Site-specific integration of bacteriophage VWB genome into Streptomyces venezuelae and construction of a VWB-based integrative vector

Lieve Van Mellaert, Lijuan Mei, Elke Lammertyn, Sabine Schacht and Jozef Anné

Author for correspondence: Jozef Anné. Tel: +32 16 33 73 75. Fax: +32 16 33 73 40. e-mail: Jozef.Anne@rega.kuleuven.ac.be

The temperate bacteriophage VWB integrates into the chromosome of Streptomyces venezuelae ETH14630 via site-specific integration. Following recombination of the VWB attP region with the chromosomal attB sequence, the host–phage junctions attL and attR are formed. Nucleotide sequence analysis of attP, attB, attL and attR revealed a 45 bp common core sequence. In attB this 45 bp sequence consists of the 3' end of a putative tRNAArg(AGG) gene with a 3'4erminal CCA sequence which is typical for prokaryotic tRNAs. Phage DNA integration restores the putative tRNAArg(AGG) gene in attL. However, following recombination the CCA sequence is missing as is the case for most Streptomyces tRNA genes described so far. Adjacent to VWB attP, an ORF encoding a 427 aa protein was detected. The C-terminal region of this protein shows high similarity to the conserved C-terminal domain of site-specific recombinases belonging to the integrase family. To prove the functionality of this putative integrase gene (int), an integrative vector pKT02 was constructed. This vector consists of a 2.3 kb HindIII-SphI restriction fragment of VWB DNA containing attP and int cloned in a non-replicative Escherichia coli vector carrying a thiostrepton-resistance (tsr) gene. Integration of pKT02 was obtained after transformation of Streptomyces venezuelae ETH14630 and Streptomyces lividans TK24 protoplasts. This vector will thus be useful for a number of additional Streptomyces species in which a suitable tRNA gene can be functional as integration site.

Keywords: Streptomyces, bacteriophage VWB, integration, tRNA, integrative vector

INTRODUCTION

The temperate phage VWB was originally isolated from soil using Streptomyces venezuelae ETH14630 as indicator strain (Anné et al., 1984). Its dsDNA genome is 47.3 kb in size and has a GC content of 69.3 mol%. VWB DNA has cohesive ends, implying a site-specific staggered cutting of concatemeric DNA during DNA packaging. Phages with such a non-headful packaging system are suitable as cloning vectors as reported, e.g. φC31 (Hopwood et al., 1987) and phage VWB. The latter phage can package at least 3.5–4 kb foreign DNA in addition to its own genome (Anné et al., 1990a).

Streptomyces integrative plasmids and temperate phages (including two plasmids of Saccharopolyspora erythraea, previously classified as Streptomyces erythraeus) most often integrate site-specifically into the host chromosome (Bar-Nir et al., 1992; Boccard et al., 1988; Brasch et al., 1993; Brown et al., 1990, 1994; Gabriel et al., 1995; Kendall & Cullum, 1986; Kuhstoss & Rao, 1991; Shirai et al., 1991; Sosio et al., 1989). In general, such site-specific integration is catalysed by a site-specific recombinase. Streptomyces integrative plasmids encode a protein belonging to the integrase family showing similarities with the phage λ integrase. Streptomyces phages have different types of proteins that cause site-specific integration. For example, Streptomyces phage φC31 encodes p68 (Kuhstoss & Rao, 1991), which could be aligned to the resolvase/DNA invertase family of site-specific recombinases (Leschziner et al., 1995). In phage R4, sre encodes a protein similar to the resolvase/invertase family (Matsuura et al., 1996). The lysogenic
Streptomyces rimosus RP3 phage requires a phage-encoded protein belonging to the integrase family (Gabriel et al., 1995).

Integration of phages and plasmids of *Streptomyces* which utilize an Int family recombinitase occurs into a tRNA gene (Bar-Nir et al., 1992; Brown et al., 1990, 1994; Mazodier et al., 1990; Reiter et al., 1989; Vogtli & Cohen, 1992). *attP* contains the 3’ end of the tRNA gene so that after the recombination event the chromosomal tRNA is restored in *attL*.

