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Gao, Hong, Taylor, Gabrielle, Evans, Stephanie K. et al. (2 more authors) (2020) Application of serine integrases for secondary metabolite pathway assembly in Streptomyces. Synthetic and Systems Biotechnology. pp. 111-119. ISSN 2405-805X

https://doi.org/10.1016/j.synbio.2020.05.006

Synthetic and Systems Biotechnology 5 (2020) 111–119

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: http://www.keaipublishing.com/synbio

Application of serine integrases for secondary metabolite pathway assembly in *Streptomyces*

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ARTICLE INFO

Keywords:
Serine integrase
Pathway assembly
Streptomyces
Erythromycin

ABSTRACT

Serine integrases have been shown to be efficient tools for metabolic pathway assembly. To further improve the flexibility and efficiency of pathway engineering via serine integrases, we explored how multiple orthogonally active serine integrases can be applied for use *in vitro* for the heterologous expression of complex biosynthesis pathways in *Streptomyces* spp., the major producers of useful bioactive natural products. The results show that multiple orthogonal serine integrases efficiently assemble the genes from a complex biosynthesis pathway in a single *in vitro* recombination reaction, potentially permitting a versatile combinatorial assembly approach. Furthermore, the assembly strategy also permitted the incorporation of a well-characterised promoter upstream of each gene for expression in a heterologous host. The results demonstrate how site-specific recombination based on orthogonal serine integrases can be applied in *Streptomyces* spp.

1. Introduction

Phage-encoded serine integrases catalyse site-specific integration of DNA into bacterial host chromosomes in a highly controllable and predictable way [1], making these proteins powerful tools for molecular genetics. Since the first serine integrases were discovered and described in the 1990s, integrases such as those from *Streptomyces* phage ϕC31 [2] and ϕBT1, and mycobacteriophage Bxb1, have been used as genome integration vectors in bacteria and in other organisms, including humans, mice and yeasts [3–6]. Moreover, the pool of available integrase proteins is still expanding, driven by the recent upsurge in synthetic biology applications of this protein family [7], such as in genetic memory devices [8].

Serine integrases bind to specific sites in the DNA that are 40–60 bp in length, bring these sites together, cut them, and then rejoin the sites to produce a recombinant product. Integration occurs between the so-called attachment sites derived from the phage (*attP*) and bacterial chromosome (*attB*) to yield the recombinant sites (*attL* and *attR*). The mechanism has been described in detail in previous publications [9–11]. During recombination the DNA is cut in both strands at specific locations in both *attP* and *attB* to yield 2 bp overhangs; as this 2 bp overhang is identical in both *attP* and *attB*, complementarity is maintained in the recombinants and the DNA backbone can be rejoined. Studies have shown that this 2 bp sequence can be changed to other sequences and recombination efficiency is unaffected, but only if both *attP* and *attB* have the same 2-bp sequence [12,13]. This central 2-bp site specificity has permitted the use of a single integrase to assemble DNA fragments together in a predictable order in a single recombination reaction [11,14]. This is evident in the study of Colloms et al., who developed the serine integrase recombinational assembly (SIRA) method that used ϕC31 to assemble up to 5 genes into a functional pigment biosynthetic pathway; the efficiency dropped from 87% for the 3-gene assembly to 18% for the 5-gene assembly [11]. This is because integrase attempts and fails to complete recombination between sites with non-identical crossover sites but recombination only proceeds to completion in reactions where the central 2-bp site is identical in both *attP* and *attB*.

As different integrases only recombine their cognate recombination sites, their activities are expected to be entirely separate and independent of each other, that is, an integrase should not recognise or recombine the substrate sites of the other integrases. This orthogonality permits the use of different integrases in the same recombination reaction, yielding entirely predictable recombinants depending on the location of recombination sites. Moreover, there should not be any loss

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Peer review under responsibility of KeAi Communications Co., Ltd.

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https://doi.org/10.1016/j.synbio.2020.05.006
Received 18 March 2020; Received in revised form 27 May 2020; Accepted 28 May 2020

Table 1
Plasmids used in this study.

Plasmid	**Description**[a]	Reference
pBF20	TG1 *int/attP, actIp-eryAI, tsr, ori/bla, aac3(IV), oriT*	16
pBF22	SV1 *int/attP, actIp-eryAIII, tsr, ori/bla, aphII, oriT*	16
pBF24	ϕBT1 *int/attP, actIp-eryAII, tsr, ori/bla, ermE, oriT*	16
pBF27C	ϕC31 *int/attP, tsr, ori/bla, hygB, oriT*	16
pHG1	ϕC31 *int/attP, tsr, bla/ori, actIIorf4/actIp-eryF, hygB, oriT*	This study
pHG1A	TG1 *int/attP, actIIorf4/actIp-eryF, tsr, ori/bla*	This study
pHG2A	*act1p, ori/bla*	This study
pHG2B0	Backbone vector for *eryB* genes assembly; ϕC31 *int/attP, tsr, bla/ori, actIIorf4/actIp-eryF, hygB, oriT, SPBc attP-lacZα-TG1-attP*	This study
pHG2B2	Plasmid carrying *eryBII* under *act1p* promoter (derived from pHG2A)	This study
pHG2B3	Plasmid carrying *eryBIII* under *act1p* promoter (derived from pHG2A)	This study
pHG2B45	Plasmid carrying *eryBIV* and *eryBV* under *act1p* promoter (derived from pHG2A)	This study
pHG2B6	Plasmid carrying *eryBVI* under *act1p* promoter (derived from pHG2A)	This study
pHG2B7	Plasmid carrying *eryBVII* under *act1p* promoter (derived from pHG2A)	This study
pHG2R2	ϕC31 *int/attP, actIp-eryBIV-eryBV, actIp-eryBVI, actIp-eryBIII-eryBII, actIp-eryBVII, tsr, ori/bla, actIIorf4/actIp-eryF, hygB, oriT, (BglII)*	This study
pHG3C1	Plasmid carrying *eryCI* under *act1p* promoter (derived from pHG2A)	This study
pHG3C23	Plasmid carrying *eryCII* and *eryCIII* under *act1p* promoter (derived from pHG2A)	This study
pHG3C45	Plasmid carrying *eryCIV* and *eryCV* under *act1p* promoter (derived from pHG2A)	This study
pHG3C6	Plasmid carrying *eryCVI* under *act1p* promoter (derived from pHG2A)	This study
pHG3F	ϕC31 *int/attP, actIp-eryBIV-eryBV, actIp-eryBVI, actIp-eryBIII-eryBII, actIp-eryBVII, tsr, ori/bla, actIIorf4/actIp-eryF, hygB, oriT, (the recognition sites of AsiSI and NsiI was inserted into pHG2R2)*	This study
pHG9A	Backbone vector for *eryC* genes assembly; ϕC31 *int/attP, actIp-eryBIV-eryBV, actIp-eryBVI, actIp-eryBIII-eryBII, actIp-eryBVII, tsr, ori/bla, actIIorf4/actIp-eryF, hygB, oriT, SPBc attP-lacZα-TG1-attP*	This study
pHG22A	ϕC31 *int/attP, actIp-eryBIV-eryBV, actIp-eryBVI, actIp-eryBIII-eryBII, actIp-eryBVII, actIp-eryCI, actIp-eryCII-eryCIII, actIp-eryCIV-eryCV, actIp-eryCVI, tsr, ori/bla, actIIorf4/actIp-eryF, hygB, oriT, SPBc attP-lacZα-TG1-attP*	This study
pJET1.2/blunt	Cloning vector, *bla*	Thermo Fisher
pLT57- *lacZα*	ϕBT1 integrating plasmid, carrying multiple *attP* sites for ϕC31, Bxb1, SPBc and TG1 integrases	Lab stock

