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A flexible, modular and versatile functional part assembly toolkit for gene cluster engineering in *Streptomyces*

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ABSTRACT

Streptomyces has enormous potential to produce novel natural products (NPs) as it harbors a huge reservoir of uncharacterized and silent natural product biosynthetic gene clusters (BGCs). However, the lack of efficient gene cluster engineering strategies has hampered the pace of new drug discovery. Here, we developed an easy-to-use, highly flexible DNA assembly toolkit for gene cluster engineering. The DNA assembly toolkit is compatible with various DNA assembling approaches including Biobrick, Golden Gate, CATCH, yeast homologous recombination-based DNA assembly and homing endonuclease-mediated assembly. This compatibility offers great flexibility in handling multiple genetic parts or refactoring large gene clusters. To demonstrate the utility of this toolkit, we quantified a library of modular regulatory parts, and engineered a gene cluster (*act*) using characterized promoters that led to increased production. Overall, this work provides a powerful part assembly toolkit that can be used for natural product discovery and optimization in *Streptomyces*.

1. Introduction

Streptomyces is one of the most industrial genera used to produce natural products, such as antitumor drugs, immunosuppressants, herbicides and especially antibiotics [1]. Recent advances in genome sequencing have revealed a vast unexploited resource of gene clusters (BGCs) within *Streptomyces*, holding great potential for the discovery of novel bioactive compounds [2]. Strategies to activate these gene clusters involve cloning and refactoring, and heterologous expression in well-characterized *Streptomyces* hosts, aiming to overcome limitations posed by individual activation methods [3,4]. However, this approach is currently limited by the lack of standard and versatile toolkits and assembly technologies. With the rapid development of synthetic biology, the construction of complex synthetic biological systems has developed from monocistronic gene expression to multi-step pathways or even chromosomes [5,6]. Accordingly, the demands of DNA assembly have shifted from single fragments to more intricate multi-fragments or large gene clusters. To address this need, several modern assembly techniques have been developed, including BioBrick [7], BglBrick [8], Golden Gate [9] and Gibson assembly [5]. Notably, Cas9-Assisted Targeting of CHromosome segments (CATCH) [10] and Transformation-Associated Recombination (TAR) [11] have proven effective for cloning large gene clusters. Moreover, yeast homologous recombination-based methods such as mCRISTAR [12], *mi*CASTAR [13] and mpCRISTAR [14] have been developed for multi-site gene cluster editing.

Although genetic engineering approaches have long been employed to boost the secondary metabolite production in *Streptomyces* for decades, and several vector platforms are commonly used, such as the pIJ

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family [1,15]. However, these vector systems are primarily limited to operate single gene, which limits their application in multi-gene biosynthetic pathways. In addition, these platforms are incompatible with the advanced standard and modular assembly approaches such as Biobrick and Golden Gate. Additionally, their reliance on φC31or φBT1 integrated system restricts efficient combinatorial engineering in *Streptomyces*. Moreover, the size of many BGCs of natural products exceeds the capacity to process them. Although several derivative vectors such as pStreptoBAC [16]and pSBAC [17] have been developed for cloning large gene clusters, they are not standard and modular vectors, and present challenges for subsequent modification. Recent efforts have led to the development of a suite of 45 orthogonal integration vectors based on different site-specific integration systems for heterologous biosynthetic pathways in *Streptomyces venezuelae* [18]. However, these vectors are designed around traditional restriction sites and pMB1 *Escherichiacoli* replicon, making them unsuitable for assembling large gene clusters. Similarly, a set of 12 standardized modular plasmids has been designed to facilitate the assembly of natural product BGCs, but only allow the iterative assembly of genes (or gene cassettes) using the BioBrick assembly method. In addition, they are all based on the *E. coli* p15A replicon, which limits the capacity of the cloned natural product BGCs [19].

In this work, we design a flexible and modular DNA assembly strategy that is easily to exchange plasmid copy number, selection marker gene, integration site, regulatory and catalytic parts of gene cluster. More importantly, it enables cloning and editing of different-sized gene clusters using various cloning methods such as CATCH and yeast homologous recombination-based DNA assembly. Base on the assembly toolkit, we have developed a high-throughput experimental pipeline for testing regulatory elements, and identified a library of modular promoters. In addition, we have demonstrated the advantage of the assembly strategy to enhance the production of a large gene cluster.

2. Materials and methods

2.1. Strains, plasmids, and media

Escherichia coli EPI300 was used for molecular cloning and plasmid propagation throughout this study. *E. coli* ET12567/pUZ8002 was used for *E. coli-Streptomyces* conjugation. *E. coli* strains were grown at 37 °C in LB medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) with the corresponding antibiotic. *S. coelicolor, S. albus and S. lividans* strains were culture on the MS medium (20 g/L soybean flour, 20 g/L mannitol, and 20 g/L agar) for sporulation and conjugation. *S. venezuelae* strains were culture on the MYM medium (4.2 g/L (D-(+)-maltose monohydrate, 4 g/L yeast extract, 4 g/L malt extract, and 20 g/L agar) for sporulation and conjugation. The antibiotics used in this work were ampicillin (100 μg/mL for *E. coli*), thiostrepton (30 μg/mL for *Streptomyces*), apramycin (50 μg/mL for *E. coli*, 50 μg/mL for *Streptomyces*), hygromycin (150 μg/mL for *E. coli*, 50 μg/mL for *Streptomyces*), spectinomycin (80 μg/mL for *E. coli*, 120 μg/mL for *S. coelicolor* and *S. venezuelae,* and 200 μg/mL for *S. albus*).

