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Resistance mechanisms adopted by a *Salmonella* Typhimurium mutant against bacteriophage

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ABSTRACT

Bacteriophages have key roles in regulating bacterial populations in most habitats. A *Salmonella* Typhimurium mutant (N18) with impaired sensitivity to phage fmb-p1 was obtained and examined, the adsorption efficiency of fmb-p1 to N18 was reduced to 6%, compared to more than 97% for wild type *S.* Typhimurium CMCC50115. Reduced adsorption was accompanied by a reduction of 90% in the LPS content compared to wild type. Electron microscopy showed phage scattered around N18 with minimal engagement, while the phage were efficiently adsorbed to the wild type with tails oriented towards the bacterial surface. Evidence suggests fmb-p1 can slightly infect N18 and this does not give rise to an increase of phage titer. RT-qPCR data show that several *Salmonella* genes involved in lipopolysaccharide synthesis and five virulence related genes were down-regulated upon exposure of N18 to phage fmb-p1. In contrast, phage resistance related genes such as the SOS response, restriction-modification (RM), and Cas1 gene were up-regulated in N18. These data suggest that although inefficient adsorption and entry is the primary mechanism of resistance, transcriptional responses to phage exposure indicate that alternative resistance mechanisms against phage infection are also brought to bear, including digestion of phage nucleic acids and activation of the SOS. These findings may help develop strategies for biocontrol of *Salmonella* where multi-resistant bacteria are encountered or emerge in applications for food production, bioremediation or wastewater treatment.

1. Introduction

Salmonella is one of the most important pathogens that frequently causes serious foodborne disease worldwide (CDC, 2017; EFSA, ECDC, 2017; Song et al., 2018). Decades of the overuse of antimicrobial agents in food animal production is regarded as one of the underlying reasons for the emergence of antimicrobial resistance in *Salmonella* (Hvistendahl, 2012). Multidrug resistance phenotypes are often observed in *Salmonella* isolated from food products (Cai et al., 2016; Zhu et al., 2017), which pose a serious threat to public health. Bacteriophages (phages) are viruses that specifically kill target bacterial strains, and generally do not attack non-target bacterial species (Salmond and Fineran, 2015). Increasing antibiotic-resistance worldwide (Li et al., 2013; Panzenhagen et al., 2016; Yang et al., 2016) has led to a re-evaluation of phage to control these bacteria in food, medical and environmental applications (Endersen et al., 2013; Akhtar et al., 2014; Mostafa et al., 2016). The global incidence of *Salmonella* has provoked a number of studies using phage infecting members of the genus, such as *Salmonella* phage SE07 (*Podoviridae*) and *Salmonella* phage Felix-O1 (*Myoviridae*) (Thung et al., 2017; Yeh et al., 2017). To date, more than 300 *Salmonella* phage genomes have been completely or partly sequenced (NCBI Database, 2019).

The detailed mechanism of interaction between phage and host bacteria is understood for relatively few phages but an exemplar *Salmonella* phage P22 (Andres et al., 2010; Wang et al., 2019). The phage infection process begins with the specific adsorption of the phage to a receptor on the host surface. The repetitive O-antigen structure in *Salmonella* lipopolysaccharide (LPS) was found to be the receptor of phage P22. Phage P22 binds to the receptor via six homotrimeric tailspikes, which possess endoglycosidase activity, hydrolyzing the O-antigen polysaccharide, before binding a secondary cell receptor (Schmidt et al., 2016; Wang et al., 2019). Thereafter, the phage injects its genome into the target cell to direct host cell resources to complete the life cycle of the phage, culminating in host cell lysis and the emergence of new

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virions.

However, host bacteria develop antiphage strategies to prevent cell lysis, for example *Salmonella* modify the structure of LPS to block the adsorption of phage P22 (Steinbacher et al., 1997). Bacterial strains can contain multiple antiphage barriers, which are of concern for phage therapy applications (Brockhurst et al., 2017). Phage resistance mechanisms in bacteria have been reported at various stages in the phage life cycle, including the prevention of phage adsorption (Kim and Ryu, 2012), the prevention of phage DNA entry (Labrie et al., 2010), targeted cleavage of phage nucleic acids (Bondy-Denomy and Davidson, 2014; Kim et al., 2012), abortive infection systems or quorum sensing (Silpe and Bassler, 2019; Refardt et al., 2013) and prophage mediated mechanisms that prevent super-infection (Davis and Waldor, 2002). However, the impact of multiple mechanisms in the same bacterial cell has rarely been assessed to date. Furthermore, the mechanism of interaction between a *Siphoviridae* phage and *Salmonella* host are not well documented.

This study aimed to provide insights into the mechanisms of phage resistance that occur in *Salmonella* Typhimurium when phage fmb-p1 and *Salmonella* coexist. The expression of LPS biosynthetic genes, recognized virulence genes of *S.* Typhimurium in a phage resistant mutant have been determined in this work. The molecular mechanisms and strategies of how *S.* Typhimurium becomes resistant to phage fmb-p1 are analyzed and discussed.

2. Material and methods

2.1. Phage, bacterial strains and culture

Phage fmb-p1 morphologically resembles the *Siphoviridae* exhibiting a virulent life cycle (no evidence of lysogeny) that was isolated from sewage in 2014. The genome of fmb-p1 is composed of a 43,327-bp double-stranded DNA molecule with 60 open reading frames. Phage fmb-p1 lyses seven serovars of *Salmonella* (*S.* Typhimurium, *S.* Enteritidis, *S.* Anatum, *S.* Miami, *S.* Agona, *S.* Saintpaul and *S.* Paratyphi-C), and remains stable over a range of temperatures (40–75 °C), pH (4–10) and NaCl solutions (1–11%). The latent period of fmb-p1 was approximately 20 min, and the burst size was 77 ± 4 PFU/cell (Wang et al., 2017a). Phage fmb-p1 was stored in SM buffer (10 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris–HCl, pH 7.5) at −20 ℃ prior to experiment.

S. Typhimurium CMCC50115 (wild type) and *S.* Typhimurium N18 (mutant) were used in this study. *S.* Typhimurium N18 was isolated after infection of a *S.* Typhimurium culture CMCC50115 (10^9 CFU/mL) in LB broth with phage (10^2 PFU/mL), after 7 days at 25 °C. Ten single colonies were recovered from an aliquot of the culture spread on LB agar at 37 °C for 24 h. After purification of the presumptive resistant strains on LB agar, the stability of the phage resistance phenotype was verified by five consecutive sub-cultures in the presence of fmb-p1 and two consecutive sub-cultures in the absence of fmb-p1.

