



Recent achievements in the generation of stable genome alterations/mutations in species of the genus *Streptomyces*

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Abstract

The bacteria of the genus *Streptomyces* are the most valuable source of natural products of industrial and medical importance. A recent explosion of *Streptomyces* genome sequence data has revealed the enormous genetic potential of new biologically active compounds, although many of them are silent under laboratory conditions. Efficient and stable manipulation of the genome is necessary to induce their production. Comprehensive studies in the past have led to a large and versatile collection of molecular biology tools for gene manipulation of *Streptomyces*, including various replicative plasmids. However, biotechnological applications of these bacteria require stable genome alterations/mutations. To accomplish such stable genome editing, two major strategies for streptomycetes have been developed: (1) integration into the chromosome through Att/Int site-specific integration systems based on *Streptomyces* actinophages (Φ C31, Φ BT1, VWB, TG1, SV1, R4, Φ Joe, μ 1/6) or pSAM2 integrative plasmid; (2) integration by homologous recombination using suicidal non-replicating vectors. The present review is an attempt to provide a comprehensive summary of both approaches for stable genomic engineering and to outline recent advances in these strategies, such as CRISPR/Cas9, which have successfully manipulated *Streptomyces* strains to improve their biotechnological properties and increase production of natural or new gene-manipulated biologically active compounds.

Keywords CRISPR/Cas9 · Gene disruption · Genome editing · Homologous recombination · Site-specific recombination · *Streptomyces*

Introduction

The genus *Streptomyces* belongs to one of the largest bacterial phylum *Actinobacteria*, which are represented by Gram-positive bacteria with high G + C DNA content. In general, *Streptomyces* species are saprophytic soil-dwelling aerobic filamentous bacteria that play a key role in soil ecology by effectively hydrolyzing a wide range of polysaccharides (cellulose, chitin, xylan, and agar) and other natural macromolecules. This biodegradation efficiency is based on the secretion of potent enzymes such as cellulases, xylanases, and chitinases. Streptomycetes undergo a complex process of morphological differentiation, somewhat resembling that of eukaryotic fungi. In the presence of water and nutrients, their

life cycle begins with germination of the spores to form hyphae that grow by tip extension and branching to form a vegetative substrate mycelium. In response to a variety of signals, special reproductive aerial hyphae, similarly growing by tip extension, emerge from the mycelium. Upon cessation of their growth, the aerial mycelium undergoes septation into unigenomic prespore compartments, which finally mature into ovoid spore chains that allow survival and dispersal. This process of morphological differentiation is accompanied and associated with the so-called physiological differentiation, which represents the production of biologically active secondary metabolites, many of which have antibacterial, antifungal, herbicidal, immunosuppressive, antihelminthic, and antitumor properties. *Actinobacteria* produce about two-thirds of all known antibiotics and other biologically active secondary metabolites, most of which are produced by the *Streptomyces* genus (Barka et al. 2016; Dyson 2011). However, these bacteria with versatile metabolism are also effective suppliers of many enzymes for biotechnological applications (Spasic et al. 2018). In addition, some *Streptomyces* strains, such as

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S. lividans, have been used to efficiently secrete heterologous proteins directly into growth media and are considered valuable hosts for the production of heterologous proteins (Anne et al. 2012; Hamed et al. 2018). So, the bacteria of the genus *Streptomyces* are clearly of great importance in the field of biotechnology and medicine.

Secondary metabolites are synthesized by complex biosynthetic pathways of subsequent chemical reactions, resulting in a final biologically active product that is specifically exported outside the cell. Biosynthetic genes of secondary metabolites are physically clustered together with other genes encoding regulatory and resistance proteins. This clustering facilitates cloning of entire gene clusters for secondary metabolites into various vectors for production in heterologous hosts and genetic manipulations. Previously, most *Streptomyces* strains were thought to produce one or only a few different bioactive secondary metabolites. However, robust genomic sequencing of *Streptomyces* strains revealed a wealth of biosynthetic gene clusters (from 20 to 50) for unknown secondary metabolites. However, only a small proportion of those metabolites are produced under laboratory controlled conditions. The remaining “silent” gene clusters represent a pool of novel metabolites that await “awakening.” *Streptomyces* strains therefore possess a great potential for synthesis of new compounds. In order to awaken these silent clusters or to increase the production of secondary metabolites, efficient and stable gene manipulation techniques are required for deleting specific genes/gene clusters or for inserting heterologous genes/gene clusters into *Streptomyces* chromosomes (Baltz 2016; Gross 2007; Chen et al. 2016; Li et al. 2017b). Similarly, heterologous high-level production of recombinant proteins in streptomycetes requires stable genetic manipulations of biotechnologically productive strains (Hamed et al. 2018).

The aim of this review is to summarize approaches for preparing stable genome alterations/mutations in *Streptomyces* species. However, in many cases, they could also be applied to other actinomycetes. Although a number of reviews have already been published on parts of this subject (Baltz 2012; Deng et al. 2017; Myronovskiy and Luzhetskyy 2013; Siegl and Luzhetskyy 2012), we have described stable genome alterations/deletions with emphasis on new approaches, including our recently published effective system for stable markerless genome alterations (Rezuchova et al. 2018), to create stable *Streptomyces* strains useful for biotechnology applications.

Genetic manipulations of *Streptomyces*

The enormous biotechnological potential of *Streptomyces* strains, as mentioned above, logically provoked great interest in the genetic manipulations of these bacteria. More than fifty years of genetic and molecular biology studies in

Streptomyces have brought a comprehensive and versatile collection of molecular biology tools for the gene manipulations of these bacteria, many of which are also useful for biotechnology applications (Hopwood 2007; Kieser et al. 2000).

Unlike efficient transformation systems in Gram-negative bacteria such as *Escherichia coli*, incorporation of DNA into streptomycetes is difficult due to strong cell wall and restriction modification systems. Nevertheless, several procedures have been developed to introduce DNA to these bacteria. The first used is protoplast transformation. This method, originally developed by Okanishi et al. (1974), was later modified to enhance the efficiency of transformation by the addition of polyethylene glycol (Bibb et al. 1978). Although this procedure has been adapted to a variety of *Streptomyces* species, this procedure is rather laborious and needs to develop specific conditions for the formation and regeneration of protoplasts in various *Streptomyces* strains (Kieser et al. 2000; Kormanec et al. 1993). Later, electroporation was developed to introduce plasmid DNA directly into mycelial fragments of some *Streptomyces* species without the need for protoplast preparation (Pigac and Schrempf 1995). Another and currently the most widely used strategy is the introduction of DNA into *Streptomyces* strains by easy and efficient conjugation from *Escherichia coli*. This method allows for the transfer of plasmids containing *cis*-acting origin of transfer *oriT* of a broad-host-range IncP-group plasmid RK2 from *E. coli* donor strains containing the functions of transfer RP4 (IncP) delivered *in trans* into the *Streptomyces* strains as a recipient. Conjugation is usually much more effective than protoplast transformation and is applicable to many *Streptomyces* strains. In addition, it allows easy cloning in *E. coli* and transfer of finished constructs transferred to *Streptomyces* only by mixing the donor *E. coli* with spores of the *Streptomyces* acceptor strain (Bierman et al. 1992; Mazodier et al. 1989). The main limitation of these methods is that many streptomycetes possess restriction-modification systems that can drastically reduce transformation efficiency. In some strains, they can be circumvented by the use of methylation-deficient *E. coli* hosts for conjugation and for DNA isolation for transformation, or by heat treatment of protoplasts prior to transformation. The frequency of transformation can also be increased by using single-stranded plasmid DNA. In this regard, conjugation is more efficient because restriction barriers can be circumvented by the transfer of single-stranded concatemers of plasmid DNA during the process (Kieser et al. 2000).

Numerous vector systems are available for genetic manipulation of *Streptomyces*, including replicative vectors containing the replication origin of *Streptomyces*, integrating vectors that site-specifically integrate into the genome using bacteriophage or plasmid integrase systems, and suicidal non-replicating vectors that can integrate into the *Streptomyces* genome by homologous recombination (Kieser et al. 2000). The replicative vectors used for genetic manipulations in

streptomycetes were mainly constructed on the basis of four native *Streptomyces* plasmids: pIJ101, pJV1, pSG5, and SCP2. The high-copy number plasmid pIJ101 with 8830 bp from *S. lividans* ISP5434 occurs at approximately 300 copies per chromosome (Kieser et al. 1982). It was used for the construction of the most popular non-transmissible replicative cloning vectors pIJ486 and pIJ702 (Kieser et al. 2000). However, the major limitation of these *Streptomyces* replicative plasmids is that they do not contain *E. coli* replication origin. Thus, gene manipulations must be performed in *S. lividans*, which is rather complicated. In addition, they can only be transferred to streptomycetes by laborious protoplast transformation. Although several pIJ101-*E. coli* shuttle plasmid vectors have been constructed, these are structurally unstable (Kieser et al. 2000; J. Kormanec, unpublished data). Another high-copy number plasmid pJV1 of 11,143 bp from *S. phaeochromogenes* occurs at approximately 150 copies per chromosome and was also used to construct several *Streptomyces* replicative cloning vectors (Bailey et al. 1986). In addition, it was used to construct stable and versatile *Streptomyces*-*E. coli* shuttle cosmid vector pFD666 (Denis and Brzezinski 1992). The medium copy number (20–50 copies per chromosome) plasmid pSG5 of 12,208 bp from *S. ghanaensis* 5/1B has temperature-sensitive replication (stops above 34 °C) and was used to construct temperature-sensitive plasmid vectors for gene disruptions (Muth et al. 1989). The low-copy number plasmid SCP2 of 31 kb from *S. coelicolor* (Bibb et al. 1977) was used to construct several stable low-copy number vectors (e.g., pIJ916, pIJ940), including stable *Streptomyces*-*E. coli* cloning shuttle vectors pIJ698 (Kieser et al. 2000), pKC1218, and pOJ446 (Bierman et al. 1992). Based on many years of experience, there are several most useful antibiotic resistance marker genes used for selection of the plasmids in *Streptomyces*. One of the first used is the thiostrepton resistance *tsr* gene of *S. azureus*, but thiostrepton partially inhibits morphological differentiation and interferes also with antibiotic production. One of the most useful resistance markers is the *aac(3)IV* gene of *Klebsiella pneumoniae*, encoding aminoglycoside acetyltransferase inactivating apramycin, which has the advantage of selecting plasmids in both *E. coli* and *Streptomyces*. The same applies to the *neo* gene encoding aminoglycoside phosphotransferase inactivating neomycin, kanamycin, and gentamicin from the *E. coli* transposon Tn5 and the *hyg* gene of *S. hygroscopicus*, encoding hygromycin phosphotransferase (Kieser et al. 2000). Detailed genetic manipulation protocols of *Streptomyces* are described in Kieser et al. (2000) and Rebets et al. (2017).

Streptomyces stable genome alterations/mutations

A major drawback of the *Streptomyces* replicative plasmids is that their host range is usually restricted to native *Streptomyces* strains and their closest relatives. In addition,

they need a permanent antibiotic selection for stable maintenance in streptomycetes (Kieser et al. 2000). This complicates their use in biotechnology applications. Despite these disadvantages, several replicative plasmids have been successfully used, especially for their high-copy number, for overproduction of many biotechnologically relevant heterologous proteins in streptomycetes (Anne et al. 2012). However, these stability and selection problems can be overcome by using two other types of vectors: (i) integrating vectors containing a DNA segment encoding attachment/integration functions (Att/Int) for site-specific integration into specific sites of the *Streptomyces* genome (an *attB* site for specific integrase), (ii) suicidal non-replicating vectors that integrate into any neutral genome region by homologous recombination. In addition, the non-replicating vectors can also be used for stable deletion of genes or whole gene clusters. The main advantage of these vectors is their stability, especially in the introduction of large DNA fragments covering whole gene clusters for secondary metabolites that range from several kb to more than 100 kb. They can be used to awaken “silent” gene clusters for new secondary metabolites, by deleting cluster-situated negative regulatory genes or by inserting strong promoters upstream of cluster-situated positive regulatory genes. They may also facilitate the heterologous production of new “silent” biologically active secondary metabolites or allow increased yields of natural compounds originally produced in low amount in their natural hosts. In addition, they can also functionally decipher huge metagenome data related to gene clusters for new secondary metabolites found by genome mining. Since antibiotic production could also be limited by the availability of precursors, the deletion of relevant biosynthetic genes or their genetically manipulated overexpression could also help in this regard (Bachmann et al. 2014; Baltz 2016; Bekiesch et al. 2016; Huo et al. 2019; Li et al. 2017b; Nah et al. 2017). Similarly, they have great potential for stable heterologous high-level production of recombinant proteins in streptomycetes (Hamed et al. 2018). Another advantage of these two types of vectors is that they are bifunctional and facilitate the engineering of genes/gene clusters in *E. coli*, followed by conjugation to streptomycetes. Both types of vectors, their advantages, their disadvantages, and their use in several applications are described in the following part of the paper.

Att/Int site-specific integrative vectors

To date, at least eight site-specific integration systems have been characterized and used to construct integration vectors in streptomycetes. Most of them are based on various actinomycete temperate bacteriophages (actinophages) identified in *Streptomyces* species, including Φ C31 (Lomovskaya et al. 1972, 1980), Φ BT1 (Gregory et al. 2003), VWB (Van Mellaert et al. 1998), TG1 (Morita et al. 2009), SV1 (Fayed et al. 2014), R4 (Chater and Carter 1979), Φ Joe (Fogg et al.

