Homology-dependent recombination of large synthetic pathways into E. coli genome via λ-Red and CRISPR/Cas9 dependent selection methodology

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Abstract
Background: Metabolic engineering frequently needs genomic integration of many heterologous genes for biosynthetic pathway assembly. Despite great progresses in genome editing for the model microorganism Escherichia coli, the integration of large pathway into genome for stabilized chemical production is still challenging compared with small DNA integration.

Results: We have developed a λ-Red assisted homology-dependent recombination for large synthetic pathway integration in E. coli. With this approach, we can integrate as large as 12 kb DNA module into the chromosome of E. coli W3110 in a single step. The efficiency of this method can reach 100%, thus markedly improve the integration efficiency and overcome the limitation of the integration size adopted the common method. Furthermore, the limiting step in the methylerythritol 4-phosphate (MEP) pathway and lycopene synthetic pathway were integrated into the W3110 genome using our system. Subsequently, the yields of the final strain were increased 106 and 4.4-fold compared to the initial strain and the reference strain, respectively.

Conclusions: In addition to pre-existing method, our system presents an optional strategy for avoiding using plasmids and a valuable tool for large synthetic pathway assembly in E. coli.

Keywords: Metabolic engineering, Chromosomal integration, Lambda Red, CRISPR-Cas9, Escherichia coli
modules (>2000 bp) and chromosomal integration of DNA module which was larger than 2500 bp using λ-Red promoted HDR was very difficult [5]. In particular, the elimination of antibiotic marker was inconvenient, and the residual FRT sites might bring about unexpected recombination in the genome of the edited strain. Various chromosomal integration strategies based on λ-Red system have been established for large DNA modules integration, including I-SceI cleavage-facilitated recombination [6], knock-in/knock-out (KIKO) vector mediated integration [7] and pSB1K3(FRTK) vector aided insertion [8]. However, the integration efficiency was significantly decreased when the size of the DNA modules was gradually increased. Currently, the maximum length of the integrated DNA modules was about 10 kb assisted by the λ-Red promoted HDR [5]. As an exceptional case, DNA modules (~15 kb) were divided into four segments (each ~3 kb) and then iteratively integrated into E. coli genome [9]. Furthermore, a 50 kb DNA module from B. subtilis 168, divided into seven segments (each ~6–7 kb), was inserted into E. coli genome through iterative integration [9]. This method could integrate large DNA fragment, which however required many rounds of integration to achieve large synthetic pathways integration.

In recent years, CRISPR/Cas9 has become the most widely used technology for genome editing in a variety of organisms [10]. In E. coli, the CRISPR-Cas9 technology was generally accomplished assisted by the λ-Red promoted HDR for chromosomal integration [11]. For example, DNA modules as large as 7 kb could be integrated in E. coli chromosome with >60% efficiency using CRISPR-based technique [12]. Another example, the 10 kb isobutanol biosynthetic pathways were able to scarlessly insert into the genome with an efficiency of 50% [13]. However, the efficiencies and the fragments size of these integrations do not meet the requirement of some engineering goals.

In this study, we developed a new strategy for integration of large synthetic pathways into E. coli W3110 genome (Fig. 1). To achieve this, we harnessed λ-Red and CRISPR/Cas9 system to increase recA-mediated HDR efficiency and delete redundant sequences, respectively. To demonstrate the feasible with which this system could be applied to genetical applications, we integrated the optimized synthetic pathways which combined limiting step in MEP pathway and lycopene synthetic pathway into E. coli W3110 genome using our system (Fig. 2). This approach enabled integration of synthetic pathways as large as 12 kb with efficiency of 100%. Consequently, we were able to obtain a strain capable of producing lycopene in a single step and the yields were increased 106 and 4.4-fold compared to the initial strain and the reference strain, respectively. Concerning the integrated DNA fragment size and the integrated efficiency, we ensured that our platform will be useful for metabolic engineering and synthetic biology in E. coli.

