Use of orthogonal serine integrases to multiplex plasmid conjugation and integration from *E. coli* into *Streptomyces*

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Abstract

Some major producers of useful bioactive natural products belong to the genus *Streptomyces* or related actinobacteria. Genetic engineering of these bacteria and the pathways that synthesize their valuable products often relies on serine integrases. To further improve the flexibility and efficiency of genome engineering via serine integrases, we explored whether multiple integrating vectors encoding orthogonally active serine integrases can be introduced simultaneously into *Streptomyces* recipients via conjugal transfer and integration. Pairwise combinations of *Escherichia coli* donors containing vectors encoding orthogonal serine integrases were used in each conjugation. Using donors containing plasmids (of various sizes) encoding either the \(\phi_{BT1}\) or the \(\phi_{C31}\) integration systems, we observed reproducible simultaneous plasmid integration into *Streptomyces coelicolor* and *Streptomyces lividans* at moderate frequencies after conjugation. This work demonstrated how site-specific recombination based on orthogonal serine integrases can save researchers time in genome engineering experiments in *Streptomyces*.

INTRODUCTION

Phage-encoded serine integrases are a family of recombinases that mediate integration or excision of the phage genome into or out of the bacterial host chromosome [1]. Since the first serine integrases were described, such as those from *Streptomyces* phages \(\phi_{C31}\) and \(\phi_{BT1}\) [2, 3], and mycobacteriophage Bxb1 [4], these site-specific recombination systems have been used to develop genome integration vectors in bacteria and other organisms, including human and mammalian cell lines, plants and fungi [5–9]. Integration is the recombination between a specific sequence in an incoming circular DNA molecule (the \(att\)P site) and a specific site in the recipient genome (normally the endogenous \(att\)B site). Serine integrases bind to these sites, which are just 40–60 bp in length, and bring the sites together through protein–protein interactions. Within this complex the DNA sites are cut by integrase, maintaining the high-energy phosphate bond in the form of a phospho-serine link, and then reconfigure so that inteprase can then rejoin the DNA strands but in a recombinant format. The end product is an integrated plasmid flanked by the recombinant sites, \(attL\) and \(attR\). In some synthetic biology applications the excision reaction is also a useful feature [10–12]. Excision is the recombination, mediated by the serine integrase in the presence of a recombination directionality factor or RDF, of \(att\)L and \(att\)R to yield the reconstituted \(att\)B site and the \(att\)P site located on excised DNA. The mechanism of integration and excision by serine integrases has been described in detail in previous publications [8, 13, 14]. The efficiency, specificity and highly controllable nature of serine integrase-mediated recombination has led to these systems being widely applied in molecular genetics.

Although the mechanism of serine integrases has only been studied in a few members of this family, serine integrases can be detected in many phages and prophages. The pool of available \(int/att\)P sites is expanding, and the rate-limiting step to their use in heterologous systems has been the detection of the \(att\)B site, although this is made easier if the source of the \(int/att\)P locus is a phage. The surge in synthetic biology applications of this protein family [15], such as in genetic memory devices [10–12], has resulted in a greatly increased number of characterized serine integrase systems.

*Streptomyces* spp. are known for their production of bioactive secondary metabolites or natural products, which are important in both healthcare and agriculture. Biosynthetic gene

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Abbreviations: BGC, biosynthetic gene cluster; 6\-DEB, 6\-deoxyerythronolide B; LB, Luria–Bertani; MS, mannitol soya flour; MSGE, multiplexed site-specific genome engineering; PCR, polymerase chain reaction; PKS, polyketide synthase; SAGE, serine-integrase assisted genome engineering; TSB, tryptic soy broth.

Two supplementary figures are available with the online version of this article.

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clusters (BGCs) of natural products are large and complex with multiple layers of regulation. Synthetic biology offers a way of exploring BGCs for which the product has not yet been characterized, that is, cloning the genes of the BGC into vectors that can be integrated into a well-studied heterologous Streptomyces host such as Streptomyces coelicolor or Streptomyces lividans and under the control of well-characterized promoters. These tractable hosts have worked well in the biosynthesis of complex natural products.

