Appl Microbiol Biotechnol (2013) 97:4701–4712
DOI 10.1007/s00253-013-4866-1

MINI-REVIEW

Genome engineering in actinomycetes using site-specific recombinases

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Received: 13 September 2012 / Revised: 18 March 2013 / Accepted: 19 March 2013 / Published online: 16 April 2013

Abstract The rational modification of the actinomycetes genomes has a variety of applications in research, medicine, and biotechnology. The use of site-specific recombinases allows generation of multiple mutations, large DNA deletions, integrations, and inversions and may lead to significant progress in all of these fields. Despite their huge potential, site-specific recombinase-based technologies have primarily been used for simple marker removal from a chromosome. In this review, we summarise the site-specific recombination approaches for genome engineering in various actinomycetes.

Keywords Site-specific recombinases · Actinomycetes · Genome engineering · Heterologous expression

Introduction

Site-specific recombinases (SSRs) catalyse recombination between two specific DNA sequences, resulting in DNA excision, inversion, integration, and translocation (Fig. 1). Because of their efficiency and simplicity, SSRs have become widespread tools for the genomic engineering of many bacteria and higher organisms (Baltz 2012; Branda and Dymecki 2004; Kuhn and Torres 2002; Leibig et al. 2008; Marx and Lidstrom 2002). The classification of SSRs as either tyrosine or serine enzymes depends on the catalytic amino acid that cleaves the DNA molecule. Serine recombinases (e.g. IntphiC31) are usually unidirectional, mediating the integration of a circular DNA molecule into the genome. Serine recombinases recognise non-identical *attP* and *attB* sites (Thorpe and Smith 1998; Gregory et al. 2003; Baltz 2012). The tyrosine family enzymes (e.g. Cre) are usually bidirectional, acting reversibly on a DNA molecule and recognising target sites sharing very limited DNA sequence identity (Rajeev et al. 2009). The positions of the recombination target sites determine the recombination outcome. If two target sites are identically oriented, SSRs perform excision of the DNA between them. SSRs change the directionality of a DNA fragment between two inversely oriented target sites. The integration of a circular DNA fragment into the genome will occur if both molecules contain the corresponding target sites (Fig. 1).

SSR technology has been used in actinomycete genome manipulation for more than 20 years (Bierman et al. 1992; Baltz 2012). However, the utilisation of SSRs in actinomycetes has been primarily limited to the use of the *phi*C31 integration system. Although using *phi*C31-based vectors has allowed tremendous progress in actinomycete genetics, the potential of other SSRs has been underexplored. Recently, several additional SSRs (Cre, Dre, Flp, and Int-VWB) have been used to expand the suite of genome manipulation strategies. Currently, SSR-based genome engineering approaches include the construction of unmarked mutants (Suzuki et al. 2005), targeting of heterologous DNA to the chromosome (Kuhstoss et al. 1991), marker-free expression of foreign genes (Schweizer 2003), deletion of large genomic fragments (Suzuki et al. 2005), and in vivo cloning of genomic DNA regions (Schweizer 2003).

Recombinant DNA integration into the actinomycete chromosome

SSR-mediated integration can be used to insert a foreign DNA molecule into a predetermined location within an actinomycete genome. This approach has been intensively used to heterologously express antibiotic biosynthetic gene clusters

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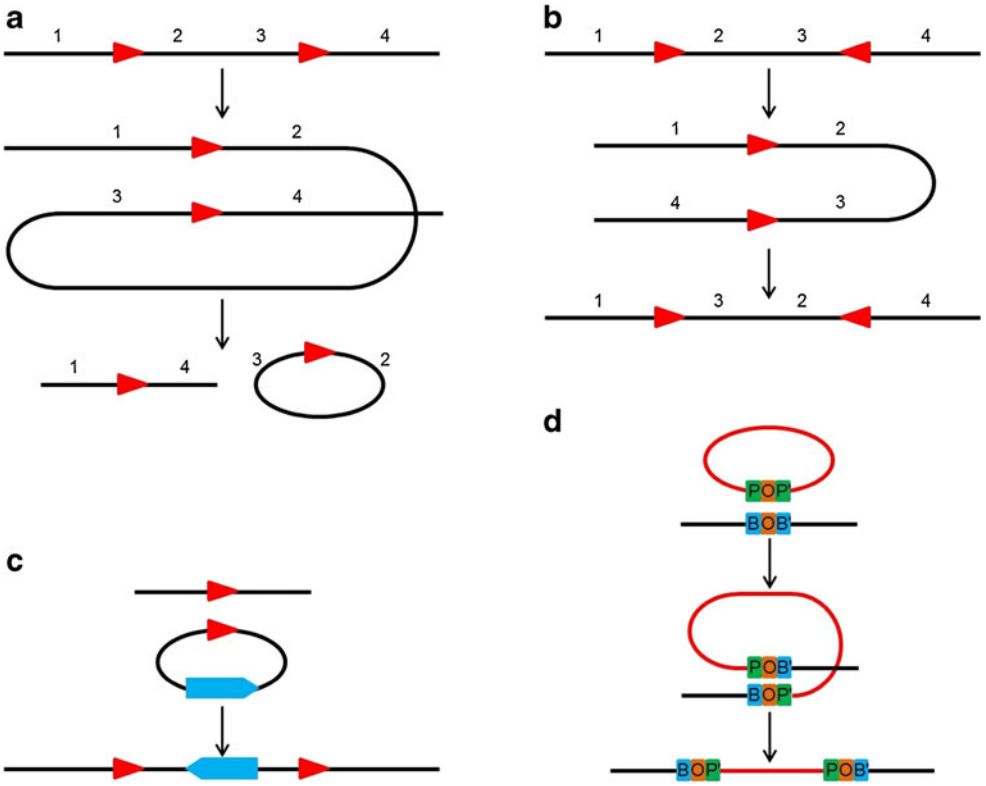

Fig. 1 Reactions catalysed by recombinases. **a** Excision of DNA fragment flanked by two recombination sites, located in the same orientation. **b** Inversion of DNA fragment located between two recombination sites, located in the opposite orientation. **c** Integration of circular DNA molecule into linear. **d** Integration of phage DNA into chromosome by recombination between *attP* and *attB* sites, with subsequent formation of *attL* and *attR* sites

to produce novel molecules. Several integration systems used in actinomycete genetics are reviewed here.

