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CRISPR/Cas9-Mediated Genome Editing of T4 Bacteriophage for High-Throughput Antimicrobial Susceptibility Testing

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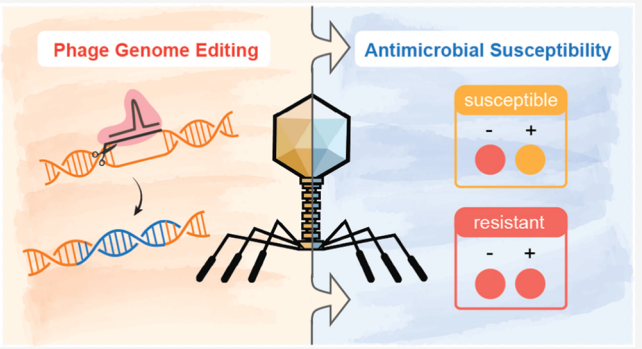
ABSTRACT: The accurate and effective determination of antimicrobial resistance is essential to limiting the spread of infectious diseases and ensuring human health. Herein, a simple, accurate, and high-throughput phage-based colorimetric sensing strategy was developed for antimicrobial susceptibility testing (AST). Taking advantage of the CRISPR/Cas9 system, the genome of the T4 phage was modularly engineered to carry lacZ-α (lacZa), a marker gene encoding the α-fragment of β-galactosidase (β-gal). $T4_{lacZa}$ phages were identified by blue-white selection and then used for a biosensing application. In this strategy, the bacterial solution is exposed to the $T4_{lacZa}$ phage, causing target bacteria to overexpress β-gal. Upon the addition of a colorimetric substrate, the β-gal initiates an enzymatic reaction, resulting in a solution color change from yellow to red. This sensing strategy offers a visual way to monitor bacterial growth in the presence of antibiotics, enabling the determination of bacterial antimicrobial susceptibility. As a proof of concept, our developed sensing strategy was successfully applied to identify 9 different multidrug-resistant *Escherichia coli* (*E. coli*) in urine samples with 100% specificity. Compared with conventional disk diffusion susceptibility tests, the engineered phage-based sensing strategy can shorten the detection time by at least half without losing detection sensitivity, providing an alternative high-throughput method for AST in clinical diagnosis.



INTRODUCTION

The emergence of antimicrobial-resistant bacteria poses a great threat to human health. According to statistics from the Centers for Disease Control and Prevention (CDC), there are over 700,000 deaths per year attributed to antimicrobial resistance.[1−3] It is predicted that the number of deaths may increase to 10 million by 2050 if no effective intervention is taken.[4] In response to this critical challenge, antimicrobial susceptibility testing (AST) has emerged as an indispensable tool. AST not only facilitates the accurate identification of antimicrobial-resistant bacteria but also enables healthcare providers to tailor antibiotic therapies effectively. Moreover, AST is critical to the development of comprehensive public health strategies aimed at mitigating the spread of antimicrobial-resistant bacteria and infectious diseases. Consequently, advancing technologies and innovation in AST remain imperative to effectively address the evolving challenges of antimicrobial resistance.

Currently, conventional culture-based methods are regarded as the gold standard of AST, including broth dilution, disk diffusion, and epsilometer test (E-test).[5−7] The principles of these methods are mainly based on the phenotypic responses of bacteria toward different antimicrobial drugs. Even though culture-based methods provide a straightforward way to determine the viability of bacteria during drug exposure, they are limited by time-consuming incubation. These methods typically take 16−24 h from sample to result, which is not conducive to urgent antibiotic therapy. As alternatives, some genotypic methods are developed to detect known resistance genes without the need for incubation, such as polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), and rolling circle amplification (RCA).[8−10] The genotypic methods greatly shorten the detection time compared to that of culture-based methods. However, the lack of a universal resistant mechanism and the huge genetic variability hinder the widespread applications of genotypic methods. For example, there are more than 800 resistance genes for β-lactam class antibiotics alone.[11] To achieve rapid and sensitive detection, some research developed impedance-based microfluidic platforms for AST.[12−14] For instance, microfluidic impedance cytometry has been constructed to analyze individual bacteria following antibiotic exposure, where

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the measured electrochemical signals reflect the phenotypic response of bacteria to specific antibiotics.[12] This approach reduces detection time to around 30 min, significantly faster than conventional methods. However, the limited number of electrochemical stations and the high cost of electrodes pose challenges for high-throughput AST by using electrical impedance-based techniques. To effectively combat multidrug-resistant bacteria, a high-throughput AST method that leverages the phenotypic response of bacteria to antimicrobial drugs would be highly beneficial for practical applications.

Recently, several studies have utilized engineered bacteriophage (phage) for AST.[15−17] For instance, the T7 phage was engineered to carry the lacZ gene and then used to detect ampicillin-resistant *Escherichia coli* (*E. coli*) strains.[15] Compared with the above-mentioned AST methods, the engineered phage-based method utilizes the phenotypic response of bacteria while shortening the detection time. While the engineered phage holds the potential to improve current AST methods, assembly of specific genes into the phage genome is still a challenge. For now, the recombination engineering method is the most common approach to generate engineering phage *in vivo*.[18] The phage genome was assembled with a cytosolic recombination template through homologous recombination. However, the low recombination frequencies (approximately $10^{-8} - 10^{-5}$) make the isolation of engineered phages a very laborious process.[19−21] As an emerging technology, cell-free transcription−translation (TXTL) has been developed to construct recombinant phages in vitro.[22,23] In this method, PCR is used to amplify overlapping fragments of the phage genome containing the desired mutations. These fragments are then assembled into a complete phage genome using a Gibson assembly. The recombinant phage can then be rebooted through in vitro transcription and translation within a cell-free expression system, bypassing the need for a live bacterial host for phage propagation. While the TXTL method allows multiplex simultaneous mutations and offers high flexibility in phage genome engineering, its efficiency tends to decrease as the genome size increases. This limitation could pose challenges in engineering the phage with a larger genome or incorporating more complex genetic modifications.[24] As an alternative, the clustered regularly interspaced short palindromic repeats (CRISPR) system was developed for engineering the larger phage genome.[25−27] The CRISPR system was designed to recognize and cleave the target region in the phage genome, followed by homologous recombination between the cleaved phage genome and donor plasmid to generate the recombinant phage. Taking advantage of high programmability and efficiency, the CRISPR system integrated with homologous recombinant has become a promising method for phage genome editing.