Strategies to manipulate BGCs in vivo have depended on the use of integration vectors derived from serine integrase int/attP loci. Haginaka et al. constructed two integrating plasmids, both containing the whole gene cluster of goadsporin and encoding either the φC31 int/attP locus or the TG1 int/attP locus. Introducing the two plasmids in consecutive conjugation experiments into the genome of the goadsporin producer, Streptomyces sp. TP-A0584, yielded a strain with two extra copies of the gene cluster. The recombinant strain was able to produce 2.25-fold more goadsporin than the wild-type strain [16]. Li et al. applied a multiplexed site-specific genome engineering (MSGE) strategy to increase the production of pristinamycin II. In their work, two additional attB sites for φC31 and one additional attB site for φBT1 were introduced into the pristinamycin II producer, Streptomyces pristinaespiralis, using CRISPR/Cas9 technology. In consecutive conjugation experiments, additional copies of the pristinamycin II gene cluster were introduced into S. pristinaespiralis via plasmids encoding the cognate integrating plasmids, resulting in a total of five copies of the BGC in the genome; two located in the innate attB sites and three in the additional attB sites. Notably, the production of pristinamycin II was elevated by four times in a 5 L bioreactor [17]. Elmore and colleagues designed a strategy called SAGE (Serine-integrate Assisted Genome Engineering), which enables iterative, site-specific integration of up to 10 different DNA constructs, into a poly-attB cassette that contains attB sequences for 10 serine integrases and has been inserted into host bacteria beforehand [18].

Previously our laboratory has used the erythromycin biosynthesis pathway as a model system to express BGCs in Streptomyces heterologous hosts [19]. 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 the integration, 6-deoxyerythronolide B (6-DEB), the first intermediate of the fermentation broth, can be detected in the fermentation broth. The results demonstrated that the sequential integration of multiple orthologous integrating vectors is a reliable method to clone large genes required to synthesize natural products.

In all these previous works (Table 1), researchers used different serine integrases and/or multiple integration loci (attB sites) to enhance hosts’ ability to accept more genes, but the integration processes were all carried out in an iterative format, that is, the integrating plasmids were introduced via consecutive conjugations. 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 does not recognize or recombine the substrate sites of the other inteigrases. This orthogonality permits the use of different integrases in the same cell or in vitro recombination reaction, yielding entirely predictable recombinants, depending on the location of recombination sites. Moreover, the presence of orthogonal integration systems should not affect the efficiency of recombination of each one of those systems. The dynamics of conjugation to introduce pairs of different integrating plasmids into the same cell compartment, however, might affect efficiency.

Here we tested a new strategy in which integrations could be multiplexed, thus introducing orthogonal integrating plasmids in a single conjugation. The results demonstrate that this strategy has the potential to be employed in synthetic biology and for natural product discovery, to engineer genomes more efficiently.

### METHODS

#### Bacterial strains and culture conditions

Escherichia coli strain Top10 (F·mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 supG1 recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str<sup>§</sup>) endA1 λ<sup>−</sup>) 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. The E. coli strains were grown in Luria–Bertani broth (LB) or on LB agar at 37°C.

*S. coelicolor* M1152 [Δact Δred Δcpk Δada rpoB(C1298T)] [20] and *S. lividans* TK24 (str-6 SLP2<sup>−</sup> SLP3<sup>−</sup>) [21] were used as the recipients in conjugation. *Streptomyces* spore stocks were prepared from lawns grown on mannitol soya flour (MS) agar at 30°C, harvested in 20% glycerol, and then frozen and stored at −38°C. Prior to conjugation, *Streptomyces* spore stocks were diluted and spread on MS agar; after

| Reference | Hosts investigated | Integrate/attB sites employed |
|-----------|-------------------|------------------------------|
| [16]      | *Streptomyces* sp. TP-A0584 | Native φC31 and TG1 |
| [17]      | *Streptomyces* pristinaespiralis | Native/additional φC31 and φBT1 |
| [18]      | *Pseudomonas* fluorescens, *Rhodopseudomonas palustris* | Cloned attB sites for Bxb1, φBT1, φC31, RV, TG1, R4, B13, A118, MR11 and φ370 |
| [19]      | *Streptomyces* coelicolor, *Streptomyces* lividans | Native φBT1, SV1 and TG1 |