[a] For clarity the *attL* and *attR* sites resulting after the assembly of the *eryB* and *eryC* genes have been left out of the description.

of efficiency of recombination, provided the integrases are sufficiently active in the recombination buffer used. Some integrases have already been used in pairs (*e.g.*, ϕC31 and Bxb1 integrase [15]) to investigate orthogonality, but information on the expected efficiency of using more than two integrases is not yet available.

Streptomyces is the primary producer of natural products, which have played important roles in healthcare all over the world. Biosynthetic gene clusters (BGCs) of natural products are large and complex (even more than 100 kb) with multiple layers of regulation. Synthetic biology offers a way of exploring BGCs for which the product has not yet been characterised, that is, cloning the genes of the BGC into vectors that integrate into a heterologous *Streptomyces* host such as *S. coelicolor* or *S. lividans* and under the control of well-characterised promoters. These tractable *Streptomyces* hosts have worked well in the biosynthesis of the complex natural products. Cloning each gene downstream of a promoter is a laborious and time-consuming process, especially when the BGC contains more than 20 genes. We propose a new strategy in which the genes are amplified using primers incorporating a promoter and assembled by *in vitro* fragment assembly using orthogonal integrases.

Previously our lab has used the erythromycin biosynthesis pathway as a model system for the expression of BGCs in a heterologous host [16]. The three polyketide synthase (PKS) genes *eryAI*, *eryAII* and *eryAIII* in the erythromycin BGC were cloned into three orthologous integrating plasmids, which were based on the *int/attP* loci from phages TG1, SV1 and ϕBT1 respectively. Following integration, 6-deoxyerythronolide B (6-dEB), the first intermediate produced by the three PKS enzymes, could be detected in the fermentation broth. The results demonstrated sequential integration of multiple orthologous integrating vectors is a reliable method to clone large genes required to synthesise many natural products.

Here we set out to demonstrate that multiplexed integration could be an option to improve the efficiency of recombination in the assembly of metabolic pathways. In this paper, results are presented for the use of orthogonal serine integrases for pathway level gene assembly. The results demonstrate improved strategies for the manipulation of BGCs in synthetic biology and for natural product discovery.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

E. coli strain Top10 (F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL*(Str^R) *endA1* λ^-) was used for plasmid propagation and subcloning. *E. coli* strain ET12567(pUZ8002) was used as the donor host in plasmid conjugations from *E. coli* to *Streptomyces* as described previously [17]. The *E. coli* strains were grown in Luria-Bertani broth (LB) or on LB agar at 37 °C.

Streptomyces coelicolor M1152 [*Δact Δred Δcpk Δcda rpoB*(*C1298T*)] [18] and *Streptomyces lividans* TK24 (*str-6* SLP2$^-$ SLP3$^-$) [19] were used as the recipients in conjugation. The strains were maintained on Soya Mannitol (SM) agar at 30 °C. Conjugations were performed on SM containing 10 mM $MgCl_2$, and tryptic soy broth (TSB) medium was used for the preparation of genomic DNA [19]. Antibiotic concentrations for *E. coli* were 150 μg/ml hygromycin, 50 μg/ml apramycin, 50 μg/ml kanamycin and 100 μg/ml ampicillin; and for *Streptomyces* were 50 μg/ml hygromycin, 50 μg/ml apramycin, 50 μg/ml kanamycin, 20 μg/ml erythromycin (in DMSO, 120 μg/ml erythromycin for *S. lividans* TK24) and 25 μg/ml nalidixic acid for selection.

2.2. DNA manipulation

E. coli transformation and gel electrophoresis were carried out as described previously [20]. Plasmid DNA extraction from *E. coli* was performed using a QIAprep spin miniprep kit (Qiagen, Germany) according to the manufacturer's protocol. Restriction enzymes were purchased from New England BioLabs (NEB, USA), and were used according to the manufacturer's instructions. In-Fusion® cloning (Clontech®, USA) and TA cloning (CloneJET PCR Cloning Kit, Thermo Scientific, USA) were used for joining DNA fragments. Genomic DNA preparation from *Streptomyces* was performed following the salting out procedure in the *Streptomyces* manual [19]. Polymerase Chain Reaction (PCR) was carried out using Phusion® High-Fidelity DNA Polymerase (NEB, USA) according to the manufacturer's instructions. The plasmids

