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Paper Title: A Study on the In Vitro Combination

Strategy of Phage and Kanamycin

Against Drug Resistant Escherichia

coli

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A Study on the In Vitro Combination Strategy of Phage and Kanamycin Against Drug-Resistant *Escherichia coli*

Abstract: With the widespread use of antibiotics, the problem of bacterial resistance has become increasingly serious, and the emergence of drug-resistant *Escherichia coli* poses a severe threat to human health. This experiment aims to isolate phages of *Escherichia coli* MG1655 from sewage, determine their biological characteristics, and then explore the optimal strategy for the combined application of phage and kanamycin, laying a foundation for subsequent verification of the feasibility of this strategy in drug-resistant clinical strains. In this experiment, strains M8 to M14 were selected from 14 isolated and purified phage strains for subsequent studies. The phage titer, optimal multiplicity of infection, and minimum inhibitory concentration of kanamycin were determined to explore the optimal strategy for combined bacteriostasis. The results showed that the MIC of kanamycin against *Escherichia coli* MG1655 transformed with pET-28a plasmid was 800 µg/mL; phage M9 showed no obvious synergistic pattern, with the optimal combination being 10³ PFU/mL phage and 2 µg/mL kanamycin. It was found that medium-concentration phage M10 exhibited excellent potential for combined application with 64 to 128 µg/mL kanamycin, and the optimal combination was 10⁶ PFU/mL phage and 16 µg/mL kanamycin.

Key words: bacteriophage, kanamycin, *Escherichia coli*, combined application, bacterial resistance

1 Introduction

1.1 Research background

1.1.1 *Escherichia coli* and its drug resistance mechanism

According to the latest estimates from the Global Research on Antimicrobial Resistance (GRAM), approximately 1.14 million deaths worldwide were directly attributable to bacterial resistance in 2021, and about 4.71 million deaths were associated with bacterial resistance^[1]; it is projected that by 2050, the number of deaths directly attributable to bacterial resistance will increase to approximately 1.91 million per year^[1]. *Escherichia coli* (*E. coli*) is a Gram-negative short bacillus with blunt ends, motile, and non-spore-forming^[2]. It is widely present in the intestines of mammals and poultry, sewage, and soil, and can be transmitted through various routes. *Escherichia coli* can infect humans, poultry, pigs, sheep, and other animals. Pathogenic *Escherichia coli* has been reported to be associated with human hemorrhagic diarrhea and hemolytic uremic syndrome (HUS), and is recognized as one of the main bacterial causes of infection and foodborne diarrhea^[3]. Data from the China Antimicrobial Surveillance System (CHINET) in 2024 showed that the resistance rates of clinical *Escherichia coli* isolates to the third-generation cephalosporin ceftriaxone and the fluoroquinolone ciprofloxacin were 52.2% and 62.8%, respectively^[4]. Antibiotics such as fluoroquinolones and aminoglycosides have bacteriostatic effects on *Escherichia coli*. Fluoroquinolones exert their bacteriostatic effect by inhibiting the activities of DNA gyrase and topoisomerase IV, thereby affecting bacterial DNA replication and transcription^[5]. The binding of the 30S ribosomal subunit to tRNA plays a key role in protein synthesis. All aminoglycoside antibiotics discovered to date use the 16S rRNA of the 30S ribosome as their main target site, and kill bacteria by inhibiting protein synthesis^[6]. Due to the advantages of low cost, easy access, and obvious effects, antibiotics are widely used in the treatment of bacterial infections. However, with the widespread application of antibiotics, bacteria have developed widespread resistance through methods such as gene mutation, and microbial antimicrobial resistance is increasing, leading to the emergence of "superbugs". At present, there are four main drug resistance mechanisms of *Escherichia coli*: (1) mutation of the target site of antimicrobial drugs; (2) modification and destruction of antibiotics by enzymes; (3) active efflux system; (4) changes in bacterial outer membrane permeability^[7]. The above resistance genes can be horizontally transmitted between strains through plasmids, accelerating the spread of drug resistance. Alleviating drug resistance is imminent, and people have to conduct research and exploration on new antibiotics and other therapies.

1.1.2 Bacteriophages and phage therapy

Among many new antibacterial strategies, phage therapy has attracted much attention due to its unique mechanism of action. Phages are viruses that specifically infect prokaryotic microorganisms such as bacteria, actinomycetes, and spirochetes^[8]. They have the ability to dynamically adjust and precisely target the host,

high species specificity, good biocompatibility, and can lyse drug-resistant bacteria [9].

Phages can be divided into virulent phages and temperate phages, with different proliferation strategies. The lytic cycle is the main strategy for phage proliferation, which ultimately leads to the lysis and death of the host bacterium. Virulent phage particles specifically adsorb to receptors on the bacterial surface and inject their genetic material into the cell. Subsequently, the phage uses the host's system resources to replicate its own nucleic acid in large quantities and synthesize structural protein components, assemble into a large number of progeny virus particles, and lyse the host cell under the action of lysozyme, thereby completing release and spreading the infection. In contrast, temperate phages do not enter the lytic cycle; they integrate their genomes into the host chromosome and remain latent, replicating along with the host DNA and being passed on to progeny cells. They can be activated under specific conditions, leading to host cell lysis [10,11].

Compared with the research and development cycle of antibacterial drugs, which often takes years or even decades, phages have the advantages of short research and development cycle and low input cost [12]. Zhang et al. isolated a virulent phage UDF157lw (genus *Rogunavirus*) targeting *E. coli* O157:H7. The phage has a latent period of about 14 min and a burst size of 110 PFU/cell, and remains stable in the range of pH 4–11 and 4–60°C; the depolymerase ORF26 encoded by its genome can degrade the O-antigen polysaccharides of various *E. coli* O157:H7 strains, the corresponding plaques are significantly enlarged, and biofilms formed at 24 h and 48 h can be significantly reduced after 1 h of treatment [13]. However, although phages have a strong ability to specifically lyse bacteria, it is difficult for them to eradicate biofilms [14]. In addition, there is a risk of developing phage-resistant phenotypes during treatment, and the host range of phages is relatively narrow, making it impossible to cope with the high diversity of biofilm communities [10,11].

1.1.3 Combined application of phage and antibiotics

Due to the limitations of using antibiotics alone and phage therapy alone, the combined application of phage and antibiotic may be a more efficient method. First, a single pressure can be completely replaced by two sufficient and different pressures. Because the mechanisms of action are different, strains resistant to antibiotics can be easily lysed by phages, and strains resistant to phages can still be cleared by antibiotics. Second, since bacterial drug resistance is reduced by phages, the dosage of antibiotics can be reduced, alleviating the antibiotic resistance crisis. Third, combined application can reduce the emergence of phage-resistant strains and make bacteria more sensitive to phages.

The combined application of phage and antibiotic may produce a synergistic effect. The advantage of synergistic effect is that it reduces the concentration of each antibacterial component, alleviating the development of drug resistance while reducing toxicity [15]. However, the effect of the combined application of phage and antibiotic varies with the type of pathogenic bacteria, drug category, concentration ratio, and administration sequence, and there is currently no universally applicable rule. In particular, systematic

experimental data on the compatibility of sub-inhibitory concentration antibiotics and phages are still limited. This study aims to accumulate data for elucidating the conditions and mechanisms of the synergistic effect of *Escherichia coli* phage and kanamycin through in vitro combination experiments.

