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Excisive Recombination of the SLP1 Element in *Streptomyces lividans* Is Mediated by Int and Enhanced by Xis

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The functions mediating site-specific recombination of the SLP1 element have been mapped to a 2.2-kb region that includes the site of integration (*attP*), a gene (*int*) that specifies a function both necessary and sufficient for integration of SLP1, and an open reading frame, *orf61*, suspected of encoding a protein, Xis, that shows limited similarity to the excisionases of other site-specific recombination systems. Here we describe experiments that investigate the respective roles of *orf61* and *int* in the excision of SLP1. We constructed derivatives of the high-copy-number *Streptomyces* plasmid pIJ101 that express *orf61*, *int*, or both *orf61* and *int* from transcriptional fusions to the Tn*5 aph* gene and tested the ability of these constructs to promote excision of an adventitious *attP*-containing plasmid that had been integrated site-specifically into the *attB* site of the *Streptomyces lividans* chromosome. Expression of the *int* gene product alone from an exogenous promoter was sufficient for excision of the integrated plasmid. This result indicates that the SLP1 *int*-encoded protein can carry out excisive, as well as integrative, recombination. The *orf61* gene product, when expressed from an exogenous promoter, inhibited *int*-mediated integration at the chromosomal *attB* site. Moreover, under conditions in which excision and transfer normally occur, precise excision of SLP1 was enhanced by the *orf61*-encoded protein. On the basis of these findings, we here designate the *orf61* gene as *xis*.

SLP1, a 17.2-kbp DNA segment indigenous to the chromosome of *Streptomyces coelicolor*, is the prototype of a family of transmissible chromosomally integrating plasmidogenic genetic elements of the actinomyces (reviewed in references 1, 2, and 23). In the accompanying report (7), we demonstrate that a 2.2-kb segment of SLP1 contains all of the functions required for site-specific integration of SLP1 into the *Streptomyces lividans* chromosome. Nucleotide sequence analysis of this DNA revealed two open reading frames, one of which encodes a putative 50.5-kDa protein having substantial similarity to a family of site-specific recombinases that includes the *Escherichia coli* bacteriophage λ integrase. The product of this open reading frame was shown to be both necessary and sufficient for integration of SLP1 in *S. lividans* and accordingly has been designated as the Int protein. The second open reading frame, *orf61*, which lies immediately 5′ to *int*, corresponds to a 61-amino-acid basic peptide showing limited similarity to the excisionase (*xis*) gene products of other site-specific recombination systems.

In other recombination systems that encode proteins resembling the *int* and *orf61* gene products, integration requires, in addition to host factors, only integrase, whereas excision ordinarily requires both integrase and an excisionase protein (1, 2, 14, 19). We report here the results of studies that analyze the respective roles of the SLP1 *int* and *orf61* genes in excisive recombination. We find that, under conditions in which the integrase is expressed under the control of an adventitious promoter, no other SLP1-encoded gene product is required for excision. However, under growth conditions in which the excision and conjugal transfer of SLP1 normally occur, the *orf61* gene product, which we now designate Xis, modulates the direction of the recombination reaction (integrative or excisive) and inhibits reintegration of excised SLP1.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study were *E. coli* BRL2288, a *recA56* derivative of MC1061 [F^- *araD139* Δ(*ara-leu*)7679 Δ(*lac*)*X74 galU galK hsdR2*(r_K^- m_K^-) *mcrB1 rpsL* (Str^r)] obtained from Life Technologies, Inc. (Gaithersburg, Md.), and, for the isolation of undermethylated DNA, RM1602, a nonmucoid derivative of GM33 (*dam-3* F^-) obtained from R. J. Meyer. The *S. lividans* 66 strains used were TK64 (*pro-2 str-6*) and TK23 (*spc-1*) (11).

Bacteriological methods. Conditions for growth and transformation of *E. coli* and *S. lividans* and the protocols used for isolation of plasmid and total DNA from *E. coli* and *S. lividans* as well as the conditions used for Southern blot analysis have been described elsewhere (7). The antibiotic G418 was used at a final concentration of 100 μg/ml for selection in *S. lividans*. For matings between *S. lividans* strains, approximately 10^7 donor spores and 10^9 recipient spores were combined, centrifuged, suspended in 20 μl of 0.3 M sucrose, and spotted onto an MY agar plate (10 g of malt extract, 4 g of yeast extract, 20 g of Bacto Agar per liter). Incubation was at 32°C for 24 h, after which the mating patch was excised from the plate, macerated, and used for the preparation of total DNA as described elsewhere (7).

Plasmid constructions. Numbers in brackets refer to restriction site positions in Fig. 1a. The plasmid pSUM317 is pBluescript SK^+ (Stratagene, La Jolla, Calif.) containing, at the *Sac*I-*Sma*I sites, a 487-bp *attP* (nucleotide [nt] 1689; see Fig. 3b in reference 7)-*Sca*I[12] fragment generated by exonuclease III-mung bean nuclease digestion of the 2-kb *Hin*dIII[3]-*Sca*I[12] fragment cloned into *Hin*dIII-*Sma*I sites