2017), and $\Phi 1/6$ (Farkasovska and Godany 2012). Another is based on the natural integrative plasmid pSAM2 found in *S. ambifaciens* (Boccard et al. 1989; Kuhstoss et al. 1989). Temperate bacteriophages (and integration vectors prepared using their functional elements) are integrated into the host chromosome at a specific site by a recombination process that requires specialized attachment sites, *attP* in the phage (or in the integration vector) and *attB* in the host chromosome. Both have 40 to 50 bp in size with different inverted repeat sequences flanking the 2- to 12-bp complementary core sequence that includes the recombination crossover point. This single DNA crossover between these two attachment sites is catalyzed by phage-encoded integrase and generates two junction sites, *attL* and *attR*, consisting of hybrid *attP* and *attB* sequences (Fig. 1). There are two evolutionary distinct families of site-specific recombinases distinguishable by sequence similarity and mechanism of action that contain a specific catalytic amino acid residue: tyrosine and serine recombinases (Fogg et al. 2014). Interestingly, actinophage integrases belong to both families. The natural integration plasmid pSAM2 has a similar recombination system but with a 26-bp-long *attB* site and a large 360-bp *attP* site (Raynal et al. 2002). Most of these integrases are unidirectional and no additional factors are needed for the integration process. Since the excision reaction in which *attL* and *attR* are recombined to regenerate *attB* and *attP* can occur only in the presence of recombination directionality factor (RDF) or Xis, integration vectors containing only the *attP* site together with the respective integrase gene remain

stably integrated (Khaleel et al. 2011). Therefore, these bacteriophage (or pSAM2-based) attachment/integration (Att/Int) systems (the *attP* site together with the respective integrase gene) are widely used to develop various integration vectors for stable genetic engineering in *Streptomyces*. Some of these Att/Int systems have already been reviewed (Baltz 2012; Myronovskyi and Luzhetskyy 2013). The following sections briefly describe individual Att/Int systems and their use for stable *Streptomyces* gene manipulations and update new information for these systems.

Φ C31 Att/Int system Integration plasmids derived from bacteriophage Φ C31, isolated from *S. coelicolor* (Lomovskaya et al. 1972, 1980), represent the most common integration vectors used in *Streptomyces*. As mentioned above, only the *attP* and *int* gene encoding serine family recombinase are essential for unidirectional site-specific integration. Φ C31-based vectors integrate into the *attB* site (Table 1) located in the pirin-like gene (*SCO3798* in *S. coelicolor*) encoding a pirin homolog that is highly conserved in prokaryotes and eukaryotes. However, after a deletion of the main *attB* site, the vectors can also integrate with dramatically reduced efficacy into pseudo-*attB* sites (Baltz 2012; Combes et al. 2002).

The *att/int* Φ C31 locus was used to construct several convenient Att/Int integration vectors for use in a broad range of *Streptomyces* species. One of the first developed and widely used were the conjugative integration plasmid pSET152 and the cosmid vectors pOJ436 and pOJ444, each containing an

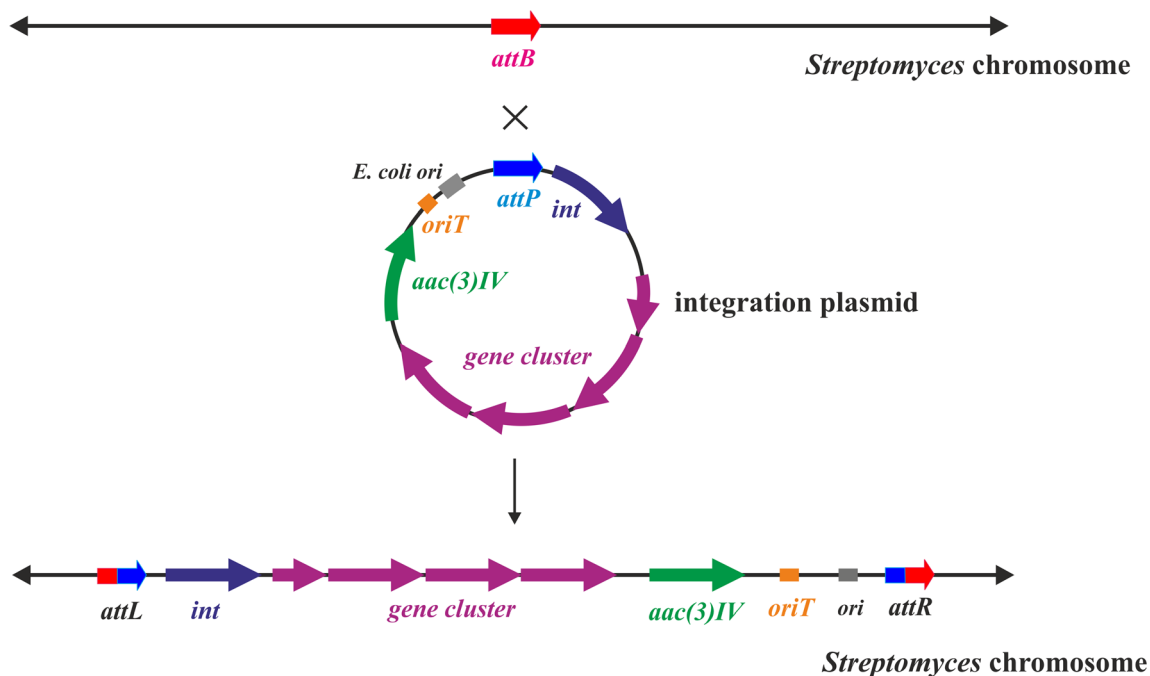


Fig. 1 Scheme of site-specific integration of the integration plasmid containing the biosynthetic gene cluster (pink arrows as an example) into the *Streptomyces* chromosome. *int*, integrase gene (dark blue arrow); *oriT*,

origin of conjugal transfer; *aac(3)IV*, the apramycin resistance marker gene used for selection as an example (green arrow)

Table 1 Individual *attB* sites for the Att/Int systems and their positions in the *S. coelicolor* A3(2) genome (acc. no. AL645882)

Att/Int system	Sequence (5' to 3')	Target gene	Position	Reference
Φ C31	CGGTGCGGGTGCAGGGCGTGCCCTT GGGCTCCCCGGGCGCTACTCCACC	<i>SCO3798</i>	4,178,697–4,178,747	Combes et al. 2002
Φ BT1	CCAGGTTTTTGACGAAAGTGATCCAG ATGATCCAGC	<i>SCO3798</i>	5,279,940–5,279,905	Zhang et al. 2008
VWB	CTCTCCTAAAGCGGGTGTGCGAGGTT CGAATCCTGCCGGGGGCAC	tRNA ^{Arg} (AGG)	3,602,638–3,602,682*	Van Mellaert et al. 1998
TG1	GATCAGCTCCGCGGGCAAGACCTTCT CCTCACGGGGTGGAAAGGTC	<i>SCO3658</i>	4,037,399–4,037,444#	Morita et al. 2009
R4	AGTTGCCCATGACCATGCCGAAGCAG TGGTAGAAGGGCACCCGGCAGACAC	<i>SCO6196</i>	6,806,607–6,806,558#	Miura et al. 2011
SV1	CATCAGGGCGGTCAGGCCGTAGATGT GGAAGAACGGCAGCACGGCGAGGACG	<i>SCO4383</i>	4,800,588–4,800,537	Fayed et al. 2014
Φ Joe	ATCTGGATGTGGGTGTCCATCTGCGG GCAGACGCCGAGTCGAAGCACGG	<i>SCO2606</i>	2,827,448–2,827,497#	Fogg et al. 2017
μ 1/6	TCTTGTAAGCGGGGGTCGTCCGGTTC GAACCCGACAGGGGGCTCAG	tRNA ^{Thr} (ACA)	4,646,562–4,646,607*#	Farkasovska and Godany 2012
pSAM2	GCGCGCTTCGTCGGGACGAAGAGGT	tRNA ^{Pro} (CCG)	3,963,133–3,963,158	Raynal et al. 1998

*Central conserved part of the *attB* site is shown; # contains mismatches

RK2 replicon *oriT* for easy conjugal transfer from *E. coli* to *Streptomyces* species (Bierman et al. 1992). Both plasmid and cosmid vectors have a cloning capacity of about 15 and 45 kb, respectively. Many Φ C31-based Att/Int cosmid libraries from various *Streptomyces* strains have been successfully constructed using these cosmid vectors or derivatives thereof to clone biosynthetic gene clusters (e.g., for daptomycin, nikkomycin, moenomycin A, chlorobiocin, novobiocin, coumerymycin A1, caprazamycins) and to express them in heterologous *Streptomyces* strains (Myronovskiy and Luzhetskyy 2013). However, the size of many gene clusters for bioactive secondary metabolites is too large (greater than 100 kb) to fit within the cloning capacity of these cosmid vectors. To solve this problem, two Φ C31-based Att/Int vectors were constructed: bacterial artificial chromosome (BAC) vector pStreptoBAC V based on *E. coli* F factor and pEASAV13 vector derived from P1 artificial chromosome (PAC). Both can carry DNA inserts of 100–300 kb. Both contain the RK2 *oriT* replicon for conjugal transfer from *E. coli* to *Streptomyces* and have been successfully used to construct libraries from various *Streptomyces* strains and for subsequent heterologous expression of biosynthetic gene clusters for daptomycin, isomigrastatin, roseoflavin, FK506, salinomycin, anthracinomycin, chaxamycin, symocyclinone, azalomycin, and vancoresmycin (Tocchetti et al. 2018). Another system for cloning large gene clusters is represented by the Φ C31-based Att/Int integration vector pTARA. It utilizes a useful transformation-associated recombination (TAR) system of the yeast *Saccharomyces cerevisiae* for cloning and reassembling overlapping DNA fragments to combine the whole biosynthetic gene clusters (Kim et al. 2010). This system has recently been used for cloning and efficient heterologous expression of the metagenomic biosynthetic gene cluster

for new calcium-dependent antibiotic malacidins, which acts against multidrug-resistant Gram-positive pathogens (Hover et al. 2018). Two other TAR-based Φ C31 Att/Int integration vectors, pCAP01 and pCAP03, have also been successfully used for cloning and heterologous expression of biosynthetic gene clusters for antibiotics marinopyrrole, taromycin A, enterocin, nataxozole, ammosamides A–C, thiolactomycin, and tiotetroamide (Tocchetti et al. 2018).

The insertion of Att/Int integrative vectors into the Φ C31 *attB* site of several *Streptomyces* strains has been shown to be neutral in most cases in terms of individual secondary metabolite production. However, the insertion of large inserts significantly reduced this production. Similarly, insertion of the *tylF* gene encoding a macrocin O-methyltransferase into *S. fradiue* using Φ C31-based Att/Int vectors to increase tylosin production resulted in its decreased yield (Baltz 1998). The reported reductions in antibiotic synthesis could be due to insertional mutagenesis into a pirin-like gene. Indeed, a recent report revealed that a significant decrease in spiramycin production following insertion inactivation of the pirin-like *pirA* gene using the Φ C31 Att/Int system-based vector in *S. ambofaciens* ATCC 23877 was due to the marked effects of this *pirA* inactivation on central carbon and energy metabolism, high sensitivity to oxidative damage, and repression of production of polyketide antibiotics. PirA has been shown to be a novel redox-sensitive negative modulator of very long-chain acyl-CoA dehydrogenase that catalyzes the first committed step of the beta-oxidation pathway. Most of these effects could therefore be traced to the inability of the *pirA*-defective strain to modulate beta-oxidation pathway, leading to an unbalanced supply of precursors for polyketide biosynthesis (Tala et al. 2018). These results suggest that although insertion into the PhiC31 *attB* site may in many cases be

almost neutral, sufficient for some applications, insertional inactivation of *pirA* homologs may have substantial metabolic defects that may not be adequate for the construction of producing strains.

Φ BT1 Att/Int system A suitable alternative to the Φ C31-Att/Int system are vectors derived from Φ BT1 phage that is related to Φ C31 (Gregory et al. 2003). Despite extensive similarities between the genomes of both phages, their *attP/int* loci are poorly conserved, although Φ BT1 integrase also belongs to the serine recombinase family. Φ BT1 integrates into a different attachment site than Φ C31 (Table 1). The Φ BT1 *attB* site is located within the *SCO4848* gene encoding a conserved integral membrane protein in the genome of *S. coelicolor* or its ortholog in other streptomycetes (Gregory et al. 2003; Zhang et al. 2008). Three integration plasmids, pRT801, pRT802, and pMS82, were constructed with different resistance marker genes (for apramycin, kanamycin, and hygromycin, respectively), all containing *oriT* for *E. coli*–*Streptomyces* conjugation. These Φ BT1 Att/Int integrative vectors have been shown to have a wide host range and are fully compatible with those based on the Φ C31 Att/Int system (Gregory et al. 2003).

The Φ BT1 Att/Int system was also used to construct the conjugative BAC vector pSBAC which was used to clone the 90-kb meridamycin gene cluster and its *ermEp**-driven heterologous expression in *S. lividans* (Liu et al. 2009). This BAC vector was also successfully used for cloning and efficient heterologous expression of two other large gene clusters for tautomycetin and pikromycin (Tocchetti et al. 2018). In addition, it has been modified for cloning and heterologous expression of whole gene cluster for calcium-dependent lipopeptide antibiotic A54145 (Alexander et al. 2010).

Although Φ BT1 Att/Int-derived vectors have good stability and broad utility in *Streptomyces* (Baltz 2012), there are minor drawbacks using this system. The integration process between Φ BT1 *attP* and *attB* is very efficient, but was not completely unidirectional as the one for Φ C31. It can also partially act as excisionase. A low but significant frequency of excision recombination between *attL* and *attR* was observed in the presence of the Φ BT1 integrase alone (Zhang et al. 2008). Initially, the insertion of Φ BT1 Att/Int integrative vectors was thought to be neutral for *Streptomyces* strains (Gregory et al. 2003). However, later work showed that disruption of *SCO4848* in *S. coelicolor* caused a delay in spore germination. This disruption affected the expression of *SCO4849*, which is cotranscribed with *SCO4848* and also encoded a putative transmembrane protein. The detected phenotype was complemented with *SCO4849*. To overcome the problem, four plasmids were generated, pNG1, pNG2, pNG3, and pNG4, which modified the Φ BT1 pMS82 integrative vector to restore *SCO4849* expression. They all produced a neutral phenotype when integrated into *Streptomyces* (Gonzalez-Quiñonez et al. 2016).

