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Original Research Article

Multiplexed site-specific genome engineering in *Mycolicibacterium neoaurum* by Att/Int system

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ABSTRACT

Genomic integration of genes and pathway-sized DNA cassettes is often an indispensable way to construct robust and productive microbial cell factories. For some uncommon microbial hosts, such as *Mycolicibacterium* and *Mycobacterium* species, however, it is a challenge. Here, we present a multiplexed integrase-assisted site-specific recombination (miSSR) method to precisely and iteratively integrate genes/pathways with controllable copies in the chromosomes of *Mycolicibacteria* for the purpose of developing cell factories. First, a single-step multi-copy integration method was established in *M. neoaurum* by a combination application of mycobacteriophage L5 integrase and two-step allelic exchange strategy, the efficiencies of which were ~100% for no more than three-copy integration events and decreased sharply to ~20% for five-copy integration events. Second, the R4, Bxb1 and ΦC31 bacteriophage Att/Int systems were selected to extend the available integration toolbox for multiplexed gene integration events. Third, a reconstructed mycolicibacterial Xer recombinases (Xer-cise) system was employed to recycle the selection marker of gene recombination to facilitate the iterative gene manipulation. As a proof of concept, the biosynthetic pathway of ergothioneine (EGT) in *Mycolicibacterium neoaurum* ATCC 25795 was achieved by remodeling its metabolic pathway with a miSSR system. With six copies of the biosynthetic gene clusters (BGCs) of EGT and pentose phosphate isomerase (PRT), the titer of EGT in the resulting strain in a 30 mL shake flask within 5 days was enhanced to 66 mg/L, which was 3.77 times of that in the wild strain. The improvements indicated that the miSSR system was an effective, flexible, and convenient tool to engineer the genomes of *Mycolicibacteria* as well as other strains in the *Mycobacteriaceae* due to their proximate evolutionary relationships.

1. Introduction

In the development of microbial cell factories for large-scale industrial applications, genome engineering is preferred over plasmid engineering due to the better genetic stability and tunable copy numbers [1]. However, the genome-based expression methods are severely limited in *Mycolicibacterium* species as well as related mycobacteria [2]. The genus *Mycolicibacterium* is a new genus recently divided from the former genus *Mycobacterium* [3], some nonpathogenic species of which are often used as a security model to investigate the pathogenesis of pathogenic mycobacteria [4], and can be developed to biotransform sterols to valuable steroidal synthons [5]. The difficulties in gene editing in the

Abbreviations: Att/Int, attachment/integration; miSSR, multiplexed integrase-assisted site-specific recombination; AE, allelic exchange; Xer-cise, Xer recombinases; EGT, ergothioneine; BGCs, biosynthetic gene clusters; TB, tuberculosis; HR, homologous recombination; PAM, protospacer adjacent motif; DSBs, double-strand breaks; NHEJ, nonhomologous end-joining; CRISPR, clustered regularly interspaced short palindromic repeats; MSGE, multiplexed site-specific genome engineering; aMSGE, advanced multiplex site-specific genome engineering; EGFP, enhanced green fluorescent proteins; HPLC, high-performance liquid chromatography; attP, phage attachment site; attB, bacterial attachment site.

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genomes of *Mycolicibacteria* have seriously slowed their research. Recently, significant progress has been made in the genome editing of *Mycolicibacterium* species by CRISPR-Cas12a-based methods [6], but the iterative and multiplexed site-specific integration of large DNA fragments in their genomes, including multi-copy gene integration, is still a challenge.

Over the past decades, a number of mycobacterial gene editing methods have been developed [2]. Currently, two-step allelic exchange (AE) system is still broadly used in both fast- and slow-growing mycobacteria to generate mutant due to its ease of implementation [7]. However, there are some inherent problems in this system, such as tedious operating steps, the unstable editing efficiency, and the poor integration ability of large DNA segment. These problems are ascribed to the notably low efficiency of homologous recombination (HR) and the low introduction rate of plasmid DNA into the mycobacterial cells [2,7]. Nowadays, CRISPR-Cas systems have also been developed for site-directed editing of the genomes of *Mycolicibacteria* and *Mycobacteria* [6,8]. However, to our best knowledge, it is still difficult to develop the CRISPR-Cas systems as efficient tools for integrating large DNA fragments into the genomes of these species due to their weak HR activity but relatively strong activity of non-homologous end-joining (NHEJ) [6]. Compared with the above methods, the site-specific recombination (SSR)-mediated integration is another excellent tool to construct gene cassettes in genomes due to its high efficiency, substrate specificity, simple operation, and great usability [9,10]. Specifically, it doesn't depend on HR events, no degradation of DNA, and no endogenous host factors or cofactors for DNA repair [10]. In most cases, however, there are one extra copy of DNA fragments can be introduced by a single SSR system [11], and thus to integrate genes/pathways with multiple copies into genomes by SSR methods remains difficult.

Up to now, SSR-mediated integration has been successfully applied in some important industrial microorganisms, such as *Actinomycetes* [11–13]. Generally, the applicable site-specific integrases come from temperate bacteriophages, the working mechanism of which is to catalyze a recombination event between two specific DNA sequences, attP and attB, locating in phage DNAs or donor DNA fragments and bacterial chromosomes, respectively, followed by generating two new sites called attL (left) and attR (right) [9,10]. Based on this principle, Li et al. proposed an "one integrase-multiple attB sites" concept and accordingly developed a simple and efficient multiplexed site-specific genome engineering (MSGE) technique to realize the single-step multi-copy chromosomal integration of some biosynthesis gene clusters (BGCs) in some *Streptomyces* species, such as the five-copy chromosomal integration of the BGC of pristinamycin II in *S. pristinaespiralis* and four-copy chromosomal integration of the BGC of chloramphenicol in *S. coelicolor*, respectively [11]. Subsequently, Li et al. then proposed a "multiple integrases-multiple attB sites" concept, which was developed as a more advanced multiplex site-specific genome engineering (aMSGE) technique for multi-copy integration of BGCs in *Streptomyces* species. This method involved five compatible SSR systems, which could mediate the simultaneous insertion of target DNA fragments into corresponding native attB sites in *Streptomyce* genomes. With aMSGE, up to four copies of the 5-oxomilbemycin BGC (72 kb) could be integrated into the genome of *S. hygroscopicus* and the generated strains were genetically stable in five generation [12]. In *Mycolicibacterium* and *Mycobacterium* species, a simple SSR system derived from mycobacteriophage L5 has also been used. Based on this system, a well-known *Mycobacterium* integrating tool pMV306hsp has been widely used [14], and a method to integrate two independent plasmids into the genome of *M. smegmetis* has been developed by using a pMV306hsp carrying an additional attB site [15]. Nevertheless, two copies possibly contribute little to construct a complex metabolic network in developing microbial cell factories based on multiple genes with multiple copies. Therefore, it is necessary to develop a more advanced SSR system facilitating multiplexed genome engineering for the construction and optimization of complex metabolic networks in *Mycolicibacteria*.

