*Article*

# Identification of The Role of O-antigen of Nonmucoid *Klebsiella pneumoniae* During Bacteriophage NJS1 Infection

**Guijuan Hao [1], Yuanqi Jia [1], Chaoqun Yuan [1], Ming liu [1], Suqin Zhao [1], Haijian Zhou [2], Shuhong Sun [3], Hui Wang [1,*]**

[1] Department of Microbiology, Nanjing Agricultural University, Nanjing 210095, China; muyue807@126.com (G.H.); 18975658870@163.com (Y.J.); 2018116079@njau.edu.cn (C.Y.); 2017216023@njau.edu.cn (M.L.); 2019116077@njau.edu.cn (S.Z.); wanghui@njau.edu.cn (H.W.)

[2] State Key Laboratory for Infectious Disease Prevention and Control, Chinese Center for Disease Control and Prevention, Beijing 102206, China; zhouhaijian@icdc.cn (H.Z.)

[3] College of Animal Science and Technology, Shandong Agricultural University, Taian, Shandong 271018, China; sunshuhong@sdau.edu.cn (S.S.)

* Correspondence: wanghui@njau.edu.cn; Tel.: +86-25-8439-6645 (H.W.)

**Abstract:** The use of bacteriophage is reemerging as a tool for combatting multi-drug resistant bacterial infections. In our previous study, we showed that colistin resistant carbapenem-resistant *Klebsiella pneumoniae* (Col$^R$-CRKP) is more susceptible to killing by lytic tailed phages, including ΦNJS1 specific for nonmucoid *K. pneumoniae*. Although we demonstrated that alteration on surface charges of Col$^R$-CRKP promotes phage adherence and infection, the receptor for ΦNJS1 was still unknown. In current study, we identified O-antigen was involved in the reversible adsorption, and outer membrane protein (OMP) FepA may be served as one of the irreversible receptors for ΦNJS1. We firstly found accelerated reversible phage adsorption to Col$^R$-CRKP cells, and that periodate treatment of bacteria inhibited the phage binding, indicating LPS may be involved in phage reversible adsorption. ΦNJS1-resistant bacterial mutants screening revealed that mutants in Δ*wecG*(mTn5) and Δ*wecA*(mTn5), two genes responsible for LPS biosynthesis, affected phage adsorption capacity and phage infectivity. The loss of *wzyE* encoding O-antigen polymerase showed no significant difference in phage adsorption but increased phage infectivity, suggesting the long chain length of O-antigen may also be a barrier for bacteriophage infection. Among four OMP mutants including Δ*fepA*, Δ*fhuA*, Δ*ompA* and Δ*ompC*, only Δ*fepA* slowed phage lysis rate, suggesting FepA may be as one of irreversible receptors for ΦNJS1. The results are helpful to better understand why Col$^R$-CRKP sensitizes phage infection and to combat multi-drug resistant *K. pneumoniae* infections in the future.

**Keywords:** *Klebsiella pneumoniae*; nonmucoid; O-antigen; outer membrane protein; phage receptor

## 1. Introduction

*Klebsiella pneumoniae* (*Kp*), a Gram-negative bacillus of the Enterobacteriaceae family, is a leading cause of nosocomial infections globally, such as pneumonia, meningitis, and liver abscess. The emergence of *K. pneumoniae* strains that have acquired additional genetic traits and become either hypervirulent or antibiotic resistant has raised concerns [1]. Polymyxins (polymyxin B and colistin) are generally used as a last resort for the treatment of severe infections caused by multi-drug resistant (MDR) *K. pneumoniae* [2]. However, polymyxins resistance have been increasing

quickly between different countries and bacterial species especially since the discovery of the first plasmid-borne polymyxin resistance gene *mcr-1* [3, 4]. Therefore, new strategies are urgently required to reverse colistin resistance in Gram-negative pathogens.