1.2 Purpose and significance of the study

This study aims to isolate phages of *Escherichia coli* MG1655 from sewage, determine their biological characteristics, and explore the optimal strategy for the combined application of phage and kanamycin. The experimental results can provide reference data for the mechanistic study of phage-antibiotic combination strategies and lay a foundation for subsequent verification of the feasibility of this strategy in drug-resistant clinical strains.

2 Experimental materials and methods

2.1 Experimental materials

2.1.1 Kanamycin-resistant *Escherichia coli* and phage sample materials

2.1.1.1 Kanamycin-resistant *Escherichia coli*

Escherichia coli MG1655 competent cells and pET-28a plasmid were both obtained from the laboratory of Prof. Jie Feng, Institute of Microbiology, Chinese Academy of Sciences.

2.1.1.2 Phage sample materials

Phages were isolated from sewage, which was collected from Guanlan Wastewater Purification Plant, Shixia Wastewater Purification Plant, Gongming Wastewater Purification Plant, and Yantian Wastewater Purification Plant.

2.1.2 Reagent and drugs

2.1.2.1 Medium preparation

(1) LB medium: Weigh 10 g of tryptone powder, 5 g of yeast extract, and 10 g of sodium chloride, dilute to 1 L with distilled water to prepare 1 L of LB liquid medium. After sterilization, dispense 4 mL of medium per portion into test tubes.

On the basis of LB liquid medium, add 6 g/L and 15 g/L of agar respectively to obtain LB semi-solid medium and LB solid medium. In a disposable petri dish, first pour the lower layer of solid medium, then pour the upper layer of semi-solid medium, and air-dry to obtain LB double-layer plate medium. If it needs to be stored for a long time, the medium should be sealed with materials such as plastic wrap to reduce water evaporation.

(2) MH medium : Weigh 22 g of MH medium powder, dilute to 1 L with distilled water.

The LB liquid medium, LB semi-solid medium, LB solid medium, and MH medium mentioned above

should be mixed, sealed, and sterilized in a high-temperature and high-pressure steam sterilizer at 121°C and 103.4 kPa for 25 minutes.

2.1.2.2 Preparation of kanamycin diluent

Weigh 24 mg of kanamycin with a precision analytical balance, dissolve it in distilled water, and dilute to 1 mL to obtain 1 mL of 24 mg/mL kanamycin diluent. Weigh 0.5 mg of kanamycin with a precision analytical balance, dissolve it in distilled water, and dilute to 10 mL to obtain 1 mL of 50 µg/mL kanamycin diluent. The prepared kanamycin diluent is filtered and sterilized through a 0.22 µm filter membrane, then dispensed and stored frozen in a -20°C refrigerator.

2.1.2.3 Other reagents and drugs

SM buffer, ice, distilled water

2.1.3 Apparatus and materials

0.22 µm filter membrane, pipettes, pipette tips, centrifuge tubes, EP tubes, disposable petri dishes, 96-well plates, plastic wrap.

2.1.4 Instruments and equipment

METTLER TOLEDO balance (Hitachi, Japan), temperature-controlled shaker (Shanghai Zhichu Instrument Co., Ltd.), electrothermal constant temperature incubator (Shanghai Yiheng Scientific Experiment Co., Ltd.), -80°C low-temperature freezer (Zhongke Meiling Cryogenic Technology Co., Ltd.), 4°C low-temperature refrigerator (Zhongke Meiling Cryogenic Technology Co., Ltd.), biosafety cabinet (Beijing Donglian Haer Instrument Manufacturing Co., Ltd.), automatic growth curve analyzer (BIOSCREEN, Finland), microwave oven (Galanz Microwave Electrical Appliance Co., Ltd.), centrifuge (Thermo Fisher)

2.2 Experimental methods

2.2.1 Experimental operation flow chart

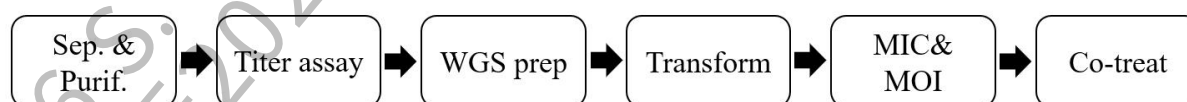


Fig.1 Experimental operation flow chart

2.2.2 Bacteriophage isolation and purification

(1) Wastewater pretreatment: Centrifuge the wastewater at 6000 rpm for 30 minutes, then filter the supernatant through a 0.22 µm filter membrane to remove large particulate impurities and bacteria.

(2) Co-culture: 1 mL wastewater + 1 mL bacterial liquid co-cultured for 6 h, basic clarification, filtration

(0.22 μm) to concentrate the supernatant, preliminarily obtaining a phage mixture.

(3) Separation and purification: Use SM buffer to dilute the phage mixture with a 10-fold gradient to 10^4 and 10^6 , 100 μL bacterial suspension + 100 μL diluent + 8–10 mL LB soft agar medium poured onto double-layer plates, incubate at 37°C for 6 h. Search for individual plaques, preliminarily differentiate different phages based on plaque morphology, the selected individual plaques are crushed in 500 μL SM buffer, filtered through 0.22 μm membrane to obtain the first purified phage solution. Repeat the above operation four times, finally obtaining 14 phage solutions, named M1 to M14 respectively.

2.2.3 Phage Amplification and Titer Determination

2.2.3.1 Expansion

The purified 14 phages were diluted 10, 100 and 1000 times, and 100 μL dilution and 200 μL bacterial solution were mixed with 8 - 10 mL soft agar medium, poured double-layer plates, and cultured for 6 h. Add 8 - 10 mL SM buffer to the plate, shake on the shaker for 3 h, and rinse out the phage. The liquid in the plate was filtered to obtain the expansion solution, which was refrigerated at 4°C .

2.2.3.2 Determination of titer

The expansion medium of 14 phages was diluted 10-fold gradient to 10^{-2} to 10^{-8} concentrations, and 2 μL dilution was inoculated on a double-layer plate of 200 μL bacterial solution mixed with 15 mL soft agar medium for drying. After 5 hours of culture, the plaque was observed, and the estimated titer range was above 10^6 to 10^8 PFU / mL. The point plate method can be used to estimate the range of titer and reduce the amount of work in the subsequent double-layer plate method.

Then, the double-layer plate method was used to dilute the phage solution to a concentration of 10^{-6} to 10^{-8} by gradient method. 100 μL of each concentration diluent was taken, and 100 μL of bacterial solution and 8-10 mL soft agar medium were added to pour the double-layer plate. After 6 h of culture, the number of plaques at each concentration gradient was counted and the titer was calculated.

The titer calculation formula is : phage titer (PFU / mL) = $10 \times$ plaque number $\times$ dilution multiple.

2.2.4 Genome extraction and sequencing

Viral genomic DNA extraction kit was used to extract M1 to M14 phage DNA. Sent to the company for sequencing.

2.2.5 Sequence assembly and annotation

Sequence assembly was performed on the website of the National Microbial Science Data Center and annotated with the PHASTEST website.

2.2.6 Plasmid transfer

(1) Take competent cells of MG1655 stored at -80°C , and thaw slightly on ice for 3 min. Pipette 10 μL of pET-28a into the EP tube containing competent cells, mix gently, and incubate on ice for 30 min.