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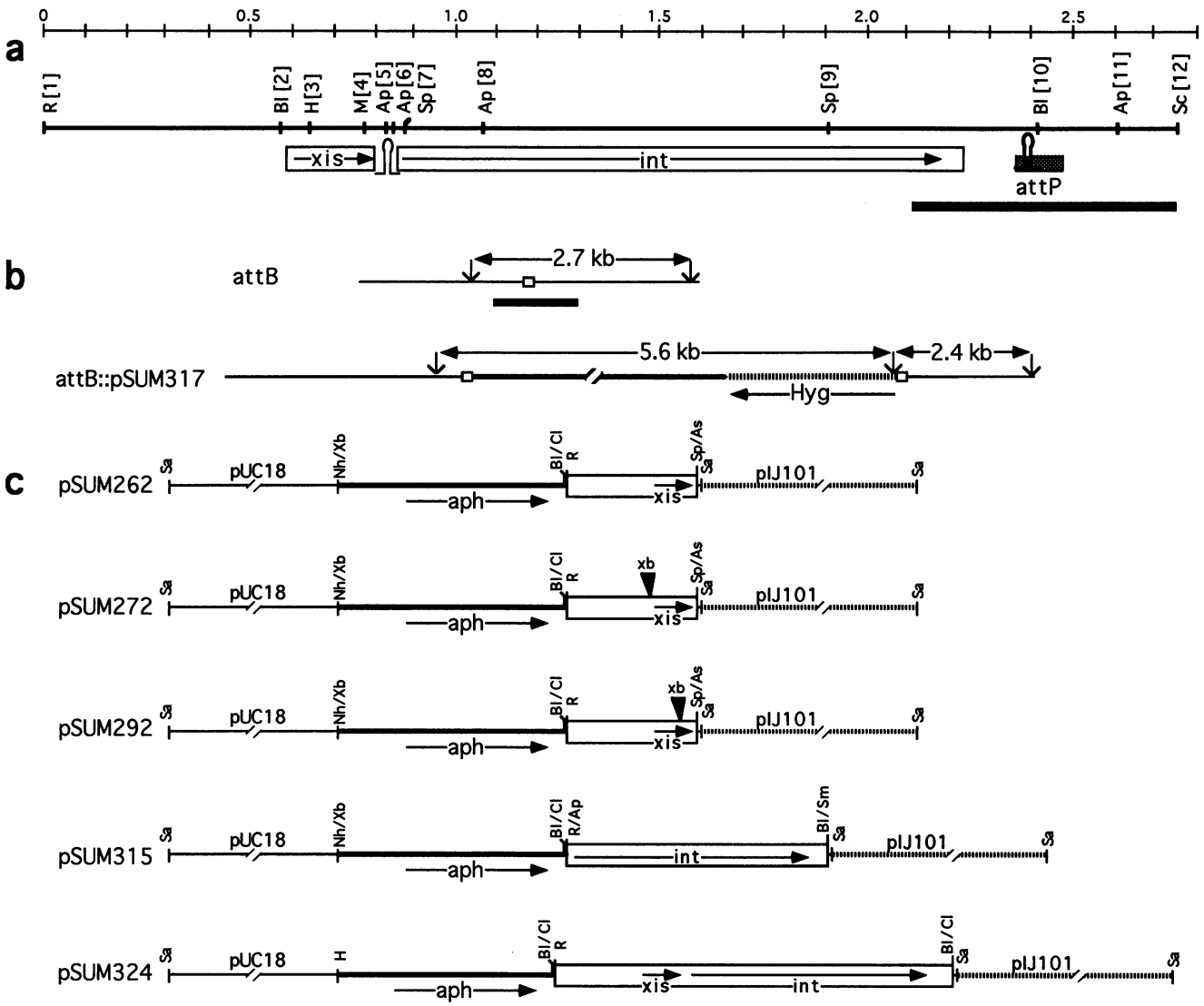

FIG. 1. Genetic organization and restriction maps of plasmids used in this study. (a) The genetic organization of SLP1 DNA involved in site-specific recombination is shown. Relevant restriction sites are shown with bracketed numbers for reference in the text, and a kilobase scale is shown above. Rectangles below the map indicate the location and direction of transcription (arrows) of genes encoding Xis (also referred to as Orf61 in the text) and integrase (Int) as well as the 112-bp segment of homology between *attP* and *attB* present (hatched rectangle) (7, 22). Stem-loop structures represent the location of putative transcription terminators. The SLP1 fragment functioning as *attP* in pSUM317 is shown by the black rectangle. (b) Positions of *Pst*I restriction sites (vertical arrows), sizes predicted for chromosomal DNA (thin line) containing the unoccupied *attB* site (open square), integrated pSUM317 that includes *E. coli* vector pBluescript SK (thick line), and a gene encoding hygromycin resistance (Hyg; hatched line). The black rectangle below *attB* shows the location of the 950-bp *Sal*I fragment cloned in pMOV110-1 and used as a probe in this study. (c) Organization of Xis- and Int-expressing plasmids. The location of *E. coli* vector pUC18 (thin lines); the *aph* gene from Tn*5* (thick lines); DNA involved in SLP recombination (open rectangles), indicating relevant genes and their direction of transcription (arrows); and *Streptomyces* plasmid pIJ101 (hatched lines) are shown. Filled triangles in pSUM272 and pSUM292 show the location of the *Xba*I linker insertion. Restriction site abbreviations: Ap, *Apa*I; As, *Asp*718; BI, *Bst*BI; Cl, *Cla*I; H, *Hin*dIII; M, *Mlu*I; Nh, *Nhe*I; R, *Eco*RI; Sa, *Sac*I; Sc, *Sca*I; Sm, *Sma*I; Sp, *Spl*I; Xb, *Xba*I.

(Fig. 1a), and, at the *Asp*718-*Hin*dIII sites, a 1.7-kb *Asp*718-*Hin*dIII fragment containing the hygromycin resistance gene.