2.2. Characterization of S. Typhimurium N18

A fresh working culture of N18 was prepared by inoculating the stock into 50 mL LB broth (tryptone 10.00 g/L: brand oxoid, yeast extract 5.00 g/L: brand oxoid, sodium chloride 10.00 g/L, pH 7.0 ± 0.2) and incubated at 37 °C for 12 h with constant, gentle shaking (180 r/min) to obtain cell concentrations of ~10^9 CFU/mL. The culture was subcultured to LB agar and *Salmonella* Chromogenic Medium plates (Luqiao, Beijing, China) and incubated at 37 °C for 24 h. The characteristics of N18 were tested using a *Salmonella* dehydration biochemical identification kit according to the manufacturer's specifications (Luqiao, Beijing, China). The phage sensitivity of N18 was tested by the spot test method as described by Wang et al. (2017b). The efficiency of plating (EOP) was tested according to the method of Kropinski (2009). The genomic DNA (gDNA) of N18 was extracted and purified using a bacterial gDNA extraction kit (Shenggong, Shanghai, China). The concentration of gDNA was measured using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, USA). DNA samples were stored at −20 °C for PCR. The gDNA of N18 was tested using a specific primer pair for *S.* Typhimurium gene STM4494 and a specific primer pair for phage fmb-p1gene P28 (Table 1). The PCR amplification mixtures contained 12.5 μL 2 × Taq Master Mix (Vazyme Biotech, Nanjing, China), 1 μL of each primer (10 mmol/L), 1 μL gDNA (50 ng/μL) and sterile distilled water up to 25 μL. A reaction mixture with sterile distilled water instead of template DNA was used as a negative control, the gDNA of CMCC50115 and phage fmb-p1 were used as positive controls. The PCR cycling program consisted of an initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s), and a final extension step at 72 °C for 10 min. The PCR products were separated on 1% agarose gel electrophoresis and visualized using a UV transilluminator. All the experiments were performed in triplicate.

2.3. Determination of phage adsorption

Phage adsorption was determined according to the method of Kropinski (2009) with minor modifications. Briefly, a mid-log phase bacterial culture was infected with a phage suspension to achieve a multiplicity of infection (MOI) of 0.01. The mixture was allowed to adsorb for 15 min at room temperature before centrifugation at 10,000 × *g* for 2 min. The supernatant was used for the determination of the unadsorbed phage titer using the agar overlay method (Hungaro et al., 2013). The phage adsorption efficiency was calculated as following:

phage adsorption efficiency = (total phage titer − unadsorbed phage titer)/total phage titer × 100%

2.4. Determination of phage adsorption using transmission electron microscopy

Bacterial cultures (CMCC50115 or N18) were prepared by inoculating the stock into 50 mL LB broth and incubated at 37 °C for 12 h with constant, gentle shaking (180 r/min) to obtain cell concentrations of ~10^9 CFU/mL. The culture was diluted to 10^7 CFU/mL with SM buffer in a 1.5 mL tube, then a 100 μL dilution solution and 100 μL phage stock (10^{10} PFU/mL) were mixed together in a new tube and kept static for 10 min at room temperature. Negative staining method was used to image phage adsorption structures (Kropinski, 2009). Phage/host morphologies were examined using a H-7650 electron microscope (Hitachi, Japan) operated at 80 kV.

2.5. Extraction, purification and determination of LPS

Salmonella without phage were inoculated into 100 mL LB broth and incubated at 37 °C for 12 h with constant shaking (180 r/min) to obtain ~10^9 CFU/mL prior to experiment. The culture was centrifuged with Eppendorf centrifuge at 8000 × *g* at 4 °C for 10 min. The pellet was washed twice with normal saline, washed once with ddH_2O, and finally re-suspended in 10 mL ddH_2O. The cell suspension was sonicated using a Scientz-IID Ultrasonic unit (Ningboxinzhi, China) for 1 h until the solution clarified. The treated solution was added as the bulk volume with 90% phenol, churned at 68 °C for 30 min, put on ice overnight and then centrifuged with 5000 × *g* at 4 °C for 20 min. The supernatant was collected, and the phenol saturated with ddH_2O before centrifuging again. The aqueous supernatants were collected and dialyzed with flow water for 24 h, and then dialyzed with ddH_2O for 72 h (until no purple emerged using a $FeCl_3$ test). The solution was concentrated to a quarter of the original volume in a fume hood. The concentrate contained the crude LPS extract.

Table 1
The primers of PCR assay in this study.

Target genes	Coding protein	Sequence of primers (5′–3′)	Fragment sizes (bp)	Reference
16 s rRNA	16S ribosomal RNA	GTTACCCGCAGAAGAAGCAC CACATCCGACTTGACAGACC	123	Zheng et al. (2011)
lpxA	UDP-N-acetylglucosamine acyltransferase	AAGCGTCACCATTCATCGTG GATGAACTGCCGTCATACCG	200	This study
lpxB	lipid-A-disaccharide synthase	TGGGCATTGTTGAAGTGCTC AACGGACGGGCTGACATAAT	188	This study
lpdT	LPS-assembly protein LptD	AACGTCTGGGAAGGCGATTA GATGACTTCACTCCCCACCA	165	This study
rfaG	alpha-1,3-glucosyltransferase	GATGACTGAACCATTCCGCC TAATATCCGCGGCCTTCTCC	152	This study
rfaI	lipopolysaccharide 1,3-galactosyltransferase	CTGAAGGCGAGTTGGAATGG GCGCTGTACTACCTCTGGAT	173	This study
rfaJ	alpha-1,2-glucosyltransferase	GCTGCGGTCGTAAAAGATGT TCAGCCTCTTTACCTGCCAA	176	This study
rfaK	lipopolysaccharide 1,2-N-acetylglucosaminetransferase	CACCGTTCTCTTGTATGCCG ATCTGGTCAGGAGATTGCCC	231	This study
rfaL	O-antigen ligase	ATGGCGCTATCATCAGGGAA GCCAGCAGAAAACCGGTAAT	172	This study
rfbA	glucose-1-phosphate thymidylyltransferase	ACGGTGTGGTTGAGTTTGAC CCCCATCATAGCGACAGACA	221	This study
kdtA	3-deoxy-D-manno-octulosonic-acid transferase	ATTCCGGTACTGATGGGTCC CGCGCCCTGATTTTGATACA	195	This study
msbA	lipid A export ATP-binding /permease	CTGGTTGTTTTAGCGCCGAT TGACGAGGCAGAGACCATTT	237	This study
pagP	lipid A palmitoyltransferase PagP	AATTTTCGCCTTGGACTGGG GCATCCAGGCGAAATAGACG	175	This study
wzzB	polysaccharide antigen chain length regulator	ACAGTTATGGCGTGGGAAGA GTTGAGCGCGTTGGTATAGG	160	This study
lexA	LexA repressor	CGCGAGGTATCCGTCTGTTA CGTTTTATGTACCGCCAGCA	221	This study
recA	recombination protein RecA	GATATCCGTCGTATTGGCGC CCGTTGTAGCTGTACCATGC	215	This study
mgtC	Mg(2 +) transport ATPase protein C (Gall et al., 2018)	TTCTGAGCTCCATGACGACA AGAATAATGATCGTCGCCGC	217	This study
invA	attachment/invasion protein (Ye et al., 2018)	ACCGTGGTCCAGTTTATCGT GCTTTCCCTTTCCAGTACGC	177	This study
sopB	Type III secretion system effector protein (Truong et al., 2018)	GCTCGCCCGGAAATTATTGT GGTCCGCTTTAACTTTGGCT	208	This study
spvC	*Salmonella* plasmid virulence protein (Mazurkiewicz et al., 2008)	ATTTGCCGGTGACAAGTTCC GGAGAAACGACGCACTGTAC	224	This study
sseL	Type III secretion system effector protein, deubiquitinase (Geng et al., 2019)	GCCCCTTCCAGATTACTTTATATG TGCTTAATATATTTTCTTTGGTGG	268	This study
Cas1	CRISPR/Cas system-associated protein Cas1	GCAAAGCTGGCGTTAGATGA GATCCTTCAATACCGCGCAG	122	This study
hsdM	type I restriction enzyme methylase	TGTATGACCTGCGGACCAAT AAGCTCCACTCCCCTTCTTC	136	This study
hsdR	endonuclease R	AGCGGTAGATAAGCAGGTCC ATTGAAAATCTCGGCGCTCC	124	This study
hsdS	type I restriction enzyme specificity protein	GTGTTCCTGTCCCACCTCTT TGGGATTTGCTCAAGACGTG	104	This study
mod	type III restriction-modification system methyltransferase	CCGAAACCGACCGCATTATT TTCATTCAGCGCCATTACCG	120	This study
res	type III restriction-modification system DNA helicase	ATTCGTGACAATAAGCCCGC CTACGCTCGAATTGCAGCAT	119	This study
STM4494	sugar/spermidine/putrescine ABC transporter ATPase	CAACTAAAAGAGAAATACCCACAGG TACCGTAAACAGCATAATCAGCAC	534	Zhai (2015)
P1	amidase	ACATCAAGAGGGAGGAACGG TGCGCTCAATCATCCGACTA	193	This study
P11	putative endolysin	TGCCAAACCGAAACATCAGT GCCGTAGTGCCCATAACCTA	125	This study
P28	tailspike protein	GTTTCGGCATGGATGGGAAA AGTGAATACCGTTTCGTGCG	101	This study

