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Integration Site for *Streptomyces* Phage ϕBT1 and Development of Site-Specific Integrating Vectors

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Despite extensive similarities between the genomes of the *Streptomyces* temperate phages ϕC31 and ϕBT1, the *attP-int* loci are poorly conserved. Here we demonstrate that ϕBT1 integrates into a different attachment site than ϕC31. ϕBT1 *attB* lies within SCO4848 encoding a 79-amino-acid putative integral membrane protein. Integration vectors based on ϕBT1 integrase were shown to have a broad host range and are fully compatible with those based on the ϕC31 *attP-int* locus.

The *attP-int* locus from ϕC31 has been heavily exploited in the construction of versatile, low-copy-number, and convenient vectors for use in a broad range of *Streptomyces* species (5, 10). Despite their wide use and clear advantages, it has been reported that integration of these vectors into the ϕC31 *attB* site can cause detrimental effects on antibiotic production in some strains (2). ϕC31 integrates intragenically into SCO3798, a highly conserved gene in prokaryotes and eukaryotes but is not essential for the growth of *Streptomyces coelicolor* in the laboratory (8). Although some phages can regenerate a functional gene after insertion (7), there is no evidence that this is the case with ϕC31. Furthermore, a vector, pSET152 containing the ϕC31 *attP-int* locus, introduced by conjugation from *Escherichia coli* can integrate into secondary or pseudo-*attB* sites in both *S. coelicolor* and *Streptomyces lividans* (8). The reported reductions in antibiotic synthesis could be caused by insertional mutagenesis into SCO3798 or by integration into one of the pseudo-*attB* sites or some other factor. Another potential problem with integrating vectors could be the absence of an efficiently recognized *attB* site in some streptomycete strains. Indeed, *Saccharopolyspora erythraea* appears to lack a ϕC31 *attB* site (P. Leadlay, personal communication). For these reasons and as many workers would like to use two compatible integrating vectors in the same organism, we have investigated the integration site of the *Streptomyces* phage ϕBT1, a homoimmune relative of ϕC31. We demonstrate that ϕBT1 does indeed integrate into a different *attB* site in *S. coelicolor*, and we have constructed novel integrating vectors derived from the ϕBT1 *attP-int* locus.

The organization of the ϕBT1 genome is highly similar to that of ϕC31, and the majority of gene products are closely related (9). There is evidence, however, of mosaicism between the two genomes where DNA has been inserted and/or deleted in one genome but not in the other, and there are sudden transitions in the level of sequence similarity (9). One of the most noticeable differences is the relatively poor sequence similarity of *int* and the three genes upstream, genes 26 to 28. ϕBT1 integrase and gp26 to gp28 exhibit 26% and 10 to 18% identity to their ϕC31 homologues, respectively. Despite this poor similarity, ϕBT1 integrase is clearly a member of the large serine recombinase family, as it contains conserved motifs present in other members of this group (12). Furthermore, no significant similarity could be detected between the ϕC31 *attP* site and any ϕBT1 sequence. These observations strongly suggest that ϕBT1 encodes a site-specific recombination system that has a different specificity from that in ϕC31 and therefore integrates into a different *attB* site in the *S. coelicolor* genome. Southern blots of DNA from an *S. coelicolor* J1929 ϕBT1 lysogen (strain J1929 contains Δ*pglY* conferring sensitivity to ϕC31 and ϕBT1 [3]) probed with DNA encoding the ϕC31 *attB* site indicated that ϕC31 *attB* was intact, suggesting that ϕBT1 was integrated elsewhere in the genome (data not shown). To test this further and to identify the ϕBT1 *attB* site, we performed vectorette PCR (Sigma-Genosys) extending outwards from the ϕBT1 DNA into the host DNA in an *S. coelicolor* ϕBT1 lysogen. This procedure is designed to isolate unknown flanking sequences from a known integrated sequence (1). One product was obtained using *S. coelicolor* J1929 ϕBT1 DNA digested with *Bcl*I as the original template. The DNA sequence of this product read outwards from the ϕBT1 genome into a segment of the *S. coelicolor* genome sequence and finally into the vectorette linker sequence. The *S. coelicolor* genome sequence matched part of cosmid SC5G8 (4). To confirm the site of ϕBT1 integration, primers were designed against this region of SC5G8 and used to amplify *attB* from *S. coelicolor* J1929 DNA and *attL* and *attR* from J1929 ϕBT1 lysogen DNA. The *attB* sequence was most similar to the genomic sequence coordinates 5279863 to 5280017 (contained within SC5G8), and the *attL* and *attR* sequences indicated that the recombination had occurred between 9 bp of identical sequence between *S. coelicolor* coordinates 5279923 to 5279915 and ϕBT1 (Fig. 1). We confirmed that this locus is the major integration site by Southern blotting of genomic DNA isolated from *S. coelicolor* J1929 containing integration vector pMS81 or pMS82 described below; no bands other than those predicted as a consequence of integration via the *attB* site were observed (data not shown).