A prominent way among these alternatives is phage therapy, the use of bacteriophages to kill or otherwise control the bacterial populations in infected hosts. The tailed phage makes specific contacts with surface receptors on its bacterial prey, using its tail or tail fibers, then coordinates with irreversible binding that ultimately leads to phage DNA release. Bacterial receptors belong to various biochemical families and are mainly represented by outer membrane proteins (OMP), capsule polysaccharides (CPS) and lipopolysaccharides (LPS) [5]. In the case of phage T4, the long tail fibers interact with LPS of *Escherichia coli* for reversible binding, while the short fibers are responsible for irreversible binding onto the heptose moiety of the host's LPS [6]. For phage T1, the initial reversible binding to LPS could allow the phages to glide across the cell surface until they find protein FhuA involved in ferrichrome uptake, then initiate irreversible binding in a TonB-dependent way [7]. In such cases, the phage's ability to mediate the irreversible adsorption by first binding reversibly to cell surface moieties, which are more exposed and easier to access, provides a beneficial advantage and effective way for phage infection [8].

The development of antibiotic resistance often comes with a cost to biological fitness. In our previous study, we focused on the fitness costs of the membrane modifications associated with colistin resistance. We had demonstrated that both colistin and carbapenem resistant *K. pneumoniae* ($Col^R$-CRKP) was significantly more sensitive to phage infection due to alteration of bacterial surface charges, and colistin resistance-enhanced phage infectivity is spread across $Col^R$-CRKP strains and their phages. ΦNJS1, a member of *Tunavirinae*, *Siphoviridae* family, is one of these well characterized phages specific for nonmucoid *K. pneumoniae* with an infection rate of 46.88% (15/32) [9]. However, the receptors for ΦNJS1 is still poorly understood. In the present study, we sought out to identify the receptors for ΦNJS1 by transposon libraries screen and phage tail fiber protein bioinformatics analysis. We found that O-antigen of LPS may not only a key but also be a barrier for bacteriophage infection.

## 2. Results

### *2.1 Accelerated reversible phage adsorption to $Col^R$-CRKP cells*

Phage NJS1 was showed more infectious toward $Col^R$-CRKP due to altered LPS among $Col^R$-CRKP promoted phage adsorption [9]. To further test if the increased adsorption on *Kp*(*mcr-1*) is reversible or irreversible attachment under this condition, we tested desorption rate of phage particles that had adsorbed to cells. As shown in Figure 1, about 60% percent phage particles adsorbed to *mcr-1* cells release at 37℃ but not at 0℃ (released particles <1.5%, data not shown), indicating that the LPS modification caused by *mcr-1* accelerates reversible adsorption.

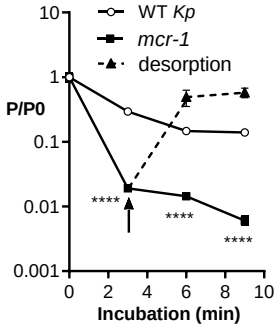


**Figure 1.** Adsorption and desorption of ΦNJS1. Bacterial cells ($10^8$ CFU/ml) were incubated with $10^7$ PFU/ml phages in LB in a 37°C water bath for 10 min. Samples were withdrawn at the time points indicated and filtered through 0.22μm-pore-size filters. The PFU of free phages in filtrates was then determined by titration on *Kp* cells. Desorption of phage from the resuspended cells with 0℃

buffered saline was followed by diluting 100-fold into saline, kept at 37℃ or 0℃ for control. At the times indicated, samples were withdrawn and centrifuged at 4℃ and the free phages released into the supernatant were determined. The mean of three independent assays is shown and error bars represent the standard deviation. ****: P<0.0001 (2-way analysis of variance [ANOVA]).

**Table 1** Bacteriophage, strains and plasmids used in this study.

| Bacteriophage/strains/plasmid | Genotype/relevant feature | Source/reference |
|---|---|---|
| Bacteriophage | | |
| ΦNJS1 | Phage specific for nonmucoid *Kp* with enhanced infectivity on Col$^R$-CRKP | [9] |
| Baterial strains | | |
| *K. pneumoniae* | | |
| A2312NM | nonmucoid phenotype strain from feces, ST11, K47, KPC-2, Sm$^R$, Amp$^R$ | [9] |
| Δ*wecG*(mTn5) | transposon inserted in *wecG* at +709/+622 (741 bp), Km$^R$, derived from A2312NM | this study |
| Δ*wecA*(mTn5) | transposon inserted in *wecA* at +953 (1104 bp), Km$^R$, derived from A2312NM | this study |
| Δ*wzyE* | in-frame deletion *wzyE* replaced with Km gene, derived from A2312NM | this study |
| Δ*fepA*<br>Δ*fhuA*<br>Δ*ompA*<br>Δ*ompC* | Km$^R$, insertional mutant with pVIK112 derived from A2312NM | this study |
| A1806M | mucoid phenotype strain from feces, ST1493, NDM-1 | [9] |
| A1806NM | nonmucoid variants derived from A1806M | this study |
| A1806(Δ*wza*) | derived from A1806M, nonmucoid phenotype, Km$^R$ | this study |
| Plasmids | | |
| pVIK112 | Suicide vector, Km$^R$ | [10] |
| pRL27 | Km$^R$ with mTn5 transposon | [11] |
| pACBSR-Hyg | Cloning vector, hygromycin resistance. | [12] |
| pFLP-Hyg | Cloning vector, hygromycin resistance. | [12] |