2.2. Conjugation of the vectors in Streptomyces species

All the vectors constructed were introduced into four *Streptomyces species* by conjugation according to the standard procedure [20]. Exconjugants were selected on MS or MYM agar containing the appropriate antibiotic and 25 μg/mL nalidixic acid.

2.3. Characterization of modular promoters

To test the T7 promoters, two plasmids pTHS-XGSN* and pPAS-PT were used as basic vectors to carry the T7 RNA polymerase and T7 promoters. To efficiently express the T7 RNAP in *Streptomyces,* the four rare TTA $_{leu}$ codons were replaced by CTC $_{leu}$, and the mutated T7 RNAP* sequence was listed in Table S1. The T7 RNAP* fragment amplified with T7 RNAP*-F and T7 RNAP*-R primers was cloned into pTHS-XGSN* using the Goden Gate method. All primers and oligonucleotides were synthesized by Generay Biotechnology (Table S2).The pPAS-$P_{T7(X)}$ series plasmids were constructed via Oligonucleotide Linkers-Mediated Assemble (OLMA) approach developed in our lab [21]. Promoter DNA fragments were acquired by annealed and phosphorylated oligonucleotides, which have sticky ends complemented with the backbone pPAS-PT. The pTHS-T7 RNAP* cassette was integrated into the chromosome of *S. albus* J1074, and the pPAS-P_{T7} series plasmids carrying the T7 promoters were introduced into the resulting strain. All the strains grown in TSB medium (30 g/L tryptic soy broth (BD)) with the addition of 10 μM cumate switched on the expression of the T7 RNAP* expression. The *kasO*p* promoter was inserted into pPAP-PT to generate the pPAS-*kasO*p* plasmid as a positive control. Quantitation of sfGFP expression was performed as described previously [22]. All enzymes and buffers for DNA assembly techniques were obtained from New England Biolabs (NEB) uncless otherwise noted.

2.4. Cloning and refactoring the act gene cluster

The *act* gene cluster from *S. coelicolor* M145 was cloned directly using a previously described method [10]. Briefly, mycelia of *S. coelicolor* M145 were collected after 2 days of cultivation. The preparation of the genomic DNA plugs was carried out according to the CHEF genomic DNA plug kit (Bio-Rad, Hercules, CA, USA). The DNA templates of sgRNA-actF and sgRNA-actR were generated by overlap extension of sgRNA-actF/sgRNA-actR and guide RNA-F plus guide RNA-R, respectively (Table S2). The *S. pyogenes* Cas9 enzyme was expressioned in *E.coli* BL21(DE3) cells, then purified by Ni-NTA affinity chromatography cation exchange. *In vitro* transcription of sgRNAs was performed using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB). Then, the DNA plugs were digested with 500 ng Cas9 enzyme, 500 ng sgRNA-F and 500 ng sgRNA-R at 37 °C for 2 h. The digested DNA was then precipitated with ethanol and resuspended in 20 μL DNase-free water. The linearized capture vector pPAB-HR was constructed to introduce two ~30 bp overlaps with the corresponding ends of the *act* gene cluster fragment and digested with *Aar*I. Approximately 50 ng of the pPAB-HR backbone and 1 μg of digested genomic fragments were assembled using Gibson assembly and introduced into *E. coli* EPI300 by electroporation. The correct recombinant plasmid was verified by PCR using the PF-1 & PR-1, PF-2 & PR-2 primers (Table S2). The recombinant plasmid was further confirmed by restriction enzyme digestion with I-*Sce*I (NEB).

For *act* gene cluster editing, four CRISPR target sequences were selected from three promoter regions (1, 2 and 3) in the *act* cluster, and one target sequence (4) for insertion an auxotrophic marker in the backbone of pPAB. sgRNA-1, sgRNA-2, sgRNA-3, sgRNA-4 were generated as described above. pPAB-act plasmid (10 μg) was digested with Cas9 guided by sgRNAs. After digestion, DNA was precipitated with ethanol and resuspended in 50 μL water. Promoter cassettes (Table S3) were synthesized by Generay Biotechnology. The yeast autotrophic marker (URA) and promoters were amplified by PCR using primers URA-F and URA-R (Table S1). Purified PCR products (150–300 ng) and the digested pPAB-act fragments (1 μg) were transformed into *Saccharomyces cerevisiae* VL6-48 [11] using the Frozen-EZ Yeast Transformation II Kit (Zymo Research). The correct promoter insertion was screened by PCR using primers upstream and downstream of the target site (Table S2). The correct plasmids were isolated and transferred into *S. albus* J1074 by conjugation.

3. Results and discussion

3.1. Design and construction the modular DNA assembly toolkit

One of the barriers to activate the silent gene clusters is the lack of ability to manipulate the multiple regulatory and multiple catalytic

parts in a cross-species manner. Here, we developed a flexible, modular and versatile DNA assembly toolkit for gene cluster engineering. All vectors in the assembly toolkit include a cargo part (part 1) and an essential part (part 2). The cargo part (part 1) was composed by the cloning sites for the regulatory parts and catalytic pats of the gene clusters, while the essential part (part 2) was responsible for the replication origins of the plasmid, selection marker genes in *E. coli*, yeast and *Streptomyces* (Fig. 1).