An alignment of the ϕBT1 *attB* site and the phage *attP* site

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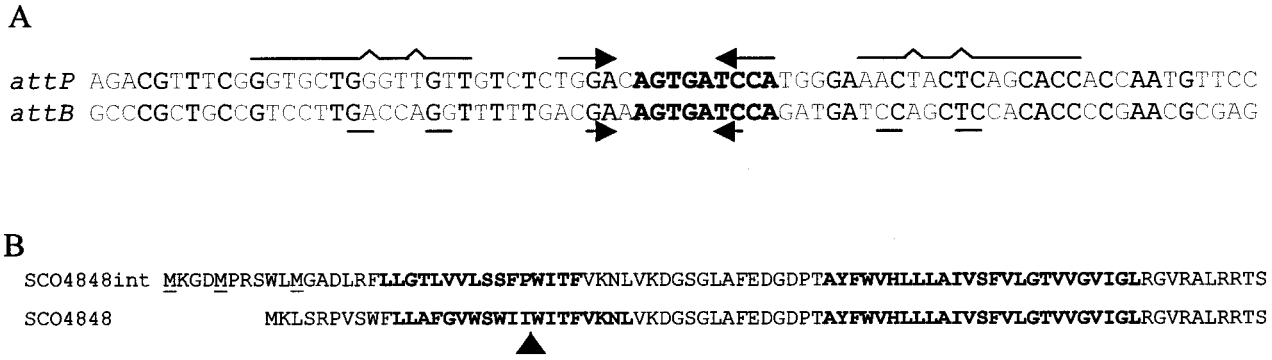

FIG. 1. Integration of ϕBT1 in *S. coelicolor* A3(2). (A) DNA sequences of ϕBT1 *attP* and *attB* sites. Bases that are identical in the *attB* and *attP* sites are shown in black type. The imperfect inverted repeats are indicated by arrows. The crossover site occurs within the region shown in black bold type. The GenBank accession number for the complete ϕBT1 genome is AJ550940. (B) Consequence of ϕBT1 integration on the predicted protein sequence of SCO4848 containing *attB*. SCO4848 is the native predicted protein sequence, and SCO4848int is the sequence after ϕBT1 integration. In SCO4848int, the N-terminal 29 amino acids are predicted from the antisense strand of the *int* gene, and the site of interruption of SC04848 is shown as a vertical arrowhead. Three possible initiating methionines in SCO4848int are underlined. The amino acids in bold type are predicted by PHDhtm (11) to be membrane-spanning alpha helices. The functional consequences of ϕBT1 integration on SCO4848 have not been tested experimentally.

indicated that, like the ϕC31 recombination system, the *attB* and *attP* sequences are quite different. The ϕBT1 *attP* site contains an imperfect inverted repeat centered around the dinucleotide 5′GT within the 9-bp core sequence (Fig. 1). In other phages or prophages encoding serine integrases, the *attP* sites also contain imperfect inverted repeats that in ϕC31, TP901-1, and ϕRv1 are symmetrical around the 2-bp sequence at which crossover occurs (6, 8).

The ϕBT1 *attB* site lies approximately 1 Mb to the right of *oriC* compared to the ϕC31 *attB* site, which is approximately 90 kbp to the left of *oriC*. The ϕBT1 *attB* site lies within SCO4848 coding for a putative integral membrane protein. This protein contains two predicted membrane-spanning alpha helices (Fig. 1). Analysis of the *attL* sequence indicates that an alternative protein coding sequence, SCO4848int, is generated after ϕBT1 integration, which still retains an N-terminal putative membrane-spanning alpha helix (Fig. 1). Thus, if SCO4848int is transcribed, an active gene product may produced even after ϕBT1 integration and which may, therefore, confer a neutral phenotype.