*2.2 ΦNJS1 infects restricted nonmucoid strains*

All *K. pneumoniae* isolates among the host range of ΦNJS1 produce a nonmucoid phenotype with a small black colony on Congo red agar plate (CRA), whereas their mucoid strain that cannot be lysed by ΦNJS1formed white or pink colonies (Figure 2A). Moreover, the CPS mutant Δ*wza* derived from A1806M strain, and the nonmucoid variants isolated from static growth condition, were both can be lysed by ΦNJS1, whereas their mucoid strain A1806M was insensitive to ΦNJS1 infection (Figure 2B). We can speculate that CPS may block receptors to be exposed on the cell surface for phage attachment.

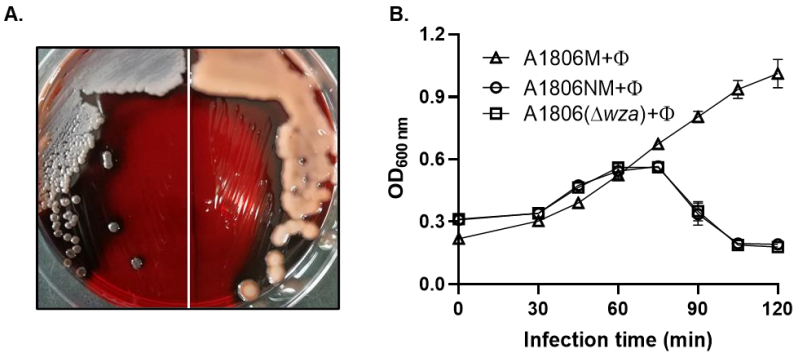


**Figure. 2 (A)** Representative colony phenotype of nonmucoid (left, black colonies) and mucoid (right, white or pink colonies) *Kp* grew on Congo-red agar plate. The strains were inoculated in streaks and incubated at 37 °C under aerobic conditions for 18 hr. **(B)** Phage NJS1 infectivity on mucoid, nonmucoid and a CPS mutant Δ*wza* of CRKP A1806 isolate. Approximately 108 CRKP cells alone, or CRKP cells mixed with phages (MOI=10-4), were incubated aerobically at 37°C. Samples were withdrawn at the time points indicated, and OD600 was measured.

*2.3 LPS may be as the receptor for ΦNJS1*

Since colistin resistance-mediated *Kp* surface modification sensitizes ΦNJS1 infection, it was important to test whether the degradation of cell surface proteins, LPS or CPS could destroy the ΦNJS1 receptors. To better differentiate the adsorption capacity of ΦNJS1 after proteinase K or periodate treatment, *Kp*(*mcr-1*) was used as control for ΦNJS1 increased adsorption ability on it. As seen in Figure 3A, the adsorption capacity to *Kp*(*mcr-1*) cells were not affected after proteinase K treatment, suggesting protein structure of bacteria surface may not affect the reversible adsorption of ΦNJS1. However, incubation of *Kp*(*mcr-1*) cells in the presence of 50 mM sodium acetate or 10 mM periodate abolished ΦNJS1 binding ability significantly. Acetic acid results in mild hydrolysis of LPS and the release of the complete core, while periodate degrades carbohydrates containing a 1, 2-diol motif in bacteria structure [13, 14]. So, we can speculate that the reversible receptor for ΦNJS1 is most likely a carbohydrate structure in LPS core or the O-antigen.

