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REVIEW

Streptomyces temperate bacteriophage integration systems for stable genetic engineering of actinomycetes (and other organisms)

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Abstract ϕC31, ϕBT1, R4, and TG1 are temperate bacteriophages with broad host specificity for species of the genus *Streptomyces*. They form lysogens by integrating site-specifically into diverse *attB* sites located within individual structural genes that map to the conserved core region of streptomycete linear chromosomes. The target genes containing the ϕC31, ϕBT1, R4, and TG1 *attB* sites encode a pirin-like protein, an integral membrane protein, an acyl-CoA synthetase, and an aminotransferase, respectively. These genes are highly conserved within the genus *Streptomyces*, and somewhat conserved within other actinomycetes. In each case, integration is mediated by a large serine recombinase that catalyzes unidirectional recombination between the bacteriophage *attP* and chromosomal *attB* sites. The unidirectional nature of the integration mechanism has been exploited in genetic engineering to produce stable recombinants of streptomycetes, other actinomycetes, eucaryotes, and archaea. The ϕC31 attachment/integration (Att/Int) system has been the most widely used, and it has been coupled with the ϕBT1 Att/Int system to facilitate combinatorial biosynthesis of novel lipopeptide antibiotics in *Streptomyces fradiae*.

Keywords Actinomycete · Genetic engineering · ϕBT1 · ϕC31 · Phage R4 · Phage TG1 · Site-specific integration · *Streptomyces*

This review is dedicated to the memory of Dr Eugene T (Gene) Seno who passed away on June 19, 2011. Among the many contributions that Gene made at the John Innes Institute and at Eli Lilly and Company, he developed the plasmid vector pSET152 [14] that utilizes the ϕC31 integration system discussed in this review. pSET152 has been used successfully in many laboratories around the world to engineer streptomycetes and other actinomycetes, and I will think of Gene and his contributions whenever I see new citations to pSET152.

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Introduction

Streptomyces species are best known for their propensity to produce secondary metabolites for use as antibiotics, antitumor agents, immunomodulators, anthelmintic agents, and insect control agents. More recently they have become an important source of genetic tools applicable to a variety of biological systems. This stems from fundamental work on actinomycete bacteriophages (actinophages), particularly on ϕC31, a temperate phage for *Streptomyces* species. Among the temperate actinophages, there are two distinct mechanisms for integration: the coliphage λ-like tyrosine recombinases that integrate into tRNA genes [1, 28, 99]; and the serine recombinases (used by ϕC31 and others discussed here) that integrate into diverse, unrelated structural genes. ϕC31 was first developed as a means to insert cloned DNA into streptomycete genomes, but the unique nature of the Att/Int system rendered it desirable for universal use in diverse cellular systems, including eukaryotes and archaea. The universal utility derives from the unidirectionality [90, 96], and subsequent stability, imparted by the serine recombinase mechanism. ϕC31 is one of several streptomycete temperate phages that have integration mechanisms catalyzed by large serine recombinases. In all cases, the pairs of *attP* and *attB* sites share little sequence identities, and the integration and excision mechanisms differ from those of temperate actinophages that utilize

tyrosine recombinases. In the present review, I describe the discovery and development of ϕC31 and other streptomycete temperate phages that utilize large serine recombinases, and discuss their applications for stable cloning and expression of genes in streptomycetes and other actinomycetes. The integration systems employing ϕC31, R4, and other serine recombinases have also been used to engineer human cells (e.g., see [32, 45, 56, 60, 80, 104]), and ϕC31 Int has been applied broadly in other eucaryotes, including lower mammals, *Drosophila melanogaster*, *Xenopus laevis*, zebrafish, Asian tiger mosquito, *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Schizosaccharomyces pombe* (e.g., see [4, 17, 25, 39, 41, 53, 57, 63, 64, 74, 83, 94, 95, 102, 105]), and in the methanogenic archaean *Methanosarcina acetivorans* [16, 34], but the details of these studies are beyond the scope of this review. Also, readers are referred to excellent reviews on the molecular mechanisms of integration catalyzed by tyrosine and serine recombinases which are not reviewed here [18, 33, 89, 90].

Temperate bacteriophages that utilize large serine recombinases

At least four temperate bacteriophages that utilize large serine recombinases have been isolated on different *Streptomyces* species. The best studied phages are ϕC31, R4, TG1, and ϕBT1 (see below). Temperate phages utilizing large serine recombinases have been described from other Gram-positive microorganisms, including *Mycobacterium* and *Lactococcus* species [29, 33]. A hallmark of these bacteriophages is that the large serine recombinases require no additional phage or host functions for site-specific integration, and that integration is unidirectional in the absence of additional factors [33, 89]. Recombination between *attP* and *attB* sites generates hybrid *attL* and *attR* sites which are generally not substrates for excision by Int alone. The excision process has been studied in detail with the mycobacteriophage Bxb1 [29]. In this case excision requires a phage-encoded protein called recombination directionality factor (RDF). Although there is no homolog of the mycobacteriophage RDF in the ϕC31 genome, an RDF has recently been characterized that binds to ϕC31 Int to change its specificity from insertion to excision [46]. A key feature of the *Streptomyces* phage integration systems is that each has a unique *attB* site, and the individual *attB* sites are located in unrelated genes (Table 1).

