exclusion [25]. This was confirmed by the lysogenic induction assay, showing that prophages were induced from the BIST^{WT} and BIST^{LAB} after mitomycin C treatment (**Figure S4**).

Numerous *Salmonella*-specific bacteriophages that use LPS as a receptor can modify LPS to protect from superinfection when host cells are lysogenized [26, 27]. Recently, two copies of lipopolysaccharide modification acyltransferase and GtrA are found on the genome of lysogenic P22-like bacteriophage [28]. Although the modification of LPS protects the lysogeny from superinfection by LPS targeting bacteriophage, the lysogeny is still susceptible to bacteriophages that target other receptors such as

flagella [28] (**Figure S3**). The multiple resistance of BIST^{CIP} might be attributed to the mutation in genes encoding bacteriophage-binding receptors [24]. The loss of bacteriophage-binding receptors is directly associated with the decrease in lytic ability of bacteriophages [8].

The highest similarity indicates that bacteriophage-binding receptors were not altered at the mutants. However, the least similarity suggests that bacteriophage-binding receptors were changed after bacteriophage-induced mutation. P22-B1 (76%), PBST-10 (78%), and PBST-35 (85%) might share common receptors on the BSST^{WT} and BIST^{WT}. PBST-10 (68%) and PBST-13 (86%) might share common receptors on the BSST^{CIP} and BIST^{CIP}, P22-B1 (77%) and PBST-13 (95%) on the BSST^{CIP} and BIST^{CIP}, and PBST-13 (99%) and PBST-35 (95%) on the BSST^{MDR} and BIST^{MDR}. The adsorption similarities between BS and BI *S. Typhimurium* strains were well corresponded to the lytic activities with the exception of BSST^{WT} and BIST^{WT} against P22-B1, BSST^{WT} and BIST^{WT} against PBST-13, BSST^{LAB} and BIST^{LAB} against P22-1, and BSST^{CIP} and BIST^{CIP} against PBST-12 (**Figure S3**). The results might be due to the alteration of bacteriophage-binding receptors, resulting in the change in binding affinity. The bacteriophage-insensitivity and antibiotic resistance contributed to the alteration of bacteriophage-binding receptors on the host cells, which may ultimately result in the decrease in bacteriophage adsorption rate and lytic activity [29]. However, a high adsorption rate does not always linked to a lytic activity [30]. The bacteriophage adsorption rate to the host cells having various receptors are not much affected by their alteration [31]. The antibiotic susceptibility patterns of bacteriophage-insensitive mutants are more likely to increase in the BIST^{CIP}, BIST^{LAB}, and BIST^{MDR} (**Figure 3**). The bacteriophage resistant mutants produce an evolutionary trade-off in antibiotic-resistant bacteria, which can change the phage binding receptors and efflux pump system, resulting in increased susceptibility to several classes of antibiotic [23].

The decrease in relative expression of *btuB*, *fhuA*, *fliK*, *fljB*, *ompC*, *ompF*, *rfaL*, and *tolC* was well corresponded to the low adsorption rates of bacteriophages to BIST^{CIP} and BSST^{MDR} (**Figure 4**). Bacteriophage tail proteins bind to the host surface proteins, polysaccharides, and lipopolysaccharides, responsible for host specificity and range [32]. BtuB (vitamin B12 transporter), FhuA (Ferrichrome outer membrane transporter), FliK (flagella hook), OmpC (outer membrane protein), OmpF (outer membrane protein), RfaL (O-antigen ligase), and TolC (innate efflux pump) can serve as surface receptors for bacteriophages [28, 29, 32]. The OmpC, OmpF, and TolC contribute to multidrug-resistance in bacteria. This confirms the antibiotic susceptibilities were increased against BIST^{CIP}, BIST^{LAB}, and BIST^{MDR} when compared to the BSST^{CIP}, BSST^{LAB}, and BSST^{MDR} (**Figure 3**). Bacteria can adapt to the selective pressure imposed by bacteriophages, leading to antibiotic resistance. This suggests that the bacterial resistance to bacteriophages is related to the antibiotic resistance [33]. The overexpression of *sieA* in BIST^{WT} and BIST^{LAB} (**Figure 4**) implies that these mutants might be due to the lysogenic conversion, preventing the entry of superinfecting bacteriophage DNA into the host [34]. The product of *sieA* is an inner membrane protein of P22-lysogenic *Salmonella* Typhimurium [14]. The suppression of *stn* in the BIST^{CIP} and BIST^{MDR} is in a good agreement with the previous observations that the bacteriophages-insensitive mutants exhibited the reduced virulence in antibiotic-resistant bacteria [35].