(2) Place the EP tube in a 42°C water bath for 90 seconds, then immediately transfer to ice for incubation for 3 minutes, avoiding significant shaking.

(3) Add 1 mL of liquid LB culture medium to an EP tube, vortex and re-suspend twice, then incubate in a 37°C , 180 rpm shaking incubator for 1 hour to revive the bacteria and facilitate the expression of the kanamycin resistance gene on the plasmid.

(4) The cells were centrifuged at 5000 rpm for 3 min; part of the supernatant was discarded, leaving 100 μL for resuspension. Then, 100 μL of the resuspended solution was spread onto a Kan 50 resistance plate and incubated inverted for 14 h.

(5) After 14h incubation, single colonies were observed. Pick single colonies and inoculate into 4mL LB liquid medium containing 4 μL of 50 $\mu\text{g/mL}$ kanamycin dilution solution to ensure the kanamycin resistance plasmid is not lost during bacterial growth. Preserve the bacteria after culture.

2.2.7 Determination of minimum inhibitory concentration (MIC) of kanamycin

The kanamycin diluent at 24mg/mL was diluted by gradient method to concentrations of 2400, 1600, 1200, 800, 600, 400, 300, 200, 150, 100, 75, and 50 $\mu\text{g/mL}$, with 200 μL per well, resulting in final kanamycin concentrations of 1200, 800, 600, 400, 300, 200, 150, 100, 75, 50, 37.5, and 25 $\mu\text{g/mL}$ in each well. Then, 200 μL of bacterial suspension was added to each well. Each group was repeated three times, with positive and negative controls included. After 20 hours of culture, the average OD values were measured and judgments were made through visual observation.

2.2.8 Determination of the Optimal Multiplicity of Infection (MOI) for Bacteriophage

(1) Culture bacteria and adjust OD: Culture the bacteria to the logarithmic phase. Adjust the bacterial OD value to 1.0, which corresponds to approximately 10^9 CFU/mL, then dilute it to 10^7 CFU/mL.

(2) Dilute each of the M8 to M14 expansion media to a phage dilution solution concentration of 10^9 to 10^3 PFU/mL.

(3) 500 μL bacterial culture + 500 μL phage diluent + 4ml LB liquid medium, co-cultured for 5h and then filtered to obtain the co-culture liquid.

(4) Dilute the co-culture solution to 10^7 -fold using SM Buffer, mix 200 μL bacterial culture + 100 μL diluted co-culture solution + 8-10mL LB soft agar medium, pour plates, incubate at 37°C for 5h, count the number of plaques, and calculate the MOI for each group.

2.2.9 Combined application

(1) *E.coli* was cultured to logarithmic phase, and then diluted to 10^6 CFU / mL with MH medium.

(2) In 96-well plates, kanamycin was diluted by double dilution method. 50 μ L MH medium was added to each well from column 1 to column 10, 100 μ L 512 μ g / mL kanamycin diluent was added to column 11, 50 μ L to 10 columns were sucked with a multichannel pipette, and so on, so that each well contained 50 μ L kanamycin diluent.

(3) Bacteriophage diluents with titers of 10^3 - 10^8 were prepared, and 50 μ L phage diluents with concentrations of 10^3 - 10^8 PFU / mL were added to the second to seventh rows of 96-well plates.

(4) 100 μ L bacterial liquid was added to each hole, so that each hole contained 200 μ L liquid.

(5) Negative and positive controls were set at the same time. The positive controls were antibiotics and *E.coli* as well as phages and *E.coli*. The negative controls were MH medium and MH medium and *E.coli*. The negative control of MH medium was added with 200 μ L, and the negative controls of MH medium and *E.coli* were added with 100 μ L each.

(6) The 96-well plate was placed in the growth curve instrument for the determination of 12h OD value.

(7) Calculate antibacterial efficiency: antibacterial efficiency = $(OD_{\text{control}} - OD_{\text{experiment}}) / OD_{\text{control}} \times 100\%$.

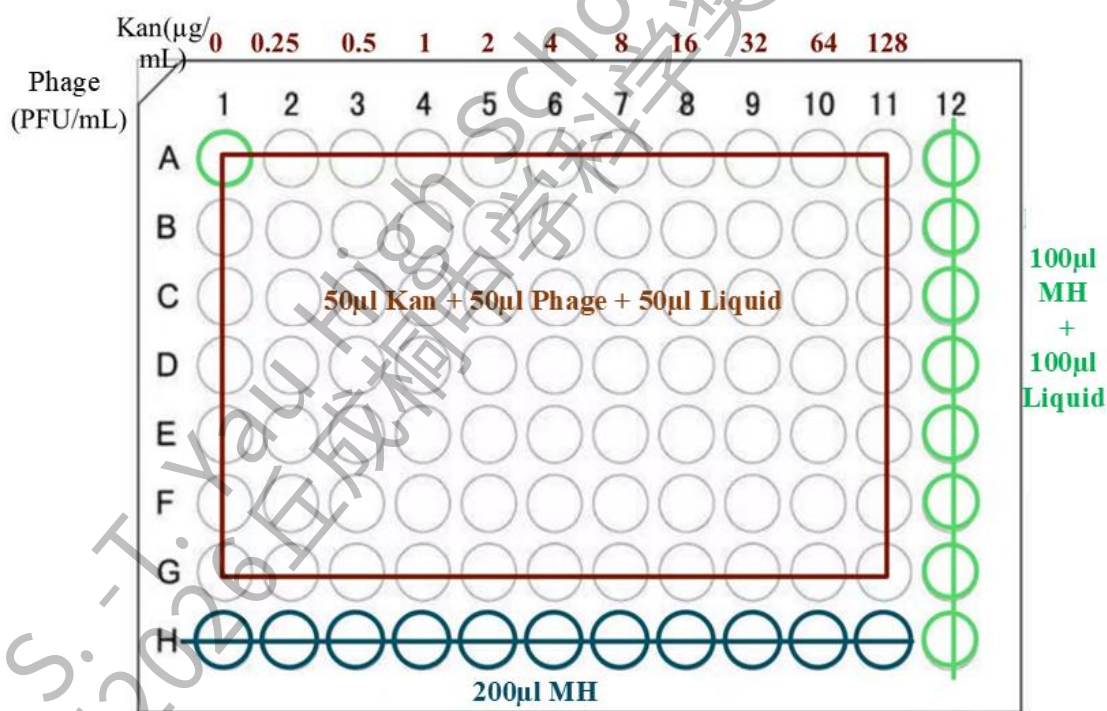


Fig.2 The schematic diagram of phage combined with kanamycin in 96-well plate

3 Results and analysis

3.1 Phage isolation and titer determination

Fourteen *E.coli* phages were isolated and named M1 to M14. Determination of its titer statistics are as follows :

Table 1 Statistical table of phage titer from M1 to M14

Phage Number	Titer (PFU/mL)
M1	5×10^{11}
M2	3×10^{11}
M3	4×10^{11}
M4	2×10^{11}
M5	8×10^{11}
M6	5×10^{11}
M7	1×10^{12}
M8	8×10^{11}
M9	1×10^{12}
M10	1×10^{12}
M11	1×10^{11}
M12	3×10^{11}
M13	6×10^{11}
M14	2×10^{11}

The phage titers of M1 to M14 were in the range of 10^{11} to 10^{12} , which was at a high level.