Results

Design of the λ-Red-assisted homology-dependent recombination for large synthetic pathway integration

The workflow of present strategy was illustrated in Fig. 1. The genome editing procedure was performed via plasmid pRC-IS5 and pCas. The plasmid pRC-IS5 contained the fragmentary IS5 sequence, the narrow-host-range replicon R6K, the chloramphenicol marker, the large synthetic pathways and a gRNA recognition region (N20PAM). The N20PAM sequence from Saccharomyces cerevisiae was used for reducing the off-target frequency. The plasmid pCas containing the λ-Red and CRISPR/Cas9 system [14]. When conducted the editing processes, the vector pRC-IS5 was inserted into the E. coli W3110 genome by recA-mediated HDR assisted by λ-Red. Then a Double-stranded break (DBS) which induced λ-Red promoted HDR by donor template (90 bp synthetically single-stranded primer) was created by Cas9 nuclease to accomplish the editing processes (Fig. 1). Finally, the redundant sequences including IS5 sequence, chloramphenicol marker and R6K were deleted.

To facilitate this platform for genome editing, λ-Red recombinases (Exo, Beta, and Gam) were expressed to facilitate the recA-mediated HDR. The plasmid pCas (MolecularCloud Cat. No.: MC_000011) was used to fulfill this function, in which λ-Red was induced via the inducible promoters pBAD and the CRISPR/Cas9 system was controlled by the native promoter. The λ-Red system was induced by 0.2% l-arabinose for the chromosomal insertion of the pRC-IS5, while the CRISPR/Cas9 system was expressed for the generation of a DSB at the universal N20PAM to remove the redundant sequences.

Construction of a model synthetic pathway for integration

The biosynthesis of lycopene was extensively studied and the synthetic pathway for lycopene was usually used as the model pathway in metabolic engineering and synthetic biology [15, 16]. Thus, the production of lycopene was chosen as a model pathway in this work. Many studies have demonstrated that the first two and last two steps of the MEP pathway were the limiting steps for lycopene production [17–20]. Based on the metabolic regulation of MEP pathway [21] (Fig. 2a), we divided the model synthetic pathways into three modules which comprised feedforward module including dxs (Gene ID: 938609) and dxr (Gene ID: 939636), feedback module including idi (Gene ID: 938985) and crtE and lycopene synthetic module including crtI, crtE and crtB (GenBank: CP002191) (Fig. 2b). Many previous
studies have proved that the carbon and energy flux were directed to cell growth in the early growth phase and later redirected to synthetic pathways to support target product formation in growth regulated pathways [22, 23]. The transcriptome analysis along with the growth phase has been done by a previous work [24]. In order to construct growth regulated pathways, we have picked the promoters which maintained low expression at exponential phase and strong upregulated when cultured to the end of exponential phase and held high expression latterly for these three modules based on the transcriptome data along with the growth phase (GSE102672). We defined each promoter as the 600 bp upstream of the ribosome-binding site (RBS) of its corresponding coding sequence, since these regions generally contained most regulatory sequences [25]. To avoid the influence by RBS site, the same Shine–Dalgarno sequence was used for each module.

As shown in Fig. 3, the trc (from pTrc99a [26]) was the best promoter for lycopene synthetic module, indicating that stronger promoter was needed for lycopene production. Similar result from previous work suggested that efficient lycopene production relied on maintaining high levels of lycopene synthase [27]. Based on the shake flask fermentations, the PphoR and PyejG were the best promoters for feedforward and feedback modules,
respectively. Thus, promoters *trc*, *PphoR* and *PyejG* were chosen for the construction of the model synthetic pathways.

**Integration of a 12 kb DNA module into E. coli W3110 genome**

To verify the efficacy of the designed platform for integration of large fragments, we used the above synthetic pathways (~12 kb) as a model module to integrate it into *E. coli* W3110 genome. We divided the plasmid pRC-IS5 into four segments including three modules and the vector backbone. Firstly, we obtained the integrative vector pRC-IS5 through Gibson assembly method (Additional file 1: Fig. S1) [28]. Subsequently, pRC-IS5 was integrated into the *IS5* locus through recA-mediated HDR assisted by λ-Red. Consequently, the optimized lycopene synthetic pathways (~12 kb) was integrated into *E. coli* W3110 genome. The correct integration was verified by the red color and colony PCR, and the edited strain was designated as EC-IS5. We found that all the colonies on the plates were red colored with λ-Red and there was no colony without adding arabinose to induce λ-Red (Additional file 1: Fig. S2). Then the red colonies were further verified by colony PCR (Fig. 4c). This result indicated that the λ-Red system was crucial for recA-mediated HDR when generated large pathway integration. Strain EC-IS5 produced 9 mg/g CDW of lycopene in the shake flask fermentation, whereas the plasmid-based strain EC101 and EC401 produced 0.086 and 2.1 mg/g CDW of lycopene, respectively (Fig. 5). EC-IS5 produced 105-fold increase of lycopene yield through integrating the optimized lycopene synthetic pathways into genome compared with the initial strain (EC101). These results indicated that this strategy which combined modular pathway engineering and integrated strategy represented a remarkable synergy.