Phage phiC31 The temperate phage *phi*C31 was isolated from *Streptomyces coelicolor* in 1971 (Lomovskaya et al. 1971). The genome of *phi*C31 is approximately 41.5 kb. After infection of the cells, the phage integrates into a conserved gene that encodes a pirin homologue and is located approximately 90 kb upstream of the *S. coelicolor oriC* (Gregory et al. 2003). Less efficient integration in additional pseudo-*attB* sites has also been reported (Combes et al. 2002). The analysis of *attB*- and *attP*-containing regions revealed imperfect inverted repeats around the site of the crossover (Kuhstoss and Rao 1991). The minimal *att* sites required for efficient recombination were found (Table 2). Both sites contain a TT dinucleotide in the centre, where the crossover occurs (Thorpe and Smith 1998). Any nucleotide substitutions in this dinucleotide region completely abolish the recombination efficiency. Recombination between *att* sites is catalysed by the integrase protein, which belongs to a family of serine recombinases (Table 1). Interestingly, *attP* is located close to the 5′-end of the *int-phi*C31 gene, downstream of the *int-phi*C31 promoter. This configuration should lead to the spatial separation of the *int-phi*C31 gene and its promoter during the process of phage integration (Kuhstoss and Rao 1991). After recombination between the *attB* and *attP* sites and integration of the phage, the *attL* and *attR* sites are formed (Fig. 1). It is postulated that the integrase alone cannot recombine the *attR* and *attL* sites or non-permissive pairs of sites, such as *attP*/*attP*, *attB*, and *attL* (Thomason et al. 2001; Thorpe and Smith 1998). Furthermore, the TT dinucleotides in the centre of the *att* sites were shown to be responsible for determining the polarity of the attachment sites during recombination. The substitution of the TT dinucleotide in one of the *att* sites by AA results in the formation of an antiparallel synapse and the formation of L_B–L_P and R_P–R_B sites, where the left side of *attB* is joined with the left side of *attP*, and subsequently, the right side of *attP* is joined with the right side of *attB* (Smith et al. 2004). Several *phi*C31-based integrative vectors were constructed, which turned out to be the most popular in streptomycetes genetics. The pSET152 integrative plasmid vector contains *int-phi*C31 and *attP*, an apramycin-resistance gene *aac(3)IV*, the RK2 origin of conjugal transfer *oriT*, *lacZα* with a multiple cloning site and the pUC origin of replication in *Escherichia coli*. The cosmid vector pOJ436 contains all of the aforementioned elements as well as three *cos* sites for in vitro packaging (Bierman et al. 1992). The construction of a pTO1 plasmid, which is less popular than the two former vectors, was also reported. This plasmid contains a pBR322 origin for replication in *E. coli*, *int-phi*C31 and *attP* fragments from *phi*C31, the RK2 origin of conjugal transfer *oriT*, the ampicillin-resistance gene *bla* for selection in *E. coli* and the thiostrepton-resistance gene *tsr* for selection in *Streptomyces*. Recently, the construction of another *phi*C31-based

integrative vector, pTES, for marker-free expression in actinomycetes was reported. This vector contains the apramycin-resistance gene *aac(3)IV*, the ColE1 origin for replication in *E. coli*, the RK2 origin of conjugal transfer *oriT* and the integrase gene *int-phi*C31. The *phi*C31 *attP* with a multiple cloning site was placed between two *loxP* sites. After the gene of interest was cloned in the vector and the resulting plasmid was integrated into the *phi*C31 *attB* site, the backbone of the plasmid, containing *aac(3)IV*, ColE1 *ori*, *int-phi*C31, and *oriT*, could be removed. This removal is accomplished by the expression of Cre recombinase from an additional vector and subsequent recombination between two *loxP* sites (Herrmann et al. 2012).

*phi*C31-based integrative plasmids have a broad host range, including not only the *Streptomyces* genus but also *Actinomadura*, *Arthrobacter*, *Micromonospora*, *Nocardia*, *Rhodococcus*, *Nonomuraea*, and *Actinoplanes* (Voeykova et al. 1998; Stinchi et al. 2003; Li et al. 2003; Ha et al. 2008; Baltz 2012). Among *Streptomyces*, the successful integration of *phi*C31-based plasmids has been reported for the following strains: *Streptomyces albus*, *Streptomyces antibioticus*, *Streptomyces aureofaciens*, *Streptomyces bambergiensis*, *Streptomyces fradiae*, *Streptomyces griseus*, *Streptomyces hygroscopicus*, *Streptomyces lavendulae*, *Streptomyces lividans*, *Streptomyces purpureus*, *Streptomyces rimosus*, *Streptomyces venezuelae*, *Streptomyces viridochromogenes*, *Streptomyces virginiae*, *S. coelicolor*, *Streptomyces peucetius*, *Streptomyces diastatochromogenes*, *Streptomyces ansochromogenes*, and *Streptomyces ambofaciens* (Voeykova et al. 1998; Eustaquio et al. 2005; Paranthaman and Dharmalingam 2003; Luzhetskyy et al. 2007; Liao et al. 2010; Kim et al. 2008; Baltz 2012). In most cases, the inheritance of the *phi*C31-based integrative plasmids had no effect on morphology or antibiotic production, but some negative effects on antibiotic production were observed (Baltz 1998; Luzhetskiĭ et al. 2001).