Table 2
Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′-3′)
pHG1A-for	CGAACGCATCGATTAATTAAGGAGGATCGTATGACGACCGTTCCCG
pHG1A-rev	CGTGGTGGGCGCTAGCCTCCTCTAGTCATCCGTCG
pHG1-for	ACTAGAGGAGGCTAGCTTCAATGGAGGAGATGATCGAGG
pHG1-rev	GCAGGTCGACTCTAGATCTCGCTACGCCGCTACG
pHG3F-for	CCCCGGCGGCAACCCTCAGCGATCGCCTAGGATGCATGGATGCCCCGGGGCTTCAC
pHG3F-rev	TTCTTGTAGATCACCTCAGCGGCGAAGCCCGCAATA
pHG9A-for	GCCCCGGGGCATCCATGCATTCAACCCCGTTCCAGCCCAA
pHG9A-rev	CGGCAACCCTCAGCGATCCTGCAGGTCGACTCTAGATC
pHG2B0-for	CGGCGTAGCGAGATCTCAACCCCGTTCCAGCCCAA
pHG2B0-rev	GGTCGACTCTAGATCATATGGCTATGACATGATTACGAATTCGA
pHG2B2-for	CGATTATCTTTCTAGAGGAGGAGCCCATATGACCACCGACGCCGCGAC
pHG2B2-rev	TAGGAGATCTTCTAGGAGCTCTTGTGCTCACTGCAACCAGGC
pHG2B3-for	CGATTATCTTTCTAGAGGAGGAGCCCATGTGGGACTAGGCAAATGC
pHG2B3-rev	TAGGAGATCTTCTAGGAGCTCGTCATACGACTTCCAGTCG
pHG2B45-for	CGATTATCTTTCTAGAGGAGGAGCCCATGTGAATGGGATCAGTGATTCC
pHG2B45-rev	TAGGAGATCTTCTAGAGCTCGGACGGACTTGTCGGTC
pHG2B6-for	CGATTATCTTTCTAGAGGAGGAGCCCATATGGGTGATCGGACCGGC
pHG2B6-rev	TAGGAGATCTTCTAGGAGCTCAGCGCGCGTTTCATCCG
pHG2B7-for	CGATTATCTTTCTAGAGGAGGAGCCCATTTGCGAGTACGTGAGCTGGCGG
pHG2B7-rev	TAGGAGATCTTCTAGGAGCTCTCGGGACCCGTCACCTG
pHG2A-for	ACGGTGAGAAGGTGCTCGTGTAG
pHG2A-rev	AATCGATGCGTTCGTCCGGTGG
TG1 attB F	**TCGATCAGCTCCGCGGGCAAGACCTTCTCCTTCACGGGGTGGAAGGTCGG**TGAGAAGGTGCTCGTGT
Bxb1 attB R	**CCGGATGATCCTGACGACGGAGACCGCCGTCGTCGACAAGCCGGCC**CGCCACCTACAACGGTTCCT
Bxb1 attP F	**GTGGTTTGTCTGGTCAACCACCGCGGTCTCAGTGGTGTACGGTACAAACCCA**CGGTGAGAAGGTGCTCGTGTA
Int9 attB R	**CAAATTTTAATTGGCGGATGAGGTATCCAGTTACCTCGTTCGCCAATTATTTTTCGCAATATAAA**GCCACCTACAACGGTTCCTGAT
Int9 attP F	**GTGGTTGTTTTTGTTGGAAGTGTGTATCAGGTATCTGCATAGTTATTCCGAACTTCCAATTA**ACGGTGAGAAGGTGCTCGTGTA
Int4 attB R	**AATCATCGCCTTTACACAAGCAGCAGTCTTATTCAAATTTCAGGTCGCGTTGGGCGCTCTTTGGAA**CGCCACCTACAACGGTTCCTGAT
Int4 attP F	**AAAAATTACAAAGTTTTCAACCCTTGATTTGAATTAGCGGTCAAATAATTTGTAATTCGTTT**CGGTGAGAAGGTGCTCGTGTAGCA
Int7 attB R	**AACGGACGACCCGGTCATCAACTTTGCCCAACTGACCCAGACGGACGGAACGTTTCTCGTCT**CGCCACCTACAACGGTTCCTGAT
Int7 attP F	**GTGTTATAAACCTGTGTGAGAGTTAAGTTTACATGCCTAACCTTAACTTTTACGCAGGTTCAGCTT**CGGTGAGAAGGTGCTCGTGTAGCA
SPBc attB R	**CACCCATTGTGTTCACAGGAGATACAGCTTTATCTGTACTGATATTAATGACATGCTGCACT**CCACGCCACCTACAACGGTTCCTG
pHG3C1-for	CGATTATCTTTCTAGAGGAGGAGCCCATATGGACGTCCCCTTCCTG
pHG3C1-rev	TAGGAGATCTTCTAGTCAAGCCCCAGCCTTGAGG
pHG3C23-for	CGATTATCTTTCTAGGGAGGAGCCCATATGACCACGACCGATCGC
pHG3C23-rev	TAGGAGATCTTCTAGAGGTCATCGTGGTTCTCTCCTTCC
pHG3C45-for	CGATTATCTTTCTAGAGGAGGAGCCCATGGATGAAACGCGCGCTGAC
pHG3C45-rev	CAGTTTCCTGAAGCTTCACCTTCCGCGCAGGAA
pHG3C6-for	CGATTATCTTTCTAGAGGAGGAGCCCATGGATGTACGAGGGCGGGTTCGC
pHG3C6-rev	TAGGAGATCTTCTAGGGTCATCCGCGCACACC

Restriction enzyme recognition sites were underlined.
The attB/attP sites were indicated in bold letters.

used in this study are listed in Table 1 and the primers used are listed in Table 2.

2.3. Plasmids

2.3.1. Construction of *eryF* expression plasmid pHG1

The *eryF* gene was amplified from *Saccharopolyspora erythraea* BIOT-0666 genomic DNA using the primer pair pHG1A-for/pHG1A-rev, and inserted by In-Fusion cloning into pBF20 [16] cut with NheI and PacI to form the plasmid pHG1A. The 3785 bp fragment containing the ϕC31 *int/attP* and hygromycin resistance gene was amplified from plasmid pBF27C [16], using the primer pair pHG1-for and pHG1-rev. Plasmid pHG1A was digested with XbaI and NheI, and the 5668 bp fragment was ligated with the 3785 bp PCR fragment from pBF27C by In-Fusion cloning to give the plasmid pHG1 (Fig. 1).

2.3.2. Backbone vectors for DNA assembly

The fragment containing TG1 *attP-lacZα*-SPBc *attP* was amplified from the plasmid pLT57-*lacZα* (Table 1) using the primer pair pHG2B0-for and pHG2B0-rev, then cloned into pHG1 cut with BglII by In-Fusion to form the plasmid pHG2B0 (Fig. 1). The plasmid pHG2B0 contains the *eryF* gene, and the *lacZα* reporter gene flanked with the TG1 *attP* [21] and SPBc *attP* [22] sequences. The plasmid pHG2B0 was used as the vector backbone for the assembly of the *eryB* genes.

One of the correctly assembled plasmids was named pHG2R2 (Fig. 1) and this plasmid was used to construct the backbone vector, pHG9A (Fig. 1), for receipt of the assembled *eryC* genes. To construct pHG9A, the *lacZα* reporter gene flanked with the TG1 *attP* and SPBc *attP* sequences was inserted between the hygromycin resistance gene and the ϕC31 *attP*/int locus in pHG2R2, allowing the *attP* sites to be again used to integrate the DNA fragment assembly, in this case the *eryC* genes. Plasmid pHG9A was therefore constructed as follows: First, to insert restriction enzyme sites AsiSI and NsiI, the primer pair pHG3F-for and pHG3F-rev was used to amplify a fragment of approximate 1 kb size from pHG2R2, which was then cloned back into pHG2R2 digested by BbvCI (two sites, located at 7385 and 8395 bp separately) to give plasmid pHG3F (Fig. S1). Next, the fragment TG1 *attP-lacZα*-SPBc *attP* was amplified from the plasmid pHG2B0 using the primer pair pHG9A-for and pHG9A-rev, then cloned into pHG3F cut with AsiSI and NsiI by In-Fusion to form the plasmid pHG9A.