VWB Att/Int system Another alternative to Φ C31 integration system are the vectors based on phage VWB identified in *S. venezuelae*. In contrast to both previous Att/Int systems, the VWB integrase belongs to the tyrosine recombinase family and the VWB *attB* site is located in the putative tRNA^{Arg} (*AGG*) gene conserved in *Streptomyces* species (Table 1). Phage integration restores the gene at the *attL* site, but lacks the CCA terminus, typical of prokaryotic tRNAs. However, most *Streptomyces* tRNA terminals also do not have this terminal CCA. Using the VWB Att/Int system, the integrative plasmid pKT02 was constructed but lacked the *oriT* origin of conjugation transfer. It has been successfully introduced into strains of *S. lividans* and *S. venezuelae* by protoplast transformation, but with less efficacy than the Φ C31-derived integration vectors (Van Mellaert et al. 1998). To improve the properties of this vector, its variant pSOK804 containing *oriT* was constructed. It is integrated into single site in *S. noursei* with an efficiency of approximately 2 orders of magnitude greater than pSET152 (Sekurova et al. 2004).

The VWB Att/Int system was used to construct the conjugative TAR vectors pCLY10 and pCLY11 based on p15a and F-factor replicons, respectively, for cloning large gene clusters of secondary metabolites. The vectors were successfully used to assemble a greccocycline biosynthetic gene cluster from *Streptomyces* sp. Acta 1362 and its heterologous expression in *S. albus* J1074 (Bilyk et al. 2016). Recently, we used the pCLY10 vector for cloning and efficient heterologous expression of the biosynthetic gene cluster for antitumor polyketide mithramycin A in engineered *S. lividans* RedStrep strains. The yield of mithramycin A was up to 3 g/L of medium, which is sixfold higher than the amount of the overproducing mutant strain for production of mitramycin A, *S. argillaceus* (Novakova et al. 2018).

TG1 Att/Int system Another Att/Int system is based on the bacteriophage TG1 isolated from *S. cattleya*. Its integrase belongs to the serine recombinase family and the TG1 *attB* site is located within the *SCO3658* gene encoding conserved aminotransferase in the *S. coelicolor* genome or its ortholog in other streptomycetes (Table 1). Using the TG1 Att/Int system, the integration plasmid pKU462 was constructed, which also contains *oriT* for *E. coli*–*Streptomyces* conjugation and was successfully integrated into *S. avermitilis* (Morita et al. 2009). *SCO3658* orthologs and TG1 *attB* sites have been found in various *Streptomyces* strains (Baltz 2012; Morita et al. 2009). Compatibility with other phage-based integration systems has been demonstrated by constructing another TG1 Att/Int conjugative integration plasmid pRF10 that was efficiently introduced to several *Streptomyces* strains. Individual parts of the erythromycin biosynthetic gene cluster were cloned into four compatible integration plasmids, including pFF10. The other three Att/Int integration systems were derived from Φ C31, Φ BT1, and SV1. All four integration plasmids with

individual parts of the erythromycin biosynthetic gene cluster were integrated into two heterologous host strains, *S. coelicolor* and *S. lividans*, and robust production of various erythromycin intermediates was achieved. This multiplex integrative approach has provided a useful platform for polyketide synthase enzyme engineering for combinatorial biosynthesis of novel compounds (Fayed et al. 2015).

R4 Att/Int system This system is based on a broad-host-range R4 bacteriophage isolated from soil on *S. albus* J1074 (Chater and Carter 1979). Its unidirectional integrase belongs to the serine recombinase family and its *attB* site is located within the *SCO6196* gene encoding the acyl-CoA synthetase homolog in *S. coelicolor* or its ortholog in other *Streptomyces* species (Table 1) (Baltz 2012; Matsuura et al. 1996; Miura et al. 2011). Despite its well-characterized nature, the R4 Att/Int system has not been widely used in *Streptomyces* integration systems, although it has been successfully used to integrate into *E. coli*, cyanobacteria, and human cells (Miura et al. 2011, 2014; Olivares et al. 2001). However, the R4 Att/Int system has recently been used to construct the conjugative integration vector pLR4 and efficiently integrated into three *Streptomyces* strains. This vector was a part of five compatible integration systems (derived from Φ C31, Φ BT1, R4, SV1, and TG1) for the multiplex site-specific genome engineering system for single-step, multi-locus integration of biosynthetic gene clusters for bioactive secondary metabolites into streptomycetes (Li et al. 2019).

SV1 Att/Int system This system is based on bacteriophage SV1 isolated from *S. venezuelae*. Its unidirectional integrase belongs to the serine recombinase family and its *attB* site is located within the *SCO4383* gene encoding a 4-coumarate:CoA ligase homolog in *S. coelicolor* or its ortholog in other *Streptomyces* species (Table 1). The SV1 Att/Int system was used to construct a conjugal pBF3 vector that integrates efficiently into several *Streptomyces* species; however, it does not integrate into its native host, *S. venezuelae* (Fayed et al. 2014). As described above, the SV1 Att/Int-derived pBF3 plasmid was successfully used together with other three compatible Att/Int integration systems (derived from Φ C31, Φ BT1, and TG1) for cloning and heterologous expression of individual parts of the erythromycin gene cluster (Fayed et al. 2015). Similarly, the SV1 Att/Int system was also used to construct the conjugative integration vector pLSV1 as part of the five compatible integration systems (Φ C31, Φ BT1, R4, SV1, TG1 Att/Int-derived) for the multiplex site-specific genome engineering system (Li et al. 2019).

Φ Joe Att/Int system This system is based on recently described bacteriophage Φ Joe isolated from soil on *S. coelicolor* (Fogg et al. 2017). Its integrase belongs to the serine recombinase family. The Φ Joe Att/Int system was used

to construct the conjugal vector pCMF92, which integrated with very low potency into *S. coelicolor* and *S. lividans*, but with dramatically higher potency in *S. venezuelae* (160-fold greater than *S. coelicolor* and 1600-fold greater than *S. lividans*). This difference was solved after examining the *attB* integration site for the Φ Joe Att/Int system. Two integration sites were identified in *S. coelicolor*, separated by a mobile genetic element containing *SCO2603*, encoding putative serine integrase with 68% identity to Φ Joe integrase. Plasmid integration was ineffective because the ancestral optimal *attB* site is occupied by the *SCO2603* encoding element. These two integration sites in *S. coelicolor* were called *attLsc* and *attRsc* to reflect the origin of the sites containing the mobile element. However, a single *attB* site (Table 1), very similar to the reconstituted *attB* site from two halves *attLsc* and *attRsc*, was identified in *S. venezuelae*. Bioinformatic analysis showed that the *attB* site for Φ Joe integrase is located at the end of the conserved *SCO2606* gene in *S. coelicolor* (or *SVEN_2383* in *S. venezuelae*) encoding the predicted B12 binding domain-containing radical *S*-adenosylmethionine protein. In several *Streptomyces* species, this site was occupied, similarly to *S. coelicolor*, by a mobile element that differs in gene content and size. Unlike previous serine phage integrases, Φ Joe integrase showed significant excision in the absence of its RDF. This bidirectional property of this system could be applied in several new ways in genome engineering (Fogg et al. 2017).

μ 1/6 Att/Int system The last phage-derived Att/Int system under investigation is based on the bacteriophage μ 1/6 isolated from *S. aureofaciens*. Its integrase belongs to family of tyrosine recombinases. The μ 1/6 *attB* site is located at the 3'-end of the tRNA^{Thr} (ACA) gene conserved in *Streptomyces* species, but the tRNA gene was completely restored after phage-integration (Table 1). The μ 1/6 Att/Int system was used to construct the pCTint integration vector, but lacked the *oriT* origin of conjugal transfer. The plasmid was effectively integrated into three *Streptomyces* strains (Farkasovska and Godany 2012).

pSAM2 Att/Int system The last characterized Att/Int system is based on the 11-kb natural integration plasmid pSAM2 identified in *S. ambofaciens* and able to site-specifically integrate into the genome of various *Streptomyces* species (Boccard et al. 1989; Kuhstoss et al. 1989). Its integrase belongs to the tyrosine recombinase family and is encoded by the *int* gene translationally coupled with the upstream *xis* gene encoding excisionase. Both constitute an operon (Sezonov et al. 1998). The pSAM2 *attB* site is located at the 3'-end of the tRNA^{Pto} (CCG) gene conserved in *Streptomyces* species and the tRNA gene was restored upon integration. Both the *attP* and *attB* regions share an identical 58-bp segment, but the minimum *attB* site length is 26 bp (Table 1). Interestingly, a

relatively large 360-bp *attP* site is required for integration (Raynal et al. 1998, 2002). The pSAM2 Att/Int system was used to construct two vectors, the integration plasmid pKC824 and the cosmid pKC767, both of which lacked *oriT* for conjugation. Both vectors were able to integrate into various *Streptomyces* strains, but their integration efficiency was significantly lower (60-fold and 600-fold) than their Φ C31 Att/Int-derived counterparts (Kuhstoss et al. 1991). Another pPM927 conjugative vector, based on the pSAM2 Att/Int system, was efficiently and stably integrated into one site in *S. lividans* (Smokvina et al. 1990). Its successful integration has been reported in several other *Streptomyces* species (Myronovskiy and Luzhetskyy 2013), indicating its broad host specificity and use for stable site-specific integration in *Streptomyces*. For example, the pSAM2 Att/Int-derived vector was successfully used to introduce the gene cluster *fkfG-K* for the methoxymalonyl-ACP biosynthetic pathways into *S. fradiae* to dramatically increase (more than 1 g/l) heterologous production of macrolide polyketide midecamycin (encoded by the *mdm* gene cluster), compared with *S. fradiae* with the *mdm* gene cluster integrated using the Φ C31-based Att/Int system (Rodriguez et al. 2004).

As described above, these Att/Int systems have found widespread use in gene manipulations in streptomycetes and, in particular, in the stable integration of biosynthetic gene clusters for many biologically active secondary metabolites into heterologous hosts. Many vectors based on these integration systems have been prepared and successfully used for efficient heterologous expression of various bacterial biosynthetic pathways producing new biologically active natural products or known products with increased yield compared with the original hosts. More than 100 different biologically active agents, including 21 completely new compounds, prepared by this approach are summarized in a recent review article (Huo et al. (2019)). The great advantage of these Att/Int systems, particularly in connection with the conjugative *oriT* origin, is high efficiency of their integration into the chromosome of *Streptomyces* strains (Baltz 2012). Another useful feature of these integration systems is that each has a unique *attB* site located at a different position in the particular *Streptomyces* genome. These Att/Int systems with their *attB* sites in the *S. coelicolor* genome are shown in Table 1. Another great advantage of the Att/Int integration vectors is that they can be multiplexed without interference during recombination or conjugation and without loss of stability. Thus, more copies of a gene cluster or more genetic elements can be stably inserted into individual *attB* sites (Fayed et al. 2015; Gregory et al. 2003; Li et al. 2019).

Despite all the benefits of these Att/Int systems, they also have some limitations. As mentioned above, some results suggested that insertion into the *attB* site may result in a substantial reduction of secondary metabolite production (Baltz 1998; Tala et al. 2018). Although Att/Int-derived vectors have good

stability and broad applicability in *Streptomyces*, not all *Streptomyces* strains contain *attB* integration sites for these vectors (Baltz 2012; Myronovskiy and Luzhetskyy 2013). For example, our model strain *S. lavendulae* subsp. *lavendulae* CCM 3239 (Busche et al. 2018) can only efficiently integrate Φ C31 and pSAM2 Att/Int-derived integrative plasmids (Novakova et al. 2011; J. Kormanec, unpublished results). However, an artificial *attB* site could be inserted into such strains to allow efficient integration, as demonstrated in several cases (Li et al. 2017a, 2019; Manderscheid et al. 2016). The other disadvantage of these systems is that they integrate the entire integration plasmid with the *E. coli* replicon, the integrase gene and the resistance marker gene (Fig. 1). It is a significant biotechnological obstacle, as biotechnological production strains should avoid the antibiotic resistance genes. Similarly, the expression of the integrase gene and the *E. coli* plasmid replication gene can affect the stability of the constructs. In fact, the instability of several Att/Int systems has been reported in several *Streptomyces* strains (Baltz 2012; Fayed et al. 2014; Myronovskiy and Luzhetskyy 2013; Siegl et al. 2010; Siegl and Luzhetskyy 2012). We also previously found that an *E. coli* origin of replication caused dramatic instability after integration in some *Streptomyces* chromosomes (Kormanec et al. 1993). Although the utility of these Att/Int systems is evident for many gene manipulations and applications in streptomycetes, this integration method may not be suitable for the construction of stable biotechnological production strains. These problems can be overcome by another method of stable integration into the *Streptomyces* chromosomes by homologous recombination, as will be described in the following section of this article.

Genome editing toolkit based on homologous recombination

Homologous recombination (HR) is a highly conserved process common to both prokaryotic and eukaryotic cells, which is essential to maintain genome stability and plays an important role in genome evolution. It is also an effective mechanism of DNA repair necessary for cell survival when DNA suffers from various types of damage, including double-strand breaks. The HR process requires energy and assistance from conserved mediator and recombinase proteins and involves aligning similar DNA sequences to form mobile forks between four DNA strands called Holliday junctions, and ultimately complete exchange of two complementary strands (Costes and Lambert 2013; Wright et al. 2018).

HR-based methodology is a powerful tool for stable genome engineering, including deletion, replacement, and insertion of genomic sequences. HR gene modifications in *Streptomyces* are usually based on suicidal non-replicating vectors that carry DNA fragments surrounding the target gene(s) to be deleted with the antibiotic resistance marker

gene. Such a mutant allele on the vector is used to replace the chromosomal copy of the target gene(s) via two crossovers, on both sides of the antibiotic resistance marker gene, resulting in a stable mutation. These two crossovers may occur simultaneously, resulting in the replacement of the target gene(s) with the antibiotic resistance marker gene, or may occur sequentially via a single crossover intermediate in which the entire vector is integrated into the chromosome through one of the homologous regions. The vectors therefore also carry a counterselectable marker (a second different antibiotic resistance marker gene). The final replacement of the desired gene(s) in the *Streptomyces* chromosome is accomplished by a double crossover event, usually after two rounds of sporulation, by selecting only for the antibiotic resistance marker inserted into the target gene(s) prior to spore plating for the individual colonies. The double crossover is subsequently identified by replica plating to select a loss of the counterselectable marker (second antibiotic resistance marker gene). However, these techniques are time consuming and require screening of thousands of clones by replica plating (Kieser et al. 2000) (Fig. 2).