*2.4 ΦNJS1-resistant bacterial mutants*

To determine the host receptor of ΦNJS1, we constructed CRKP A2312NM transposon-insertion library for phage-resistant mutant screening. After substantial efforts, we got two mutants among $10^7$ clones: Δ*wecG*(mTn5), which was inserted by mTn5 transposon at two sites from two libraries, and Δ*wecA*(mTn5) which was completely resistant to ΦNJS1. Both two genes are involved in LPS biosynthesis: *wecA* catalyzes the synthesis of Lipid I (GlcNAc-PP-C55) and provides receptors for the subsequent addition of O antigen sugar groups [15]. Gene *wecG* encodes UDP-N-acetyl-D-mannosyltransferase, which catalyzes the synthesis of Lipid II [16]. The EOP of ΦNJS1 on Δ*wecG* was still around 1(data not shown), indicating Δ*wecG* affected the infection rate of ΦNJS1 but not the progeny phage particles. The adsorption capacity of ΦNJS1to both Δ*wecG*(mTn5) and Δ*wecA*(mTn5) reduced significantly compared with that to WT CRKP by counting unadsorbed phages in the filtrates (Figure 3B). As shown in Figure 3C, ΦNJS1 lysed Δ*wecG*(mTn5) considerably slower than WT CRKP, and no decrease in $OD_{600}$ was observed on group of Δ*wecA*(mTn5) treated with phage. These results suggested that LPS of CRKP was involved in ΦNJS1 adsorption and O-antigen may be essential for ΦNJS1 reversible adsorption.

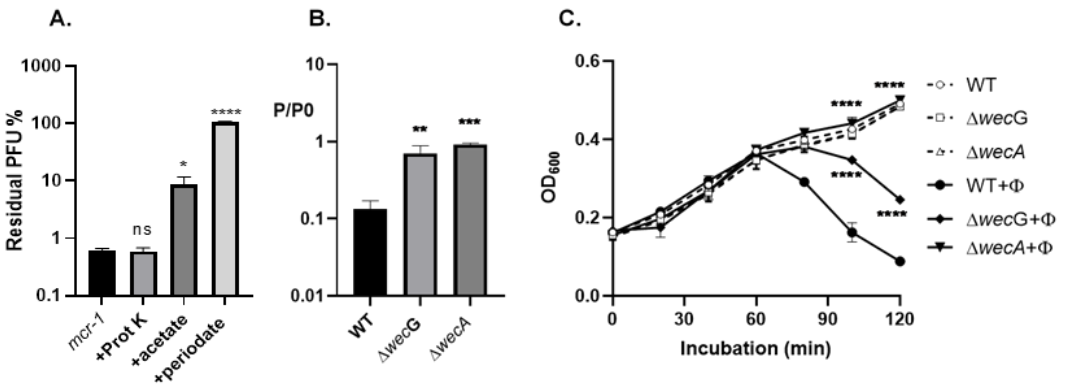


**Figure 3 (A).** Effects of different treatments of bacteria on ΦNJS1 adsorption. Stationary cells were treated with proteinase K (0.2 mg/ml; Promega), sodium acetate (50 mM; pH 5.2) or periodate (10 mM) for 2 hr. The cells were washed with saline and suspended in LB, then the phage adsorption assay was carried out as described above. *: $P$<0.05 (one-way ANOVA); ****: $P$<0.0001; ns: no significance. **(B).** ΦNJS1 adsorption to Δ*wec*A and Δ*wec*G. Bacterial cells ($10^8$ CFU/ml) were incubated with phages ($10^7$ PFU/ml) at 37°C for 5 min in LB medium. Binding was stopped by filtration and the number of unbound phages in the supernatant was determined by titration on *Kp* cells. **: $P$<0.005; ***: $P$<0.001 (one-way ANOVA). **(C).** ΦNJS1 infectivity dynamics on Δ*wec*A and Δ*wec*G. Approximately $10^8$ *Kp* cells alone, or *Kp* cells mixed with phages (MOI=$10^{-4}$), were incubated aerobically at 37°C. Samples were withdrawn at the time points indicated, and $OD_{600}$ was measured. ****: $P$<0.0001(2-way ANOVA).