In this study, we developed a CRISPR/Cas9-mediated gene editing platform for phage engineering. The lacZ-α (lacZa) gene was inserted into the T4 phage genome at a high recombination frequency through the CRISPR/Cas9 system. The recombinant phage ($T4_{lacZa}$ phage) could be screened by blue-white selection and further applied to identify antibiotic-resistant bacteria. The $T4_{lacZa}$ phage-based AST achieves 100% specificity for the identification of 9 different multidrug-resistant *E. coli* in urine samples. The results of the $T4_{lacZa}$ phage-based AST were compared with those of conventional disk diffusion susceptibility tests. To the best of our knowledge, this is the first study to utilize a CRISPR/Cas9-based platform to engineer phage for AST. The developed platform provides a versatile and efficient way for generating and selecting engineered phages, which have great potential in clinical diagnostic and therapeutic applications.

MATERIALS AND METHODS

Bacterial Strains, Phages, and Chemicals. *Escherichia coli* NEB5α (*E. coli* NEB5α) was purchased from New England Biolabs (Ipswich, WA). *E. coli* bacteriophage T4 was purchased from ATCC (Manassas, VA). The T4 ligase, PCR master mix, restriction enzymes, and DNA purification kit were purchased from New England Biolabs (Ipswich, WA). All DNA primers were synthesized by Integrated DNA Technologies (Coralville, IA). The chlorophenol red-β-D-galactopyranoside (CPRG) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Fisher Scientific (Hampton, NH).

Plasmid Construction. The Cas9 plasmid was constructed by cloning the crRNA protospacer sequence into the pCas9 plasmid (Addgene plasmid #42876). The pCas9 plasmid was digested with the BsaI enzyme first and then ligated with the crRNA protospacer sequence. The crRNA protospacer was designed to target the soc gene of T4 phage with the sequence TGTGAACGTCAGAATAAAGA. The oligoes for the construction of crRNA protospacer were listed in Table S1. The ligation products were transformed into *E. coli* NEB5α chemically competent cells for amplification.

The pCRISPR plasmid (donor plasmid) was constructed using a Gibson assembly, which consisted of four parts, the backbone, two homologous arms, and the lacZ-α (lacZa) sequence. All primers for the construction of donor plasmid were listed in Table S1. The backbone of the donor plasmid was amplified from pCRISPR (Addgene plasmid #42875). The homologous arms were cloned from the T4 phage genome. The lacZa sequence was amplified from the M13KE plasmid (New England Biolabs, Ipswich, WA). All fragments were assembled into the donor plasmid using Gibson assembly prior to transformation into *E. coli* NEB5α chemically competent cells. The sequence of all inserts was confirmed by Sanger sequencing.

CRISPR/Cas9-Based Genome Editing for T4 Bacteriophage. *E. coli* NEB5α cell containing both Cas9 plasmid and donor plasmid was used for genome editing of T4 phage. The wild-type *E. coli* NEB5α cell was used as a control. One single colony of *E. coli* NEB5α containing Cas9 plasmid and donor plasmid was inoculated into LB media (containing 25 μg/mL chloramphenicol and 50 μg/mL kanamycin) and incubated at 37 °C overnight. The overnight culture (300 μL) was mixed with wild-type T4 phage (100 μL, 10^4 PFU/mL) and then incubated for 7 min at 37 °C. After that, the top agar (3 mL, with 25 μg/mL chloramphenicol and 50 μg/mL kanamycin) was added into the bacteria-phage mixture and then poured onto an LB agar plate containing chloramphenicol and kanamycin. The plate was incubated at 37 °C overnight. The efficiency of plating was calculated by dividing the number of phages produced from the infection of *E. coli* NEB5α containing both the Cas9 plasmid and donor plasmid by the number of phages generated from the infection of wild-type *E. coli* NEB5α. The recombinant phages were identified by blue-white selection and Sanger sequencing.

Transformation One-Step Phage Growth. A one-step phage growth experiment was carried out as previous report.[28] In brief, the *E. coli* NEB5α (2×10^8 CFU/mL) at the log phase was infected with T4 phages (2×10^7 PFU/mL) at 37 °C for 5

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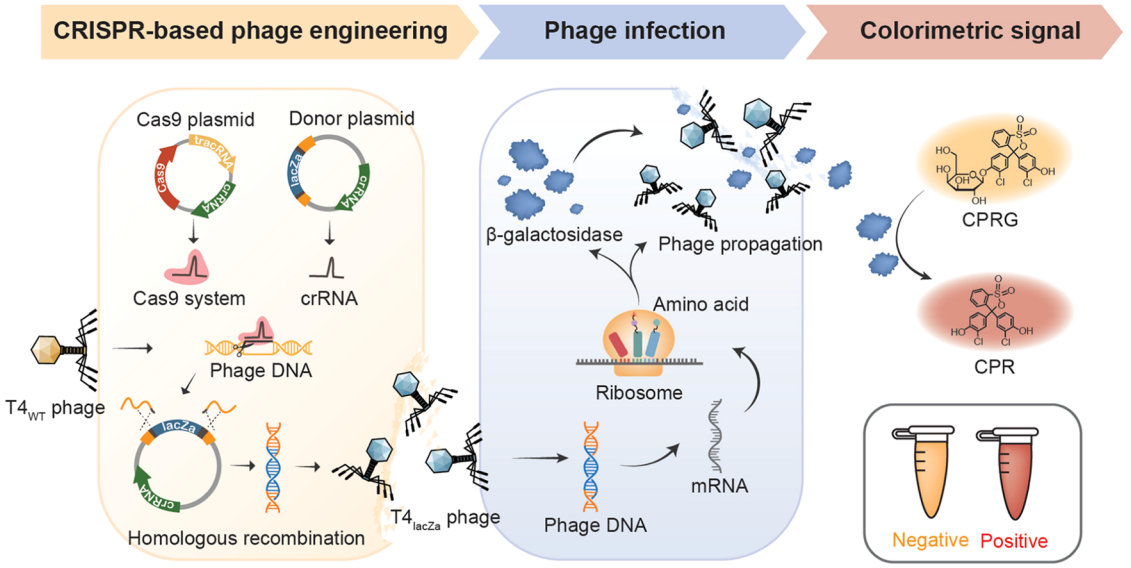