Bacteriophage ϕC31

ϕC31 is a temperate bacteriophage originally described by Lomovskaya and colleagues [61, 62] that displays broad host specificity for *Streptomyces* species (but not for other actinomycetes) [47, 100]. As with many other streptomycete bacteriophages, the ϕC31 host range within streptomycetes is limited primarily by type II restriction endonuclease barriers [24, 36, 37, 100]. The biology of ϕC31 and its interaction with streptomycete hosts has been widely studied, but most of these studies are beyond the scope of this review.

Table 1 Genes that contain primary bacteriophage *attB* sites and their gene products in *S. roseosporus*

Phage	Target gene size (nt)	Gene product	Annotation
ϕC31	948	ZP_04709706	Pirin-like protein
ϕBT1	246	ZP_06586584	Integral membrane protein
R4	1,644	ZP_04712391	Acyl-CoA synthetase
TG1	1,209	ZP_04710111	Aminotransferase

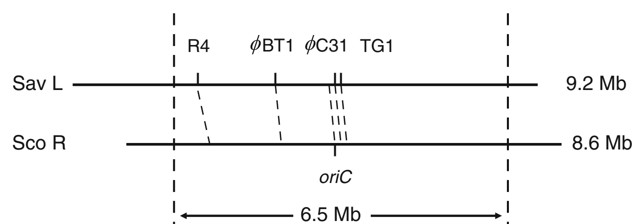

Fig. 1 Genetic map locations of the *attB* sites for bacteriophages R4, ϕBT1, ϕC31, and TG1 in *S. avermitilis* (*Sav*) and *S. coelicolor* (*Sco*) relative to *oriC*. All four sites reside in the 6.5-Mb core regions that contain genes highly conserved across *Streptomyces* sp. [13, 21, 42, 49]

ϕC31 integrates via a large serine recombinase into an *attB* site located in a pirin-like gene (Table 1) located about 85 and 92 kb to the right of the *oriC* in *S. avermitilis* and *S. coelicolor*, respectively (Fig. 1). This lies in the center of an approximately 6.5-Mb region of the linear chromosomes that contain mainly highly conserved genes dedicated to primary metabolism, stress responses, macromolecule biosynthesis, and developmental biology including sporulation [13, 21, 42, 49]. (The *S. coelicolor* orientation [13] has been reversed to line up with the *S. avermitilis* genome in Fig. 1). The mechanism of integration of ϕC31 into streptomycete chromosomes has been characterized [51, 52, 59, 69, 70, 85, 86, 96] and reviewed [18, 33, 89, 90], and the ϕC31 genome sequence is known [88]. The minimal *attP* and *attB* sites comprise 39 and 34 bp, respectively [32], and they share a 3-bp common sequence at the site of conservative crossing-over [51].

BLASTP analysis with ϕC31 Int was carried out in October 2011, and five full-length hits were obtained (Table 2). Two of the hits were to actinophages TG1 and ϕBT1 Int proteins, and three were to proteins encoded by *Kitasatospora setae*, *Streptomyces violaceusniger*, and

Table 2 Homology relationships between streptomycete bacteriophage large serine recombinases or integrases (Int) determined by BLASTP

BLASTP subject (amino acids)	Amino acid identities (%) with BLASTP query		
	ϕC31 Int	TG1 Int	ϕBT1 Int
ϕC31 CAA07153 (605)	605/605 (100)	305/614 (49.7)	157/614 (25.6)
TG1 BAF03600 (619)	305/621 (49.1)	619/619 (100)	164/639 (25.7)
ϕBT1 CAD80152 (594)	162/629 (25.8)	160/642 (24.9)	594/594 (100)
K. setae BAJ29918 (571)	312/587 (53.2)	282/610 (35.8)	166/612 (27.1)
S. violaceusniger ZP_07610497 (595)	221/601 (36.8)	222/620 (35.8)	169/631 (26.8)
S. zinciresistens ZP_08805684 (574)	262/579 (45.3)	263/588 (44.7)	151/631 (25.6)

Streptomyces zinciresistens. The Int homolog encoded by *K. setae* shows highest sequence similarity to ϕC31 Int (53.2%), and its gene maps to a region central to the linear chromosome (about 4.59 Mb into the 8.78-Mb genome) located just downstream of a truncated pirin-like gene. Just downstream of the *int* homolog is a gene that encodes a potential Xis function that shows 37.7% amino acid identity to the gp3 protein encoded by ϕC31 ([46]; see below). Further downstream of these genes is a large portion of the pirin-like gene missing in the truncated gene upstream of *int* that may have been generated by an integration event. Adjacent to the downstream truncated pirin-like gene is a complete pirin homolog which may contain the target for pSET152 integration [20]. BLASTP analysis with ϕC31 gp11, a DNA polymerase that has homologs encoded by the streptomycete phages ϕBT1 [30] and phiSASD1 [101], and by many mycobacterial phages, revealed no homolog in *K. setae*. BLASTP analysis of *S. violaceusniger* and *S. zinciresistens* also revealed no homologs to ϕC31 gp3 or gp11. In summary, there is no evidence for complete prophage insertions in the vicinity of the *int* homologs in *K. setae*, *S. violaceusniger*, or *S. zinciresistens*.

