Site-Specific Integration in *Saccharopolyspora erythraea* and Multisite Integration in *Streptomyces lividans* of Actinomycete Plasmid pSE101

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An 11.3-kilobase-pair plasmid, designated pSE101, exists in *Saccharopolyspora erythraea* NRRL 2338 as an integrated sequence (pSE101\(^{\text{int}}\)) at a unique chromosomal location and in the free form in less than an average of 1 copy per 10 chromosomes. The plasmid sequence is missing from *S. erythraea* NRRL 2359. Restriction maps of the free and integrated forms of pSE101 showed point-to-point correspondence. Plasmid pECT2 was constructed by ligation of pSE101, pBR322, and the gene for thiostrepton resistance (*ttr*). When introduced by polyethylene glycol-mediated transformation into protoplasts of *S. erythraea* NRRL 2359, all thiostrepton-resistant regenerants examined were found to carry a single copy of pECT2 in the integrated state at a single chromosomal site. The chromosomal site of pECT2 integration in strain NRRL 2359 (attB) corresponded to the chromosomal location of pSE101\(^{\text{int}}\) in strain NRRL 2338. The plasmid crossover site (attP) was mapped to the plasmid site that corresponded to the site of interruption of the plasmid sequence in the host carrying pSE101\(^{\text{int}}\), indicating that site-specific integrative recombination had occurred. An additional 2.8-kilobase-pair chromosomal sequence homologous to a segment of pSE101 was also observed in strains NRRL 2338 and NRRL 2359. After introduction of pECT2 into *Streptomyces lividans*, approximately half of the transformants examined were found to carry the plasmid as a stable, autonomously replicating element. The other half carried a single copy of pECT2 as an integrated sequence, but the location of pECT2\(^{\text{int}}\) in *Streptomyces lividans* varied from one transformant to another. In each case, integrative crossover used the attP site. A model is proposed to account for the determination of the particular state of pSE101 in *Streptomyces lividans*.

Several plasmids of actinomycetes, the filamentous bacteria that produce greater than 60% of all known antibiotics, have been shown to exist in both autonomously replicating and chromosomally integrated states. For some plasmids, only the integrated state was observed in one host, but the autonomous state was produced after conjugal transfer of the plasmid to a plasmid-free host (1, 9, 10, 20, 23). In other instances, both free and integrated states were observed for plasmids in particular hosts (4, 20, 23) but it was not established whether both forms were present in the same mycelium or whether the plasmids could replicate autonomously in a cell that contained an integrated copy. The demonstration of conversion from the integrated to the autonomous state within a given host has also not yet been reported.

In a number of cases studied carefully, an undeleted, uninterrupted, linear integrated sequence of the plasmid at a single chromosomal location was demonstrated either in naturally occurring hosts that contained the integrated plasmid or in hosts into which the plasmid was introduced and which had undergone site-specific integration (1, 18, 21, 25). Short regions of cognate plasmid-chromosome homology have been identified as crossover sites for the integration of two integrative plasmids (18, 22). No example of plasmid integration at more than one chromosomal site has yet been reported.

In this paper, we present a characterization of both integrated and autonomous forms of an 11.3-kilobase-pair (kb) plasmid designated pSE101 from the erythromycin-producing organism, *Saccharopolyspora erythraea* NRRL 2338 (formerly called *Streptomyces erythraeus* [16]) and demonstrate site-specific integration at a single site in *S. erythraea* and at multiple chromosomal sites in *Streptomyces lividans*. Autonomous replication of pSE101 in *Streptomyces lividans* is also demonstrated.

(A preliminary report of this work has appeared previously by S. D. Chiang J. Tuan, D. Brown, and L. Katz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H193, p. 140. We have changed the name of the plasmid from pSE1 to pSE101 to avoid confusion with a plasmid described elsewhere [27].)