Unlike the site-specific Att/Int-based integrations, HR is much less effective and depends on the size of crossover homologous regions. Generally, the lower limit is about 500 bp, but for some strains, much larger (several kb) homologous DNA fragments are needed (Kieser et al. 2000). To increase the efficacy of HR, a PCR-targeted system (REDIRECT) has been developed for efficient gene deletions in *Streptomyces*. The strategy used μ -Red-mediated recombination in *E. coli* (Datsenko and Wanner 2000) between PCR-amplified antibiotic resistance cassette flanked by 39-nt homologous arms and the large *Streptomyces* DNA (about 40 kb) on cosmid where the target gene(s) are replaced with this cassette. The amplified cassette contains the origin of transfer *oriT* for *E. coli*–*Streptomyces* conjugation and is flanked by yeast F1p recombinase target sequences *FRT* for the removal of the antibiotic resistance marker gene. Such a mutated cosmid is subsequently used to be introduced into *Streptomyces* to replace the target gene(s) with the cassette. Due to the large homologous DNA fragments flanking the cassette, the recombination process between the cosmid and *Streptomyces* chromosome is more effective (Gust et al. 2003). In addition to gene deletions, this REDIRECT system has also been successfully used for insertions into the *Streptomyces* genome, such as the insertion of a strong *ermEp** promoter upstream a “silent” gene cluster for a secondary metabolite allowing its activation (Novakova et al. 2011; Zheng et al. 2007). However, this system requires an ordered cosmid library that may be a hindrance to *Streptomyces* strains that have not been genetically well characterized, and also does not overwhelm the screening of large number colonies by replica plating to distinguish between single and double crossover.

In order to avoid these laborious and time-consuming steps, several gene inactivation systems with positive selection of double crossovers have been developed. One of these is a system based on the *glkA* counterselectable gene encoding glucose kinase which confers a sensitivity to the glucose analog 2-deoxyglucose. Thus, after selection on plates containing this toxic compound, only double crossovers lacking *glkA* can grow. However, this system requires a *Streptomyces* strain that carries a *glkA* deletion to allow counterselection of *glkA* in the vector. If the *glkA* mutation interferes with further analysis, the final gene disruption can always be crossed into a *glkA*⁺ genetic background (van Wezel and Bibb 1996). The second counterselectable gene used for positive selection of double crossovers is *rpsL*, which encodes S12 ribosomal protein. It confers streptomycin sensitivity in selected spontaneous streptomycin-resistant *Streptomyces* strain. The *rpsL* gene was cloned into a plasmid vector containing a *Streptomyces* pSG5-based temperature-sensitive replication origin and a temperature shift from 29 to 39 °C was used to select double crossovers. However, this system also requires a mutated *Streptomyces* strain that carries a *rpsL* mutation to allow counterselection of *rpsL*⁺ on the vector (Hosted and Baltz 1997). Another counterselectable system utilizes the synthetic *codA* gene encoding cytosine deaminase. CodA enzyme converts 5-fluorocytosine into a highly toxic 5-fluorouracyl. After selection in 5-fluorocytosine-containing plates, only double crossovers lacking *codA* can grow (Dubeau et al. 2009). The use of a suitable chromogenic selectable marker gene to distinguish between single crossover and double crossover mutants by producing colonies of different colors could simplify the procedure and significantly reduce selection time. The first such gene was *gusA* encoding β -glucuronidase that is absent in *Streptomyces* strains. The GusA enzyme hydrolyzes a wide variety of β -glucuronides, including commercially available chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-glucuronide, which is converted to blue products. This system enables to distinguish between blue single crossover and white double crossover clones. It has been successfully used for deletion of several secondary metabolite gene clusters in *S. albus*. However, the disadvantage of this system is the relatively high cost of this substrate (Myronovskyi et al. 2014). Thus, all the above counterselectable systems require either mutated *Streptomyces* host strains or external supply of substrates. Therefore, we have developed a novel efficient gene deletion system based on the positive selection of double crossovers using a counterselectable *bpsA* gene from *S. aureofaciens* CCM 3239, which encodes a protein for the synthesis of blue pigment indigoidine. The suicidal non-replicative deletion plasmid pAMR4 contains the apramycin resistance marker gene with *oriT* for easy *E. coli*–*Streptomyces* conjugation. The resistance marker gene was flanked by yeast F1p

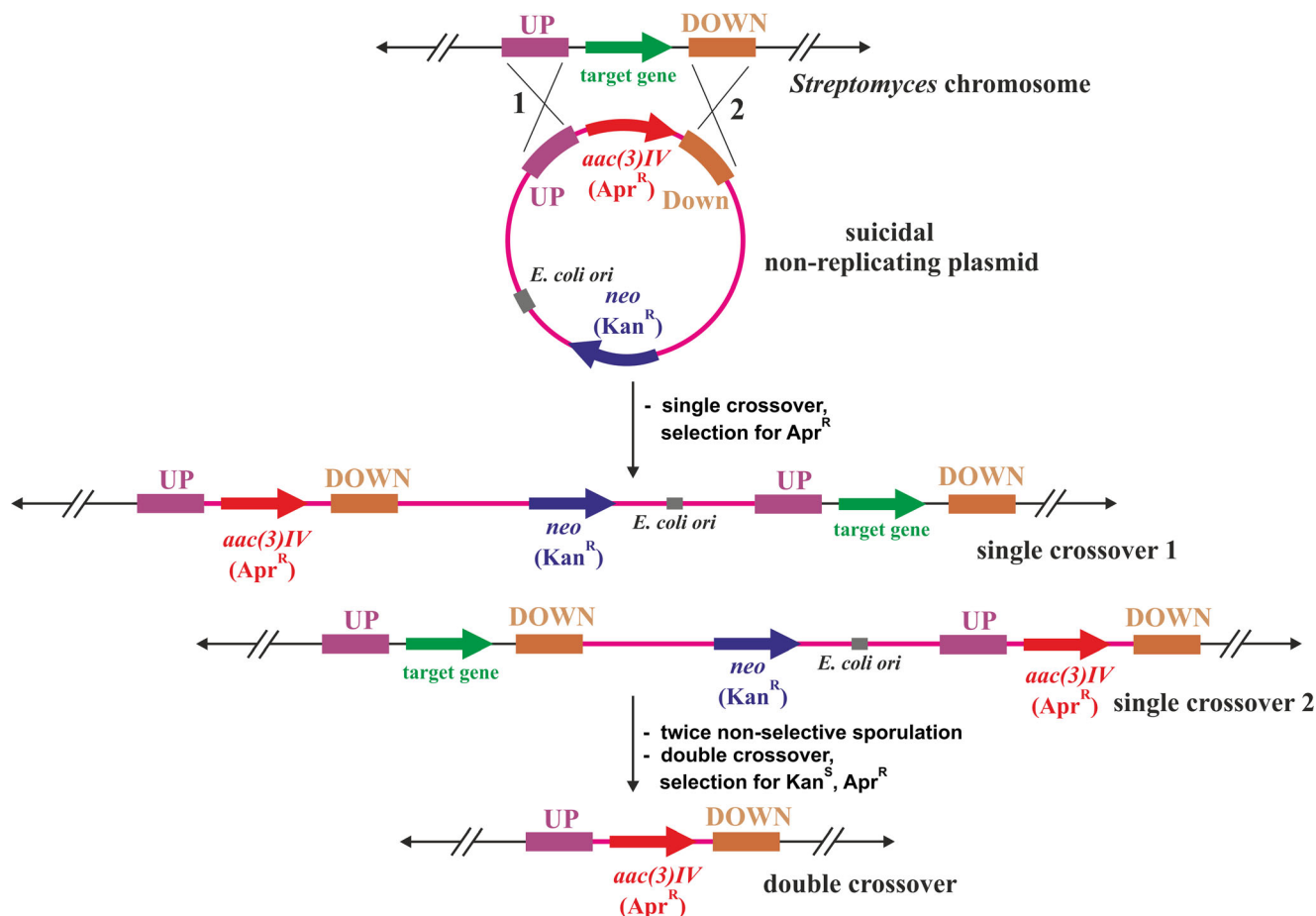


Fig. 2 Scheme of deletion of a target gene (green arrow) with a conventional inactivation system using a suicidal non-replicating plasmid. UP, upstream homology arm; DOWN, downstream homology

arm; *aac(3)IV*, the apramycin resistance marker gene used to replace the target gene as an example (red arrow); *neo*, the Tn5 counterselectable kanamycin resistance marker gene as an example (dark blue arrow)

recombinase target sequences *FRT* for removal of the antibiotic resistance marker gene. In addition, this apramycin resistance marker cassette was flanked by two polylinkers with several unique sites for rare restriction enzymes in streptomycetes to clone two homologous regions for crossovers. The plasmid contains an additional counterselectable kanamycin resistance gene which also directs the expression of the *bpsA* gene (Knirschova et al. 2015). After conjugation of the recombinant plasmid containing the two PCR-amplified DNA fragments with the homologous regions flanking the gene(s) to be deleted into the *Streptomyces* strain, blue colonies (presence of blue-pigmented indigoidine produced by BpsA) and the uncoloured colonies (absence of BpsA) allow easy detection of single and double crossover recombination, respectively (Fig. 3). The system was successfully tested by the deletion of the 21.2 kb *act* gene cluster responsible for the biosynthesis of polyketide antibiotic actinorhodin in *S. lividans* TK24 (Knirschova et al. 2015). A similar blue-white deletion system was independently developed using the homologous indigoidine biosynthetic *idgS* gene from *S. lavendulae* CGMCC 4.1386 (Li et al. 2015).

Marker-free genome editing tools

However, all these gene deletion strategies lead to the replacement of the target gene(s) with a selectable antibiotic resistance marker gene that may have a polar effect on the expression of downstream genes. In addition, multiple gene deletions in the same *Streptomyces* strain are restricted to a limited number of antibiotic resistance marker genes. Therefore, several strategies were introduced to remove this resistance marker gene. In the REDIRECT PCR-targeting method, the Flp/*FRT* recombination system is used as an assistant tool to remove resistance markers. First, a temperature-sensitive replication plasmid pCP20 containing the thermoinducible Flp recombinase gene is introduced into *E. coli*, which contains the cosmid with the replaced target gene(s) by the antibiotic resistance marker gene cassette flanked by *FRT* sites. Expression of the FLP recombinase removes the central part of the *FRT*-flanked disruption cassette from the disrupted gene leaving an 81-bp “scar” sequence that contains a single *FRT* site. Subsequently, the resulting cosmid containing the markerless deletion is introduced into the *S. coelicolor* mutant

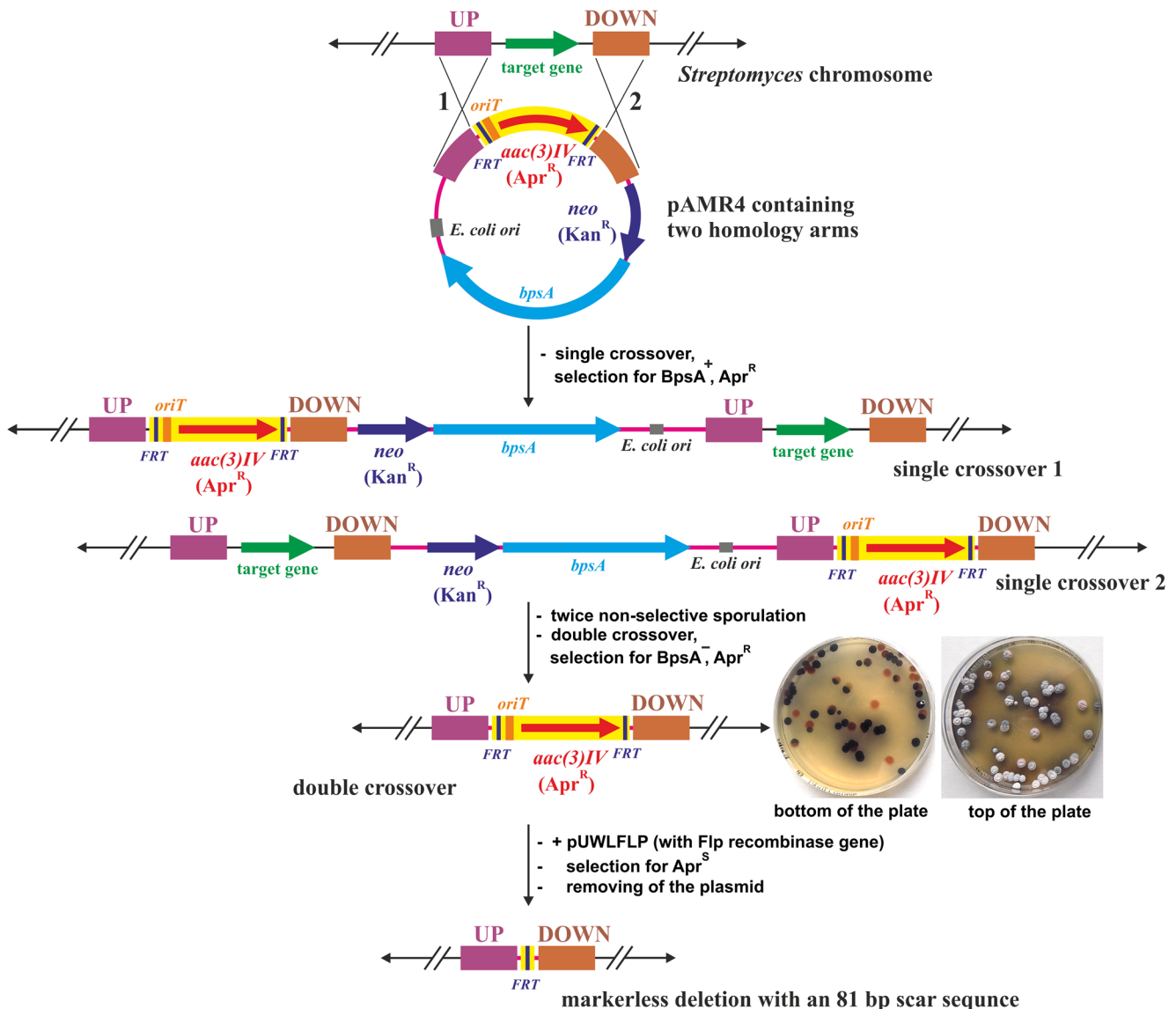


Fig. 3 Scheme of deletion of a target gene (green arrow) with the gene deletion system based on the positive selection of double crossovers using the counterselectable *bpsA* gene that encodes a protein for the synthesis of blue pigment indigoidine (Knirschova et al. 2015). The suicidal non-replicating plasmid pAMR4 contains two homologous arms surrounding the gene (UP and DOWN) to be deleted, the promoterless indigoidine biosynthetic gene (*bpsA*) and the Tn5 kanamycin resistance marker gene (*neo*) directing *bpsA* expression. The yellow box indicates the cassette which contains the *aac3(IV)* apramycin resistance marker gene, the *oriT*