2.4. DNA assembly

2.4.1. Assembly of *eryB* genes

All the *eryB* genes were amplified from *S. erythraea* BIOT-0666 genomic DNA using the primer pairs listed in Table 2 (pHG2B2-for/rev, pHG2B3-for/rev, pHG2B45-for/rev, pHG2B6-for/rev and pHG2B7-for/rev). As *eryBIV* and *eryBV* are adjacent genes in the native erythromycin biosynthesis gene cluster *S. erythraea* BIOT-0666 and apparently translationally coupled, they were amplified together so that their

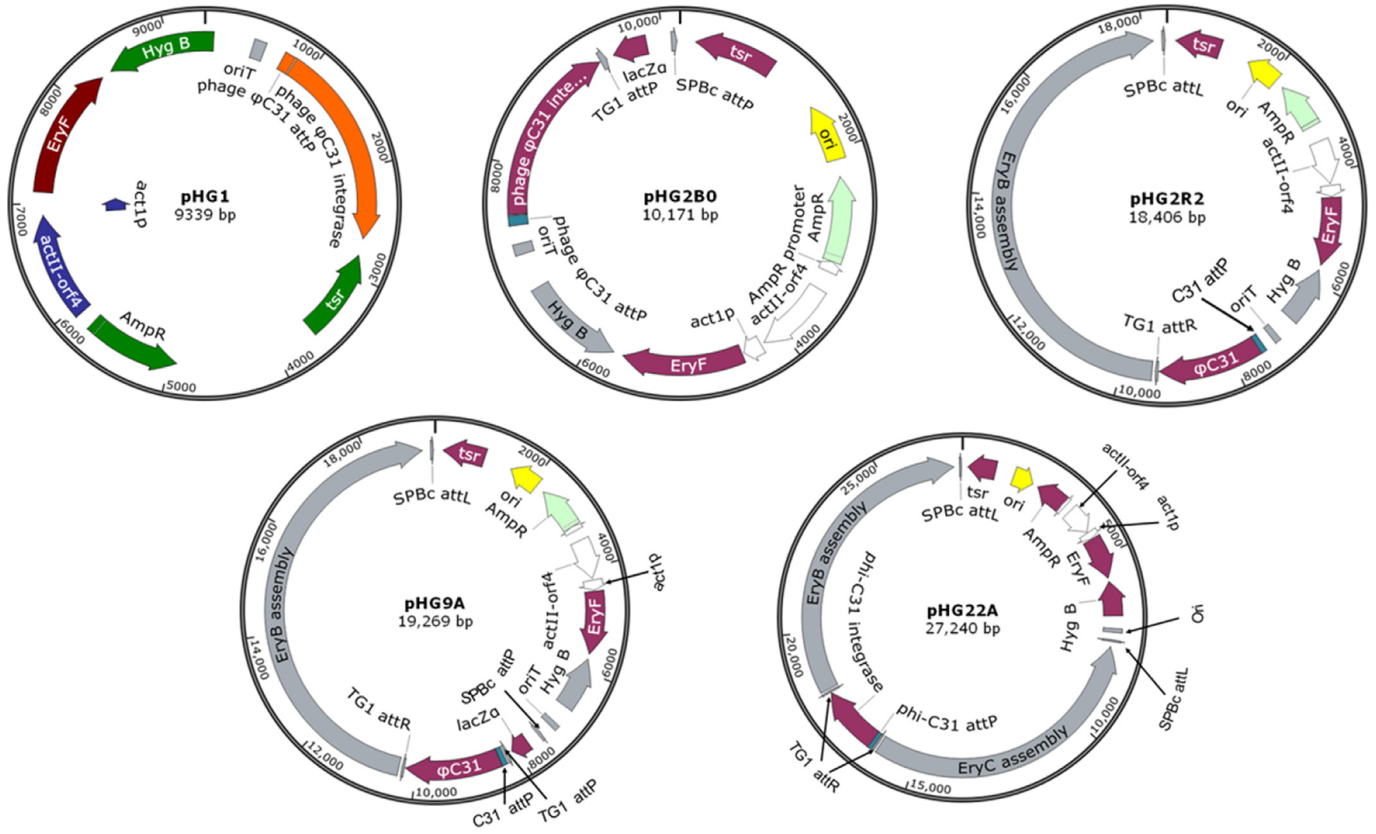


Fig. 1. Plasmids used for integration in this study. In pHG2R2, pHG9A, and pHG22A, the *eryB* genes were assembled in the order of B45–B6–B3–B2–B7, and integrases used are TG1-Int7-Int9-Int4-Bxb1-SPBc, *act1p* was cloned at the upstream of each gene fragment (B45, B6, B3, B2 or B7). In pHG22A, the *eryC* genes were assembled in the order of C1–C23–C45–C6, the integrases used are TG1-Bxb1-Int9-Int7-SPBc, and *act1p* was cloned at the upstream of each gene fragment.

coupled status was maintained.

Firstly, an *act1p* promoter fragment was amplified using the primer pair pHG2A-for/pHG2A-rev (Table 2), then cloned into pJET1.2/blunt, to generate pHG2A (Fig. S1). Then, the five gene fragments: *eryBII*, *eryBIII*, the fragment containing *eryBIV* and *eryBV*, *eryBVI*, and *eryBVII*, were cloned individually into pHG2A downstream of the *actIp* promoter (Fig. 2), to give plasmids pHG2B2, pHG2B3, pHG2B45, pHG2B6 and pHG2B7, respectively. Gene cassettes, each encoding one of the *eryB* genes or the *eryBIV/V* operon and the upstream *actIp* promoter were amplified from plasmids pHG2B2 - pHG2B7 using the primer pairs listed in Table 2 (TG1 attB F, Bxb1 attB R, Bxb1 attP F, Int9 attB R, Int9 attP F, Int4 attB R, Int4 attP F, Int7 attB R, Int7 attP F and SPBc attB R). Each primer contains the sequence of an *attP* or *attB* site and, at the 3’ end, a sequence that binds to vector derived DNA. The amplified gene cassettes can thus be tagged with different *attP/B* sites. *In vitro* recombination between all matching *attP-attB* pairs using purified integrases joins the cassettes together end-to-end in a specific order and inserts the resulting linear assembly into the pHG2B0 vector, replacing the *lacZα*. This system is extremely versatile as the use of the vector binding sites in the primer sequences allows the researcher to generate different orders of assembled genes and thus optimise expression of the gene assembly. The strategy to amplify the DNA fragments to be used for assembly is shown in Fig. 2A.

The assembly strategy for the assembly of *eryB* genes is shown in Fig. 2B. In this assembly, six integrases were used: TG1 [23], Bxb1 [24], Int9, Int4, Int7 [8] and SPBc [22], each recognising only their cognate *attP* and *attB* sites located in the amplified gene cassettes.