*phi*C31-based integrative constructs were used for the heterologous production of the following antibiotics: nikkomycin, moenomycin A, coumermycin A1, chlorobiocin, caprazamycins, novobiocin, aranciamycin, A54145, and daptomycin (Liao et al. 2010; Ostash et al. 2007; Flinspach et al. 2010; Eustaquio et al. 2005; Luzhetskyy et al. 2007; Alexander et al. 2010; Penn et al. 2006). Notably, in the case of novobiocin and chlorobiocin, the heterologous expression of wild-type biosynthetic clusters in *S. coelicolor* led to production levels comparable with those of native producers. After the modification of the novobiocin gene cluster by the introduction of the strong inducible promoter *tcp830* upstream of the cluster, the level of antibiotic production was 3.4-fold higher than that with an unmodified cluster. *S. lividans* derivatives with the same expression constructs were at least five times less productive (Eustaquio et al. 2005; Dangel et al. 2010). In the case of nikkomycin, the introduction of an additional copy of its biosynthetic cluster in its native producer *S. ansochromogenes* led to a fourfold enhancement in the production of nikkomycin X and a 1.8-fold enhancement in the production of nikkomycin Z (Liao et al. 2010). Heterologous expression of the *lpt* gene cluster in *S. ambofaciens* BES2074 resulted in average yields of 385 mg/l of the fully modified A54145 B-core lipopeptides. This yield amounts to 92 % of the *S. fradiae* high-producer control (Alexander et al. 2010).

Phage phiBT1 The *phi*BT1 phage is a homoimmune relative of *phi*C31, and despite the high level of similarity between their genomes, integrase genes *int-phi*BT1 and

Table 1 Classification and features of SSR used in actinomycetes genome engineering

Recombinase	Type		Features and application	Recognition sites
	Tyrosine	Serine		
Int-phiC31		+	Irreversible, vector integration	Non-identical
Int-phiBT1		+	Might be reversible, vector integration, DNA assembly	Non-identical
Sre (Int-R4)		+	Irreversible, vector integration	Non-identical
TG1		+	Irreversible, vector integration	Non-identical
Int-pSAM2	+		Irreversible, vector integration, marker excision	Non-identical
Cre	+		Reversible, DNA integration and excision	Identical
Dre	+		Reversible, DNA excision	Identical
Flp	+		Reversible, DNA excision	Identical
Int-VWB	+		Irreversible, vector integration	Exactly not determined
Int-μ1/6	+		Irreversible, vector integration	Exactly not determined
Int-pMLP1	+		Vector integration	Exactly not determined

*int-phi*C31 share only 26 % identity. Similar to Int-phiC31, Int-phiBT1 integrase belongs to the family of large serine recombinases (Table 1). *phi*BT1 *attP* and *attP* from *phi*C31 share no significant homology. Similar to *phi*C31, the *attB* and *attP* of *phi*BT1 are quite different and contain imperfect inverted repeats (Gregory et al. 2003). The minimal sizes of *phi*BT1 *attB* and *attP* have also been determined. The minimal size of *attB* for in vitro integration is 36 bp, while the minimal size of *attP* is 48 bp (Table 2). Both *att* sites share a GT dinucleotide in the centre (Zhang et al. 2008). In *S. coelicolor*, *phi*BT1 *attB* maps within *sco4848*, which encodes a putative integral membrane protein. This gene lies approximately 1 Mb to the right of *oriC* (Gregory et al. 2003). In the mouse and human genomes, multiple pseudo *attB* and *attP* sites were found, indicating that *phi*BT1-based integrative plasmids can integrate into pseudo *attB* sites in *Streptomyces* chromosomes in the same manner as *phi*C31-based integrative plasmids (Chen and Woo 2005, 2008). In 2010, an article devoted to the identification of 16 pairs of non-compatible *phi*BT1 *attB* and *attP* recombination sites was published (Zhang et al. 2010). As a result of this discovery, a new approach for DNA assembly, called site-specific recombination-based tandem assembly was developed (Zhang et al. 2011). The plasticity of *phi*BT1 integrase can be used for the introduction of additional incompatible *phi*BT1 *attB* sites in various chromosome regions with the subsequent integration of *phi*BT1-based vectors containing corresponding *attP* sites in the desired region of the chromosome or for the creation of multiple insertions.

Interestingly, there is evidence that *phi*BT1 integrase, with its low efficiency, catalyses the in vitro recombination between *attL* and *attR* (Zhang et al. 2008). This result represents the first example of serine recombinase catalysing a reverse reaction because serine recombinases were strongly believed to mediate *attB–attP* recombination only in a unidirectional manner (Thorpe and Smith 1998). This property of *phi*BT1 integrase might result in the instability of integrated plasmids in vivo, though no experimental evidence of such instability was reported (Baltz 2012).

Because the utilisation of *phi*C31-based integrative vectors is restricted in a number of strains owing to a lack of *attB* sites in non-streptomycetes actinomycetes or to detrimental effects on the level of antibiotic production, the *phi*BT1 phage was used for the construction of integrative vectors that should serve as good alternatives to *phi*C31-based vectors (Baltz 1998, 2012; Gregory et al. 2003).

Vector pRT801 was constructed by the substitution of a *phi*C31 *attP*/*int-phi*C31-containing BamHI/SphI fragment of pSET152 with a *phi*BT1 *attP*/*int-phi*BT1 fragment. The constructed vector contains the apramycin-resistance gene *aac*(*3*)*IV*, the origin of conjugal transfer *oriT*, a multiple cloning region, a *phi*BT1 *attP* and the *int-phi*BT1 gene. Additionally, derivatives of pRT801 with a resistance gene for hygromycin instead of apramycin were created by the ligation of a hygromycin-resistance-encoding HpaI fragment from Tn5099-10 with a 4.415 kb Ecl136II fragment of pRT801. The resulting plasmids, which differed only in the orientation of the hygromycin-resistance gene, were named pMS81 and pMS82 (Gregory et al. 2003).

