JOURNAL OF BACTERIOLOGY, May 1988, p. 2174–2184
0021-9193/88/052174-11$02.00/0

Vol. 170, No. 5

Site-Specific Insertion of Biologically Functional Adventitious Genes into the *Streptomyces lividans* Chromosome

CHARLES A. OMER,[1,2] DAVID STEIN,[1] AND STANLEY N. COHEN[1,3]*

Departments of Genetics[1] and Medicine,[3] Stanford University School of Medicine, Stanford, California 94305, and Central Research and Development Department, E. I. du Pont de Nemours & Company, Inc., Wilmington, Delaware 19898[2]

Received 15 October 1987/Accepted 9 February 1988

We report that transformation of *Streptomyces lividans* with cloned DNA of the SLP1 genetic element results in integration of the element at the same chromosomal locus (*attB*) normally occupied by SLP1 in its original host, *Streptomyces coelicolor*, and in *S. lividans* that has received SLP1 by mating. We constructed SLP1 derivatives that can integrate foreign DNA at the *attB* site and used these to introduce adventitious DNA sequences into the *S. lividans* chromosome. We also identified three regions of SLP1 essential for its integration and demonstrated that integration of the SLP1 element does not require expression of functions necessary for stable maintenance or transfer of extrachromosomal forms of SLP1.

Transmissible genetic elements are widespread in the actinomycetes (2, 4, 9, 14, 18, 27, 31, 36, 38, 43, 47). These elements encode a variety of biologically important traits, including synthesis of and resistance to antibiotics and the ability to synthesize products affecting secondary metabolism and cell differentiation (14, 43, 48). Certain transmissible genetic elements of the actinomycetes also mediate chromosome fertility (2, 4, 26), and some have been shown to exist as autonomously replicating plasmids that are capable of integrating into the chromosome of the host cell (4, 9, 17, 31, 32, 36); the most extensively studied of the chromosomally integrating transmissible genetic elements is the *Streptomyces coelicolor* A3(2) element SLP1 (4, 32–34).

It previously has been reported that the integrated SLP1 sequence (i.e., SLP1^{int}) is transferred between strains as a transiently existing, physically autonomous 17-kilobase (kb) plasmid produced by site-specific excision of SLP1 from the chromosome (32–34). When this plasmid enters an SLP1$^-$ *Streptomyces lividans* cell by mating, it integrates into the chromosome of the recipient cell at a site identical in location and structure to the site of excision of SLP1^{int} from the donor chromosome. Integration and excision occur site specifically within homologous DNA segments 112 base pairs in length; these *att* loci flank SLP1^{int} and also are found individually on the transiently existing 17-kb SLP1 plasmid (*attP*) and on the chromosome (*attB*) (34). Transfer of SLP1 from donor bacteria plated on a lawn of SLP1$^-$ cells results in localized areas of growth inhibition (termed "pocks") in the lawn (2, 4). Cells within the pock are recipients of the SLP1 element.

When an *S. coelicolor* strain carrying SLP1^{int} is mated with an SLP1$^-$ *S. lividans* strain, about 90% of the recipient *S. lividans* cells are found to contain physically autonomous SLP1-derived plasmids, 11 to 14 kb in size; these extrachromosomal replicons, which have been designated SLP1.1, 1.2, 1.3, etc., result from deletion of segments of the 17-kb SLP1 sequence (4, 33). The deletion derivatives lack the *attP* locus or other regions required for integration or for maintenance or both of SLP1 in the integrated state. In the majority of cases, intraspecies transfer of SLP1^{int} between *S. lividans* strains yields only recipient strains that contain the integrated SLP1 sequence (32). Analogous intraspecies transfer of SLP1^{int} in *S. coelicolor* has not been analyzed, as no *S. coelicolor* strains lacking SLP1^{int} have been found.

We are interested in understanding further the mechanism of integration and excision of SLP1^{int}, in identifying the genes involved in these processes, and in studying the control of their expression. Here we report that the 17-kb (full-length) SLP1 sequence that has been cloned in *Escherichia coli* can integrate site specifically into the *S. lividans* chromosome following its introduction by transformation into an SLP1$^-$ strain. This finding has enabled us to identify SLP1 regions essential for integration and to construct SLP1-derived vectors that can accomplish the integration of cloned segments of foreign DNA into the *S. lividans* chromosome.

MATERIALS AND METHODS

Bacterial strains, culture media, matings, and bacterial transformations. The bacterial strains used are listed in Table 1. *S. lividans* C28 was made by a fusion of JI3198 and TK71 (19). *Streptomyces* cultures were grown on R2YE plates or in yeast extract-malt extract liquid media (16). Thiostrepton (Th) (a gift from S. J. Lucania, E. R. Squibb & Sons, New Brunswick, N.J.) was used for selection of certain *Streptomyces* strains at concentrations of 50 μg/ml in R2YE plates and 5 μg/ml in yeast extract-malt extract liquid media. Viomycin (kindly provided by The Upjohn Co., Kalamazoo, Mich.) was used at a concentration of 30 μg/ml in R2YE plates. *E. coli* cultures were grown on L agar or in L broth. Ampicillin was used in *E. coli* media at a concentration of 50 μg/ml. *Streptomyces* strains were mated by plating approximately 10^7 spores of the donor and recipient on an R2YE plate and allowing them to grow at 30°C until sporulation occurred. The spores were then harvested by adding 5 ml of sterile water to the plate and scraping. The spores were passed through filter tubes (16) to remove mycelial fragments, pelleted, and then suspended and stored in 20% glycerol at −20°C until analyzed. *Streptomyces and E. coli* transformations were carried out as described previously by Bibb et al. (3) and Cohen et al. (10), respectively.

Isolation of total cellular DNA and plasmid DNA. A modification of the method of Marmur was used to isolate total cellular DNA from *Streptomyces* spp. (30). *E. coli* and

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Markers	Reference
E. coli		
MC1061	*araP139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK hsdR hsdM*$^+$ *rpsL*	6
CE106	MC1061(pCAO106)	This work
CE115	MC1061(pCAO115)	This work
CE153	MC1061(pCAO153)	This work
CE170	MC1061(pCAO170)	This work
CE175	MC1061(pCAO175)	This work
CE178	MC1061(pCAO178)	This work
CE180	MC1061(pCAO180)	This work
CE190	MC1061(pCAO190)	This work
CE200	MC1061(pCAO200)	This work
CE205	MC1061(pCAO205)	This work
S. lividans		
C14	Wild-type isolate of *S. lividans* JI1326	32
C21	*str-1*, SLP^{int} tandem duplication	32
C28	*str-6 his-2 ilv-3*	This work
C37	Derivative of C14 containing single copy of pCAO106 chromosomally integrated	This work
C50	*S. lividans* containing plasmid pSRG04	This work
CAO153-1	C14 containing single copy of pCAO153 chromosomally integrated	This work
CAO153A1	C14 containing single copy of pCAO153 *afsB* chromosomally integrated	This work
CAO153A2	C14 containing single copy of pCAO153 *afsB* chromosomally integrated	This work
CAO153A3	C14 containing single copy of pCAO153 *afsB* chromosomally integrated	This work
CAO153A4	C14 containing single copy of pCAO153 *afsB* chromosomally integrated	This work
CAO153A5	C14 containing single copy of pCAO153 *afsB* chromosomally integrated	This work
C28-BH5-3	Str^r *afsB* Ilv$^+$	This work
C28-BH5-153	C28-BH5-3 containing single copy of pCAO153 chromosomally integrated	This work
C28-BH5-153A1	C28-BH5-3 containing single copy of pCAO153 *afsB* chromosomally integrated	This work
C28-BH5-JAS01 *afsB*	C28-BH5-3 containing pJAS01 *afsB*	This work
C14-JAS01	C14 containing pJAS01	23
C14-JAS01 *afsB*	C14 containing pJAS01 *afsB1*	This work
M180	SLP1.2	4
M192	SLP^{int}	4
TC21	pIJ13	45
S. coelicolor		
A3(2) BH5	*S. coelicolor* A3(2) *afsB* Ilv$^+$	21

Streptomyces plasmid DNA was isolated by the alkaline lysis method, with subsequent cesium chloride-ethidium bromide density gradient centrifugation as summarized by Maniatis et al. (29).

Enzymes. Restriction endonucleases, DNA polymerase I, T4 DNA ligase, calf intestinal alkaline phosophatase and DNase I were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.; New England BioLabs, Inc., Beverly, Mass.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; and Worthington Diagnostics, Freehold, N.J. Restriction enzymes were used as recommended by the manufacturers. Ligations were performed with vector DNA treated with calf intestinal alkaline phosphatase (29). Nick translation was carried out as described previously (39).

