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Cloning and Expression in *Streptomyces lividans* of Antibiotic Resistance Genes Derived from *Escherichia coli*

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Hybrid plasmids that replicate in both *Escherichia coli* and *Streptomyces lividans* were constructed in vitro by joining the *E. coli*-derived plasmid pACYC184 or pACYC177, at their *Bam*HI or *Pst*I restriction site, respectively, to *S. lividans* plasmid pSLP111. After introduction of the composite replicons into *S. lividans* by transformation, chloramphenicol (Cm) resistance encoded by pACYC184 and kanamycin resistance encoded by pACYC177 were phenotypically expressed in the *S. lividans* host. A *Sau*3A restriction endonuclease-generated deoxyribonucleic acid fragment from pACYC184 containing the entire structural gene for the Cm acetyltransferase enzyme, but lacking the nucleotide sequence ordinarily serving as the Cm resistance gene promoter, also specified resistance to Cm when introduced in either orientation into the *Bam*HI or *Bcl*I endonuclease cleavage site of pSLP111 or into corresponding sites of the analogous plasmid pSLP101. These findings make it unlikely that the biologically active Cm acetyltransferase was being made in *S. lividans* as part of a fused protein, but instead indicate that the ATG start codon used for initiation of translation of the Cm resistance gene in *E. coli* was also utilized in *S. lividans*. In contrast, the synthesis of messenger ribonucleic acid that encodes the Cm acetyltransferase in *S. lividans* was, in at least one instance, apparently initiated at nucleotide sequences within the *S. lividans* plasmid vector, with resulting transcriptional read-through into the *E. coli*-derived deoxyribonucleic acid segment.

Streptomyces spp. are gram-positive, mycelial organisms that undergo a complex developmental life cycle and produce a variety of medically important antibiotics as secondary metabolites (3). Recently, the establishment of an efficient transformation system for *Streptomyces* spp. (6) and the development of endogenous plasmids and phages as cloning vehicles (5, 29, 30) have provided the tools for investigating the molecular genetics of these organisms. Moreover, it is now possible to use DNA cloning to examine the organization and regulation of antibiotic production pathways in *Streptomyces* spp. in an effort to produce new and possibly more-effective antibiotics.

Hybrid plasmids able to replicate in both *Escherichia coli* and *Streptomyces* species, and containing selectable genes capable of phenotypic expression in each of the hosts, could greatly facilitate the structural analysis of *Streptomyces* genes and the study of factors that regulate the expression of genetic information transplanted between these hosts. Whereas the expression of genes derived from gram-positive organisms has been shown to occur in gram-negative bacteria (8, 11, 12), the reverse is not generally true (12, 13). One possible barrier to gene expression between different bacterial species may result from the known differences in the guanine-plus-cytosine content of their DNA (e.g., 73 mol% for *Streptomyces lividans* and 48 mol% for *E. coli*) and the consequent failure of regulatory signals (e.g., A+T-rich sequences of promoters [24]) to function effectively.

During investigations intended to identify DNA sequences that accomplish transcriptional and translational control in *Streptomyces* spp. we observed that two antibiotic resistance genes derived from *E. coli* were expressed in *S. lividans*. The present report describes these findings and presents data indicating that the promoter that normally accomplishes transcription of the chloramphenicol (Cm) resistance gene in *E. coli* is not responsible for its expression in *S. lividans* and that the ATG start codon for this gene is utilized as the translational start signal in the new host.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strain C600 (*hsdR hsdM*$^+$) (2), *S. lividans* strain 1326, and a Cm^s derivative of 1326, M252, were used as the host

organisms for all of the transformations described in this paper. Cm^s derivatives of 1326 arose spontaneously at a frequency of about 5% (data not shown). M252 was chosen as a suitable Cm^s isolate because of its relatively very low reversion frequency to resistance ($<10^{-8}$ [14]), and it was used as the recipient for all *S. lividans* transformations involving the *E. coli* Cm resistance gene. *S. lividans* strain M233 harbors plasmid SCP1 (17, 32), which encodes production of and resistance to methylenomycin A (Mm), and was used in the selection and confirmation of Mm^r transformants as previously described (5).