**Deletion of redundant sequences with CRISPR-Cas9 system**

We next cultivated EC-IS5 in medium supplemented with kanamycin at 30 °C to maintain the pCas plasmid and made the competent cell washed by 10% glycerol. To obtain marker-free strains, the competent EC-IS5 was transformed with 90 bp synthetically single-stranded donor template and plasmid pTargetF-delete (constructed based on pTargetF-cadA [14]) using electroporation and then were spread on the LB plate with kanamycin and spectinomycin. The deletion efficiency of the redundant sequences was exceeded 70% after an overnight incubation (Additional file 1: Fig. S3). This feature might facilitate iterative genome editing. Then the
The final strain EC-IS5 (ΔCm) was used for lycopene production without antibiotic maintenance. As shown in Fig. 5, strain EC-IS5 (ΔCm) produced 9.1 mg/g CDW lycopene, which was 4.4-fold of the reference strain (EC401). This result confirmed that chromosomal integration shown great advantage than plasmid-based method.

**Discussion**

In a previous study, we developed a platform for chromosomal integration (~ 1.5 kb) in *E. coli* for xylitol production using recA-mediated HDR [29]. In preliminary experiment before this study, we attempted to integrate a larger pathway (~ 7 kb, only including *crtI*, *crtB* and *crtE*) into *E. coli* chromosome using recA-mediated HDR. Nevertheless, we could not get any colonies using the above method and CRISPR/Cas9 system [14]. Alonso-Gutierrez and colleagues attempted to integrate a synthetic pathway comprised terpene synthase (~ 12 kb) into *E. coli* DH1 genome using assistant plasmid which could mediate chromosomal integration by λ-Red promoted HDR [7]. However, they could not get the expected integration through this system. They claimed that the large size of the synthetic pathway and the complicacy of the pathway might be the most probable explanation for these failing attempts. As an alternative, they divided the synthetic pathway into three segments (shorter than 5 kb) and integrated them through three rounds of integration to achieve the final integration [30].

*Escherichia coli* was highly dependent on a homologous recombination to repair DBS in the chromosome. λ-Red promoted HDR successfully supplemented the low efficiency of the *E. coli* native repair system and, thus, succeeded in genome editing, while single DSB could not be repaired without λ-Red [14]. The recA-mediated HDR is another form of allelic exchange [31]. However, this recombination is insufficient for large pathway integration (Fig. 4b). Fortunately, we successfully rescued the low efficiency of recA-mediated HDR by using λ-Red. RecA is one of DNA strand exchange proteins which are essential for homologous recombination. In vivo, RecA preferentially binds to ssDNA (double stranded DNA (dsDNA) breaks or ssDNA gaps in replication forks stall), and then the assembly of a presynaptic filament of RecA on the ssDNA was generated during homologous recombination, which in turn uses the ssDNA sequence to search for a homologous region in the dsDNA [32]. Meanwhile, the λ-Red system consists of several genetic components (Exo, Beta, and Gam) and Beta also binds to the ssDNA [2]. Although we do not know what is the real mechanism, we speculated that Beta would likely facilitate rescuing the low activity of recA-mediated HDR for large synthetic pathway.
Fig. 4 Integration of a 12 kb DNA module into E. coli W3110 genome. a The optimized lycopene synthetic pathways in pRC-ISS. b Colony forming unit (CFU, indicated the number of colonies on the selective plates with 34 μg mL⁻¹ chloramphenicol after one experiment of integrating optimized lycopene synthetic pathways into E. coli W3110) and integration efficiency with or without adding arabinose to induce λ-Red. c PCR confirmation of the integration of the optimized lycopene synthetic pathways using primers IS5-Q-P1 and dxs-dxr-P2 for feedforward module (6521 bp), IEB-P1 and IS5-Q-P2 for lycopene synthetic module (4811 bp), idi-crtE-P1 and idi-crtE-P2 for feedback module (3042 bp). M: DNA marker; CK: E. coli W3110, 1, 2, 3, 4, 5, 6: colonies from the plates after chromosomal integration.
Recent years, CRISPR/Cas9 based genome editing has been obtained great progresses in E. coli. However, chromosomal integration of large DNA modules was still limited by low efficiency and integration size compared with chromosomal integration of DNA modules shorter than 2000 bp. To alleviate these issues, Li and colleagues developed a platform with multiple step integration of divided segments [33]. They found that the integration efficiency decreased gradually with the increasing size of the modules, and the efficiency for a 7 kb DNA module was about 10% which was similar to that in E. coli MG1655 [34]. To facilitate the high efficiency and the preparation of the DNA modules, about 3–4 kb of the entire DNA module was optimal. They could insert a 15.4 kb synthetic pathway which contained several crucial genes for uridine biosynthesis into E. coli W3110 genome with five steps of integration [33]. However, this strategy needed many rounds of integration to access the final goals.