3.2 Genome sequencing

M1 to M14 were sent to sequencing. The genomes of M1 to M7 were damaged or contaminated, and no results were obtained. The next experiments were based on M8 to M14 phages. The brief results of gene sequencing are shown in Table 2.

Table 2 Brief results of M8 to M14 gene sequencing

	M8	M9	M10	M11	M12	M13	M14
Genome Length	87929	86437	86911	87530	87736	86437	87736
GC Content	38.89	40.01	38.96	38.99	38.97	40.01	38.97
Integrity	100	98.15	100	100	100	98.15	100
Integrity Assessment	DTR	AAI-based	DTR	DTR	DTR	AAI-based	DTR
Method							
ORFs	145	149	149	147	153	149	153
tRNAs	26	24	25	26	24	24	24
Closest Related Phage	<i>Felixounavirus</i>	<i>Suspvirus</i>	<i>Felixounavirus</i>	<i>Felixounavirus</i>	<i>Felixounavirus</i>	<i>Suspvirus</i>	<i>Felixounavirus</i>
Similarity	88.22	92.2	91.51	90.11	89.91	92.2	89.91
Predicted Family	<i>Chaseviridae</i>	<i>Chaseviridae</i>	<i>Chaseviridae</i>	<i>Chaseviridae</i>	<i>Demereciviridae</i>	<i>Chaseviridae</i>	<i>Demereciviridae</i>

The M8 to M14 phage sequences were assembled and annotated, and the whole genome map was obtained as follows :