DNase and RNase were added into the LPS crude extract to final concentrations of 100 μg/mL, respectively. The suspension was then incubated at 37 °C for 4 h. After this, proteinase K was added into the suspension to final concentrations of 100 μg/mL, and then incubated at 37 °C for 3 h. The suspension was then heated at 100 °C for 10 min, centrifuged at 5000 × *g* for 30 min after cooling to room temperature. The supernatant was added to two-fold volume of acetone and kept overnight. The solution was centrifuged with 10,000 × *g* for 10 min, and the precipitate dried in a fume hood. The dried precipitate represents purified LPS.

The purified LPS was dissolved in ddH_2O for further detection. The concentration of LPS solution was determined using phenol-sulfuric acid method (Gabriela et al., 2003) with slight modifications as indicated below. The LPS solution (1.0 mL) and the phenol solution (0.5 mL) were added to screw cap tubes (13 × 150 mm), which were capped and vortex-stirred. Then 3.0 mL of concentrated sulfuric acid was added slowly down the side of the tube. The tubes were then closed, vortex-stirred for 5 s and incubated at 100 °C for 20 min, and

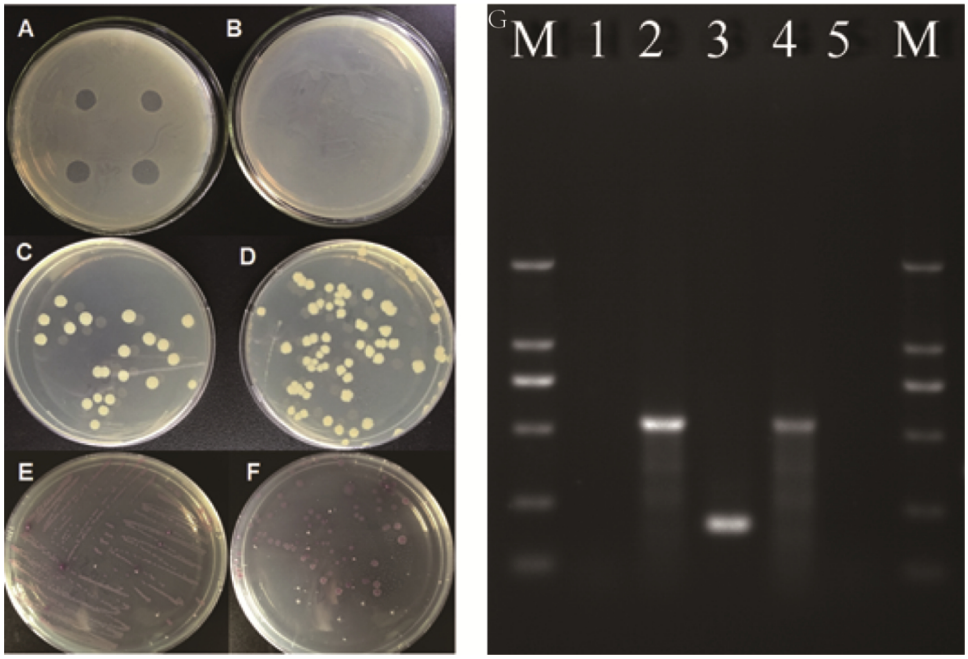

Fig. 1. The characterization of *S.* Typhimurium N18.
(A): Phage fmb-p1 spotted on *S.* Typhimurium CMCC50115 on a LB top-agar plate; (B): Phage fmb-p1 spotted on *S.* Typhimurium N18 and on a LB top-agar plate; (C): *S.* Typhimurium CMCC50115 colony morphology on LB-agar plate; (D): *S.* Typhimurium N18 colony morphology on LB-agar plate; (E): *S.* Typhimurium CMCC50115 on *Salmonella* Chromogenic Medium; (F): *S.* Typhimurium N18 on *Salmonella* Chromogenic Medium; (G): Agarose gel electrophoresis of PCR amplification products. M : DS2000 DNA marker, 1: negative control, 2: product of *S.* Typhimurium CMCC50115 gDNA (gene STM4494), 3: product of phage fmb-p1 gDNA (phage gene P28), 4: product of N18 gDNA (gene STM4494), 5: product of N18 gDNA (phage gene P28).

then cooled to room temperature with flow water, before reading the absorbance at 490 nm using distilled water as blank in a UV-2600 UV/Visible spectrophotometer (Shimadzu, Japan).

2.6. LPS as a decoy for phage adsorption

The extract solutions of *Salmonella* LPS were diluted to the same concentration (400 μg/mL) with sterile distilled water prior to the experiment. An overnight *Salmonella* culture of 100 μL (10^5 CFU/mL) was added into 6 sterilized tubes with cap (20 mL). Then, either 100 μL or 400 μL of LPS solution from either wild type (50115LPS) or mutant (N18LPS) were added to the first four of the tubes. Fresh LB medium was added to each of the six tubes to a total volume of 9.9 mL. Finally, 100 μL of phage (10^{11} PFU/mL) was added into 5 of the above tubes, and 100 μL of SM buffer was added to the last tube as a negative control. All tubes were shaken and gently mixed and incubated at room temperature for 15 min. The *Salmonella* count of each test tube was determined after incubation at 37 °C for 4 h. All experiments were performed in triplicate.