2.4.2. Assembly of eryC genes

The *eryC* genes were assembled using a similar strategy to that used for the *eryB* genes. The *eryC* genes were amplified from *S. erythraea* BIOT-0666 genomic DNA using the primer pairs listed in Table 1 (pHG3C1-for/rev, pHG3C23-for/rev, pHG3C45-for/rev and pHG3C6-for/rev). Among them, *eryCII* and *eryCIII*, and *eryCIV* and *eryCV* are located adjacent to each other and are possibly translationally coupled in the native erythromycin biosynthesis gene cluster. Their putative operon organisation was maintained for the assembly and the two gene pairs were amplified generating two separate fragments. Thus, four DNA fragments: *eryCI*, the fragment containing *eryCII* and *CIII*, the fragment containing *eryCIV* and *eryCV*, and *eryCVI* were cloned individually into pHG2A, to give plasmids pHG3C1, pHG3C23, pHG3C45 and pHG3C6. In each plasmid, the gene fragment containing the *eryC* gene(s) was under the control of the *act1p* promoter. Each cassette used in assembly was amplified from plasmids pHG3C1 – pHG3C6 using the primer pairs listed in Table 2 (TG1 attB F, Bxb1 attB R, Bxb1 attP F, Int9 attB R, Int9 attP F, Int7 attB R, Int7 attP F and SPBc attB R). As for the assembly of the *eryB* genes, the primers contain the *attP* or *attB* sites and a region at the 3′end that binds to vector sequences derived from pHG2A. *In vitro* recombination between all matching *attP-attB* pairs via the integrases joins the cassettes together end-to-end in a pre-determined order and inserts the resulting linear assembly into the pHG9A vector, replacing the *lacZα*.

The assembly strategy is shown in Fig. 3A. In the *eryC* assembly, five integrases were used: TG1 [23], Bxb1 [24], Int9, Int7 [8] and SPBc [22], each recognising only its cognate *attP* and *attB* sites located in the amplified gene cassettes.

2.4.3. Assembly reactions

The enzymes were prepared as previously described [21,25]. Recombination reactions contained one of four different buffers: Buffer 1 (ϕC31 RxE buffer [26]), buffer 2 (10 mM Tris-HCl [pH 7.5], 5 mM spermidine·3HCl, 2 mM dithiothreitol, 0.1 mM EDTA, 0.1 mg/ml

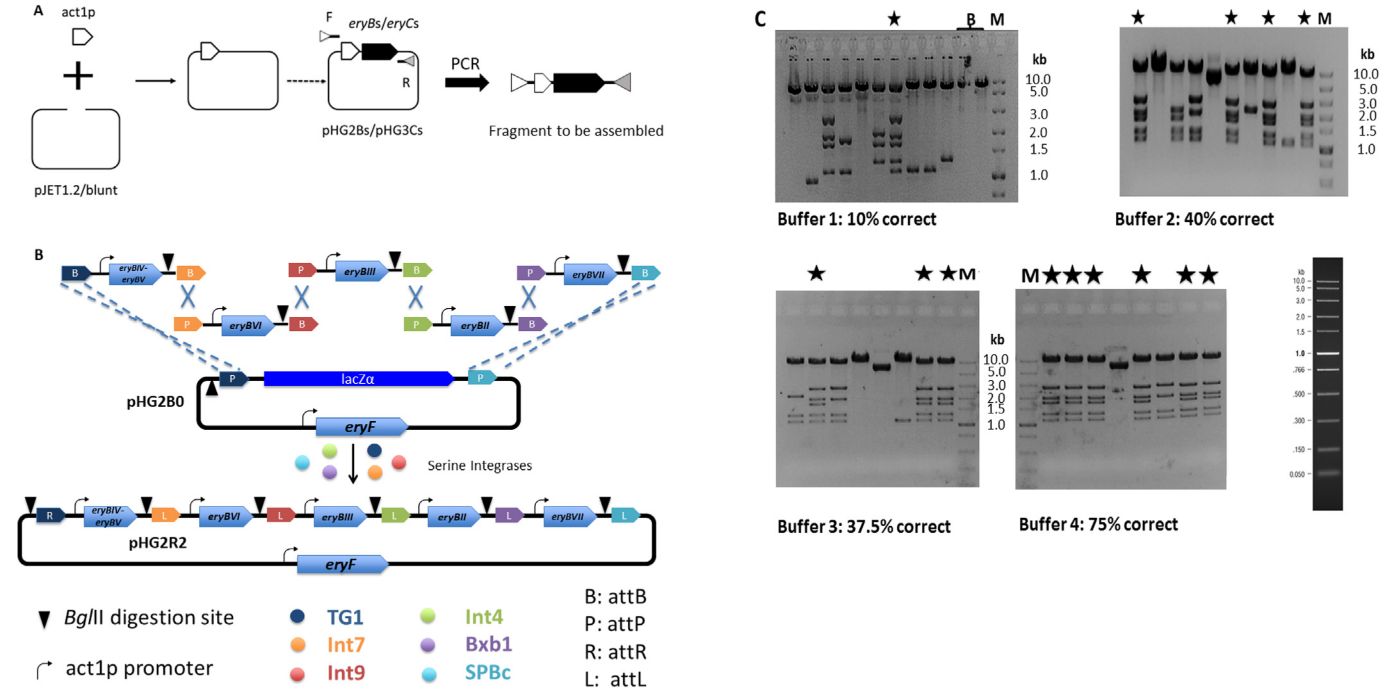


Fig. 2. The assembly of DNA fragments. (A) The construction of DNA fragments to be assembled. The *eryB* or *eryC* genes were cloned into pJET1.2/blunt, under the control of the promoter *act1p*. Next, primer pairs (F: forward primer; R: reverse primer) contained *attB* or *attP* sites (indicated using triangle) at the 3′ end were used to amplify the DNA fragment used in the assembly reaction. The primers only contain the sequence complemented with the sequences in the backbone vector flanking the target DNA fragment, rather than the specific *eryB* or *eryC* genes. So these primers are universal and could be used to amplify any gene inserted in the backbone vector in the same way. (B) The assembly strategy of pHG2R2. The length of each fragment is: pHG2B0 (10.2 kb), eryBII (1.4 kb), eryBIII (1.7 kb), eryBIV-BV (2.7 kb), eryBVI (1.9 kb) and eryBVII (1.0 kb); (C) The assembly reactions were carried out in four buffers, and the assembled plasmids were checked by digesting with BglII. Stars mark the clones have been assembled correctly. B: pHG2B0, M: NEB Fast DNA Ladder (from https://international.neb.com/products/n3238-fast-dna-ladder#Product%20Information). 0.8% agarose gel was used to check the digested bands.

bovine serum albumin and 4.5% glycerol, adapted from Refs. [11]), buffer 3 (Bxb1 RxE buffer [26]) and buffer 4 (TG1 RxE buffer [26]), in 20 μl final volume. The final concentrations of integrases were: 200 nM for TG1 and Bxb1, 100 nM for Int9, Int4 and Int7, and 50 nM for SPBc. Recombination substrates were used at 50 ng each per reaction. Reactions were incubated at 30 °C overnight and then heated (10 min, 75 °C) to denature integrases. The reaction mixtures were transformed into *E. coli* Top10 and blue-white screening was used to select colonies containing assembled plasmids.

2.5. Production and analysis of erythromycin intermediates

Strains were cultured in 25 ml of R2YE medium [19] as seed culture medium for 3 days at 30 °C, then 1.25 ml of inoculum was transferred to 25 ml of R2YE medium fed with 0.2 ml of propan-1-ol at 30 °C for fermentation. The extraction process was carried out as described previously [16].