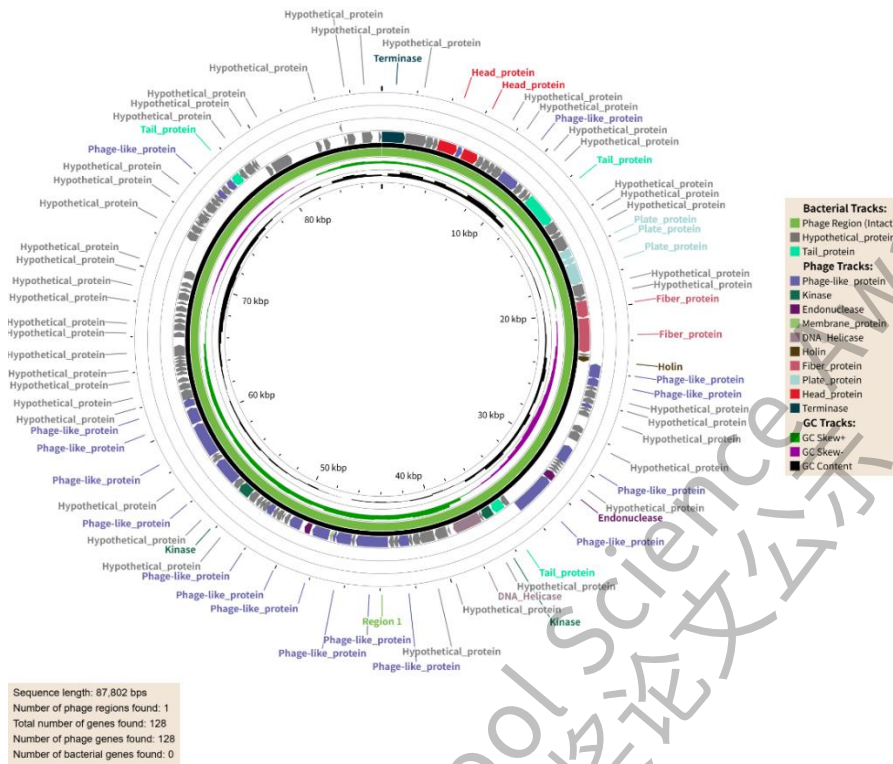


Fig. 3 Whole genome map of M8 phage

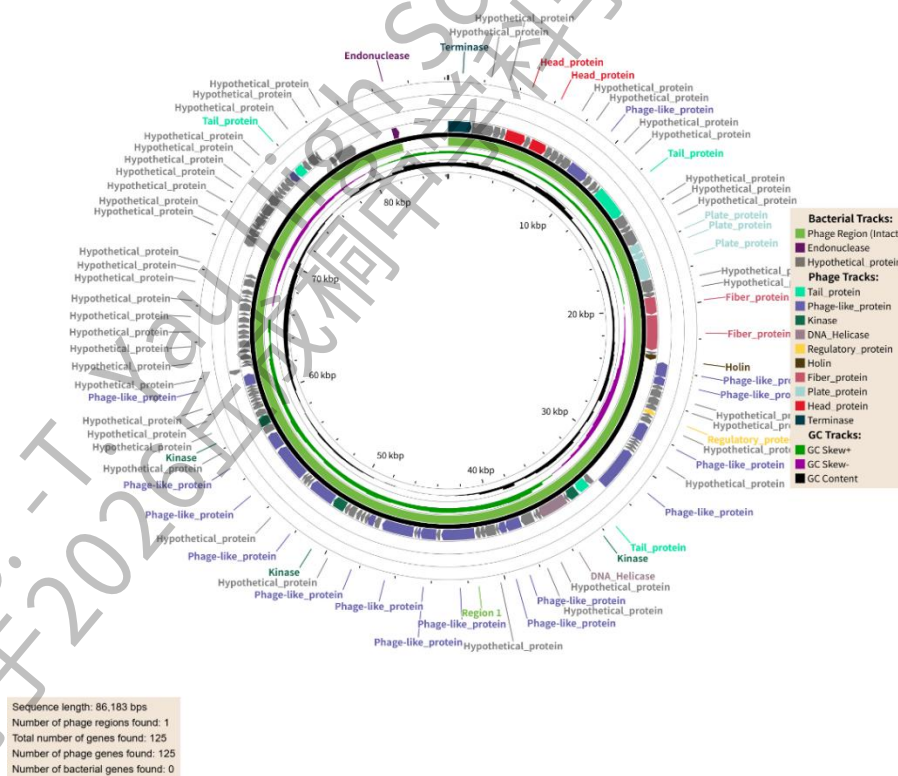


Fig. 4 Whole genome map of M9 phage

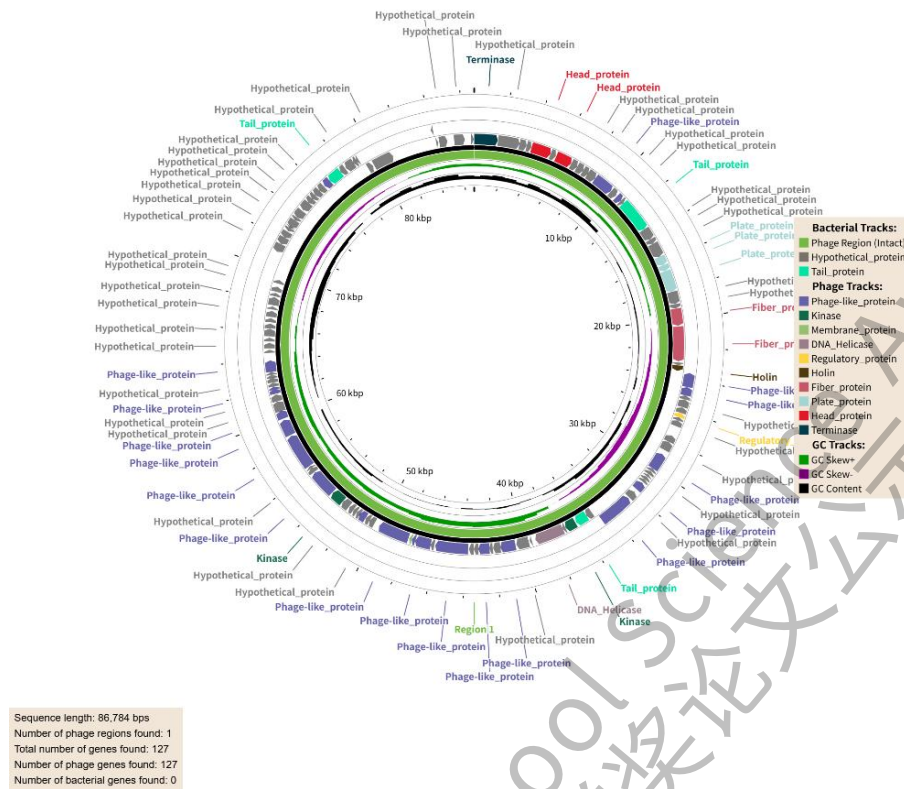


Fig. 5 Whole genome map of M10 phage



Fig. 6 Whole genome map of M11 phage

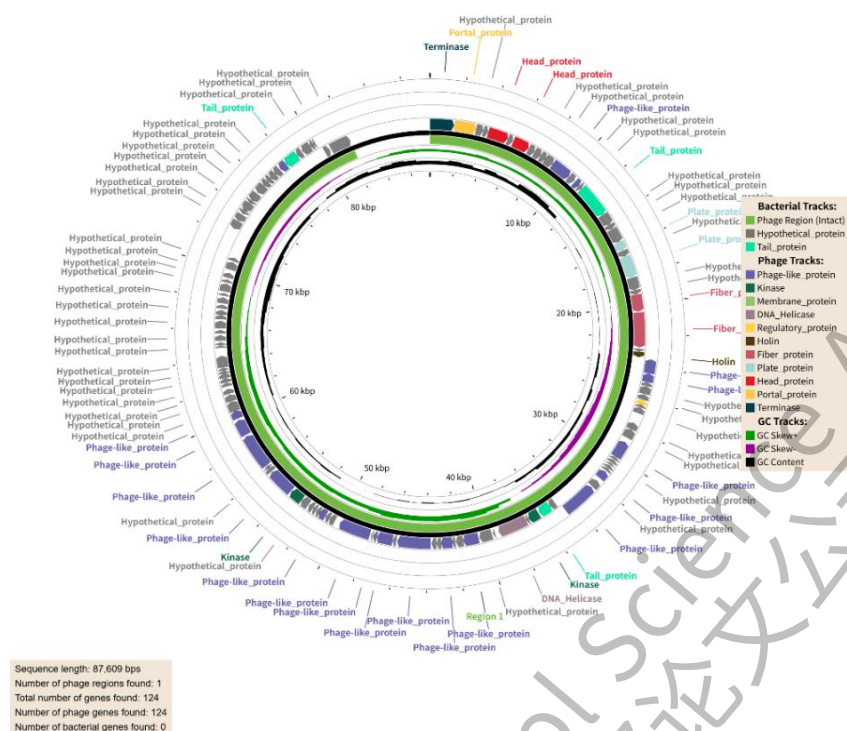


Fig.7 Whole genome map of M12 phage

3.3 Plasmid transformation

When the *E.coli* transformed with pET-28a was coated on the resistance plate, the plate was obtained after 14 hours of culture as follows :

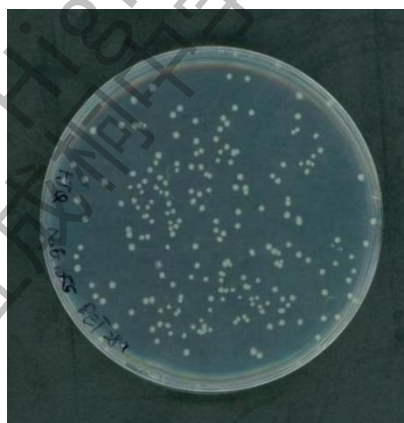


Fig. 8 Spread plate of *E. coli* transformed with pET-28a on a Kan-resistant plate

It was found that the single colony could prove that the pET-28a plasmid was successfully introduced into *E.coli*.

3.4 Determination of minimum inhibitory concentration of kanamycin

Use a microplate reader to measure the OD values of 14 groups with 42 wells after 20 hours of culture, calculate the average OD value of each group, and draw a table as follows:

Table 3 Average OD value of each group

Group	kanamycin concentration ($\mu\text{g/mL}$)	Average OD value of group
1	1200	0.007
2	800	-0.004
3	600	0.244
4	400	0.472
5	300	0.537
6	200	0.634
7	150	0.848
8	100	1.045
9	75	1.058
10	50	1.001
11	37.5	1.058
12	25	1.048
13	0(+)	1.016
14	0(-)	0.000

At the same time, it was observed that the holes in groups 1,2 and 14 were clear, and the holes in groups 3 to 13 were turbid.

Combined with the measurement of OD value and visual observation, the MIC of kanamycin was 800 $\mu\text{g} / \text{mL}$.

3.5 The optimal multiplicity of infection from M8 to M12 was determined.

The average and standard deviation of phage titers at each MOI value of M8 to M12 phages were plotted as follows :

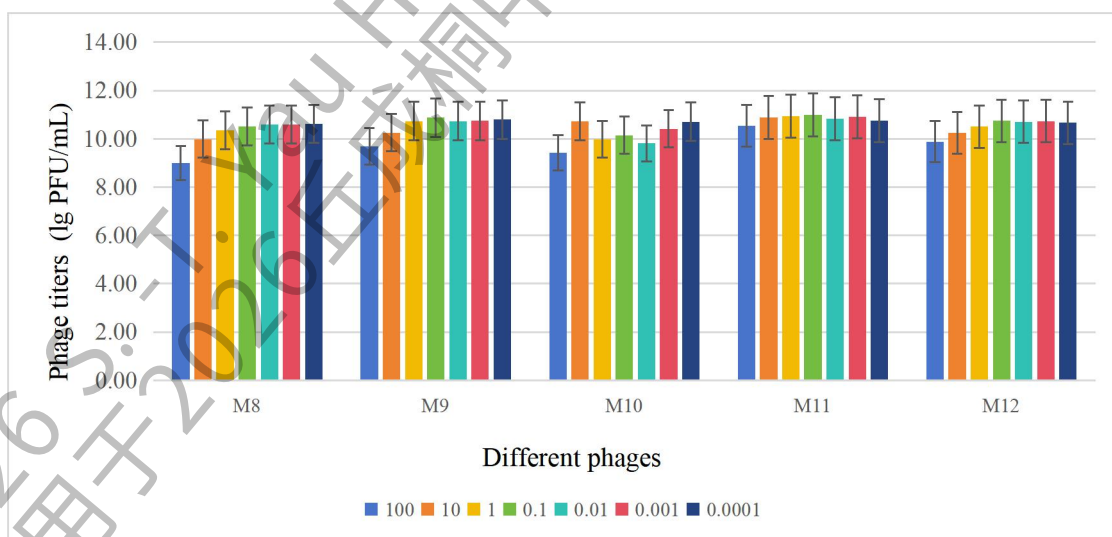


Fig. 9 The titers of different phages at different MOI values

The titers of each phage at different MOIs were in the range of 10^9 to 10^{11} , which was at a high level. The standard deviation is large, and more repetition is needed.