Recent studies have characterized protein gp3 encoded by ϕC31 as the RDF or Xis protein required for excision of integrated ϕC31. Protein gp3 binds directly to Int in 1:1 stoichiometry and changes the recombinational specificity from *attP* and *attB* to *attL* and *attR* [46]. The gp3–Int complex also catalyzes recombination between two *attL* or two *attR* sites. These findings should further extend the utility of the ϕC31 integration (and now excision) system for genetic engineering applications [91].

A number of cloning vectors employing ϕC31 have been developed [47], and those employing only the *att/int* functions coupled with *oriT* from RP4 for conjugation from *E. coli* were developed by Bierman et al. [14]. Notably, pSET152, which lacks replication functions for streptomycetes, has gained wide acceptance as an insertion vector to generate stable recombinants. More recently, bacterial artificial chromosome (BAC) vectors containing ϕC31 *att/int* and *oriT* functions have been used to stably insert large secondary metabolite gene clusters into the chromosomes of heterologous hosts [2, 8, 9, 71, 82]. pSET152 and other ϕC31-based conjugal insertion vectors have utility in many streptomycetes and other actinomycetes. The frequencies of transconjugant formation range from 1.6×10^{-4} to 1.4×10^{-2} in many *Streptomyces* species (Table 3). In some cases where the recipient host restricts modified DNA, conjugation requires the use of an *E. coli* host defective in Dam/Dcm methylation. The generally high transconjugant frequencies in streptomycetes can be attributed to three factors: (1) conjugation bypasses type II restriction enzyme barriers [8, 14, 67]; (2) the ϕC31 *attB* site is located in a gene encoding a pirin-like protein that is widely distributed within *Streptomyces* sp. [23] (Tables 4, 5); and (3) ϕC31 integration is generally very efficient. In addition to the primary *attB* site, *Streptomyces* sp. can have pseudo-*attB* sites for ϕC31 integration. The frequency of transconjugant formation in *S. coelicolor* dropped from 1.5×10^{-3} to 5×10^{-6} when the primary *attB* site was deleted (Table 3), and the insertions mapped to three pseudo-*attB* sites that showed some sequence homology to authentic *attB* sites [23].

Some other actinomycetes are recipients for transconjugation, protoplast transformation, or electroporation with pSET152 or other ϕC31-based integration vectors (Table 3). In some cases transconjugant frequencies in non-streptomycetes were high (e.g., in *Actinoplanes teichomyceticus* and *Nonomuraea* sp. 40027), but in other cases they were very low. For instance, in *Saccharopolyspora spinosa*, which lacks a pirin-like gene, transconjugants were obtained at a frequency of 10^{-7}, and integrations occurred in two pseudo-*attB* sites [67]. In *Saccharopolyspora erythraea*, which also lacks a pirin-like gene [81] and is normally a poor recipient for conjugation, insertion of a portable streptomycete *attB* site converted it into a high frequency recipient for the integration of transgenes [84]. In *Mycobacterium smegmatis*, *Mycobacterium bovis*, and *Mycobacterium tuberculosis*, low frequencies of recombinants were obtained by electroporation with pIJ8600 [77]. *Mycobacterium smegmatis* MC^2-155 has a pirin-like gene,

Table 3 Actinomycete host range of ϕC31-based vectors

Strain	Vector	Insertion site	Transconjugant frequency[a] ($\times10^{-5}$)	References
Amycolatopsis japonicum	pSET152	*attB?*	2.4	[92]
A. japonicum	pSET152	–	<0.01	[15]
A. teichomyceticus	pSET152	*attB*	610	[35]
Arthrobacter aurescens	pTOL1	ND[b]	1	[100]
Kitasatospora setae	pSET152	*attB*	0.1	[20]
Micromonospora aurantiaca	pTOL1	ND	1	[100]
M. griseorubida	pSET152	–	NO[b]	[98]
M. griseorubida	pSET152	*strep-attB*	NR[b]	[98]
M. rosaria	pSET152	pseudo-*attB?*	NR (low)	[5]
Micromonospora sp. 40027	pSET152	pseudo-*attB?*	NR	[54]
Mycobacterium smegmatis	pIJ8600	pseudo-*attB?*	Low[c]	[77]
Nonomuraea sp. ATCC 39727	pSET152	*attB*	~100	[65, 93]
Pseudonocardia autotrophica	pSET152	*attB?*	NR	[43]
Rhodococcus equi	pSET152	*attB*	High[d]	[40]
S. albus G	pTO1	*attB*	50	[100]
S. ambofaciens	pSET152	*attB*	1,400	[48]
S. antibioticus ATTC 23879	pTO1	*attB*	300	[100]
S. aureofaciens VKPM AC 755	pTO1	*attB*	50	[100]
S. avermitilis	pSET152	*attB*	NR[b,e]	[49, 55]
S. bambergiensis ATCC 13879	pTO1	*attB*	1	[100]
S. clavuligerus	pSET152	*attB*	NR	[97]
S. coelicolor	pSET152	*attB*	210	[15]
S. coelicolor	pSET152	*attB*	150	[23]
S. coelicolor (ΔattB)	pSET152	pseudo-*attB*	0.5	[23]
S. diastatochromogenes	pSET152	*attB*	16	[15]
S. fradiae (tyl)	pSET152	*attB*	10,000	[14]
S. fradiae (A54145)	pStreptoBAC V	*attB*	NR (high)	[2]
S. griseus Kr.15	pTO1	*attB*	20	[100]
S. hygroscopicus ATCC 21705	pTO1	*attB*	100	[100]
S. hygroscopicus ATCC 10976	pTO1	*attB*	20	[100]
S. lividans	pSET152	*attB*	530	[15]
S. lividans TK64	pTO1	*attB*	100	[100]
S. pristinaespiralis	pSET152	*attB*	136	[44]
S. purpureus ATCC 21405	pTO1	*attB*	10	[100]
S. rimosus ATCC 23955	pTO1	*attB*	10	[100]
S. roseosporus	pStreptoBAC V	*attB*	NR (high)	[22]
S. toyocaensis	pOJ436	*attB*	20	[66]
S. venezuelae ATCC 10595	pTO1	*attB*	20	[100]
S. virginiae ATCC 13161	pTO1	*attB*	200	[100]
S. viridochromogenes Tu 494	pTO1	*attB*	30	[100]
Sacc. erythraea	pSET152	pseudo-*attB*	NR (low)	[84]
Sacc. erythraea	pSET152	*strep-attB*	NR (high)	[84]
Sacc. spinosa	pOJ436	pseudo-*attB*	0.01	[67]

