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

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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 tRNA^{Arg}(AGG) gene with a 3'-terminal CCA sequence which is typical for prokaryotic tRNAs. Phage DNA integration restores the putative tRNA^{Arg}(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. ϕ C31 (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 ϕ C31 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

The EMBL accession numbers for the sequences reported in this paper are AJ000047–AJ000050.

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 recombinase occurs into a tRNA gene (Bar-Nir *et al.*, 1992; Brown *et al.*, 1990, 1994; Mazodier *et al.*, 1990; Reiter *et al.*, 1989; Vöggtli & 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; Abremski & 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 pBluescriptIIKS(+) (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*–*HindIII* 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 [³⁵S]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 (Genoft, IntelliGenetics)

Construction of the integrative vector. For the construction of the integrative vector pKT02 (see Fig. 6), *E. coli* vector pIC20R (Marsh *et al.*, 1984) was used, in which the *tsr* gene was cloned by inserting the 1.1 kb *BclI* fragment of pIJ702 (Katz *et al.*, 1983) into the *BamHI* site. The resulting pKTO1 vector was linearized with *PstI*, blunted in a T4 DNA polymerase reaction, next digested with *HindIII* and finally ligated to the 2.3 kb *SphI* (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 265 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

Fig. 1. (a) Schematic representation of the *SphI*-B fragment of the VWB genome which contains the *int* gene (hatched box) and *attP* locus (solid black box), followed by a very stable stem-loop structure (open circle). The relevant restriction sites are indicated (B, *Bam*HI; H, *Hind*III; P, *Pst*I; S, *Sph*I). (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 *tRNA^{Arg}* gene is indicated by open arrows. Arrows under the sequence show an inverted repeat with one allowed G-U bp (□).

*Bam*HI-H1 restriction fragment. Since both the *SphI*-B and the *Bam*HI-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 *Bam*HI-*SphI* fragment that is on the right side of the *SphI*-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-Düby *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 ΔG (25 °C) of the stem-loop for the RNA molecule is calculated to be $-50.8 \text{ kcal mol}^{-1}$ ($-212.5 \text{ kJ mol}^{-1}$).

Further sequencing of the ORF (located on the upstream 1.6 kb *Hind*III-*Bam*HI 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-Düby *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-Düby *et al.*

site Tyr (position 400), two highly conserved aa, namely Gly₃₈₉ and His₃₉₀. 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 & Scoocca (1997).

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 ϕ 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).

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attB CGGCCTTCGTAGCTCAGGGGATAGAGCACCGCTCTCCTAAAGCGGGTGTCGCAGGTTCGAATCCTGCCGGGGGCACAGTTTCAG
attL CGGCCTTCGTAGCTCAGGGGATAGAGCACCGCTCTCCTAAAGCGGGTGTCGCAGGTTCGAATCCTGCCGGGGGCACACCTTGCA
attR GACTCACTGAGGCTCATGATCGCTTACGTTCTCTCCTAAAGCGGGTGTCGCAGGTTCGAATCCTGCCGGGGGCACAGTTTCAG
attP GACTCACTGAGGCTCATGATCGCTTACGTTCTCTCCTAAAGCGGGTGTCGCAGGTTTCGAATCCTGCCGGGGGCACACCTTGCA

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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 tRNA^{Arg} 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.

Nucleotide sequence of *attB*, *attL* and *attR*

Phage VWB integrates into its host chromosome by recombination with the host chromosomal *attB* locus. This recombination regenerates two hybrid sites, *attL* and *attR*. These three *att* loci were isolated from genomic libraries representing wild-type and VWB-lysogenized *S. venezuelae* strains. Chromosomal DNA of *S. venezuelae* and the lysogen was digested with *SphI* and *BclI*, respectively. On hybridization with the digoxigenin-labelled 0.7 kb *BamHI*–*SphI* VWB fragment containing the *attP* site, clear hybridization signals could be detected. The *SphI* 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 *SphI* or *BamHI*. *E. coli* MC1061 transformants containing DNA fragments homologous to *attP* were selected by colony hybridization with the 0.7 kb *BamHI*–*SphI* VWB fragment as probe. Positive inserts were subcloned and subsequent hybridization experiments revealed the presence of *attB* on a 0.4 kb *FspI*–*SmaI* fragment. The recombinant *att* loci (*attL*, *attR*) were localized on a 0.5 kb *BclI*–*SphI* and a 0.6 kb *DraIII* 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 tRNA^{Arg} 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 tRNA^{Arg}(AGG) gene with a 5'CCU anticodon. The tRNA^{Arg} 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 sequence 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 nucleotidyltransferase. Only one of the 18 characterized *S. lividans* tRNA genes, i.e. *cysT*, encodes the CCA terminus as is the case for *bldA* [a

Fig. 5. Cloverleaf structure of the tRNA^{Arg} 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*.

tRNA^{Leu}(TTA) gene] from *Streptomyces coelicolor* and *Streptomyces griseus* (Sedlmeier *et al.*, 1994).