Using our platform, we were able to obtain a strain capable of producing lycopene in a single step and the production yields were increased 106 and 4.4-fold compared to the initial strain (EC101) and the reference strain (EC401), respectively (Additional file 1: Fig. S4). This study clearly demonstrate that our platform was quite feasible and useful for constructing microbial cell factories which needed large synthetic pathways. Therefore, we concluded that the recA-mediated HDR integration aided by λ-Red in this study was relatively practical for metabolic engineering (Table 1). Furthermore, we speculated that our platform facilitated integration of large synthetic pathways could be applied to other prokaryotic microorganism to achieve stable strains for chemical production, for that insertion sequences were widely distributed in many microorganism [35].

Conclusions
We have developed a useful platform for integration of large synthetic pathways into E. coli W3110 genome. Taking advantage of the λ-Red promoted HDR and the Cas9 nuclease, only the integrative vector is needed to construct for each round of integration. Another characteristic is that stable strains can be obtained by integration of large synthetic pathways that are responsible for valuable chemicals biosynthesis. In order to verify the feasibility of our platform, a 12 kb DNA module contained several key genes for lycopene biosynthesis was integrated into the E. coli W3110 chromosome. The production yields were increased 106 and 4.4-fold compared to the initial strain (EC101) and the reference strain (EC401), respectively. Our platform has been proven to be practical in E. coli and would be adapted for the production of valuable chemicals.

Methods
Strains and culture medium
Strains and plasmids used in this study are listed in Table 2. E. coli strain DH5α and DH5α λpir (pir+ for propagating R6K ori) were used for the construction of the plasmids. E. coli W3110 was used for chromosomal integration. Strains were cultured in Luria–Bertani (LB) medium supplemented with 100 μg mL⁻¹ ampicillin, 50 μg mL⁻¹ kanamycin, 50 μg mL⁻¹ spectinomycin or 34 μg mL⁻¹ chloramphenicol when needed at 37 °C or 30 °C with shaking at 200 rpm.

Plasmid construction
Primers for construction of various plasmids are listed in Additional file 1: Table S1. Plasmids for expression of
heterologous lycopene synthesis pathway are based on pCDFDuet-1 or pET-30a-trc, plasmids for expression of feedforward module and feedback module were based on pACYCDuet-1. Plasmid containing the large synthetic pathways is divided into several small fragments, including three modules and the vector backbone. All the plasmids were constructed according to the protocol of ClonExpress MultiS One Step Cloning Kit (Vazyme, China).