[a] Transconjugants per recipient cell unless reported otherwise

[b] *ND* not determined, *NO* none observed, *NR* not reported

[c] Low frequency of recombinants obtained by electroporation

[d] 8.5×10^4 CFU/μg DNA by electroporation

[e] Plasmid introduced by protoplast transformation

but the recombinant analyzed by Murry et al. [77] localized the insertion in a pseudo-*attB* site. *Mycobacterium tuberculosis* and *M. bovis* do not have pirin-like genes, and insertions were in pseudo-*attB* sites.

BLASTP and BLASTN surveys of ten streptomycetes identified pirin-like genes in each case. In typical streptomycete orthologs, the ratio of the number of mutations causing non-synonymous amino acid substitutions (d*N*) to

Table 4 Homologs in streptomycetes and other actinomycetes to genes containing bacteriophage *attB* sites and *glnA* from *S. roseosporus*

Strain	Homolog to target gene (% nt identity)[a]				
	C31	BT1	R4	TG1	GlnA
Streptomyces albus	85.4	84.0	80.9	77.3	91.3
S. avermitilis	84.4	89.0	81.0	78.5	91.4
S. clavuligerus	85.4	86.1	83.2[b]	76.9	92.1
S. coelicolor	84.7	86.2	80.3	79.8	89.1
S. flavogriseus	88.4	83.5	85.7	76.8	93.9
S. ghanaensis	85.3	85.1	82.3	79.2	90.6
S. griseoflavus	85.2	81.9	82.1	78.3	90.6
S. griseus	95.7	99.2	92.0	91.0	98.1
S. viridochromogenes	85.0	88.8	81.2	78.4	90.7
S. sp. SPB78	83.6	83.5	79.7	79.9	92.2
Streptomyces ave	86.3	86.7	82.8	79.6	92.0
Amycolatopsis mediterranei	–	72.1	78.5	70.5	79.4
Catenulispora acidiphila	72.6	65.4	73.7	75.4	82.1
Frankia sp. EAN1pec	70.0	–	64.4[b]	65.5	77.2
Micromonospora aurantiaca	78.1	–	63.4[b]	74.6	80.3
Mycobacterium smegmatis	71.7	–	71.9	70.9	78.1
Rhodococcus erythropolis	69.8	–	69.7	67.3	77.3
Saccharomonospora viridis	–	71.0	73.7	64.9	75.5
Saccharopolyspora erythraea	–	62.3	76.4	68.6	80.8
Salinispora arenicola	–	–	–	71.7	78.8
Streptosporangium roseum	74.8	–	77.2	69.6	81.3
Non-streptomycete ave	72.8	67.7	74.4[c]	69.9	79.1

[a] Nucleotide searches were carried out using search parameters adjusted to a word size of 16, match/mismatch scores of 2, −3, and gap costs of 2 for existence and 2 for extension, using the BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

[b] BLASTN aligned only 67–75% of these nt sequences, whereas BLASTP (Table 5) aligned 95–100% of the aa sequences

[c] *Frankia* sp. EAN1pec and *M. aurantiaca* were excluded in the calculation because of the lack of full-length sequence in the BLASTN analysis

the number causing synonymous amino acid substitutions (d*S*) is about 0.4–0.9 ([10]; this report). The d*N*/d*S* ratios for paralogs tend to be about 1.0 or higher. Because of the high G+C content of streptomycete genes, d*N*/d*S* ratios for orthologs translate into a situation where the percent change in amino acid identities diverges at nearly the same rate as the percent change in nucleotide identities. Inspection of the amino acid and nucleotide identities for the pirin-like homologues in the ten streptomycetes indicates that both are drifting at about the same rates; the d*N*/d*S* ratio calculated for the average of all ten was 0.4. By comparison, the d*N*/d*S* ratio calculated for the average of ten *glnA* genes (Tables 4, 5) was also 0.4. Thus it appears that the pirin-like genes are orthologs. Inspection of several pirin-like gene sequences indicated that the actual 45-nucleotide *attB* sequence is present in all cases, and that it is generally even more conserved than the overall gene sequence. For instance, the *S. avermitilis attB* shared 93.3% nucleotide identities with the *S. roseosporus attB*, whereas the complete genes showed 84.4% nucleotide identities (Table 4). Likewise, the *S. griseus attB* showed 100% nucleotide identities to *S. roseosporus attB* and their genes shared 95.7% identities. It is clear from these data that the presence of ϕC31 *attB* sites can be surveyed efficiently by initially doing BLASTP analysis, followed by confirmation at the gene and *attB* site level using BLASTN.