3.6 The best strategy for the combination of M9 and M10 phages with kanamycin

The OD value of 96-well plate after 12 h was measured, and the antibacterial efficiency of each well was calculated, and Table 4 and Table 5 were obtained. Different colors are used to represent the antibacterial efficiency interval, and the antibacterial efficiency heatmap is drawn as shown in Fig.10 and Fig.11.

Table 4 M9 96-well plate antibacterial efficiency table

	1	2	3	4	5	6	7	8	9	10	11	12
A	18%	19%	11%	10%	19%	4%	7%	21%	13%	26%	38%	31%
B	43%	26%	34%	40%	49%	29%	30%	19%	22%	35%	48%	28%
C	14%	16%	-25%	6%	0%	-5%	12%	-7%	21%	3%	27%	15%
D	11%	33%	-1%	19%	27%	8%	22%	26%	-17%	11%	25%	0%
E	10%	6%	12%	-2%	-10%	46%	21%	20%	18%	-1%	23%	-7%
F	24%	19%	-17%	51%	27%	20%	23%	16%	12%	25%	16%	-9%
G	7%	9%	10%	53%	6%	18%	37%	10%	8%	11%	10%	-22%
H	55%	62%	51%	51%	58%	50%	58%	49%	50%	46%	54%	-53%

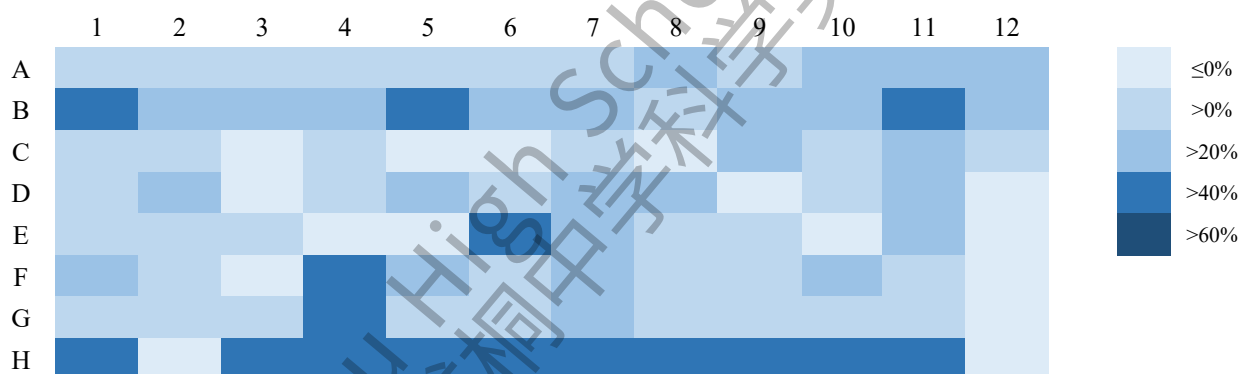


Fig.10 Heatmap of antibacterial efficiency of M9 96-well plate

Table 5 M10 96-well plate antibacterial efficiency table

	1	2	3	4	5	6	7	8	9	10	11	12
A	16%	20%	12%	351%	434%	409%	427%	474%	12%	22%	25%	-4%
B	55%	46%	29%	26%	50%	37%	41%	40%	38%	36%	52%	-6%
C	13%	24%	57%	18%	27%	47%	9%	31%	36%	43%	33%	0%
D	56%	29%	12%	27%	17%	-18%	11%	27%	22%	22%	51%	6%
E	55%	14%	42%	22%	65%	25%	21%	66%	34%	36%	64%	-5%
F	14%	24%	60%	32%	55%	20%	24%	63%	26%	64%	20%	-4%
G	33%	56%	62%	20%	51%	56%	22%	61%	26%	41%	53%	4%
H	70%	33%	68%	66%	67%	67%	69%	69%	68%	67%	69%	-6%

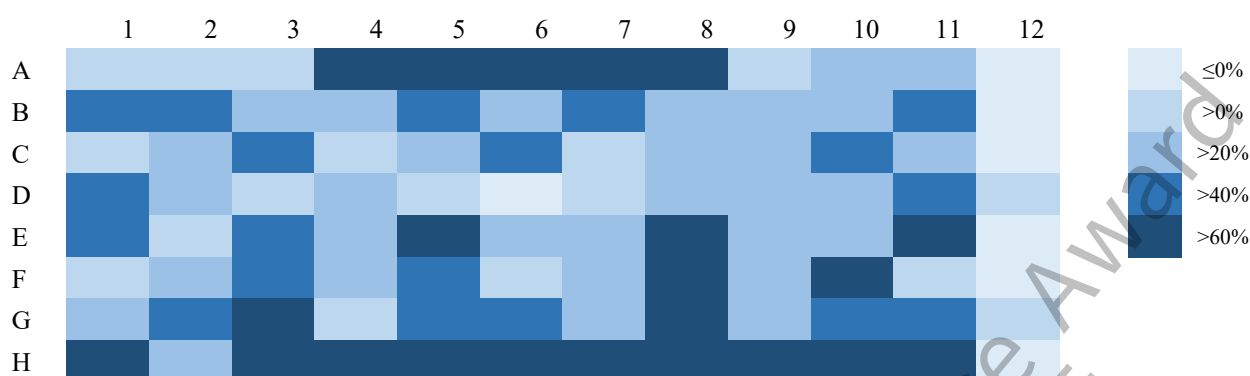


Fig.11 Heatmap of antibacterial efficiency of M10 96-well plate

In the combined application plate of M9 and M10 phages, row H showed high bacteriostatic efficiency, which increased the credibility of the experiment. The antibacterial efficiency of column 12 was low, which ensured the high activity of the strain and rapid growth in MH medium.

3.7 Spot plate analysis of phage receptor binding sites

To preliminarily determine whether the receptors of M9 and M10 depend on O antigen or the LPS core region, this experiment plates M9 and M10 phages separately onto different *E. coli* strains with differing O antigen backgrounds and LPS core region integrity, with results illustrated in Fig.12-16:

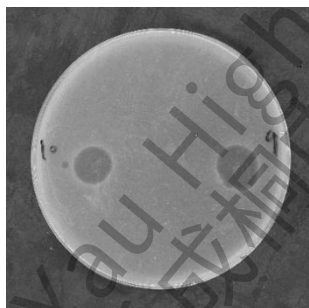


Fig.12 MG1655

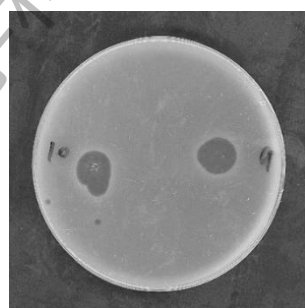


Fig.13 MG1655/p15A-WBBN

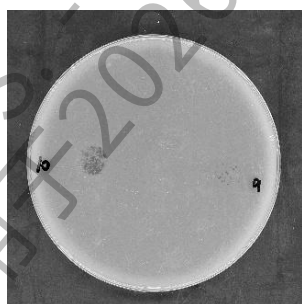


Fig.14 BL21

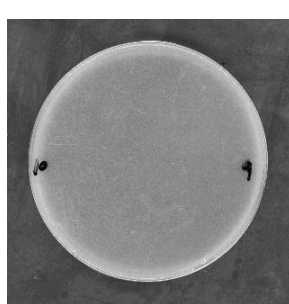


Fig.15 BL21ΔwaaG

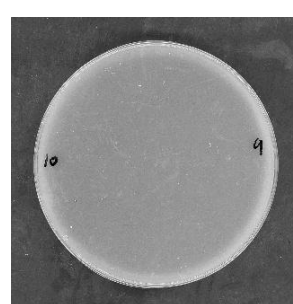


Fig.16 BL21ΔABE

On MG1655 wild-type (Fig.12) and complemented O antigen strain (Fig.13), M9 and M10 can both

form plaques, and there is no significant difference in plaque morphology and size between the two bacterial strains, indicating that the infection of the two phages does not depend on the O antigen.