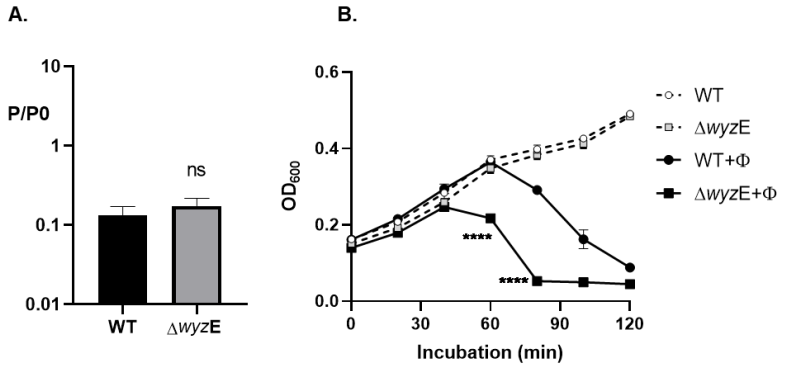


**Figure 4** ΦNJS1 adsorption capacity and infectivity on Δ*wzyE*. (**A**). ΦNJS1 adsorption to Δ*wzyE*. (**B).** ΦNJS1 infection dynamics on wildtype and Δ*wzyE*. The host bacteria were infected with phages at a MOI of 0.0001 at 37℃. Samples were withdrawn at the time points indicated, and $OD_{600}$ was measured. ****: $P$<0.0001(2-way ANOVA).

*2.5 O-antigen long chain protects bacteria from phage infection*

To examine the role of O-antigen in ΦNJS1 infection, we engineered an in-frame deletion mutant in *wzyE* gene which encodes [17]. The inactivation of WzyE leads to a single trisaccharide unit attached to the lipid A core. We then assayed phage adsorption ability and infectivity on the mutant Δ*wzyE*. The results showed that the loss of O-antigen polymerase did not affect the reversible adsorption capacity of ΦNJS1(Figure 4A). However, Δ*wzyE* was lysed by phage more efficiently than the wildtype (Figure 4B), suggesting irreversible adsorption ability on it may be enhanced for shorter O-antigen chain allowing phage tail fiber to attach second receptors more efficiently.

*2.6 Identification of phage irreversible receptor*

Some T1-like phages can utilize outer membrane protein (OMP), such as transporter protein FhuA [18], FepA [19], OmpA [20] and OmpC [21], as their receptor. Since we did not obtain any related OMP mutants after substantial efforts in screening transposon libraries, we then constructed above four OMP mutants and examined ΦNJS1 infectivity on them. All the EOP of ΦNJS1 on these four OMP mutants were still around 1(data not shown). Phage lysis assay (Figure 5) showed that Δ*fhuA*, Δ*ompA* and Δ*ompC* had no influence on phage infectivity except for Δ*fepA* which just slowed phage lysis rate, suggesting FepA may be as an irreversible receptor for ΦNJS1.

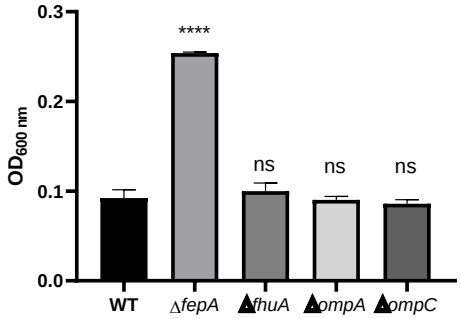


**Figure 5** ΦNJS1 infection dynamics on different OMP mutants. Bacterial cells ($10^8$ CFU/ml) were incubated aerobically with $10^7$ PFU/ml phages in LB at 37°C. Samples were withdrawn at the time points, and $OD_{600}$ was measured. ****: $P<0.0001$; ns: no significance. (one-way ANOVA).

*2.7 Phage tail fiber sequence analysis*

For ΦNJS1 belongs to the Tunavirinae subfamily, we then compared the central fiber protein (CFP, S1_041) and side fiber protein (SFP, S1_042) sequence of ΦNJS1 with PSI-BLAST. The CFP, S1_041) responsible for irreversible phage binding to receptor, including baseplate hub protein domain (BHP), unknown tail domain and receptor binding protein domain (RBP), shows 56.02% sequence identity with fibA of T1. However, its SFP showed only 31.36% sequence identity with that of phage T1, but 70.86% sequence identity with *Escherichia* phage T5 of L-shaped tail fiber (LTF, pb1) (Figure 6). This unknown tail domain which is found in various bacteriophage host specificity proteins, can be found in tip attachment protein J from *Enterobacteria* phage lambda. It attaches the virion to the host receptor LamB, inducing viral DNA ejection [22]. Bacteriophage T5 binds to the polymannose O-antigens of *E. coli* LPS [23] and the structure of the receptor-binding carboxy-terminal domain of LTF is well studied [24], providing support for O-antigen as ΦNJS1 reversible receptor.