A BLASTP survey of ten non-streptomycete actinomycetes genomes identified ϕC31 *attB* potential targets in six strains. In all cases, pirin-like genes were present, and *attB* sites were confirmed in the two strains examined in detail. In *Frankia* sp. EAN1pec, the *attB* site showed 84.4% nucleotide identities with the *S. roseosporus attB*, and the *M. smegmatis attB* showed 73.3% identities. The average d*N*/d*S* for the six pirin homologs was 0.6, suggesting that most or all are orthologs to the *S. roseosporus* pirin-like gene. For comparison, the *glnA* genes from the non-streptomycetes have diverged from the *glnA* gene of *S. roseosporus* at an average d*N*/d*S* ratio of 0.9.

Notably, a pirin-like gene was absent from *S. erythraea*, and the closest homolog encoded a protein with only 31.7% amino acid identity to the pirin-like protein of *S. roseosporus* (Table 5). The combined genetic and bioinformatic data indicate that ϕC31-based vectors are widely applicable for streptomycetes, and suggest that they may be useful in certain other actinomycetes. The potential utility can be determined a priori by genome sequencing to determine if a pirin-like gene is present. If a ϕC31 *attB* site is not present, then a portable *attB* site might be inserted to increase the

Table 5 Homologs in streptomycetes and other actinomycetes to proteins encoded by genes containing bacteriophage *attB* sites and GlnA from *S. roseosporus*

Strain	Homolog to target protein (% aa identity)				
	ϕC31	ϕBT1	R4	TG1	GlnA
Streptomyces albus	89.2	80.0	78.0	72.7	92.3
S. avermitilis	85.7	84.4	80.5	75.4	91.7
S. clavuligerus	88.6	89.6	77.7	70.3	95.1
S. coelicolor	87.3	83.5	80.3	74.2	89.3
S. flavogriseus	90.8	83.1	87.2	73.3	96.8
S. ghanaensis	86.7	75.9	80.0	74.7	90.6
S. griseoflavus	87.9	74.4	80.5	71.4	91.0
S. griseus	96.5	100	92.1	90.5	99.1
S. viridochromogenes	88.3	79.7	80.0	73.5	94.5
S. sp. SPB78	84.1	83.1	76.1	80.2	92.1
Streptomyces ave	88.5	83.4	81.3	75.6	93.3
Amycolatopsis mediterranei	–	61.3	68.5	58.7	70.0
Catenulispora acidiphila	69.8	46.9	63.8	71.9	77.2
Frankia sp. EAN1pec	72.0	51.9	40.6	43.2	69.6
Micromonospora aurantiaca	70.7	–	68.2	67.4	73.4
Mycobacterium smegmatis	65.4	–	60.3	55.4	69.1
Rhodococcus erythropolis	68.8	–	65.2	55.2	70.0
Saccharomonospora viridis	–	59.0	68.7	58.5	70.7
Saccharopolyspora erythraea	–[a]	49.4	67.0	58.9	74.7
Salinispora arenicola	–	–	–	71.7	72.8
Streptosporangium roseum	66.4	–	68.2	59.4	71.9
Non-streptomycete ave	68.9	54.2[b]	66.0[c]	60.0	71.9

Protein searches were carried out using the BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

[a] Closest match, 31.7%

[b] This calculation does not include *Frankia* sp. EAN1pec for which no BLASTN hit was obtained

[c] *Frankia* sp. EAN1pec and *M. aurantiaca* were excluded in the calculation because of the lack of full-length sequence in the BLASTN analysis. See footnote c in Table 4

efficiency of genetic manipulations, as demonstrated in *S. erythraea* [84] and *Micromonospora griseorubida* [98]. This concept has already been generalized to engineer eucaryotes (e.g., see [18, 56, 60, 74, 103]) and archaea [16, 34], and should be applicable to any organism that is amenable to genetic manipulation.

Bacteriophage ϕBT1

ϕBT1 is a temperate phage related to ϕC31 [30]. Like ϕC31, it integrates via a large serine recombinase, and its 73-nucleotide *attP* and *attB* sites are quite different from each other. However, they have core 12-nucleotide sequences nearly identical to each other (11 of 12 identities) where crossing-over occurs. Importantly, ϕBT1 integrates into a gene annotated to encode an integral membrane protein unrelated to the pirin-like gene for ϕC31 integration (Table 1). The ϕBT1 Int is distantly related to ϕC31 Int, showing only 26% amino acid identities in reciprocol BLASTP analyses (Table 2). It has similar low sequence identities to the three other proteins that gave significant hits to ϕC31 Int, and had no other significant hits (Table 2). In *S. coelicolor* and *S. avermitilis*, the ϕBT1 *attB* site is located about 1 Mb to the left of *oriC*, and within the 6.5 Mb core region (Fig. 1). The mechanism of insertion was studied in vitro where it was shown that the minimal *attB* and *attP* sites comprise 36 and 48 bp, respectively [106]. The integration process was very efficient with *attB* and *attP* substrates, but was also measurable with *attL* and *attR* sequences, implying that Int might excise ϕBT1 in vivo at some frequency in the absence of other factors. Further mechanistic studies have been reported recently [107]. Although no studies have been carried out to characterize an Xis or RDF protein, BLASTP analysis with the 244-aa gp3 RDF from ϕC31 gave a top hit of 84.8% amino acid identities to a 247-aa gp3 protein from ϕBT1 (this report). It is likely that this protein serves an Xis or RDF function for ϕBT1. If so, it could extend the potential utility of the ϕBT1 integration system.