The cargo part contains a multiple cloning site (MCS) and two transcription terminators (T1 and T2). In order to be compatible with a number of standard cloning systems, the MCS was designed as follows: I-*Sce*I–*Eco*RI–*Xba*I–*Bsa*I-*Bsa*I–*Spe*I–*Pst*I–I-*Sce*I. The *Eco*RI, *Spe*I, *Xba*I and *Pst*I sites allow biobrick assembly, and two *Bsa*I sites allow Golden Gate assembly. The two homing endonucleases I-*Sce*I sites, which recognize long DNA sequences (18 bp) are suitable for exchanging large fragments. To avoid unwanted transcriptional read-through into other elements of the backbone, two transcriptional terminators, T1 and T2 were inserted into the up and down of MCS (Fig. 1).

The essential part consists of four modules, which are arranged in the same order in all vectors (1) integration/replication module in *Streptomyces*; (2) antibiotic markers; (3) replication module in *E. coli*; (4) replication module in yeast (Fig. 1). To ensure the ability to accommodate large gene clusters, the vectors are mainly based on medium or low copy number replicons, including p15A, pSC101 and BAC in *E. coli*, and ARS/CEN origin in yeast. For the hierarchical construction of gene clusters, more than one vector should be used in a single host. Therefore, four antibiotic marker cassettes (Apr, Hyg, Spn, and Tsr + Amp), four integration/replicon elements (φC31, TG1, VWB and pIJ101) for four commonly used *Streptomyces* hosts (*Streptomyces coelicolor* M1154, *Streptomyces lividans* TK24, *Streptomyces albus* J1074 and *S. venezuelae* ISP5230) were included (Fig. 1, Fig. S1).

As a result, a total of 10 basic vectors were constructed (Table 1). In general, large DNA gene clusters are more stable in a low-copy vector. In our toolkit, the pPAB with BAC replicon is primarily designed for assembling large sized gene clusters, especially >50 kb, the pPAS, pVHS and pTHS with pSC101 replicon are designed for the assemble medium sized gene clusters (<50 kb), the pPAP, pVSP, pTSP and pIATP with p15A replicon are used to assemble <30 kb gene clusters, and the pIATU with pUC replicon is suable to small size gene clusters (<10 kb). The detailed information of all vectors maps and sequences can be found in Fig. S2 and Table S4.

Table 1
List of 10 basic plasmids and 5 derivatives.

Plasmid	*Streptomyces* integrate/ replicon	*E. coli* replicon	Selection marker (s)	Cargo
Basic plasmids				
pPAB	φC31 int-attP	BAC	Apr	MCS
pPAS	φC31 int-attP	pSC101	Apr	MCS
pPAP	φC31 int-attP	p15A	Apr	MCS
pPAU	φC31 int-attP	pUC	Apr	MCS
pVHS	VWB int-attP	pSC101	Hyg	MCS
pVSP	VWB int-attP	p15A	Spn	MCS
pTHS	TG1 int-attP	pSC101	Hyg	MCS
pTSP	TG1 int-attP	p15A	Spn	MCS
pIATP	pIJ101	p15A	Amp,Tsr	MCS
pIATU	pIJ101	pUC	Amp,Tsr	MCS
Derived plasmids				
pPAS-PT	φC31 int-attP	pSC101	Apr	*sfGFP*
pTHS-XGk	TG1 int-attP	pSC101	Hyg	*kasOp**
pTHS-XG23	TG1 int-attP	pSC101	Hyg	SP23
pTHS-XGe	TG1 int-attP	pSC101	Hyg	*ermEp**
pTHS-XGSN*	TG1 int-attP	pSC101	Hyg	P_{cymR}*

To test the conjugation frequencies of the 10 basic vectors in different *Streptomyces* hosts, they were conjugated from *E. coli* into *S. coelicolor* M1154, *S. lividans* TK24, *S. albus* J1074 and *S. venezuelae* ISP5230, respectively. At the same time, commonly used plasmid pIJ8660 was used as a positive control. As shown in Fig. 2, the conjugal frequency of the majority of the plasmids was greater than 10^{-6}, with the exception of the following cases. (1) *S. lividans* TK24 was found to be resistant to spectinomycin. Therefore, the plasmids with the spectinomycin antibiotic marker cannot be used in it. (2) *S. albus* J1074 has a high level of tolerance to spectinomycin. Therefore, the concentration of spectinomycin used in *S. albus* J1074 is higher than that used in other hosts.

3.2. Demonstration of compatible molecular operations

The BioBrick and Golden Gate are the most widely used DNA assembly methods due to their robustness and modularity. BioBrick provides a straightforward way to combine standardized biological components, and Golden Gate assembly allows scarless, multi-part DNA assembly. However, they have the limitation that all the parts used must