2.7. Gene expression analysis

To detect the gene expression of *Salmonella* exposed to phage, total RNA was extracted immediately after phage addition, after 10 min and 24 h of incubation. *Salmonella* infection was initiated by adding 100 μL of phage (10^9 PFU/mL) and 100 μL of overnight cultures of *Salmonella* N18 and CMCC50115 (10^9 CFU/mL) to 100 mL of LB broth at 37 °C. All extractions were performed in triplicate.

Total RNA was isolated from *Salmonella* cells using a Bacterial RNA Kit (Omega Bio-tek, USA) according to the manufacturer's instructions, and then each RNA sample was treated with recombinant DNase I (Takara, Japan). The DNase-treated RNA was reverse-transcribed using the First strand cDNA synthesis kit (Vazyme, China), according to manufacturer's specifications (5 min at 25 °C, 15 min at 50 °C, 5 min at 85 °C). The concentrations of cDNA were determined using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The cDNA samples were then divided into small volumes and stored at −20 °C until use.

Real-time PCR assays were prepared as follows (20 μL final volume per sample): 10 μL of SYBR® Green Master Mix (High ROX Premixed, Vazyme), 0.4 μL of each primer (10 mM, Table 1), 2 μL of cDNA template (100 ng/uL), and 7.2 μL of RNase-free water. A thermocycler (ABI StepOnePlus™ system) was programmed as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 10 s at 95 °C, 30 s at 60 °C, with a single fluorescence measurement; a final melting curve program of 15 s at 95 °C and 1 min at 60 °C, followed by 15 s at 95 °C. A cDNA template-free negative control was included in each run to confirm that there was no background contamination, and a housekeeping gene for 16S rRNA was used as an endogenous control since it is constitutively expressed under a wide range of conditions. The mean values of curve thresholds (Ct) were considered to calculate the relative expression of target genes by the comparative method using the $2^{-\Delta\Delta CT}$ equation (Livak and Schmittgen, 2001). PCR efficiency was determined and melting curve analysis was performed to ensure that a single gene product was amplified for each target gene prior to experiment.

2.8. Statistical analysis

Statistical significances were determined by analysis of variance (ANOVA) in SAS, and Duncan's new-multiple range test of SPSS 13.0 (SPSS Inc, Chicago, IL, USA). The level of statistical significance was $p < 0.05$.

3. Results

3.1. Characterization of S. Typhimurium N18

S. Typhimurium N18 was isolated and purified post-infection of *S.* Typhimurium CMCC50115 with phage fmb-p1. Plaque assays indicated strain N18 was resistant to phage fmb-p1 (see Fig. 1B compared to wild type 1A). The EOP of phage fmb-p1 on N18 plate was 10^{-4}, this result indicated that N18 was impaired sensitivity and not completely resistant to phage fmb-p1. Fig. 1D shows the N18 mutant produces an atypical colony morphology with irregular borders on LB solid medium compared to the regular round colonies of wild type *S.* Typhimurium CMCC50115 (Fig. 1C). However, N18 retains the diagnostic purple color on *Salmonella* chromogenic medium plate as *S.* Typhimurium CMCC50115 (Fig. 1E, F) and the same biochemical profile as the wild type. Genomic DNA of N18 could be PCR amplified with specific primers designed on the *S.* Typhimurium CMCC50115 strain but did not

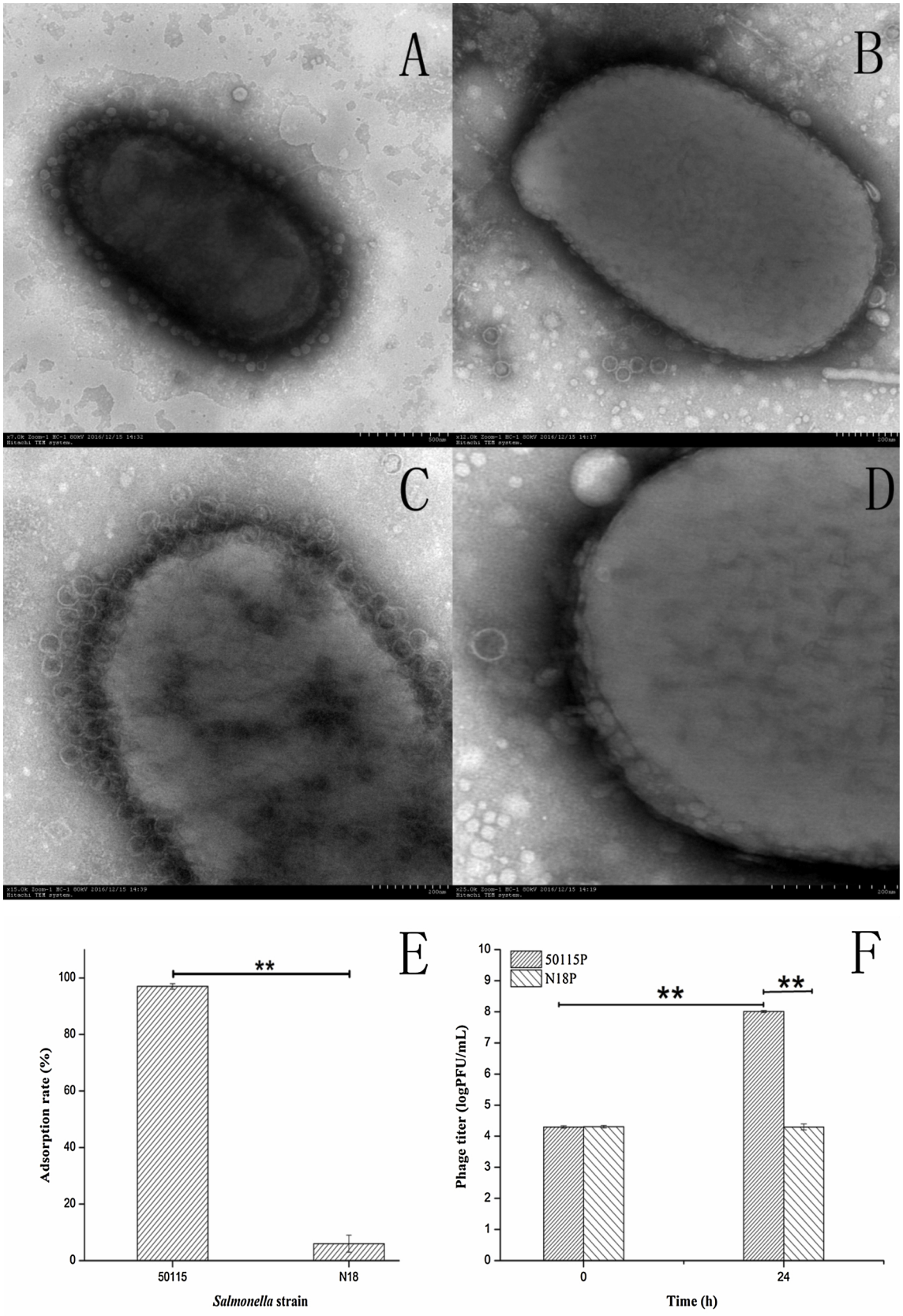

Fig. 2. Adsorption rate and transmission electron micrographs of phage fmb-p1 binding *S*. Typhimurium CMCC50115 and N18.
(A and C): *S*. Typhimurium CMCC50115 and phage fmb-p1; (B and D): *S*. Typhimurium N18 and phage fmb-p1; (E): The adsorption efficiency of phage fmb-p1 to *S*. Typhimurium CMCC50115 and *S*. Typhimurium N18; (F): Replication of phage fmb-p1 at 37 °C over 24 h with either *S*. Typhimurium CMCC50115 or *S*. Typhimurium N18. * indicates significant differences between treatments, $p < 0.05$, ** indicates significant differences between treatments, $p < 0.01$.