**Chromosomal integration procedure**

Briefly, the host strain *E. coli* W3110 was transformed with pCas and then was prepared for competent cells with λ-Red recombinase induction by L-arabinose according to the protocol [36]. Immediately, 100 μL of the competent cells was mixed with 300 ng of plasmid pRC-IS5 in MicroPulser (Eppendorf). After electroporation (2.5 kV, 5 ms), the competent cells were suspended in 1 mL LB broth quickly. After 6 h incubated at 30 °C, cells were centrifuged and resuspended in 0.1 mL sterile water. Then the cells were spread on LB agar plates with chloramphenicol. After genome editing, the colonies on the plates were identified for red color and colony PCR with primers IS5-Q-P1, dxs-dxr-P2, idi-crtE-P1, idi-crtE-P2, IEB-P1 and IS5-Q-P2 which straddling the synthetic pathway on chromosome (Additional file 1: Table S1). The editing efficiency was calculated as the number of colonies with red color divided by the number of all the colonies. The correct strain was transferred into LB broth with kanamycin, and was prepared as electrocompetent cells with the expression of Cas9 nuclease and λ-Red proteins. Donor template (90 bp synthetically single-stranded primer) and plasmid pTarget-delete (including the gRNA sequence) were electroporated into the competent cells and then the cells were spread on the LB plate with kanamycin and spectinomycin. Deletion of the redundant sequences was identified by chloramphenicol sensitive and colony PCR using primers IS5-check-P1 and IS5-check-P2 (Additional file 1: Table S1).

| Method                  | Technique feature                              | Integration efficiency (%) | Integration sites | Maximum integration size (kb) | Rounds of integration | Marker left or markerless | Reference |
|------------------------|------------------------------------------------|----------------------------|-------------------|-------------------------------|----------------------|--------------------------|-----------|
| I-SceI endonuclease    | Combined λ-Red and I-SceI Cleavage             | 19.2–100                   | Desired locus     | 7                             | One                  | Markerless               | [5]       |
| λ-Red recombination    | λ-Red assisted                                 | 0–50                       | Desired locus     | 7                             | One                  | Markerless               | [7]       |
| λ-Red recombination    | Combined λ-Red and CRISPR/Cas9                | 10                         | Desired locus     | 7                             | One                  | Markerless               | [33] [9] |
| λ-Red recombination    | λ-Red assisted                                 | 60                         | Not provide flIK   | 15                            | Five                 | Markerless               |           |
| CRISPR/Cas9            | Combined λ-Red and CRISPR/Cas9                | 60                         | Desired locus     | 5                             | Four                 | Markerless               | [12]      |
| CRISPR/Cas9            | Combined λ-Red and CRISPR/Cas9                | 50                         | Desired locus     | 10                            | One                  | Markerless               | [13]      |
| RecA homologous recombination | Combined λ-Red, RecA and CRISPR/Cas9 | 100                        | IS5               | 12                            | One                  | Markerless               | This study |

**Shake flask cultures and analysis of lycopene**

For shake flask fermentation, a single colony selected from a fresh LB agar plate was grown overnight in 5 mL of LB broth in a shaker at 37 °C for overnight growth, then 1 mL of the preculture was inoculated into 250 mL shake flask containing 50 mL 2×TY medium with 4% glycerol and grown at 30 °C for 48 h. Individual flasks were stopped at regular times to determine biomass and lycopene yields. Extraction of carotenoid was as described by literature with some modifications [37]. Briefly, cells were harvested by centrifugation at 8000g for 5 min, and then were suspended in 1 mL of acetone. The lysate was incubated at 55 °C for 15 min and centrifuged at 12,000g for 20 min. The acetone supernatant was transferred into a clean tube for measuring lycopene. The lycopene content of the extracts was determined by UV/Vis spectrometer (PerkinElmer Lambda 45) at 470 nm. Spectra was recorded in acetone using an A 1% 1 cm of 3450 [38]. The yields of lycopene were expressed as mg per g cell dry weight (mg/g CDW).
Table 2  *Escherichia coli* strains and plasmids used in this study