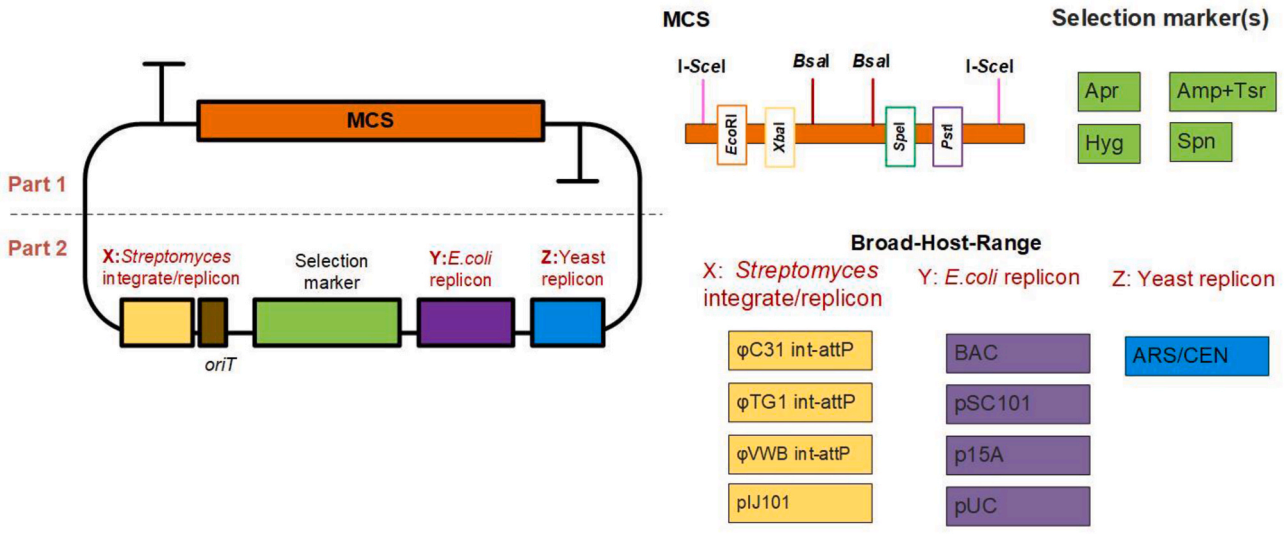

Fig. 1. The modular DNA assembly toolkit. Left part shows the structural organization of the vectors in the toolkit. The right part depicts the details of the MCS, selection marker(s), *Streptomyces* integrate/replicon, *E. coli* and yeast replicon. Apr, apramycin; Amp, ampicillin; Hyg, hygromycin; Spn, spectinomycin; Tsr, thiostrepton.

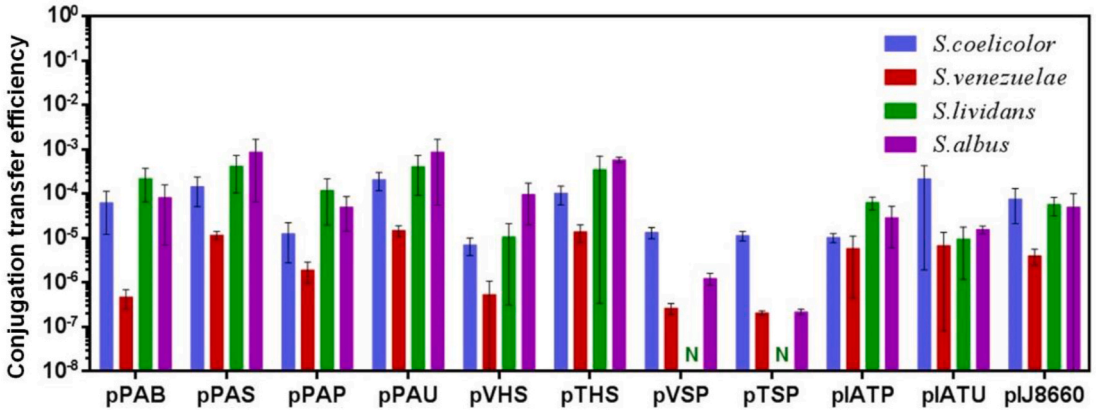

Fig. 2. Conjugation frequencies of 10 basic vectors in four *Streptomyces* strains including *S. coelicolor* M1154, *S. venezuelae* ISP5230, *S. lividans* TK24 and *S. albus* J1074. Conjugation frequency was calculated as the number of exconjugants per input donor cell. Data were obtained from three biological replicates. Error bars represent standard deviation. "N" means no true exconjugant in these groups.

be free of the enzymes used, which makes them unsuitable for assembling gene clusters due to the frequent presence of restriction sites in the target sequence. Therefore, some CRISPR/Cas9-based methods developed to clone and refactor large gene clusters. In addition, I-*Sce*I-based assembly is suitable for exchanging large gene clusters due to the long recognition sequence.

The assembly toolkit in our work is compatible with a wide range of DNA assembly approaches mentioned above. All vectors in the toolkit support the assembly of standard BioBrick parts according to the procedure shown in Fig. 3A. For example, an anther fragment can be easily ligated to the standard plasmids containing a fragment as follows: The first step is to digest the donor part with *Spe*I and *Eco*RI and the target vector with *Eco*RI and *Xba*I. Then, the donor part and the target vector are ligated using T4 DNA ligase to generate the recombinant plasmid. For Golden Gate assembly, PCR products or fragment inserts in donor vectors flanked by specific four-base overhangs and *Bsa*I restriction sites, or double-stranded DNA oligos with appropriate overhangs could be assembled into the vector backbone containing two *Bsa*I restriction sites (Fig. 3B). In addition, the plasmid toolkit allows rapid assembly of gene clusters using the CATCH method. As shown in Fig. 3C, the first step is to introduce homologous arms corresponding to the two ends of the gene cluster into the vector backbone using the Golden Gate assembly. The resulting plasmid was then linearized with *Aar*I. Finally, the linearized capture vector was assembled with the gene cluster fragment from Cas9-treated bacterial genomic DNA by Gibson assembly. Furthermore, all vectors contain a yeast ARS/CEN origin to support gene cluster editing in yeast (Fig. 3D). Recombinant plasmids carrying gene clusters are easily edited in yeast using homologous recombination-based methods such as mCRISTAR, *mi*CASTAR or mpCRISTAR. Moreover, all vectors carry two I-*Sce*I recognition sites. This means that a gene cluster in one vector can be easily assembled into another vector as follows: the gene cluster was digested with I-*Sce*I from original plasmid, and the destination vector was digested with *Bsa*I. The digested gene cluster was then assembled into the linearized destination vector using Gibson assembly (Fig. 3E).