Figure 1. Overview of CRISPR/Cas9-mediated genome editing of T4 phage for colorimetric detection of bacteria. (1) The $T4_{WT}$ phage is engineered with a lacZa gene through the CRISPR/Cas9 system. The *E. coli* strain containing both Cas9 plasmid and donor plasmid express Cas9/sgRNA complex *in vivo*. During the phage infection, the phage genome is cleaved by the Cas9/sgRNA complex and then triggers the homologous recombination between the broken phage genome and homologous arms of the donor plasmid, producing the recombinant phage ($T4_{lacZa}$ phage). (2) The $T4_{lacZa}$ phage is used for further detection of bacteria. The infection of the $T4_{lacZa}$ phages triggers phage propagation and overexpression of β-galactosidase (β-gal). Subsequently, both $T4_{lacZa}$ phage and β-gal are released upon cell lysis. (3) The detection signal is generated under the enzymatic reaction between β-gal and chlorophenol red-β-D-galactopyranoside (CPRG). The detection results can be determined through colorimetric signals.

min. The bacteria-phage infection mixture was centrifuged (10,000*g*, 2 min) and washed with an LB medium to remove the unabsorbed phage. The infection mixture was diluted 10^5 times and incubated at 37 °C under agitation of 250 rpm. The number of infection centers is determined by the amount of phage in the initial diluted mixture. Every 5 min, samples were withdrawn from the infection mixture and measured by plaque assay. Burst size is defined as the amount of progeny phage produced per infection center.

Colorimetric Detection of Bacteria Using $T4_{lacZa}$ Phage. The bacteria solutions (10^6 CFU/mL) were added into LB broth and preincubated at 37 °C for 3 h with an agitation of 250 rpm. Afterward, the chlorophenol red-β-D-galactopyranoside (CPRG) solution (24 mM) and $T4_{lacZa}$ phage (10^3 PFU/mL,) solution were added and reacted for another 5 h. The absorbance intensity of different solutions was measured every hour at a wavelength of 573 nm using a Synergy HTX multimode reader (BioTek Instruments, Winooski, VT).

Antimicrobial Susceptibility Testing (AST) for Urine Samples. Normal human urine was purchased from BioIVT (Westbury, NY) and filtered by a 0.22 μm pore size membrane. The *E. coli* NEB5α was transformed with plasmids carrying different antibiotic resistance genes, including the AmpR for ampicillin, KanR for kanamycin, SmR for spectinomycin, CmR for chloramphenicol, and GmR for gentamicin. To simulate the application scenarios, the *E. coli* NEB5α (1×10^6 CFU/mL) with different antibiotic resistance genes were spiked into the normal human urine sample and distributed into six groups within a 96-well plate. Each group was treated with a different antibiotic: 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, 10 μg/mL gentamycin, 50 μg/mL kanamycin, or 50 μg/mL spectinomycin. The sterile water was used as a negative control. The samples were then preincubated at 37 °C with agitation at 250 rpm for 3 h. The following steps were the same as the colorimetric detection of bacteria using $T4_{lacZa}$ phage. The wild-type *E. coli* NEB5α without any antibiotic resistance gene was used as a positive control.

Disk Diffusion Susceptibility Test. Five different antibiotic disks were prepared by dropping a certain amount of antibiotic (10 μg ampicillin; 5 μg spectinomycin; 5 μg kanamycin; 2.5 μg chloramphenicol; or 1 μg gentamicin) onto the filter paper disk and drying it at room temperature.[29] The fresh cultures of multidrug-resistant *E. coli* NEB5α (200 μL) were plated on LB agar plates individually. The antibiotic disks were then fixed to the surface of the LB agar plates using sterile tweezers. The plates were incubated at 37 °C for 18 h. The filter paper disk without antibiotics was regarded as a control.

RESULTS AND DISCUSSION

Principle of T4 Phage Genome Editing Using CRISPR/Cas9 System. As shown in Figure 1, the principle of CRISPR/Cas9-mediated T4 phage genome editing and the detection of bacteria are illustrated. The wild-type T4 phages ($T4_{WT}$) are engineered with a lacZa gene through the CRISPR/Cas9 system, generating $T4_{lacZa}$ phages. After that, the $T4_{lacZa}$ phages are used to infect bacteria, resulting in the overexpression of β-galactosidase (β-gal) *in vivo*. Upon cell lysis, β-gal is released from bacteria and generates the detection signal with the colorimetric substrate.

The CRISPR/Cas9 system consists of two plasmids: Cas9 plasmid and the donor plasmid. The Cas9 plasmid includes the coding sequence of Cas9 nuclease, *trans*-activating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA) targeting the phage genome. The donor plasmid contains the lacZa gene flanked by homologous arms along with the crRNA cassette.

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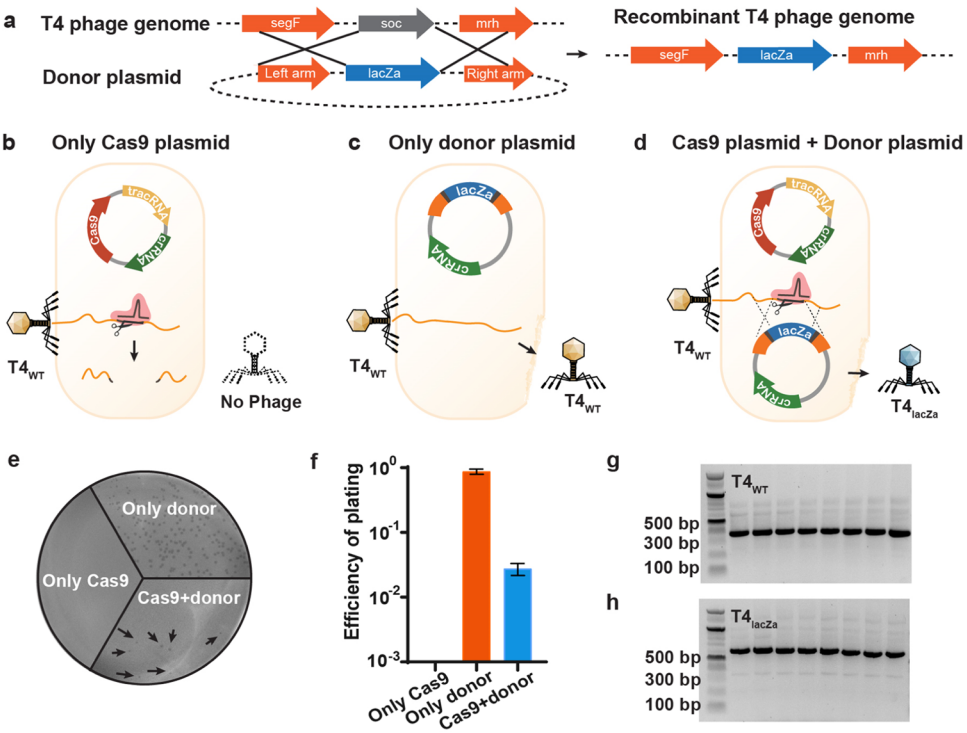