The broad host range of *phi*BT1-based vectors was demonstrated by the introduction of pRT801 into *Streptomyces avermitilis*, *Streptomyces cinnamonensis*, *S. fradiae*, *Streptomyces lincolnensis*, *Streptomyces nogalater*, *Streptomyces roseosporus*, *S. venezuelae*, *Streptomyces ghanaensis*, and other *Streptomyces* strains. The frequencies of exconjugant formations with pRT801 and pSET152 were also comparable. The stability of *phi*BT1-integrative vectors was shown in *S. coelicolor* with pMS81/82 plasmids during two rounds of sporulation under non-selective conditions (Gregory et al. 2003; Ostash et al. 2009).

All of the above information indicates that *phi*BT1-based vectors are as convenient as *phi*C31-based vectors. Nevertheless, *phi*BT1-based integrative vectors have been much less popular in *Streptomyces* genetics than *phi*C31-based vectors. The most interesting examples of the application of the *phi*BT1 integrative system include the expression of biosynthetic clusters of the daptomycin-related compound A54145 in *S. fradiae* and of meridamycin in *S. lividans*. In the case of A54145, a BAC harbouring the entire cluster was modified by the insertion of a cassette containing *oriT*, *aac*(*3*)*IV*, and *phi*BT1 *attP*/*int-phi*BT1. The integration of the biosynthetic cluster at the *phi*BT1 *attB* resulted in a production level greater than or equal to that observed in the case of integration at the *phi*C31 *attB* site. Notably, in both cases of the ectopic expression of A54145, involving integration at the *phi*C31 and *phi*BT1 *attB* sites, the production level was higher than in the parental strain (Alexander et al. 2010). Expression of the meridamycin gene cluster in *S. lividans* was not as successful as in the previous example. After the integration of the BAC containing the meridamycin gene cluster at the *phi*BT1 *attB* site, no production of the compound was detected. The transcription of the meridamycin structural genes occurred at a much lower level than in the natural host. Detectable amounts of meridamycin were produced only after the introduction of a strong *ermE* promoter in front of the structural genes (Liu et al. 2009).

Phage VWB The temperate bacteriophage VWB was isolated in 1984 using *S. venezuelae* as a test strain (Anné et al. 1984). Unlike *phi*C31 and *phi*BT1, integrase of phage VWB belongs to the integrase family of tyrosine recombinases (Table 1) showing homology to the phage lambda integrase. The integration site of the VWB phage is located in a putative $tRNA^{Arg}$ gene (Van Mellaert et al. 1998). Intriguingly, the native $tRNA^{Arg}$ gene contains a CCA terminus,

Table 2 Nucleotide sequences of the SSR target sites

Recombinase	Recombination site	Sequence	Reference
Int-phiC31	*attB*	GGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCG	Groth et al. (2000)
	attP	GTAGTGCCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGGCGTAG	
Int-phiBT1	*attB*	CCAGGTTTTTGACGAAAGTGATCCAGATGATCCAGC	Zhang et al. (2008)
	attP	TGCTGGGTTGTTGTCTCTGGACAGTGATCCATGGGAAACTACTCAGCA	
Int-VWB	*attB*	CGGCCTTCGTAGCTCAGGGGATAGAGCACCGCTCTCCTAAAGCGGGTG TCGCAGGTTCGAATCCTGCCGGGGGCACCAGTTCAG	Van Mellaert et al. (1998)
	attP	GACTCACTGAGGCTCATGATCGCTTTACGTTCTCTCCTAAAGCGGGTGTC GCAGGTTCGAATCCTGCCGGGGGCACAACCTGCA	
Int-pSAM2	*attB*	GCGCGCTTCGTTCGGGACGAAGAGGT	Raynal et al. (1998, 2002)
	attP	GGACGGCATCTTCAAGCGGGGTCCGGCGCGATAGTCACGCAGATAGACAC GCACAGAAAACAGGTGAGGCAGACCGTAACGTTACGGTCTGCCTCACC TGGTGTTTCTCTGTCGGGGTGGCGGGATTTGAACCCACGACCTCTTCGT CCCGAAGCAACTTGGGTGGGCGCTCGACCTTGGGCCAGTGTGCTTCTCA CCTGCGGCGCCAGTCCATGGGTGCTCGCGGTTGTCCGCCGCTGTGCGCT GGCGTTGTCACGCAGTTAGACACTCACCTTCCCA	
Sre (R4)	*attB*	TGGGAGGTGGCGCCCAAGTTGCCCATGACCATGCCGAAGCAGTGGTAGAA GGGCACCGGCAGACACACCCGGTCCTGCT	Miura et al. (2011)
	attP	CCGAGGCATGTTCCCCAAAGCGATACCACTTGAAGCAGTGGTACTGCTTG TGGGTACACTCTGCGGGTGATG	
Int-μ1/6	*attB*	GGCCACCGTGCCTCCTTAGCTCAGCTGGCCAGAACACCGCTCTTGTAAAGC GGGGGTCGTCGGTTCGAACCCGACAGGGGGCTCAGTGGTGGAAGGGCC GGCTCGATGATCGAGCCG	Farkašovská and Godány (2012)
	attP	TCTCTGAGCCTCTCTCAAGCAGCCACTGAATGACAGCTGTTCTTGTAAAG CGGGGGTCGTCGGTTCGAACCCGACAGGGGGCTCAGGCCTTTGACCAGG CAAAAGGGCTCGAACGGA	
Cre	*loxP*	ATAACTTCGTATAGCATACATTATACGAAGTTAT	Branda and Dymecki (2004)
Dre	*rox*	TAACTTTAAATAATTGGCATTATTTAAAGTTA	Sauer and McDermott (2004)
Flp	*frt*	GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC	Branda and Dymecki (2004)
TG1	*attB*	GATCAGCTCCGCGGGCAAGACCTTCTCCTTCACGGGGTGGAAGGTC	Morita et al. (2009a, b)
	attP	CCAGCCCAACAGTGTTAGTCTTTGCTCTTACCCAGTTGGGCGGG	
pMLP1	*attB*	GCATGGTGGCTGTAGCTCAGTTGGCAGAGCACCGGGTTGTGGTCCCGGTTGT CGTGGGTTCAATTCCCATCAGTCACCCGTACACGAAGGCCCCCTCCACTCGG AGGGGGCCTTCGGCGTTCCTGAGGGTTCGCGGTCAGGCGGT	Alexander et al. (2003)
	attP	CGGAGCAACCAGCAGGTCAGGTGGCCTGTTGACCCCCTGACCAGGGCCCCGG TACGGGTTCAATTCCCATCAGTCACCCGGCAAGTGGATCTACTCCACAGCAGA TCAGGCCCCCTCCGAAGAGGGGGCCTGATGCGTCATAGGG	