**MATERIALS AND METHODS**

**Bacteria, bacteriophage, and plasmids.** *Escherichia coli* HB101 (3), used where indicated, plasmids pBR322 and pBR325 (2), and phage T1 1059 (12) were obtained from commercial sources. *Streptomyces lividans* and *S. erythraea* strains and plasmids used are listed in Table 1.

**Media, culture conditions, and transformation.** *Streptomyces lividans* was grown in YEME liquid medium (8) containing 0.2% glucose or on Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.). *S. erythraea* was grown in SGGP (0.4% peptone [Difco Laboratories, Detroit, Mich.], 0.4% yeast extract [Difco], 0.05% MgSO\(_4\)·7H\(_2\)O, 1% glucose, 0.2% glycine, 10 mM KPO\(_4\) buffer [pH 7.0]). R3M medium contained 2.0% (wt/vol) agar, 0.4% Bacto-tryptone, 0.4% yeast extract (Difco), 0.4% Casamino Acids, 0.025 M Tris hydrochloride (pH 7.2), 0.05% KH\(_2\)PO\(_4\), 0.05 M MgCl\(_2\), 0.05 M CaCl\(_2\), 0.025% K\(_2\)SO\(_4\), 0.0025 M NaOH, 1% d-glucose, 10.3% sucrose, and trace elements as described by Hopwood et al. (8). PT buffer is P buffer (8) lacking KH\(_2\)PO\(_4\). Transformation of *S. erythraea* protoplasts by using selection for thiostrepton resistance was performed essentially as described by Hopwood et al.
TABLE 1. Strains and plasmids used

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant properties or method of construction</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharopolyspora erythraea</em> NRRL 2338</td>
<td>Source of pSE101</td>
<td>Abbott collection</td>
</tr>
<tr>
<td><em>Saccharopolyspora erythraea</em> NRRL 2359 SE2001-SE2012</td>
<td>Devoid of pSE101</td>
<td>Abbott collection</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em> 66 AL301-AL345</td>
<td>Devoid of pSE101 sequences</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
<td>tsr gene contained on 1.05-kb BclI fragment</td>
<td>13</td>
</tr>
<tr>
<td>pJ704</td>
<td>11.3 kb, isolated from <em>Saccharopolyspora erythraea</em> NRRL 2338 (Fig. 1)</td>
<td>This work</td>
</tr>
<tr>
<td>pSE101</td>
<td>Ligation of EcoRI-digested pBR325 and pSE101</td>
<td>This work</td>
</tr>
<tr>
<td>pEC5</td>
<td>Ligation of EcoRI-digested pBR322 and pSE101; contains single <em>BamH</em>I site</td>
<td>This work</td>
</tr>
<tr>
<td>pEC9</td>
<td>Insertion of 1.05-kb <em>BamH</em>I tsr fragment of pJ704 in <em>BamH</em>I site of pEC9 (Fig. 2, map B)</td>
<td>This work</td>
</tr>
<tr>
<td>pECT2</td>
<td>Rescued in <em>Escherichia coli</em> AL301; approx 25 kb; pECT2 + adjacent chromosomal DNA (Fig. 2, map C)</td>
<td>This work</td>
</tr>
<tr>
<td>pDPB11</td>
<td>Rescued in <em>Escherichia coli</em> AL303; approx 22 kb; pECT2 + adjacent chromosomal DNA (Fig. 2, map D)</td>
<td>This work</td>
</tr>
<tr>
<td>pDPB31</td>
<td>Rescued in <em>Escherichia coli</em> AL307; approx 27 kb; pECT2 + adjacent chromosomal DNA (Fig. 2, map E)</td>
<td>This work</td>
</tr>
<tr>
<td>pDPB37</td>
<td>Rescued in <em>Escherichia coli</em> AL344; approx 26 kb; pECT2 + adjacent chromosomal DNA (Fig. 2, map F)</td>
<td>This work</td>
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<tr>
<td>pDPB44</td>
<td></td>
<td></td>
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</table>

(8) but used PT, as well as R3M regeneration medium and thiotrepton at a concentration of 40 μg/ml in the overlay medium. Growth and transformation of *E. coli* were performed as described previously (19).