origin of conjugal transfer and is flanked by yeast Flp recombinase target sequences *FRT* (Gust et al. 2003). To remove the antibiotic resistance marker gene, a plasmid pUWLFLP containing the Flp recombinase gene (Fedoryshyn et al. 2008a) was introduced into the double crossover mutant. A solid Bennet medium plate with colonies after two rounds of non-selective sporulation is shown to distinguish between double-crossover Apr^R clones (white colonies) and single-crossover Apr^S clones (blue colonies)

carrying the marked deletion in the target gene. Replacement of the marked deletion in the *Streptomyces* chromosome with the markerless deletion in the cosmid is screened by replica plating for the loss of antibiotic resistance marker gene and the counterselectable antibiotic resistance marker gene (kanamycin resistance in the case of SuperCos 1 cosmid) (Gust et al. 2003). The drawback of this REDIRECT system is that Flp/FRT recombination must be performed in *E. coli* and the selection procedure in *Streptomyces* with unmarked cosmid, which is a very laborious and time-consuming process. To

overcome these limitations, two *Streptomyces* replicative plasmids, pUWLFLP and pALFLP, were constructed with pIJ101-based and the temperature-sensitive pGS5-based replicons, respectively. Both contain a synthetic gene encoding Flp recombinase with GC content optimized for expression in *Streptomyces* under the control of a strong *ermEp** promoter and the origin of transfer *oriT*. After conjugation of these replicative plasmids, the apramycin resistance marker gene flanked by *FRT* sites was removed from *Streptomyces* chromosome with 40% efficiency (Fedoryshyn

et al. 2008a) (Fig. 3). The other two site-specific recombination systems, *Cre/loxP* from the phage P1 and *Dre/rox* from the phage D6, were also successfully used to remove the resistance marker gene in a similar manner using pIJ101-based or pSG5-based *Streptomyces* replicative plasmids pUWLCRE/pUWLDRE and pNLCRE/pALDRE, respectively. Similarly, they contain synthetic genes encoding Cre or Dre recombinases with codon usage optimized for expression in *Streptomyces*. After conjugation of these plasmids, the apramycin resistance marker gene flanked by *loxP* or *rox* sites was removed from *Streptomyces* chromosome with 100% efficiency. Because all three site-specific recombination systems are strictly specific to their target sites, they could be used sequentially in multiple mutations in a single *Streptomyces* strain (Fedoryshyn et al. 2008b; Hermann et al. 2012).

All three site-specific recombination systems described above have one limitation, they cannot be used iteratively because they leave an active recognition site in the *Streptomyces* genome. However, the stable manipulation of *Streptomyces* genomes requires the manipulation of dozens of loci that can improve their biotechnological properties, e.g., in the preparation of minimal *Streptomyces* genomes for efficient chassis for the discovery and overproduction of natural product or for the integration of multiple genes (Liu et al. 2018). Thus, there is a need for a site-specific recombination system that can be iteratively used for such multiple genome editing purposes. Such a system, called iterative marker-free excision system (IMES), has been developed. It uses Φ C31 integrase and its mutated *att* sites *B-CC* and *P-GG*. These mutated sites are derivatives of the Φ C31 *attB* and *attP* sites by substituting the central core TT dinucleotide with CC and GG. IMES can be used to effectively remove antibiotic resistance marker gene flanked by inversely oriented *B-CC* and *P-GG*. The resulting *RR* site, which evolves as a result of the deletion, is the joining product of the right arms of *B-CC* and *P-GG* and is unable to recombine with *P-GG* and *B-CC*. Several cassettes carrying synthetic genes of the apramycin, spectinomycin, or hygromycin resistance markers, flanked by the *P-GG* and *B-CC* sites, have been generated for this strategy. They were efficiently excised from the *Streptomyces* chromosome containing the replaced target genes by expression of the Φ C31 integrase from the pIJ101-based *Streptomyces* replicative pUWLint31 plasmid. Thus, IMES can be used for multiple genomic engineering without the risk of unwanted DNA recombination (Myronovskyi et al. 2014). IMES has recently been used for the successful deletion of all 15 secondary metabolite gene clusters from *S. albus* J1074 chromosome, resulting in a cluster-free *S. albus* Del14 chassis strain that was subsequently used to improve heterologous expression of several secondary metabolite gene clusters (Myronovskyi et al. 2018).

Using this INES strategy, we have modified our previous effective deletion system based on the positive double

crossovers selection with the *bpsA* gene responsible for the blue pigment indigoidine production. To reduce unwanted recombination in successive multi-step genomic deletions, new non-replicative plasmid vectors, pAMR12 and pSPR12, were prepared by replacing the apramycin resistance marker gene in pAMR4 with synthetic apramycin and spectinomycin resistance genes, respectively. Both cassettes were flanked by recombination sites *P-GG* and *G-CC* for Φ C31 recombinase and contain the conjugation origin *oriT* of transfer. Similarly, the two new cassettes were flanked by two polylinkers with several unique sites for rare restriction enzymes in streptomycetes to clone two homologous regions for crossovers. Using this new modified system, we prepared a collection of *S. lividans* RedStrep strains with sequentially deleted gene clusters for five interfering secondary metabolites: *act* for actinorhodin, *red* for undecylprodigiosin, *cdc* for calcium-dependent antibiotic, *cpk* for coelimycin, *mel* for brown pigment melanin. In addition, the *hrdD* gene for the stationary phase sigma factor and the *scbAR* genes for the γ -butyrolactone system were deleted. In these strains, we demonstrated a dramatic increase in the heterologous production of the antitumoral polyketide mitramycin A, encoded by the *mtm* gene cluster (up to 3 g/L medium), compared with *S. lividans* TK24 with the *mtm* gene cluster integrated using the VWB-based Att/Int system. It demonstrated the biotechnological impact of *S. lividans* RedStrep strains on heterologous production of biologically active substances (Novakova et al. 2018).

All the markerless systems described above have one disadvantage. In order to remove the antibiotic resistance marker gene, the site-specific recombinase gene must be delivered to the *Streptomyces* strain in the replicative plasmid (pIJ101-based or temperature sensitive pGM5-based). In addition to this additional time-consuming step, it is also possible that some *Streptomyces* strains may not accept these replicative plasmids. Although pIJ101 derivatives have a wide host range within the genus *Streptomyces*, only 13 out of the 18 strains have been successfully transformed (Kieser et al. 1982). Similarly, we were unable to introduce these pIJ101 derivative plasmids into our model *S. lavendulae* subsp. *lavendulae* CCM 3239 (Busche et al. 2018; Kormanec et al. 1993). In addition, the scar sequence left after removal of the resistance marker gene contains an integration site (e.g., an 81-bp *FRT* site for Flp recombinase) with a longer inverted repeat that can affect adjacent genes. To simplify the markerless deletions and to reduce the time needed, we prepared a new efficient markerless deletion system based on the positive selection for double crossovers with the blue pigment indigoidine *bpsA* gene. A new suicidal non-replicative plasmid vector pAMR18 containing a large polylinker with many unique restriction sites flanked by the apramycin and kanamycin resistance genes (used for single crossover selection) and the *bpsA* gene for double crossover selection were created. To delete the

target gene(s), two PCR-amplified DNA fragments from its flanking regions can be cloned into the large polylinker of pAMR18A, leaving just one restriction site between them. Since pAMR18A does not have the replication origin of *Streptomyces*, it will be integrated into the *Streptomyces* genome by HR of one of the cloned *Streptomyces* DNA fragments. After two rounds of non-selective sporulation, it is possible to readily select double crossover mutant or wild-type reverse colonies based on colony color (blue or white) and then examined by PCR to distinguish between these two events (Fig. 4). This system was successfully tested by the deletion of the 21.2-kb *act* gene cluster for polyketide antibiotic actinorhodin in *S. lividans* TK24 with 30% efficacy (3 out of 10 selected white colonies contained correctly deleted *act*) or by effective deletion of the *matAB* operon for mycelial aggregation in selected *S. lividans* RedStrep strains with 40% efficiency. In addition, the system was used for the markerless insertion of the *mCherry* gene under the control of the subtilisin inhibitor protein secretory signal sequence into the chromosome of *S. lividans* TK24 (with 30% efficacy), resulting in efficient secretion of mCherry into the culture medium. Thus, this markerless system can also be used to stably insert foreign genes or gene clusters into any neutral position of the *Streptomyces* chromosome (Rezuchova et al. 2018).

Compared with the site-specific integrative Att/Int systems, HR-based genome editing systems have several advantages. They are more stable because they leave only the deleted or inserted gene(s) with a short scar sequence (50 to 80 bp) or a single restriction site (6 bp) as opposed to the Att/Int systems that integrate the entire integrative plasmid together with the *E. coli* replicon, integrase gene, and the resistance marker gene. In addition, they can stably integrate foreign DNA into any neutral chromosome position. However, the main disadvantage of HR-based genome editing systems is their low integration efficiency, depending on the size of the homology arms. In addition, it differs from *Streptomyces* strain to strain. Two systems have been developed to increase HR efficiency.

I-SceI endonuclease-based genome editing system

The first is based on the yeast mitochondrial homing endonuclease I-SceI, which specifically recognizes and cleaves the 18-bp DNA sequence TAGGGATAACAGGGTAAT. The efficacy of HR could be improved by introducing double-strand DNA breaks (DSBs) into the desired DNA region. In the presence of a homology template, these breaks can be effectively repaired by HR. This strategy is used in this I-SceI-based system to perform markerless gene mutations. Theoretically, the I-SceI recognition sequence appears once in 70 billion bases of a random sequence, and *Streptomyces* genomes analyzed to date are free of I-SceI recognition sites. To perform a markerless mutation, the mutant gene is cloned

into a suicidal non-replicative plasmid that contains the antibiotic resistance marker gene and the I-SceI recognition site. The plasmid is introduced into the *Streptomyces* strain and integrated into the chromosome by HR. Subsequently, the I-SceI endonuclease gene is expressed in the resulting mutant upon conjugation with one of the *Streptomyces* replicative plasmids, pALSCE or pUWLHSCE, containing pIJ101 or the temperature sensitive pGS5 replicons, respectively. Both contain a synthetic gene encoding I-SceI recombinase using codons optimized for *Streptomyces* under the control of a strong *tipAp* or *ermEp** promoter, respectively. The DSB introduced by I-SceI stimulates intramolecular recombination between wild-type and mutant gene. Chromosome repair and viability can only lead to HR between duplicated regions of the genome, providing selection for a second crossover event that provides a mixture of both the original and the mutant gene. They can be easily identified by PCR analysis. Finally, the replicative plasmids containing the I-SceI endonuclease gene are removed from the mutant strain after several rounds of non-selective sporulation (pUWLHSCE) or increased temperature (pALSCE). This procedure significantly increased efficacy of HR leading to markerless mutation and could be used for various manipulations of genome. In addition, the marker-free nature of the mutations facilitates the generation of multiple gene deletions in one genomic background (Siegl et al. 2010). A similar I-SceI-based system was used to efficiently delete the *red* gene cluster for the antibiotic undecylprodigiosin in *S. coelicolor* (Fernandez-Martinez and Bibb 2014). However, as in the previous cases described above, the use of *Streptomyces* replicative plasmids to introduce I-SceI expression into *Streptomyces* is a disadvantage of the I-SceI endonuclease recombination system, since some *Streptomyces* strains may not accept these replicative plasmids.