M10 forms a clear plaque on BL21 wild-type bacteria (Fig.14), while M9 produces a weaker plaque. However, on the BL21 $\Delta waaG$ strain (Fig.15), which has a defect in the outer core of LPS, and the BL21 ΔABE strain (Fig.16), which has a defect in LPS core synthesis, both M9 and M10 plaques completely disappeared. This indicates that an intact LPS core is essential for infection by both phages.

4 Discussions

4.1 Discussion on Phage Sequencing Results

Analysis of basic genomic characteristics showed that all successfully sequenced phages had relatively large genomes and relatively low GC content, which are consistent with members of multiple phage families within the class *Caudoviricetes* under the current ICTV classification system^[17].

Within this framework, the closest related genus of M8, M10, and M11 is *Felixounavirus*, whose type member Felix O1 has a genome of approximately 86 kb and a GC content of about 39%^[18], which is highly consistent with the corresponding phages measured in this experiment (genome 86.4–87.9 kb, GC 38.89–38.99%). The closest related genus of M9 and M13 is *Suspvirus*; the closest related genus of M12 and M14 is the family *Demerecviridae*^[19].

Among them, M9 and M13, as well as M12 and M14, are completely consistent in all independent characteristics such as genome length, GC content, completeness, number of ORFs, number of tRNAs, and sequence similarity with the closest related phages. This high consistency of multiple features strongly indicates that they are respectively repeated isolations of the same phage isolate during the experimental operation. Therefore, in subsequent experiments, we only conducted in-depth studies on the representative M8 to M12 to reduce the workload.

When conducting the combined application experiment of phages and antibiotics, M9 and M10 were selected as typical representatives. They belong to two different groups (*Suspvirus* and *Felixounavirus*), have high genome completeness, and their sequence similarity with their respective closest related phages is the highest in their groups (92.2% and 91.5%, respectively), showing good group representativeness.

4.2 Minimum Inhibitory Concentration of Kanamycin

The MIC of kanamycin was determined by two methods: measuring the OD value with a microplate reader and visually observing whether bacteria grow. The control group provided strong support for the experiment. The OD value of the positive control group was as high as 1.016 and was obviously turbid, proving that the bacteria had vigorous reproductive capacity. The OD value of the negative control group was 0 and was clear, excluding the interference of contamination. When the kanamycin concentrations were 1200 $\mu\text{g/mL}$ and 800 $\mu\text{g/mL}$, the OD values were around 0, indicating that kanamycin could completely

inhibit the growth of *Escherichia coli* at these two concentrations. When the kanamycin concentration was 600 µg/mL, the OD value increased significantly to 0.244. After that, the OD value showed an upward trend with the decrease of kanamycin concentration, rising significantly in groups 3 to 5, and then tending to level off. Visual observation revealed that starting from the third group, the liquid in the wells became turbid, marking the massive growth of bacteria. This indicates that 800 µg/mL is the minimum concentration at which kanamycin can completely inhibit the growth of *E. coli*, so 800 µg/mL was determined as the MIC of *E. coli*.

Numerous studies have concluded^[20,21] that the MIC of Gram-negative bacilli and *E. coli* is usually below 100 µg/mL. The measured MIC value is significantly higher than the normal level, which reflects that *E. coli* may be insensitive or resistant to kanamycin. The abnormally high MIC is caused by the introduction of the pET-28a plasmid.

4.3 Optimal Multiplicity of Infection of Phages M8 to M12

In this study, by comparing the average value and standard deviation of phage titers at different MOI values, the MOIs of phages M8 to M12 were determined to be 1, 10, 0.001, 1, and 0.1, respectively. At these MOIs, the phage titers were higher than those at other MOI values of the same phage, which were 2.3×10^{10} , 1.8×10^{10} , 2.6×10^{10} , 8.7×10^{10} , and 5.5×10^{10} , respectively.

The average titer of most phages showed a trend of first increasing and then decreasing with the decrease of MOI value. At a low MOI, each bacterial cell contacts fewer virus particles, resulting in a reduced infection probability and failure to fully utilize the infection potential of the cells, so the titer is low. When the MOI reaches the maximum value, the vast majority of cells are infected, giving full play to the ability of phages. When the MOI is high, excessive phage particles may lead to the production of toxicity among phages, forcing host cells to lyse prematurely, thereby shortening the viral replication cycle and reducing the total replication amount. At the same time, there may be competition for limited cellular receptors or resources among virus particles, leading to a decrease in infection efficiency.

Determining the MOI value can not only improve efficiency in phage production and application but also provide a reference for the specific dosage of drugs in subsequent combined application experiments.

4.4 Analysis of Phage Receptor Binding Sites

First, the receptor does not depend on O-antigen. In the MG1655 strain, since the K-12 lineage naturally lacks the O-antigen gene cluster^[22,23], the infection status of M9 and M10 phages with and without O-antigen was tested respectively. The results showed that both phages could infect wild-type MG1655 (without O-antigen) and the strain that restored O-antigen after complementing the *WBBN* gene through the p15A vector, indicating that the presence or absence of O-antigen does not affect the receptor binding of the two, and the possibility of O-antigen as a receptor is excluded.

Second, the receptor is located in the intact LPS core region. *waaG* encodes the first glycosyltransferase in the outer core region of LPS, which catalyzes the transfer of UDP-glucose to inner core heptose II; the LPS of the $\Delta waaG$ mutant is truncated at the initiation of the outer core, retaining only the Kdo-inner core heptose structure^[24]. In this experiment, the plaques of M9 and M10 on BL21 $\Delta waaG$ completely disappeared, and the phenotype was consistent with that of the LPS core region synthesis-deficient strain ΔABE , indicating that the infection of both phages completely depends on the intact LPS core region structure.

It should be noted that BL21 belongs to the *E. coli* B lineage, which naturally lacks the O-antigen gene cluster and does not synthesize O-antigen^[25]. Therefore, the observed infection difference in the BL21 group is unrelated to O-antigen, further confirming the judgment that the receptor is located in LPS. The difference between the two phages is reflected in infection efficiency rather than receptor dependence: on BL21 wild-type with intact LPS, M10 forms obvious plaques while M9 has weak plaques; however, both completely lose infectivity in core-deficient strains. This suggests that both depend on the same type of receptor structure (intact LPS core region), but there are differences in receptor utilization efficiency or adsorption capacity.