To assess the functional properties of the *orf61* (*xis*) and *int* genes, we generated a collection of structurally similar plasmids based on the high-copy-number *Streptomyces* plasmid pIJ101 and containing various fragments of SLP1 DNA placed 3′ of the Tn*5* *aph* gene (Fig. 1c). The Tn*5* *aph* gene functions efficiently in both *E. coli* and *S. lividans* and encodes resistance to the antibiotic G418 in *S. lividans*. Insertion of this gene as a 2-kb *Nhe*I-*Bam*HI fragment from Tn*5* into the *Bam*HI site upstream of the *xylE* reporter gene in vector pXE4 (13) results in catechol dioxygenase activity 58-fold higher than that which occurs with the vector alone, when assayed in *S. lividans* TK64 (data not shown).

The Xis (Orf61)-expressing plasmid pSUM262 was constructed as follows: pSUM11 is pUC18 (30) containing, at the *Xba*I site, a 3.6-kb *Nhe*I fragment from Tn*5* (3–5) that includes the kanamycin resistance (*aph*) gene and promoter. The 0.9-kb *Eco*RI[1]-*Spl*I[7] fragment from SLP1.2 was

inserted into the *Bst*BI-*Asp*718 sites of pSUM11 as a *Cla*I-*Spl*I fragment with the polylinker from pSP72 (Promega Corp., Madison, Wis.). The resulting construct, pSUM260, contains SLP1 DNA between *Eco*RI and *Spl*I immediately downstream of the Tn*5* *aph* gene. The high-copy-number *Streptomyces* plasmid pIJ101 (15) was linearized by treatment with *Sac*I and ligated to *Sac*I-digested pSUM260, generating pSUM262. The plasmids pSUM272 and pSUM292 are identical to pSUM262 except that prior to ligation with pIJ101, a 14-bp *Xba*I nonsense linker (CTAGTCTAGACTAG) purchased from U.S. Biochemical, Cleveland, Ohio (no. 70882), was inserted into pSUM260 at the *Bst*BI site [2] or *Mlu*I site [4] that had been blunt ended with the Klenow fragment of DNA polymerase I. The plasmid pSUM297 is pSP72 containing, at the *Sma*I site, SLP1 DNA from the *Mlu*I[4]-*Bst*BI[10] sites that had been made blunt ended with the Klenow fragment of DNA polymerase I. A potential transcriptional terminator between the *xis* and *int* genes (Fig. 1a) (7) was removed by digesting pSUM297 with *Eco*RI and partially with *Apa*I and then incubating the plasmid with T4 DNA polymerase prior to ligation. One resulting construct, pSUM306, was found by nucleotide sequence analysis to possess an *Eco*RI-SLP1 junction (GAATT-GAGGCG) at nt 346 (see Fig. 3b in reference 7), 6 nt upstream of the *Apa*I[6] site. The SLP1 DNA from pSUM306 was inserted, as a *Cla*I-*Bst*I fragment, into the *Bst*BI-*Bst*I sites of pSUM11 (above), placing the SLP1 *int* gene (nt 346 to *Bst*BI[10]) immediately 3′ of the Tn*5* *aph* gene. This plasmid, pSUM307, was linearized with *Sac*I and ligated to *Sac*I-digested pIJ101, giving pSUM315. A plasmid expressing both *xis* and *int*, pSUM318, was constructed by inserting SLP1 DNA *Bst*BI[2]-*Bst*BI[10] as an *Eco*RV-*Sac*I fragment (with the polylinker from plasmid pSP72) into the *Sma*I-*Sac*I sites of pSUM11 and then inserting pIJ101 at the *Sac*I site. However, we were unable to introduce pSUM318 into protoplasts of *S. lividans* TK64, presumably because of overexpression of Xis and/or Int. We therefore inserted DNA upstream of the *xis* gene by replacing a 1.5-kb *Hin*dIII fragment that includes the Tn*5* *aph* gene and SLP1 DNA *Bst*BI[2]-*Hin*dIII[3] with that from pSUM262 (above). The resulting plasmid, pSUM324, contains SLP1 DNA *Eco*RI[1]-*Bst*BI[10] immediately downstream of the Tn*5* *aph* gene and inserted at the *Sac*I site of pIJ101.

The plasmid pCAO153 is SLP1 into which pACYC177 (9) and a thiostrepton resistance gene were inserted at the unique *Bam*HI site (24). pSUM343 is pCAO153 containing the 14-bp *Xba*I nonsense linker (above) inserted at the *Mlu*I[4] site. An *Eco*RI-*Bam*HI fragment from pCAO153 that contains 4.3 kb of SLP1 DNA, which had been inserted into the *Eco*RI-*Bam*HI sites of pSP72, was linearized at the unique *Mlu*I[4] site, treated with the Klenow fragment of DNA polymerase I, and ligated to the *Xba*I linker. The *Eco*RI-*Bam*HI fragment from this plasmid, which now contains the *Xba*I linker at *Mlu*I[4], was exchanged with that from pCAO153. pSUM352 was constructed similarly: pSUM49 is pBR322 (6) containing, at the *Eco*RI-*Bam*HI sites, a 4.3-kb *Eco*RI-*Bam*HI segment of SLP1 DNA that contains the Int-encoding region. This plasmid was digested with *Hin*dIII and *Mlu*I and treated with the Klenow fragment of DNA polymerase I and T4 DNA ligase. An *Eco*RI-*Spe*I fragment from the resulting plasmid, pSUM349, containing a deletion within the *xis* gene, was used to replace the *Eco*RI-*Spe*I fragment of pCAO153, giving pSUM352.