Phage VWB has a narrow host range, i.e. among six *S. venezuelae* strains, only the ETH14630 strain in addition to *Streptomyces exfoliatus* ATCC 12672 could be infected. Nevertheless, VWB could be introduced by transfection into several non-infectible *Streptomyces* strains, including *Streptomyces lividans*, with the formation of lysogens (Anné et al., 1990b). Bacteriophage VWB integrates site specifically into the chromosome of its host *S. venezuelae* ETH14630 at the attachment site *attB* (Anné et al., 1990b).

In this study, the *att* sites involved in the integration of VWB into the *S. venezuelae* ETH14630 chromosome were characterized and an ORF (int) upstream of *attP* encoding a 427 aa protein resembling the recombinases of the integrase family (Argos et al., 1986; Abramski & Hoess, 1992; Esposito & Scocca, 1997; Nunes-Düby et al., 1998) was identified. By introducing deletions in the *int* gene, we proved the requirement of the gene for site-specific recombination of the *attP* with the *attB* site. Furthermore, a functional integrative vector was constructed using an *Escherichia coli* replicon containing the VWB *attP* site, the *int* gene and a thiostrepton-resistance (tsr) marker.

**METHODS**

**Bacterial strains, plasmids, growth conditions and transformation experiments.** *S. venezuelae* ETH14630 was used as host strain for phage VWB propagation (Anné et al., 1984). Integration of VWB DNA or VWB-based vectors was tested both in *S. venezuelae* ETH14630 and in *S. lividans* TK24 (Hopwood et al., 1985), grown in phage medium (Korn et al., 1978) supplemented, when necessary, with 10 μg thiostrepton ml⁻¹. Protoplast transformation was carried out as previously described (Anné et al., 1990c; Hopwood et al., 1985). Regeneration of protoplasts and growth occurred on MRYE medium (Anné et al., 1990c) and transformants were selected by means of an overlay with 2.5 ml soft MRYE medium containing 75, 150 or 300 μg thiostrepton ml⁻¹ 20–24 h after transformation.

A genomic bank of *S. venezuelae* or its VWB lysogen was made in *E. coli* MC1061 (Wertman et al., 1986) using pACYC184 (Chang & Cohen, 1978) as cloning vector. *E. coli* TG1 (Sambrook et al., 1989) was used for subcloning of DNA fragments into pUC18/19 (Yanisch-Perron et al., 1985) or pBluescriptII KS(−) (Stratagene). *E. coli* strains were grown in LB medium, and when applicable, ampicillin (50 μg ml⁻¹) or chloramphenicol (12.5 μg ml⁻¹) was added.

**DNA manipulations.** Total DNA of *Streptomyces* cells was isolated as described by Hopwood et al. (1985). Plasmid DNA isolation from *E. coli* cells, restriction enzyme digestions and other DNA manipulations were carried out by standard protocols (Sambrook et al., 1989). Restriction DNA fragments were eluted from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN).

**Hybridization experiments.** To identify the *attP* region, 2 μg *S. venezuelae* DNA, 2 μg lysogenic DNA and 0.5 μg VWB DNA were digested with different restriction enzymes. The DNA fragments were subsequently separated by agarose gel electrophoresis and blotted on Hybond-N membrane (Amersham) using a VacuGene system (Pharmacia). The UV cross-linked DNA fragments were hybridized with an appropriate digoxigenin-labelled probe as described by Engler-Blum et al. (1993). Hybridization signals were detected with 0.25 mM CDP-Star (Tropix) according to the method of Hoeltke et al. (1995).