Figure 2. Construction of $T4_{lacZa}$ recombinant phages by CRISPR/Cas9 system. (a) Schematic illustration of homologous recombination. (b) $T4_{WT}$ phage infection of *E. coli* containing only Cas9 plasmid. (c) $T4_{WT}$ phage infection of *E. coli* containing only donor plasmid. (d) $T4_{WT}$ phage infection of *E. coli* containing both Cas9 plasmid and donor plasmid. (e) Phage plaques from various infection scenarios. (f) Efficiency of plating of various infection scenarios. (g) Characterization of phage plaques after the infection with *E. coli* containing only donor plasmid. (h) Characterization of phage plaques after the infection with *E. coli* containing both Cas9 plasmid and donor plasmid.

Two plasmids are cotransformed into *E. coli* NEB5α and then express CRISPR system (Cas9:tracrRNA:crRNA complex) *in vivo*. Upon infection with the $T4_{WT}$ phage, the CRISPR/Cas9 system recognizes and cleaves the phage genome. This cleavage can be repaired through homologous recombination with donor plasmid, leading to the integration of the lacZa sequence into the phage genome. The recombinant $T4_{lacZa}$ phage is generated and purified for further biosensing applications.

The lacZa gene encodes the α-fragment of β-galactosidase (β-gal), a glycoside hydrolase enzyme commonly utilized as an indicator in enzymatic reactions.[30] Consequently, an enzymatic biosensor is developed using the $T4_{lacZa}$ phage. Engineered to function as a sensing element, the $T4_{lacZa}$ phage specifically targets viable *E. coli*. During infection, the $T4_{lacZa}$ phages attach to bacteria and insert their genome into the bacteria cell. Afterward, the inserted DNA takes over bacterial fundamental mechanisms to express the phage particles, inducing the overexpression of β-gal. Following the lysis cycle, the cell membranes are destroyed, releasing the phages and β-gal. In the presence of the colorimetric substrate (chlorophenol red-β-D-galactopyranoside, CPRG), β-gal catalyzes the hydrolysis of CPRG to result in a color change from yellow to red. The absorbance measurement of the reaction solution allows us to derive the colorimetric signal and assess bacterial growth.

Construction of $T4_{lacZa}$ Recombinant Phage. For the CRISPR/Cas9-mediated gene editing, the Cas9:tracrRNA:crRNA complex functions as a molecular scissor, targeting and cleaving the phage genome. Subsequently, the donor plasmid facilitates the repair of the cleaved genomes through homologous recombination. Homologous recombination is a genetic process characterized by the exchange of nucleotide sequences between two DNA molecules that share a significant similarity or identity. As shown in Figure 2a, the *soc* gene is selected as the target for gene editing, wherein it is replaced by the lacZa gene utilizing the CRISPR/Cas9 system. The *soc* gene encodes the small outer capsid protein, a nonessential protein of T4 phage, with approximately 870 copies per capsid.[31] The donor plasmid was designed to contain the lacZa gene flanked by approximately 1000 bp homologous arms derived from the sequence adjacent to the *soc* gene (SegF and Mrh). Using the CRISPR/Cas9 system, the *soc* gene was substituted with the lacZa gene within the phage genome, resulting in the overexpression of the β-gal protein during phage infection.

Based on the mechanism of the CRISPR/Cas9 system, three infection scenarios were compared in Figure 2b–d. First, if the bacteria possess only the Cas9 plasmid, the Cas9:tracrRNA:crRNA complex would cleave the $T4_{WT}$ phage genome upon infection (Figure 2b). As a result, none of the phages would survive in the plaque assay. Conversely, in the presence of only the donor plasmid, the bacteria would lack resistance to phage infection. All phages could survive after phage infection, and most of them retain the wild-type genome (Figure 2c). In this case, it is important to note that recombinant phages may be generated through homologous recombination between the phage genome and the donor plasmid. Previous research indicates that the efficiency of spontaneous homologous recombination ranges from 10^{-8} to 10^{-5}.[19] Finally, if the bacteria contain both the Cas9 plasmid and the donor plasmid, the Cas9 system would facilitate the replacement of a targeted phage gene with the lacZa gene from the donor plasmid. In this case, only recombinant phages carrying the lacZa gene would survive in plaque assay (Figure 2d).

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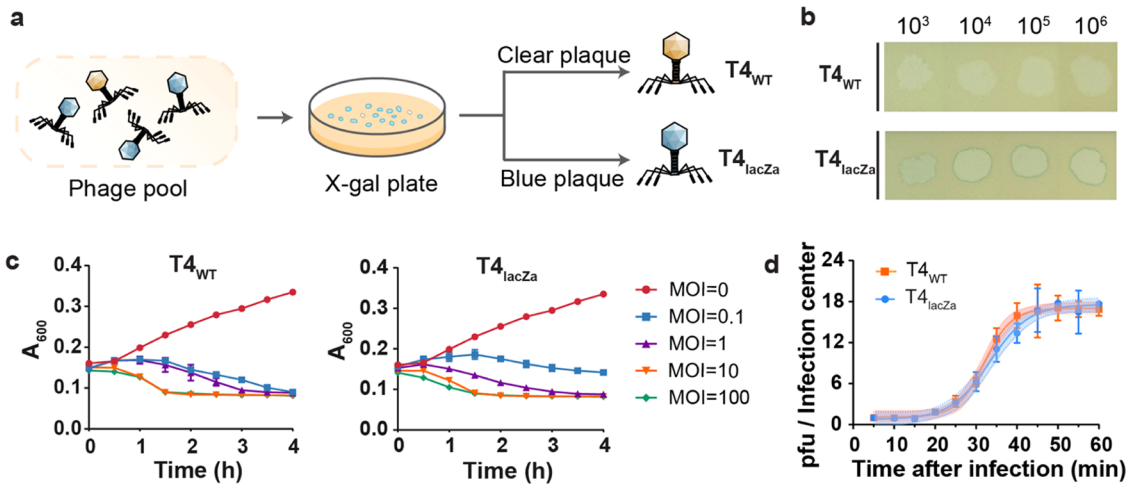

Figure 3. Characterization of $T4_{lacZa}$ recombinant phages. (a) Workflow for blue/white selection of engineered phages. (b) Photograph of blue/white selection for engineered phages. (c) Inhibitory effect of the $T4_{WT}$ and $T4_{lacZa}$ phage on *E. coli* cells. (d) One-step growth curve of $T4_{WT}$ and $T4_{lacZa}$ phage.