RESULTS

SLP1 Int alone can mediate excision as well as integration. Figure 1 summarizes the genetic organization of the segment of SLP1 involved in site-specific recombination. We have shown that the *int* gene encodes a product necessary for integration and have speculated that the open reading frame *orf61* encodes a protein implicated in excision (7). To test directly the roles of *orf61* and the *int* gene in SLP1 excision, we expressed Int and Orf61 both separately and concurrently in *S. lividans* and monitored excision of a nonreplicating hygromycin resistance (Hyg^r) plasmid that had been integrated site-specifically at the SLP1 *attB* site. Expression was accomplished by constructing transcriptional fusions to the *aph* gene from Tn*5* (3, 4) and inserting the resulting cassette, which also contains the *E. coli* vector pUC18 (30), into the high-copy-number *Streptomyces* plasmid pIJ101 (15). The genetic organization of these plasmids is presented in Fig. 1c.

To assay excision, we constructed a strain that has a replication-deficient plasmid integrated site-specifically at the SLP1 chromosomal attachment site, *attB*. This plasmid, pSUM317, is a Hyg^r derivative of *E. coli* vector pBluescript SK$^+$ containing a 485-bp segment of SLP1 DNA (nt 1689 to *Sca*I[12]; see Fig. 3b in reference 7 and Fig. 1a above) that includes *attP*. Approximately 2.5 μg of pSUM317 DNA was introduced together with 2 μg of pSUM307 DNA (the precursor to pSUM315 [Fig. 1c] that expresses Int but lacks pIJ101 and is therefore unable to replicate in *S. lividans*) into protoplasts of TK64. Using this protocol, in which Int is expressed transiently, we obtained approximately 1,500 Hyg^r transformants.

Six independent transformants were analyzed by Southern blot hybridization to *Pst*I-digested total DNA, by using as a probe a 950-bp *Sal*I fragment containing a segment of *S. lividans* chromosomal DNA that includes *attB* (29) and that had previously been cloned in pUC19 (pMOV110-1). As seen in Fig. 2 (lane 7), the unoccupied *attB* site generated a 2.7-kb hybridizing band; this band was lost upon integration of pSUM317 into *attB*, and two new bands corresponding to *attL* (5.6 kb) and *attR* (2.4 kb), the sites flanking the integrated SLP1 element, were observed. DNA from three transformants showed hybridizing bands of the size predicted for integration of pSUM317 into *attB* (Fig. 2, lanes 3, 4, and 6). Of the remaining three isolates, two showed hybridization patterns consistent with tandem integration of two copies of pSUM317 into *attB* (i.e., fragments of the size predicted for *attL* and *attR* and an additional band migrating at a position predicted for linear pSUM317 [5.2 kb, lanes 1 and 2]). One shows integration of pSUM317 into *attB* but contains an altered *attR* fragment (lane 5). Control experiments in which pSUM317 was introduced in the absence of pSUM307 failed to produce hygromycin-resistant transformants. A representative strain, MAB.S159, shown in Fig. 2, lane 4, was used for subsequent experiments.

The above results indicate that the *int* gene is expressed functionally from pSUM307 and that transient expression of SLP1 Int in *trans* was sufficient to promote site-specific integration of a nonreplicating circular DNA fragment containing *attP*. During the course of the above experiments, we were repeatedly unsuccessful in attempts to integrate a plasmid similar to pSUM317 but containing a segment that includes only the 112-bp homology between *attP* and *attB* instead of the 485-bp *attP* fragment (pSUM4). This observation is considered further in the Discussion.

Protoplasts of strain MAB.S159 were prepared, and plas-

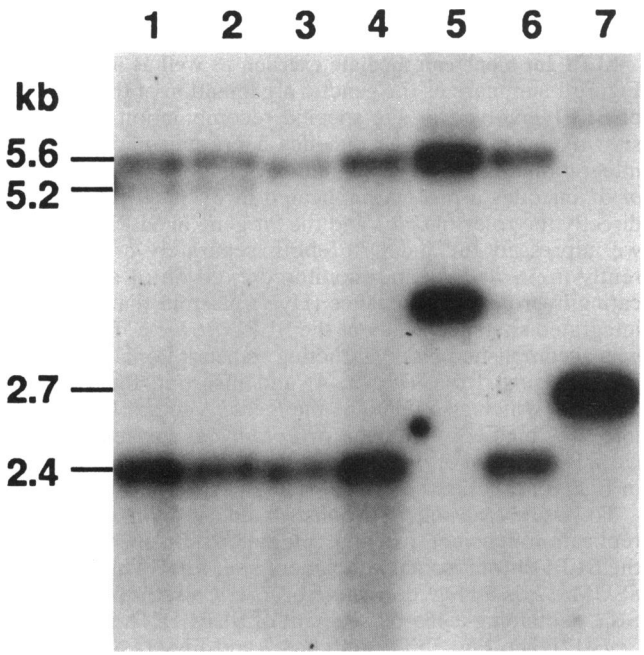

FIG. 2. Site-specific integration of pSUM317. Southern blot analysis of *Pst*I-digested total DNA from Hyg^r transformants of TK64 cotransformed with pSUM317 and pSUM307. The predicted *Pst*I restriction fragments for the free *attB* site and integrated pSUM317 are shown in Fig. 1b. Lanes 1 to 6, independent Hyg^r transformants; lane 7, TK64. The positions of *attB* (2.7 kb), *attL* (2.4 kb), *attR* (5.6 kb), and linear pSUM317 (5.2 kb) are indicated. The probe was a ^{32}P-labeled 950-bp *Sal*I *attB* fragment (29) from pMOV110-1 (Fig. 1b).