which is removed after the integration of the phage. Because this CCA terminus is often missing in *Streptomyces* tRNA genes, the question of whether the $tRNA^{Arg}$ gene retains its functionality after the integration of the phage needs to be answered. DNA fragments containing the *attP* site from VWB and the *attB* site from *S. venezuelae* were analysed (Table 2). Alignment of the DNA sequences containing the *attB*, *attP*, *attL*, and *attR* sequences revealed a 45-bp fragment common to all of the sites (Van Mellaert et al. 1998). A VWB *attB* site from *S. ghanaensis* was also revealed. This site was located in the $tRNA^{Arg}$ gene, which differs from that of *S. venezuelae* only in the lack of a CCA terminus. Genome sequence analysis of *S. ghanaensis* did not reveal any other $tRNA^{Arg}$ gene, indicating its essentiality (Ostash et al. 2009).

Using the integrase gene *int*-VWB and *attP* site of the VWB phage, the integrative plasmid pKT02 was constructed. This plasmid also contains the backbone of the *E. coli* vector pIC20R and the thiostrepton-resistance gene *tsr*. The main drawback of this plasmid is the absence of the *oriT* origin of conjugal transfer; thus, this plasmid can only be introduced into *Streptomyces* cells by protoplast transformation. This vector was successfully transformed into *S. lividans* and *S. venezuelae* cells. Notably, the frequency of transformant formation with pKT02 was much lower than with *phi*C31- and pSAM2-based plasmids (Van Mellaert et al. 1998).

Another VWB-based integrative vector, known as pSOK804, was created. This vector was constructed by the ligation of a 2.3 kb SphI/HindIII fragment from pKT02 to a 3.0 kb SphI/HindIII fragment of pSET152; therefore, this vector contains the origin of conjugal transfer *oriT* and an apramycin-resistance gene *aac(3)IV* that is functional in both *E. coli* and *Streptomyces* (Sekurova et al. 2004). The possibility of transferring pSOK804 by conjugation makes this vector more convenient than pKT02.

VWB-based integrative plasmids have been reported to be successfully, but occasionally with low efficiency, introduced into the following strains: *S. natalensis*, *S. fradiae*, *S. sioyaensis*, *S. lividans*, *S. albus*, *S. coelicolor*, and *Actinoplanes teichomyceticus* (Herrmann et al. 2012; Enríquez et al. 2006; Butler et al. 2002; Myronovskyy et al. 2009; Horbal et al. 2012). Challenges have also been encountered in working with pSOK804. Together with different replicative plasmids, the introduction of pSOK804 into *S. verticillus* has not been possible so far, whereas the introduction of *phi*C31- and *phi*BT1-based plasmids has been successful. The failure to introduce the pSOK804 vector or replicative vectors into *S. verticillus* is not surprising because this strain has long been known to be refractory to all means of introducing foreign DNA (Galm et al. 2008).

Recently, another VWB-based integrative vector was constructed, namely, pTOS. This vector was designed for the so-called marker-free expression of genes in actinomycetes. Vector pTOS contains two *rox* sites flanking *attP* and the multiple cloning site. After the gene of interest is cloned in a marker-free expression vector and integrated into the chromosome of the desired strain, the plasmid backbone with the antibiotic-resistance marker can be deleted. This deletion is accomplished by the expression of Dre recombinase from a second replicative vector, which will recombine two *rox* sites and delete the redundant sequence located between them. The utility of this vector was shown in *S. lividans* (Herrmann et al. 2012).

pSAM2 plasmid pSAM2 is an 11 kb integrated element found in *S. ambofaciens* ATCC15154 that can also persist in a form of cccDNA. This element can replicate, is self-transmissible, and mobilises chromosomal markers (Kuhstoss et al. 1989). pSAM2 resembles temperate bacteriophages with its site-specific recombination system involved in integration. The DNA sequence of pSAM2 contains a gene *int* showing homology to the site-specific recombinases of the integrase family (Table 1). The introduction of point or frameshift mutations in this gene completely abolished the integrative function of pSAM2 (Boccard et al. 1989b). The putative excisionase gene *xis* is located upstream of the integrase gene. The stop codon of *xis* overlaps with the start codon of integrase, and together they constitute an operon (Sezonov et al. 1998).

pSAM2 integrates site specifically in a unique site of the *S. lividans* genome, whereas several integration sites have been reported for *S. ambofaciens* (Boccard et al. 1989a). Integration of the plasmid occurs through recombination between the *attB* and *attP* sites. In *S. ambofaciens*, the *attB* site of pSAM2 overlaps with the proline tRNA gene. A comparison of the *attB* and *attP* regions revealed a 58-bp similarity between the two sequences. Southern hybridisation showed that the genomes of *S. lividans*, *Streptomyces bikiniensis*, *Streptomyces glaucescens*, *Streptomyces actuosus*, *S. coelicolor*, *S. antibioticus*, and *Streptomyces parvulus* contain an *att* sequence in a conserved chromosomal region (Boccard et al. 1989a). The minimal fragment of pSAM2 *attB* sufficient for the successful recombination corresponds to the 26-bp long anticodon stem and loop of $tRNA^{Pro}$ (Table 2; Raynal et al. 2002). Analysis of the pSAM2 *attP* organisation revealed two arm-type sites consisting of 17-bp direct repeats that lie between positions −100/−116 and +102/+118 according to the centre of the core sequence at position 0 (Raynal et al. 2002).