E. coli plasmids pACYC184, which encodes resistance to Cm and tetracycline (Tc), and pACYC177, which encodes resistance to kanamycin (Km) and ampicillin (Ap) (9), were used for the construction of the hybrid plasmids. pACYC184 was also used as the source of the *Sau*3A restriction endonuclease-generated DNA fragment that encodes the structural gene for the Cm acetyltransferase (1).

Derivatives of the SLP1.2 plasmid isolated from *S. lividans* and used for the cloning experiments described in this paper have been described previously (5). pSLP111 encodes resistance to Mm and elicits the lethal zygosis phenotype. pSLP101 is identical to pSLP111 but lacks the *Pst*I-generated fragment that encodes resistance to Mm.

Culture conditions and transformation procedures. Luria broth (23) and Penassay base agar (antibiotic medium no. 2; Difco Laboratories) were used for the cultivation of strain C600. When antibiotic selection for *E. coli* derivatives was necessary, the following concentrations were used: Cm, 25 μg/ml; Tc, 10 μg/ml; Km, 25 μg/ml; Ap, 25 μg/ml. The procedure for transformation of *E. coli* by $CaCl_2$ treatment has been described previously (10).

For the isolation of plasmid DNA, the *S. lividans* strains were grown in yeast extract-malt extract medium containing 34% sucrose and 0.1% $MgCl_2$ (YEME [4]). For the preparation of protoplasts, mycelia were grown in S medium (19) containing 1% glycine and 0.1% yeast extract. Transformation of *S. lividans* protoplasts, using polyethylene glycol, was essentially as described previously (6), except that 0.1% yeast extract was added to the regeneration medium. Selection for Mm^r bacteria was done on complete medium agar (16), and the lethal zygosis phenotype was tested on regeneration medium (R2, reference 19). Cm^r transformants were selected on R2 containing 0.1% yeast extract and Cm at 5 μg/ml unless otherwise indicated. Km^r transformants were selected on minimal medium (16) containing 0.1% yeast extract and Km at 0.5 or 1 μg/ml.

DNA isolation and manipulation. The isolation of covalently closed circular DNA from *E. coli* (31) and *S. lividans* (4) by using ethidium bromide-cesium chloride density gradients has been described previously. A mini-lysate procedure for analysis of plasmid DNA from *Streptomyces* spp. was used for screening purposes essentially as described previously (7). YEME cultures (10 ml each) were harvested after growth at 30°C for 24 to 36 h, and the mycelia were suspended in 1 ml of 20% glycerol. The lysozyme treatment was done at 37°C for 30 min, using 0.10 to 0.25 ml of the resuspended cells.

Restriction endonucleases and the bacteriophage T4 DNA ligase were purchased from New England Biolabs and used as the manufacturer recommended. Enzyme reactions were terminated by heating at 65°C for 5 min and were monitored for completion by agarose gel analysis.

Agarose gel electrophoresis was performed either with vertical slab gels in 40 mM Tris–5 mM sodium acetate–1 mM EDTA (pH 7.8) or with horizontal slab gels in Loening buffer (15) (36 mM Tris, 30 mM NaH_2PO_4, and 1 mM EDTA; pH 7.8). Acrylamide gels were run as vertical slabs in 100 mM Tris-borate–2 mM EDTA (pH 8.3) by the method of Maniatis et al. (21). After electrophoresis, the gels were stained in 1-μg/ml ethidium bromide for 20 min, and the DNA bands were visualized under UV light.