In this study, an upgraded multiplexed integrase-assisted site-specific recombination (miSSR) system was developed to facilitate the genome engineering of *Mycolicibacterium* species. As a proof of concept, an ergothioneine (EGT) overproduction cell factory based on *M. neoaurum* ATCC 25795 was developed. To enable the system to be multiplexed, three effective integrases were screened from bacteriophages R4, Bxb1, and ΦC31 and a mycolicibacterial Xer recombinases (Xer-cise) system was reconstructed to recycle the selection marker for further gene recombination. In addition, the number of the specific attB attachment sites in genome were regulated by an optimized two-step AE strategy, so that the integration number of target DNA fragments could be flexibly adjusted. The test results indicated that 6 copies of BGC (6.1 kb) for EGT synthesis could be efficiently introduced to the specific attB sites by two rounds of operations. The titer of EGT in *M. neoaurum* ATCC 25795 was increased from 17.5 mg/L to 66 mg/L. These results clearly demonstrated that the developed miSSR system was an effective and user-friendly tool to facilitate the genome engineering of *Mycolicibacterium* species and might be applied in the other strains of *Mycobacteriaceae*.

2. Materials and methods

2.1. Strains, media, reagents, primers, and synthesized DNA fragments

Escherichia coli DH5α grown at 37 °C in Luria-Bertani (LB) medium was used for plasmid construction. Kanamycin (50 μg/mL) and/or hygromycin (150 μg/mL) was added to the medium as required. The mycobacteria used in this study was *M. neoaurum* ATCC 25795, which could grow in LB medium and MYC/01 medium at 30 °C or on LB agar plates at 37 °C [16]. The genomes of *M. neoaurum* and *M. smegmetis* were extracted with Column Myco DNAOUT kit (TIANDZ, Beijing, China). KOD One™ PCR Master Mix (Toyobo, Osaka, Japan) and PrimeSTAR® Max DNA Polymerase (Takara, Dalian, China) were used for high-fidelity DNA amplification. 2xEs Taq MasterMix (CWBIO, Jangsu, China) and rTaq (Takara, Dalian, China) were used for PCR analysis of constructed plasmids or the genotypes of wild-type and mutant strains derived from *M. neoaurum*, respectively. Plasmids were constructed with restriction enzymes and T4 DNA ligase (Thermo Fisher Scientific, America) or Hieff Clone® Plus One Step Cloning Kit (YEASEN, Shanghai, China). DNA purification and plasmid extraction were implemented with corresponding Kits purchased from Magen (Guangzhou, China). R4, Bxb1 and ΦC31 integrase genes (Table S1) were synthesized by GenScript (Nanjing, China) [17–19]. DNA sequencing was performed by Personalbio (Shanghai, China) and all primers (Table S1) used in this study were synthesized by Generay (Shanghai, China).

2.2. Plasmid construction

All plasmids (Tables S2 and S3) were constructed according to the procedure described in Supporting Information.

2.3. Transformation of M. neoaurum by electroporation

Electrocompetent cells of *M. neoaurum* were prepared according to normative protocol [20]. *M. neoaurum* grew in 30 mL of MYC/01 medium. When the optical density at 600 nm (OD600) of the MYC/01 medium containing *M. neoaurum* was between 1.5 and 2.0, 3 mL 20% (v/v) glycine was added in the MYC/01 medium. Then, *M. neoaurum* grew under shaking conditions (220 rpm) for 18–24 h, followed by cell collection by centrifugation (4 °C, 4000×*g*, 10 min). Collected cells were washed three times with pre-cooled 10% (v/v) glycerol (4 °C, 4000×*g*, 10 min), and finally resuspended in 1 mL of pre-cooled 10% glycerol. Then 100 μL of cells were mixed with 500 ng of pure plasmid DNA and incubated on ice for 20 min. Electroporation was implemented in a 2-mm electric shock cup (Bio-Rad, Shanghai, China) with a Bio-Rad MicroPulser™ under the following electrical settings: 2.5 kV and

electric shock frequency of 4–5 ms^{-1}. After electroporation, 600 μL of LB medium was added to transfer cells to a 1.5-mL Eppendorf tube, which was incubated at 30 °C for 4 h. The properly diluted electroporated cells were plated onto LB medium agar containing corresponding antibiotics or other additives.

2.4. Ergothioneine production

M. neoaurum fermentation was implemented as described previously except that MYC/01 medium was used as the fermentation medium [4,16]. The fermentation cultures were harvested in the 1st, 3rd and 5th day, respectively. A total of 2 mL M. *neoaurum* cultures was centrifuged (12000×*g*, 2 min), and washed twice with PBS buffer. The cells were resuspended in 1 mL 70% (v/v) acetonitrile and disrupted with magnetic beads. After centrifugation (12000×*g*, 20 min), the supernatant was used for product analysis on a HPLC system (Agilent 1260) equipped with a Diamonsil Plus 5 μm C18-A column (250 × 4.6 mm) and the UV/VIS signals were detected at 254 nm. The mobile phase consisted of acetonitrile-water (3:97 v/v) with a flow rate of 1 mL/min at 40 °C. The standard metabolite of EGT was dissolved in 70% acetonitrile to prepare the standard curves. The contents of metabolites were presented as mg per milliliter (mg/mL).

3. Results and discussion

3.1. Mycobacteriophage L5 integrase-based multiple site-specific integration in Mycolicibacteria

A mycobacteriophage L5 integrase-based attachment/integration system (L5-att/int) has been widely used for genomic integration of DNA fragments in mycobacteria [14,15,21]. Based on this principle, pMV306hsp carrying the elements of mycobacteriophage L5 integrase and corresponding phage attachment (attP) sites was developed as a common integration tool for genomic engineering of *Mycolicibacteria* [14]. Most species of *Mycolicibacteria*, including *M. neoaurum* ATCC 25795, however, contain only one L5 attB site in their genomes for pMV306hsp-mediated integration [21]. Therefore, it is still difficult to achieve targeted and multiple site-specific genome engineering in *Mycolicibacteria* with the existing L5-att/int based system.

In this study, the single-step and multi-copy targeted integration of DNA fragments in genome was firstly investigated based on the available L5-att/int based system. This strategy was to introduce multiple L5 attB sites into the target genome loci of *M. neoaurum*, so that multi-copy targeted integration events between these attB sites and attP sites of the pMV306hsp could be achieved in one time. Therefore, we attempted to introduce additional L5 attB sites into the *M. neoaurum* genome by a