CRISPR/Cas-based genome editing systems

Another system to increase HR efficacy in the markerless genome manipulations in *Streptomyces* is a CRISPR/Cas-based genome editing tool that has revolutionized genomic engineering in almost every organism over the past six years, including *Streptomyces* species (Tao et al. 2018; Tong et al. 2018). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas (CRISPR-associated proteins) is an adaptive immune system to defend against exogenous DNA or RNA. Three different CRISPR/Cas systems have been identified. Type I and type III require multiple Cas proteins to induce cleavage of their target DNA. However, type II systems require a single Cas9 endonuclease. The best studied example of type II systems is *Streptococcus pyogenes* CRISPR/Cas9, which has allowed its widespread use in genome editing applications. This system consists of three elements: Cas9 endonuclease, short crRNA that confers target

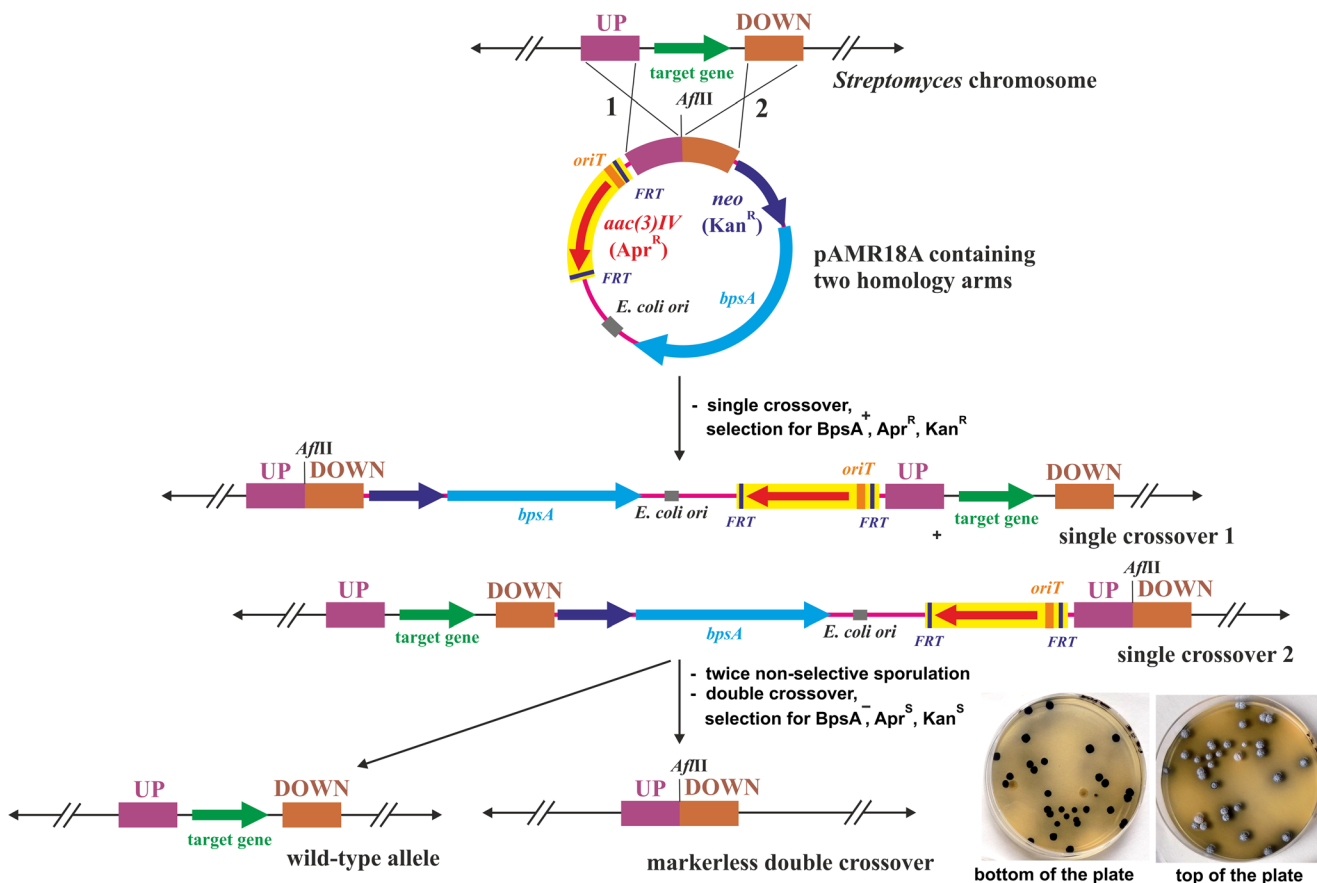


Fig. 4 Scheme of deletion of a target gene (green arrow) using the markerless gene deletion system with the *bpsA* indigoidine biosynthetic gene (Rezuchova et al. 2018). The suicidal non-replicating plasmid pAMR18A contains two homologous arms flanking the deleted gene (UP and DOWN), the promoterless indigoidine biosynthetic gene (*bpsA*) and the Tn5 kanamycin resistance marker gene (*neo*) driving *bpsA* expression. The yellow box indicates the cassette which contains

the *aac3(IV)* apramycin resistance marker gene, the *oriT* origin of conjugal transfer and is flanked by yeast F1p recombinase target sequences *FRT* (Gust et al. 2003). A solid Bennet medium plate with colonies after two rounds of non-selective sporulation is shown to distinguish between double-crossover clones or wild-type revertant clones (white colonies) and single-crossover clones (blue colonies)

site specificity, and short tracrRNA facilitating crRNA processing and recruitment. The crRNA–tracrRNA duplex forms a complex with Cas9 and scans the target genome for the presence of PAM (Protospacer adjacent motif 5'-NGG-3'), in which 5' sequence is complementary to the spacer sequence in the crRNA–tracrRNA duplex. Once the complex binds this specific target sequence, Cas9 specifically cleaves DNA resulting in a lethal DSB. These two small RNAs can be artificially fused into a single synthetic guide RNA (sgRNA) that retains full functionality (Fig. 5). The DSB must be repaired by rejoining the DNA ends. In the absence of a homologous template DNA, this can be achieved by activation of a non-homologous end joining (NHEJ) pathway. In the presence of a homologous template DNA, the ends can be efficiently joined by HR. In eukaryotes, NHEJ is the dominant pathway for DSB repair. However, NHEJ does not widely occur in bacteria, including *Streptomyces*. Therefore, the repair of bacterial DSB relies mainly on HR (Selle and Barrangou 2015; Tao et al. 2018; Tong et al. 2018).

To date, four *S. pyogenes*-based CRISPR/Cas9 systems have been developed and applied to rationally edit the *Streptomyces* genome, including the following delivery plasmid vectors: pCRISPomyces-2 (Cobb et al. 2015); pCRISPR-Cas9 (Tong et al. 2015); pKCcas9dO (Huang et al. 2015); and pWHU2059 (Zeng et al. 2015). All these four systems share some common designs and applications. They use a one-vector strategy that combines the synthetic *S. pyogenes cas9* gene with optimum codon usage for *Streptomyces* and the sgRNA expression cassette, both under the control of the promoters active in these bacteria, and the homology repair template for HR on a single delivery plasmid. Systems specifically generates DSB at a target site using Cas9 under the guidance of a sgRNA harboring a custom-designed spacer, and then repairs it by HR repair in the presence of a homology repairing template, resulting in an accurate markerless deletion of the gene or large gene cluster with efficacy ranging from 50 to 100% in several *Streptomyces* strains. Using these systems, the multiplex

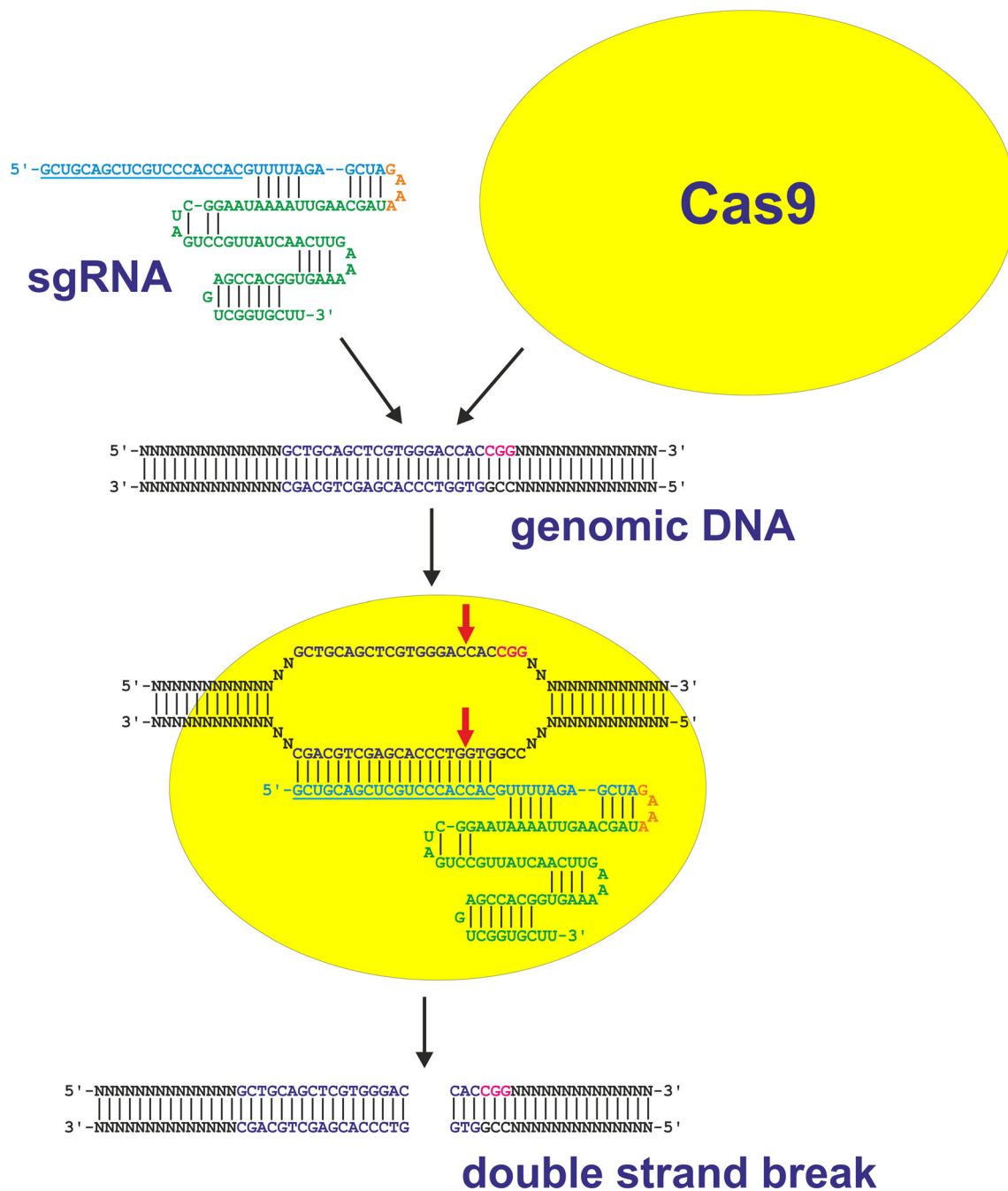


Fig. 5 Scheme of the class II *Streptococcus pyogenes* CRISPR/Cas9 system. The crRNA (blue) and tracrRNA (green) are fused by a short loop (orange) to create a sgRNA. The 20 nucleotides programmable sequence in the sgRNA to recognize the target sequence (dark blue) in

genome is underlined. PAM sequence is in red font. The CRISPR-associated protein 9 (Cas9 nuclease) is indicated by yellow oval. The positions of the cleavage of the target DNA strands by Cas9 nuclease are indicated by red arrows

genome editing has also been achieved by equipping multiplex sgRNA cassettes and corresponding homology repairing templates (Cobb et al. 2015; Huang et al. 2015). Besides, CRISPR/Cas9 was also used to introduce nucleotide point mutations into the *rpsL* gene to generate a specific streptomycin resistant mutation (Huang et al. 2015). The first three systems are based on the *Streptomyces* temperature-sensitive pSG5 replicon to facilitate multiple

rounds of editing (Cobb et al. 2015; Tong et al. 2015; Huang et al. 2015). The latter (pWHU2059), is based on a segregation-unstable *sti⁻* pIJ101-derived *E. coli*-*Streptomyces* high-copy number shuttle vector producing large amounts of single-stranded plasmid DNA and hence the amount of template DNA. The result was a substantially higher HR efficiency and target mutant frequency. In addition, this CRISPR/Cas9 system utilizes the D314A cytosine

Table 2 A summary of HR-based stable genome editing methods in streptomycetes

Methods	Principles	Advantages	Disadvantages	Reference
Classical insertional inactivation	Two ARM gene strategy (one for replacement, the other one for counterselection) using non-replicating plasmid vectors	Simple, universal	Low efficiency, laborious and time-consuming selection of DC inserted ARM gene.	Kieser et al. 2000
PCR-targeted system (REDIRECT)	μ Red recombination in a cosmid library clone followed by HR in <i>Streptomyces</i>	Efficient, markerless deletion/insertion	Necessary ordered cosmid library, laborious and time consuming removing of ARM gene, scars	Gust et al. 2003
Site-specific recombination systems	<i>FliP/FRT</i> , <i>Cre/loxP</i> , <i>Dre/rox</i> site-specific recombination systems to remove ARM gene flanked by <i>FRT</i> , <i>loxP</i> , <i>rox</i> sites	Efficient, markerless deletion/insertion	Delivery of the recombinase genes by replicative plasmids, scars, cannot be used iteratively	Fedoryshyn et al. 2008a, b; Hermann et al. 2012
IMES system	ϕ C31-based integrase/ <i>B-CC</i> , <i>P-GG</i> site-specific recombination system to iteratively remove ARM gene flanked by <i>B-CC</i> , <i>P-GG</i> sites	Efficient, markerless deletion/insertion can be used iteratively	Delivery of the ϕ 31 gene by replicative plasmid, scars	Myronovskiy et al. 2014
GlkA counterselection	Positive selection of DC with glucose kinase <i>glkA</i> gene conferring sensitivity to toxic 2-deoxyglucose	Efficient	Necessary <i>glkA</i> mutant, needed crossing of the deletion into a <i>glkA</i> ⁺ strain, inserted ARM	van Wezel and Bibb 1996
RpsL counterselection	Positive selection of DC with S12 ribosomal protein <i>rpsL</i> gene conferring sensitivity to streptomycin	Efficient	Necessary <i>rpsL</i> mutant, inserted ARM gene	Hosted and Baltz 1997
CodA counterselection	Positive selection of DC with cytosine deaminase <i>codA</i> gene conferring sensitivity to toxic 5-fluorouracyl	Efficient, markerless deletion	Necessary 5-fluorocytosine, necessary strain resistant to 5-fluorocytosine	Dubeau et al. 2009
GusA counterselection	Positive selection of DC with β sitive selectio <i>gusA</i> gene conferring blue/white selection of DC	Efficient, markerless deletion	High cost of GusA substrate, delivery of the ϕ gh gene by replicative plasmid, scars	Myronovskiy et al. 2014
BpsA counterselection	Positive selection of DC with blue pigment indigoidine synthase <i>bpsA</i> gene conferring blue/white selection of DC	Simple, efficient, markerless deletion/insertion, no scar	Necessary absence of active <i>bpsA</i> homolog in strain used	Rezuchova et al. 2018
I-SceI system	Formation of DSB in target sequence by I-SceI nuclease repaired by HR, resulting in markerless deletion/insertion	Efficient, markerless deletion/insertion	Necessary to introduce I-SceI of I-SceI by replicative recognition site in vector, delivery	Siegl et al. 2010; Fernandez-Martinez and Bibb 2014
CRISPR/Cas9 system	sgRNA-based formation of DSB in target sequence by Cas9 nuclease repaired by HR using homology template, resulting in markerless deletion/insertion	Efficient, fast, markerless deletion/insertion	Non-specific off-target effects, delivery of <i>cas9</i> by replicative plasmids	Tong et al. 2018

ARM, antibiotic resistance marker; DC, double crossover

deaminase mutant CodA(sm), which converts 5-fluorocytosine to highly toxic 5-fluorouracil, as a counterselection approach to select delivery plasmid loss that significantly accelerates the screening process (Zeng et al. 2015). These studies have facilitated rapid progress in the modifications of the *Streptomyces* genome. For example, the CRISPR/Cas strategy using the pCRISPOmyces-2 vector was used to efficiently insert strong promoters into several unknown silent biosynthetic gene clusters to activate their production in five *Streptomyces* species, resulting in the production of unique metabolites (Zhang et al. 2017). As noted above, cloning of large clusters (> 100 kb) is still an obstacle to heterologous expression of antibiotic gene clusters. However, this CRISPR/Cas9 approach in combination with Gibson assembly and TAR cloning allowed cloning of such large gene clusters (Jiang et al. 2015; Wang et al. 2015; Lee et al. 2015). Therefore, the CRISPR/Cas9-based methodology appears to be a very promising tool for discovering novel bioactive secondary metabolites in *Streptomyces* and other *Actinobacteria*. All other applications of these CRISPR/Cas systems in the modification of the *Streptomyces* genome are described in two recent reviews (Tao et al. 2018; Tong et al. 2018).