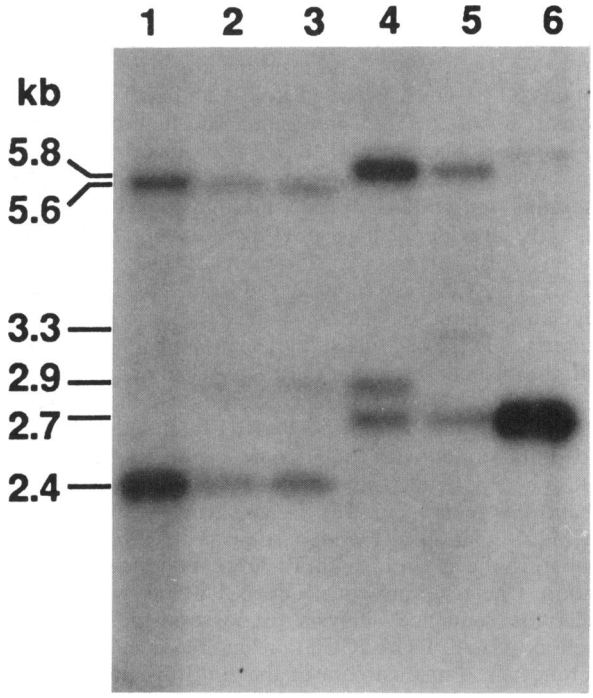

FIG. 3. SLP1 Int promotes excision. Southern blot analysis of *Pst*I-digested total DNA from pooled transformants of MAB.S159 containing the indicated expression plasmid. Lane 1, MAB.S159; lane 2, MAB.S159(pSUM262); lane 3, MAB.S159(pSUM292); lane 4, MAB.S159(pSUM315); lane 5, MAB.S159(pSUM324); lane 6, TK64. The probe was a ^{32}P-labeled 950-bp *Sal*I fragment of pMOV110-1 that contains *attB*. The positions of *attB* (2.7 kb), *attL* (2.4 kb), and *attR* (5.6 kb) of the integrated pSUM317 are indicated. Bands at 2.9 kb (lanes 2 to 4) and 3.3 kb (lane 5) are due to hybridization of the pUC18 portion of the expression plasmids with contaminating pUC19 sequences in the probe, and those at 5.8 kb (lanes 4 and 5) are due to hybridization of the *attP* site on the expression plasmids to homologous sequences in *attB*.

mids pSUM262 (Orf61), pSUM292 (identical to pSUM262 but containing within *orf61* a 14-bp linker having nonsense codons in each reading frame), pSUM315 (Int), and pSUM324 (Orf61 and Int) were introduced by transformation. Resulting G418-resistant transformants were pooled by scraping colonies from regions of confluency, patched to MY agar plus G418, and then inoculated into liquid medium plus G418 for isolation of total DNA. The result of Southern blot hybridization to *Pst*I-digested total DNA is shown in Fig. 3. MAB.S159 (Fig. 3, lane 1), containing either the *orf61*-expressing plasmid pSUM262 (Fig. 3, lane 2) or control plasmid pSUM292 (Fig. 3, lane 3), retained the integrated pSUM317 (bands corresponding to *attL* [5.6 kb] and *attR* [2.4 kb]), indicating that Orf61 alone was insufficient to promote excision. That *orf61* is expressed from pSUM262 is demonstrated below. In contrast, excision of pSUM317 was observed following the introduction of plasmid pSUM324, which expresses both Orf61 and Int: bands corresponding to *attL* (5.6 kb) and *attR* (2.4 kb) are lost, and the *attB* band (2.7 kb) is regenerated. Excision of pSUM317 was also observed in the presence of pSUM315, expressing Int alone (lane 4). These data indicate that the product of the SLP1 *int* gene is sufficient to promote both integrative and excisive recombination.

The *orf61* gene product inhibits SLP1 Int-mediated integration. The finding that the SLP1 Int protein alone can promote excision prompted us to further examine the role of the *orf61* gene product in SLP1-mediated site-specific recombination. To investigate whether expression of the Orf61 protein in *S. lividans* can inhibit Int-promoted integration, as does the Xis protein of bacteriophage λ in *E. coli* (2, 20, 21, 28) we used the plasmid pSUM154 (7), a derivative of the SCP2-based vector pOJ160 that carries a segment of SLP1 DNA including *int* and *attP* (*Hin*dIII[3]-*Sca*I[12], Fig. 1a). It is shown elsewhere (7) that pSUM154 exists autonomously as well as integrated at the SLP1 *attB* site. However, because this plasmid is an unstable replicon, strains containing pSUM154 eventually lose the autonomous form during culture (Fig. 4, lane 6).

Three plasmids were tested for their ability to inhibit pSUM154 integration; the *orf61*-expressing plasmid pSUM 262 and the control plasmid pSUM292 are described above and shown in Fig. 1c. To confirm the proposed ATG translational start codon assignment for the Orf61 protein, we also constructed pSUM272, which is analogous to pSUM262 but has the 14-bp nonsense linker used in the construction of pSUM292 inserted at the *Bst*BI site [2] 50 nt upstream of the predicted start of *orf61* (Fig. 1a). Each of these plasmids was introduced separately into *S. lividans* TK64 by transformation; selection for G418 resistance was carried out, and protoplasts of each resulting strain were prepared and transformed to thiostrepton resistance (Tsr^r) with plasmid pSUM154. Total DNA prepared from representative independent transformants was digested with *Bcl*I and electrophoresed on a 0.8% agarose gel for Southern blot hybridization with ^{32}P-labeled pSUM154 as a probe. The