To isolate *attB*, *attL* and *attR*, a genomic bank of *S. venezuelae* and of its VWB lysogen was made following digestion of the chromosomal DNA with *SphI* and *BclI*, respectively. The digested DNA was subsequently ligated to *SphI*- or *BamHI*-digested pACYC184. After transformation of *E. coli* MC1061, colonies were lifted on Hybond-N⁺ membranes (Amersham) following the manufacturer’s instructions. Next, a digoxigenin-labelled *attP*-specific probe was used for selection of the desired subclones.

To demonstrate the integration of the VWB-based integration vector into the *S. venezuelae* or *S. lividans* chromosome, 2 μg chromosomal DNA isolated from strains with or without the integrated vector, and 1 ng vector were digested and blotted on Hybond-N membrane as described above. The digoxigenin-labelled 0.5 kb *BamHI*-HindII fragment of VWB containing the *attP* locus was used as probe.

**Sequencing and computer-assisted analyses.** Nucleotide sequence analysis was performed by manual sequencing using [³²P]dATPαS (NEN Life Science Products) or by automated sequencing on ALFexpress (Pharmacia) using CY5-labelled oligonucleotides. Sequencing was repeatedly carried out on both strands. Computer-assisted analyses of sequences occurred with programs of PC/GENE (GenoSoft, IntelliGenetics).

**Construction of the integrative vector.** For the construction of the integrative vector pKT02 (see Fig. 6), *E. coli* vector pC20R (Marsh et al., 1984) was used, in which the *tsr* gene was cloned by inserting the 1.1 kb *BclI* fragment of pJ702 (Katz et al., 1983) into the *BamHI* site. The resulting pKT01 vector was linearized with *PstI*, blunted in a T4 DNA polymerase reaction, next digested with *HindIII* and finally ligated to the 2.3 kb *PstI* sticky ends removed by T4 DNA polymerase–*HindIII* VWB fragment. pKT02 thus contained the *tsr* gene, the *attP* locus and the *int* gene of VWB.

In addition, two derivatives of pKT02 were made. In pKT03, the 1.6 kb *HindIII*–*BamHI* fragment (Fig. 1) containing the 3’-terminal part of the *int* gene was removed. In pKT04, a 0.6 kb internal *PstI* fragment of the *int* gene (Fig. 1) was deleted so that only the first 263 N-terminal aa of the integrase protein remained.

**RESULTS AND DISCUSSION**

**Characterization of *attP* and *int* gene of VWB**

In a previous report, *attP* of phage VWB was localized on the left side of the 9.6 kb *SphI*-B fragment (Anné et al., 1990b). Further comparisons between hybridization patterns of phage VWB and its prophage, using more sensitive methods for the detection of hybridizing bands, revealed that *attP* is located within the 1-9 kb VWB
Site-specific integration of bacteriophage VWB

**Fig. 1.** (a) Schematic representation of the SpBl-B fragment of the VWB genome which contains the int gene (-----) and attP locus (-----), followed by a very stable stem-loop structure (>). The relevant restriction sites are indicated (B, BamHI; H, HindIII; P, PstI; S, SphI). (b) Fragments cloned in pKT01 to investigate the functionality of the int gene and resulting in integration of the integrative vector (+) or not (−).

**Fig. 2.** (a) Nucleotide sequence downstream of the int gene containing the attP sequence. The identical sequence is given in bold characters. The C-terminal amino acid sequence of Int is shown below the int sequence. Arrows under the sequence show direct, and indirect repeats and mismatches within the repeats are indicated by small circles. (b) Nucleotide sequence of the S. venezuelae attB region with the common sequence indicated by bold characters. The tRNAArg gene is indicated by open arrows. Arrows under the sequence show an inverted repeat with one allowed G-U bp (O).