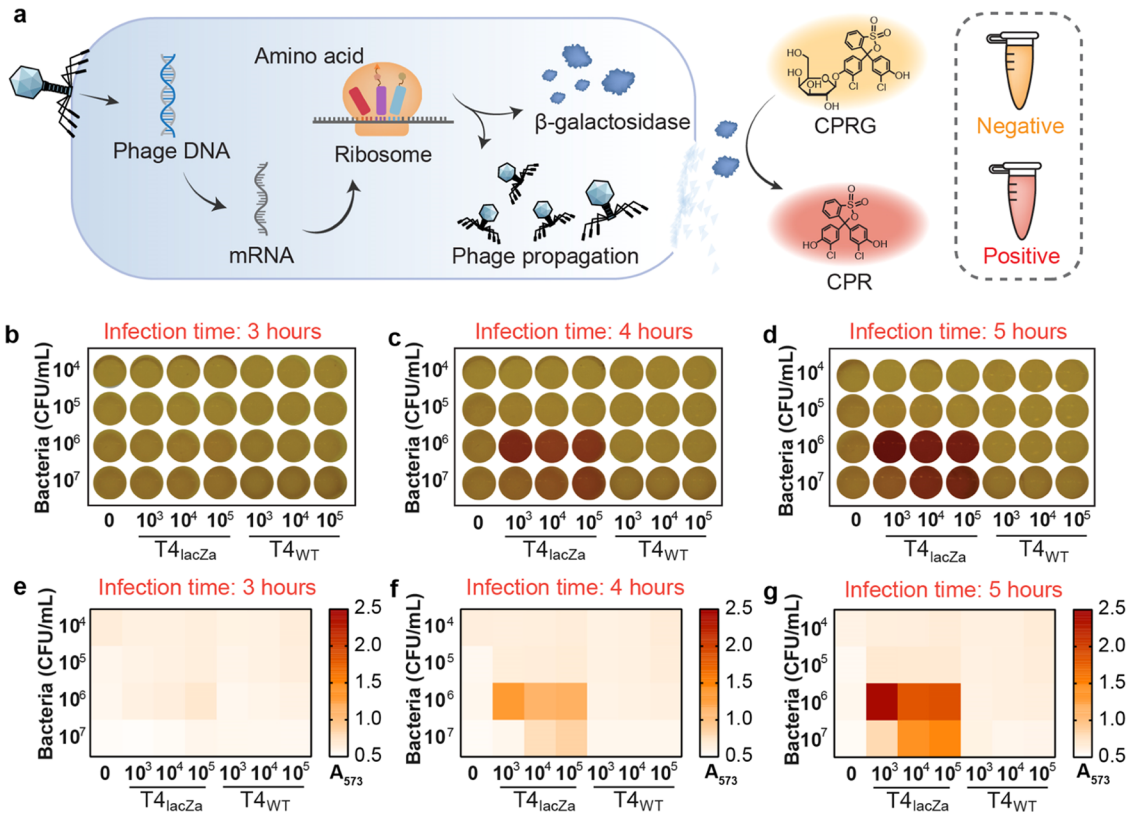

Figure 4. Colorimetric detection of *E. coli* using $T4_{lacZa}$ phages. (a) Schematic illustration of the principle of the colorimetric biosensor for the detection of *E. coli*. Digital photographs of the reaction solution for 3 h (b), 4 h (c), and 5 h (d) of infection. Heatmaps of colorimetric response for 3 h (e), 4 h (f), and 5 h (g) of infection. Colorimetric response based on absorbance intensities at 573 nm (A_{573}).

The plaque assay and polymerase chain reaction (PCR) were used to verify the proposed hypothesis. $T4_{WT}$ phages were incubated with *E. coli* NEB5α processing various plasmid combinations. Subsequently, the top agar was added to the mixture and poured on the LB plates. As shown in Figure 2e, no plaque was observed after the T4 phage infected the bacteria containing only Cas9 plasmid, demonstrating that all phages were unable to survive due to cleavage by Cas9:tracrRNA:crRNA complex. In contrast, when the T4 phage infected the bacteria containing only the donor plasmid, a significant number of plaques were produced. The efficiency of plating was calculated by dividing the number of plaques produced from the bacteria containing various plasmid combinations by the number of plaques produced from the bacteria without any plasmid. It was found that the efficiency of plating was 87.3% during the infection of bacteria containing only donor plasmid (Figure 2f). Eight plaques were randomly selected from the plate for PCR (Figure 2g). It is shown that all plaques kept the wild-type genome, which was further confirmed by Sanger sequencing. Furthermore, the plaque