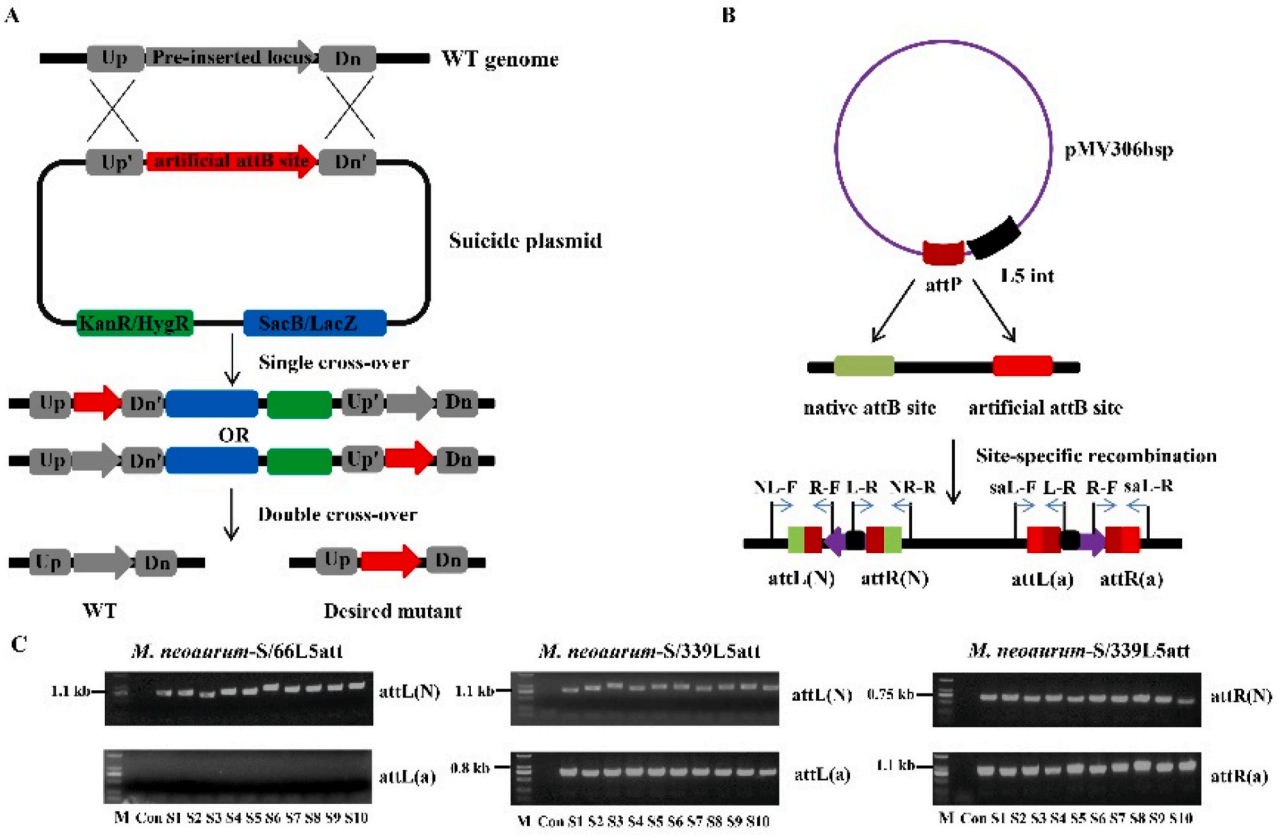


Fig. 1. Mycobacteriophage L5 integrase-based multiple site-specific integration in *Mycolicibacteria*. (A) In the two-step AE procedure, the entire suicide plasmid, carrying additional attB site, was integrated into *M. neoaurum* genome in the single cross-over. Subsequently, the double cross-over event was drived by using the negative selection marker *SacB*, generating the wild-type strain or the desired mutant. Up: the upstream DNA sequence of pre-inserted locus. Dn: the downstream DNA sequence of pre-inserted locus. Up': upstream homologous arm. Dn': downstream homologous arm. KanR/HygR: kanamycin/hygromycin. SacB/LacZ: negative selection marker/visual reporter gene. WT: wild-type. (B) The mycobacteria integrating vector pMV306hsp, carrying L5 integrase/attP elements, was simultaneously integrated into the native attB site and the artificial attB site through site-specific recombination of attP (pMV306hsp) and attB (host genome) attachment sites, generating attL (left) and attR (right) sites. N: Native attB site. a: additional attB site. The attL(N) and attR(N) recombination was verified by using the primers NL-F/R–F and L-R/NR-R, respectively. The attL(a) and attR(a) recombination was verified by using the primers saL-F/L-R and R–F/saL-R, respectively. (C) The PCR screening of the desired recombination events in *M. neoaurum*-S/339L5att electroporated with the plasmid pMV306hsp. The PCR-amplified electrophoretic bands indicated that the desired recombination events have happened in corresponding genomic locus. M: DNA ladder marker. Con: Control. S1-10: Randomly selected sample1-10.

method of two-step AE strategy, which was carried out by the toolkit of p2NIL and pGOAL19 (Fig. 1A). The desired mutant, carrying 339 bp additional L5 attB site in *sal* gene (Supplementary information), was named as *M. neoaurum*-S/339L5att. Then, pMV306hsp was used to evaluate the integration efficiencies simultaneously at the native and additional attB sites. After electroporation and screening on resistant plates, the recombinants were selected by PCR with four pairs of primers annealing to the flanking regions of attL and attR generated after attB/attP site-specific integrative recombination (Fig. 1B). As expected, the whole pMV306hsp (4373 bp) was simultaneously integrated into the native L5 attB site and additional 339 bp L5 attB site with ~100% efficiency in a single round of electroporation, which was further confirmed by DNA sequencing (Figs. 1C and S1A). The above result demonstrated that the two-copy site-specific integration could be effectively realized by adding additional attB loci in the genome of *Mycolicibacteria*.

Since two-copy integration is successful, the integration of more copies in one time was attractive. Therefore, the mutant *M. neoaurum*-2S/L5 (two L5 attB in *sal* gene), *M. neoaurum*-2S1Ka/L5 (two L5 attB in *sal* gene, one L5 attB in *KasB* gene), *M. neoaurum*-2S2H/L5 (two L5 attB in *sal* gene, two L5 attB in *HAL* gene) were constructed and tested for multi-copy integration of plasmid p306-EGFP, respectively. Encouragingly, three-copy integration events in the genome were achieved in *M. neoaurum*-2S/L5 with ~100% efficiency, while five-copy integration events were achieved in *M. neoaurum*-2S2H/L5 with ~20% efficiency (Table 1 and Fig. S2). It was confirmed that the integration of more copies in one time by the combination application of L5-att/int and two-step AE strategy was feasible. However, it was worth noting that a single integrase had a lower efficiency to realize four or more copies of target DNA fragment in a single round as described previously [11]. Therefore, it is valuable to introduce novel bacteriophage Att/Int systems into *M. neoaurum* for expanding site-specific recombinase toolbox and further increasing integrated copy numbers. In addition, no three-copy, four-copy integration event was detected with the same integration method in *M. neoaurum*-2S1Ka/L5 (Table 1 and Fig. S2). It was speculated that the deletion of *KasB* gene had a negative impact on cell survival and integration efficiency [22,23].

3.2. Improving the usability of the L5 integrase-based multiple site-specific integration

To achieve multiple site-specific integration, multiple attB sites need to be introduced into the genome in advance. However, the introduction of attB sites into the genome one by one is a time-consuming and labor-intensive process. We attempted to develop an "one-time multiple attBs" strategy to introduce multiple L5 attB sites into the genome of *M. neoaurum* in a single round (Fig. 2A). The generated transformants with two and three additional attB sites, named as *M. neoaurum*-S2/L5 and *M. neoaurum*-S3/L5, respectively, was tested with plasmid pMV306hsp. Consistent with the previous experiment, three-copy integration events of pMV306hsp were achieved in *M. neoaurum*-S2/L5 with ~100% efficiency (Fig. 2B). This result demonstrated that "one-time two attB" strategy was functional in *M. neoaurum* and it took only half the time to insert multiple attB sites compared to the previous two-step AE method. However, although three-copy integration events had been detected, four-copy integration events could not be detected in *M. neoaurum*-S3/L5, which might be retarded by the clustered homologous DNA fragments.