**BamHI-H1 restriction fragment.** Since both the SpBl-B and the BamHI-H1 VWB restriction fragments contained the attP locus, it could be localized on the common part of these fragments, i.e. a 0-7 kb BamHI-Sphl fragment that is on the right side of the SpBl-B fragment (Fig. 1). Sequencing this 0-7 kb fragment revealed an incomplete ORF encoding a polypeptide sequence that could be aligned to the conserved C-terminal domain of the recombinases of the integrase family (Argos et al., 1986; Esposito & Scocca, 1997; Nunes-Duby et al., 1998). The ORF is followed by three direct repeats of 8 bp of which the function is unknown and one inverted repeat of 12 bp with two mismatches (Fig. 2a). This inverted repeat may serve as a transcriptional terminator of the ORF. Approximately 170 bp downstream of the TGA stop codon, a sequence with similarities to the 3' end of known tRNA genes (Sprinzl et al., 1991) was found. tRNA genes are frequently utilized as attachment sites for integrative elements (Reiter et al., 1989) and this sequence was therefore presumed to be part of the VWB attP locus. A perfect inverted repeat composed of 17 bp arms interrupted by five unpaired bases followed this putative attP sequence (Fig. 2). The free energy AG (25 °C) of the stem-loop for the RNA molecule is calculated to be −50.8 kcal mol⁻¹ (−212.5 kJ mol⁻¹).

Further sequencing of the ORF (located on the upstream 1-6 kb HindIII-BamHI fragment, Fig. 1) revealed a complete ORF of 1284 nt encoding a putative integrase (Int). The int start codon is an ATG, which is preceded by a Shine–Dalgarno sequence (AGGTGA). The int gene can encode a protein of 427 aa with a deduced molecular mass of 49.1 kDa. This protein displays the features of an integrase as concluded from the homology of its C-terminal region to recombinases of the integrase family (Argos et al., 1986; Esposito & Scocca, 1997; Nunes-Duby et al., 1998).

Although this family exhibits a large diversity of sequences, within the C-terminal catalytic domain two Arg residues and a Tyr residue are absolutely conserved whilst a His residue is highly conserved. These four conserved residues form the active site and can be placed in three boxes (A, B and C) showing high similarity among nearly all proteins of the integrase family (Esposito & Scocca, 1997). Recently, Nunes-Duby et al.
Gly and Glu residues, at positions 231, 229 and 234, respectively. In patch 11, a conserved Lys residue is found in the catalytic domain of the proteins of the Int family. In patch I a group of acidic amino acids and two highly conserved aa, namely Gly\textsubscript{362} and His\textsubscript{396}. They belong to the Leu-Leu-Gly-His consensus sequence found in this domain. The spacing between the two conserved Arg residues in VWB Int consists of 136 aa, which is rather long in comparison with other members of the integrase family. The distance between the second conserved Arg and the active-site Tyr is 32 aa as in the majority of the proteins analysed by Esposito & Scocca (1997).

Furthermore, Int has a calculated pi value of 10.2. The basic protein contains 20.3% basic amino acids and only 12.6% acidic residues, which is typical for DNA-binding proteins. In addition, int is located close to the att\textsubscript{P} locus as found for most other integrases.

For many int genes described, the ORF is immediately preceded by or overlaps an ORF which is defined in most instances as, or speculated to be, the excisionase (xis) gene. Up to now, no ORF sharing similarity with the described Streptomyces excisionase proteins (Boccard et al., 1989; Brasch et al., 1993; Brown et al., 1990, 1994; Gabriel et al., 1995) could be detected in the sequence surrounding VWB int.

It is remarkable that all Streptomyces plasmids that can integrate into the host chromosome code for an integrase belonging to the Int class of site-specific recombinases. The use of a site-specific recombinase of the integrase family is, however, not common for Streptomyces phages. Like VWB, RP3 encodes an integrase (Gabriel et al., 1995), but \#C31 and R4 use a recombinase with a resemblance to the proteins of the resolvase/invertase family (Leschziner et al., 1995; Matsuura et al., 1996).