Table 2
LPS yields from *S.* Typhimurium CMCC50115 and N18.

LPS	Sample volume	LPS mass/mg
50115LPS	100 mL (5.0×10^9CFU/mL)	4.67 ± 0.26
N18LPS	100 mL (5.0×10^9 CFU/mL)	0.40 ± 0.12

produce amplicons with fmb-p1 phage specific primers (Fig. 1G). These results suggest that N18 is a phage resistant non-lysogenic derivative of *S.* Typhimurium CMCC50115.

3.2. Differences in phage adsorption between N18 and CMCC50115

Transmission electron microscopy showed that phages gathered in an orderly fashion upon encountering *S.* Typhimurium CMCC50115 cells with phage tails observed near the host cell (Fig. 2A, C). However, there were only a few non-adherent phage particles scattered around N18 cells, and even fewer adsorbed with tails orientated towards the N18 cell (Fig. 2B, D). These images suggest that the primary interaction of phage fmb-p1 with N18 has been compromised. The phage adsorption rate to *S.* Typhimurium N18 was reduced in comparison to *S.* Typhimurium CMCC50115, with only 6% of the applied titer adsorbed by N18 as against 97% for CMCC50115 (Fig. 2E). Phage replication was not evident after 24 h incubation with N18, while the phage titer of wild type *S.* Typhimurium CMCC50115 increased by 4 $\log_{10}$ PFU/mL (Fig. 2F). As shown in Table 2, the LPS content of N18 was determined as only 10% of that for *S.* Typhimurium CMCC50115, which may account for the lack of adsorption.

Fig. 3 shows the ability of LPS to act as decoy for phage fmb-p1 binding to *S.* Typhimurium CMCC50115. The phage adsorption and infection were interfered with LPS, and the degree of the fall in the viable count was reduced. In the absence of LPS, the viable count of *S.* Typhimurium CMCC50115 falls by $\log_{10}$ 3.3 CFU/mL post exposure to 10^{10} PFU/mL fmb-p1. LPS extracted from wild type *S.* Typhimurium acts an effective decoy, the viable count of *S.* Typhimurium CMCC50115 falls by 0.9 or 0.3 $\log_{10}$ CFU/mL post exposure to 10^{10} PFU/mL fmb-p1 cultures containing respectively low (100 µL) or high (400 µL) concentrations of 50115LPS. The N18 LPS extract was not as effective as LPS extracted from wild type *S.* Typhimurium, the *Salmonella* count reduces by 2.1 or 1.5 $\log_{10}$ CFU/mL in phage cultures containing respectively either low (100 µL) or high (400 µL) concentrations of N18LPS.

3.3. Effect of phage on Salmonella LPS synthesis gene expression

The biosynthetic pathway and export mechanisms of LPS are common to most Gram-negative bacteria (Wang and Quinn, 2010). The expression of the majority of LPS-related synthesis genes in the absence of phage was not significantly different between the wild type strain and phage-resistant isolate N18 in LB broth. However, there were four key exceptions to this: *lpxA, rfaL, pagP* and *wzzB* that show reduced expression in N18 (Fig. 4). Fig. 5A shows 13 LPS synthesis genes of *S.* Typhimurium N18 were significantly down-regulated ($p < 0.01$) upon exposure to phage with most showing down-regulation greater than 3 $\log_2$ fold, compared to the control. Genes *lpxA*, *lpxB*, *pagP* and *msbA* have key functions in lipid A biosynthesis. These four genes of N18 were down-regulated (3.9-, 3.7-, 3.4-, and 3.8- $\log_2$ fold, respectively), suggesting that the lipid A biosynthesis was reduced in phage-exposed *Salmonella*. Genes *rfaG*, *rfaI*, *rfaJ*, *rfaK* and *rfaL* play an important role in core oligosaccharide biosynthesis. These five genes of N18 strain were also down-regulated by phage (3.7-, 3.6-, 3.8-, 3.5-, and 3.7- $\log_2$ fold, respectively), indicating that the core oligosaccharides biosynthesis was similarly affected. Genes *rfbA*, *kdtA*, *wzzB* and *lpdT* are the major genes responsible for O-antigen biosynthesis. These four genes of N18 strain were down-regulated by phage (3.8-, 3.8-, 3.6-, and 3.4- $\log_2$ fold, respectively), indicating that the O-antigens biosynthesis were modified in *Salmonella*. These results were consistent with the reduction of LPS content observed for N18 (Table 2). However, seven genes of *S.* Typhimurium CMCC50115 were down-regulated by phage fmb-p1 exposure. Under these circumstances (10 min post-infection at MOI = 1) *S.* Typhimurium CMCC50115 is phage infected with demonstrable transcription of the representative phage genes *p1, p11* and *p28*. In contrast, phage gene transcription in the phage resistant mutant N18 at 10 min was undetectable but was evident by 24 h; accordingly, the differences recorded for *S.* Typhimurium CMCC50115 were significantly less than that determined for N18.