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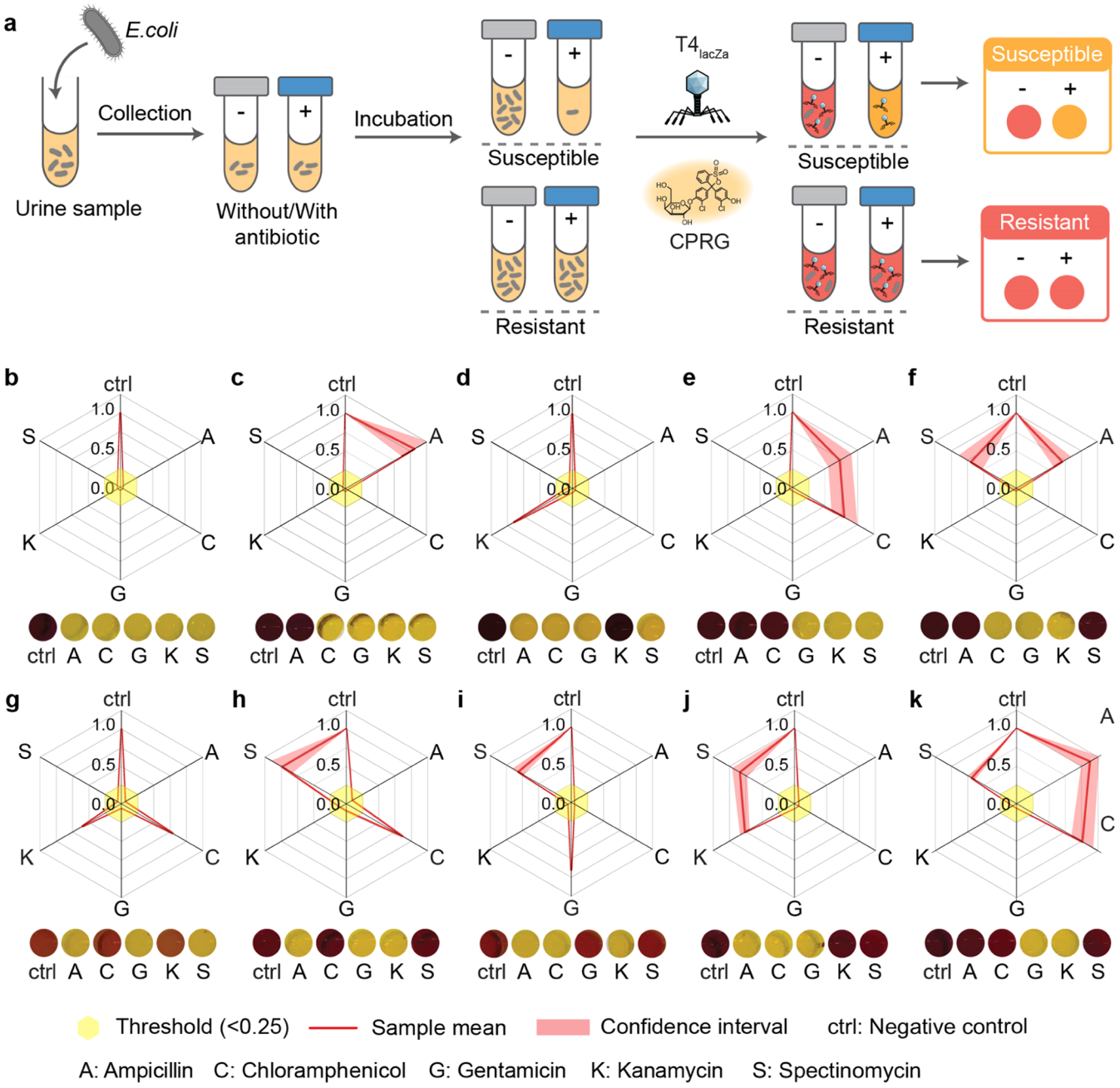

Figure 5. High-throughput antimicrobial susceptibility testing using $T4_{lacZa}$ phages. (a) Workflow of the colorimetric AST in urine samples. (b–k) Photographs and radar chart of antimicrobial susceptibility testing results. The bacteria from left to right: (b) wild-type *E. coli* (positive control), (c) ampicillin-resistant *E. coli*, (d) kanamycin-resistant *E. coli*, (e) ampicillin- and chloramphenicol-resistant *E. coli*, (f) ampicillin- and spectinomycin-resistant *E. coli*, (g) kanamycin- and chloramphenicol-resistant *E. coli*, and (h) chloramphenicol- and spectinomycin-resistant *E. coli* (i) gentamicin- and spectinomycin-resistant *E. coli* (j) kanamycin- and spectinomycin-resistant *E. coli* (k) ampicillin-, chloramphenicol- and spectinomycin-resistant *E. coli*. The value is calculated by dividing the A_{573} of the experimental group by the A_{573} of the negative control group. The concentration of bacteria was 10^6 CFU/mL, while the concentration of phage was 10^3 PFU/mL. All experiments replicated three times.

assay results were investigated when the T4 phage infected the bacteria containing both Cas9 and donor plasmids. The efficiency of plating is 2.7%, which is about 32 times lower than that of the bacteria carrying only donor plasmid. Similarly, the PCR and sequencing results demonstrated that 8 randomly selected phage plaques contain the lacZa gene (Figure 2h). All of these results indicate that the $T4_{WT}$ phage was successfully engineered into the $T4_{lacZa}$ phage using the CRISPR/Cas9 system.

Characterization of Recombinant Phages. Leveraging the unique characteristics of the lacZa gene, a blue-white selection was designed to rapidly differentiate the $T4_{lacZa}$ phage from the $T4_{WT}$ phage. The blue-white selection relies on the enzymatic reaction between β-gal and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), an organic compound consisting of galactose linked to a substituted indole. In the presence of the $T4_{lacZa}$ phage, the overexpressed β-gal hydrolyzes X-gal, releasing the substituted indole. This indole then spontaneously dimerizes, forming an insoluble, intensely

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blue product in the phage plaque. Using the blue-white selection method, the $T4_{lacZa}$ phage can be visually identified on the X-gal plate (Figure 3a). The plaques of the $T4_{WT}$ phage and $T4_{lacZa}$ phage at different concentrations on the X-gal plate are shown in Figure 3b. It is observed that all $T4_{WT}$ phages formed clear plaques, whereas all $T4_{lacZa}$ phages produced blue plaques. Subsequently, phage plaques from different infection scenarios were transferred onto X-gal plates for blue-white selection. In each infection scenario, 24 plaques were selected. Figure S1 demonstrates that plaques generated from bacteria containing only donor plasmid exhibit colorless plaques, whereas those picked from bacteria containing dual plasmids (Cas9 plasmid and donor plasmid) appeared blue, indicating that all recombinant phages carried the lacZa gene. Therefore, the blue-white selection method is a sophisticated approach that effectively addresses the issues of time-consuming and labor-intensive processes during recombinant phage screening. In addition, although we did not observe any blue plaques when screening phages generated from the bacteria containing only the donor plasmid, there remains a very slight possibility (frequencies range from 10^{-8} to 10^{-5}) that phages could be produced through spontaneous homologous recombination.[19]