Southern blot analysis of *S. lividans* strains transformed with SLP1 constructs. *S. lividans* transformants detected by either their pocking phenotype or Th resistance were patched onto R2YE plates and allowed to sporulate. Spores were harvested from the patches and used to inoculate yeast extract-malt extract liquid media, and cultures were grown at 30°C for 2 to 3 days. DNA was isolated from the cells as described above. A 1-μg portion of restriction endonuclease-digested total cellular DNA or 1 ng of marker plasmid DNA was electrophoresed per lane in a 0.7% agarose gel (20 by 20 by 0.8 or 15 by 15 by 0.8 cm) for approximately 12 to 16 h at 1.5 V/cm. The gel was then transferred to nitrocellulose as described before (42). Filters were prehybridized for 6 to 12 h at 42°C in 50% (wt/vol) formamide–5× SSPE (1× SSPE is 0.18 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, pH 7.4)–100 μg of denatured herring sperm DNA per ml–5× Denhardt solution (11). Filters were hybridized for 12 to 16 h in the same solution as for prehybridization, except that approximately 10^6 cpm of nick-translated probe DNA (specific activity, approximately 1×10^8 to 3×10^8 cpm/μg) per ml was added. After hybridization, the filters were washed four times for 5 min each in 2× SSPE at 23°C and two times for 5 min each in 0.5× SSPE at 42°C. The washed filters were exposed to Kodak XAR-5 X-ray film at room temperature or at −70°C with DuPont Cronex Lightning-Plus intensifying screens.

Restriction mapping. The restriction maps of pCAO106 and other plasmids were determined by digesting the DNA with a combination of restriction endonucleases, subsequent analysis by gel electrophoresis, and sizing of the resulting DNA fragments in comparison with known marker DNAs. Also, some restriction mapping was done by the method of Smith and Birnstiel (41).

Construction of an *S. lividans* strain having a mutation in the *afsB* gene. Construction of an *S. lividans* strain having a mutation in *afsB* was carried out by transfer of a mutated *afsB* locus from *S. coelicolor* A3(2) BH5 (kindly provided by S. Horinouchi) into *S. lividans* C28 by fusion of protoplasts of the two species (19, 20). C28 contains the auxotrophic markers *his-2*, *str-6*, and *ilv-3*; since the *afsB* locus maps

very close to the *ilvB* locus on the *S. coelicolor* chromosomal linkage map, we hypothesized that the *ilvB* and *afsB* loci would be similarly linked in *S. lividans*, which has a map almost identical to that of *S. coelicolor* (19). Following interspecific protoplast fusion, protoplasts were plated on R2YE medium, allowed to regenerate for 64 h, and replica plated onto media supplemented with histidine and 100 μg of streptomycin per ml. Fifteen Str^r Ilv^+ colonies were obtained from the BH5/C28 fusion, whereas growth of the A3(2) BH5 and C28 strains alone yielded no Ilv^+ Str^r colonies. Three colonies were found to be deficient in pigment formation, and further testing indicated that they did not produce actinorhodin or undecylprodigiosin, although they appeared to sporulate normally. Colony morphology suggested that the strains produced no agarase and thus were isolates of *S. lividans*; this was confirmed by the ability of the strains to support extrachromosomal replication of an SLP1.2-derived plasmid, which will not replicate in *S. coelicolor*. One clone (C28–BH5-3) was selected for use in the subsequent investigations described in Results.

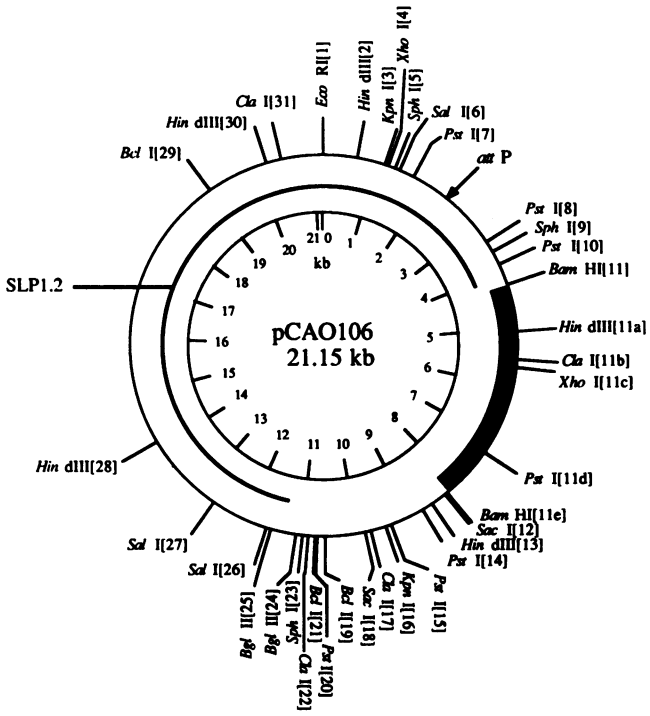
RESULTS

Integration capabilities of the cloned 17-kb SLP1 element. Previously we isolated the full-length (~17-kb) SLP1 element by cloning it in *E. coli* from the DNA of *S. lividans* C21, which contains a head-to-tail tandem duplication of $SLP1^{int}$ [$(SLP1^{int})^2$] (32, 34, 35). A 17.2-kb *Bam*HI fragment that consists of a circularly permuted copy of $SLP1^{int}$ was inserted into the *E. coli* plasmid pACYC177, yielding the 21.15-kb construct pCAO106 (Fig. 1).

Our previously proposed model for transfer of $SLP1^{int}$ predicts that a circularized 17-kb SLP1 molecule will be capable of integrating chromosomally when introduced into $SLP1^-$ *S. lividans* (32). To test this prediction and potentially to establish a system for investigating the genetic requirements for SLP1 integration, we introduced pCAO106 DNA into $SLP1^-$ *S. lividans*. Transformants were obtained, as identified by the pocking phenotype, using either the intact pCAO106 plasmid or pCAO106 DNA that had been digested with *Bam*HI and ligated under dilute conditions (3-μg/ml DNA concentration) to separate the SLP1 and pACYC177 components of the plasmid. DNA from these transformants was treated with *Hin*dIII and analyzed by Southern blot hybridization to a radioactively labeled SLP1.2 plasmid DNA probe.

A restriction endonuclease cleavage map of the integrated SLP1 element is shown in Fig. 2. The hybridization pattern of DNA from *S. lividans* that has been transformed with the *Bam*HI-digested and religated pCAO106 plasmid (Fig. 3, lane 3) indicates that SLP1 had integrated at the same chromosomal location occupied by $SLP1^{int}$ in an *S. lividans* strain that has received the 17-kb SLP1 sequence by mating. (i.e., M192, Fig. 3, lane 6; see also Table 2 and reference 32). In cells that had received the full-length SLP1 sequence by either route, a 5.1-kb *Hin*dIII junction fragment was seen; the second *Hin*dIII-generated junction fragment, which is >30 kb in length, is not defined in these gels. In addition, a 4.3-kb SLP1.2-hybridizing band identical in size to a band observed in DNA from *S. lividans* C21 (which is known to contain tandemly duplicated $SLP1^{int}$ [Fig. 3, lane 5]) was observed in DNA obtained from transformants, suggesting that some cells transformed with *Bam*HI-cut and religated pCAO106 DNA contain tandemly duplicated copies of the integrated plasmid (32).

The hybridization pattern of *Hin*dIII-digested DNA from *S. lividans* transformed with the uncleaved pCAO106 plasmid (Fig. 3, lane 2) showed 5.5- and 4.7-kb *Hin*dIII fragments hybridizing to the SLP1.2 probe instead of the 5.1- and 4.3-kb *Hin*dIII fragments found in DNA obtained from cells transformed with the intact pCAO106 plasmid (Fig. 3, lane 3). This gel pattern is consistent with the fact that pACYC177 was inserted into the *Bam*HI 17-kb SLP1 element to form pCAO106; a *Hin*dIII site is located 0.8 kb from the *Bam*HI site of pACYC177, yielding two *Hin*dIII fragments 0.4 kb longer than the corresponding *Hin*dIII fragments obtained from *S. lividans* $SLP1^{int}$. Together these results are consistent with the notion that genetic functions carried by the 17-kb SLP1 plasmid are capable of integrating, site specifically, *E. coli* plasmid DNA that has been linked to SLP1.



[1] *Eco*RI 0, 21.15 kb	[11b] *Cla*I 5.55 kb	[20] *Pst*I 10.7 kb
[2] *Hin*dIII 0.6	[11c] *Xho*I 5.65	[21] *Bcl*I 10.75
[3] *Kpn*I 1.05	[11d] *Pst*I 7.3	[22] *Cla*I 10.85
[4] *Xho*I 1.1	[11e] *Bam*HI 8.2	[23] *Sph*I 10.95
[5] *Sph*I 1.3	[12] *Sac*I 8.3	[24] *Bgl*II 11.05
[6] *Sal*I 1.35	[13] *Hin*dIII 8.6	[25] *Bgl*II 11.55
[7] *Pst*I 1.65	[14] *Pst*I 8.75	[26] *Sal*I 11.6
[8] *Pst*I 3.4	[15] *Pst*I 9.4	[27] *Sal*I 12.65
[9] *Sph*I 3.6	[16] *Kpn*I 9.5	[28] *Hin*dIII 14.15
[10] *Pst*I 3.85	[17] *Cla*I 9.85	[29] *Bcl*I 19.0
[11] *Bam*HI 4.25	[18] *Sac*I 9.9	[30] *Hin*dIII 20.15
[11a] *Hin*dIII 5.1	[19] *Bcl*I 10.55	[31] *Cla*I 20.4

FIG. 1. Restriction endonuclease map of pCAO106. The locations of restriction endonuclease sites in plasmid pCAO106 are indicated, as is the segment of pCAO106 included in SLP1.2. The thickened line on the map of pCAO106 indicates the segment derived from pACYC177; the remainder is the SLP1-derived segment. The position of the *attP* site is shown. The restriction endonuclease sites of the SLP1-derived segment of pCAO106 are numbered in sequence, while those within the pACYC177-derived segment are indicated as 11a, b, c, etc. Below the diagram is a listing of the restriction sites, and their locations are given in kilobases from the single *Eco*RI site on pCAO106.