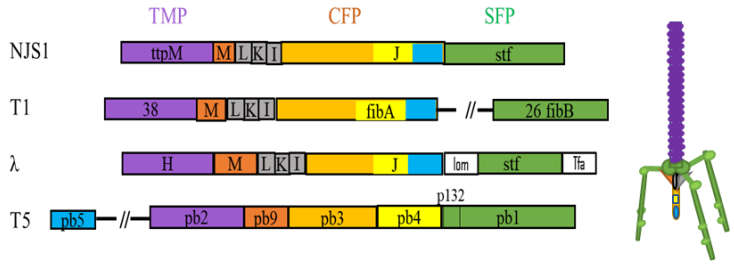


**Figure 6** Arrangement of the tail tip genes in the siphophages NJS1, T1, λ and T5. Genes or part of them predicted to encode the same functions are depicted in the same color: tape measure protein (TMP), violet; Distal tail proteins (Dit), orange; central fiber protein (CFP): including BHP (orange yellow), unknown tail domains (yellow) and RBP (cyan); SFP (Stf or fibB) side tail fibers, green. The proteins L, K, and I (gray) are known to be part of the tail tip complex, but their location is unknown. Schematic representation of NJS1 tail architecture based on the description of λ and T5 structural proteins (right).

## 3. Discussion

*K. pneumoniae* is an important nosocomial pathogen that causes a wide range of community- and healthcare-associated infections. We previously reported that the covalent modifications of LPS for colistin resistance accelerated the rate of phage adsorption to Col$^R$-CRKP [9]. The process of phage adsorption is a combination of physical diffusion, biochemical surface interactions, and reaction-induced conformational changes in receptor proteins [25]. Based on the data before, the model of ΦNJS1 adsorption to CRKP or Col$^R$-CRKP changed from sequential model to the adsorption efficiency mode, we then firstly suggested that the significantly decreased phage particles in the filtrates was due to increased reversible adsorption level. The LPS modification caused by colistin resistance results in lowering the level of electrostatic repulsion between the phage and bacteria, thereby promoting phage adherence and infection [9].

Since all *K. pneumoniae* isolates that can be lysed by ΦNJS1 produce a nonmucoid phenotype including a CPS mutant Δ*wza*, we therefore hypothesized CPS may block receptors to be exposed on the cell surface for phage attachment. Some work has suggested that *K. pneumoniae* strains with the K1, K10, and K16 antigens can mask their LPS [1]. As an attempt to further characterize the receptors needed for ΦNJS1 adsorption, bacteria treated with periodate prior to the phage adsorption assay showed that a carbohydrate structure is the receptor for ΦNJS1. Since the enterobacterial common antigen cannot be destroyed by periodate treatment and CPS may block phage recognition, this carbohydrate is mostly likely LPS. The loss of WecA and WecG involved in LPS biosynthesis affected phage adsorption capacity and phage infectivity, whereas the O-antigen long chain shortened to trisaccharide repeat units for the loss of *wzyE* results in increased infectivity of ΦNJS1. The *Klebsiella* O-antigen comprises polymannans, polygalactans or heteropolymers with a repeat unit structure, and there have been only 9 different O-antigen types identified in *K. pneumoniae* isolates [1]. The inactivation of WecA results in the loss of polymer and "primer" consisting of undecaprenyl pyrophosphoryl (und-PP)-linked GlcNAc, indicating the O-antigen plus the GlcNAc acceptor were indispensable for phage adsorption and subsequent infection. Besides, the mutation of *wecG* slowed the adsorption capacity and infection rate of ΦNJS1 significantly, demonstrating GlcNAc-ManNAcA carbohydrate may play an important role in phage attachment. The LPS O-chain length is crucial for the biology of *Klebsiella* because it contributes to resistance to complement-mediated serum killing [26]. Interestingly, in the current study, we found that the O-antigen long chain shortened to the trisaccharide unit for the loss of *wzyE* results in increased infectivity of ΦNJS1. For phage adsorption capacity was not affected, demonstrating a new role of long chain length of O-antigen was to provide an effective barrier against phage infectivity. To our knowledge, only O-acetylation was reported to provide protection of the host cells against attack by phages [27]. These results indicate O-antigen of LPS may not only a key but also be a barrier for bacteriophage infection. Maybe the identification of O-antigen type in the future could help to understand the host range of ΦNJS1 and be beneficial for phage therapy application in nosocomial infections.