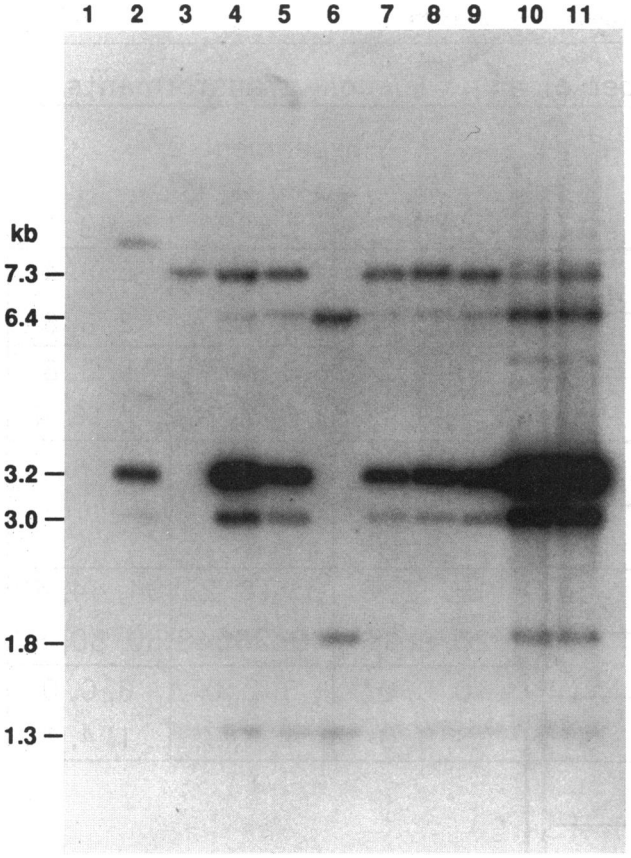

FIG. 4. Inhibition of pSUM154 integration by *orf61*. Southern blot analysis of total DNA digested with *Bcl*I from the following sources is shown. Lane 1, TK64; lane 2, TK64(pSUM262); lane 3, pSUM154 plasmid DNA from *E. coli*; lanes 4 and 5, independent isolates of TK64(pSUM262)(pSUM154); lane 6, TK64(pSUM154); lanes 7 to 9, independent isolates of TK64(pSUM272)(pSUM154); and lanes 10 and 11, independent isolates of TK64(pSUM292)(pSUM154). The probe was ^{32}P-labeled pSUM154. The structures and predicted restriction fragment sizes of autonomous and integrated pSUM154 have been published (7). The structures of the expression plasmids are shown in Fig. 1c. Note that the ratio of the integrated form of pSUM154 (6.4 and 1.8 kb) to the autonomous form (7.3 kb) is much lower in the presence of pSUM262 and pSUM272 than in the presence of pSUM292.

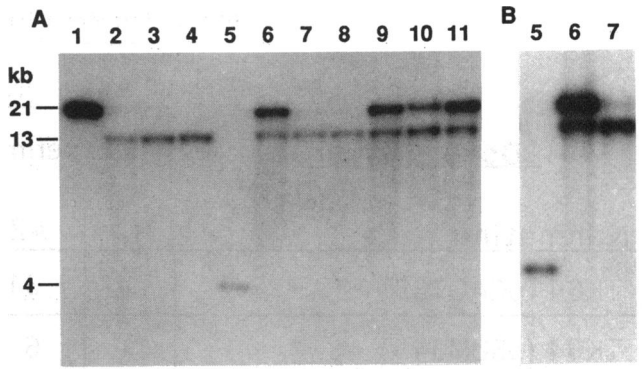

FIG. 5. Site-specific integration of plasmids containing mutated *xis* genes. (A) Southern blot hybridization of *Bgl*II-digested total DNA from TK64 transformed with pSUM343, pSUM352, or pCAO153. Lane 1, pCAO153 purified from *E. coli* and digested with *Bgl*II; lanes 2 to 4, independent isolates of TK64(pSUM343); lane 5, TK64; lanes 6 to 8, independent isolates of TK64(pCAO153); lanes 9 to 11, independent isolates of TK64(pSUM352). Hybridization signals corresponding to *attB* (4-kb) and the *attL* (12-kb) and *attR* (13-kb) fragments of the integrated elements are indicated, as is the position of linear pCAO153 (ca. 21 kb). (B) An independent Southern blot analysis using the DNA samples from panel a and showing lanes 5 to 7 that reveals in lane 7 the presence of free *attB* sites (4 kb) and autonomous pCAO153 (ca. 21 kb) as considered in the Discussion. The probe is ^{32}P-random-primer-labeled pMOV110-1.

predicted restriction patterns of autonomous (7.3-, 1.3-, and 0.2-kb bands) and integrated (6.4-[*attL*], 1.8-[*attR*], 1.3-, and 0.2-kb bands) pSUM154 have been described (see Fig. 2a in reference 7). The resulting blot (Fig. 4) shows that while the majority of pSUM154 DNA existed in the integrated state in the presence of control plasmid pSUM292 (lanes 10 and 11), which contains an inactive *orf61*, the ratio of integrated plasmid (6.4 [*attL*] and 1.8 [*attR*] kb) to autonomous plasmid (7.3 kb) was greatly reduced in the presence of both pSUM262 (lanes 4 and 5) and pSUM272 (lanes 7 to 9). These data indicate that the product of the *orf61* gene inhibits SLP1-specific Int-promoted integration.

The *orf61*-encoded protein increases the number of excision events in strains containing integrated SLP1. Excision of plasmid pSUM154 from the *S. lividans* chromosome to regenerate autonomous plasmids has not been observed, implying that notwithstanding the ability of Int to promote excision, recombination in nonmating cells is strongly in the direction of integration. However, excision of SLP1 normally is induced by mating, which results in transiently existing circular forms of SLP1. If these transfer intermediates include an *E. coli* replicon, they can be detected by transformation of *E. coli* with total DNA prepared from mating *Streptomyces* cells (18). We used this strategy to examine the role of the Orf61 protein in excision during mating of strains containing integrated SLP1.