Identification of DNA sequences required for integration of SLP1. Correlation of Southern blot data with an analysis of certain SLP1 functions (Fig. 4; Tables 2 and 3) has enabled us to identify regions necessary for the site-specific integra-

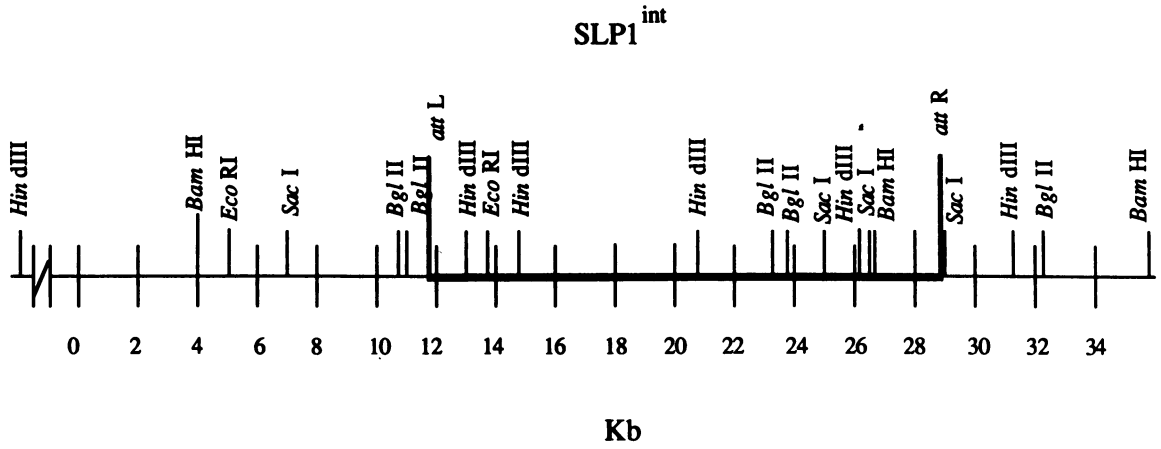


FIG. 2. Restriction map of SLP1^{int} region of the *S. lividans* SLP1^{int} or *S. coelicolor* chromosome. The map is derived from a compilation of data from Southern blot analyses of DNA isolated from strains containing SLP1^{int} and from restriction maps of cloned DNAs derived from these chromosomal regions (32, 34). The maps for SLP1^{int} and the immediately surrounding region of the chromosome are identical for *S. coelicolor* and *S. lividans*.

tion of SLP1 into the *S. lividans* chromosome and also to extend previous observations (16) regarding the SLP1 regions required for transfer and stable maintenance of physically autonomous SLP1-derived replicons. The plasmid constructs used for these studies are diagrammed in Fig. 5. As found previously (34), the *attP* locus, which resides on a 1.75-kb DNA fragment located between *Pst*I sites [7] and [8], is one of the regions required for integration. When this locus (which recombines with *attB* during the integration event) is deleted (as in pCAO115), the resulting plasmid is unable to integrate (Fig. 3, lane 1; Tables 2 and 3).

The SLP1.2 replicon, which is the largest SLP1-derived extrachromosomal element generated during interspecies mating, has not been observed to integrate into the chromosome. While this plasmid contains the *attP* locus, it lacks an approximately 3-kb segment that extends on the full-length SLP1 element from a position between *Bam*HI site [11] and *Sac*I site [12] to a position between *Bgl*II sites [24] and [25] (Fig. 1). Thus, functions other than the *attP* locus of the plasmid appear to be required for stable integration. To localize these functions, we used pCAO106, its derivative pCAO153 (which contains a Th resistance gene that allows the identification of transformants even in the absence of pocking), and certain other plasmid constructs that are derivatives of pCAO153 or pCAO106.

Whether integration had occurred was determined by comparing the SLP1-hybridizing restriction enzyme fragmentation pattern actually observed for total DNA isolated from transformants (Fig. 4) with the fragmentation pattern expected upon integration of each of the SLP1 derivatives analyzed (Table 2). Transforming capabilities of plasmids (Table 3) were assessed by the ability of the recipients to form pocks on a lawn of SLP1$^-$ cells or to express the plasmid-borne Th resistance gene. (Transformants can contain either integrated SLP1 or SLP1-derived plasmids.) Transfer capabilities were assessed by either pock formation or transfer of Th resistance by procedures indicated in Table 3.

One of the functions required for stable integration of SLP1 is encoded, at least in part, by the 1.65-kb *Sac*I[18] to *Bgl*II[25] segment of SLP1 lacking in SLP1.2. This was shown by the chromosomal integration of an SLP1.2 derivative (i.e., pIJ13) when a 4.5-kb DNA fragment that encompasses the region lacking in SLP1.2 is present in *trans* on a

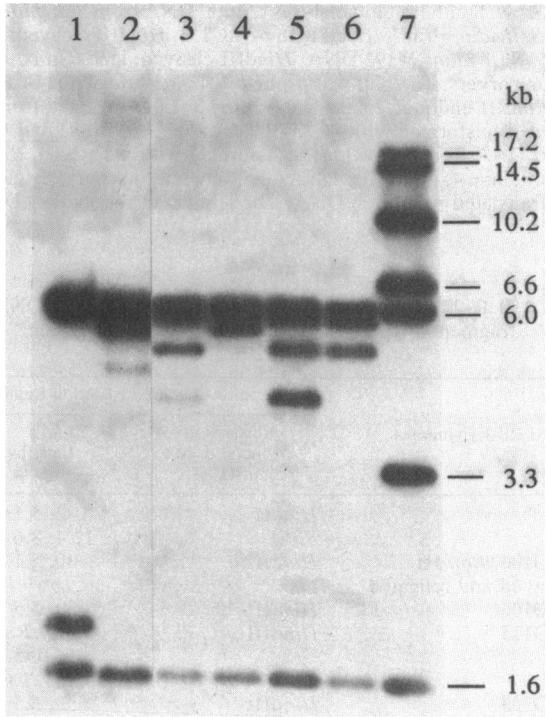


FIG. 3. Southern blot analysis of total DNA from *S. lividans* strains containing various SLP1 derivatives. Lane 1. *Hin*dIII-cleaved DNA from *S. lividans* that has been transformed with the pCAO115 plasmid, which lacks the *attP* site (Fig. 5; see text); lane 2, *Hin*dIII-cleaved DNA from *S. lividans* transformed with intact pCAO106; lane 3, *Hin*dIII-cleaved DNA from *S. lividans* transformed with pCAO106 DNA that has been cut with *Bam*HI and self-ligated under dilute conditions (3 μg/ml); lane 4, *S. lividans* C37 DNA, *Hin*dIII cleaved; lane 5, *S. lividans* C21 DNA, *Hin*dIII cleaved; lane 6, *S. lividans* M192 DNA, *Hin*dIII cleaved; lane 7, marker, SLP1.2 DNA that has been cleaved individually with *Hin*dIII, *Eco*RI, or *Sal*I endonuclease and *Bam*HI-digested pCAO106 DNA. The blot was prepared as indicated in Materials and Methods and was probed with nick-translated SLP1.2 DNA. The lengths of the marker DNA fragments are indicated.