3.3. Development of an extensible assembly toolkit and parts library for gene cluster engineering

Controlled gene expression is important for improvement of secondary metabolite production. However, there is currently no easy-to-use DNA assembly toolbox that enables controlled gene expression in *Streptomyces*. In order to facilitate optimization gene expression at the desired level, we developed a rapid, simple, and standardized gene expression assembly vectors based on pTHS, including three constitutive expression vectors and one inducible expression vector compatible with the Goden gate cloning method. Of these, pTHS-XGe, pTHS-XGk and pTHS-XG23 carry constitutive promoters with different strengths (*ermE*p*, *KasO*p*and SP23) [22], and pTHS-XGSN* contains a tight cumate-inducible promoter P_{cymR*} [23] (Fig. 4A).

In order to fine-tune and enable combinatorial expression each gene (operon) of gene cluster, regulatory elements with a broad range of strengths need to be implemented. To rapid assembly and characterizing of regulatory elements, we constructed a sfGFP reporter vector pPAS-PT (Fig. 4A). The T7 bacteriophage-derived T7 expression system, consisting of T7 RNA polymerase (T7 RNAP) and T7 promoter, is one of the most widely used expression systems. It is simple, efficient and orthogonal to most hosts. Using pTHS-XGSN* and pPAS-PT, we identified a panel of modular T7 promoters, where pTHS-XGSN* expresses T7 RNA polymerase (T7 RNAP*), and pPAS-PT allows easy assessment of T7 promoters strength. The T7 RNAP* encoding gene was cloned into pTHS-XGSN* using Golden gate assembly, resulting in pTHS-T7RNAP*. As well as, 41 chosen T7 promoters [23], were cloned into pPAS-PT using Golden gate assembly, resulting in pPAS-$P_{T7(X)}$ (Fig. 4B and C) (Table S5). The desired constructs were generated by Golden Gate assembly with 100 % efficiency (Table S6). pTHS-T7RNAP* was first introduced into *S.albus*, resulting in *S.a*/T7RNAP*.The pPAS-$P_{T7(X)}$ plasmids were then introduced into *S.a*/T7RNAP*. The promoter activities were quantified by flow cytometry. As shown in Fig. 4D, the T7 promoter exhibits a very strong activity in *S.albus*, and 15 promoters were found to be stronger than the commonly used strong promoter *kasO*p*. Particularly, the wild-type T7 promoter was the strongest of these promoters, reaching approximately 10-fold *kasO*p* (Fig. 4D). At the same time, we found that the activities of T7 promoters in *Streptomyces* showed a good linear correlation with the expression level in *E. coli* host ($R^2 = 0.92$), demonstrating the highly modular feature of the T7 promoters (Fig. 4E). These results indicated that our toolkit is effective for high-throughput analysis of regulatory elements, and the corresponding characterized modular promoters could be used to express genes or gene clusters at different levels as required in *Streptomyces*.

3.4. Applying the assembly toolkit to clone and refactor gene cluster

Actinorhodin (ACT) is a blue pigment produced by *S. coelicolor* M145 via a type II polyketide synthase (PKS)-mediated biosynthetic pathway encoded by the *act* gene cluster. ACT has served as a favored model for studies of antibiotic biosynthesis and regulation. To illustrate the powerful capabilities of our toolkit for gene clusters engineering, we cloned the 26 kb *act* gene cluster in pPAB vector using the CATCH method as follows. First, two 30 bp DNA fragments from each side of the *act* were cloned into pPAB to generate pPAB-HS. Then, the 26 kb *act* BGC