T1-like phages recognize bacterial hosts via adhesin tips of their long tail fibers with reversible binding to cells prior to infection. The tail fiber adhesin sequences are highly variable in both their sequence and specificity for bacterial receptors [28]. Although ΦNJS1 belongs to T1-like genus, the CFP responsible for irreversible adsorption is homologous to that of phage T1, but its SFP shows high sequence identity to that of phage T5. Interaction of the receptor-binding protein (RBP, pb5) of T5 with its specific receptor, ferrichrome outer membrane transporter (FhuA), triggers DNA ejection. Phage T5 has three additional L-shaped tail fibers that consist of pb1 binding to polymannose O antigen to increase adsorption [24, 29, 30]. In T5-like phages, such as coliphages DT57C and DT571/2, this interaction is required for their CFP to successfully reach vitamin B12 transporter BtuB [31]. The CFP of ΦNJS1 is highly homologous to *Klebsiella* phage GH-K3, and OmpC is essential for GH-K3 infection [21]. However, the loss of OmpC has no significant difference in ΦNJS1 infection. FepA serves as the surface receptor for Escherichia virus H8 that also requires TonB for infectivity (16). Here we found the inactivation of FepA just slowed the infection rate of phage, so other OMPs which may be important for bacteria growth were likely to be targeted by ΦNJS1. Another evidence

was that no any OMP mutants were found in our substantial efforts of phage-resistant screen. There is a disadvantage of using transposon technology to screen genes related to phage receptors, that is if two or more receptors can be utilized by phage, it is difficult to screen the suspected genes. In fact, it has been demonstrated that phages can use more than one receptor to recognize hosts. For instance, Shigella Sf6 infection utilizes either OmpA or OmpC, with OmpA being the preferred receptor [20].

## 4. Materials and Methods

### *4.1 Bacterial strains and growth conditions*

The bacteriophage, bacterial strains, and plasmids used in this work are described in Table 1. All *K. pneumoniae* strains were cultured in LB media unless otherwise stated. When appropriate, antibiotics were used as follows: ampicillin: 100 μg/ml, streptomycin: 100 μg/ml, kanamycin: 50 μg/ml, polymyxin B: 2 μg/ml in liquid culture and 5 μg/ml on agar plate. For transconjugant selections, LB agar plates supplemented with the appropriate antibiotics were used. Unless otherwise stated, *K. pneumoniae* and phage cultures were incubated at 37℃.

### *4.2 Bacteriophage adsorption and desorption assays*

Bacteriophage adsorption experiments were carried out at 37℃ in LB medium with a little modification as described previously [32]. Briefly, stationary *K. pneumoniae* cells were resuspended into LB medium at desired concentration. Phages ($2\times10^7$ PFU/ml) were incubated together with host cells $2\times10^8$ CFU/ml at 37°C in LB medium. Samples were withdrawn at the indicated time points and filtered with 0.22 μM filters immediately. Unadsorbed phages in the filtrates were determined by titration on the double-layer agar.

Desorption of phage from the resuspended cells with 0℃ buffered saline was followed by diluting 100-fold into saline, kept at 37 ℃ or 0℃ for control. At the times indicated, samples were withdrawn and centrifuged at 4℃ and the free phages released into the supernatant were determined.

### *4.3 Differentiation of mucoid and nonmucoid isolates*

To differentiate the mucoid and nonmucoid phenotype isolates, strains were inoculated in streaks on BHIA (Difco) with 0.08% (w/v) Congo red (Sigma-Aldrich, Germany) supplemented with 5% (w/v) sucrose and incubated at 37°C under aerobic conditions for 18 hours [33].