The study of the function of the *int* and *xis* gene products revealed that the Int of pSAM2 is the only protein required for the site-specific integration of the plasmid, whereas Int and Xis are both required for its excision (Raynal et al. 1998).

Based on pSAM2, the pPM927 integrative vector was constructed. This vector contains the pBR329 replicon-,

thiostrepton-, and spectinomycin-resistance genes, the origin of conjugal transfer, pSAM2 *int* and *xis* genes and *attP*. In addition, the *fd* transcriptional terminator is followed by the *tipA*-inducible promoter for the regulated expression of cloned genes (Smokvina et al. 1990). The presence of the *xis* gene in pPM927 is redundant because Int alone is sufficient for integration in *E. coli* (Raynal et al. 1998). This requirement is supported by the successful integration of pSAM2-based plasmids containing only *attP* and the *int* gene in *Mycobacterium smegmatis* (Seoane et al. 1997). Utilisation of the *xis-int* operon for the construction of pPM927 raises the question of plasmid stability in *Streptomyces*. Nevertheless, no excision event could be detected (Smokvina et al. 1990).

The comparison of the transformation efficiencies of replicative plasmids and the pSAM2-based vector in *S. ambofaciens* shows at least a 100-fold decrease in the latter (Smokvina et al. 1990). *phi*C31-based integrative vectors also transform with higher efficiencies than pSAM2-based vectors: 60-fold higher with cosmid vectors and up to 600-fold higher for plasmid vectors (Kuhstoss et al. 1991). The successful integration of pSAM2-based plasmids has been reported in the following *Streptomyces* strains: *S. ambofaciens*, *Streptomyces griseofuscus*, *Streptomyces lipmanii*, *S. lividans*, *Streptomyces toyocaensis*, *S. hygroscopicus*, *S. avermitilis*, *S. aureofaciens*, *S. coelicolor*, and *S. fradiae* (Kuhstoss et al. 1991; Ichinose et al. 2001; Rodriguez et al. 2004; Wang et al. 2009; Novakova et al. 2010; Zhao et al. 2010, 2011; Liu et al. 2010). All of the data indicate that pSAM2-based integrative plasmids have broad host specificity and can be used when the utilisation of superior *phi*C31-based vectors is not wanted.

Phage R4 R4 is a broad host *Streptomyces* bacteriophage isolated from soil samples in 1979 (Chater and Carter 1979). The integration and excision functions are performed by the product of the single gene ORF469 (*sre*). R4 integrase belongs to a serine recombinase family (Table 1), showing homology to Tn2501 resolvase and SpoIVCA of *Bacillus subtilis* (Matsuura et al. 1996; Miura et al. 2011). The *attB* and *attP* sites share a 12 bp common core sequence, and the *attP* site is located 27 bp upstream of the integrase translational start codon (Miura et al. 2011). The integrative functions of R4 integrase and its cognate *att* sites were demonstrated using *E. coli*, *S. parvulus*, human cells, and insect cells (Miura et al. 2011; Matsuura et al. 1996; Olivares et al. 2001; Chompoosri et al. 2009). Although the minimal *attB* and *attP* sites determined in *E. coli* assays were 50 and 49 bp (Table 2), respectively, the longer 64 bp *attP* and 295 bp *attB* resulted in enhanced recombination efficiency in human cells (Miura et al. 2011; Olivares et al. 2001). R4 integrase was also shown to successfully perform not only intermolecular but also intramolecular recombination. Unfortunately, despite its well-characterised functionality, the R4 integrative system has not yet found application in *Streptomyces* genetics.

Phage μ1/6 Recently, a new site-specific integrase system based on phage μ1/6 was established. Originally, this phage was discovered in the *S. aureofaciens* genome as a DNA fragment of 38,194 bp (Farkasovská et al. 2007). A bioinformatics analysis of the μ1/6 genome sequence revealed a gene encoding a putative integrase. This basic protein of 416 amino acids belongs to the tyrosine recombinase/integrase family, which also includes lambda integrase, bacteriophage P1 Cre recombinase and bacterial XerD/C recombinases (Table 1). Downstream of the integrase gene, an *attP* site was detected (Table 2). Within its sequence, multiple direct and inverted repeats were observed, which most likely act like binding sites for the integrase and other factors that participate in recombination. The *attB* site (Table 2) for phage μ1/6 is located in the 3′-end of the putative $tRNA^{Thr}$ gene. After phage integration in its cognate *attB* site, the sequence of the tRNA gene was completely restored. Although the sequence alignment of all attachment sites revealed a common 46-bp region, minimal attachment sites were not determined (Farkašovská and Godány 2012).

Based on μ1/6, the integrative vector pCTint was constructed. This vector contains a chloramphenicol marker for selection in *E. coli*, a thiostrepton marker for selection in *Streptomyces*, the *E. coli* origin of replication and the integrase- and *attP*-containing DNA fragment of μ1/6. This vector contains no origin for conjugal transfer; thus, it can be introduced in *Streptomyces* cells only by transformation. Using this vector, thiostrepton-resistant transformants of *S. aureofaciens*, *S. lividans*, and *S. coelicolor* were successfully obtained. The functionality of μ1/6 integrase was also demonstrated in an *E. coli* model, indicating the potential to use this integrational system with a broad host range. However, experiments in vitro revealed that in addition to integrase, some host factors are most likely also required for efficient recombination between the *attB*/*P* sites (Farkašovská and Godány 2012).