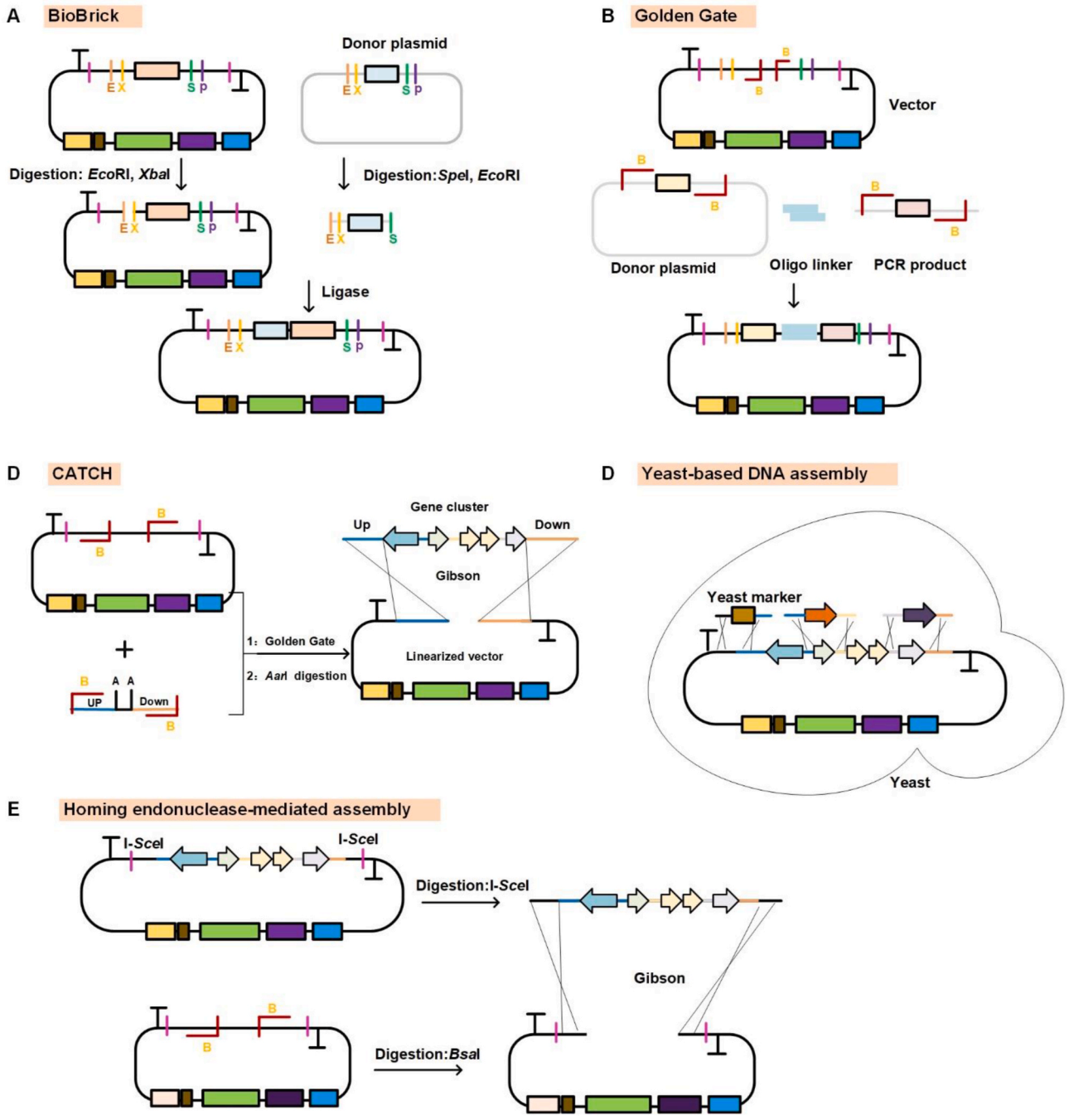

Fig. 3. Demonstration of compatible molecular operations. A. Biobrick standard assembly. E, *Eco*RI; X, *Xba*I; S, *Spe*I; P, *Pst*I. B. Golden Gate assembly. B, *Bsa*I. C. Direct cloning gene cluster using CATCH method. B: *Bsa*I, A: *Aar*I. D. Schematic of homologous recombination-based gene cluster editing in yeast. E. Vector exchange for large gene cluster based on I-*Sce*I-mediated assembly.

digested from *S. coelicolor* M145 genome by CRISPR/Cas9 was cloned directly into the pPAB-HS using Gibson, resulting in recombinant plasmid pPAB-act (Fig. 5A). The procedure takes 2–3 days to complete using standard laboratory equipment at a reasonable cost, dramatically simplifying and accelerating the efficiency of cloning large clusters compared to traditional genomic library construction methods. Using the method mentioned above, we obtained a total of 8 positive colonies by patching 10 colonies with an 80 % positive rate (Table S6). The correct recombinant plasmid was verified by PCR and restriction enzymes digestion (Fig. S3). Promoter engineering strategy enables overproduction of valuable metabolites. The *act* gene cluster is predicted to contain five biosynthetic operons and two regulatory genes encoding the pathway-specific transcriptional regulator actII-ORF4 and the actinorhodin export regulator actII-ORF1. In order to improve the production of actinorhodin, the regulatory network of *act* gene cluster was refactored by inserting well-characterized promoters (P1–P2) to control the actVA and *act*VI operons, P3 promoter to control the *act*II-orf2, and (P4–P5) to control the *act*III and *act*I operons (Fig. 5B). For *act* gene cluster editing, the pPAB-act was first digested at the native promoter sites of gene cluster with Cas9 and sgRNAs *in vitro*. Then the digested fragments, the DNA cassettes containing synthetic promoters with homology arms matching each digestion site in the gene cluster, and the URA marker were co-transformed into yeast. Transformants were plated on yeast synthetic dropout media missing uracil, and verified by PCR.