### Fig. 3. Alignment of amino acid sequences of six domains of the VWB integrase protein with amino acids in the same domains of the \(\lambda\) Int, the prototypical member of the integrase family, and other Streptomyces integrases. Boxes A, B and C correspond to the three major clusters with global similarities among the proteins of the integrase family described by Esposito & Scocca (1997). Patches I, II and III refer to the three additional patches of conserved sequence among the integrase family proteins found by Nunes-Duby et al. (1998). The numbers of amino acids spanning the different domains are indicated by the numbers in parentheses. Bold characters indicate similar or identical residues at a particular position in five of the seven aligned integrase proteins; asterisks indicate perfectly conserved residues and dots indicate well-conserved residues among the aligned proteins. The arrows indicate the four active-site residues of the integrases.

### Table: Conserved Residues in VWB Int

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<tr>
<td>VWB</td>
<td>1-268</td>
<td>PKSNAG--FRTPV (35)</td>
<td>ELVRFGP (41)</td>
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<td>pSAM2</td>
<td>1-242</td>
<td>QPAWQ-HGCDDP (99)</td>
<td>GGTVFGQ (29)</td>
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<tr>
<td>pSE11</td>
<td>1-239</td>
<td>QRTWK-HGCCDP (91)</td>
<td>GEKTVFGQ (29)</td>
</tr>
<tr>
<td>pSAM2</td>
<td>1-247</td>
<td>TKTWSKRTALHP (27)</td>
<td>HLSFVSQ (32)</td>
</tr>
<tr>
<td>SLP1</td>
<td>1-321</td>
<td>LKFRPKAVWTPV (21)</td>
<td>GRFRFGQ (35)</td>
</tr>
<tr>
<td>RP3</td>
<td>1-286</td>
<td>TRGQR-RAAVLP (25)</td>
<td>GNNRTSF (31)</td>
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<td>(\lambda)</td>
<td>1-233</td>
<td>SRTGV---KIAR (25)</td>
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(1998) identified three additional sequence patches (I, II and III). The Tyr is involved in the formation of a phosphotyrosyl bond to the cleaved 3' end of the DNA. This covalent protein-DNA linkage conserves the bond energy, which is later transferred to the DNA during ligation. The three other active-site residues Arg, His and Arg form a basic triad and are structurally clustered in the active-site cleft as shown by crystal structure analysis of four integrase proteins (Yang & Mizuuchi, 1997). In the VWB Int protein, the four conserved residues all are present and its C-terminal sequence can be aligned (Fig. 3) to the mentioned boxes and patches found in the catalytic domain of the proteins of the Int family. In patch I a group of acidic amino acids and precisely spaced hydrophobic residues, LT-EEV--LL, are conserved. The VWB Int sequence, however, is not common for the Int family (Leschziner et al., 1995).
WB DNA integrates into a putative tRNAAq gene

Secondary structure in sequences revealed the presence of a tRNA cloverleaf the four tRNA genes are often the sites at which the recombinant events with bacterial integrative elements occur. Positive inserts were subcloned and subsequent hybridization experiments revealed the presence of containing the homologous to the DNA sequences of the four loci on a 0.4 kb fragment, respectively. On hybridization with the digoxigenin-labelled 0.7 kb lysogenized S. chromosomal DNA was digested with Sph1 and BclI, respectively. On hybridization with the digoxigenin-labelled 0.7 kb BamHI–Sph1 VWB fragment containing the attP site, clear hybridization signals could be detected. The Sph1 fragment of S. venezuelae DNA containing the attB locus was about 4 kb; the BclI fragments with the recombinant att loci (attL, attR) were 0.8 kb and more than 8 kb. These digests were ligated to pACYC184 digested with Sph1 or BamHI. E. coli MC1061 transformants containing DNA fragments homologous to attP were selected by colony hybridization with the 0.7 kb BamHI–Sph1 VWB fragment as probe. Positive inserts were subcloned and subsequent hybridization experiments revealed the presence of attB on a 0.4 kb FspI–Smal fragment. The recombinant att loci (attL, attR) were localized on a 0.5 kb BclI–Sph1 and a 0.6 kb DraI fragment, respectively.

DNA sequences of the four att loci were aligned (Fig. 4). This alignment revealed a 45 bp sequence common to the four att sites without duplications of the chromosomal sequences in the integrated state.