Phage pMLP1 Temperate bacteriophage pMLP1 was found in *Micromonospora carbonacea* var. *africana*. This phage can persist in both as a replicative element and an integrated into a chromosome. Detailed analysis of the pMLP1 sequence revealed the DNA region responsible for an integrative function. This region contains an integrase gene *intM*, an excisionase gene *xisM* and *attP*. 322 aa integrase shows similarity to the integrase family of proteins (Table 1). It contains three highly conserved regions (boxes A, B, and C) typical for tyrosine recombinases. *xisM* is located upstream of *intM* and encodes a putative protein similar to excisionases. *attP* is located downstream of *intM*.

Integration of the bacteriophage occurs into the $tRNA^{His}$ gene, which is regenerated at *attL*. Comparison of the *attB* sites of *M. carbonacea* var. *africana* and *M. halophytica* var. *nigra* with *attP* revealed 24 and 25 bp identical segments, respectively (Table 2). Based on pMLP1, two integrative vectors pSPRH840 and pSPRH910 were constructed. Vector pSPRH840 contains backbone of pUC19 with *xisM*, *intM*, and *attP* of pMLP1 as well as *oriT*, apamycin-, and hygromycin-resistance genes. pSPRH910 differs from pSPRH840 only in absence of hygromycin-resistance gene. Successful integration of pSPRH840 was shown for both *M. carbonacea* var. *africana* and *M. halophytica* var. *nigra*. The frequency of exconjugant formation of *M. carbonacea* var. *africana* with the vector pSPRH910 was higher than 1×10^{-3}. Also it was shown that pSPRH840 and pSET152 do not interfere one with another when integrated in the same strain (Alexander et al. 2003).

Phage TG1 Temperate bacteriophage TG1 was isolated from soil samples on *Streptomyces cattleya*. It was reported that TG1 lysogenizes several other *Streptomyces* strains including *S. avermitilis* and *S. coelicolor*. Sequencing of the lysogenized and nonlysogenized strains of *S. avermitilis* led to the identification of the TG1 attachment sites. The *attB* site of TG1 is located within the *dapC* gene encoding *N*-succinyldiaminopimelate aminotransferase. Nevertheless, disruption of *dapC* by integration of the phage does not result in lysine or diaminopimelate auxotrophy. TG1 *attB* site in *S. coelicolor* was found in the gene *sco3658*, also encoding aminotransferase. The *attP* site was deduced from comparative studies of TG1 lysogenized and nonlysogenized strains of *S. avermitilis*. The *attP* site is located upstream of the *int*-TG1 gene encoding integrase. The integrase of TG1 shows high similarity to the integrase of *phi*C31, which belongs to the serine recombinase family (Table 1). Both, *attB* and *attP* sites contain incomplete inverted-repeat sequences with central TT dinucleotide where crossing-over occurs (Morita et al. 2009a). In vitro studies demonstrated that the minimal sequences of *attP* and *attB* required for recombination by the TG1 integrase are 43 and 39 bp, respectively. However, longer *attP* and *attB* sequences of 44 and 46 bp, respectively (Table 2) are required for recombination without loss of efficiency compared to the recombination efficiency of the full-length sites. Experiments in vitro also demonstrated that TG1 integrase does not mediate recombination between *attL* and *attR* (Morita et al. 2009b). Genome database survey with TG1 *attB* from *S. avermitilis* as a query revealed presence of respective homologous sequences in *Streptomyces scabies* 87.22, *Salinispora tropica* CNB-440, *Salinispora arenicola* CNS-205, *S. griseus* subsp and others. Indeed, successful TG1-based integration in *S. scabies* and *S. griseus* was reported (Morita et al. 2012). Construction of the TG1-based integrative vector pKU462 was reported. This vector contains *int* and *attP* of TG1, kanamycin-resistance gene (*aphII*), *oriT*, and pMB1 origin of replication in *E. coli* (Morita et al. 2009a). This vector was successfully used for DNA integration in *S. avermitilis* (Jiang et al. 2009).

Generation of the marker-free mutants

Gene inactivation methods most often retain a selectable marker within the genome. Because the number of available resistance markers for actinomycete genome manipulation is limited, their reutilisation within the same genome is highly desirable. In addition, resistance markers integrated into a chromosome may result in polar effects on the expression of genes located upstream and downstream. The expression of selectable markers may also influence bacterial fitness. Therefore, the elimination of the resistance marker after genome manipulation is very important. SSR was among the first methods to allow elimination of the resistance marker after mutant selection in a range of organisms (Branda and Dymecki 2004; Schweizer 2003; Fedoryshyn et al. 2008a, b; Zelyas et al. 2009). The SSR-mediated excision of the antibiotic-resistance cassette leaves a ‘scar’ in the chromosome and generates a single target site (Fig. 1). The length of this scar is usually less than 50 bp and does not exhibit polar transcriptional effects. Marker rescue and reuse offers a simple and efficient way to introduce multiple gene deletions. However, consecutive deletions of additional genes in the same genetic background may be limited because of undesired DNA rearrangements generated by the multiple target sites left after each excision. Therefore, different recombinases should be used for the generation of multiple gene deletions.