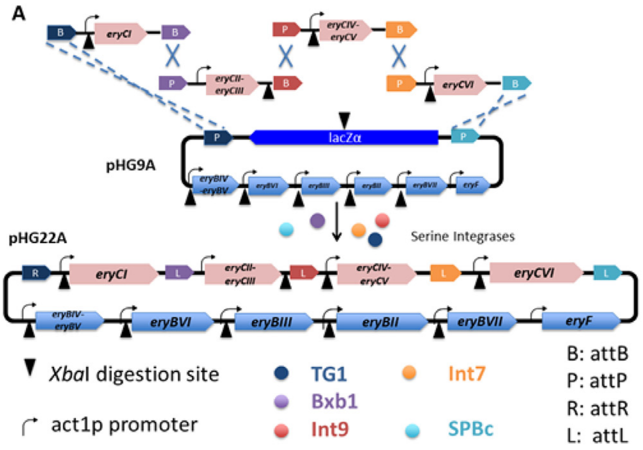
Dried extracts were re-suspended in methanol (200 μl), and an aliquot (5 μl) was injected for analysis by high-pressure liquid chromatography (Acquity I class; Waters, Elstree, UK) on a reverse-phase BEH C18 column (100 by 2.1 mm, 1.7 μm, Waters; including a Waters security guard; flow rate 0.4 ml·min-1; 40 °C). Solvents A (0.1% [vol/vol] formic acid in water) and B (0.1% [vol/vol] formic acid in methanol) were mixed to give 40% B initially and held at 40% B upon injection for 0.2 min, and then a linear gradient was applied from 40 to 100% B over 3.8 min. The solvent was then held at 100% B for 0.34 min prior to reversion back to 40% B and equilibration for 1.32 min before the next injection. Total method duration was 5.7 min. Samples contained 5 μM internal standard (IS) Roxithromycin (Rox; Sigma-Aldrich, Gillingham, UK). Calibration standards containing a fixed amount of the internal standard, Rox (5 μM), and increasing amounts of Erythromycin A (EryA; Sigma Aldrich, Gillingham, UK), namely 0.2–100 μM, were prepared and used to quantify Erythronolide B (EB), 6-dEB and 3-*O*-alpha-L-Mycarosylerythronolide B (MEB). Both EB and 6-dEB were provided by Isomerase Therapeutics, purified from fermentations with blocked mutants of *S. erythraea*. Standards for EB and 6-dEB were used for qualitative but not quantitative purposes. Analytes were identified by their mass spectra and MS/MS fragmentation profiles using electrospray ionization mass spectrometry (Fusion Orbitrap, Thermo Fisher Sci, Loughborough, UK; positive-ion mode; capillary voltage 3500 V; mass range, 150–1700 *m/z*; Orbitrap resolution: 240000; HCD fragmentation).

Values for analytes were calculated using Xcalibur software (v. 4.0.27.10, Thermo Fisher Sci, Loughborough, UK) by plotting normalised areas of extracted ion chromatograms (EICs) corresponding to the sodium ion adducts of the molecular ion of 6-dEB ([M+Na]+ = 409.256 *m/z*), EB ([M+Na]+ = 409.256 *m/z*), MEB ([M+Na]+ = 596.330); and the protonated ions of EryA ([M+H]+ = 734.468) and Rox ([M+H]+ = 837.531).

3. Results

3.1. Production of the erythromycin intermediate EB

The biosynthesis pathway of erythromycin A is shown in Fig. 4. This study was designed to assemble *eryB* and *eryC* genes using orthogonal integrases. A strain producing EB was needed to be the starting strain, and then the production of MEB or erythromycin D could be tested after the assembly of *eryB* or *eryC* genes, to confirm the assembled pathways are functional. So the *eryF* expression plasmid pHG1 was introduced by conjugation into the 6-dEB producers constructed previously; *S. coelicolor* M1152:pBF20:pBF22:pBF24 (M1) and *S. lividans* TK24:pBF20:pBF22:pBF24 (T1) [16]. Genomic DNA from the recipient strains was extracted, and PCR was used to confirm the integration.



B

Size (kb)	Fragment
9.2	Containing eryCVI, eryF and eryBVII
3.9	Containing eryCI and eryCII-CIII
3.2	eryCIV-CV
2.7	eryBVI-BV
2.6	Between eryBVI-BV and eryCI
2.0	eryBVI
1.7	eryBIII
1.5	eryBII
0.5	Between eryCII-CIII and eryCIV-CV

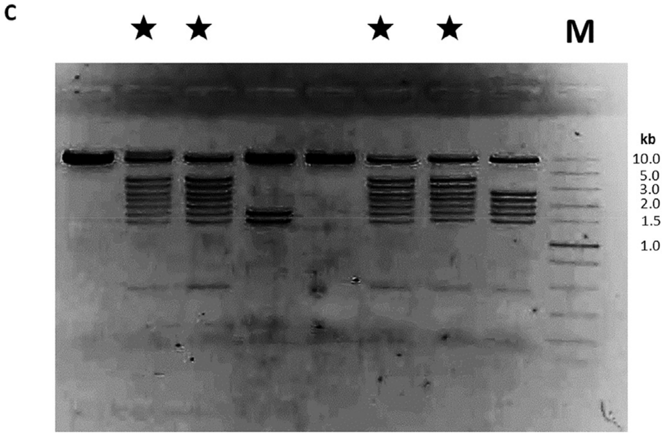


Fig. 3. Assembly of *eryC* genes. (A) The assembly strategy of pHG22A. The length of each fragment is: pHG9A (19.3 kb), eryCI (1.5 kb), eryCII-CIII (2.7 kb), eryCIV-CV (3.1 kb) and eryCVI (1.1 kb); (B) The assembled plasmids were checked by digesting with XbaI. NEB Fast DNA Ladder was used to assess the size of digested fragments. From (A), XbaI could digest the correctly assembled plasmids into these fragments: Stars mark the clones have been assembled correctly. M: NEB Fast DNA Ladder. 0.8% agarose gel was used to check the digested bands.

In both *S. coelicolor* M1152:pBF20:pBF22:pBF24:pHG1 (M2) and *S. lividans* TK24:pBF20:pBF22:pBF24:pHG1 (T2), the expected ion (*m/z* = 425.3) indicative of EB could be found (peak 2, Fig. 5). The yield of 6-dEB produced in M1 was lower than that in T1 [16], but M2 and T2 achieved similar levels of EB production (~10 mg/L) and biomasses (0.06 g and 0.08 g [dry weight] per 5 ml of culture, respectively). Furthermore, in both M2 and T2, only a tiny amounts of 6-dEB (~1 mg/L) could be detected, suggesting high efficiency of the hydroxylase encoded by *eryF* gene in converting 6-dEB to EB. Thus, pHG1 can be modified into a backbone vector for the assembly of *eryB* genes with the aim of generating a strain that produces MEB.