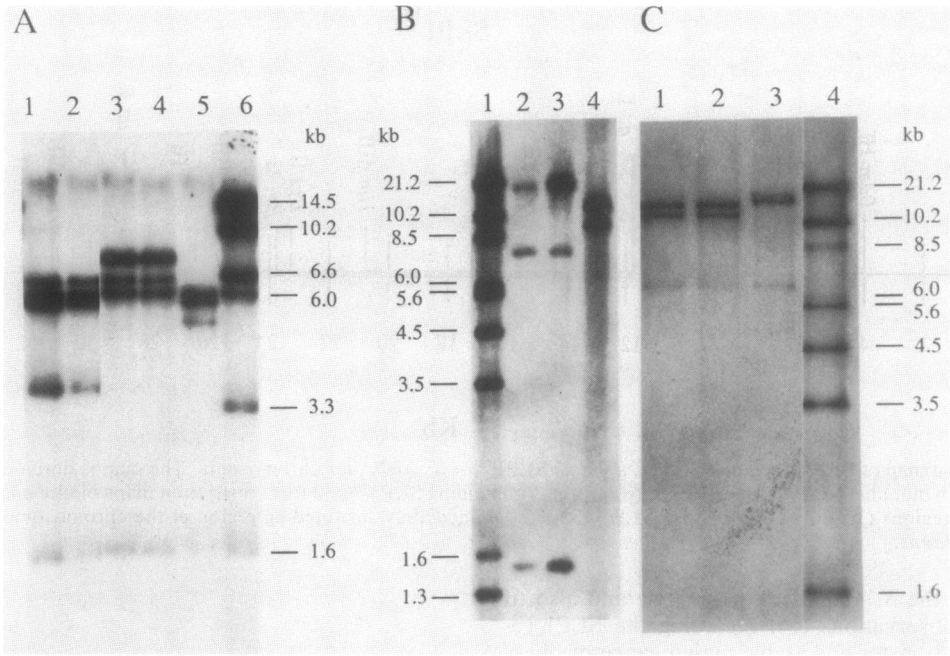


FIG. 4. Southern blot analyses of total DNA from *S. lividans* strains containing various SLP1 derivatives. (A) Lanes 1 and 2, DNA isolated from *S. lividans* transformed with pCAO153, *Hin*dIII cleaved; lanes 3 and 4, DNA of *S. lividans* transformed with pCAO170, *Hin*dIII cleaved; lane 5, *S. lividans* M192 DNA, *Hin*dIII cleaved; lane 6, mixture of SLP1.2 DNA that has been cleaved with *Eco*RI, *Sal*I, or *Hin*dIII for use as size markers. The blot was probed with nick-translated SLP1.2 DNA. (B) Lane 1, pCAO106 DNA that has been cleaved with *Eco*RI, *Sal*I, and *Hin*dIII endonucleases; lanes 2 and 3, DNA isolated from *S. lividans* transformed with pCAO180, *Sac*I cleaved; lane 4, DNA from *S. lividans* transformed with pCAO190, cleaved with *Bgl*II and *Eco*RI. The blot was probed with nick-translated pCAO106 DNA. (C) Lanes 1 and 2, DNA from *S. lividans* transformed with pCAO200, *Bgl*II cleaved; lane 3, DNA from *S. lividans* transformed with pCAO205, *Bgl*II cleaved; lane 4, pCAO106 DNAs that have been separately cleaved with *Eco*RI, *Sal*I, and *Hin*dIII endonucleases. The blot was probed with nick-translated pCAO106 DNA. The lengths of the marker DNA bands are indicated.

TABLE 2. Lengths of SLP1-chromosomal junction DNA fragments expected upon chromosomal integration of various SLP1 derivatives[a]

SLP1 derivative	Integration-specific restriction fragments	
	Restriction enzyme(s) used to cut DNA	Lengths (kb)
SLP1^{int}	*Hin*dII	>30, 5.1
	*Bgl*II	12.4, 8.6
pCAO106, *Bam*HI cleaved and religated	*Hin*dIII	>30, 5.1
pCAO106	*Hin*dIII	>30, 5.5
pCAO115	*Hin*dIII	No *attP* site (6.0, 5.65, 2.3, 1.6)
pCAO153	*Hin*dIII	>30, 6.5
	*Hin*dIII + *Bam*HI	9, 6.5
pCAO153 *afsB*	*Hin*dIII + *Bam*HI	9, 6.5
pCAO170	*Hin*dIII	>30, 6.5
pCAO175	*Hin*dIII	>30, 6.5
pCAO178	*Hin*dIII	>30, 6.5
pCAO180	*Sac*I	21, 7.4
pCAO190	*Eco*RI + *Bgl*II	11.8, 3.4 (12.3, 9.6, 0.5)
pCAO200	*Bgl*II	12, 6.4
pCAO205	*Bgl*II	16, 6.4

[a] The expected sizes were determined from the restriction maps in Fig. 1, 2, and 3. The sizes of the non-junction DNA fragments are not listed. For pCAO115 and pCAO190, the sizes of the fragments for the autonomously replicating forms of these plasmids are given in parentheses after the sizes of the integration-specific bands.

plasmid unrelated to SLP1 (i.e., pIJ922int4.5) (Fig. 6). The data shown in Fig. 6, lanes 3 and 4, indicate that pIJ13 has integrated chromosomally, as demonstrated by the presence of 12.4- and 8.0-kb SLP1.2-hybridizing *Bgl*II DNA restriction fragments in DNA from these strains compared with the

TABLE 3. Ability of SLP1 derivatives to transform, chromosomally integrate, and transfer when introduced into *S. lividans*[a]

SLP1 derivative	Transform	Integrate	Transfer
pCAO106	+	+	+
pCAO106, *Bam*HI cut and ligated to remove pACYC177 DNA	+	+	+
pCAO115	+	−	+
pCAO153	+	+	+
pCAO170	+	+	+
pCAO175	−	ND	ND
pCAO178	−	ND	ND
pCAO180	+	+	−
pCAO190	+	−	+
pCAO200	+	+	−
pCAO205	+	+	−

[a] Transformants were identified either by the presence of growth inhibition zones (pocks) in lawns of cells from a transformation mix or, when a plasmid carried the gene for Th resistance, by selecting for Th-resistant cells. The ability to integrate chromosomally was determined by the presence of the integration-specific bands indicated in Table 2 in Southern blots of transformants (Fig. 4 and 5). The ability to transfer was determined by the ability to either form pocks on SLP1$^-$ cells (for pCAO106 and pCAO115) or transfer Th resistance into SLP1$^-$ cells. ND, Not determined, because the plasmids are unable to transform *S. lividans*.

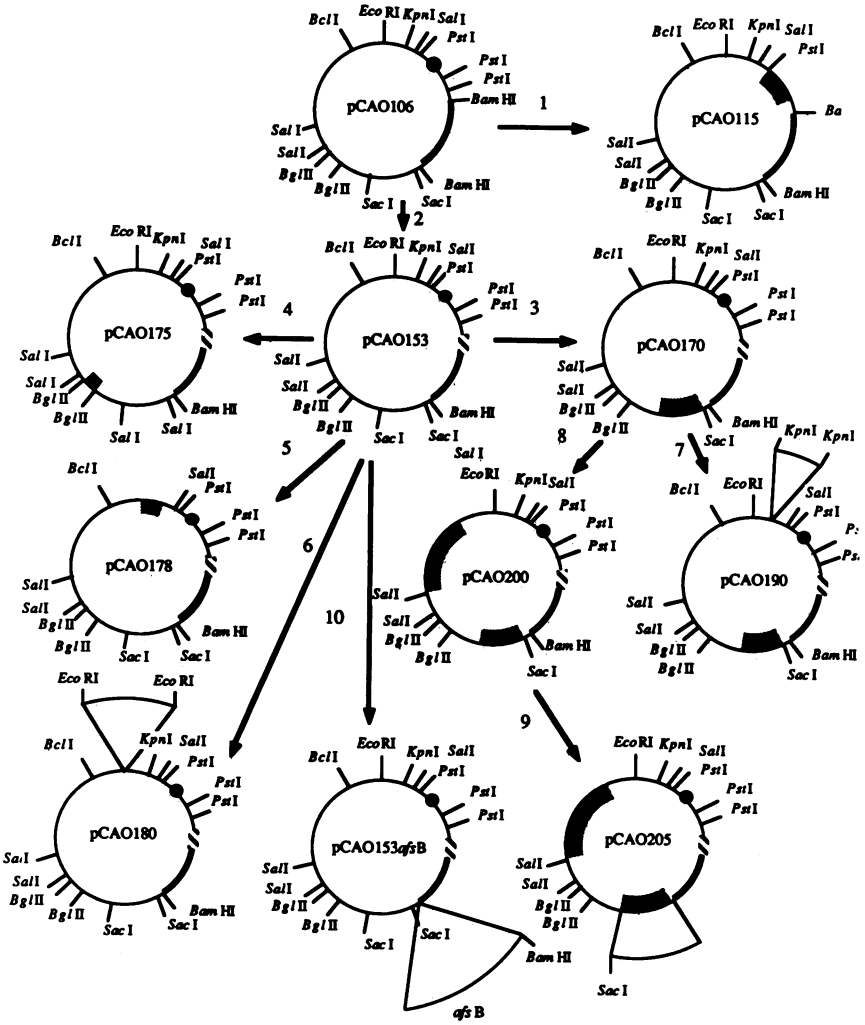


FIG. 5. Plasmid constructions. The *attP* site is indicated as a filled circle. The Th resistance gene from *Streptomyces azureus* is indicated as a thick hatched line. The pACYC177 segment of the constructs is indicated by a thick solid line. Deletions from the pCAO106 parent plasmid are indicated as internally located shaded arcs. Insertions are indicated as externally located arcs, with lines to the plasmid indicating the endpoints of the insertions. Parental plasmid pCAO106 was constructed previously (34). All numbering of restriction sites in the constructs is as shown in Fig. 1 for pCAO106. pCAO115 was constructed (arrow 1) from pCAO106 by deletion of the 2.2-kb *Pst*I[7] to *Pst*I[10] fragment. pCAO153 was made (arrow 2) by inserting the 1.05-kb *Bcl*I fragment carrying a gene for Th resistance from *S. azureus* (44) into *Bam*HI site [11] of pCAO106, which is proximal to *attP*. pCAO170 was derived (arrow 3) from pCAO153 by deleting the 1.6-kb *Sac*I[12] to *Sac*I[18] fragment. pCAO175 was constructed (arrow 4) from pCAO153 by deleting the fragment between *Bgl*II sites [24] and [25]. pCAO178 was constructed (arrow 5) from pCAO153 by removing the 1.05-kb *Eco*RI site [1] to *Kpn*I site [3] fragment, blunting the DNA ends, and religating the plasmid DNA. pCAO180 was constructed (arrow 6) by inserting a 4-kb *Eco*RI *S. lividans* DNA fragment into the *Eco*RI site [1] of pCAO153. pCAO190 was made (arrow 7) by inserting a 1.6-kb *Kpn*I bacteriophage λ DNA fragment into *Kpn*I site [3] of pCAO170. pCAO200 was derived from pCAO170 (arrow 8) by replacing the 6.35-kb DNA fragment between *Sal*I site [27] and *Bcl*I site [29] with the *Sal*I to *Bam*HI segment of the polylinker of pUC9 (46). pCAO205 was constructed (arrow 9) by replacing the 0.1-kb *Bam*HI[11e] to *Sac*I[18] fragment of pCAO200 with the 4.0-kb *Bgl*II to *Sac*I fragment from pCAO109 (34). pCAO153 *afsB* was constructed by inserting a 10-kb *Bam*HI-*Bgl*II fragment containing the *S. lividans afsB*$^+$ gene into the *Bam*HI[11e] site of pCAO153.