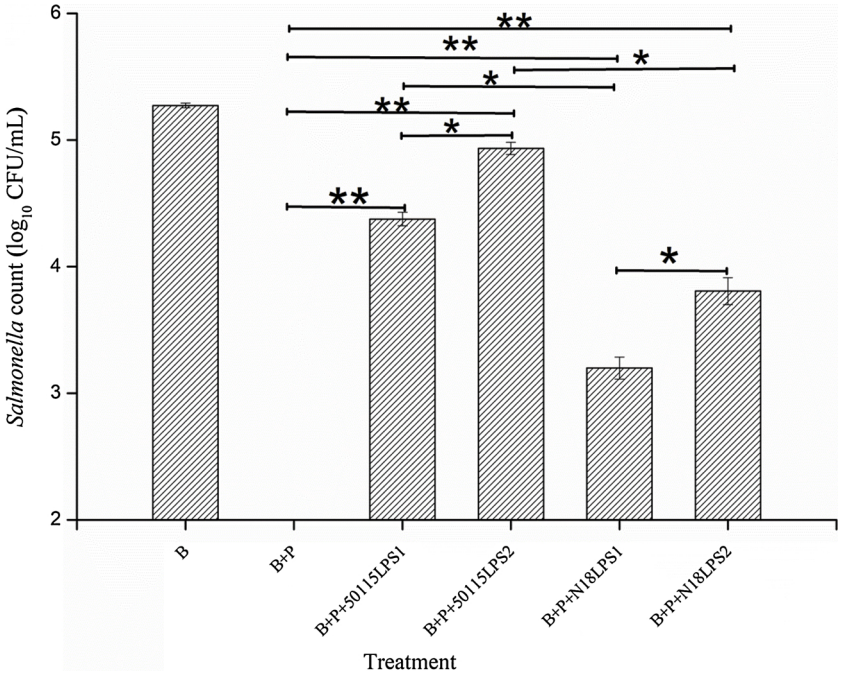

Fig. 3. Protective effects of *Salmonella* lipopolysaccharide on the lysis of *S.* Typhimurium CMCC50115.
The extract solutions of *Salmonella* LPS were diluted to the same concentration (400 µg/mL) with sterile distilled water prior to the experiment. *S.* Typhimurium CMCC50115 cells were incubated at 37 °C in LB broth for 4 h with the following: B is control bacteria with SM; B + P bacteria treated with phage fmb-p1; B + P + 50115LPS1 bacteria treated with phage fmb-p1 and 100 µL *S.* Typhimurium CMCC50115 LPS; B + P + 50115LPS2 bacteria treated by phage fmb-p1 and 400 µL *S.* Typhimurium CMCC50115 LPS; B + P + N18LPS1 bacteria treated by phage fmb-p1 and 100 µL *S.* Typhimurium N18LPS; B + P + N18LPS2 bacteria treated by phage fmb-p1 and 400 µL *S.* Typhimurium N18LPS. * indicates significant differences between treatments, $p < 0.05$, ** indicates significant differences between treatments, $p < 0.01$.

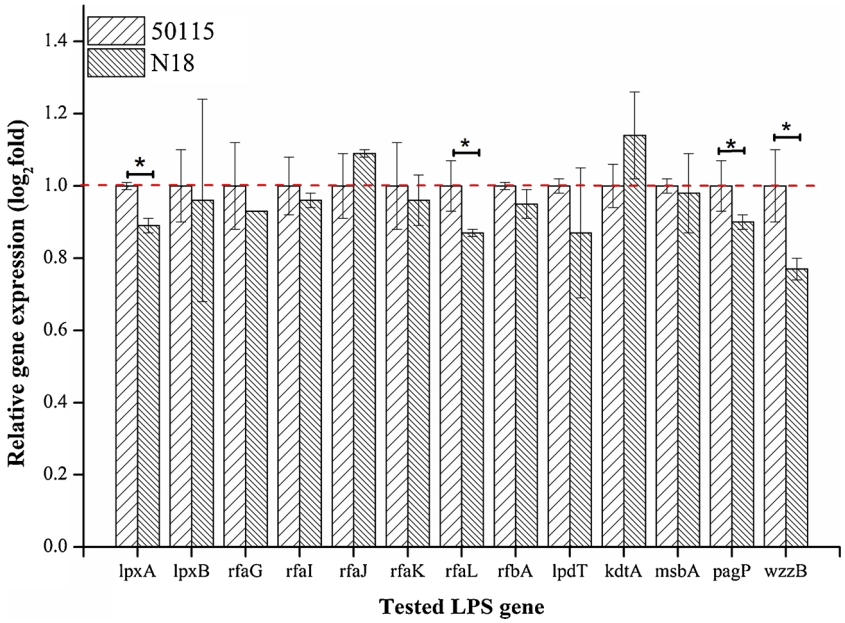

Fig. 4. The relative expression of LPS synthesis genes.
50115: *S.* Typhimurium CMCC50115, N18: *S.* Typhimurium N18. * indicates significant differences between treatments, $p < 0.05$.

3.4. Effect of phage on the expression of Salmonella virulence genes

The virulence of *Salmonella* is of general concern as it represents a serious threat to the health of domestic animals and humans worldwide. In the present study, expression of five host virulence genes (*invA, sseL, mgtC, sopB* and *spvC*) was tested during phage infection. Fig. 5B shows the expression of five virulence genes to be significantly down-regulated by phage in *S.* Typhimurium CMCC50115 and N18.

3.5. Effect of phage on gene expression of phage resistance in Salmonella

Phage infection up-regulated gene expression of *lexA, recA* and *cas1* in *S.* Typhimurium CMCC50115 by 1.5-, 1.3- and 2.9-$\log_2$ fold, respectively (Fig. 6A). These changes were greater than the corresponding values observed for the phage treatment of N18 (0.9-, 0.7-, and 1.4-$\log_2$ fold, respectively). As shown in Fig. 6B, genes *hsdR, hsdM* and *hsdS* comprising the type I RM system were up-regulated by phage in strain N18 (1.7-, 1.1-, and 1.4-$\log_2$ fold, respectively). In contrast, *hsdM* was up-regulated by phage in *S.* Typhimurium CMCC50115, and *hsdS* was slightly down-regulated after phage infection. Two genes representing the type III RM system were significantly up-regulated ($p < 0.05$) by phage in both *S.* Typhimurium CMCC50115 and N18 (Fig. 6C).

4. Discussion

Recently, there has been renewed interest in phage-bacteria interactions as phages have the potential to treat multi-drug resistant bacteria in medicine and agriculture. However, there are still obstacles to phage application, notably phage resistance. Phage resistance mechanisms include the inhibition of phage attachment to cell surface receptors, cleavage of the invading phage genome, replication interference exerted by bacterial CRISPR–Cas systems and even the inductive abortion of phage infection (Brockhurst et al., 2017; Labrie et al., 2010; Samson et al., 2013).

The *S.* Typhimurium CMCC50115 derivative N18 was largely but not completely resistant to infection by phage fmb-p1. Phage gene transcription was detected in N18 at 24 h post treatment albeit at low levels. Low-level transcription may have arisen as a consequence of mutant instability permitting phage access to a subpopulation of bacteria, or that a genetic subpopulation of phage can achieve entry and initiate transcription or simply that sufficient phage host interactions can give rise to infection by stochastic process. These interactions do not give rise to an increase in phage titer either because the replication does not complete or that the subpopulations supporting phage infection are insufficient to increase phage titer against phage loss due to inactivation. Among these reasons, expression of phage genes in the mutant strain later than those in the wild type strain is most probably caused by a reduced production of LPS by N18, and therefore a reduced availability of the receptor of the phage for adsorption.