The plasmid pCAO153 and its derivatives pSUM343 and pSUM352, which contain a nonsense codon and deletion, respectively, in *orf61* (see Materials and Methods), were separately introduced by transformation into protoplasts of TK64. Total DNA prepared from three representative Tsr^r transformants from each transformation was digested with *Bgl*II and examined by Southern blot hybridization with ^{32}P-labeled pMOV110-1 as a probe. In all cases, the input plasmid was found integrated at *attB*; a 4-kb *Bgl*II fragment corresponding to the unoccupied *attB* site (Fig. 5a, lane 5) was lost, and two new fragments that migrate as a doublet of approximately 13 kb, corresponding to the *attL* and *attR* sites of the integrated SLP1 derivative, were observed. An additional band was also observed at a position predicted for *Bgl*II-digested pCAO153 (21 kb, lanes 1, 6, and 9 to 11). These bands presumably result from tandem integration of an additional copy (or more) of the input plasmid at *attB*.

Transfer intermediates were detected by mixing approximately 10^7 spores of the donor strains examined for Fig. 5 with 10^9 spores of recipient strain TK23 and allowing these cells to mate for 24 h on MY plates. At this point, aerial mycelia were just beginning to form in some of the mating patches. Cells from these matings were scraped from the plate, macerated, and used directly for the preparation of total DNA. *E. coli* BRL2288 was transformed to ampicillin resistance (Amp^r) with 1-μg aliquots of total DNA prepared from mating cells or from mock matings, which were performed in the absence of recipient cells with the independently derived donor strains examined above. The result of

TABLE 1. Transformation of *E. coli* to ampicillin resistance

Donor Strain	Number of amp^r *E. coli* transformants					
	Experiment 1[a]			Experiment 2[b]		
Nonmating	#1	#2	#3	#1	#2	#3
TK64 (pCAO153)	50	31	21	1, 2, 5	13, 13, 3	6, 8, 4
TK64 (pSUM343)	5	6	23	0, 0, 0	16, 15, 6	2, 6, 6
TK64 (pSUM352)	0	8	36	3, 4, 2	6, 7, 3	5, 5, 8
TK23	0			1, 0, 1	5, 5, 2	9, 16, 4
Mating						
TK64 (pCAO153) x TK23	88	110	242	19, 13, 8	35,18, 25	54, 44, 40
TK64 (pSUM343) x TK23	0	0	5	23, 9, 16	40, 23, 46	50, 30, 52
TK64 (pSUM352) x TK23	6	18	14	0, 0, 0	3, 2, 0	6, 0, 0
100 pg pCAO153	5.8×10^6			3, 3, 0	1, 1, 3	1, 4, 1
1 ug TK23 + 1ng pCAO153	2.6×10^5					

[a] Total DNA prepared from mating patches in which approximately 10^7 spores from each of three independent donors per strain (no. 1, 2, and 3) were plated alone (nonmating) or together with approximately 10^9 recipient spores (mating) was used to transform *E. coli* BRL2288 to ampicillin resistance (Amp^r). Following heat shock, the cells were incubated at 37°C for 90 min prior to plating. The total number of Amp^r transformants obtained from 1 μg of total DNA per transformation is shown. Purified pCAO153, isolated from *E. coli* by isopycnic centrifugation, was used to assess the transformation efficiency of the *E. coli* cells.

[b] Aliquots of total DNA (1 μg) from donors containing pCAO153 or pSUM352 obtained in experiment 1 were mixed with competent cells and immediately divided into three tubes. The procedure was performed in duplicate to give six independent platings per donor. The numbers of Amp^r transformants from each of the three platings corresponding to 1 μg of input total DNA are shown in rows. Conditions were as described in footnote *a*.

transformation using total DNA from mating mixtures (Table 1, experiment 1) shows that the number of transformants obtained from *orf61*$^+$ donors increased 2- to 10-fold over that of nonmating donors. In contrast, while transformants were observed for both mating and nonmating *orf61* mutant donors, the number of colonies obtained was not affected by mating.

The Amp^r colonies obtained are the product of various recombination mechanisms that generate transformants, i.e., recircularization of sheared total DNA, homologous recombination, and site-specific excision, etc. To determine the fraction of transformants that specifically result from precise excision, the above experiment was repeated with total DNA from donor strains containing pSUM352 (*orf61* mutant) and pCAO153 (*orf61*$^+$), except that, following mixing of the DNA and competent cells, the cultures were split into three tubes to control for sibling transformants and the experiment was done in duplicate to provide 6 independent transformants per mating and a maximum of 18 nonsibling transformants per donor strain. As a control, we also used total DNA from MAB.S159 (above) to assess the frequency of excision in the absence of both Orf61 and Int. The result of these transformations is summarized in Table 1, experiment 2.

Physical examination of independent transformants from mating donors showed that precise excision had occurred in 18 of 18 transformants from the *orf61*$^+$ donor and in 4 of 11 transformants from *orf61* mutant donors. Restriction analysis of 15 independent transformants from mock matings with *orf61*$^+$ donors showed that 11 of 15 contained plasmids having structures consistent with precisely excised molecules while only 1 of 17 from *orf61* mutant donors did. We also obtained seven transformants from one plating using DNA from MAB.S159; none of these had structures predicted for precisely excised pSUM317 (data not shown). These data indicate that precisely excised molecules are present in nonmating cells and that the prevalence of these molecules is substantially enhanced in the presence of *orf61*. The relatively low frequency with which we observe precise excision of *orf61* mutant donors during mating indicates that Xis-independent excision of SLP1 is not common under conditions in which transfer of SLP1 normally occurs. On the basis of these data and results described above, we designate *orf61* as *xis*.