Table 1
Efficiency of multi-copy integration mediated by a single L5 integrase.

Chassis strains	Number of PCR	three-copy events	four-copy events	five-copy events
M. neoaurum-2S/L5	10	10/10	No	No
M. neoaurum-2S1Ka/L5	10	0/10	0/10	No
M. neoaurum-2S2H/L5	10	4/10	2/10	2/10

No represents no PCR amplification and sequencing verification.

In addition, as an attractive alternative, the method to integrate two independent plasmids into the mycobacterial chromosome had been confirmed in *M. smegmatis* by using a mycobacterial integrating vector carrying an additional attB site as described previously [15]. Thus, a specific integrating vector p306-L5att was constructed by adding a L5 attB site in the plasmid pMV306hsp (Fig. 3A). Using *M. neoaurum*-S2/L5 as a chassis, three copies of p306-L5att could be integrated into the genome with the formation of three new attB sites by ~10% efficiency (Fig. 3B). This lower integration efficiency was due to the attP × attB recombination competition between the attP site with the plasmid p306-L5att attB or *M. neoaurum* native attB sites as described previously [15]. Subsequently, the second-round integration was performed with a hygromycin-resistant plasmid p306-hyg in the above three copies of p306-L5att transformant. Disappointingly, no newly desired PCR-amplified electrophoretic bands were simultaneously confirmed in all examined transformant colonies, except the hygromycin resistance fragment amplified with the primers hyg-F/hyg-R, indicating that all examined transformant colonies contained at least one copy of plasmid p306-hyg (Fig. 3C). The lower integration efficiency might be ascribed to the limited capacity of a single integrase and the clustered repetitive DNA fragments of attB sites.

3.3. Developing multiplexed site-specific integration systems in *M. neoaurum* by introducing heterogenous bacteriophage Att/Int toolboxes

To develop a cell factory, different copy numbers of DNA fragments often need to be integrated in the genome, the requirement of which cannot be easily met by only one L5-based Att/Int system in *M. neoaurum*. Therefore, it is necessary to develop multiplexed site-specific integration systems using different Att/Int toolboxes. Considering the diversity of bacteriophages and their broad host ranges, some other bacteriophage-derived Att/Int systems were tested in *M. neoaurum* to expand the available Att/Int toolboxes, including Bxb1, R4, and ΦC31 Att/Int system (Supplementary information). Similar to the development of the L5 based Att/int toolbox, the toolboxes from Bxb1, R4, and ΦC31 were constructed and their useable attB sequences was tested as 50 bp, 348 bp, and 414 bp, respectively. After the introduction of one copy of these attB sequences in the genome of *M. neoaurum* by two-step AE, the strains of *M. neoaurum*-S/50Bxatt, *M. neoaurum*-S/348R4att and *M. neoaurum*-S/414C31att were constructed, respectively. Meanwhile, the integration vectors with an eGFP-encoding gene as a marker, pLB-eGFP, pLR-eGFP and pLC-eGFP, carrying the corresponding integrase and attP sequence, were constructed, respectively. The resulting transformant colonies on the LB agar plate were examined by PCR screening, further DNA sequencing, and fluorescence intensity measurement (Supplementary information). Encouragingly, three newly introduced bacteriophage Att/Int systems were hyperactive to mediate site-specific recombination with ~100% efficiency in *M. neoaurum* (Figs. 4A, S1B, S1C and S1D), which was also confirmed by the comparison results of the fluorescence intensity of eGFP between these strains and the control (Fig. 4B). The above result demonstrated that the Bxb1, R4, and ΦC31 site-specific integration could be effectively realized by adding corresponding attB loci in the genome of *Mycolicibacteria*.

To examine the multiplexing of these Att/Int toolboxes, the multiplexed site-specific integration was tested in *M. neoaurum* (Fig. 4D). The transformants *M. neoaurum*-2S/L5-Ks/Bx, carrying two additional L5 attB sites and one Bxb1 attB site in the loci of *sal* and *KshB*, respectively, was constructed (Supplementary information). Subsequently, the integrating plasmids p306-hyg and pLK306-Bxb1 were successively introduced into *M. neoaurum*-2S/L5-Ks/Bx to obtain the strains with multiplex integration events. Encouragingly, desired integration events at the three L5 attB sites and one Bxb1 attB site were achieved with

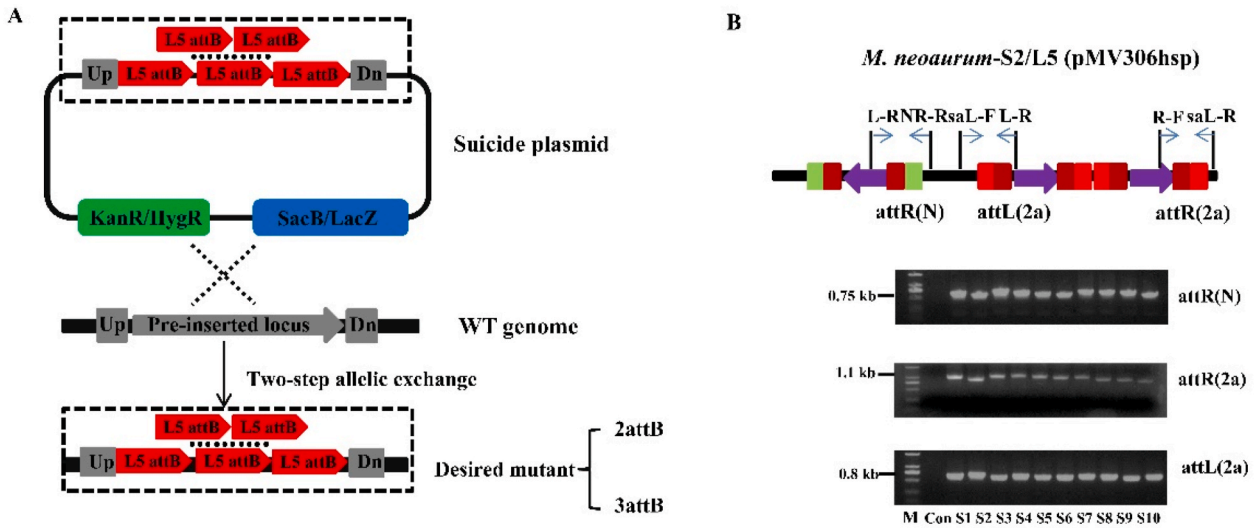


Fig. 2. "One-time multiple attBs" strategy. (A) The modified suicide plasmids, carrying two, three additional attB sites, were used to introduce multiple attB sites into *M. neoaurum* genome through two-step AE in a single round of electroporation. (B) The PCR screening of the desired recombination events in *M. neoaurum*-S2/L5 electroporated with the plasmid pMV306hsp was implemented by using the primers L-R/NR-R, saL-F/L-R and R–F/saL-R, respectively. 2a: 2 additional attB sites.