**Isolation of plasmid and total cellular DNA.** Plasmid DNA was prepared either by the method of Guerry et al. (6) or by scaleup of the “miniprep” procedure (14) followed by centrifugation in cesium chloride-ethidium bromide density gradients for purification. Total cellular DNA was obtained from protoplasts of *Streptomyces lividans* or *S. erythraea* either by the method described by Hintermann et al. (7) or by centrifugation of cell lysates in cesium chloride-ethidium bromide density gradients.

**Southern hybridizations.** Chromosomal DNA (1 to 10 μg) and plasmid DNA (1 to 50 ng), cleaved with the appropriate restriction enzymes, was subjected to agarose gel electrophoresis and subsequently transferred to Durapore membranes (Millipore Corp., Boston, Mass.) as described previously (26). After transfer, the membranes were baked at 65°C for a minimum of 2 h. DNA probes labeled with [32P]dCTP were prepared by nick translation with DNA polymerase I as described by Rigby et al. (24). For hybridization, the membranes were preincubated in the hybridization solution (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt solution [5], 10 mM KPO₄, buffer [pH 7.2], 0.1% sodium dodecyl sulfate [SDS], and 250 μg of denatured salmon sperm DNA per ml) at 65°C for at least 2 h before addition of the probe. Hybridization was allowed to proceed at 65°C overnight. Unless otherwise indicated, membranes were washed twice at room temperature for 30 min each with 2× SSC-0.1% SDS, followed by two 30-min washes at 65°C with 0.1× SSC-0.1% SDS, and then dried and subjected to autoradiography.

**RESULTS**

**Isolation and restriction analysis of pSE101.** Two plasmids, approximately 11 and 18 kb, were isolated from a CsCl-ethidium bromide density gradient that contained a lysate of *S. erythraea* NRRL 2338 prepared from a 600-ml culture grown for 5 days in SGGP medium. The 11-kb plasmid was designated pSE101. The 18-kb plasmid, designated pSE211, was determined to be not related by sequence to pSE101 and is the subject of another report (D.P.B., J.S.T., and L.K., manuscript in preparation). In repeated attempts to isolate pSE101, it was generally observed that less than 1 μg of plasmid DNA could be recovered from 1 liter of culture. To obtain enough plasmid DNA for subsequent work, pSE101 was cloned into pBR325 as described in Table 1. The resulting hybrid, pEC5, was purified from *E. coli* and used to construct the restriction map shown in Fig. 1. Plasmid pSE101 is approximately 11.3 kb and contains single restriction sites for the enzymes *BamH*I, EcoRI, XhoI, and *Xho*I. It should be noted that pSE101 does not contain a site for cleavage by *BamH*I.

The integrated form of pSE101 in *S. erythraea*. Preliminary Southern blots of *BamH*I-digested genomic DNA indicated that bands of approximately 22 and 5 kb from *S. erythraea* NRRL 2338 and approximately 10 kb from *S. erythraea* NRRL 2359 hybridized to pSE101 DNA used as a probe (data not shown). To further identify the chromosomal sequences that hybridized to the plasmid, a library of *S. erythraea* NRRL 2338 total DNA, partially digested with the restriction enzyme *Sau3A*I, was constructed in bacteriophage λ 1059 by the procedure of Karn et al. (12) and individual plaques were probed with 32P-labeled pSE101. Phage was propagated from a number of plaques which hybridized to the plasmid (19), and the DNA was isolated and restriction maps were generated. Two different chromosomal sequences were found. One sequence did not correspond in restriction map to the plasmid. Further description of this sequence is provided below. The sequence corresponding to pSE101 was obtained in a second clone. A restriction map of pSE101 DNA and adjacent chromosomal sequences in this clone is shown in Fig. 2, map A. From the
correspondence of the restriction endonuclease cleavage map of plasmid pSE101 and the extensive homology between the two sequences (data not shown), it is concluded that the sequence designated pSE101" in Fig. 2, map A, is an integrated form of pSE101 in S. erythraea NRRL 2338. By examination of map A in Fig. 2, it can be seen that the site of interruption of the pSE101 sequence in pSE101" lies between BglII site 18 [BglII(18)] and Clal site 19 [Clal(19)] on the plasmid map (Fig. 1). Both pSE101 and pSE101" are absent from S. erythraea NRRL 2359. The chromosomal DNA band from this strain that hybridized to pSE101 is described below.