The infection efficiency of T4 phage was then evaluated by the cell density at different multiplicity of infection (MOI), defined as the ratio of phage to host bacteria. Various concentrations of phages were individually mixed with *E. coli* cells (10^8 CFU/mL) and incubated for 4 h. Absorbance intensities at 600 nm (A_{600}) were recorded every 30 min. As shown in Figure 3c, the $T4_{WT}$ and $T4_{lacZa}$ phages exhibited similar infection responses. The infection efficiency increased with a higher MOI value. Additionally, a one-step growth curve experiment was performed to compare the infectivity between the $T4_{WT}$ and $T4_{lacZa}$ phages (Figure 3d). Interestingly, the $T4_{lacZa}$ phage generated a similar burst size of progeny phage as the $T4_{WT}$ phage. These results demonstrated that the *soc* gene is a nonessential gene on the T4 phage genome. Moreover, the engineered phage can incorporate functional protein without losing infectivity, making it an ideal sensing element for biosensing methods.

Colorimetric Detection of Bacteria Using $T4_{lacZa}$ Phage. Taking inspiration from previous results, we aimed to design an engineered phage-based biosensor leveraging the unique properties of the $T4_{lacZa}$ phage. As illustrated in Figure 4a, with the assistance of the $T4_{lacZa}$ phage, a colorimetric biosensor was established between viable bacteria and detection signals. Upon attachment to the bacteria, the $T4_{lacZa}$ phage injected its genome into the bacterial cytoplasm, triggering the overexpression of phage particles within the host cell. This process includes the expression of lacZa, the gene encoding β-gal. Following a lytic cycle, progeny phages and β-gal were released from the host cell, initiating the enzymatic reaction with the colorimetric subtract (CPRG). As the galactoside analog, CPRG was degraded into galactose and chlorophenol red, leading to a solution color change from yellow to red. The resulting color change serves as a visual indicator of bacterial presence, providing a sensitive and reliable detection method for various applications, including viable bacterial detection and antimicrobial susceptibility testing (AST).

Infection time is one of the main factors that influence the colorimetric results of the engineered phage-based biosensor. The bacteria were pre-enriched at different concentrations in a 96-well plate for 3 h. Subsequently, the CPRG substrate and phages at different concentrations were added to the bacteria and the samples were incubated under agitation. The absorbance at 573 nm (A_{573}) and the photograph of 96-well plates were collected every hour. The $T4_{WT}$ phage is regarded as the control. As the infection time increased, the color of the bacteria-phage solution changed from yellow to red, reaching a dark red after the incubation of 5 h (Figure 4b–d). Therefore, the infection time was optimized to be 5 h.

The concentration of the phage and bacteria is the other key factor of the colorimetric biosensor. The phages at high concentrations could rapidly kill all bacteria in early lytic cycles, resulting in a lower colorimetric signal. Conversely, a low phage concentration may require more time to infect the bacteria and produce sufficient β-gal for colorimetric detection. Therefore, there is a trade-off between the bacteria and phage concentrations. Various combinations of bacteria and phages were investigated during infection to find the optimal balance. The heatmaps in Figure 4e–g visualized the absorbance of the different bacteri-phage combinations. It was found that the optimized bacteria concentration is 10^6 CFU/mL while the optimized phage concentration is 10^3 PFU/mL.

Antimicrobial Susceptibility Testing (AST) for Urine Samples. Since the emergence of multidrug-resistant strains, the treatment of bacterial infections (such as urinary tract infections) with empirical antimicrobial therapies is becoming increasingly challenging. Disk diffusion, a conventional method for antimicrobial susceptibility testing (AST), has been widely utilized to determine susceptibility in acute urinary tract infections. However, its lengthy culturing time (>16 h) delays prompt antimicrobial therapy. To shorten the detection time of AST, the engineered phage-based colorimetric biosensor was applied to detect multidrug-resistant bacteria in urine samples.

The workflow of colorimetric AST is depicted in Figure 5a. In brief, the bacteria were spiked into a urine sample and pre-enriched with antibiotics for 3 h. Following exposure to antibiotics, antibiotic-resistant bacteria survive, whereas antibiotic-susceptible bacteria perish. After that, the $T4_{LacZa}$ phage and CPRG were added to the bacterial sample, triggering the colorimetric reaction. The bacteria grow in the urine sample without any antibiotic as a negative control (the group labeled with "-" in Figure 5a). Based on this, the susceptibility of bacteria was determined by the different colors between the control and experiment groups. For susceptible strains, after being incubated with antibiotics, the inactive bacteria would be unable to trigger the color change with the $T4_{lacZa}$ phage and CPRG, resulting in a significant difference in solution absorbance compared to the negative control.

The feasibility of an optimized colorimetric AST was demonstrated for 9 antibiotic-resistant bacteria in urine samples, including 2 single-drug-resistant bacteria and 7 multidrug-resistant bacteria. Five antibiotics broadly used in the therapy for bacterial infections (ampicillin, chloramphenicol, gentamicin, kanamycin, and spectinomycin) were chosen to provide different resistance profiles. The sterile water was used as the negative control for each bacterium. Plasmids containing antibiotic-resistant genes were transformed into bacteria to confer resistance. The bacteria without any antibiotic resistance were chosen as a positive control. After the antibiotic exposure and phage-based colorimetric detection, we took pictures and recorded the A_{573} for each reaction solution. The detection signal was calibrated by dividing the A_{573} of the experiment group by that of the negative control