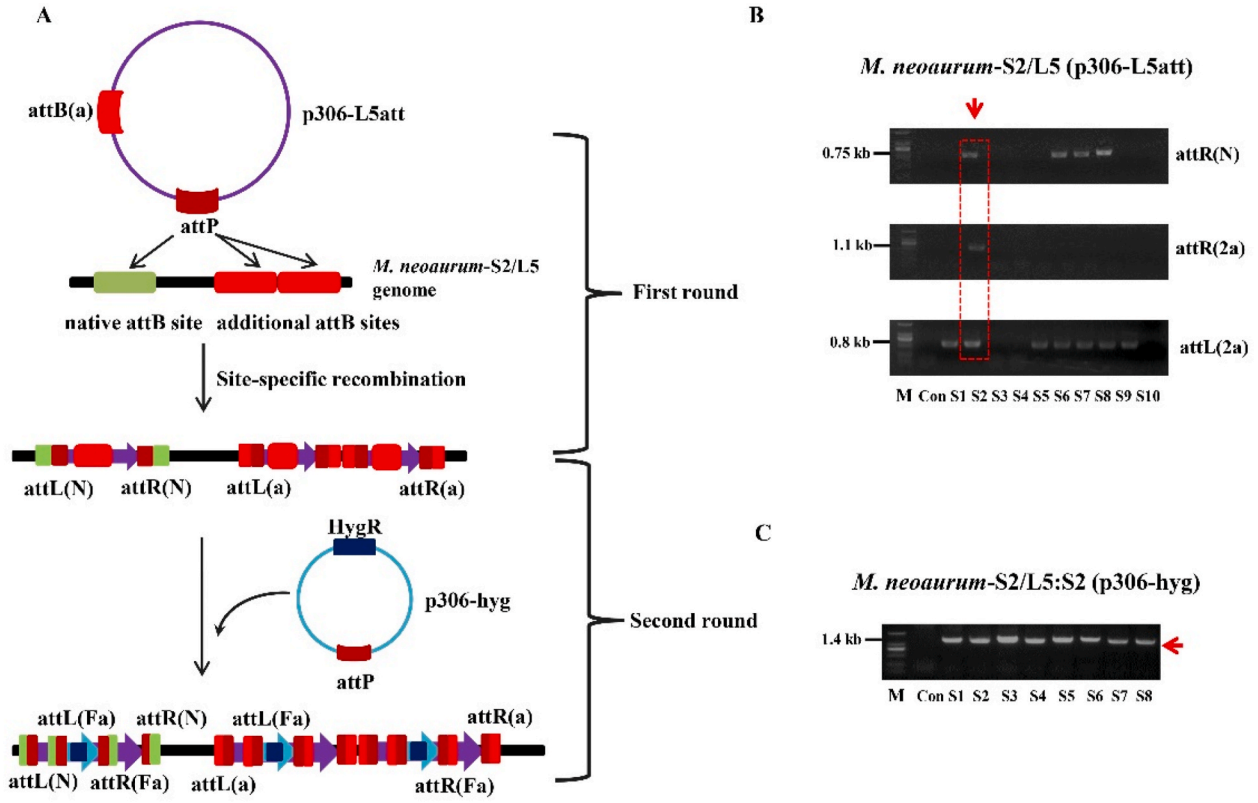
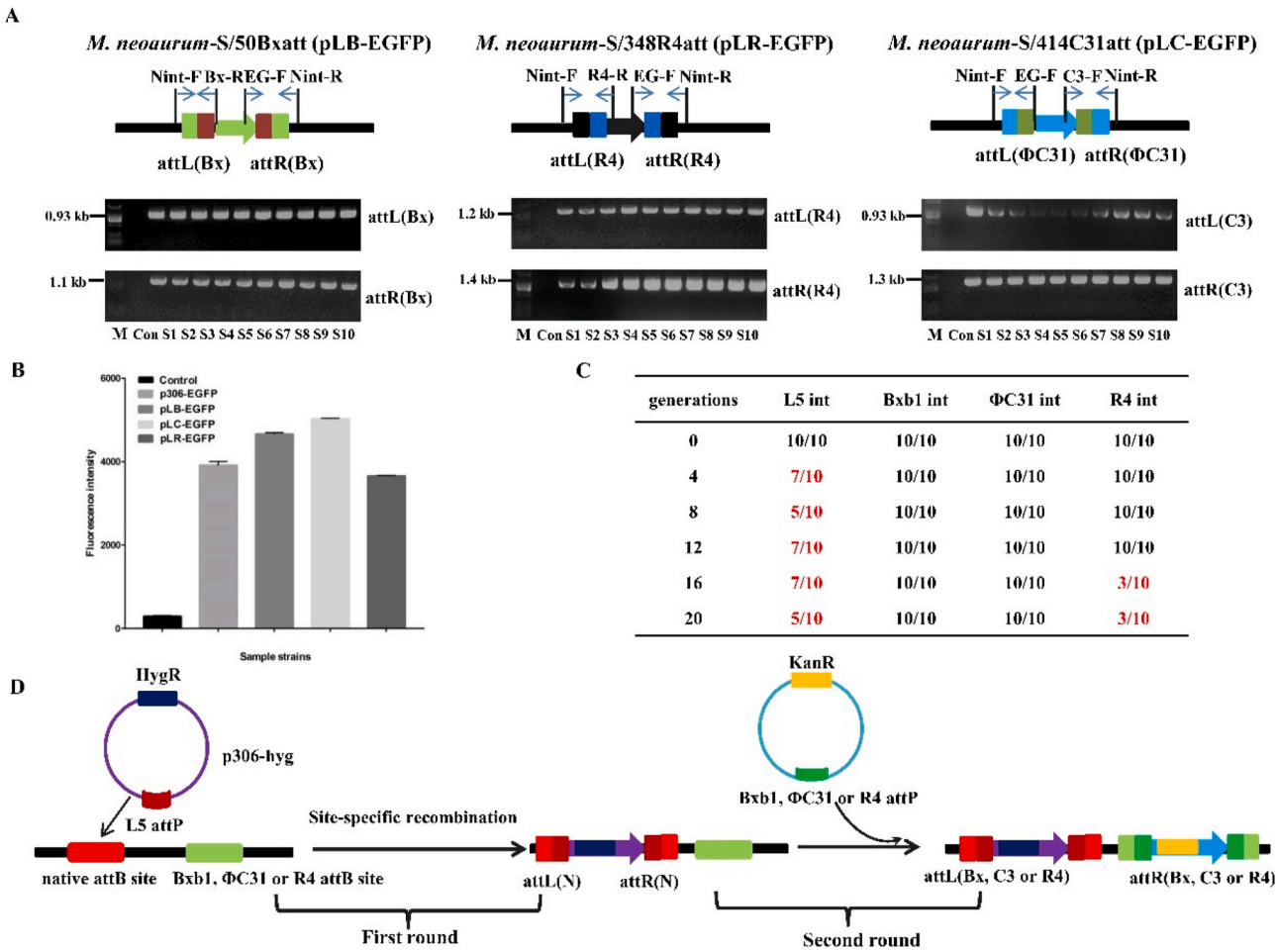


Fig. 3. "two-round multiple attBs" strategy. (A) Two specific mycobacteria integrating vector with different antibiotic resistance, p306-L5att and p306-hyg, were applied to the two-round integration of multiple plasmids. Fa: Fast additional attB site (B) The PCR screening of *M. neoaurum*-S2/L5 electroporated with the plasmid p306-L5att was implemented by using the same primers L-R/NR-R, saL-F/L-R and R–F/saL-R, respectively. Red arrow represents correct electrophoretic band. (C) The PCR verification of second round integration of the plasmid p306-hyg into *M. neoaurum*-S2/L5:S2 (S2: Sample 2) genome by using the primers hyg-F/hyg-R.