12.4- and 8.6-kb SLP1.2-hybridizing *Bgl*II-cleaved DNA fragments seen in *S. lividans* M192 (SLP1^{int}) (Fig. 6, lane 2). This shows expression in *trans* of a function required for SLP1 integration that is missing from SLP1.2 (pIJ13); we have termed this function *intA*. The SLP1.2 hybridization band at approximately 16 to 16.5 kb of *Bgl*II-cleaved DNA from these strains could be due to either tandem duplications of chromosomally integrated pIJ13 or segments of autonomous pIJ13, other than the 16.5-kb *Bgl*II fragment of pIJ922-int4.5 that would hybridize to SLP1.2. While SLP1.2 also lacks the 1.7-kb *Bam*HI[11] to *Sac*I[18] region, this segment is not required for integration of SLP1 derivatives, as indicated by the ability of pCAO170, pCAO200, and pCAO205 to integrate (Table 3). Results obtained with another plasmid, pCAO175, also are consistent with the *Sac*I[18] to *Bgl*II[25] segment being involved with integration of SLP1. pCAO175, which lacks the 0.6-kb *Bgl*II[24] to *Bgl*II[25] fragment internal to the *Sac*I/*Bgl*II segment, can neither chromosomally integrate nor be maintained as an autonomously replicating plasmid (Table 3). The inability of pCAO175 to be maintained as an autonomously replicating plasmid is because it has deleted part of a region previously shown to be necessary for SLP1 plasmid maintenance (16).

A third SLP1 region required for integration spans the

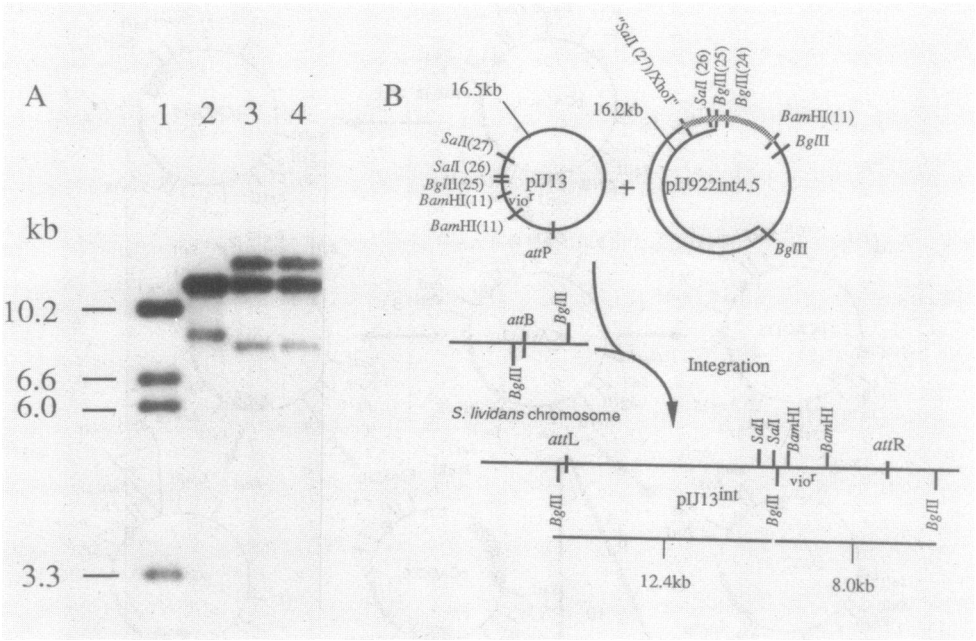


FIG. 6. *trans* complementation of the *intA* function. The 4.5-kb *Bam*HI[11e] to *Sal*I[27] fragment of pCAO106 was introduced into *Bam*HI + *Xho*I-cleaved pIJ922 (28) to make plasmid pIJ922-int4.5. An *S. lividans* strain containing pIJ922-int4.5 (C50) was mated with *S. lividans* TC21, which contains pIJ13, a derivative of SLP1.2 that contains a gene for viomycin resistance cloned into the *Bam*HI site. Cells containing both plasmids were isolated by plating spores from the mating onto R2YE agar plates containing Th and viomycin. The DNA from such doubly resistant cells was isolated, and Southern blot analysis was performed with SLP1.2 as the hybridization probe as shown in panel A. Lane 1, Mixture of SLP1.2 DNAs cut with either *Sal*I or *Hin*dIII; lane 2, DNA from M192 (SLP1^{int}) cut with *Bgl*II; lanes 3 and 4, DNAs cut with *Bgl*II from cells resistant to Th plus viomycin from two independent matings as described above. The lengths of the SLP1.2 DNA fragments are indicated. (B) Diagram of the two plasmids used in the mating and sizes of the SLP1.2-hybridizing *Bgl*II fragments expected from either of the two plasmids or from pIJ13 when it chromosomally integrates at the *S. lividans attB* site. The numbers of the restriction sites on the plasmids correspond to the numbering of the sites in Fig. 1. The site labeled "*Sal*I[27]/*Xho*I" is a hybrid site that is not cut by either *Sal*I or *Xho*I.

*Kpn*I[3] site, as demonstrated by the failure of pCAO190 to integrate. This function, which we have designated *intB*, is contained within this region that stops short of the *Eco*RI[1] site, as pCAO180 (which has an insertion in this site) can integrate. *Eco*RI site [1] is within a region believed to be required for replication or maintenance of the autonomous plasmid derivatives of SLP1 (16). Results obtained with plasmid pCAO178 are consistent with the view that a function required for integration of SLP1 is present in the vicinity of the *Kpn*I[3] site: pCAO178 has deleted the 1.05-kb *Eco*RI[1] to *Kpn*I[3] segment, and it does not integrate. Like pCAO180, pCAO178 has disrupted part of a region known to be required for replication or maintenance of SLP1 plasmids, and it also fails to replicate autonomously. pCAO200 and pCAO205, which lack a 6.35-kb segment between *Sal*I site [27] and *Bcl*I site [29] that previously has been identified with SLP1 transfer functions (16), can integrate. However, transformants carrying pCAO200 and pCAO205 do not transfer these integrated plasmids to SLP1$^-$ cells (Table 3).

Use of SLP1 vectors to chromosomally integrate adventitious DNA segments. The data presented above indicate that SLP1-derived plasmids cloned in another bacterial host species can integrate at the *attB* site of the *S. lividans* chromosome when introduced by transformation; the data also map certain functions required for integration. Because several restriction enzyme cleavage sites are located in regions found to be dispensable for integration of SLP1, these sites (*Eco*RI site [1], *Bam*HI site [11], and *Sac*I sites [12] and [18]) are available for cloning of adventitious DNA fragments and potentially for the site-specific integration of foreign DNA into the *S. lividans* chromosome.

As noted above, chromosomal DNA isolated from *S. lividans* transformants that have received an SLP1 derivative linked to an *E. coli* plasmid shows a hybridization pattern suggesting that a foreign DNA segment (i.e., pACYC 177) has been integrated into the *S. lividans* chromosome along with SLP1 (Fig. 3). To further investigate this possibility, we utilized an *S. lividans* gene that appears to be an analog of an *S. coelicolor* gene identified as *afsB* by Horinouchi et al. (21). A DNA fragment containing this gene had been isolated (Stein and Cohen, unpublished data) by cloning a *Sau*3A partial digest of genomic DNA from *S. lividans* C14 into an SLP1.2-derived plasmid (23) and introducing this ligation mixture into D32, a pigment-deficient mutant of *S. lividans* C14, and subsequently identifying pigmented transformants. A 9.4-kb *Bam*HI-*Sau*3AI DNA fragment containing a gene(s) that enables pigment production by D32 cells was found (Stein and Cohen, unpublished data) to have a restriction endonuclease fragmentation pattern similar to that reported for the *S. coelicolor afsB* gene (21). Also, this fragment hybridized to a locus in the *S. coelicolor* genome having a restriction pattern identical to that reported for the *afsB* gene for all restriction endonucleases tested (21). A 10-kb *Bam*HI-*Bgl*II fragment from pJAS01 *afsB* that includes the *afsB*-complementing fragment was introduced into the unique *Bam*HI site of the pCAO153 plasmid (Fig. 5),

yielding plasmid pCAO153*afsB*, which was propagated in *E. coli*. The *afsB*-containing plasmid was introduced by transformation into *S. lividans*, and five Th-resistant clones were isolated for further study; these were designated CAO 153A1, CAO153A2, CAO153A3, CAO153A4, and CAO 153A5.