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group. To minimize the background noise, the threshold was set to 0.25. Values below 0.25 were interpreted as negative results, indicating the susceptibility of the bacteria to the antibiotic. In contrast, values above 0.25 were classified as positive results, showing the resistance of the bacteria to the antibiotic. The wild-type *E. coli*, which lacks any antibiotic resistance genes, was used as the positive control in the experiment. The radar chart in Figure 5b shows that the positive control group only has signal response in the absence of antibiotics. In contrast, when *E. coli* carries the ampicillin-resistant gene, the engineered phage-based biosensor generated significant colorimetric signals in both the control channel and the ampicillin channel (Figure 5c). Based on this mechanism, the proposed colorimetric biosensor successfully determined 9 antibiotic-resistant bacteria with 100% specifically (Figure 5d−k). Furthermore, the disk diffusion method was performed for the same antibiotic-resistant bacteria in urine samples (Figure S2). The inhibition radius of antibiotic disks is presented in Figure S3. Based on the inhibition radius generated through the negative control group, values larger than 1.5 mm indicate susceptibility toward the corresponding antibiotic. The bacterial susceptibility pattern using the disk diffusion method perfectly matched the previous phage-based results, proving the reliability of the engineered phage-based colorimetric method. However, there are key differences between the two methods. The disk diffusion method is a signal-off approach, where inhibition zones are formed only when bacteria are sensitive to the antibiotic, resulting in a clear area around the disk. In contrast, our proposed biosensor is a signal-on method, generating a detectable colorimetric signal only when bacteria are resistant to the antibiotic. This makes the signal-on method more straightforward for screening antibiotic-resistant bacteria, as it directly indicates resistance without requiring manual measurement of the inhibition zones. Furthermore, the colorimetric signal is visually clearer, eliminating the potential for human error in the radius measurement. Importantly, our colorimetric biosensor also reduces the detection time by at least half compared to the disk diffusion method without compromising specificity, making it a more efficient and user-friendly option for clinic applications.

Although the engineered phage-based detection method has been successfully applied in AST, its sensitivity and efficiency are limited by the expression level of the lacZa gene in the T4 phage. In the future, there are many strategies that could be used to address this issue. First, multiplex gene editing could enhance the expression of the lacZa gene. In this study, we chose the soc gene locus for phage gene editing as a demonstration. Another potential target is the hoc gene, which encodes a nonessential capsid protein and is commonly used in phage display.[32] Inserting the lacZa gene at both the hoc and soc loci using the CRISPR/Cas9 system could increase the copy number of the lacZa gene, thereby boosting β-gal production during phage infection. Second, optimizing the reaction process could reduce detection time. Currently, our AST protocol involves a two-step process: bacterial preenrichment followed by phage infection. Given that the T4 phage lytic cycle is approximately 30 min,[33] similar to the *E. coli* replication cycle (30−40 min), it may be feasible to combine the bacteria, T4 phages, and substrate into a single step, thereby shortening the reaction time. Third, the detection efficiency could be enhanced by utilizing alternative phage strains. For instance, the T7 phage, with its simpler replication system and more compact genome, could offer higher protein expression efficiency during infection compared to the T4 phage.[34] Besides these potential solutions, we also applied the proposed method for the detection of *E. coli* containing lacZa gene. The results were compared to bacteria lacking the lacZa gene (Table S2). It was found that the bacteria with the lacZa gene generated a signal in 3 h. When the infection time was fixed at 5 h, these bacteria exhibited a higher background signal compared to those without the lacZa gene. Therefore, the infection time could be further reduced when applying the colorimetric biosensor to bacteria containing the lacZa gene.

CONCLUSIONS

Taking advantage of high efficiency and specificity, the CRISPR systems are driving a gene editing revolution in different species. In this study, we developed a CRISPR/Cas9-mediated gene editing platform for T4 phage engineering. The engineered phage was equipped with enzymatic markers, making it easy to select from the wild-type phage. The innovation of the CRISPR/Cas9 platform breaks down the conventional barriers of phage engineering, offering a versatile and efficient method of customizing phages for biosensing applications. Compared to previous CRISPR-based phage engineering methods,[28,35] our approach is specifically designed for rapid screening of recombinant phages. Using blue-white selection, we can easily distinguish recombinant phages from wild-type phages, simplifying the screening process. Our method provides a more efficient and streamlined solution, addressing the time-consuming and labor-intensive steps often encountered in previous phage engineering workflows. However, the system is not without challenges. For example, the CRISPR/Cas9 system is highly dependent on the PAM sequence. to recognize the target and cleave the target region in the phage genome. Designing crRNA can be difficult if there are few PAM sites available in the target gene sequence. To address this limitation, protein engineering methods, such as directed evolution, could be employed to develop PAM-independent Cas9 nucleases, thereby expanding the capabilities of the CRISPR/Cas9 system and overcoming the current constraints.

As a demonstration of feasibility, the engineered phage was applied to antimicrobial susceptibility testing in urine samples, generating a colorimetric signal after 5 h of infection. Compared with the conventional culture-based AST methods, the engineered phage-based colorimetric biosensor shortens the detection time while maintaining comparable specificity. Furthermore, the liquid-phase reaction facilitates bacterial enrichment and phage-to-bacteria reactions, allowing for simultaneous high-throughput screening of antimicrobial-resistant bacteria. This innovative biosensor holds promise for accurate and efficient detection of viable bacteria with potential applications in clinical diagnostics and biomedical therapy.

Looking to the future, this work has great potential for applications in a variety of fields. The CRISPR system could be adapted to different phage strains, contributing to phage therapy. Except for the T4 phage, there are many phages that have great potential for AST applications, such as the KEP10 and T1 phages. These phages could be used to detect and hinder the growth of *E. coli* during urinary tract infections.[36,37] However, these two phages have not been explored for phage engineering due to the time-consuming process of recombinant phage screening. Through changing the crRNA and homologous arms on the Cas9 plasmid and donor plasmid, we

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could use our proposed CRISPR/Cas9-mediated phage genome editing method to edit different phage genomes and efficiently screen recombinant phages. These engineering phages could be used as phage cocktail to expand the applicability of the proposed colorimetric biosensor for AST. Additionally, the engineering phage-based biosensing method could be extended to the minimum inhibitory concentration (MIC) assay. The MIC assay is used to determine the lowest concentration of an antimicrobial agent that prevents the visible growth of bacteria.[38] Traditionally, MIC assays rely on assessing bacterial turbidity after overnight culture under antibiotic exposure, but our biosensor could offer a faster alternative by using colorimetric signals. Integrating smartphone-based color analysis could further enable portable, on-the-spot detection, making this method more efficient and practical for widespread use.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.4c05177.

Primers used to construct the donor plasmid; comparison of the colorimetric results for bacteria without lacZa gene and bacteria containing lacZa gene; blue-white selection method is used to identify phages from plaques formed after infecting bacteria containing different plasmids; comparison between the engineered phage-based colorimetric biosensor and disk diffusion method for antimicrobial susceptibility testing in urine samples; heatmaps of disk diffusion method and the engineered phage-based colorimetric biosensor for antimicrobial susceptibility testing in urine samples; and sequence of homologous recombination cassette (PDF)

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Notes

The authors declare no competing financial interest.

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