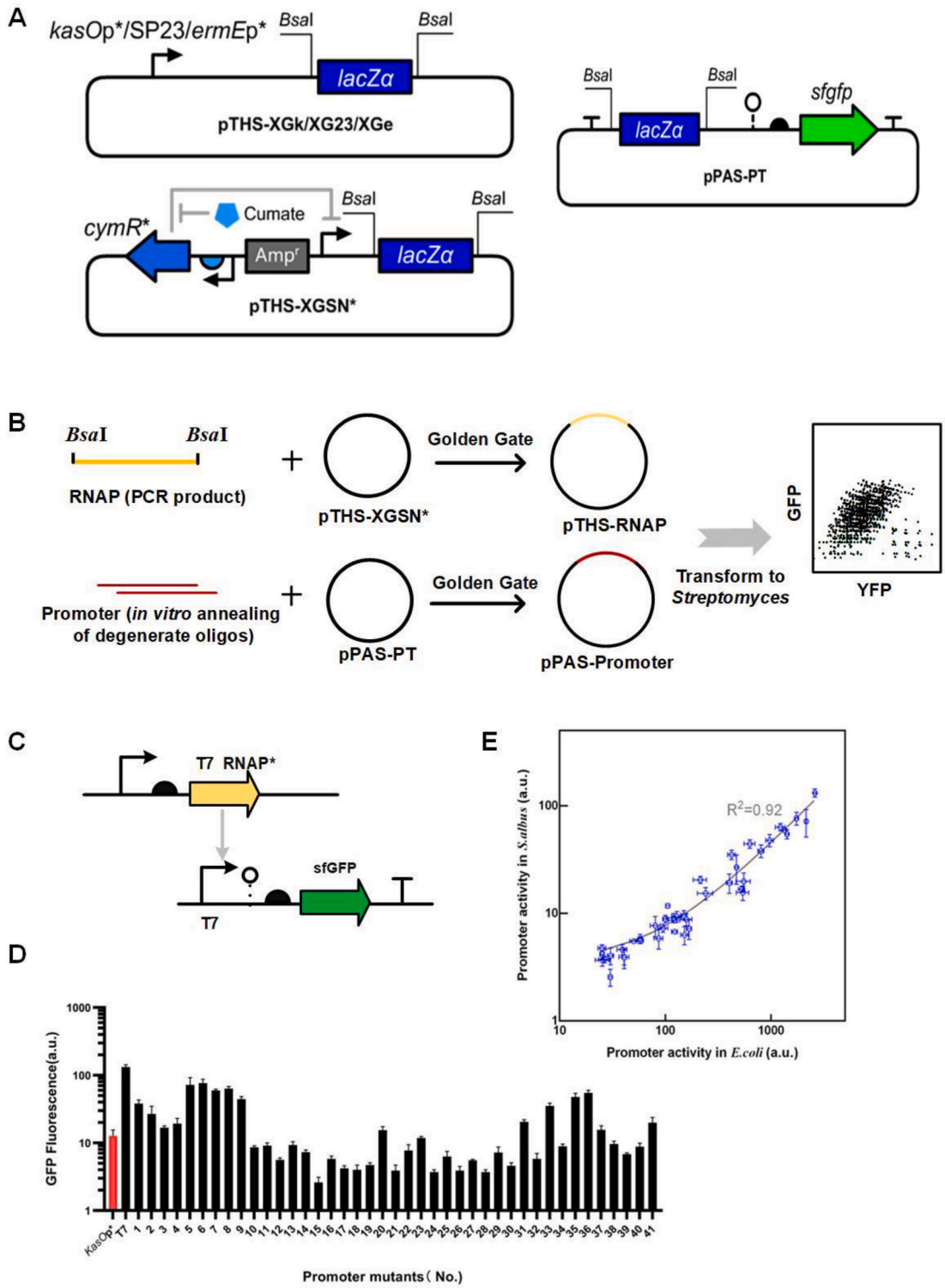

Fig. 4. Derived vectors and their application for promoter activity analysis. A. The maps of three constitute expression plasmids, one inducible expression plasmid and one reporter plasmid. B. promoter library was constructed on the sfGFP reporter vector (pPAS-PT), and RNA polymerase (RNAP) was constructed on modularized expression vector (pTHS-XGSN*) using Golden Gate assembly. C. The structure of the genetic circuit used to characterize the T7 orthogonal transcription system. D. Analysis of fluorescence intensities of the T7 promoter library using flow cytometry-based quantitative method. *KasOp** was used as the positive control(red). E. Correlation between T7 promoter activities in *S.albus* (plotted on the y-axis) and *E.coli* (plotted on the x-axis).

The process takes 3–4 days and positive clones are confirmed by patching 15 colonies. Approximately 67 % efficiency of insertion of three marker-free promoter cassettes was confirmed, a significant improvement over previous *mi*CASTAR and mCRISTAR methods (Table S6) [12,13,24]. The corrected recombinant plasmid pPAB-act2 and original plasmid pPAB-act were introduced into *S. albus* J1074 to generate *S.a/act2* and *S.a/act*, respectively. As shown in Fig. 5B, the production of actinorhodin by *S.a/act2* resulted in a more intense blue pigment than that of *S.a/act*. These results demonstrate that our assembly toolkit is effective for gene cluster engineering, and shows