### *4.4 Periodate and proteinase K treatments*

To test whether proteinase K treatment or periodate can affect ΦNJS1 adsorption, 1 ml of stationary *Kp* cultures in saline ($10^8$ CFU/mL) was treated with proteinase K (>=600 U/ml; Takara) at 37°C for 1 h and washed with saline twice, and the phage adsorption assay was performed as described above. In order to study whether periodate can destroy the phage receptor, 1ml of stationary *Kp* culture ($10^8$ CFU/mL) was centrifuged at 10,000 g for 2 min, and the bacterial pellet was suspended into 1 ml sodium acetate (50 mM; pH 5.2) or 10 mM periodate. The cells were incubated for 1 h (protected from light), then centrifuged as described above. After washing and centrifuging again, cells were resuspended in saline. The phage adsorption assay was carried out as described above. To confirm that the possible effect was not due to incubation at 37°C, a control without any reagent addition was included.

### *4.5 Phage resistant mutants screen*

To screen phage resistant mutants, *E. coli* BW20676 harboring pRL27 [11] and carbapenem-resistant *K. pneumoniae* A2312NM were used as donor and recipient strains, respectively. Kanamycin-resistant transconjugants were grown in LB broth containing phage of $10^5$ PFU/mL at 37 ℃ for 5-7 hours, then cells were centrifuged and resuspended into new LB media for another 5-7 hours of phage lysis. Survival mutants were spread on selected LB agar plates. *K. pneumoniae* A2312NM treated with phage lysis using above the same method was used as control.

Suspected colonies were purified by streaking on the plate and confirmed by spot tests with phage suspensions. The transposon insertion site of the mutant was determined by arbitrary PCR and sequencing.

*4.6 Construction of LPS and OMP mutants*

Gene deletions in *K. pneumoniae* were constructed by replacing the gene of interest with a kanamycin resistance cassette FRT-Km-FRT by recombineering using pACBSR-Hyg as described in [12, 34]. Plasmid pVIK112 was used as the template for the FRT-Kan-FRT segment for the Δ*wzyE* strain. Antibiotic resistance cassettes flanked by FLP recombinase target (FRT) sites were removed using pFLP-Hyg. All OMP mutants were constructed by cloning internal fragments of interest gene into pVIK112 [10]. The resulting plasmids were then introduced into CRKP A2312NM by conjugation and integrated into the interest gene locus.

*4.7 Phage lytic activity*

Overnight cultures of *K. pneumoniae* wild type and mutants were inoculated at 1:1000 into LB medium with appropriate antibiotics and grew at 37°C 180 rpm for 6 hours. Bacterial cells were centrifuged and washed with LB medium, then resuspended with fresh LB medium to approximately $10^9$ CFU/mL. Approximately $10^8$ *K. pneumoniae* cells alone, or *K. pneumoniae* cells mixed with phages (MOI=$10^{-4}$), were incubated aerobically at 37°C. Samples were withdrawn at the time points indicated, and $OD_{600}$ was measured.

## 5. Conclusions

In conclusion, this study showed that O-antigen were likely to be the reversible receptor for *Klebsiella* phage NJS1 and FepA may be as one of its irreversible receptors. We also discovered that the long chain length of O-antigen may protect bacteria from phage infection. The results also provide a reference for the phage that showed increased infectivity to colistin-resistant bacteria.

**Author Contributions:** G.H. and H.W. framed this study, G.H. Y.J. and S.J. conducted the experiments and collected the data. M.L. and S.Q. constructed the mutant strain. H.Z. provided clinical strains and S.S. helped in manuscript writing. G.H. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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## Abbreviations

| | |
|---|---|
| $Col^R$-CRKP | colistin resistant carbapenem-resistant *Klebsiella pneumoniae* |
| MDR | multi-drug resistant |
| OMP | outer membrane protein |
| CPS | capsule polysaccharide |
| LPS | lipopolysaccharide |
| CRA | Congo red agar |
| FRT | FLP recombinase target |
| TMP | tape measure protein |
| CFP | central fiber protein |
| SFP | side fiber protein |
| RBP | receptor binding protein |
| LTF | L-shaped tail fiber |
| BHP | baseplate hub protein domain |

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