Gregory et al. [30] constructed vectors derived from pSET152 by replacing the ϕC31 *att/int* with ϕBT1 *att/int*, and by exchanging antibiotic resistance genes. They showed that the ϕBT1-based vectors conjugated from *E. coli* into *S. coelicolor* and integrated at frequencies comparable to those of pSET152 (3.5×10^{-3} per recipient). Importantly, they demonstrated that an *S. lividans* transconjugant containing a ϕBT1-based vector inserted in the chromosome was an efficient recipient for conjugal transfer of pSET152. Since the ϕC31 and ϕBT1 systems are compatible, they can be used to add genes sequentially

to genetically engineer *S. lividans*, and other streptomycetes. Gregory et al. [30] investigated conjugation into other streptomycetes, and recovered transconjugants from *S. avermitilis*, *S. cinnamonensis, S. fradiae, S. lincolnensis, S. nogalater, S. roseosporus*, and *S. venezuelae*. ϕBT1-based vectors also function efficiently in the rapamycin-producing *Streptomyces hygroscopicus* [31, 50], where it was shown that insertions are neutral under the prevailing fermentation conditions (i.e., they cause no reduction in rapamycin production). This property is important for the genetic engineering of industrial production strains.

The ϕBT1 Att/Int system can also be used in conjugal BAC vectors for site-specific insertion in *Streptomyces* chromosomes. Liu et al. [58] developed a ϕBT1-based BAC and used it to clone and express the meridomycin biosynthetic gene cluster in *S. lividans*. Alexander et al. [2] modified a BAC vector to accommodate the engineering of lipopeptide biosynthetic genes in *E. coli* followed by conjugal transfer and insertion into the ϕBT1 *attB* site in *S. fradiae* strains. This system was coupled with the use of ϕ C31-based vectors to set up an ectopic transcomplementation system that allows lipopeptide biosynthetic genes to be expressed from three different locations in the chromosome to facilitate combinatorial biosynthesis [2, 3, 12, 79]. They also demonstrated that insertions into ϕBT1 and ϕC31 *attB* sites are neutral with respect to antibiotic production in *S. fradiae*, and that the complete set of A54145 biosynthetic genes can be expressed more efficiently from either *attB* site than from the native locus which is located in a potentially unstable subteleomeric region containing IS and transposase sequences [11, 72]. This approach might be applied to other streptomycetes where antibiotic biosynthetic genes are located in unstable subteleomeric regions of linear chromosomes. The compatibility of the two integration systems presents possibilities for doubling and tripling of complete secondary metabolite gene clusters for heterologous expression and strain improvement in streptomycetes [8, 9].

The apparent broad host specificity of the ϕBT1 Att/Int system is supported by recent genome sequencing studies. The integral membrane protein gene containing the *attB* site for *S. roseosporus* has apparent orthologs in all ten *Streptomyces* surveyed by BLASTN and BLASTP analyses (Tables 4, 5). Although the average d*N*/d*S* ratio for all ten was 0.8, BLASTN analysis of the *S. griseus* and *S. ghanaensis* genomes using the original 73-nucleotide *attB* site described by Gregory et al. [30] picked up full-length sequences with 92 and 96% identities to *attB* in the target genes. The *attB* sites are located in a highly conserved region in the first one-third of the gene.

Of the ten non-streptomycete actinomycetes surveyed, only four have homologous genes encoding integral membrane proteins. These include *Amycolatopsis mediterranei* and *S. erythraea*, both of which lack ϕC31 *attB* sites. The average d*N*/d*S* ratio for the four genes is 1.0, suggesting that one or more may have been under selection to evolve a paralogous function. A closer inspection of the *attB* regions in these four genes indicated that the *A. mediterranei* and *S. viridis attB* sequences, which have only 1 mismatch in the 12-nucleotide crossover region, are more highly conserved than those of *S. erythraea* and *C. acidiphila*, which have four and five mismatches in the crossover region, respectively. The lower amino acid sequence homologies relative to the *S. roseosporus* target gene product observed with the last two strains also suggests that the corresponding genes are paralogs to the streptomycete genes, and that purifying selection [10] to maintain the usually highly conserved *attB* region is relaxed in both cases.

The biological data and bioinformatic analyses indicate that ϕBT1-based vectors should have broad applicability for engineering of streptomycetes; bioinformatic data also suggest limited potential utility in other actinomycetes. As demonstrated with the ϕC31 integration system, a portable ϕBT1 *attB* site could be inserted into non-streptomycete chromosomes for genetic engineering purposes. A portable ϕBT1 *att* site has been used to demonstrate that ϕBT1 Int functions efficiently in vertebrate cells and *Schizosaccharomyces pombe*. Morover, this system has been used in conjunction with Cre to build a transgenic human–Chinese hamster hybrid cell line containing 400 kb of contiguous transgenic DNA [105].