The first barrier to phage replication in N18 is poor adsorption. Transmission electron microscopy confirmed that adsorption of fmb-p1 to the surface of N18 bacteria is disordered compared to the regular engagement observed for the wild type strain. LPS is a significant structure in this process as demonstrated by the concentration dependent decoy effect of LPS extracts to interfere phage infection of sensitive *S.* Typhimurium CMCC50115. *S.* Typhimurium N18 yielded only 10% of the extractable LPS of wild type. *S.* Typhimurium cells may decrease the density of LPS or alter the structure of the receptor critical for phage adsorption. This mechanism could be a key factor and conducive for the survival of bacteria along with phage present in the same habitat. LPS extracts of wild type *S.* Typhimurium and phage-resistant mutant can interfere with the infection and lysis of *Salmonella* by phage. The LPS of N18 is not as effective as wild type LPS in phage adsorption but is still capable of interfering with phage adsorption and lysis, suggesting that the essential structures for LPS with phage interaction are present but are limited due low overall LPS content and low specificity in the phage-resistant *Salmonella*. We also observed down-regulation of the LPS synthesis genes upon exposure to phage. These data imply a host adaptive response to phage infection that was most notable in the phage resistant *S.* Typhimurium, likely because the host was not wholly committed to phage transcription and the shifts in host metabolism that support replication. These data do not rule out external sensor signaling as a result of phage host surface interactions or early diffusible lysis products.

Bacteria have evolved a range of barriers to prevent phage adsorption, such as the blocking of phage receptors, the production of extracellular matrix and the production of competitive inhibitors (Brockhurst et al., 2017; Labrie et al., 2010; Samson et al., 2013). For example, *Escherichia coli* phage T5, produces a lipoprotein (Llp) that blocks its own receptor, ferrichrome-iron receptor (FhuA) (Pedruzzi

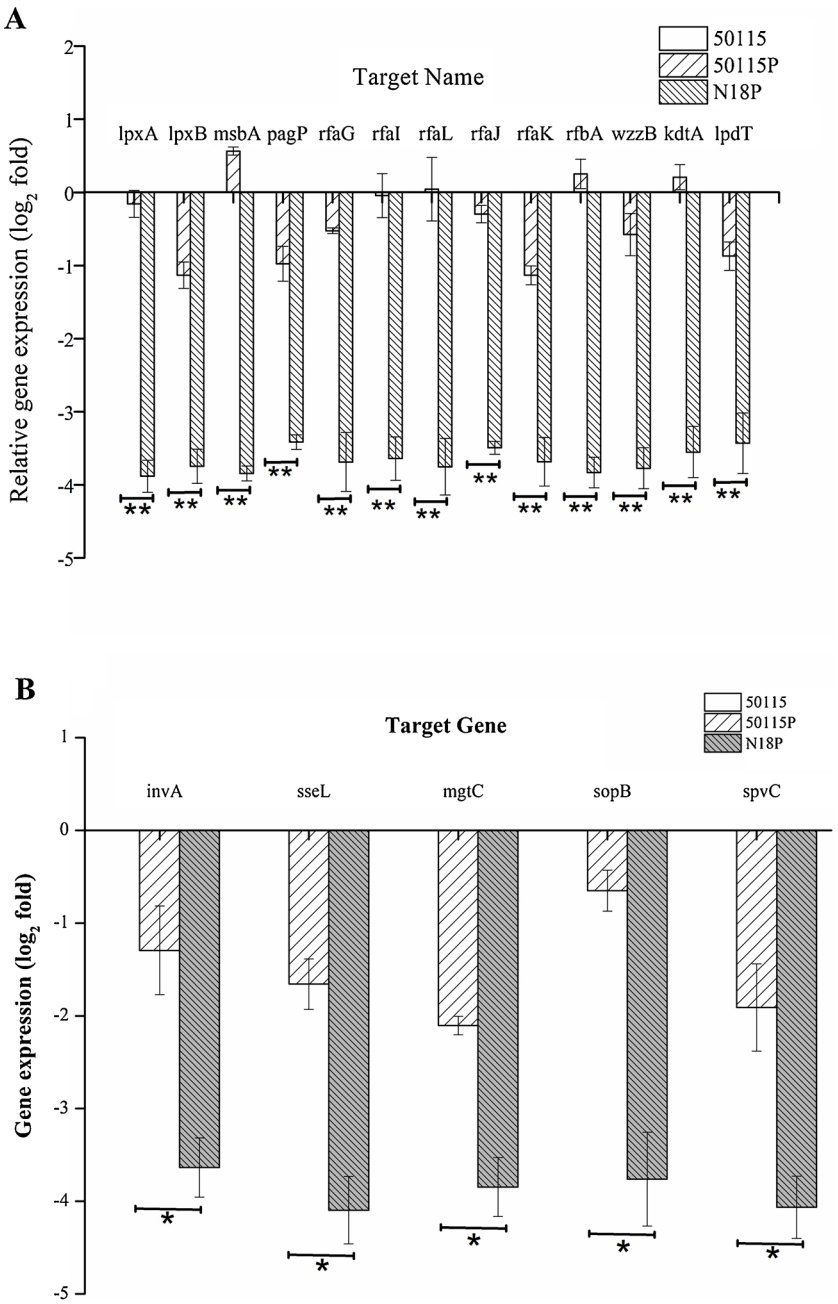

Fig. 5. The relative gene expression of LPS synthesis and virulence associated genes in *Salmonella* treated with phage fmb-p1.
(A) The response of LPS synthesis genes to phage relative to non-treated *S*. Typhimurium CMCC50115. 50115 P: *S*. Typhimurium CMCC50115 treated by phage fmb-p1; N18P: *S*. Typhimurium N18 treated by phage fmb-p1. (B) The relative gene expression of virulence genes of *Salmonella* strains treated by phage fmb-p1. 50115 P: *S*. Typhimurium CMCC50115 treated by phage fmb-p1, N18P: *S*. Typhimurium N18 treated by phage fmb-p1. ** indicates significant differences between treatments, $p < 0.01$.

et al., 1998). Phage ΦV10 possesses an O-acetyltransferase that modifies the O157 antigen of *E. coli* O157:H7 to block adsorption of ΦV10 and phages with similar specificities (Perry et al., 2009). *Salmonella* phage P22, which recognizes the O antigen, has also evolved to specifically recognize polysaccharides such as O antigens (Schmidt et al., 2016). The antimicrobial molecule microcin J25 uses FhuA as a receptor and can outcompete phage T5 for binding to FhuA (Destoumieux-Garzón et al., 2005). The changes we observed in LPS of *Salmonella* CMCC50115 upon phage infection show broad similarity to mechanisms adopted to evade phage infection.

High concentrations of LPS can induce fever, increase heart rate, and lead to septic shock and death following organ failure (Wang and Quinn, 2010). The content of LPS in N18 was much lower than that of the wild type *S.* Typhimurium strain. The selection of phage resistant types with reduced LPS content could also reduce the risk associated with endotoxicity of *Salmonella*, and given that LPS is a common feature of many Gram-negative bacteria, this may be true of other phage therapy targets (Tran and Whitfield, 2009). Attenuated virulence has been observed for phage resistant *Salmonella* that lack the O-polysaccharide chain from LPS (Santander and Robeson, 2007). However, this is in contrast to reports of temperate phage that can add to the pathogenicity or virulence of their hosts, such as *Vibrio cholera* phage CTXΦ and *E. coli* phages STX (Waldor and Friedman, 2005).