**VWB DNA integrates into a putative tRNAA⁹ gene**

tRNA genes are often the sites at which the recombination events with bacterial integrative elements occur (Reiter et al., 1989). A tRNA search in the analysed sequences revealed the presence of a tRNA cloverleaf secondary structure in attB and attL transcripts (Figs 4 and 5). The 45 bp identical sequence comprises the 3' end of a tRNA⁹ (AGG) gene with a 5'CCU anticodon. The tRNA⁹ genes present in attB and attL differ from each other in that the CCA terminus typical for prokaryotic tRNAs is present in attB, but is lacking in attL. This CCA terminus is present in most bacterial tRNA genes (Sprinzl et al., 1991), but seems to be absent in the majority of Streptomyces tRNA genes. In these cases, the CCA terminus can be added post-transcriptionally by a nucleotideyltransferase. Only one of the 18 characterized S. lividans tRNA genes, i.e. cryT, encodes the CCA terminus as is the case for bldA [a tRNA⁹(TTA) gene] from Streptomyces coelicolor and Streptomyces griseus (Sedlmeier et al., 1994).

Further experiments are required to answer the question whether the tRNA⁹ genes present in attB of S. venezuelae and in attL after VWB integration into the chromosome are transcribed and functional. It was shown in other reports that the integrated plasmid pSG1 is flanked at attL by a functional tRNA⁹ gene (Bar-Nir et al., 1992) and SLP1 integrates in a biologically functional tRNA⁹ gene that is essential for host viability (Vogtl & Cohen, 1992). The tRNA⁹ gene described in this report is followed by an energetically stable stem-loop structure in both attB and attL. The putative hairpin downstream of the tRNA⁹ gene in attB has a stem size of 22 bp with one allowed G-U base pair and a loop of 3 unpaired bases (Fig. 2b). The free energy of this structure is calculated to be -48.8 kcal mol⁻¹ (-204.2 kJ mol⁻¹). The tRNA⁹ gene in attL is followed by the previously described hairpin that is equally present downstream of the attP locus. Both

![Fig. 4. Alignment of the att loci involved in site-specific recombination of the VWB genome into the S. venezuelae chromosome. The 45 bp identical sequence is presented in bold characters and the tRNA⁹ genes found both in attB and attL are boxed. Arrows represent the two types of inverted repeats seen at the 5' end of the common att region. Dotted lines mark the small 5 bp identical box.](image)

![Fig. 5. Cloverleaf structure of the tRNA⁹ genes found in attB and attL. The anticodon 5'CCU is boxed. The nucleotides common to the attP sequence are in bold characters. Circled nucleotides correspond to conserved nucleotides in tRNAs (Sprinzl et al., 1991). The CCA sequence in parentheses is present only in the tRNA gene of attB.](image)
putative hairpins may serve as transcription terminator indicative of functionality. After integration this could prevent transcription of phage genes which are possibly toxic for the host cell.

Besides VWB, the only Streptomyces phage known that uses an integrase to recombine its genome with the host chromosome is RP3. It also integrates in a tRNA$^{	ext{Arg}}$ (AGG) gene of the S. rimosus chromosome (Gabriel et al., 1995). Both tRNA$^{	ext{Arg}}$ genes are identical with the exception of one nucleotide located in the more variable tRNA acceptor stem. Although the common att region of RP3 is described to be somewhat longer than that of VWB, it shares the total 45 bp VWB attP core sequence. There are, however, no similarities in the DNA regions flanking the two attP sequences outside the tRNA sequences. Despite the similarities of the attachment sites, the RP3 and VWB Int sequences did not display a high level of similarity.