~100% efficiency (Table 2 and Fig. S3), indicating that four-copy integration event could be easily achieved in a multiplexed way. In addition, similar result could be obtained with multiplex integration events of the remaining Att/Int toolboxes (Table 2 and Fig. S3).

As some integrases can mediate reverse excision of the integrated DNA fragments [24], the stability of site-specific integration is concerned. Therefore, the genetic stability of these recombinant strains, *M. neoaurum*-S/50Bxatt, *M. neoaurum*-S/348R4att, and *M. neoaurum*-S/414C31att, carrying corresponding integrating plasmids, were evaluated via the repeated culture in LB medium without any selective pressures over twenty generations (Supplementary information). The results indicated that the integration loci of *M. neoaurum*-S/50Bxatt and *M. neoaurum*-S/414C31att mediated by the Bxb1 and ΦC31 Att/Int toolboxes, respectively, maintained fairly stable



generations	L5 int	Bxb1 int	ΦC31 int	R4 int
0	10/10	10/10	10/10	10/10
4	7/10	10/10	10/10	10/10
8	5/10	10/10	10/10	10/10
12	7/10	10/10	10/10	10/10
16	7/10	10/10	10/10	3/10
20	5/10	10/10	10/10	3/10

Fig. 4. Multiplexed heterogenous bacteriophage Att/Int toolboxes in *Mycolicibacteria*. (A) The PCR screening of site-specific recombination mediated by pLB-EGFP, pLR-EGFP and pLC-EGFP vector at the corresponding attB sites by using the primer pairs Nint-F/Bx-R and EG-F/Nint-R, Nint-F/R4-R and EG-F/Nint-R, Nint-F/EG-F and C3–F/Nint-R, respectively. Bx: Bxb1 attB site. R4: R4 attB site. C3: ΦC31 attB site. (B) The fluorescence intensity of different sample strains. Control: Wild-type *M. neoaurum*. p306-EGFP: *M. neoaurum* electroporated with the plasmid p306-EGFP. pLB-EGFP: *M. neoaurum*-S/50Bxatt electroporated with the plasmid pLB-EGFP. pLC-EGFP: *M. neoaurum*-S/414C31att electroporated with the plasmid pLC-EGFP. pLR-EGFP: *M. neoaurum*-S/348R4att electroporated with the plasmid pLR-EGFP. The error bar represents three parallel samples of one clone, respectively. (C) the genome stability analysis of four bacteriophage Att/Int systems over twenty generations, including L5, Bxb1, ΦC31 and R4 Att/Int systems. (D) Two types of mycobacteria integrating vector, carrying different Att/Int systems and antibiotic resistance, were applied to the two-round integration of multiple plasmids.

Table 2
Efficiency of multi-copy integration mediated by multiplexed different integrases.

Chassis strains	Number of PCR	four-copy events
M. neoaurum-2S/L5-Ks/Bx	10	10/10
M. neoaurum-2S/L5-Ks/R4	10	10/10
M. neoaurum-2S/L5-Ks/C3	10	10/10

inheritance over twenty generations without any excision events, while some unexpected attL × attR recombination events were detected in the sixteenth and twentieth generations of *M. neoaurum*-S/348R4att, indicating that the integration stability mediated by the R4 Att/Int toolbox was not as good as those mediated by the Bxb1 and ΦC31 Att/Int toolboxes (Figs. 4C and S3). Meanwhile, the integration stability mediated by the L5 Att/Int toolbox was also tested, which showed to be genetically unstable and there was an excision ratio of 30–50% in the integration locus (Figs. 4C and S3). Obviously, the genetic instability significantly compromised the applicability of L5 Att/Int toolbox and R4 Att/Int toolbox for the development of robust industrial strains. Therefore, it is necessary to inactivate the expression of the corresponding integrase gene in time after the site-specific integration.

3.4. Excising the superfluous part of integration plasmids from genome by constructing a Xer recombinase system in M. neoaurum

For the site-specific integration, there is an obvious defect that is the recombination of the whole integration plasmid into the target attB sites. This recombination will inevitably hinder the iterative and multiplexed application of the integration plasmids due to the available selection markers for *Mycolicibacteria* and *Mycobacteria* are limited [25]. In addition, the existence of the integrase gene will lead to the unexpected excision events among these integrated multi-copy DNA fragments. Therefore, it is necessary to excise the superfluous part of the integration plasmids from the genome after the integration. In *M. tuberculosis* and *M. smegmatis*, a Xer recombinase system has been reported to spontaneously excise the DNA fragment flanked by *dif* sites in genome and has been used to accurately excise antibiotic resistance cassettes [26,27]. Thus, the Xer recombinase system was harnessed to precisely delete the superfluous integrase fragment and antibiotic resistance cassettes of the integration plasmids from the genome of *M. neoaurum* without damaging the target integration fragments.

Unfortunately, although there are some putative Xer recombinase encoding genes in *M. neoaurum*, no native *dif* sequence was confirmed and some heterologous *dif* sequences were not effective in this strain.

Therefore, a heterogenous Xer recombinase system derived from *M. smegmatis* was constructed in *M. neoaurum* to precisely delete the redundant DNA fragments from the genome (Fig. 5A). In this way, we designed a novel mycobacterial integrating vectors p306-28dif, which contained two direct repeat *dif* sites from *M. smegmatis* flanking the L5 integrase and kanamycin-resistance cassettes. Meanwhile, another plasmid ph261-XerC/D-oriT was constructed to carry the Xer recombinase genes (XerC/D) from *M. smegmatis* and temperature sensitive replicon oriT (Supplementary information). To verify the practicality of this tool system, p306-28dif was integrated into the native attB site of *M. neoaurum*, generating strain *M. neoaurum*-L5dif with ~100% efficiency (Fig. 5B). Subsequently, ph261-XerC/D-oriT was electroporated into *M. neoaurum*-L5dif to activate the Xer recombinase system. The direct screening results from the colonies on agar plate showed only ~10% excision ratio between the *dif* sites. Nevertheless, the remanent colonies showed to be heterozygous containing cells with correct excision (Fig. 5C). Therefore, the heterozygous colonies were further cultured in a non-resistant LB medium for 24 h and then plated on the non-resistant agar plate for 3–4 days. Encouragingly, the correct excision of the integrase and kanamycin-resistance cassettes occurred in 80–90% of the resulting colonies (Figs. 5C and S4). These results demonstrated that the reconstructed Xer recombinase tool system worked well in *M. neoaurum*, and could be used to effectively excise the superfluous part of the integration plasmids in the site-specific integration process for the development of robust mycolicibacterial cell factories.

3.5. Developing a mycolicibacterial cell factory for the production of EGT by the multiplexed site-specific genome engineering

To confirm the applicability of these Att/Int toolboxes for multiplexed site-specific genome engineering in *M. neoaurum*, the development of an EGT producing cell factory was taken as an example. EGT is a rare histidine derivative with the high nutritional value, which can be produced by *Mycolicibacteria*, but in a low level [4]. In *Mycolicibacteria*, the synthesis of EGT is a complex process involving an EGT gene cluster consisting of five genes (*egtA*, *egtB*, *egtC*, *egtD*, and *egtE*) and the synthesis of precursors, including histidine, S-adenosylmethionine, and cysteine [28]. Therefore, to develop a robust strain for the production of EGT, the synthetic pathway of EGT needs to be thoroughly modified by genome engineering, which is a challenge due to the difficulty to iteratively introduce large DNA fragments into the genome of *Mycolicibacteria*.