Conclusions

The most significant findings in this study were that (i) P22-induced BIST^{CIP} mutant had multiple resistance to P22, P22-B1, PBST-32, and PBST-35, (ii) the superinfection exclusion occurred at P22-induced BIST^{WT} and BIST^{LAB}, (iii) the adsorption rates were varied between BS and BI *S. Typhimurium* strains, (iv) antibiotic susceptibilities were increased in the BIST^{CIP}, BIST^{LAB}, and BIST^{MDR}, and (v) the virulence was reduced in the BIST^{CIP} and BIST^{MDR}. The results pointed out that the bacteriophage-binding receptors were altered in the BI mutant *S. Typhimurium* strains, which did not induce cross-resistance to antibiotics. The results provide useful information for designing effective treatments in bacteriophage alone or combination of bacteriophages and antibiotics that reduce the risk of antibiotic resistance in bacteria. However, further study is needed to understand the association between bacteriophage insensitivity and antibiotic resistance.

Declarations

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Availability of data and materials

The data supporting the conclusions are included within the manuscript and also provided in additional files.

Author's contributions

MJU conducted all experiments and also contributed to the writing and preparation of the manuscript. JA contributed to the experimental design, data interpretation, and manuscript writing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Tables

Table1. .Primer sequences used in qPCR analysis for my study

Gene	Molecular function	Primer sequence *
16S rRNA	Reference gene	F: AGGCCTTCGGGTTGTAAAGT R: GTTAGCCGGTGCTTCTTCTG
<i>btuB</i>	TonB-dependent vitamin B12 transporter	F: AGGACACTAGCCCGGATAACC R: CAGTACATGGCTGGAGTTGG
<i>fhuA</i>	Ferrichrome outer membrane transporter (<i>tonA</i>)	F: CCAGATGAACGAAAGTAAACAAACAG R: GCCGCCGAGAGTAAATACCC
<i>fliK</i>	Flagellar hook-length control	F: AGCTACTGACCCAACATGGC R: GTAAGCGTTTCATCCGTCGC
<i>fljB</i>	Flagellin, phage 2 antigen	F: TGGATGTATCGGGTCTTGATG R: CACCAGTAAAGCCACCAATAG
<i>ompC</i>	Outer membrane protein C	F: TCGCAGCCTGCTGAACCAGAAC R: ACGGGTTGCGTTATAGGTCTGAG
<i>ompF</i>	Outer membrane protein F	F: CGGAATTTATTGACGGCAGT R: GAGATAAAAAAACAGGACCG
<i>rfaL</i>	O-antigen ligase	F: GTGCTTAGCGCCATCTACCT R: ACTTCCATTGGCGGTTTCAGT
<i>sieA</i>	Superinfection exclusion protein A	F: GCTTCTCCGGGGTATCTTCC R: GCCTGTTGTTCTTTGGGTTCC
<i>stn</i>	Salmonella enterotoxin	F: GCCATGCTGTTCGATGAT R: GTTACCGATAGCGGGAAGG
<i>tolC</i>	Multidrug efflux system	F: GCCCGTGCGCAATATGAT R: CCGCGTTATCCAGGTTGTTG

* F, forward; R, reverse

Figures

Figure 1

Lytic activity of bacteriophages, P22, P22-B1, PBST-10, PBST-13, PBST-32, and PBST-35, against *Salmonella* Typhimurium ATCC 19585 (ST^{WT}), ciprofloxacin-induced *S. Typhimurium* ATCC 19585 (ST^{CIP}), *S. Typhimurium* KCCM 40253 (ST^{LAB}), and clinically isolated multidrug-resistant *S. Typhimurium* CCARM 8009 (ST^{MDR}).

Figure 2

Dendrogram based on the adsorption rates of bacteriophages (P22, P22-B1, PBST-10, PBST-13, PBST-32, and PBST-35; +) to bacteriophage-sensitive (BS) and bacteriophage-insensitive (BI) *Salmonella* Typhimurium ATCC 19585 (STWT), ciprofloxacin-induced *S. Typhimurium* ATCC 19585 (STCIP), *S. Typhimurium* KCCM 40253 (STLAB), and clinically isolated multidrug-resistant *S. Typhimurium* CCARM 8009 (STMDR).

Figure 3

Radar plot of antibiotic resistance profiles (disk diffusion in mm) of bacteriophage-sensitive (BS; ●) and bacteriophage-insensitive (BI; ○) *Salmonella* Typhimurium ATCC 19585 (STWT; A), ciprofloxacin-induced *S. Typhimurium* ATCC 19585 (STCIP; B), *S. Typhimurium* KCCM 40253 (STLAB; C), and clinically isolated multidrug-resistant *S. Typhimurium* CCARM 8009 (STMDR; D). *, **, and *** denote significance differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Figure 4

Relative gene expression of bacteriophage-insensitive (BI) *Salmonella* Typhimurium ATCC 19585 (STWT; □), ciprofloxacin-induced *S. Typhimurium* ATCC 19585 (STCIP; ■), *S. Typhimurium* KCCM 40253 (STLAB; ■), and clinically isolated multidrug-resistant *S. Typhimurium* CCARM 8009 (STMDR; ■) compared to bacteriophage-sensitive (BS) STWT, STCIP, STLAB, and STMDR, respectively. Different letters (a–c) on the bars within strains are significantly different at $p < 0.05$.

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