Site-specific integration of pSE101 in S. erythraea. A hybrid plasmid, designated pECT2, carrying the gene for thiostrepton resistance (tsr), pSE101, and pBR322 (Fig. 2, map B), was constructed as outlined in Table 1 and was used to transform S. erythraea NRRL 2359. Thiostrepton-resistant transformants were observed at a frequency of approximately 10^3/μg of DNA. The state of pSE101 DNA was determined by Southern hybridization analysis of BglII-cleaved genomic DNA from 12 transformants (strains SE2001 to SE2012) picked directly from the regeneration plates to broth (Fig. 3). Strongly hybridizing bands of 7.6 and approximately 21 kb were observed in each of the transformants (lanes 2 to 13), indicating the presence of integrated pECT2 DNA. In addition, an 8.9-kb band, which indicates the presence of autonomous plasmid (lane 1), was also observed in all of the transformants except SE2005 (lane 6) and SE2007 (lane 8). In strain SE2003, the 8.9-kb band was visible but reduced in intensity (lane 4). To determine whether both the autonomous and integrated forms of the plasmid could be maintained within the same cell, strains SE2002, SE2003, and SE2006 were plated on agar media that permitted sporulation and BglII-digested total DNA was examined by Southern hybridization for the state of pSE101 from a number of colonies of each strain that arose after two successive spore-to-spore passages. In all cases, the pECT2-hybridizing bands of 7.6 and 21 kb were observed but the 8.9-kb band was absent (data not shown), indicating that only the integrated form of pECT2 was maintained through spore-to-spore passage.

Cleavage of pECT2 with EcoRI removes the pBR322-tsr sequence from the plasmid and permits precise comparison of the location of integrated plasmids pSE101 in S. erythraea NRRL 2338 and pECT2 in S. erythraea NRRL 2359. It can be seen, in the Southern blot of EcoRI-BglII-digested genomic DNA from SE2005, SE2007, and NRRL 2338 with pSE101 DNA as a probe (Fig. 4A, lanes 3 to 5), that all three preparations displayed hybridizing bands of 7.8 and 9.6 kb. Both bands consist of pSE101 DNA and adjoining chromosomal sequences. Similarly, the Southern blot of the same preparations cut with EcoRI-BglII also displayed identical hybridizing bands of 7.6 and 14.5 kb (Fig. 4B, lanes 3 to 5). The 7.6-kb band corresponds to the BglII(1)-BglII(15) segment of the plasmid. The 14.5-kb band consists of a plasmid segment and adjoining chromosomal sequence. The absence of a second hybridizing band that contains the other plasmid-chromosome junction indicates that the crossover point on the plasmid is located sufficiently near one of the BglII sites to render the remaining plasmid sequence too short to be detected by the probe. Since the plasmid-chromosome junction fragments prepared from the three hosts exhibit the same length, it can be concluded that the plasmid site used for integration in SE2005 and SE2007 lies within the BglII(18)-Clal(19) segment of the corresponding pSE101 map (Fig. 1) and that pECT2" occupies the same chromosomal site in the NRRL 2359 transformants that is occupied by pSE101" in NRRL 2338.