For this reason and in order to provide additional integration vectors, we constructed integrating plasmids similar in design to pSET152 except that they integrate via ϕBT1 *attP-int* (3). pRT801 (Fig. 2) was constructed in two steps. PCR was used to amplify the ϕBT1 *int-attP* region from phage particles in the presence of primers RT26 (5′ ATT AGC ATG CTG GCG CCG GAC GGG GCT TCA GAC) and RT27 (5′ TAA TGG ATC CGC TCC CTG CCC GCT GTG GTG AC) and *Pfu* polymerase, using the manufacturer's recommended procedures (Stratagene). The PCR fragment and pGEM7 (Promega) were cut with *Bam*HI and *Sph*I, ligated to form pRT800, and the *attP-int* region was sequenced. The ϕBT1 *attP-int*-containing fragment was then used to replace the ϕC31 *attP-int* sequences in pSET152 using the restriction sites *Bam*HI and *Sph*I to form pRT801. The plasmids pRT801 and pSET152 were introduced into the nonmethylating *E. coli* strain ET12567 (containing pUZ8002, required to provide the transfer functions [10]), and these strains were used as donors in conjugations with *S. coelicolor* J1929. Comparable numbers of apramycin-resistant transconjugants of *S. coelicolor* J1929 were obtained with *E. coli* ET12567(pUZ8001, pRT801) and with ET12567(pUZ8001, pSET152) (Table 1).

To ensure that the ϕBT1 *attP-int*-based vectors were compatible with pSET152 and to assay the frequency of integration into the *S. lividans* genome, pRT802 was constructed containing the *aphII* gene from Tn*5* to replace the apramycin resistance marker (Fig. 2). pRT802 was constructed by inserting a 1,327-bp fragment from pNRT4 (encoding the *aphII* gene from Tn*5* and kindly provided by P. Herron, University of Swansea) cut with *Hind*III, blunt ended with Klenow fragment, and then cut with *Sac*I and inserted into pRT801 cut with *Nru*I and *Sac*I. It should be noted that during the construction of pRT802, an approximately 600-bp deletion was introduced in the *oriT* region (S. Ward, personal communication). pRT802 and pSET152 were introduced separately into *S. lividans* TK24 by conjugation from *E. coli* S17-1(pRT802) or S17-1(pSET152), and similar numbers of kanamycin- and apramycin-resistant colonies were obtained (data not shown). *S. lividans* TK24 or TK24(pRT802) was then used as the recipient for the introduction of pSET152 from *E. coli* S17-1(pSET152), and similar numbers of apramycin-resistant colonies were obtained in both recipients (Table 1). Thus, the ϕBT1 *attP-int*-containing vectors are transferred efficiently to both *S. coelicolor* and *S. lividans*, and integration of pSET152 is not inhibited by the presence of pRT802.

The broad host range of pRT801 was demonstrated by conjugation into other streptomycetes using standard methods (8). Apramycin-resistant transconjugants were recovered for many of the *Streptomyces* strains investigated, including *S. avermitilis*, *S. cinnamonensis*, *S. fradiae*, *S. lincolnensis*, *S. nogolater*, *S. roseosporus*, and *S. venezuelae*. To complete the suite of ϕBT1 *int*/*attP* vectors, we constructed hygromycin-resistant derivatives of pRT801, pMS81, and pMS82 (Fig. 2). These plasmids were constructed by ligation of a *Hpa*I fragment encoding hygromycin resistance (derived from Tn*5099-10*) (13) with the 4,415-bp *Ecl*136II fragment from pRT801 and differ only in the