An *S. lividans* strain carrying a mutation in *afsB* was constructed as described in Materials and Methods by recombination with the *S. coelicolor* mutant BH5. The resulting *S. lividans* mutants were defective in pigment production and failed to produce actinorhodin and undecylprodigiosin, although sporulation appeared normal; one *afsB* isolate of *S. lividans* C28 (C28–BH5-3) was used in subsequent studies. During the course of these studies, we observed that a restriction-fragment-length polymorphism in a DNA segment that hybridized with the cloned *afsB*-complementing gene did not move from *S. coelicolor* to *S. lividans* with the transfer of the pigment-deficient phenotype. We have also found that the *S. lividans* gene we have cloned, which seems to be analogous to the *S. coelicolor afsB* gene cloned by Horinouchi et al., complements a number of other pigmentless mutants in addition to the *afsB* mutant that Hara et al. have isolated. These observations, which will be described in greater detail elsewhere, do not affect the conclusions of the studies reported here. However, it should be noted that, while we have adopted the *afsB* designation of Horinouchi et al. for the cloned gene, this gene may not correspond to the chromosomal locus that Hara et al. have designated as *afsB*.

Figure 7 shows the results of Southern blot analysis of genomic DNA isolated from strains CAO153A1 through A5; total DNA was probed with nick-translated pSLP1.2 plasmid DNA or with a 9.3-kb *Bam*HI-*Cla*I DNA fragment containing the *afsB*-complementing gene. The detection of *Bam*HI-*Hin*dIII-generated DNA fragments of 9, 6.5, 6.2, 5.9, and 1.65 kb is consistent with the view that an additional *Bam*HI-*Bgl*II DNA fragment, 10 kb in length, had integrated

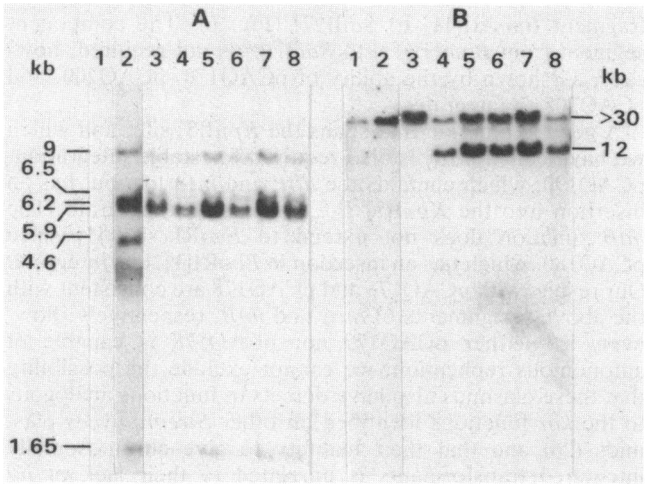


FIG. 7. Southern analysis demonstrating SLP1-mediated integration of an additional copy of the *afsB* gene into the *S. lividans* chromosome. *Bam*HI and *Hin*dIII double digests of 2 μg of chromosomal DNA were probed with nick-translated SLP1.2 DNA (A) or the nick-translated 9.3-kb *Bam*HI-*Cla*I fragment containing the cloned *afsB* gene (B). Chromosomal digests are from strains C14 (lane 1), M192 (lane 2), C14 containing pCAO153 (lane 3), C14 containing pCAO153 *afsB* derivatives; lanes 4 to 8 contain DNA from five separate isolates. Fragment mobilities were calculated by using the known circular map of pCAO153 and confirmed by comparison with *Hin*dIII-cut bacteriophage λ size markers.

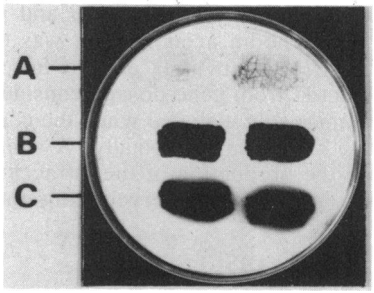


FIG. 8. Pigment production by *S. lividans* C14 strains containing different copy numbers of the *afsB*$^+$ gene. Spores were patched onto R2YE (0.2% yeast extract) containing 2.0% agar. Plates were incubated for 72 h at 30°C. Patched strains are as follows: A, strain C14 containing pCAO153; B, strain C14 containing pCAO153 *afsB*; C, strain C14 containing pJAS01 *afsB* (strain harbors about six extrachromosomal copies of the *afsB*$^+$ gene). Note the halo of excreted actinorhodin around patch C.

into the *S. lividans* chromosome along with the SLP1 and pACYC177 segments of the pCAO153-derived plasmids. Further analysis with the nick-translated *afsB* DNA probe showed a hybridizing fragment approximately 30 kb in length in strains that have received the SLP1 or pCAO153 plasmids; this *Bam*HI-*Hin*dIII band presumably results from the chromosomal copy of *afsB*$^+$ normally present in *S. lividans*. However, DNA isolated from the pCAO153 *afsB* A1 to A5 strains showed, in addition to the 30-kb band, a band 12 kb in length, consistent with the presence of the *afsB*-containing adventitious DNA fragment at the *attB* site of the chromosomes of these strains.

As the relative concentrations of regulatory proteins and the genes that they control can affect gene expression (7, 37, 40, 50), and as chromosomal genes carried by plasmids have their copy number elevated because of the multiplicity of plasmids in a bacterial cell, investigations of genetic control mechanisms potentially can be affected when a gene of chromosomal origin is carried by a plasmid. The results described above indicate that a single copy of a cloned adventitious DNA sequence can be inserted into the *S. lividans* chromosome by using SLP1-mediated site-specific integration. It was therefore of interest to determine whether the developmentally implicated *afsB*-complementing gene (21) inserted into the *S. lividans* chromosome is biologically competent to express regulatory functions and to examine whether gene dosage effects of this pleiotrophic regulatory gene are evident.

Figure 8 shows pigment production on solid media by *S. lividans* C14 cells that contain the chromosomally integrated pCAO153 or pCAO153 *afsB1* element or the extrachromosomal pJAS01-derived replicon carrying the *afsB*-complementing gene. Cells carrying the *afsB*$^+$ gene extrachromosomally show greatly increased pigment production. In addition, actinorhodin production after growth in liquid culture for 24 h was assessed spectrally for an *S. lividans* strain lacking a functional *afsB* gene, for a strain in which one copy of the *afsB*-complementing gene had been introduced into the chromosome, and for a strain containing a multicopy plasmid (pJAS01*afsB*, five to six copies per chromosome) that carries the *afsB*-complementing gene extrachromosomally (Fig. 9). Both the integrated *afsB*$^+$ gene and the plasmid-borne gene complemented the defect present in the *afsB* mutant, resulting in the production of actinorhodin (Fig. 9). Actinorhodin continued to accumulate beyond 24 h

of growth for cultures of pCAO153*afsB* and pJAS01*afsB*; however, no actinorhodin accumulation was observed for cultures of C28-BH5 even after growth for an extended period. As expected from gene dosage considerations, pigment yield was markedly elevated when the complementing gene was located extrachromosomally. A similar effect of gene dosage on the production of the intracellular pigment undecylprodigiosin was also observed (data not shown).

DISCUSSION

Our results demonstrate that the 17-kb full-length SLP1 element can be used to integrate foreign DNA into the *S. lividans* chromosome. An SLP1 element that has been cloned in another bacterial species inserts both itself and DNA that has been linked to it into the *S. lividans* chromosome at the same site occupied by an SLP1 element that has been transferred to *S. lividans* by mating.