Table 1. Previously published works using different serine integrases and/or multiple integration loci (attB sites) to engineer genomes iteratively.
being incubated at 28 °C for 5 days, the number of colonies were counted to calculate spore concentrations (c.f.u. ml⁻¹, number of colonies/volume plated×dilution factor). Counting was performed using plates with 25–250 colonies. Tryptic soy broth (TSB) medium was used for the preparation of genomic DNA [21]. The antibiotic concentrations for *E. coli* were 150 µg ml⁻¹ hygromycin, 50 µg ml⁻¹ apramycin, 50 µg ml⁻¹ kanamycin and 100 µg ml⁻¹ ampicillin, and for *Streptomyces* they were 50 µg ml⁻¹ hygromycin, 50 µg ml⁻¹ apramycin, 50 µg ml⁻¹ kanamycin and 20 µg ml⁻¹ erythromycin in DMSO for the selection of *S. coelicolor* M1152, 120 µg ml⁻¹ erythromycin in DMSO for the selection of *S. lividans* TK24 and 25 µg ml⁻¹ nalidixic acid for counterselection against *E. coli* on conjugation plates.

**DNA manipulation**

*E. coli* transformation and gel electrophoresis were carried out as described previously [22]. 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. 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. Plasmids used in this study**

| Plasmid | Description | Reference |
|---------|-------------|-----------|
| pBF20  | TG1 int/attP, actlp-eryAI, tsr, ori/bla, aac(3)IV, oriT | [19] |
| pBF22  | SV1 int/attP, actlp-eryAIII, tsr, ori/bla, aphII, oriT | [19] |
| pBF24  | φBT1 int/attP, actlp-eryAII, tsr, ori/bla, ermE, oriT | [19] |
| pHG4   | TG1 int/attP, bla/ori, aac(3)IV, oriT | [23] |
| pHG5   | SV1 int/attP, bla/ori, aphII, oriT | This study |
| pHG6   | φBT1 int/attP, bla/ori, ermE, oriT | This study |
| pHG7   | φC31 int/attB, bla/ori, hygB, oriT | This study |
| pHG2R2 | φC31 int/attP, actlp-eryBIV-eryBV, actlp-eryBVI, actlp-eryBII-eryBII, actlp-eryBVII, tsr, ori/bla, actII-orf4/actlp-eryF, hygB, oriT | [25] |

**Table 3. Oligonucleotides used in this study**

| Oligonucleotide | Sequence (5’−3’) |
|-----------------|------------------|
| pHG5-for        | CGAACGCATCGATTAATTAAGCCGCCCATATGCTAGAGGTACCGAGCTCGCTAGCAGATCTATGAAACGAGACCTACCA |
| pHG5-rev        | TGATTACGCCAAGCTTTGCAGAGAAGAATCTGCTAAAGAAGG |
| pHG6-for        | CGAACGCATCGATTAATTAAGGTACCGAGCTCCATATGCCTCAGCGCATGCAGATCTCGGCCCGGGCGTCAGGCG |
| pHG6-rev        | TGATTACGCCAAGCTTTGCAGGGCTTCCAGAAAGGCCTCTCAGHG |
| pHG7-for        | CGAACGCATCGATTAATTAAGCCGCCCATATGCTAGAGGTACCGATCGCATAGTCTATGAAACGAGACCTACCA |
| pHG7-rev        | TGATTACGCCAAGCTTTGCAGGGCTTCCAGAAAGGCCTCTCAGHG |
| pHG6-integration-for | AAGGGCAGCGATCGG |
| pHG6-integration-Sc rev | CGACAGGGCAGCCACACAG |
| pHG6-integration-SI rev | GTGCGGCTATGAGCTCCG |
| pHG7-integration-for | TCGAAGCGTAAGGGCCGCC |
| pHG7-integration-Sc rev | GCCCTGCTACAGC TGTC |
| pHG7-integration-SI rev | GGATGTCCTTGAGCG |

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The primers used in this study are listed in Table 2 and the primers used are listed in Table 3.

**Construction of plasmids**

Plasmids pHG4 [23], pHG5, pHG6 and pHG7 are all integrating vectors encoding the int/attP loci from TG1, SV1, φBT1 and φC31, respectively (Fig. 1). These four plasmids also encode compatible antibiotic resistance loci: apramycin resistance (aac(3)IV, pHG4), kanamycin resistance (aphII, pHG5), erythromycin resistance (ermE, pHG6) and hygromycin resistance (hygB, pHG7). Additionally, the plasmids all encode the expression cassette actI/p/actII-orf4 [24].