Additional chromosomal sequences homologous to pSE101. In addition to the 7.6-, 8.9-, and 21-kb BglII-digested fragments of genomic DNA from NRRL 2359 transformants that hybridized strongly to pSE101, it was also observed that each preparation contained an 18.5-kb band that hybridized to the probe as well (Fig. 3). In EcoRI-BamHI-digested or EcoRI-BglII-digested preparations of NRRL 2359, SE2005, and SE2007 genomic DNA, a pSE101-hybridizing fragment appeared as a 2.8-kb band (Fig. 4A and 4B, lanes 2 to 4). In chromosomal DNA preparations of NRRL 2338, the hybridizing fragment appeared as a 5.1-kb band after EcoRI-BamHI digestion or as a 9.0-kb band after EcoRI-BglII digestion (Fig. 4A and 4B, lane 5). Since the 2.8-kb pSE101-hybridizing EcoRI-generated band is present in the NRRL...
2359 host that was lacking the full pSE101 sequence, it represents a segment of the S. erythraea NRRL 2359 genome that is homologous to up to 2.8 kb of pSE101 sequence. Similarly, the 5.1-kb BamHI fragment that hybridized to pSE101 represents a segment of the S. erythraea NRRL 2338 genome that is homologous to up to 5.1 kb of pSE101 sequence but is distinct from the integrated pSE101* sequence.

**Integration of pSE101 sequences in Streptomyces lividans.**

pECT2 was used to transform *Streptomyces lividans* 66 protoplasts by using selection for thiostrepton resistance. Forty-five transformants were examined for the state of pECT2 by agarose gel electrophoresis and Southern hybridization of BglII-digested genomic DNA. In 7 transformants, the 7.6-kb band was present but the 8.9-kb band was replaced by a new pSE101-hybridizing band that was different in each case (Fig. 5A, lanes 2 to 8). These findings indicate that pECT2 had integrated into the chromosome in these transformants and that the location of the integrated plasmid sequence was different in each case. In 15 other transformants, a new, distinct pSE101-hybridizing band was observed, indicating the presence of integrated pECT2, but the 8.9-kb pSE101-hybridizing band was also observed (Fig. 5B, lanes 2 to 16). In several of the transformants examined further, the 8.9-kb band was lost but the 7.6-kb and the new pSE101-hybridizing bands were retained in genomic preparations from all individuals examined after two successive spore-to-spore passages (data not shown). In addition, free

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**FIG. 2.** Restriction endonuclease maps of autonomous or integrated pSE101 sequences and adjacent chromosomal DNA in *S. erythraea* or *Streptomyces lividans*. Map A, *S. erythraea* NRRL 2338 chromosomal DNA containing pSE101* as shown (map derived from a 1059 clone of total DNA); map B, plasmid pECT2; map C, plasmid pDPB11 (plasmid derived from *Streptomyces lividans* AL301); map D, plasmid pDPB31 (plasmid derived from *Streptomyces lividans* AL303); map E, plasmid pDPB37 (plasmid derived from *Streptomyces lividans* AL307); map F, plasmid pDPB44 (plasmid derived from *Streptomyces lividans* AL344). See Table 1 for descriptions of strains used. ☐, pSE101 sequences; ☒, pBR322 sequences; ☑, sequence containing tsr gene; ☐, chromosomal sequences.
plasmid was lost and the integrated copy was retained after protoplast formation and subsequent regeneration on solid medium (data not shown).

**Recovery of integrated pECT2 and adjacent chromosomal sequences in *E. coli* and determination of plasmid and chromosomal integration sites in *Streptomyces lividans***. Integrated plasmid sequences and adjoining chromosomal DNA were recovered in *E. coli* as follows. Chromosomal DNA preparations of several *Streptomyces lividans*-pECT2 transformants that appeared to carry the plasmid exclusively as an integrated sequence were digested with *Bam*HI, treated with T4 DNA ligase, and then used to transform *E. coli* cells by using selection for ampicillin resistance. Since neither pSE101 nor its derivative, pECT2, contains a site for cleavage with *Bam*HI, *E. coli* colonies produced in this manner would be expected to carry plasmids that contained the pECT2 sequence, as well as *Streptomyces lividans* chromosomal DNA adjoining the left and right ends of the integrated plasmid sequence to the nearest adjacent *Bam*HI sites. From strains AL301, AL303, AL307, and AL344, plasmids pDPB11 (ca. 25 kb), pDPB31 (ca. 22 kb), pDPB37 (ca. 27 kb), and pDPB44 (ca. 26 kb), respectively, were recovered in *E. coli* (Table 1). Restriction maps of these plasmids are shown in Fig. 2, maps C to F. In each case, the linear pECT2 sequence corresponds to the linear *S. erythraea* chromosomal pSE101" sequence (map A), with the exception of the pBR322-*trr* sequence inserted at the *Eco*RI site, indicating that integration of pECT2 in these transformants used a site between *Bgl*II(18) and *Cla*I(19) on the corresponding circular plasmid map (Fig. 1) for crossover. Differences between the restriction maps of the corresponding left and right adjoining chromosomal sequences in the four plasmids can also be seen in Fig. 2, maps C to F, clearly illustrating the different chromosomal locations of pECT2 integration in *Streptomyces lividans*.