| Strain/plasmid       | Description                                                                 | Source          |
|----------------------|-----------------------------------------------------------------------------|-----------------|
| **Strains**          |                                                                             |                 |
| DH5α                 | supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1         | Invitrogen      |
| DH5α λpir            | supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 LAMpir U169 recA1 endA1 gyrA96 thi-1 relA1 | Lab stock       |
| W3110                | Wide type, λ-F-mpR A mpR IN (mpD-mpED1)                                     | DSM5911         |
| EC101                | W3110 with plasmid pET-trc-IEB                                              | This study      |
| EC102                | W3110 with plasmid pCDF-yciG-IEB                                            | This study      |
| EC103                | W3110 with plasmid pCDF-pstA-IEB                                            | This study      |
| EC104                | W3110 with plasmid pCDF-yodA-IEB                                            | This study      |
| EC105                | W3110 with plasmid pCDF-astC-IEB                                            | This study      |
| EC106                | W3110 with plasmid pCDF-ybiM-IEB                                            | This study      |
| EC201                | W3110 with plasmid pACYC-phenI                                             | This study      |
| EC202                | W3110 with plasmid pACYC-phenF                                             | This study      |
| EC204                | W3110 with plasmid pACYC-phenC                                             | This study      |
| EC205                | W3110 with plasmid pACYC-phenD                                             | This study      |
| EC301                | W3110 with plasmid pACYC-yfL                                              | This study      |
| EC302                | W3110 with plasmid pACYC-yfF                                              | This study      |
| EC303                | W3110 with plasmid pACYC-cysP                                              | This study      |
| EC304                | W3110 with plasmid pACYC-yejG                                              | This study      |
| EC305                | W3110 with plasmid pACYC-yhcN                                              | This study      |
| EC401                | W3110 with plasmid pACYC-yhiL                                              | This study      |
| **Plasmids**         |                                                                             |                 |
| pCDF-Duet-1          | pCloDF13-derived vector; T7 promoter, Str<sup>a</sup>                      | Lab stock       |
| pET-30a-trc          | pBR322-derived vector; trc promoter, Kmr<sup>a</sup>                        | Lab stock       |
| pACYC-Duet-1         | p15A-derived vector; T7 promoter, Cmr<sup>a</sup>                          | Lab stock       |
| pTrc99a              | pBR322-derived vector; trc promoter, Amp<sup>a</sup>                       | [26]            |
| pRC43                | Including R6K ori, Cm, IS5 sequence                                         | [29]            |
| pCas                 | repA101(Ts) kan Pcas-cas9 ParaB-Red lacIq Ptrc-sgRNA-pMB1                   | [14]            |
| pTargetF-cadA        | pMB1 aadA sgRNA-cadA                                                       | [14]            |
| pET-trc-IEB          | Lycopene synthetic module under the trc promoter                            | This study      |
| pCDF-yciG-IEB        | Lycopene synthetic module under the yciG promoter                            | This study      |
| pCDF-pstA-IEB        | Lycopene synthetic module under the pstA promoter                            | This study      |
| pCDF-yodA-IEB        | Lycopene synthetic module under the yodA promoter                            | This study      |
| pCDF-astC-IEB        | Lycopene synthetic module under the astC promoter                            | This study      |
| pCDF-ybiM-IEB        | Lycopene synthetic module under the ybiM promoter                            | This study      |
| pACYC-phenI          | Lycopene synthetic module under the yciG promoter, feedforward module under the phenI promoter | This study |
| pACYC-phenR          | Lycopene synthetic module under the yciG promoter, feedforward module under the phenR promoter | This study |
| pACYC-phenF          | Lycopene synthetic module under the yciG promoter, feedforward module under the phenF promoter | This study |
| pACYC-phenC          | Lycopene synthetic module under the yciG promoter, feedforward module under the phenC promoter | This study |
| pACYC-phenD          | Lycopene synthetic module under the yciG promoter, feedforward module under the phenD promoter | This study |
| pACYC-yfL            | Lycopene synthetic module under the yciG promoter, feedback module under the yfL promoter | This study |
| pACYC-yfF            | Lycopene synthetic module under the yciG promoter, feedback module under the yfF promoter | This study |
| pACYC-cysP           | Lycopene synthetic module under the yciG promoter, feedback module under the cysP promoter | This study |
| pACYC-yejG           | Lycopene synthetic module under the yciG promoter, feedback module under the yejG promoter | This study |
| pACYC-yhcN           | Lycopene synthetic module under the yciG promoter, feedback module under the yhcN promoter | This study |
| pTargetF-delete      | gRNA for N2O0PM                                                             | This study      |
| pRC-SS               | Including R6K ori, Cm, fragmentary IS5 sequence, Lycopene synthetic module under the trc promoter, feedforward module under the phoR promoter and feedback module under the yejG promoter | This study |

*  Amp<sup>a</sup>: ampicillin; Kan<sup>a</sup>: kanamycin; Str<sup>a</sup>: Streptomycin; Cm: chloramphenicol; R:resistance
Supplementary information

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Authors’ contributions

HZ conceived the project; BS designed the experiments; BS and DS performed the experiments; BS wrote and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets and materials used during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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