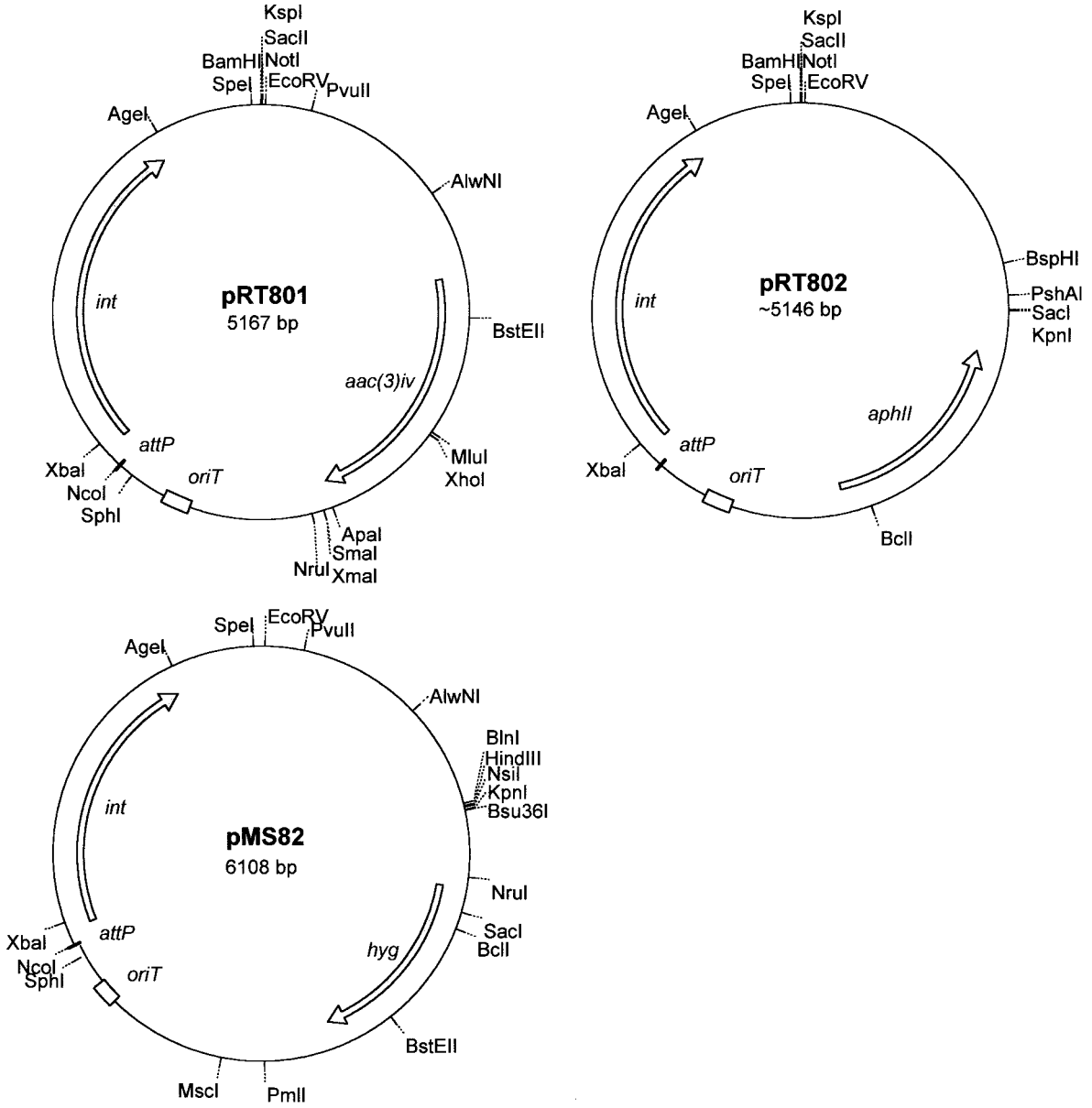

FIG. 2. Integration vectors derived from the ϕBT1 *attP-int* locus for use in *Streptomyces* species.

orientation in which the fragment encoding hygromycin resistance has inserted. The hygromycin resistance marker in *S. coelicolor* J1929(pMS81) and J1929(pMS82) was confirmed to be stable after two rounds of sporulation in the absence of selection (data not shown). We believe that all of these vectors will become a useful addition to the genetic toolbox of the streptomycete researcher.

TABLE 1. Numbers of apramycin-resistant transconjugants obtained with different *E. coli* donors and *Streptomyces* recipients

E. coli donor	*Streptomyces* recipient	No. of apramycin-resistant transconjugants/spore
ET12567(pUZ8002, pSET152)	J1929	5.0×10^{-3}
ET12567(pUZ8002, pRT801)	J1929	3.5×10^{-3}
S17-1(pSET152)	TK24	5.0×10^{-4}
S17-1(pSET152)	TK24(pRT802)	9.4×10^{-4}

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