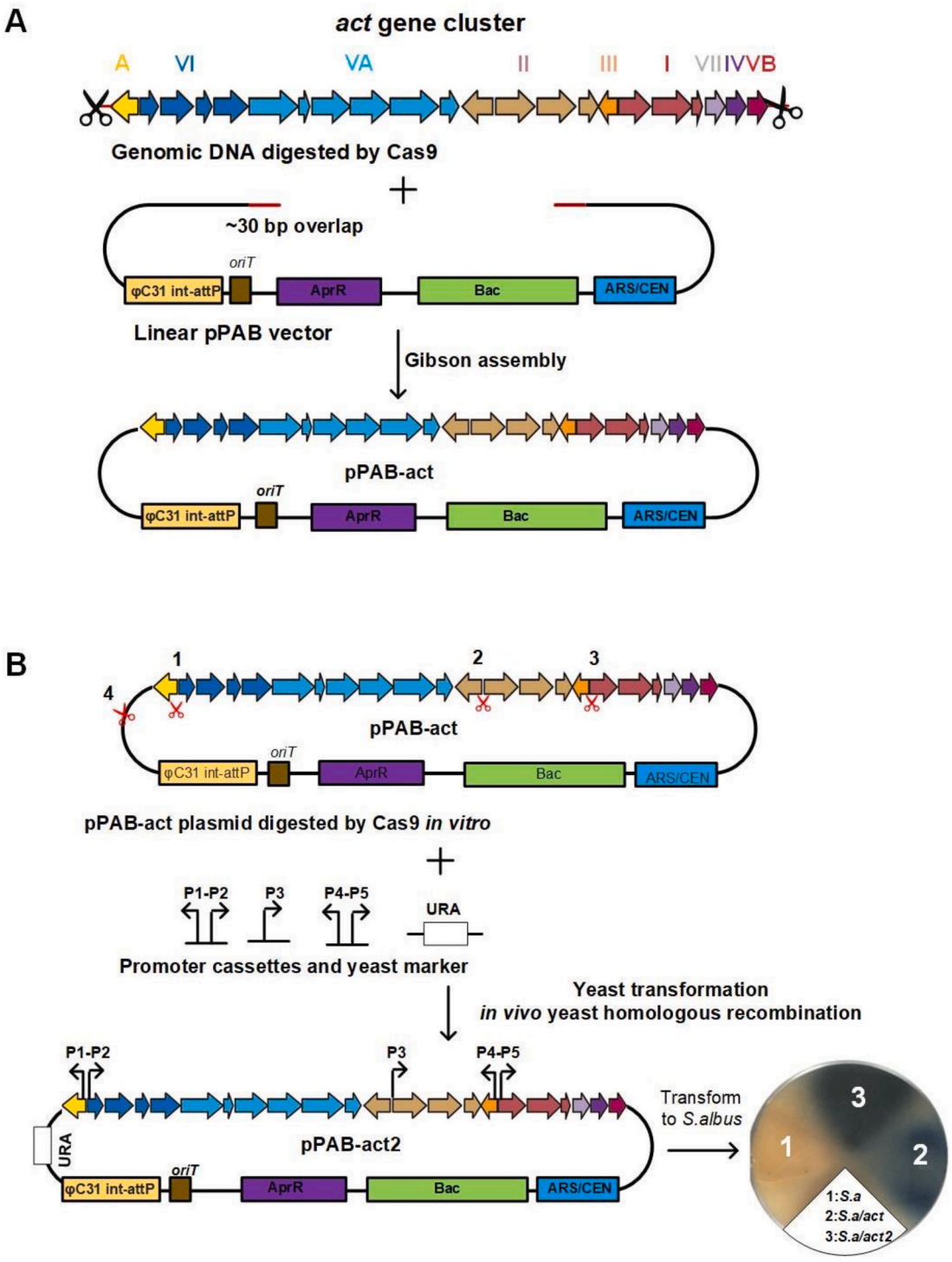

Fig. 5. Cloning and editing of the *act* gene cluster. A.The *act* gene cluster was cloned into pPAB vector using CATCH method. B. The *act* gene cluster was reconstructed with well-characterized promoters using yeast homologous recombination-based DNA assembly.

excellent performance achieved by engineering gene clusters using desired regulatory elements.

4. Discussion and conclusion

Future progress in gene cluster engineering will rely, to a great extent, on the availability of rapid, efficient DNA manipulation technologies and high quality regulatory and catalytic parts. Modern hierarchical and modular assembly methods mean that rapid assembly of multiple parts or large fragments is no longer a major limiting factor. CRISPR/Cas9 technology has enabled efficient cloning and multi-site editing of gene cluster [10,12–14]. However, current assembly toolkits are often not compatible with these requirements. For example, previous toolkits have limitations regarding the compatibility across modulator assembly methods, which typically rely on traditional enzyme cutting and ligation. They have usually been designed around the high-copy *E. coli* replicon, such as pMB1, which limits the capacity ability of large gene clusters. In addition, the application of CRISPR/Cas9 eases the editing of gene clusters at multiple sites in yeast, but previous toolkits usually lack the yeast replicon. In our work, the developed assembly

toolkit is compatible with a wide range of DNA assembly approaches, including modular cloning methods such as BioBrick, Golden Gate, and large fragment manipulation methods such as CATCH and yeast homologous recombination-based DNA assembly. Moreover, our assembly toolkit includes several plasmids for customized requirements, such as 4 *E.coli* replicons for gene clusters of different sizes, 4 antibiotic markers and 4 *Streptomyces* integration/replication modules for multiple genes or gene clusters within the same cell.

Fine-tuning gene expression is another key component of gene cluster engineering. This requires the availability of a wide range of well characterized regulation element, such as promoters [22,25]. The assembly toolkit we have developed also includes three constitutive and one inducible promoter system for precise gene expression, and an sfGFP reporter plasmid for testing regulatory elements. Using the toolkit, we have set up an efficient promoter screening system that makes promoters readily available for both assembly and testing. In the future, more strains of actinomyces strains can be tested with the present toolkit to expand application range. In addition, more orthogonal inducible promoter systems could be constructed to allow the discrete programming of multiple genes within a single cell. Other regulatory elements such as terminators also need to be developed based on the assembly toolkit.

In summary, our work presents an attractive assembly toolkit for engineering of gene cluster. Using this toolkit, a modular promoter library for tune gene expression was characterized by a rapid and efficient method. In addition, the assembly toolkit allows manipulation and optimization of large gene clusters and has been successfully used to clone and refactor the *act* BGC, resulting in improved actinorhodin production. Taken together, the modular plasmid toolkit provides a complete solution for manipulating gene clusters, and expands the plasmid toolkit for synthetic biology research in *Streptomyces*. The sequence of all the vectors can be found in the Supplementary Material. We hope that this will help to unlock the potential of the huge gene clusters from *Streptomyces* through synthetic biology and biotechnology.

CRediT authorship contribution statement

Xuejin Zhao: Data curation, Formal analysis, Methodology, Conceptualization, Paper writing. **Yeqing Zong:** Data curation, Formal analysis, Methodology. **Qiuli Lou:** Data curation. **Chenrui Qin:** Data curation. **Chunbo Lou:** Supervision, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2023.12.003.

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