Plasmids pHG5, pHG6 and pHG7 were constructed as follows: the fragment containing SV1 int/attP, oriT and the kanamycin resistance gene was amplified from plasmid pBF22 [19] using the primer pair pHG5-for/pHG5-rev; the fragment containing erythromycin resistance gene, oriT and φBT1 int/attP was amplified from plasmid pBF24 [19] using the primer pair pHG6-for/pHG6-rev; and the fragment containing hygromycin resistance gene, oriT and φC31 int/attP was amplified from plasmid pBF27C [19] using the primer pair pHG7-for/pHG7-rev. Each PCR fragment was then inserted by In-Fusion cloning into pBF22 [19] cut with HindIII and PacI separately, to form the plasmids pHG5, pHG6 and pHG7 respectively.

Plasmids pBF20, pBF22, pBF24 [19] and pHG2R2 [25] were constructed in our previously published work.

**Intergeneric conjugation**

*E. coli* ET12567 (pUZ8002) donors carrying desired plasmids were prepared as follows: a single colony was inoculated into 3 ml of LB broth with appropriate antibiotics for plasmid maintenance and incubated overnight at 37°C and 200 r.p.m. The overnight cultures were diluted 100-fold into 10 ml of LB broth with appropriate antibiotics and incubated...
Germinating *Streptomyces* spores were used as plasmid recipients. Briefly, ~10^8 spores were heat shocked in 2xYT medium (16 g l\(^{-1}\) tryptone, 10 g l\(^{-1}\) yeast extract and 5 g l\(^{-1}\) NaCl) at 50 °C for 10 min. Then prepared donor *E. coli* and recipient spores were mixed, plated onto MS agar plates containing 10 mM MgCl\(_2\) and incubated at 28 °C for 20 h. The MS plates were overlaid with water (1 ml) containing nalidixic acid (0.5 mg) plus the appropriate antibiotics for selection of exconjugants. Where required, each plate received: apramycin, 1.25 mg; kanamycin, 5 mg; erythromycin, 1.25 mg for *S. coelicolor* and 3.75 mg for *S. lividans*; and hygromycin, 1 mg. The overlaid plates were incubated at 28 °C until exconjugants could be counted. All conjugation experiments were performed in biological triplicate (n=3), and error bars denote standard deviation.

The resultant exconjugant colonies were replica plated onto MS agar containing nalidixic acid (25 µg ml\(^{-1}\)) and appropriate selective antibiotics and incubated at 28 °C until sporulation. Spores collected from individual colonies were used directly as PCR templates. Conjugation efficiency was calculated as the number of exconjugants obtained from 10^8 recipient cells.

**RESULTS**

**Optimization of the multiplexed conjugation**

A drawback of using multiple expression plasmids is that consecutive rounds of conjugations are time-consuming, so we aimed to test whether it was possible to introduce two or more plasmids into *Streptomyces* hosts by multiplexing the conjugation donors in a single step. Plasmids pHG4, pHG5, pHG6 and pHG7 (Fig. 1) are approximately 6–7 kb and only contain essential vector elements. These basic integrating vectors were used to test the efficiency of multiplexed conjugations.

First, the integrating plasmids were transferred into *S. coelicolor* individually to assay their conjugation and integration efficiencies. As *E. coli* donors containing either pHG6 (φBT1 int/attP) or pHG7 (φC31 int/attP) showed the highest efficiencies (Fig. 2a; diagonal cells), these donors were then selected to optimize the protocol for multiplexed conjugations.

For a multiplexed conjugation, the two *E. coli* donors containing either pHG6 or pHG7 were mixed together to simultaneously transfer plasmids into *S. coelicolor* M1152 in a single conjugation experiment. The effects of different ratios between *S. coelicolor* spores and *E. coli* cells on the transfer frequency were tested to find the optimal ratio (Fig. 2c). In the standard protocol, 10^8 *E. coli* cells were conjugated with 10^8 *S. coelicolor* M1152 spores. In this study, when a threefold excess of *E. coli* cells was used, the highest conjugation frequency could be achieved, and this ratio was used in all the following multiplexed mating attempts.