Here, the site-specific integration of the whole EGT gene cluster drived by the promoter *hsp60* (6.1 kb) was firstly performed by using p306-EGT in *M. neoaurum* ATCC 25795, which generated the transformant *M. neoaurum*-Egt with ~100% efficiency. As expected, the titer of EGT was enhanced by 2.7 folds (Figs. 6B and S5), indicating that the integration of a large biosynthetic gene cluster (BGC) via site-specific recombination was effective in *M. neoaurum*. Subsequently, the integration number of the EGT BGC was increased by multiplexed site-specific integration. Based on the strain *M. neoaurum*-2S/L5-Ks/Bx, two additional L5 attB sites or two Bxb1 attB sites were introduced into the *HAL* and *CYP125* loci (Supplementary information), generating two new chassis, *M. neoaurum*-2S2H/L5-Ks/Bx with five L5 attB sites and one Bxb1 attB site and *M. neoaurum*-2S/L5-Ks2C/Bx with three L5 attB sites

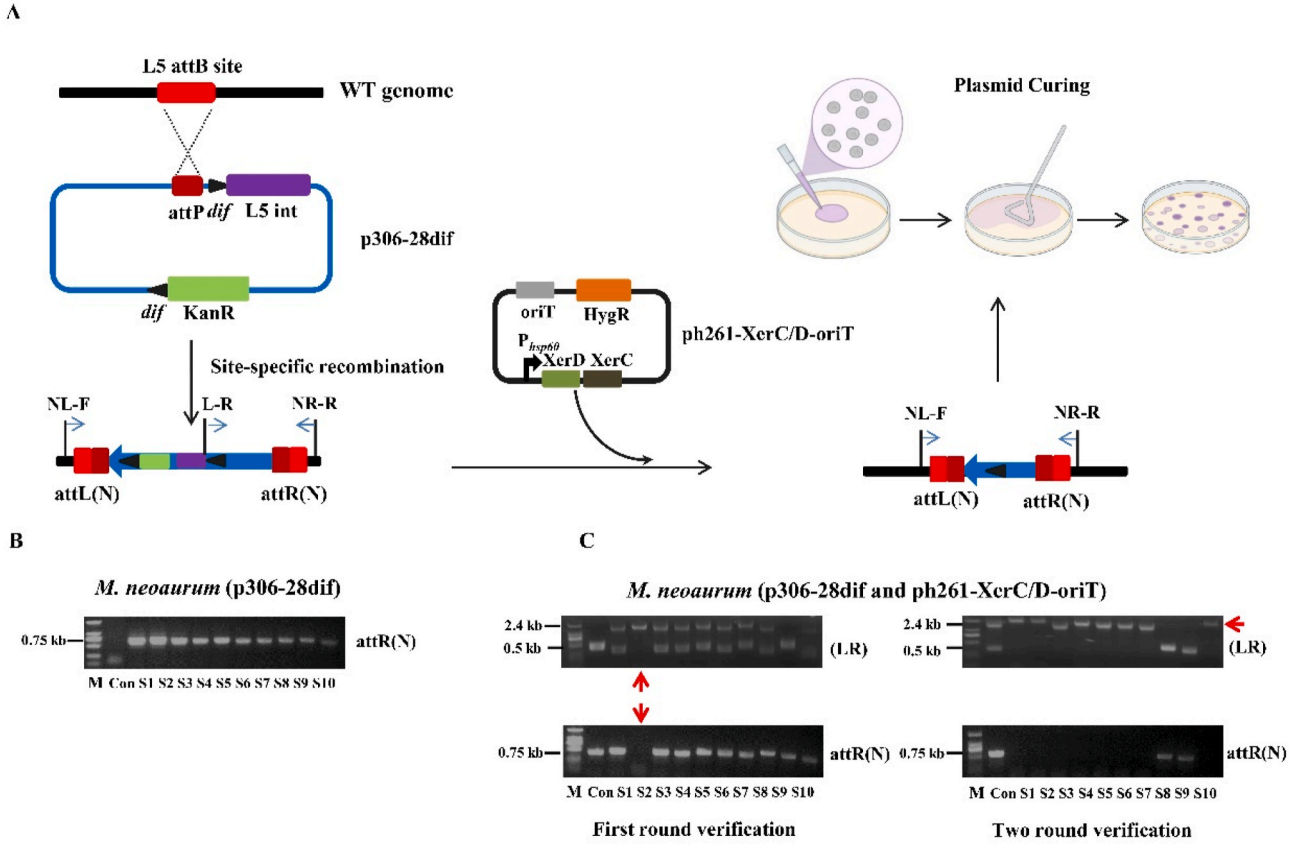


Fig. 5. Introduction of heterogenous Xer recombinases system into *M. neoaurum* for deleting targeted fragment. (A) The specific mycobacteria integrating vector p306-28dif was integrated into *M. neoaurum* genome, and subsequent removal of the targeted gene (e.g., antibiotic resistance and site-specific integrase) by using the plasmid ph261-XerC/D-oriT through Xer recombination at flanking *dif* sites, followed by eliminating the plasmid ph261-XerC/D-oriT at 40 °C for 3–4 days. (B) The PCR screening of *M. neoaurum* electroporated with the plasmid p306-28dif was implemented by using the primers L-R/NR-R. (C) Two rounds PCR verification of *M. neoaurum* electroporated with the plasmid p306-28dif and ph261-XerC/D-oriT was implemented by using the primers NL-F/NR-R and L-R/NR-R, respectively. red arrows represent correct desired electrophoretic bands. (LR):(attL-attR).

and three Bxb1 attB sites, respectively. Then, the Bxb1-based integration plasmid pLB-EGT and the L5-based integration plasmid ph306-EGT were consecutively used to introduce multiple EGT BGCs into the two chassises. Encouragingly, the strains with six copies of EGT BGCs were obtained with ~20% efficiency in *M. neoaurum*-2S2H/L5-Ks/Bx and 80% efficiency in *M. neoaurum*-2S/L5-Ks2C/Bx, respectively (Fig. S6). To our best knowledge, it was currently the highest copy number report using a similar method based on site-specific recombination, and the integration of higher copy number was also expected to be achieved with the introduction of the other two integrases. Unfortunately, the production of EGT was not as high as we expected. The strain *M. neoaurum*-2S2H/L5-Ks/Bx with six copies of EGT BGCs only produced 66 mg/L of EGT within 5 days of fermentation (Fig. 6D), lower than the strain *M. neoaurum*-2S/L5-Ks/Bx with four copies of EGT BGCs (69.5 mg/L). Meanwhile, the similar result could be obtained in another strain with six copies of EGT BGCs.