3.2. Pathway assembly

3.2.1. Choice of integrases

Since the assembled plasmids will be recombined into the chromosomes of Streptomyces hosts through ϕC31, this widely used integrase was not used in the assembly reactions. TG1 and SPBc have been demonstrated to be effective to insert complex pathways into assembly backbone vectors in our lab's previous work (unpublished data). Therefore, the attP sequences for TG1 and SPBc were used as the entry sites on the assembly backbone vectors in this study. From all the serine integrase stocks stored in our lab, Bxb1, Int9, Int4, and Int7 were chosen because they performed relatively consistently in different buffer systems and the introduction of the attB or attP sequences to PCR primers did not hamper the PCR reactions.

Here, integrases Int4, Int7 and Int9 were identified by Yang et al. [8]. Int4 was isolated from *Streptococcus equi*, Int7 was from *Geobacillus sp.*, and Int9 was identified from *Staphylococcus aureus*. They are not similar to other well-known serine integrases, including TG1, SPBc and Bxb1 (the other integrases used in this study); and the attB/attP sites (Table 2) do not share significant similarity to attB/attP sites of other well-known serine integrases as well.

3.2.2. Assembly of the eryB genes

The six *eryB* genes were assembled as described in Materials and Methods. The assembly reactions were performed in four previously described integrase buffers, referred to here as buffers 1 to 4. The *eryB* genes were ordered as B2–B3–B45–B6–B7. After the assembly reactions were stopped and introduced by transformation into *E. coli*, eight or ten white colonies were picked randomly; plasmids were extracted and digested with BglII. Six bands after digestion, as shown in the lanes marked with stars in Fig. 2C, suggested that all *eryB* genes had been incorporated. The assembly reactions showed different efficiencies in the four buffers (Fig. 2C), and using buffer 4, most (75%) of the white colonies contained the correctly assembled plasmids.

Efforts were made to assemble the *eryB* genes in different orders (Table S1). When the order of fragments was changed from the standard B2–B3–B45–B6–B7, the assembly efficiency dropped. For example, when the *eryB* genes were assembled in the order B45–B6–B3–B2–B7 (the plasmid was named pHG2R2), all the white colonies from the same reaction in the previously identified optimal buffer 4 did not contain any correctly assembled plasmids, and correctly assembled plasmids were only achieved when buffer 2 was used. The results suggested that the multiplex integration could work, but as a complex reaction including six integrases, further investigation will be required to learn the reaction kinetics, which will be extremely helpful in optimising the reactions.

Next, the assembled plasmids were transferred into M1 and T1 individually. After 7 days fermentation in R2YE medium fed with propan-1-ol, MEB production ability was tested. None of the *S. lividans* strains produced any MEB in their fermentation broth, while amongst *S. coelicolor* strains, *S. coelicolor* M1152:pBF20:pBF22:pBF24:pHG2R2 (M4) produced a very small amount of MEB (~1.4 mg/l, Fig. S2). So, the plasmid pHG2R2 was modified into pHG9A for the assembly of *eryC* genes.

3.2.3. Assembly of eryC genes

The six *eryC* genes were assembled as described in Materials and Methods in buffer 4. Then eight white colonies were picked randomly; plasmids were extracted and digested by XbaI to check if all the *eryC* genes had been incorporated. The efficiency of the assembly reaction (50%, Fig. 3B) was lower than for the assembly of *eryB* genes (75%).

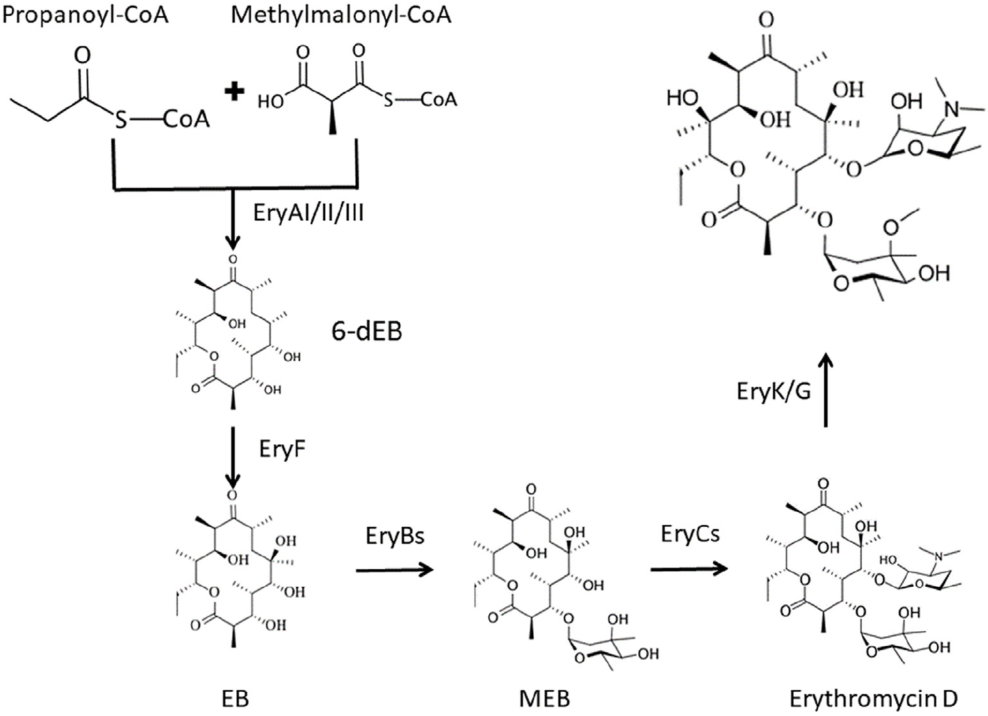


Fig. 4. The biosynthetic pathway of erythromycin A.