**Pairwise plasmid multiplex integration**

Using the optimal ratio between donor cells and spores, *E. coli* donors containing the integrating plasmids pHG4, pHG5, pHG6 or pHG7 were tested pairwise in the multiplexed...
conjugation method with *S. coelicolor* M1152 (Fig. 2a) or *S. lividans* TK24 (Fig. 2b) as recipients. As expected, the number of exconjugants containing both integrating plasmids from the multiplexed donors in the conjugations was significantly reduced compared to using each donor individually. While use of the donor pairs containing either pHG6 and pHG7 led to reliable simultaneous transfer of both plasmids, *E. coli* pairs containing pHG4 (TG1 int/attP, aac(3)IV) or pHG5 (SV1 int/attP, aphII), and pHG4 or pHG7 (φC31 int/attP, hygB) did not lead to any exconjugants that had received both plasmids.

Overall the efficiencies of multiplexed conjugations using the donors in pairwise combinations were: pHG6 and pHG7 (φBT1 int/attP + φC31 int/attP) >pHG5 and pHG7 (SV1 int/attP + φC31 int/attP) >pHG4 and pHG6 (TG1 int/attP + φBT1 int/attP) >pHG5 and pHG6 (SV1 int/attP + φBT1 int/attP) in the *Streptomyces* strains tested. To confirm that correct site-specific integration had occurred in the exconjugants obtained after a multiplexed conjugation using *E. coli* donors containing pHG6 or pHG7, primers were designed to amplify the region across the φBT1 and φC31 attL sites after recombination (Fig. 3a). The exconjugants from the multiplexed conjugation were checked by colony PCR using the primer pairs pHG6-integration-forward/pHG6-integration-Sc reverse and pHG7-integration-forward/pHG7-integration-Sc reverse (for *S. coelicolor* M1152, Fig. 3) or pHG6-integration-forward/pHG6-integration-Sc reverse and pHG7-integration-forward/pHG7-integration-Sc reverse (for *S. lividans* TK24, Fig. S1, available in the online version of this article). For *S. coelicolor* M1152-derived exconjugants, all of the 10 colonies picked randomly had pHG6 and pHG7 integrated correctly into the chromosome. For *S. lividans* TK24 exconjugants, the attL amplicons from both integrated pHG6 and pHG7 were obtained from seven out of eight colonies.

To gain insight into why the numbers of exconjugants containing both plasmids were so significantly reduced by multiplexed conjugations, we assayed the conjugation efficiency of each plasmid individually in the multiplexed experiments by selecting for just one of the plasmids being transferred. For individual plasmids, the efficiency of conjugation and integration was similar to when only one plasmid donor was used. This demonstrates that there is no interference between the donor cells.

The reliable ability to simultaneously transfer φBT1- and φC31-derived integrating vectors to *S. coelicolor* M1152 and *S. lividans* TK24 indicates that this is a practical method for future studies, saving a considerable amount of time.

**Multiplexed conjugations using larger plasmids containing biosynthetic genes**

BGCs are usually highly complex, encoding many genes and sometimes contain very large, single multifunctional genes. Consequently BGCs are encoded by large DNA fragments. As the efficiency of DNA transformation by large plasmids can be reduced, multiplexed conjugation was tested with a plasmid set that encode large biosynthetic genes, i.e. plasmids pBF20, pBF22, pBF24 [19] and pHG2R2 [25]. The size of these four plasmids is between 17 and 19 kb and they encode biosynthetic genes for erythromycin biosynthesis. With these much larger plasmids, the number of exconjugants that had received the two plasmids was much lower than the experiments using just the empty vectors. The most efficient combination, pBF24 and pHG2R2 (φBT1 int/attP + φC31 int/attP), however still showed that simultaneous transfer of two plasmids is feasible, even though the plasmids are nearly 20 kb (Fig. 4).