The results of these studies show that CRISPR-Cas9 is more efficient and faster than other methods for markerless genome editing in *Streptomyces* species. However, it has some bottlenecks. Similarly to previous methods, it also uses *Streptomyces* replicative plasmids to deliver the system to streptomycetes, which may again cause similar replication problems for these plasmids in some strains, especially those that are less studied. Another concern with this CRISPR/Cas9 system is the occurrence of non-specific off-target effects that introduce DSBs in undesired regions of the genome at sites that resemble the 20-nt guide sequence flanking the NGG sequence (PAM). In fact, non-specific loss of huge portions of the genome has been observed in some *Streptomyces* strains after engineering experiments with CRISPR/Cas9. Therefore, for all CRISPR/Cas9 applications involving DBS generation, it is recommended to check the integrity of genomes (Tong et al. 2018).

Conclusion

The bacteria of the genus *Streptomyces* are excellent sources for many bioactive compounds with antibacterial, antifungal, herbicidal, immunosuppressive, antihelminthic, and antitumor properties. In addition, some *Streptomyces* strains, such as *S. lividans*, are perfect hosts for the effective secretion of heterologous biotechnologically and clinically relevant proteins.

These bacteria are therefore of great importance in the field of biotechnology and medicine (Barka et al. 2016; Dyson 2011). It is now clear that, based on genome analysis, their

potential to produce new biologically active metabolites is enormous. However, many of them are not produced under laboratory conditions. Thus, efficient and stable genome manipulations are necessary to induce their production (Baltz 2016; Chen et al. 2016). Comprehensive studies in the past have led to an extensive and versatile collection of molecular biology tools for gene manipulation of *Streptomyces*, mainly based on their replicative plasmids, which require sustained antibiotic selection to maintain them (Kieser et al. 2000). However, biotechnological applications of these bacteria require stable genome alterations/mutation.

Two major strategies in the *Streptomyces* species have been developed to accomplish such a stable genome modification; integration into the chromosome through Att/Int site-specific integration systems based on *Streptomyces* actinophages or the integrative plasmid pSAM2, and integration through HR using suicidal non-replicating vectors. Each strategy has its advantages and limitations. Although Att/Int site-specific integration strategy is much more effective than the HR-based strategy, its biotechnology main concern is the parallel integration of the entire delivery vector, including the replication origin and the antibiotic resistance marker gene. Another limitation is the integration into a single specific *attB* site on the *Streptomyces* chromosome, which may not be present in some *Streptomyces* strains (Baltz 2012; Myronovskiy and Luzhetskyy 2013). However, both disadvantages can be overcome by using other genome editing tools (Li et al. 2017a, 2019; Siegl and Luzhetskyy 2012). Therefore, although not so effective, the HR-based genome editing system is more beneficial for biotechnological applications. Several systems for effective marker-free HR-based genome modification have also been developed (Myronovskiy et al. 2014; Rezuchova et al. 2018). Efficiency issues in HR-based methodology have been dramatically improved by introducing a revolutionary DBS-generating CRISPR/Cas9 methodology in accurate and programmable *Streptomyces* chromosome positions that HR can correct, resulting in different editing events (deletions, insertions). Therefore, this CRISPR/Cas9-based methodology appears to be a very promising tool for various stable biotechnological applications. All the HR-based genome editing systems are summarized in Table 2.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

- Alexander DC, Rock J, He X, Miao V, Brian P, Baltz RH (2010) Development of a genetic system for combinatorial biosynthesis of lipopeptides in *Streptomyces fradiae* and heterologous expression of the A54145 biosynthetic gene cluster. *Appl Environ Microbiol* 76:6877–6887
- Anne J, Maldonado B, van Impe J, Mellart v, Bernaerts K (2012) Recombinant protein production and streptomycetes. *J Biotech* 158:159–167
- Bachmann BO, van Lanen SG, Baltz RH (2014) Microbial genome mining for accelerated natural products discovery: is a renaissance in the making? *J Ind Microbiol Biotechnol* 41:175–184
- Bailey CR, Bruton CJ, Butler MJ, Chater KF, Harris JE, Hopwood DA (1986) properties of *in vitro* recombinant derivatives of pJV1, a multi-copy plasmid from *Streptomyces phaeochromogenes*. *J Gen Microbiol* 132:2071–2078
- Baltz RH (1998) Genetic manipulation of antibiotic-producing *Streptomyces*. *Trends Microbiol* 6:76–83
- Baltz RH (2012) *Streptomyces* temperate bacteriophage integration systems for stable genetic engineering of actinomycetes (and other organisms). *J Ind Microbiol Biotechnol* 39:661–672
- Baltz RH (2016) Genetic manipulation of secondary metabolite biosynthesis for improved production in *Streptomyces* and other actinomycetes. *J Ind Microbiol Biotechnol* 43:343–370
- Barka EA, Vatsa P, Sanchez L, Gaveau-Vaillant N, Jacquard C, Klenk H-P, Clément C, Ouhdouch Y, van Wezel GP (2016) Taxonomy, physiology, and natural products of *Actinobacteria*. *Microbiol Mol Biol Rev* 80:1–43
- Bekiesch P, Basitta P, Apel AK (2016) Challenges in the heterologous production of antibiotics in *Streptomyces*. *Arch Pharm Chem Life Sci* 349:594–601
- Bibb MJ, Freeman RF, Hopwood DA (1977) Physical and genetical characterization of a second sex factor, SCP2, for *Streptomyces coelicolor* A3(2). *Mol Gen Genet* 154:155–166
- Bibb MJ, Ward JM, Hopwood DA (1978) Transformation of plasmid DNA into *Streptomyces* at high frequency. *Nature* 274:398–400
- Bierman M, Logan R, O'Brien K, Seno ET, Rao RN, Schoner BE (1992) Plasmid cloning vectors for conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* 116:43–49
- Bilyk O, Sekurova O, Zotchev SB, Luzhetskyy A (2016) Cloning and heterologous expression of the grecoacycline biosynthetic gene cluster. *PLoS One* 11(7):e0158682
- Boccard F, Smokvina T, Pernodet JL, Friedmann A, Guéroux M (1989) The integrated conjugative plasmid pSAM2 of *Streptomyces ambofaciens* is related to temperate bacteriophages. *EMBO J* 8:973–980
- Busche T, Novakova R, Al'Dilaimi A, Homerova D, Feckova L, Rezechova B, Mingyar E, Csolleiova D, Bekeova C, Winkler A, Sevcikova B, Kalinowski J, Kormanec J, Rückert C (2018) Complete genome sequence of *Streptomyces lavendulae* subsp. *lavendulae* CCM 3239 (formerly “*Streptomyces aureofaciens* CCM 3239”), a producer of the angucycline-type antibiotic auricin. *Genome Announc* 6:e00103–e00118
- Chater KF, Carter AT (1979) A new, broad host-range, temperate bacteriophage (R4) of *Streptomyces* and its interaction with some restriction-modification systems. *J Gen Microbiol* 115:431–442
- Chen J, Wu Q, Hawas UW, Wang H (2016) Genetic regulation and manipulation for natural product discovery. *Appl Microbiol Biotechnol* 100:2953–2965
- Cobb RE, Wang Y, Zhao H (2015) High-efficiency multiplex genome editing of *Streptomyces* species using an engineered CRISPR/Cas system. *ACS Synth Biol* 4:723–728
- Combes P, Till R, Bee S, Smith MCM (2002) The *Streptomyces* genome contains multiple pseudo-*attB* sites for the Φ C31-encoded site-specific recombination system. *J Bacteriol* 184:5746–5752
- Costes A, Lambert SAE (2013) Homologous recombination as a replication fork escort: fork-protection and recovery. *Biomolecules* 3:39–71
- Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97:6640–6645
- Deng Y, Zhang X, Zhang Y (2017) Recent advances in genetic modification systems for Actinobacteria. *Appl Microbiol Biotechnol* 101:2217–2226
- Denis F, Brzezinski R (1992) A versatile shuttle cosmid vector for use in *Escherichia coli* and actinomycetes. *Gene* 111:115–118
- Dubeau MP, Ghinet MG, Jacques PE, Clermont N, Beaulieu C, Brzezinski R (2009) Cytosine deaminase as a negative selection marker for gene disruption and replacement in the genus *Streptomyces* and other Actinobacteria. *Appl Environ Microbiol* 75:1211–1214
- Dyson PJ (2011) *Streptomyces*: molecular biology and biotechnology. Caister Academic Press, Norfolk
- Farkasovska J, Godany A (2012) Analysis of the site-specific integration system of the *Streptomyces aureofaciens* phage μ 1/6. *Curr Microbiol* 64:226–233
- Fayed B, Younger E, Taylor G, Smith MCM (2014) A novel *Streptomyces* spp. integration vector derived from the *S. venezuelae* phage, SV1. *BMC Biotechnol* 14:51
- Fayed B, Ashford DA, Hashem AM, Amin MA, El Gazayerly ON, Gregory MA, Smith MCM (2015) Multiplexed integrating plasmids for engineering of the erythromycin gene cluster for expression in *Streptomyces* spp. and combinatorial biosynthesis. *Appl Environ Microbiol* 81:8402–9413
- Fedoryshyn M, Petzke L, Welle E, Bechthold A, Luzhetskyy A (2008a) Marker removal from actinomycetes genome using F1p recombinase. *Gene* 419:43–47
- Fedoryshyn M, Welle E, Bechthold A, Luzhetskyy A (2008b) Functional expression of the Cre recombinase in actinomycetes. *Appl Microbiol Biotechnol* 78:1065–1070
- Fernandez-Martinez LT, Bibb MJ (2014) Use of the Meganuclease I-SceI of *Saccharomyces cerevisiae* to select for gene deletions in actinomycetes. *Sci Rep* 4:7100
- Fogg PCM, Colloms S, Rosser S, Stark WM, Smith MCM (2014) New applications for phage integrases. *J Mol Biol* 426:2703–2716
- Fogg PCM, Haley JA, Stark WM, Smith MCM (2017) Genome integration and excision by a new *Streptomyces* bacteriophage, Φ Joe. *Appl Environ Microbiol* 83:e02767–e02716
- Gonzalez-Quiñonez N, López-García MT, Yagüe P, Rioseras B, Pisciotta A, Alduina R, Manteca A (2016) New Φ BT1 site-specific integrative vectors with neutral phenotype in *Streptomyces*. *Appl Microbiol Biotechnol* 100:2797–2808
- Gregory MA, Till R, Smith MCM (2003) Integration site for *Streptomyces* phage Φ BT1 and development of site-specific integrating vectors. *J Bacteriol* 185:5320–5323
- Gross H (2007) Strategies to unravel the function of orphan biosynthesis pathways: recent examples and future prospects. *Appl Microbiol Biotechnol* 75:267–277
- Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc Natl Acad Sci U S A* 18:1541–1548
- Hamed MB, Anne J, Karamanou S, Economou A (2018) *Streptomyces* protein secretion and its application in biotechnology. *FEMS Microbiol Lett* 265:fny250
- Hermann S, Siegl T, Luzhetskyy M, Petzke L, Jilg C, Welle E, Erb A, Leadlay PF, Bechthold A, Luzhetskyy A (2012) Site-specific