Bacteriophage R4

R4 is a broad-host-range streptomycete temperate bacteriophage isolated from soil on *Streptomyces albus* J1074, a mutant of *S. albus* G defective in *Sal*I restriction and modification [19]. Like many other *Streptomyces* bacteriophages, its host range is limited primarily by type II restriction enzyme barriers [19, 24, 36, 37]. R4 integrates site-specifically into the chromosome of *Streptomyces parvulus* (and presumably in other streptomycetes) to establish lysogeny [87]. Matsuura et al. [68] demonstrated that integration is catalyzed by a large serine recombinase that recognizes *attP* and *attB* sites for integration, but not *attL* and *attR* sites for excision. The 50-nucleotide *attB* site contains a 12-nucleotide common core that is also found in the *attP* site, and serves as the region for site-specific recombination [80]. The 50-nucleotide *attB* site was used to carry out BLASTN analysis in *S. roseosporus*, and a highly conserved 41-nucleotide segment containing the 12-nucleotide common core was located in a gene that encodes an acyl-CoA synthetase (Table 1). This gene and its product were used to carry out BLASTN and BLASTP analyses against ten *Streptomyces* and ten other actinomycete genomic sequences: highly conserved apparent orthologs

(average d*N*/d*S* = 0.6) were observed in all ten *Streptomyces* species (Tables 4, 5). The acyl-CoA synthetase apparent orthologs containing *attB* sites in *S. coelicolor* and *S. avermitilis* mapped to similar locations within the 6.5-Mb core regions of the linear chromosomes (Fig. 1). Homologs were also observed in nine of ten other actinomycetes, but these appear to be a mixture of orthologs and paralogs. The average d*N*/d*S* ratio for seven of the gene/protein pairs (excluding *Frankia* sp. EAN1pec and *M. aurantiaca*) was 0.9. The 50-nucleotide R4 *attB* sites were compared for four of the strains. *C. acidiphila*, *S. erythraea*, and *S. viridis* have authentic *attB* sites showing 94, 96, and 92% nucleotide identities, respectively, to the *S. roseosporus attB* site. Furthermore, the same strains had 12, 12, and 11 nucleotide identities to the 12-nucleotide crossing-over region. On the other hand, *Frankia* sp. EAN1pec, which encodes an acyl-CoA synthetase homolog that shows only 40.6 amino acid identities to the *S. roseosporus* counterpart, has a 50-nucleotide *attB* site that is only 54% identical to the *attB* of *S. roseosporus*, and it has only 6 of the conserved 12 nucleotides for crossing-over. This gene appears to be a paralog to the streptomycete R4 target genes, and probably would not serve as an efficient target for R4 integration.

Although R4 has not been used widely as a general tool for insertion of genes in actinomycetes, it has been shown to be a useful tool for engineering human cells (e.g., see [56, 60, 80]).

Bacteriophage TG1

TG1 is a temperate bacteriophage isolated on *Streptomyces cattleya*, the thienamycin producer [26]. It has a broad host range for *Streptomyces* species, but did not form plaques on *S. coelicolor* or *S. lividans* [26, 27]. Analysis of multiple lysogens indicated that it inserted into a single *attB* site in *S. cattleya* [26]. TG1 was developed as a bifunctional vector that could be engineered in *E. coli*, transfected into a streptomycete host, then transduced into other streptomycete hosts where it formed relatively stable lysogens [27].

TG1 was recently shown to integrate site-specifically by a large serine recombinase mechanism [75, 76]. BLASTP analysis with TG1 Int gave significant hits only to ϕC31 Int (49.7%) and to the four other proteins identified in BLASTP analyses with ϕC31 and ϕBT1 integrases (Table 2). In vitro studies demonstrated that the TG1 Int does not require host factors for insertion, and that it does not catalyze excision [76]. The minimal *attP* and *attB* sites were shown to comprise 43 and 39 nucleotides, respectively, and share a common dinucleotide (TT) at the site for crossing-over [76]. Recent studies have demonstrated that TG1 Int can drive efficient integration of *attB*-containing circular plasmid DNA into *E. coli* containing an *attP* sequence inserted into the chromosome by EZ-Tn5 transposition [38], a technique that might be applicable to other bacteria and other serine integrases.

The TG1 *attB* site is located in a *dapC*-like gene which may encode an *N*-succinylaminopimelate aminotransferase [75]. However, TG1 lysogens of *S. avermitilis* did not require lysine or diaminopimelate for growth, suggesting that the *dapC* annotation may be incorrect, and that the gene may encode an aminotransferase with a different function. The TG1 *attB* site in the *dapC*-like gene is located about 230 kb to the right of *oriC* in both *S. coelicolor and S. avermitilis*, or about 140 kb to the right of the ϕC31 *attB* site (Fig. 1). Apparent orthologs of the *dapC*-like gene were observed in all ten streptomycetes (average d*N*/d*S* = 0.70) (Tables 4, 5). Homologs of the *dapC*-like gene were observed in all ten other actinomycetes surveyed, but some of these are likely to be paralogs. For instance, the d*N*/d*S* ratios for *Frankia* sp. EAN1pec and *M. smegmatis* are 1.4 and 1.2, respectively. The bioinformatic data suggest that the TG1 integration system may be directly applicable to many streptomycetes and possibly to some other actinomycetes.