4. Conclusions

The genome engineering of *Mycolicibacteria* and *Mycobacteria* is widely concerned for their high values in the areas of medicine and biotechnology. However, to locus-specifically introduce large DNA fragments by a multi-copy way in their genomes is still a challenge due to their weak activities of common HR event [6]. In this study, we demonstrated that the combined utilization of bacteriophage Att/Int systems is a simple and effective method to perform multiplexed site-specific genome engineering in *Mycolicibacteria*, which might also work well in *Mycobacteria* due to their close affinity to *Mycolicibacteria* and the universality of gene manipulation tools between these two close genuses, such as two-step AE, site-specific integrase and Xer recombinases [2,3]. Certainly, the method needs to be further verified in the pathogenic mycobacteria.

In this study, two-step AE was used to facilitate the introduction of attB sites into the genome of *Mycolicibacteria*, but it was not a handy approach due to the laborious construction of suicide plasmids and time-consuming screening of single positive colony with desired modification. With the emergence of new genome editing systems, such as ORBIT (Oligo-mediated Recombineering followed by Bxb1 Integrase Targeting) that can be used to introduce large numbers of deletions, insertions or fusions in the target genome of *M. tuberculosis* and *M. smegmatis* [29], the two-step AE approach may be replaced to more easily introduce attB sites in genome. Besides the tested bacteriophages in the study, more bacteriophages can be adopted to further expand the toolbox for genome engineering among *Mycolicibacteria*. As the compatibility of these serine integrase Att/Int systems as reported anteriorly, another markerless targeted genome integration methodologies, such as “dual integrase

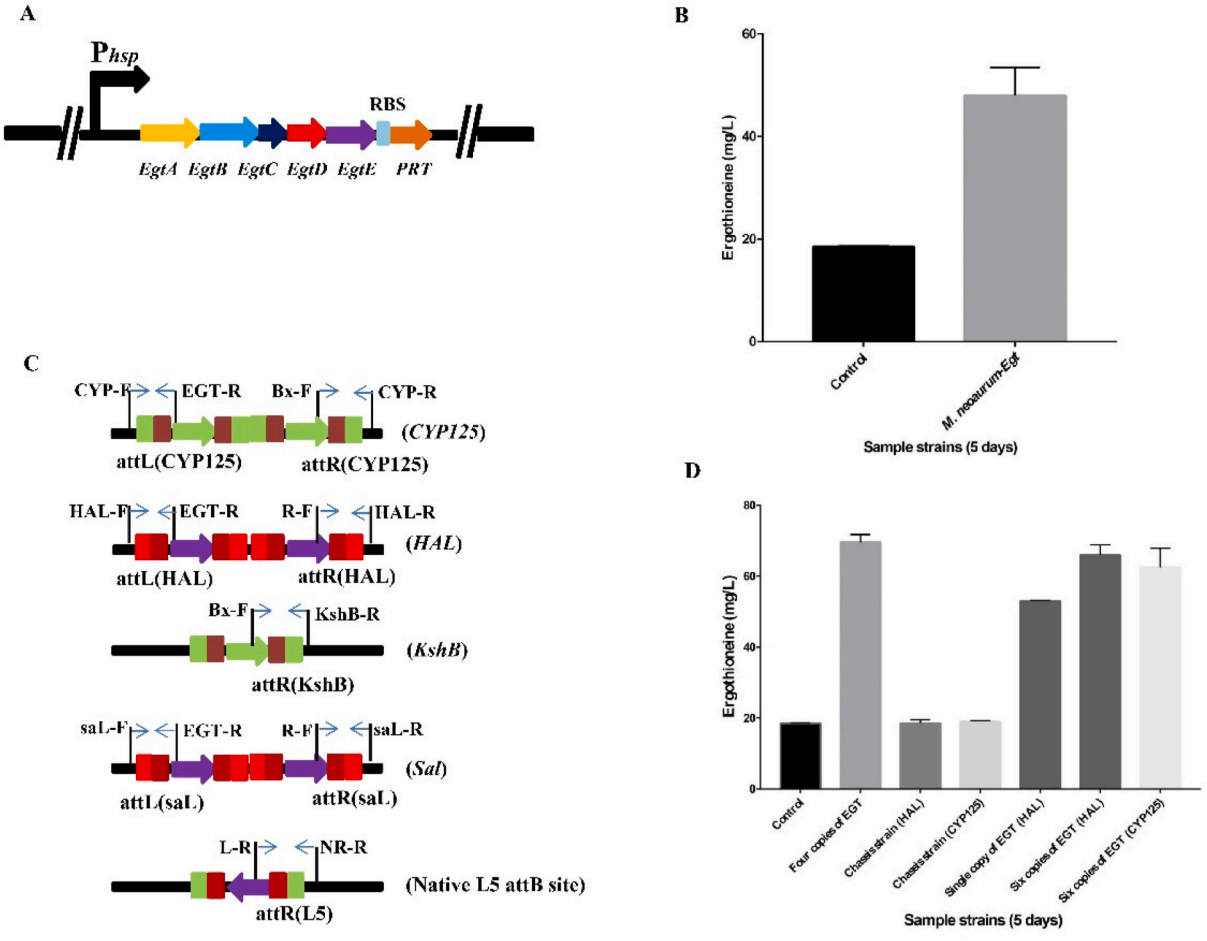


Fig. 6. The *mycolicibacterial* cell factory for the production of EGT by the multiplexed site-specific genome engineering. (A) The sketch map of EGT synthetic core gene cluster used in this work. (B) The yield of ergothioneine produced by the chassis *M. neoaurum* (control) and the engineered single copy of EGT strain (*M. neoaurum-Egt*), respectively. Data are means ± standard deviations of three independent experiments. (C) The sketch map of PCR verification of desired recombination in six copies of EGT strains by using corresponding primers. (D) The yield of ergothioneine produced by the chassis *M. neoaurum* and the corresponding engineered strain, respectively. Four copies of EGT: *M. neoaurum*-2S/L5-Ks/Bx electroporated with pLB-EGT and ph306-EGT. Single copy of EGT (HAL): *M. neoaurum*-2S2H/L5-Ks/Bx electroporated with pLB-EGT. Six copies of EGT (HAL): *M. neoaurum*-2S2H/L5-Ks/Bx electroporated with pLB-EGT and ph306-EGT. Six copies of EGT (CYP125): *M. neoaurum*-2S/L5-Ks2C/Bx, electroporated with pLB-EGT and ph306-EGT.

cassette exchange" (DICE) [10,12,13] can be further combined in the multiplexed site-specific genome engineering systems proposed in this study. In addition, given that the effects of the insertion loci of attB sites used in the study have not been well evaluated on mycolicibacterial growth and metabolism, it is necessary to further evaluate and screen more favorable attB loci for gene integration to avoid uncertain damage to the normal metabolism of the modified strains in the subsequent studies.

In conclusion, we successfully achieved the construction of engineered *M. neoaurum* which was multi-copy integrated of target DNA fragments into the pre-insertion artificial attB sites of host genome for the first time. Such a practical genetic manipulation methodology, especially multi-copy integration of large DNA fragments into the genome, has not been achieved in mycobacteria. Therefore, this technique holds great potential to promote the rapid development of the mycobacteria in many research fields. Meanwhile, this approach is of great value for the design and construction of mycobacterial cell factories for stable large-scale manufacturing.

Ethics approval and consent to participate

All the authors have read and agreed to the ethics for the publication of the manuscript.

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Consent for publication

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.05.006.

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