This result is consistent with recent literature: Kaneko et al. conducted a systematic receptor analysis of 13 *E. coli* phages and showed that phages targeting LPS lose their infectivity after mutations in LPS outer core modifying enzymes (*waaV*, *waaW*, etc.)^[16]; since the *waaG* mutation truncates the initial substrate for outer core synthesis, its effect can cover all phage receptors that depend on the outer core structure. In this experiment, the complete disappearance of plaques of M9 and M10 on the $\Delta waaG$ mutant is consistent with the above mechanism.

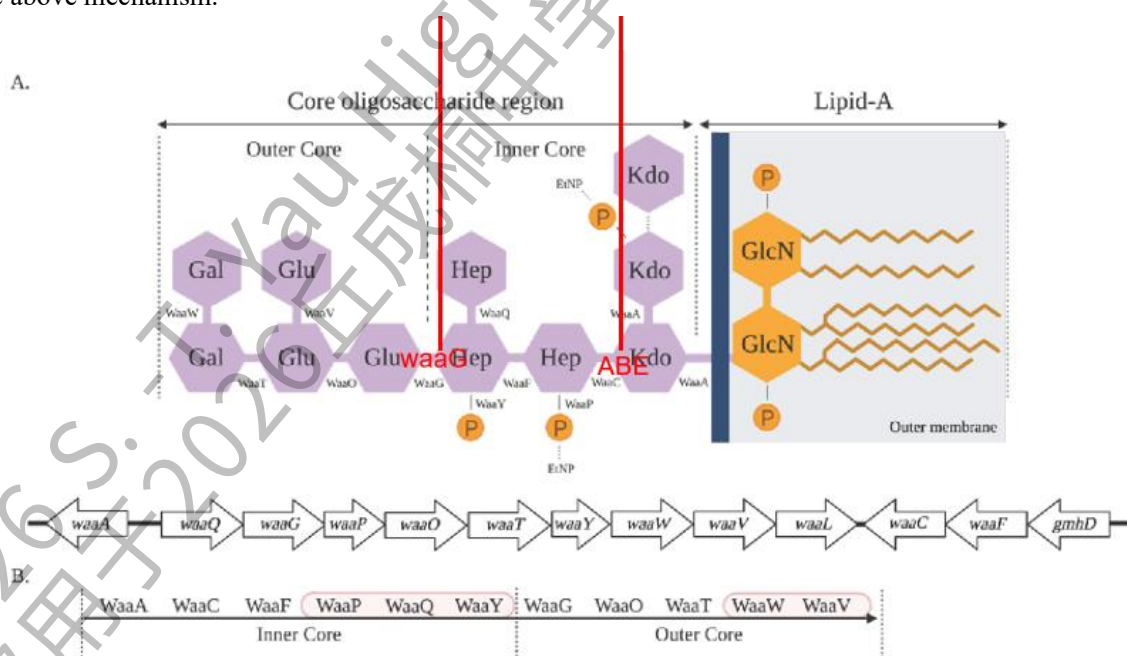


Fig.17 Lipopolysaccharide core oligosaccharide region and lipid A structure diagram

4.5 Study on the Strategy of Combined Application of M9 and M10 Phages with Kanamycin

The combined application of M9 phage at 10^3 PFU/mL and kanamycin at 2 μ g/mL exhibited the highest antibacterial effect. Although B1, B5, and B11 showed relatively high antibacterial efficiency, their distribution was uniform with no obvious synergistic pattern, suggesting that higher antibiotic concentrations may be required to achieve an antibacterial effect.

M10 phage Row A, columns 4–8 show antibacterial efficiency greater than 300%, exceeding the efficiency of columns 1–3 and markedly higher than columns 9–12; this is attributed to experimental dilution errors, instrument inaccuracies, and contamination between wells. Consequently, the data from Row A, columns 4–8 are not considered as corroborating evidence.

The antibacterial efficiency of Row E, column 5 (65%), Row E, column 8 (66%), Row F, column 8 (63%), and Row G, column 3 (62%) is close to or even superior to that of Row H containing only kanamycin (70%). The antimicrobial effects are excellent. While achieving excellent effects, its kanamycin concentration is significantly reduced (estimated at 64–128 μ g/mL, only 1/12–1/8 of MIC), realizing the synergistic effect of "low concentration kanamycin + medium concentration M10". The mechanism of phage–antibiotic synergy lies in: sub-inhibitory concentrations of antibiotics alter bacterial physiological states (such as inhibiting protein synthesis, slowing growth), thereby reducing the proportion of phage-resistant strains in bacterial populations and promoting phage adsorption and proliferation; phage lysis, conversely, can restore bacterial sensitivity to drugs, forming positive feedback synergy^[15,26]. Among these, the group with the highest inhibition efficiency is the combination of M10 phage at 10^6 PFU/mL with 16 μ g/mL kanamycin.

Compared to M10, M9 has lower antibacterial efficiency with a certain difference.

5 Conclusion

5.1 Fourteen strains of *E. coli* MG1655 phages were successfully isolated and their titers were determined, and the optimal MOIs of 5 phage strains were determined.

5.2 The minimum inhibitory concentration of kanamycin was determined and the optimal strategy for its combined application with M9 and M10 phages was explored. It is concluded that there is no obvious synergistic pattern with M9 phage, and the optimal combination is 10^3 PFU/mL phage with 2 μ g/mL kanamycin. It was found that medium-concentration M10 phage and 64 to 128 μ g/mL kanamycin have excellent potential for combined application, and the optimal combination is 10^6 PFU/mL phage with 16 μ g/mL kanamycin.

6 Innovation and Prospects

6.1 Innovations

(1) This experiment introduced a kanamycin resistance plasmid into *Escherichia coli* to simulate

kanamycin-resistant bacteria, which can reduce the demand for pathogenic bacteria and increase safety. The experiment isolated multiple *Escherichia coli* MG1655 bacteriophages and analyzed the optimal strategy for the combined application of multiple bacteriophages and kanamycin.

(2) Using bacterial strains with varying degrees of LPS integrity (MG1655, complemented strain, BL21, $\Delta waaG$), the phage receptor was localized to the intact LPS core region.

6.2 Prospects

(1) Due to time constraints, some combined application experiments were repeated insufficiently, and existing conclusions remain preliminary. Future work will increase the number of repetitions for MOI gradients and combined applications, and introduce pharmacodynamic models to quantitatively distinguish synergy, additivity, and antagonism effects. Additionally, the range of phage varieties will be expanded to test more combinations of *E. coli* phages and kanamycin, examining whether synergistic effects are generally applicable.

(2) This study has positioned the receptor to the complete LPS core region. In the future, the simultaneous effects of changes in LPS structure on phage adsorption and kanamycin uptake through the outer membrane can be directly verified by adsorption kinetics and drug accumulation quantification, indicating that a synergistic effect may exist coupling receptor targeting and drug permeation.

(3) In vitro validation can be first conducted in drug-resistant clinical isolates to confirm whether the combined effect holds true across strains; on this basis, the efficacy and safety of the combination regimen can then be evaluated in animal infection models to accumulate data for clinical applications.

(4) In current international phage research, the accumulation of genome sequences far outpaces receptor identification and phenotypic characterization, leaving a large number of sequenced phages lacking complete experimental information. This study organizes and makes publicly available the genome sequences, receptor localization, and combined-application data of the isolated phages, rendering them searchable and re-analyzable, thereby directly addressing the aforementioned gap.

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