DISCUSSION

The segment of SLP1 DNA that mediates site-specific recombination lies within a 2.2-kb region of the element and includes two *trans*-acting genes, *xis* and *int*, plus a *cis*-acting site, *attP* (Fig. 1a) (7). The product of the SLP1 *int* gene, which shows amino acid similarities with the Int protein of bacteriophage λ and related site-specific integrases, is both necessary and sufficient to promote integration of SLP1 at its chromosomal attachment site, *attB* (7). The experiments reported here indicate that the *int*-encoded protein can also mediate excisive recombination of SLP1 at the *attR* and *attL* sites that bracket the integrated element, yielding extrachromosomal SLP1 molecules; a second SLP1-encoded protein, Xis, both enhances the excisive activity of Int and inhibits reinsertion of SLP1 elements that have been excised.

Excision of bacteriophage λ from the *E. coli* chromosome ordinarily requires expression of both the Int and Xis proteins (1); however, like the Xis protein of SLP1, the Xis protein of λ may, under at least some circumstances, be dispensable for excision. Shimada and Campbell (26) observed that a λ lysogen defective for Xis, but showing increased Int activity (8) as the result of an IS*2* insertion upstream of *int* (31), cures at a frequency that, although very low, is significantly higher than the frequency of curing of an *xis* mutant lysogen that expresses *int* from its native promoter. Moreover, purified λ Int protein, in the presence of host factors, has been found to promote (albeit inefficiently) site-specific excision of λ in vitro in the absence of Xis (1). Int-promoted excision has also been reported for the *Streptococcus pneumoniae* conjugative transposon Tn*1545* (25).

While the SLP1 Xis-encoding gene is not absolutely required for excision of this genetic element, our data show that it can alter the equilibrium between integration and excision by inhibiting integration. Inhibition of integration by Xis could result from the occlusion of Int binding sites on SLP1 DNA, direct effects on Int expression or activity, or as in bacteriophage λ, formation of higher-order complexes that are not suitable structures for integrative recombination (16, 20). Functionally, the SLP1 Int and Xis proteins parallel those of bacteriophage λ (reviewed in references 17 and 27). Consequently, we suspect that the functional similarities between λ and SLP1 will extend also to the mechanisms by which these proteins promote integrative and excisive recombination.

Plasmids showing a structure consistent with precise excision were detected in mock matings in which donor cells containing *xis*$^+$ SLP1 plasmids were plated in the absence of recipient. Similar experiments reported previously (18) failed to detect these free circular molecules in nonmating cells, leading to the conclusion that excision is induced by mating. While we found a 2- to 10-fold increase in the abundance of autonomous *xis*$^+$ SLP1 molecules during mating, the presence of precisely excised SLP1 plasmids in nonmating cells indicates that mating is not required for excision. The presence of free *attB* sites (Fig. 5b, lane 7) as well as bands that comigrate with linear pCAO153 is consistent with the presence of free SLP1 plasmids in nonmating cells.

We also detected rare precisely excised SLP1 molecules that lack a functional *xis* gene in mating and nonmating cells. The mechanism by which these molecules are formed is unknown. However, their presence is noteworthy: we observed that the transfer frequency of chromosomally integrated *xis* mutant SLP1 elements is only marginally lower (5- to 10-fold) than that of similarly integrated *xis*$^+$ SLP1 elements, suggesting that once a transfer intermediate is formed, it can be spread throughout the mycelium with extraordinary efficiency. This notion is consistent with the observation that plasmid transfer originating from a single germinating spore is sufficient to form a pock, a zone of retarded recipient growth that resembles a phage plaque and is characteristic of plasmid transfer in *Streptomyces* spp. It is unclear to what extent rare excision events might contribute to SLP1 transfer in nature, where recipients may not be in vast excess and where the time and conditions for mating are not ideal.

The SLP1 *attP* site shares a 112-bp region of near identity (differing at only a single nucleotide) with chromosomal DNA containing the *attB* site (22). Consequently, integration of SLP1 generates *attL* and *attR* sites retaining homologies identical in sequence to *attP* and *attB*. It is now clear that because the *attB* site lies within the 3′ end of a gene encoding a $tRNA^{Tyr}$ essential for viability of *S. lividans* (29), at least part of the 112-bp homology is required to regenerate this gene upon integration. We have shown previously that no more than 48 bp of the 112-bp *attB-attP* homology is required for a sequence to function as an *attB* site (18). However, during construction of a substrate for excision assays, we found that, while the Int-expressing plasmid pSUM307 can promote integration of a plasmid that contains a 485-bp segment of SLP1 DNA flanking *attP* (pSUM317, Fig. 1b), we were repeatedly unsuccessful in integrating a similar plasmid containing only the 112-bp segment of homology. This observation suggests that a functional *attP* site requires SLP1 sequences outside the 112-bp region of homology between *attP* and *attB*. If this notion were correct, the *attL* and *attR* sites that result from integrative recombination would differ in structure from *attB* and *attP*. We suggest that this structural difference provides a molecular basis for allowing the determination of integration versus excision to be made. Recently, Hauser and Scocca (10) have shown that phage HP1 of *Haemophilus influenza*, which integrates into an operon containing sequences that resemble tRNA genes and which shows a 182-bp segment of sequence identity between *attP* and a DNA segment that contains *attB*, requires a 418-bp segment of phage DNA for *attP* function.

Our demonstration that expression of SLP1 Int from a transiently existing plasmid can promote stable integration of a coexisting nonreplicating *attP*-containing plasmid enables adventitious DNA segments linked only to *attP* to be introduced conveniently into the *S. lividans* chromosome or into extrachromosomal replicons that contain *attB*. Potentially, plasmids promoting excision can be used for subsequent removal and recovery of these integrated sequences.

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