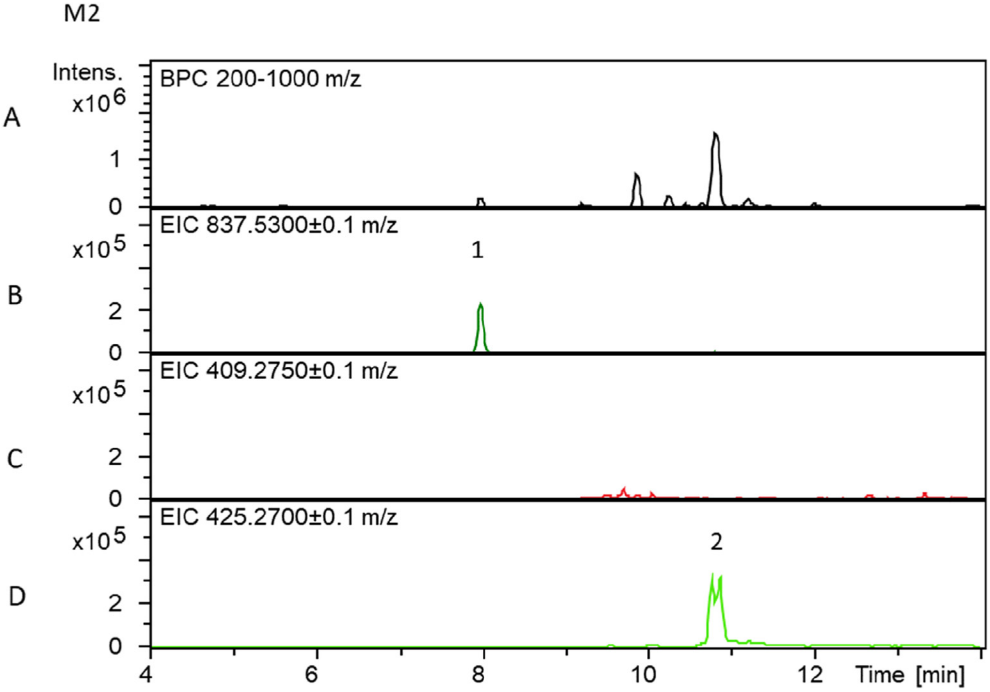


Fig. 5. Production of EB in *Streptomyces coelicolor* M1152. (A) HPLC base peak chromatogram (BPC) showing the most intense peaks for each mass spectrometry scan in the extract from a *S. coelicolor* M1152:pBF20:pBF22:pBF24:pHG1 (M2) fermentation. (B) Extracted ion chromatogram (EIC) of the internal standard roxithromycin (*m/z* 837.53; peak 1) from the same extract as in panel A. (C) Extracted ion chromatogram of 6-dEB (*m/z* 409.2750) from the same extracted as in panel A, showing almost all 6-dEB has been catalysed by EryF. (D) Extracted ion chromatogram of EB (*m/z* 425.2700, peak 2) from the same extracted as in panel A.

However, considering the size of the final plasmid increases from 19.2 kb (pHG2R2) after *eryBs* assembly to 28.1 kb (pHG22A) after *eryCs* assembly (Fig. 1), the correct plasmids' ratio is still satisfactory. However, when the *eryC* genes were assembled in different orders (Table S1), no correct clone was obtained. Three plasmids that were incorrectly assembled were picked randomly and sent for sequencing. The sequencing results showed that the SPBc *attP* site (GTAGTAAGTATCT TAATATACAGCT<u>TTA</u>TCTGTTTTTTAAGATACTTACTACTTT) frequently recombined with a sequence upstream of the *act1p* promoters (TGCTCGTGTAGCACCGGTCCGTGAACGCGGTGGAGCCCCTC<u>TTA</u>AG) through the nucleotides TTA. Since there are multiple *act1p* promoters in the backbone plasmid and the fragments being assembled, there were many opportunities for recombination between the SPBc *attP* and the off-target site. The net result was that the incorrectly assembled plasmids had unwanted deletions. However, why the recombination between the SPBc *attP* and the off-target site was much higher in some reactions and not others remains unclear; further exploration is required.

4. Conclusions and discussion

In this study, we tested and optimised protocols for multiplexing

Table 3
The examples of pathway assembled using serine integrases.

Integrase used	Product synthesised	Pathway assembled		Percentage of correctly assembly	Reference
		Size (kb)	Number of fragments		
φBT1	Epothilone	56	9	Not reported	14
φC31	Lycopene, β-carotene, and zeaxanthin	Up to 7.8	8	48% for 7.8 kb assembly	11
Bxb1	Lycopene	3.6	3	> 90%	25
Multiple integrases	EB	8.7	5	75%	This study
	MEB	8.4	4	50%	

integrases to assemble antibiotic pathway genes into integrating plasmids. The erythromycin pathway from *S. erythreae* was chosen as an example in this work. The erythromycin pathway PKS genes had already been cloned into three orthogonal integrating vectors and the production of their product 6-dEB had been demonstrated [16]. The next big challenge was to assemble the smaller genes required for glycosylation of the aglycone and to place each one downstream of a promoter that would express in a heterologous host. Using multiple integrases in an *in vitro* recombination reaction five *eryB* DNA fragments (9.0 kb total and assembled plasmid is nearly 20 kb) were initially assembled into an integrating plasmid compatible with the three already expressing the PKS genes. The four fragments encoding the remaining *eryC* genes were also inserted to generate a plasmid of around 30 kbp.

The design of the assembly pathway employed here allowed a combinatorial approach for the order of the DNA fragments being assembled. This is because the primers tagged with the different integrase *attP/B* sites bound universally to the same site in the vector backbones into which the *ery* genes had been cloned. However, when we tried to assemble the *eryB* genes in different orders, the ratio of successfully assembled vectors dropped dramatically. More research on enzyme kinetics is needed. For example, in this study, the activities of the integrases in different buffers were only tested qualitatively. In the future, the activities could be assessed quantitatively, thus to help us understand the enzyme kinetics in the multiplexed assemble reaction.

The application of serine integrases in pathway assembly has been explored in several previous publications [11,14,25], and a couple of review papers have discussed methods used for pathway assembly, including the ones based on serine integrases [10,27,28]. All the previous publications using serine integrases to assemble DNA fragments were based on using a single integrase (Table 3). There are other reports using a combination of a serine integrase and other enzymes to assembly complex pathways, usually with tyrosine integrases, *Cre* [29,30] or FLP recombinases [31]. As far as we are aware, the work presented here is the first to exploit a multiplex integration system based on using more than one integrase at a time. For five fragments assembled into a 10 kb backbone vector, 75% of colonies were the correctly assembled plasmids, and for four fragments assembled into the 20 kb backbone vector, 50% of colonies contained the desired construct. Although the reliability needs to be further improved, and more investigations are required to avoid the off-target recombination, the high efficiency of this assembly strategy still suggests that there is potential for its use in pathway assembly and synthetic biology.

To the best of our knowledge, this study is the first time that multiplex recombination based on serine integrases has been used to assemble multiple genes into pathways. These results open the way for further investigation of the application and potential of this enzyme family in synthetic biology and natural products biosynthesis.

CRediT authorship contribution statement

Hong Gao: Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Gabrielle Taylor:** Resources, Writing - review & editing. **Stephanie K. Evans:** Resources, Writing - review & editing. **Paul C.M. Fogg:** Resources, Writing - review & editing. **Margaret C.M. Smith:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Acknowledgements

This work was supported by the Biotechnology and Biological Sciences Research Council project grant BB/K003356/1. HPLC-MS analysis was performed on equipment within the Centre of Excellence in Mass Spectrometry (University of York), funded by Science City York (Yorkshire Forward, EP/K039660/1, EP/M028127/1). The strain *Saccharopolyspora erythraea* BIOT-0666 was a gift from Dr Matthew Gregory, Isomerase Therapeutics Ltd, and we also thank Professor Marshall Stalk for the proof reading of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2020.05.006.

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