Uses of site-specific insertion for genetic engineering in actinomycetes

Streptomycete phage site-specific integration systems have been used for a number of applications that require stable insertion of one or more genes into the chromosome. Industrial applications include strain improvement for early to late-stage process development, heterologous expression of cryptic secondary metabolite biosynthetic gene clusters for drug discovery, and combinatorial biosynthesis to generate novel derivatives of known secondary metabolites [6–9, 12]. For strain improvement, site-specific insertion can be used to: (1) increase gene dosage to address rate-limiting primary or secondary metabolic steps; (2) change promoters to improve the expression of regulatory and other genes; (3) alter the metabolic capability of cells by adding new functions; (4) and duplicate or triplicate complete secondary metabolite gene clusters [9]. For the discovery of novel drug candidates from cryptic secondary metabolite gene clusters discovered in genome sequencing projects, candidate gene clusters can be: (1) cloned in BAC vectors that replicate in *E. coli*; (2) transferred by conjugation from *E. coli* into streptomycete expression hosts, including those derived from industrial production strains; and (3) stably inserted at appropriate *attB* sites. Transconjugants can then be fermented in several media and screened for the expression of novel secondary metabolites. This process and the properties of key streptomycete expression hosts are discussed in more detail elsewhere [8, 9]. The use of site-specific integration vectors for combinatorial biosynthesis has

the advantage that different genes or sets of genes can be engineered separately, and then different combinations of the engineered genes can be brought together in an expression host [7, 8, 12]. Recent examples that demonstrate the power of this approach are the engineering and expression of separate nonribosomal peptide synthetase (NRPS) multienzymes, or other genes encoding amino acid modifying enzymes, by insertion into the *S. fradiae* chromosome at the ϕC31 and ϕBT1 *attB* sites to generate a large array of novel lipopeptide antibiotics with tridecapeptide structures derived from A54145 and daptomycin [2, 3, 79]. These site-specific integration systems can also be used in combination with other insertion systems, such as IS*117* [22, 73, 78].

Discussion

The bacteriophage ϕC31 Att/Int system has made a large impact on the development of robust genetic engineering tools for the industrially important *Streptomyces* and other actinomycetes. This work was initiated in Russia by the Lomoskaya laboratory, and further developed in Russia and in the UK by the Keith Chater and Margaret Smith laboratories. The work on the fundamental biology of ϕC31 provided a rich starting point for the seminal work of Kustoss, Rao, and colleagues at Eli Lilly and Company, who developed the Att/Int system into a widely useful set of cloning vectors for *Streptomyces* species [14, 51, 52]. These and their derivatives have been applied to strain improvement, combinatorial biosynthesis, and whole pathway heterologous expression. In addition to the important applications in the native actinomycetes, the unidirectional serine recombinase systems have impacted the broader field of biotechnology, providing a robust methodology for the engineering of eucaryotic cells.

ϕC31 is just one of several streptomycete temperate phages described in the literature, most of which are poorly characterized. R4, TG1, and ϕBT1 have been studied in some detail; as with ϕC31, all three have broad host ranges within *Streptomyces* species, and integrate by unidirectional serine recombinases. Importantly, they integrate into different genes that are highly conserved in *Streptomyces* species. This represents an interesting evolutionary strategy to have the potential to lysogenize any species of the genus *Streptomyces*, rather than limit the host range to one or a subset of streptomycetes by inserting into genes not conserved across the species. The host range is thus maximized, and limited primarily by host type II restriction barriers.

For applications in streptomycetes, the broad host specificity and conservation of genes containing *attB* sites for these integration systems enables sequential addition of genes for combinatorial biosynthesis, strain development, and other applications. The host restriction barriers are often easily overcome by using conjugal transfer from *E. coli* [14, 67], a process that transfers linear concatemers of single-strand DNA which are not susceptible to host-encoded type II restriction endonuclease cleavage [8]. The different *attB* genes or sequences can also be used as portable integration sites in other actinomycetes that lack *attB* sites. It is conceivable that four or more different *attB* sites could be cloned contiguously, then inserted into an actinomycete of interest at a site that is neutral for secondary metabolite production. This would provide a target for sequential addition of any number of genes for a variety purposes. This approach has already been applied to mammalian cells [56, 60, 74, 103]. In principle, this concept could be applied to other eubacteria, archaea, plants, mammals, and other eucaryotes. The applications are limited only by our current knowledge of bacteriophages that employ large serine integrases. There are undoubtedly many more temperate bacteriophages for streptomycetes and other actinomycetes that use this mechanism. Broad-host-range temperate bacteriophages are readily isolated on *Streptomyces* strains, and *Streptomyces griseofuscus* is particularly useful for bacteriophage isolations because it is non-restricting for bacteriophage plaque formation [24], and has been used to isolate temperate bacteriophages from soil [36, 37].

Although several large serine recombinase systems have already been discovered, it is not known if the best ones have been identified. There exists an untapped wealth of additional temperate actinophages yet to be discovered, and these can be isolated inexpensively from soil.

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