Further experiments are required to answer the question whether the tRNA^{Arg} 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 tRNA^{Ser} gene (Bar-Nir *et al.*, 1992) and SLP1 integrates in a biologically functional tRNA^{Tyr} gene that is essential for host viability (Vögtli & Cohen, 1992). The tRNA^{Arg} 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 tRNA^{Arg} 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 $-48.8 \text{ kcal mol}^{-1}$ ($-204.2 \text{ kJ mol}^{-1}$). The tRNA^{Arg} gene in *attL* is followed by the previously described hairpin that is equally present downstream of the *attP* locus. Both

Fig. 6. (a) A schematic map of the integrative pKT02 vector. The ampicillin-resistance gene (*bla*) is only expressed in *E. coli*. The thiostrepton-resistance gene (*tsr*) is used as selection marker in *Streptomyces*. The restriction sites indicated on the map are unique and available to clone genes of interest. (b) Site-specific integration of pKT02 in the *S. venezuelae* and *S. lividans* chromosome: 2 µg *Hind*III-digested chromosomal DNA of *S. venezuelae* (lane 1), 2 µg *Hind*III-digested chromosomal DNA of *S. venezuelae*[pKT02] (lane 2), 1 ng *Hind*III-digested pKT02 (lane 3), 2 µg *Hind*III-digested chromosomal DNA of *S. lividans* (lane 4) and 2 µg *Hind*III-digested chromosomal DNA of *S. lividans*[pKT02] (lane 5) were blotted on membrane. The Southern blot was hybridized with the digoxigenin-labelled 0.5 kb *Bam*HI–*Hind*III fragment of VWB containing the *attP* locus.

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^{Arg}(AGG) gene of the *S. rimosus* chromosome (Gabriel *et al.*, 1995). Both tRNA^{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^{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^{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 sequence, i.e. in the loop of the anticodon arm

of the tRNA^{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^{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 pKT02 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 pKT02, thiostrepton-resistant clones could readily be obtained, suggesting that the pKT02 plasmid was incorporated into the chromosome. pKT02 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 ϕ C31-based and pSAM2-based integrating vectors, the VWB-based vector pKT02 gave rather low amounts of integrants. Kuhstoss *et al.* (1991) reported that a ϕ C31-based and a pSAM2-based vector gave 3.8×10^6 and 6.4×10^3 *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 thiostrepton-resistant clones was isolated and digested with *Hind*II. After electrophoresis and blotting, restriction fragments from the integrated DNA were identified with the digoxigenin-labelled 0.5 kb VWB *Bam*HI–*Hind*II fragment as probe. The results prove that the 2.3 kb VWB fragment used to construct the integrative pKT02 vector contains all information required for integration. pKT03, containing only the 0.7 kb *Bam*HI–*Sph*I VWB fragment, and pKT04, in which the 0.6 kb *Pst*I fragment of pKT02 was deleted (Fig. 1), did not result in thiostrepton-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 ϕ C31 attachment locus. This is concluded from hybridization experiments of the VWB *attP* sequence with an *S. coelicolor* cosmid bank. The *Ase*I–E fragment of the *S. coelicolor* genome that hybridizes with VWB *attP* (H. Kieser, personal communication) does not contain the ϕ C31 attachment sequence. As a consequence, the VWB-based integrative vector could be used in conjunction with ϕ C31-based vectors, which might be useful for specific applications.

If the tRNA^{Arg} gene, the recombination site for VWB integration in *S. venezuelae*, is conserved in other *Streptomyces* spp., it might be used as a general target site to introduce genes of interest. In this respect, we are currently using this vector for the transfer to *S. lividans* of genes encoding proteins of medical potential and we will further compare the stability of the recombinant strains and their yield to plasmid-based expression/secretion systems.

ACKNOWLEDGEMENTS

This research was supported by VIS (Verkennde Internationale Samenwerking – VIS/95/10) and the Fund for Scientific Research (FWO). S.S. is a research fellow of iWT.

We thank Helen Kieser for carrying out the hybridization experiments with the *S. coelicolor* cosmid bank.

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Received 1 April 1998; revised 11 August 1998; accepted 25 August 1998.