The *attP* site of SLP1 plus two separate additional regions of the element are required for integration. The location of these regions is shown in Fig. 10. One of the required regions, the *attP* sequence, previously has been identified as the site at which integration of SLP1 occurs at the *attB* locus of the *S. lividans* chromosome (34). One of the two other plasmid regions identified as being required for stable integration is located at least partly within a segment absent in most of the naturally occurring SLP1 deletion derivatives that replicate extrachromosomally as plasmids. As noted above, we have designated the essential genetic function in this region, which has as its outside limits *Sac*I[18] to *Sal*I[27], as *intA*. SLP1.2 contains *attP* but lacks at least part

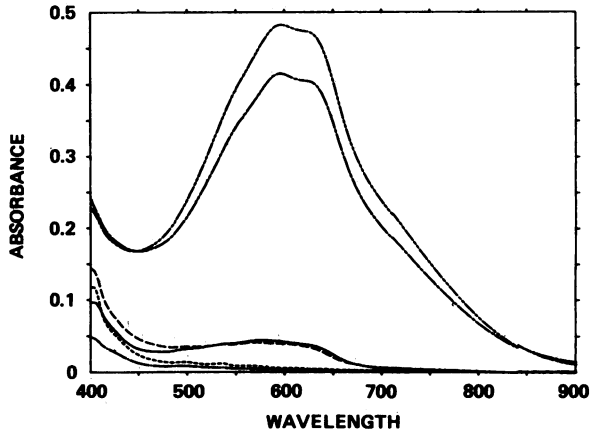


FIG. 9. Relative production of actinorhodin by *S. lividans* C28-BH5-3 containing integrated versus plasmid-borne copies of the *afsB*-complementing gene. Cultures (50 ml) of liquid R2 including 0.2% yeast extract were inoculated to a density of 10^7 spores per ml with *S. lividans* C28-BH5-3 (*afsB*) containing various SLP1-derived constructs, two of which carry the cloned *afsB*$^+$ gene. Cultures were incubated with aeration for 24 h at 30°C. A 5-ml amount of each culture was filtered through a 0.2-μm filter unit (Millipore Corp.), and absorbance was measured over the range of wavelengths from 400 to 900 nm. At culture pH, the absorbance maximum for actinorhodin occurs at about 594 nm. Two separate isolates were inoculated and scanned for each of the three strains tested, and cell densities were approximately equivalent in all six cultures at the time of sampling. Tracings represent actinorhodin produced by the following three strains: (——— · ——— · ——— · ,----) C28-BH5-3 containing pCAO153; (————,————) C28-BH5-3 containing pCAO153 *afsB*; (——— - - ——— - - ———,— - — - —) C28-BH5-3 containing pJAS01 *afsB*.

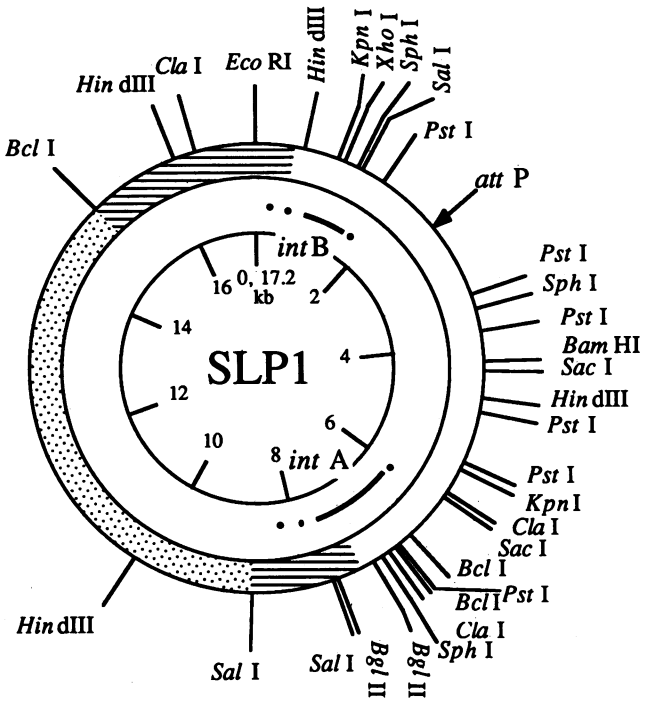


FIG. 10. Location of regions of SLP1 required for its chromosomal integration. A map of the full-length SLP1 element in its circular form is shown. The locations of the two regions previously found to be essential for autonomous SLP1 plasmid replication or maintenance are indicated as hatched arcs, and the region required for transfer of SLP1 is indicated as a stippled arc (16). The approximate locations of *intA* and *intB* are indicated as arcs. The location of the *attP* site (32) is indicated by an arrow.

of *intA* and is unable to integrate. Integration of the SLP1.2 derivative pIJ13 can be complemented in *trans* by the fragment *Bam*HI[11] to *Sal*I[27] (Fig. 6). The component segment from *Bam*HI[11] to *Sac*I[18] is not required, however, as shown by the ability of pCAO170, pCAO200, and pCAO205 to integrate.

A genetic function that spans the *Kpn*I[3] site, and which we have called *intB*, is also required for stable integration. pCAO190, which contains the *attP* and *intA* loci but has an insertion into the *Kpn*I[3] site, is unable to integrate. The *intB* function does not extend to *Eco*RI site [1] since pCAO180, which has an insertion in *Eco*RI[1], can integrate. Our results with pCAO175 and pCAO178 are consistent with the above assignments of *intA* and *intB*, respectively; however, as neither pCAO175 nor pCAO178 is capable of autonomous replication, we cannot exclude the possibility that these plasmids also have defects in functions analogous to the *kor* functions identified on other *Streptomyces* plasmids (26) and that their inability to give chromosomally integrated transformants is unrelated to their lack of *int* functions.

Our finding that chromosomal integration of SLP1 requires at least two separate plasmid regions in addition to the *attP* site implies that more than one SLP1-encoded product is required to mediate site-specific integration of the SLP1 element or that the integrated SLP1 element cannot be stably maintained in the chromosome in the absence of a gene product separate from the putative "integrase." The inability of SLP1 plasmids to replicate in a cell carrying an integrated SLP1 element and the failure to observe amplifi-

cation of SLP1 when it is integrated into the chromosome (4, 32) together suggest that SLP1^{int} synthesizes a *trans*-acting gene product that controls replication of the integrated element. Direct evidence for the existence of a repressor of the SLP1 replicon function recently has been obtained (S. R. Grant and S. N. Cohen, unpublished data).

In addition to SLP1, a number of other genetic elements encoding site-specific recombination systems have been identified in recent years. In the bacteriophage P1 *loxP* and yeast 2μ plasmid FLP systems, single genes carried by the extrachromosomal element are required for site-specific recombination with the chromosome (1, 15). Also, only one element-encoded gene is involved, together with chromosomally encoded gene products, in the site-specific DNA inversions that occur during phase variation in *Salmonella* spp. and in the G-loop and C-loop DNA rearrangements of bacteriophages Mu and P1, respectively (5, 22, 25). In contrast, integration of bacteriophage λ into the *E. coli* chromosome requires expression of the λ *int* gene protein (49), which in turn requires expression of the λ *N* gene (12). Similarly, complex mechanisms regulate expression of the λ *xis* gene, which is needed in addition to *int* for phage λ excision (12, 13, 24). The integration and excision functions of SLP1 also are regulated (S. C. Lee, C. A. Omer, and S. N. Cohen, manuscript in preparation).

The observation that plasmids lacking the capacity for interbacterial transfer can nevertheless integrate chromosomally into *S. lividans* indicates that transfer and integration are independent events. Moreover, the integration capabilities of the pCAO180 plasmid, which is defective in a region previously identified as being required for plasmid replication or maintenance or both, shows that these functions are not required for integration.

It is well recognized that the stoichiometry of repressors and activators relative to the sites of their interaction with DNA is of major importance in the regulation of gene expression (7, 37, 40, 50). Thus, investigation of genetic control mechanisms that operate during the complex developmental life cycle of *Streptomyces* species may require maintenance of the regulatory genes or the sites at which they interact at the copy number normally occurring in the chromosome. Our data indicate that the phenotypic properties of cells containing plasmid-borne copies of the cloned *afsB*-complementing gene differ from those of cells containing an integrated copy.

In the experiments reported here, we have begun to define the genetic functions required for site-specific integration of SLP1. We speculate that an integration cassette consisting of only an *attP* site plus genes included in the "essential" regions of SLP1 may be sufficient to accomplish stable integration into the *S. lividans* chromosome of a variety of DNA sequences derived from foreign sources. The ability of an SLP1 element to integrate both itself and a linked segment of adventitious DNA into the *S. lividans* chromosome potentially provides a tool for introducing single copies of various cloned genes into the chromosome of *S. lividans* and also to circumvent the propensity for structural instability that has been observed previously for genes carried by *Streptomyces* plasmids (8, 27; our unpublished observations).

The site-specific integration system of SLP1 may also be useful for stably adding long biosynthetic pathways to the *S. lividans* chromosome. While we have not determined the maximum size of adventitious DNA that can be inserted by SLP1, in the experiments reported here approximately 15 kb of adventitious DNA was delivered at high frequency into the *S. lividans* chromosome. Finally, it should be noted that pCAO153 is a conjugally competent replicon that is also an integrating vector. Thus, this plasmid can be used to transfer the DNA to be integrated between different cells or strains. When transfer of the integrating plasmid is not desired, pCAO200, which does not contain the SLP1 transfer region and thus cannot mediate conjugation between cells, can be used.

ACKNOWLEDGMENTS

Plasmid pIJ922-int4.5 was constructed by S. R. Grant.

These studies were supported by Public Health Service grant GM 26355 from the National Institutes of Health and American Cancer Society grant MV-44 to S.N.C. C.A.O. was supported by Postdoctoral Fellowship PF 2148 from the American Cancer Society during part of this work. D.S. was the recipient of a Public Health Service Predoctoral Fellowship Award from the National Institutes of Health Training Program for Ph.D. Candidates, GM07790. Certain work was carried out as Dupont Experimental Station contribution 4517.