The virulence *Salmonella* requires the expression of *Salmonella* pathogenicity islands (SPIs), 16 variants of which are currently described (Sterzenbach et al., 2013). Some of these SPIs are conserved throughout

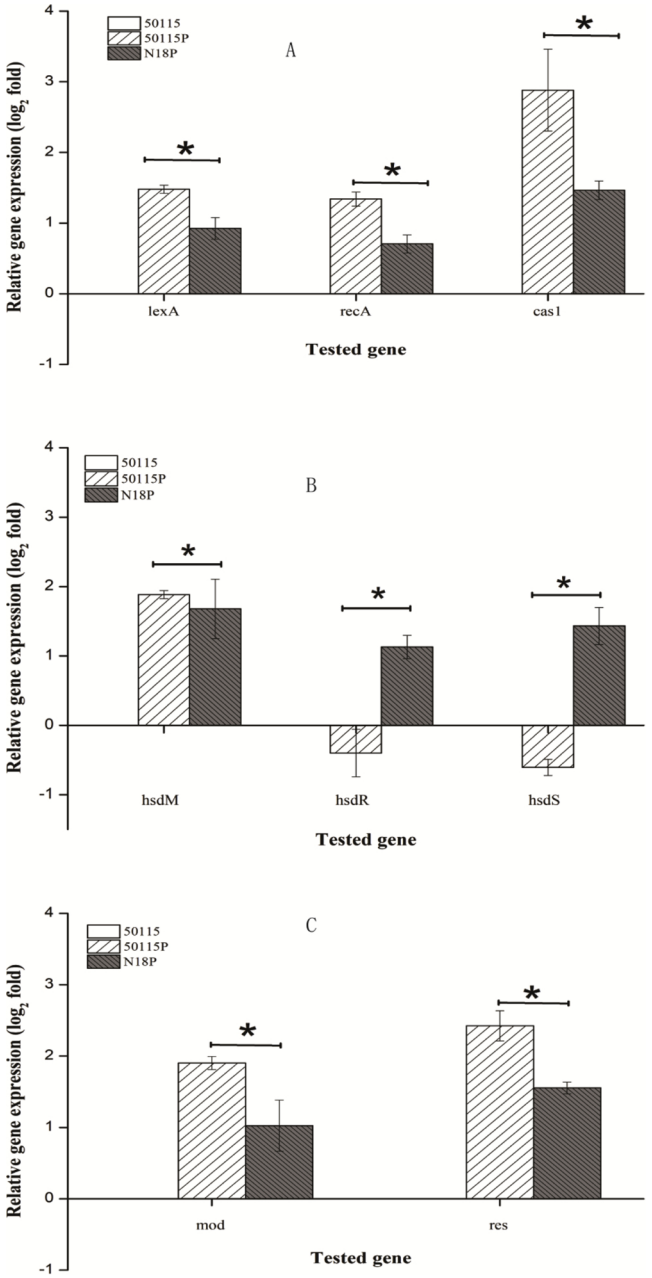

Fig. 6. The relative gene expression of phage resistance associated genes of *Salmonella* treated with phage fmb-p1.
The response of the phage resistance associated genes to phage relative to non-treated S. Typhimurium CMCC50115 are recorded. 50115 P indicates *S.* Typhimurium CMCC50115 treated by phage fmb-p1. N18 P indicates *S.* Typhimurium N18 treated by phage fmb-p1. A: SOS response genes, B: RM-I genes, C: RM-III genes. * indicates significant differences between treatments, $p < 0.05$.

the genus, and others serovar specific (Nieto et al., 2016). The *invA* gene is essential for *Salmonella* to enter cultured epithelial cells and belongs to SPI-1 (Ye et al., 2018), whereas *sseL* encodes a *Salmonella* deubiquitinase required for macrophage killing and virulence which belongs to SPI-2 (Geng et al., 2019). SPI-1 and SPI-2 encode effectors of the type III secretion systems (T3SS) that confer the major virulence traits of *Samonella* enterica (*S.* enterica), such as invasion, intracellular survival and proliferation, and enteropathogenesis (Majewski et al., 2019). The *mgtC* gene encodes a virulence factor of *Salmonella* in response to low Mg^{2+} and pH levels and belongs to SPI-3. *Salmonella* require *mgtC* to adapt to the nutritional limitations of the intraphagosomal habitat, and down regulation would adversely affect phagosomal survival (Gall et al., 2018). Gene *sopB* encodes proteins to hydrolyze inositol phosphatase and belongs to SPI-5 (Truong et al., 2018). Down-regulation of *sopB* could reduce the action of inositol phosphatase involved in triggering fluid secretion and diarrhoeal symptoms. Gene *spvC* is present on the virulence plasmid of *Salmonella* and is a T3SS effector, and which is closely related to bacterial adhesion, colonization and serum resistance factors (Mazurkiewicz et al., 2008). Down-regulation of *spvC* would reduce the capacity of *Salmonella* to cause disease. A phage resistant *Salmonella* strain called Salp572φ1R lacks the O-polysaccharide from LPS, and expression of several genes related to virulence was under-expressed. Moreover, Salp572φ1R was avirulent in mice. These examples suggest that modifications in LPS can produce phage resistance and impair virulence (Capparelli et al., 2010). In this study, five virulent genes were shown to be down-regulated by phage in *S.* Typhimurium N18.

When the first defensive line is broken, bacteria maintain an arsenal of defense mechanisms against the phage. Gene *lexA* encodes protein binding to its specific recognition motif in the promoter region of SOS genes. Gene *recA* encodes RecA protein that promotes the autocatalytic cleavage of LexA repressor (Jaktaji and Pasand, 2016). The relative gene expression level of *recA* has clearly increased in both *Salmonella* strains by phage. The SOS genes of *Salmonella* were induced by gene *recA* encoding products. This mechanism enables cell survival in the presence of extensive DNA damage. Gene *Cas1* encodes CRISPR/Cas system-associated protein Cas1, which targets invasive nucleic acid in the host cell for degradation (Silas et al., 2016). The increase of gene expression level of *Cas1* indicated that *Salmonella* cell may increase its capacity to degrade of exogenous nucleic acids during phage infection. The type I restriction-modification (RM) systems consisting of the gene products *hsdR*, *hsdM* and *hsdS* cleaves nonmethylated DNA randomly at a remote site from the recognition sequence determined by the specificity subunit. This could enable the bacterium to distinguish between its own (methylated) DNA and incoming non-methylated DNA (Roer et al., 2015). The type III RM systems, consisting of the gene products Res and Mod, hemimethylate the DNA and cleave DNA at specific sites (Rao et al., 2014). Higher gene expression of type I RM and type III RM systems in phage treated strains could be part of a stress response or a general adaptation to phage interaction.

5. Conclusion

While bacterial strains often contain multiple antiphage barriers, the conjunction of these mechanisms in a single host have rarely been assessed. This study has shown that more than one phage resistance mechanisms of *S.* Typhimurium can function together in a single strain, including the prevention of phage adsorption, the prevention phage DNA entry, awakening of the SOS system, and the targeting of phage nucleic acids. Furthermore, this study found that virulent phage fmb-p1 could decrease the content of LPS and the potential toxicity of *Salmonella* Typhimurium. These findings will assist in the development of phage biocontrol strategies for *Salmonella* when responses to multi-resistant bacteria are required.

Acknowledgements

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