- recombination strategies for engineering actinomycete genomes. *Appl Environ Microbiol* 78:1804–1812
- Hopwood DA (2007) *Streptomyces* in nature and medicine. The antibiotic makers. Oxford University Press, New York
- Hosted TJ, Baltz RH (1997) Use of *rpsL* for dominance selection and gene replacement in *Streptomyces roseosporus*. *J Bacteriol* 179:180–186
- Hover BM, Kim S-H, Katz M, Charlop-Powers Z, Owen JG, Ternei MA, Maniko J, Estrela A, Molina H, Park S, Perlin DS, Brady SF (2018) Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens. *Nat Microbiol* 3:415–422
- Huang H, Zheng G, Jiang W, Hu H, Lu Y (2015) One-step high-efficiency CRISPR/Cas9-mediated genome editing in *Streptomyces*. *Acta Biochim Biophys Sin* 47:231–243
- Huo L, Hug JJ, Fu C, Bian X, Zhang Y, Muller R (2019) Heterologous expression of bacterial natural product biosynthetic pathways. *Nat Prod Rep*. <https://doi.org/10.1039/c8np00091c>
- Jiang W, Zhao X, Gabrieli T, Lou C, Ebenstein Y, Zhu TF (2015) Cas9-assisted targeting of chromosome segments CATCH enables one-step targeted cloning of large gene clusters. *Nat Commun* 6:8101
- Khaleel T, Younger E, McEwan AR, Varghese AS, Smith MCM (2011) A phage protein that binds Φ C31 integrase to switch its directionality. *Mol Microbiol* 80:1450–1463
- Kieser T, Hopwood DA, Wright HM, Thompson CJ (1982) pIJ101, a multi-copy broad host-range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. *Mol Gen Genet* 186:223–228
- Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical *Streptomyces* genetics. The John Innes Foundation, Norwich
- Kim JH, Feng Z, Bauer JD, Kallifidas D, Cale PY, Brady SF (2010) Cloning large natural product gene clusters from the environment: piecing environmental DNA gene clusters back together with TAR. *Biopolymers* 93:833–844
- Knirschova R, Novakova R, Mingyar E, Bekeova C, Homerova D, Kormanec J (2015) Utilization of a reporter system based on the blue pigment indigoidine biosynthetic gene *bpsA* for detection of promoter activity and deletion of genes in *Streptomyces*. *J Microbiol Methods* 113:1–3
- Kormanec J, Rezuchova B, Farkasovsky M (1993) Optimization of *Streptomyces aureofaciens* transformation and disruption of the *hrdA* gene encoding a homologue of the principal sigma factor. *J Gen Microbiol* 139:2525–2529
- Kuhstoss S, Richardson MA, Rao RN (1989) Site-specific integration in *Streptomyces ambofaciens*: localization of integration functions in *S. ambofaciens* plasmid pSAM2. *J Bacteriol* 171:16–23
- Kuhstoss S, Richardson MA, Rao RN (1991) Plasmid cloning vectors that integrate site-specifically in *Streptomyces* spp. *Gene* 97:143–146
- Lee NC, Larionov V, Kouprina N (2015) Highly efficient CRISPR/Cas9-mediated TAR cloning of genes and chromosomal loci from complex genomes in yeast. *Nucleic Acids Res* 43:e55
- Li P, Li J, Guo Z, Tang W, Han J, Meng X, Hao T, Zhu Y, Zhang L, Chen Y (2015) An efficient blue-white screening based gene inactivation system for *Streptomyces*. *Appl Microbiol Biotechnol* 99:1923–1933
- Li L, Zheng G, Chen J, Ge M, Jiang W, Lu Y (2017a) Multiplexed site-specific genome engineering for overproducing bioactive secondary metabolites in actinomycetes. *Metab Eng* 40:80–92
- Li L, Jiang W, Lu Y (2017b) New strategies and approaches for engineering biosynthetic gene clusters of microbial natural products. *Biotechnol Adv* 35:936–949
- Li L, Wei K, Liu X, Wu Y, Zheng G, Chen S, Jiang W, Lu Y (2019) aMSGE: advanced multiplex site-specific genome engineering with orthogonal modular recombinases in actinomycetes. *Metab Eng* 52:153–167
- Liu H, Jiang H, Haltli B, Kulowski K, Muszynska E, Feng X, Summers M, Young M, Graziani E, Koehn F, Carter GT, He M (2009) Rapid cloning and heterologous expression of the meridamycin biosynthetic gene cluster using a versatile *Escherichia coli-Streptomyces* artificial chromosome vector, pSBAC. *J Nat Prod* 72:389–395
- Liu R, Deng Z, Liu T (2018) *Streptomyces* species: ideal chassis for natural product discovery and overproduction. *Metab Eng* 50:74–84
- Lomovskaya ND, Mkrtumian N, Gostimskaya NL, Danilenko VN (1972) Characterization of temperate actinophage Φ C31 isolated from *Streptomyces coelicolor* A3(2). *J Virol* 9:258–262
- Lomovskaya ND, Chater KF, Mkrtumian N (1980) Genetics and molecular biology of *Streptomyces* bacteriophages. *Microbiol Rev* 44:206–229
- Manderscheid N, Bilyk B, Busche T, Kalinowski J, Paululat T, Bechthold A, Petzke L, Luzhetskyy A (2016) An influence of the copy number of biosynthetic gene clusters on the production level of antibiotics in a heterologous host. *J Biotech* 323:110–117
- Matsuura M, Noguchi T, Yamaguchi D, Aida T, Asayama M, Takahashi H, Shirai M (1996) The *sre* gene (ORF469) encodes a site-specific recombinase responsible for integration of the R4 phage genome. *J Bacteriol* 178:3374–3376
- Mazodier P, Petter R, Thompson C (1989) Intergenic conjugation between *Escherichia coli* and *Streptomyces* species. *J Bacteriol* 171:3583–3585
- Miura T, Hosaka Y, Yan-Zhuo Y, Nishizawa T, Asayama M, Takahashi H, Shirai M (2011) *In vivo* and *in vitro* characterization of site specific recombination of actinophage R4 integrase. *J Gen Appl Microbiol* 57:45–57
- Miura T, Nishizawa A, Nishizawa T, Asayama M, Takahashi H, Shirai M (2014) Construction of a stepwise gene integration system by transient expression of actinophage R4 integrase in cyanobacterium *Synechocystis* sp. PCC 6803. *Mol Gen Genomics* 289:615–623
- Morita K, Yamamoto T, Fusada N, Komatsu M, Ikeda H, Hirano N, Takahashi H (2009) The site-specific recombination system of actinophage TG1. *FEMS Microbiol Lett* 297:234–240
- Muth G, Nussbaumer B, Wohlleben W, Puhler A (1989) A vector system with temperature-sensitive replication for gene disruption and mutational cloning in streptomycetes. *Mol Gen Genet* 219:341–348
- Myronovskiy M, Luzhetskyy A (2013) Genome engineering in actinomycetes using site-specific recombinases. *Appl Microbiol Biotechnol* 97:4701–4712
- Myronovskiy M, Rosenkranz B, Luzhetskyy A (2014) Iterative marker excision system. *Appl Microbiol Biotechnol* 98:4557–4570
- Myronovskiy M, Rosenkranz B, Nadmid S, Pujic P, Normand P, Luzhetskyy A (2018) Generation of a cluster-free *Streptomyces albus* chassis strains for improved heterologous expression of secondary metabolite clusters. *Metab Eng* 49:316–324
- Nah H-J, Pyeon H-R, Kang S-H, Choi S-S, Kim E-S (2017) Cloning and heterologous expression of a large-sized natural product biosynthetic gene cluster in *Streptomyces* species. *Front Microbiol* 8:394
- Novakova R, Rehakova A, Feckova L, Kutas P, Knirschova R, Kormanec J (2011) Genetic manipulation of pathway regulation for overproduction of angucycline-like antibiotic auricin in *Streptomyces aureofaciens* CCM 3239. *Folia Microbiol* 56:276–282
- Novakova R, Núñez LE, Homerova D, Knirschova R, Feckova L, Rezuchova B, Sevcikova B, Menéndez N, Moris F, Cortés J, Kormanec J (2018) Increased heterologous production of the antimicrobial polyketide mithramycin A by Engineered *Streptomyces lividans* TK24 Strains. *Appl Microbiol Biotechnol* 102:857–869
- Okanishi M, Suzuki K, Umezawa H (1974) Formation and reversion of streptomycete protoplasts: cultural conditions and morphological study. *J Gen Microbiol* 80:389–400
- Olivares EC, Hollis RP, Calos MP (2001) Phage R4 integrase mediates site-specific integration in human cells. *Gene* 278:167–176

- Pigac J, Schrempf H (1995) A simple and rapid method of transformation of *Streptomyces rimosus* R6 and other streptomycetes by electroporation. *Appl Environ Microbiol* 61:352–356
- Raynal A, Tuphile K, Gerbaud C, Luther T, Guérineau M, Pernodet JL (1998) Structure of the chromosomal insertion site for pSAM2: functional analysis in *Escherichia coli*. *Mol Microbiol* 28:333–342
- Raynal A, Friedmann A, Tuphile K, Guérineau M, Pernodet JL (2002) Characterisation of the *attP* site of the integrative element pSAM2 from *Streptomyces ambofaciens*. *Microbiology* 148:61–67
- Rebets Y, Kormanec J, Lutzhetsky A, Bernaerts K, Anné J (2017) Cloning and expression of metagenomic DNA in *Streptomyces lividans* and subsequent fermentation for optimized production. In: Streit W, Daniel R (eds) *Metagenomics. Methods and protocols. Methods in Molecular Biology*, vol 1539. Springer, New York, pp 99–144
- Rezuchova B, Homerova D, Sevcikova B, Núñez LE, Novakova R, Feckova L, Skultety L, Cortés J, Kormanec J (2018) An efficient blue-white screening system for markerless deletions and stable integrations in *Streptomyces* chromosomes based on the blue pigment indigoidine biosynthetic gene *bpsA*. *Appl Microbiol Biotechnol* 102:10231–10244
- Rodriguez E, Ward S, Fu H, Revill WP, McDaniel R, Katz L (2004) Engineered biosynthesis of 16-membered macrolides that require methoxymalonyl-ACP precursors in *Streptomyces fradiae*. *Appl Microbiol Biotechnol* 66:85–91
- Sekurova ON, Brautaset T, Sletta H, Borgos SE, Jakobsen MØM, Ellingsen TE, Strøm AR, Valla S, Zotchev SB (2004) *In vivo* analysis of the regulatory genes in the nystatin biosynthetic gene cluster of *Streptomyces noursei* ATCC 11455 reveals their differential control over antibiotic biosynthesis. *J Bacteriol* 186:1345–1354
- Selle K, Barrangou R (2015) Harnessing CRISPR–Cas systems for bacterial genome editing. *Trends Microbiol* 23:225–232
- Sezonov G, Duchêne AM, Friedmann A, Guérineau M, Pernodet JL (1998) Replicase, excisionase, and integrase genes of the *Streptomyces* element pSAM2 constitute an operon positively regulated by the *pra* gene. *J Bacteriol* 180:3056–3061
- Siegl T, Lutzhetsky A (2012) Actinomycetes genome engineering approaches. *Antonie Van Leeuwenhoek* 102:503–516
- Siegl T, Petzke L, Welle E, Lutzhetsky A (2010) I-SceI endonuclease: a new tool for DNA repair studies and genetic manipulations in streptomycetes. *Appl Microbiol Biotechnol* 87:1525–1532
- Smokvina T, Mazodier P, Boccard F, Thompson CJ, Guérineau M (1990) Construction of a series of pSAM2-based integrative vectors for use in actinomycetes. *Gene* 94(1):53–59
- Spasic J, Mandic M, Djokic L, Nikodinovic-Runic J (2018) *Streptomyces* spp. in the biocatalytic toolbox. *Appl Microbiol Biotechnol* 102:3513–3536
- Tala A, Damianoa F, Gallo G, Pinatel E, Calcagnile M, Testini M, Fico D, Rizzo D, Suter A, Renzone G, Scaloni A, De Bellis G, Siculella L, De Benedetto GE, Puglia AM, Peano C, Alifano P (2018) Pirin: a novel redox-sensitive modulator of primary and secondary metabolism in *Streptomyces*. *Metab Eng* 48:254–268
- Tao W, Yang A, Deng Z, Sun Y (2018) CRISPR/Cas9-based editing of *Streptomyces* for discovery, characterization, and production of natural products. *Front Microbiol* 9:1660
- Tocchetti A, Donadio S, Sossio M (2018) Large inserts for big data: artificial chromosomes in the genomic era. *FEMS Microbiol Lett* 365:fny064
- Tong Y, Charusanti P, Zhang L, Weber T, Lee SY (2015) CRISPR/Cas-based engineering of actinomycetal genomes. *ACS Synth Biol* 4:1020–1029
- Tong Y, Weber T, Lee SY (2018) CRISPR/Cas-based genome engineering in natural product discovery. *Nat Prod Rep*. <https://doi.org/10.1039/c8np00089a>
- Van Mellaert L, Mei L, Lammertyn E, Schacht S, Anne J (1998) Site-specific integration of bacteriophage VWB genome into *Streptomyces venezuelae* and construction of a VWB-based integrative vector. *Microbiology* 144:3351–3358
- Van Wezel GP, Bibb MJ (1996) A novel plasmid vector that uses the glucose kinase gene (*glkA*) for the positive selection of stable gene disruptants in *Streptomyces*. *Gene* 182:229–230
- Wang JW, Wang A, Li K, Wang B, Jin S, Reiser M, Lockey RF (2015) CRISPR/Cas9 nuclease cleavage combined with Gibson assembly for seamless cloning. *Biotechniques* 58:161–170
- Wright WD, Shan SS, Heyer WD (2018) Homologous recombination and the repair of DNA double-strand breaks. *J Biol Chem* 293:10524–10535
- Zeng H, Wen S, Xu W, He Z, Zhai G, Liu Y, Deng Z, Sun Y (2015) Highly efficient editing of the actinorhodin polyketide chain length factor gene in *Streptomyces coelicolor* M145 using CRISPR/Cas9-CodA(sm) combined system. *Appl Microbiol Biotechnol* 99:10575–10585
- Zhang L, Ou X, Zhao G, Ding X (2008) Highly efficient *in vitro* site-specific recombination system based on the *Streptomyces* phage Φ BT1 integrase. *J Bacteriol* 190:6392–6397
- Zhang MM, Wong FT, Wang Y, Luo S, Lim YH, Heng E, Yao WL, Cobb RE, Enghiad B, Ang EL, Zhao H (2017) CRISPR–Cas9 strategy for activation of silent *Streptomyces* biosynthetic gene clusters. *Nat Chem Biol* 13:607–609
- Zheng JT, Wang SL, Yang KQ (2007) Engineering a regulatory region of jadomycin gene cluster to improve jadomycin B production in *Streptomyces venezuelae*. *Appl Microbiol Biotechnol* 76:883–888

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