Cre recombinase The 38 kDa Cre (cyclisation recombination) originates from bacteriophage P1 and belongs to a tyrosine recombinase family (Table 1). Cre catalyses a DNA recombination between two 34-bp recognition sites named *loxP* (locus of crossing over [X] of the P1 phage). The *loxP* sequence consists of two 13-bp inverted repeats linked together by an 8-bp internal region (Table 2). The reaction performed by Cre on the *loxP* sites is very efficient and does not require any accessory proteins or high-energy cofactors, thus making it suitable for use in many different organisms. In 2006, the successful expression of Cre recombinase from the phage vector in *S. coelicolor* A(3)2 was reported (Khodakaramian et al. 2006). The frequency of resistance marker excision was from 60 to 90 %. We have reported the successful expression of the synthetic gene encoding the Cre recombinase expressed from different plasmid vectors in several members of the *Streptomyces*, *Micromonospora*, *Kitasatospora*, and *Saccharothrix* genera (Herrmann et al. 2012; Fedoryshyn et al. 2008b). The frequency of resistance marker excision by Cre expressed from

pALCRE, pNLCRE, pUWLHCRE, and pUWLCRE has been nearly 100 % in all strains we have tested. To minimise genetic instability, different heterotypic *lox* sites containing mutations within the inverted repeats (*loxLE* and *loxRE*) have been used for plants, chicken cell lines, and bacteria (Branda and Dymecki 2004; Leibig et al. 2008). The recombination of *loxLE* and *loxRE* results in a double-mutant *loxLERE* site, which is a poor substrate for Cre. We could show, however, that Cre recognises the double mutated site at very high frequency in actinomycetes. Thus, the risk of undesired chromosomal rearrangements is still very high if Cre is iteratively used in combination with *loxLE* and *loxRE* (Herrmann et al. 2012).

Flp recombinase Flp recombinase is another tyrosine recombinases (Table 1) isolated from *Saccharomyces cerevisiae*. The Flp recognition site (*frt*) consists of two 13-bp palindromic sequences separated by an 8-bp spacer region (Table 2). Flp has been used to perform gene deletions, insertions, integrations, and inversions in various organisms (Schweizer 2003). The functional expression of the synthetic gene *flp*(*a*), encoding Flp recombinase, has been reported in several actinomycetes, including *S. coelicolor M145*, *S. lividans TK24*, and *Saccharothrix espanaensis*, but not in *Micromonospora Tü6368* (Fedoryshyn et al. 2008a; Herrmann et al. 2012). However, the frequency of *frt*-flanked apramycin-resistance gene excision from the chromosome of actinomycetes is lower than those of Cre, ranging from 10 to 40 %. The native *flp* gene has also been expressed, showing an even lower marker excision frequency of approximately 2 % (Zelyas et al. 2009). However, even at this frequency, marker-free mutants can be obtained relatively easily.

Dre recombinase Recently, we reported the very efficient expression of Dre recombinase in actinomycetes. Similarly to Cre, Dre is a tyrosine-type recombinase (Table 1) recognising a target site called *rox* (Table 2). All three recombinases (Flp, Cre, and Dre) are heterospecific: Cre does not recognise *rox* or *frt*, Flp does not recognise *loxP* or *rox*, and Dre does not recognise *loxP* or *frt*. This characteristic makes Dre an ideal partner for Flp and Cre in performing multiple mutations in a single genetic background. The frequency of the marker removal from chromosomes of different actinomycetes was nearly 100 %. Thus, Dre recombinase is comparable with Cre in its high efficiency. Similar to Cre, Dre recombinase was successfully expressed in the *Saccharothrix*, *Streptomyces*, and *Micromonospora* genera (Herrmann et al. 2012).

int and xis system of pSAM2 In Prof. Pernodet's group, the *xis* and *int* genes from the pSAM2 plasmid have been used to delete antibiotic-resistance markers from the chromosomes of streptomycetes. Several cassettes that contain antibiotic-resistance genes inserted between the *attL* and *attR* sites (*attL* antibiotic-resistance *attR*) and several vectors expressing the Int and Xis proteins from pSAM2 (Raynal et al. 2006) have been generated. The successful functional expression of four different recombinases (Cre, Flp. Dre, and Xis/Int) in actinomycetes enables the construction of marker-free multi-mutant strains. At least four consecutive resistance marker deletions can be generated in actinomycetes in a single genomic background.

Generation of large-scale deletions

The deletion of large genomic DNA fragments is becoming an increasingly important way to generate bacteria with minimized genomes and improved characteristics (e.g. small molecule overproduction; Jewett and Forster 2010). In addition, the ability to delete and reintegrate large DNA fragments within a genome facilitates gene functional studies. Large genome rearrangements in many organisms have been accomplished using the Cre/*loxP* system (Branda and Dymecki 2004; Komatsu et al. 2010). Recently, Cre-mediated large fragment excisions were utilised to construct improved *S. avermitilis* host strains for the heterologous production of natural products. Cre recombinase was able to excise a fragment of greater than 1.5 Mb in one step. The obtained mutant was used for the improved production of the natural products streptomycin, cephamycin C, and pladienolide after the heterologous expression of three corresponding biosynthetic gene clusters (Komatsu et al. 2010).

Another strategy for the generation of biosynthetic gene cluster deletions based on the Cre/*loxP* system was recently reported (Herrmann et al. 2012). Two *loxP* sites should be introduced at both ends of a gene cluster as direct repeats for the subsequent recombination. The usefulness of this method has been demonstrated recently by generating three deletions spanning the α-lipomycin, phenalinolactone, and monensin biosynthetic gene clusters in *S. aureofaciens* Tü117, *S.* sp. Tü6071, and *S. cinnamonensis* A519, respectively. The successful complementation of phenalinolactone production by the introduction of the corresponding gene cluster allowed the modification of a cosmid directly in *E. coli* via recombineering techniques and subsequent fast-gene deletion in situ using mutated cosmids (Herrmann et al. 2012).

In summary, a variety of different genome-engineering strategies, including the construction of unmarked multiple mutations, marker-free expression of target genes, large-fragment deletions, and chromosomal integration of biosynthetic gene clusters, have been performed in actinomycetes using the site-specific recombinases Int-phiC31, Int-phiBT1, Int-VWB, Cre, Dre, Flp, and Int/Xis of pSAM2.

These techniques have a broad application in actinomycete genetics and will further facilitate functional studies of a large number of genes from the increasing set of sequenced genomes.

Acknowledgments The work in the laboratory of AL was supported by the BMBF (GenBioCom), DFG (Lu1524/2-1) and ERC (EXPLOGEN) grants.

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