**Autonomous replication of pSE101 in *Streptomyces lividans***. An agarose gel of *Bgl*II-digested total DNA from 4 of the remaining 23 *Streptomyces lividans*-pECT2 transformants is shown in Fig. 6A, lanes 3 and 5 to 7. It can be seen that each transformant displayed prominent bands of 7.6 and 8.9 kb which hybridized strongly to a pSE101 DNA probe in the corresponding Southern blot (Fig. 6B, lanes 3 and 5 to 7). No other hybridizing bands were seen. For comparison, similarly prepared and digested total DNA from AL301 and AL303, which carried pECT2 as an integrated sequence (see above) did not display the prominent pSE101-hybridizing bands (Fig. 6A and 6B, lanes 2 and 4). These profiles indicate that in strains AL304, AL306, AL338, and AL341, the plasmid is present exclusively in the autonomous state. Of the 45 transformants examined in this manner, 23 were found to carry pECT2 exclusively as an autonomous sequence (data not shown).

Stability of the autonomous form was examined in strains AL304 and AL306 as follows. Mixed populations of spores of each strain were passed twice on agar medium lacking
thiostrepton and then plated on medium either containing or lacking thiostrepton. Approximately the same number of colonies were found to arise on each plate. Southern blots of BgIII-digested genomic DNA from several individuals examined after the successive platings exhibited pSE101-hybridizing bands of 7.6 and 8.9 kb only (data not shown), indicating the presence of autonomous plasmid in these cells.

Plasmid DNA from strain AL304 was inserted into E. coli, extracted and purified, and reintroduced into protoplasts of Streptomyces lividans 66 or S. erythraea NRRL 2359 by using selection for thiostrepton resistance. A Southern blot of BgIII-digested genomic DNA from one of the Streptomyces lividans transformants displayed pSE101-hybridizing bands of 7.6 and approximately 14.5 kb (Fig. 7, lane 2). Similarly digested genomic DNA from several S. erythraea transformants showed intensely hybridizing bands of 7.6 and 21 kb, as well as the weakly hybridizing 18-kb band in each case (Fig. 7, lanes 3 to 5). These findings indicate that autonomously replicating pECT2 isolated from Streptomyces lividans underwent integration upon reintroduction into Streptomyces lividans or S. erythraea.