LITERATURE CITED

1. **Andrews, B. J., G. A. Proteau, L. G. Beatty, and P. D. Sadowski.** 1985. The FLP recombinase of the 2μ circle DNA of yeast: interaction with its target sequences. Cell **40**:795–803.
2. **Bibb, M. J., R. F. Freeman, and D. A. Hopwood.** 1977. Physical and genetic characterization of a second sex factor, SCP2, from *Streptomyces coelicolor* A3(2). Mol. Gen. Genet. **154**:155–166.
3. **Bibb, M. J., J. M. Ward, and D. A. Hopwood.** 1978. Transformation of plasmid DNA into Streptomyces at high frequency. Nature (London) **274**:398–400.
4. **Bibb, M. J., J. M. Ward, T. Kieser, S. N. Cohen, and D. A. Hopwood.** 1981. Excision of chromosomal DNA sequences from *Streptomyces coelicolor* forms a novel family plasmids detectable in *Streptomyces lividans*. Mol. Gen. Genet. **184**:230–240.
5. **Bruist, M. F., and M. I. Simon.** 1984. Phase variation and the *Hin* protein: in vivo activity measurements, protein overproduction, and purification. J. Bacteriol. **159**:71–79.
6. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–201.
7. **Chadwick, P., V. Pirrotta, R. Steinberg, N. Hopkins, and M. Ptashne.** 1970. The λ and 434 phage repressors. Cold Spring Harbor Symp. Quant. Biol. **35**:283–294.
8. **Chen, C. W., J. F. Y. Tsai, and S. Chuang.** 1987. Intraplasmid recombination in *Streptomyces lividans 66*. Mol. Gen. Genet. **209**:154–158.
9. **Cohen, A., D. Bar-Nir, M. E. Goedeke, and Y. Parag.** 1985. The integrated and free states of *Streptomyces griseus* plasmid pSG1. Plasmid **13**:41–50.
10. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
11. **Denhardt, D. T.** 1966. A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
12. **Gottesman, M. E., and R. A. Weisberg.** 1983. Prophage insertion and excision, p. 113–138. *In* Lambda II. R. Hendrix, J. Roberts, F. Stahl, and R. A. Weisberg (ed.), Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
13. **Guarneros, G., and H. Echols.** 1970. New mutants of bacteriophage λ with a specific defect in exision from the host chromosome. J. Mol. Biol. **47**:565–574.
14. **Hara, O., S. Horinouchi, T. Uozumi, and T. Beppu.** 1983. Genetic analysis of A-factor synthesis in *Streptomyces coelicolor* A3(2) and *Streptomyces griseus*. J. Gen. Microbiol. **129**: 2939–2944.
15. **Hoess, R. H., M. Ziese, and N. Sternberg.** 1982. P1 site-specific recombination: nucleotide sequence of the recombining sites. Proc. Natl. Acad. Sci. USA **79**:3398–3402.

16. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of Streptomyces, a laboratory manual. John Innes Foundation, Norwich, U.K.
17. **Hopwood, D. A., K. F. Chater, J. E. Dowding, and A. Vivian.** 1973. Advances in *Streptomyces coelicolor* genetics. Bacteriol. Rev. **37**:371–405.
18. **Hopwood, D. A., G. Hinterman, T. Kieser, and H. M. Wright.** 1984. Integrated DNA sequences in three streptomycetes form autonomous plasmids after transfer to *Streptomyces lividans*. Plasmid **11**:1–16.
19. **Hopwood, D. A., T. Kieser, H. M. Wright, and M. J. Bibb.** 1983. Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. J. Gen. Microbiol. **129**:2257–2269.
20. **Hopwood, D. A., H. M. Wright, M. J. Bibb, and S. N. Cohen.** 1977. Genetic recombination through protoplast fusion in *Streptomyces*. Nature (London) **286**:171–174.
21. **Horinouchi, S., O. Hara, and T. Beppu.** 1983. Cloning of a pleiotropic gene that positively controls biosynthesis of A-factor, actinorhodin, and prodigiosin in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J. Bacteriol. **155**:1238–1248.
22. **Iida, S., J. Meyer, K. Kennedy, and W. Arber.** 1982. A site-specific conservative recombination system carried by bacteriophage P1. EMBO J. **1**:1445–1453.
23. **Jaurin, B., and S. N. Cohen.** 1984. *Streptomyces lividans* RNA polymerase recognizes and uses *Escherichia coli* transcriptional signals. Gene **28**:83–91.
24. **Kaiser, A., and T. Masuda.** 1970. Evidence for a prophage excision gene in λ. J. Mol. Biol. **47**:557–564.
25. **Kamp, D., L. T. Chow, T. R. Broker, D. Kwoh, D. Zipser, and R. Kahmann.** 1979. Site-specific recombination in phage Mu. Cold Spring Harbor Symp. Quant. Biol. **43**:1159–1167.
26. **Kendall, K., and S. N. Cohen.** 1987. Plasmid transfer in *Streptomyces lividans*: identification of a *kil/kor* system associated with the transfer region of pIJ101. J. Bacteriol. **169**:4177–4183.
27. **Kieser, T., D. A. Hopwood, H. M. Wright, and C. J. Thompson.** 1982. pIJ101, a multicopy broad host range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. Mol. Gen. Genet. **185**:223–238.
28. **Lydiate, D. J., F. Malpartida, and D. A. Hopwood.** 1985. The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. Gene **35**:223–235.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
31. **Moretti, P., G. Hinterman, and R. Hutter.** 1985. Isolation and characterization of an extrachromosomal element from *Nocardia mediterranei*. Plasmid **14**:126–133.
32. **Omer, C. A., and S. N. Cohen.** 1984. Plasmid formation in *Streptomyces*: excision and integration of the SLP1 replicon at a specific chromosomal site. Mol. Gen. Genet. **196**:429–438.
33. **Omer, C. A., and S. N. Cohen.** 1985. SLP1: transmissible *Streptomyces* chromosomal element capable of site-specific integration, excision, and autonomous replication, p. 449–453. *In* D. Schlessinger (ed.), Microbiology—1985. American Society for Microbiology, Washington, D.C.
34. **Omer, C. A., and S. N. Cohen.** 1986. Structural analysis of plasmid and chromosomal loci involved in site-specific excision and integration of the SLP1 element of *Streptomyces coelicolor*. J. Bacteriol. **166**:999–1006.
35. **Omer, C. A., and S. N. Cohen.** 1987. Site-specific excision and integration of the *Streptomyces* transmissible element SLP1, p. 95–101. *In* M. Alačević, D. Hranvelí, and Z. Toman (ed.), Proceedings of the Fifth International Symposium on the Genetics of Industrial Microorganisms: Split, Yugoslavia, part A.
36. **Pernodet, J. L., J. M. Simonet, and M. Guerineau.** 1984. Plasmids in different strains of *Streptomyces ambofaciens*: free and integrated form of plasmid pSAM2. Mol. Gen. Genet. **198**: 35–41.
37. **Ptashne, M.** 1986. A genetic switch. Blackwell Scientific Publication, Palo Alto, Calif.
38. **Richardson, M. A., J. A. Mabe, N. E. Beerman, W. M. Nakatsukasa, and J. T. Fayerman.** 1982. Development of cloning vehicles from the *Streptomyces* plasmid pFJ103. Gene **20**:451–457.
39. **Rigby, P. W. J., M. Dieckman, C. Rhodes, and P. Berg.** 1977. Labelling deoxynucleic acid to high specific activity in vitro by nick translating with DNA polymerase I. J. Mol. Biol. **113**:237–251.
40. **Riggs, A. D., H. Suzuki, and S. Bourgeois.** 1970. *Lac* repressor-operator interaction. J. Mol. Biol. **48**:67–83.
41. **Smith, H. O., and M. L. Birnstiel.** 1976. A simple method for DNA restriction site mapping. Nucleic Acids Res. **3**:2387–2398.
42. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
43. **Stonesifer, J., P. Matsushima, and R. H. Baltz.** 1986. High frequency conjugal transfer of tylosin genes and amplifiable DNA in *Streptomyces fradiae*. Mol. Gen. Genet. **202**:348–355.
44. **Thompson, C. J., T. Kieser, J. M. Ward, and D. A. Hopwood.** 1982. Physical analysis of antibiotic-resistance genes from *Streptomyces* and their use in vector construction. Gene **20**:51–62.
45. **Thompson, C. J., J. M. Ward, and D. A. Hopwood.** 1982. Cloning of antibiotic resistance and nutritional genes in streptomycetes. J. Bacteriol. **151**:668–677.
46. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13 mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
47. **Vivian, A.** 1971. Genetic control of fertility in *Streptomyces coelicolor* A3(2): plasmid involvement in the interconversion of UF and IF strains. J. Gen. Microbiol. **69**:353–364.
48. **Wright, L. F., and D. A. Hopwood.** 1976. Identification of the antibiotic determined by the SCP1 plasmid of *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. **95**:96–106.
49. **Zissler, J.** 1967. Integration-negative (*int*) mutants of phage λ. Virology **31**:189.
50. **Zuber, P., J. M. Healy, and R. Losick.** 1987. Effects of plasmid propagation of a sporulation promoter on promoter utilization and sporulation in *Bacillus subtilis*. J. Bacteriol. **169**:461–469.