As before, the sites of integration for the plasmids after multiplexed conjugation were verified by colony PCR using the primer pairs pHG6-integration-forward/pHG6-integration-Sc reverse and pHG7-integration-forward/pHG7-integration-Sc reverse for *S. coelicolor* M1152 (Fig. S2a), and the primer pairs pHG6-integration-forward/pHG6-integration-Sc reverse and pHG7-integration-forward/pHG7-integration-Sc reverse for *S. lividans* TK24 (Fig. S2b). The attLs for both plasmids could be amplified from the majority of exconjugants, showing the efficiency of the simultaneous integration, even with large inserts, from a single mating experiment, indicating that this is still a practical method.
While we were preparing this paper, Ko and colleagues published their findings [26]. They constructed vectors encoding orthogonal resistances, integrases (and containing their cognate attP sites) and origins of replication and introduced these into a single E. coli donor that in a conjugation step could then be introduced into various Streptomyces hosts. In our study, the integrating vectors used contained the same origin of replication and the plasmids were introduced from combinations of E. coli donors. Both studies used a single conjugation step to deliver integrating plasmids.

Ko’s work and our study achieved similar results. As described above, conjugations in which plasmids containing the φBT1 and φC31 int/attP loci are simultaneously transferred yield the highest numbers of exconjugants. We did not test the φOZJ integration system, since it was characterized for the first time by Ko et al. However, their outcomes suggest that φOZJ might also work in our multiplexed conjugation and integration system.

We attempted multiplexed conjugations that sought to simultaneously transfer three plasmids into Streptomyces hosts. The combinations tested contained the int/attP loci from φBT1 and φC31, and int/attP from either TG1 or SV1. No exconjugants were obtained with either combination using the same procedure described for the conjugations using pairs of donors. Increasing the concentration of the donors by 10× to be in a standard bi-parental conjugation protocol also failed to produce exconjugants containing all three plasmids (data not shown). Similarly, Ko’s tetra-parental (three E. coli donors containing plasmids encoding the integration loci from φOZJ, φBT1 and φC31, into a Streptomyces host) mating attempt failed to yield exconjugants, but if the three plasmids to be transferred are all present in the same E. coli donor (via compatible origins of replication and orthogonal resistance markers), exconjugants containing all three plasmids are obtained. Based on these results, Ko et al. suggested that during conjugation multiple plasmids can be transferred via the conjugation apparatus formed in a donor–recipient interaction. Our data indicate that multiple independent conjugation events also occur, but even though the number of exconjugants may not be as high as when using a single donor, there may be advantages to using the same high copy replication origin for plasmid construction and simply mixing the two donors.

In summary, we optimized the multiplexed conjugation and integration method, which can simultaneously introduce two plasmids encoding orthogonal integrating loci into Streptomyces. We also demonstrated that the method is robust even when introducing large plasmids, a likely scenario when engineering biosynthetic gene clusters.

**DISCUSSION**

In this study, we tested the feasibility of simultaneous conjugation and integration of plasmids into Streptomyces sp. Conjugation is one of the most commonly used methods of bacterial gene transfer and in Streptomyces. It is widely used to deliver integrating plasmids that depend on the presence of a phage-derived int/attP locus. Although the simultaneous transfer and integration of two plasmids into Streptomyces in a single conjugation experiment is substantially less efficient than the transfer of each plasmid in standard bi-parental matings, the observed numbers of exconjugants obtained make a multiplexed conjugation step a viable proposition in genetic engineering methodology. Integrating plasmids based on φBT1 and φC31 int/attP loci showed the highest efficiencies of transfer in a multiplexed conjugation. The plasmids based on these int/attP sites would even allow the simultaneous conjugation and integration of very large plasmids. In previous work requiring the conjugation and integration of multiple plasmids with orthologous int/attP loci, each plasmid was introduced in series using repeated rounds of conjugative transfer. Our results showed that when the plasmids being delivered are derived from φBT1 and φC31 int/attP loci, only a single conjugation using two E. coli donors is required, obviating the need for separate rounds of conjugation.

**Fig. 4.** Conjugation frequencies of plasmids pBF20, pBF22, pBF24 and pHG2R2 (single and multiplexed conjugation) in (a) S. coelicolor M1152 and (b) S. lividans TK24. The numbers indicated the numbers of exconjugants per 10^8 spores.

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**Author contributions**

H.G. (ORCID: 0000-0002-8544-7826) contributed to conceptualization, methodology, investigation and writing of the original draft. M.C.M.S.
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