**DISCUSSION**

The results presented here clearly indicate that the circular DNA element pSE101, normally found in S. erythraea, is a plasmid that is capable of autonomous replication in Streptomyces lividans. In two independently isolated S. erythraea strains, the preferred state of pSE101 is a chromosomally integrated sequence. By restriction enzyme and Southern blot analyses of the integrated plasmids and their corresponding flanking chromosomal DNA sequences, it was found that pSE101int in strain NRRL 2338 and pECT2int in strain NRRL 2359 transformed with plasmid pECT2 occupy the same single chromosomal sites (Figs. 2 and 4), indicating that integration of pSE101-containing sequences in S. erythraea occurs by site-specific recombination. The
terms attP and attB are used to designate the plasmid and chromosomal sites involved in the process. Site-specific recombination has been established for integration of plasmids SLPI in Streptomyces lividans (1, 21, 22), pMEA100 in Nocardia mediterranei (18, 20), and pSAM2 and its derivatives in Streptomyces ambofaciens (23, 25). For pMEA100 and SLPI, integrative crossover has been demonstrated to take place between cognate attP and attB sequences that contained 47 and 111 base pairs of homology, respectively (18, 22). Preliminary DNA sequencing of the left and right plasmid-chromosome junctions of pSE101attP has revealed a 46-base-pair direct repeat of a sequence that is present between the BglII(18) and ClaI(19) segments of pSE101 (Fig. 1). (Brown et al., unpublished results). The presence of the direct repeat is consistent with an integrative crossover between sites in the homologous attP and attB regions of plasmid and chromosome. Independent confirmation of the presence of attB in strain NRRL 2359 is in progress. Precise excision of integrated plasmids that have restored cognate attB sites have been reported for plasmids pMEA100 in N. mediterranei (18) and pSAM2 in Streptomyces ambofaciens (25) and for a conjugative plasmid, pSE211, in S. erythraea (D. Brown et al., unpublished results). It is likely that the small amount of free pSE101 circular molecules found in the pSE101attP or pECT2attP-containing S. erythraea strains is due to precise excision of the plasmid sequence from the chromosome.

Integration of pSE101 by homologous recombination cannot be ruled out, but the data presented here make this possibility unlikely. As can be seen in Fig. 4, S. erythraea NRRL 2359 was found to contain a 2.8-kb chromosomal DNA segment that is highly homologous to a portion of the pSE101 sequence. Preliminary mapping has indicated that this sequence does not include the attP-containing BglII(18)-ClaI(19) region of the plasmid (unpublished results). In the S. erythraea NRRL 2359-pECT2 transformants reported here (Fig. 3 and 7), integrative recombination was determined to have used the attP site only and to have not used the 2.8-kb sequence for homologous recombination. In other work, M. Weber and R. Losick (submitted for publication) have demonstrated integration in S. erythraea through homologous recombination of plasmid pJ702 that contained chromosomal DNA segments of 400 base pairs or greater. It is yet possible that homologous recombination mechanisms are involved in crossover between pSE101 attP and attB sites, but the small extent of homology between the two sites makes this very unlikely.

The origin of the 2.8-kb chromosomal DNA sequence that is homologous to a segment of pSE101 is not known, but two observations concerning this sequence are noteworthy. (i) The sequence is homologous to the segment of pSE101 that lies between the SalI(22) and XhoI(3) sites shown on the restriction map of the plasmid but does not contain restriction sites for the enzymes EcoRI and BglII that correspond to positions 23 and 1, respectively, on the plasmid map (Fig. 1). (ii) The sequence is present at different locations in the chromosome of the two S. erythraea strains used in this study, although one of the strains, NRRL 2359, does not carry pSE101attP or pSE101 sequences but does contain the attB site for pSE101 integration. One possibility is that the homologous plasmid and chromosomal sequences are derived from an insertion sequence element that underwent sequence rearrangement after transposition. Examination of the plasmid and chromosomal sequence for inverted repeats is in progress. Inverted repeats have been demonstrated in two plasmids of the host Streptomyces lividans, but it was not determined whether the inverted repeats involved functional or defective insertion sequence elements (15).