For pSE101, it was speculated that the strand cleavage in attB and attP occurs at one end of the identical segment, corresponding to the anticodon loop of the tRNA$^{	ext{Thr}}$ gene (Brown et al., 1994). For bacteriophage HP1 of Haemophilus influenzae it was reported that the HP1 integrase introduced a staggered cleavage with a 5–7 bp overlapping region. The overlap is again identical to the loop sequence of the tRNA$^{	ext{Leu}}$ anticodon arm (Hauser & Scocca, 1992). Therefore, it is possible that the attB and attP crossover between the S. venezuelae chromosome and the VWB genome takes place at the 5' end of the 45 bp segment, i.e. in the loop of the anticodon arm of the tRNA$^{	ext{Arg}}$ gene. The exact positions of cleavage by VWB Int, however, have not been located.

Two different inverted repeats were found overlapping the 5' end of the identified common att sequence (Fig. 4). In attB and attL, the inverted repeat is 5 bp long and consists of the stem of the tRNA$^{	ext{Arg}}$ anticodon arm. In attP and attR a 7 bp inverted repeat at about the same location is seen. The inverted repeats can function as recognition regions for the VWB integrase.

The meaning of the identical 5 bp box found in the att loci (Fig. 4) is not clear. In attB and attL, this box is localized in the stem of the tRNA D-arm but there is no obvious reason for the presence of this sequence in attP and attR. It could be a recognition site for the integrase or putative accessory proteins involved in the integration or excision process of the VWB genome.

The use of attP and int to construct an integrative vector

To prove the functionality and the requirement of the described int gene for site-specific recombination of attP with attB, the integrative vector pKTO2 was constructed (Fig. 6a). This non-replicative plasmid carries the thiostrepton-resistance marker, the intact VWB int gene and the VWB attP locus. After transformation of S. venezuelae and S. lividans protoplasts with pKTO2, thiostrepton-resistant clones could readily be obtained, suggesting that the pKTO2 plasmid was incorporated into the chromosome. pKTO2 transforms S. lividans
TK24 more efficiently than it does S. venezuelae ETH-14630: per µg vector DNA 300–500 S. lividans TK24 and 10–50 S. venezuelae ETH14630 integrants could be obtained. The observed difference in transformation efficiency is probably more related to the difference in stability and regeneration capacity between S. lividans and S. venezuelae protoplasts and to a different restriction barrier in both strains, rather than to the efficiency of finding the attB target sequence. In comparison with pC31-based and pSAM2-based integrating vectors, the VWB-based vector pKT02 gave rather low amounts of integrants. Kuhstoss et al. (1991) reported that a pC31-based and a pSAM2-based vector gave $3 \times 10^5$ and $6 \times 10^5$ Streptomyces ambofaciens BES2268 transformants per µg DNA, respectively. An RP3-derived integrating vector, however, transformed S. rimosus at a frequency of 10–100 transformants per µg DNA (Gabriel et al., 1995).

Confirmation of the site-specific recombination of pKT02 in the S. venezuelae as well as in the S. lividans chromosome was obtained by Southern hybridization analysis (Fig. 6b). Total DNA of thioestrepton-resistant clones was isolated and digested with HindIII. After electrophoresis and blotting, restriction fragments from the integrated DNA were identified with the digoxigenin-labelled 0.5 kb VWB BamHI-HindII fragment as probe. The results prove that the 2.3 kb VVB fragment used to construct the integrative pKT02 vector contains all information required for integration. pKT03, containing only the 0.7 kb BamHI-SphI VVB fragment, and pKT04, in which the 0.6 kb PstI fragment of pKT02 was deleted (Fig. 1), did not result in thioestrepton-resistant colonies, proving that the int gene has to be functional to achieve site-specific recombination.

In S. lividans the recombination with the host chromosome seems to occur at a different region than the pC31 attachment locus. This is concluded from hybridization experiments of the VVB attP sequence with an S. coelicolor cosmid bank. The AseI-E fragment of the S. coelicolor genome that hybridizes with VVB attP (H. Kieser, personal communication) does not contain the conserved arginine residue in the integrase family of recombinases. We thank Helen Kieser for carrying out the hybridization experiments with the S. coelicolor cosmid bank.

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