Multisite integration has not been reported for any other actinomycete plasmid, although integration of a 2.6-kb minicircle from Streptomyces coelicolor at distinct chromosomal sites has been described previously (17). Of 22 transformants examined, at least 7 (and possibly 15) different Streptomyces lividans chromosomal sites were used for pECT2 integration (Fig. 5). Preference for particular chromosomal sites was not demonstrated. In four cases that were studied carefully, integrative recombination of pECT2 and the chromosome of Streptomyces lividans was shown to have used the attP region of the plasmid (Fig. 2). In all other cases examined, integration through crossover at attP is consistent with the data obtained in Southern blots (Fig. 5). The sequences of the chromosomal target sites are not known but are currently being examined. In Southern blots, probes consisting of either the complete pECT2 sequence or the 400-base-pair attP-containing BglII(18)-ClaI(19) segment of pSE101 failed consistently to hybridize to Streptomyces lividans chromosomal DNA, suggesting that the targets are not copies or highly conserved homologs of attB scattered throughout the chromosome. It appears, at least superficially, therefore, that multisite integration of pECT2 in Streptomyces lividans might be analogous to the secondary site attachment of bacteriophage λ in E. coli strains that have suffered deletions of λ-attB. In a related example, the multisite integration into the chromosome of Myxococcus xanthus observed for plasmid RP4 was postulated to be mediated by an insertion sequence element resident in the host (11). DNA sequencing, now in progress, should enable a greater understanding of the specificity required and mechanisms involved in pECT2 integration in Streptomyces lividans. Of particular interest, as well, is the determination of how extensive the number of sites for integration is and whether the plasmid can be used for insertional mutagenesis.
Observations made with other actinomycete plasmids generally indicate that only a single state of the plasmid is preferred both in naturally occurring plasmid-containing strains and in plasmid-free strains into which the plasmid has been transferred (1, 20, 21, 23). Thus, the finding that approximately half of the Streptomyces lividans-pECT2 transformants carried the plasmid as a free replicon, whereas the other half carried it as an integrated sequence, was quite surprising. The basis for this observation is not presently understood, but several findings reported here have led us to a proposal concerning the fate of the pSE101 sequence when introduced into Streptomyces lividans. It was observed that pECT2, extracted from a transformant of Streptomyces lividans in which the plasmid had replicated autonomously, could integrate after reintroduction into either S. erythraea or Streptomyces lividans (Fig. 7). It cannot be argued, therefore, that introduction of the plasmid into Streptomyces lividans resulted in mutation or deletion of plasmid sequences involved in integration, as had been previously observed after conjugal transfer of SLP1 from Streptomyces coelicolor to Streptomyces lividans (1). It was also observed that although some transformants of both S. erythraea and Streptomyces lividans appeared to carry both autonomous and integrated plasmids (Fig. 3 and 5), the autonomous plasmids were lost after one or two successive spore-to-spore passages or after conversion of mycelia to protoplasts and regeneration on solid media (see Results). In contrast, transformants of Streptomyces lividans that were demonstrated to carry only autonomously replicating pECT2 retained the plasmid in the autonomous state after spore-to-spore passage without selection. Furthermore, it was demonstrated that once a given plasmid state is established, it is stably maintained. Hence, strains of Streptomyces lividans that carried the plasmid autonomously did not give rise, at any appreciable rate, to cell lines that carried the plasmid as an integrated sequence. Similarly, very little, if any, free plasmid was detected in cells that had established an integrated form of pECT2. These findings are consistent with a X-like life cycle and lead us to suggest an extension of the prophage model for pSE101 that has been previously proposed for integration of SLP1 (21). We suggest that soon after the plasmid enters the cell, a decision is made to establish either the autonomous (vegetative) or integrative (prophage-like) pathway for plasmid maintenance. The pathways are mutually exclusive and, once established, not reversible. Thus, once autonomous replication is established, genes involved in integration are not expressed. Similarly, in the integrated state, autonomous replication is not permitted, perhaps through the action of a pSE101-encoded repressor on genes involved in plasmid replication. As can be seen in Fig. 6A, the visible presence of plasmid bands amid the background of chromosomal DNA indicates a minimum copy number of pECT2 of 5 to 20 in strains of Streptomyces lividans carrying the autonomous element. A mechanism must exist, therefore, to silence functions involved in plasmid replication. In S. erythraea, the elements involved favor integration. In Streptomyces lividans, different elements may apply for the determination of which pathway is used. We are currently using the two types of Streptomyces lividans-pECT2 cell lines to examine sequences involved in the establishment and maintenance of the two plasmid states.

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LITERATURE CITED