The recovery of DNA from acrylamide gels was performed essentially as described previously (22), by cutting the DNA band out of the gel while visualizing it under UV light. The gel slice was placed inside a dialysis bag with 1 to 2 ml of 5 mM Tris-borate plus 0.1 mM EDTA (pH 8.3). The DNA was electroeluted from the gel slice against 2 liters of the same buffer at 80 V for 10 h or 200 V for 2 h. The polarity of the current was reversed for 1 min to avoid DNA binding to the side of the dialysis bag. The DNA solution was removed from the dialysis bag and passed through siliconized glass wool to remove any pieces of acrylamide. The DNA was extracted once with an equal volume of phenol equilibrated against TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA; pH 8) and three times with an equal volume of ether. The DNA was precipitated with ethanol after the addition of tRNA carrier (final concentration, 50 μg/ml) and 0.3 M potassium acetate (pH 7). Two volumes of cold ethanol were added, and the mixture was incubated in a bath of dry-ice plus ethanol for 20 min. The DNA was pelleted in a Beckman JS13 swinging-bucket rotor at 10,000 rpm for 30 min, washed with 70% ethanol at −70°C, lyophilized to dryness, and suspended in TE buffer.

Assay for Cm acetyltransferase activity. Preparation of the cell extracts and assay for the presence of the Cm acetyltransferase were performed essentially as described by Shaw and Hopwood (27), except that the *S. lividans* cultures were grown in YEME at 30°C for 36 to 48 h and the *E. coli* cultures were grown in Luria broth at 37°C for 12 to 16 h.

RESULTS

Construction and analysis of hybrid plasmids pSLP120 and pSLP125. The constructions of hybrid plasmids pSLP120 and pSLP125 are illustrated in Fig. 1. Both *S. lividans* plasmid pSLP111 and *E. coli* plasmid pACYC184 contain single sites for restriction enzyme *Bam*HI within nonessential regions of the plasmid DNA (5, 9). After both plasmids were cleaved with *Bam*HI, the linear DNAs were mixed, ligated, and introduced by transformation into *E. coli* C600. Since insertion of DNA into the *Bam*HI site of pACYC184 inactivates the gene for Tc resistance (9), *E. coli* transformants containing the hybrid

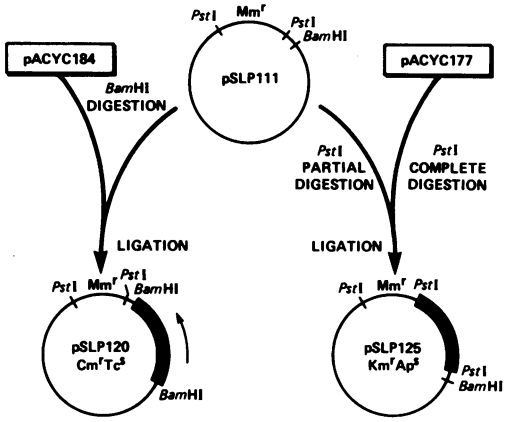

FIG. 1. *Construction of the composite replicons containing E. coli and S. lividans plasmid DNA. pSLP120 was constructed by first digesting both pACYC184 and pSLP111 with BamHI. After ligation of the two plasmids and introduction into C600 by transformation, Cm^r colonies were screened for Tc sensitivity. Digestion of the composite plasmids with HindIII determined the orientation of the pACYC184 insertion into pSLP111. The direction of transcription of the Cm resistance gene is indicated by an arrow. pSLP125 was constructed by ligating a partial PstI digest of pSLP111 with pACYC177 DNA that had been digested completely with the same enzyme and transforming C600 with the resulting ligation mix. Km^r colonies were screened for Ap sensitivity. Digestion of the composite plasmid with BamHI identified the PstI site of pSLP111 into which pACYC177 had been inserted. The heavy lines represent either pACYC177 or pACYC184 DNA, and the thin lines indicate pSLP111 DNA.*

plasmid were selected as Cm^r colonies and screened for Tc sensitivity. Plasmid DNA was isolated from the Cm^r Tc^s candidates. Digestion with *Bam*HI endonuclease and subsequent analysis by agarose gel electrophoresis revealed two fragments identical in length to that of plasmids pSLP111 and pACYC184. Digestion of the hybrid plasmid with endonuclease *Hin*dIII or *Sal*I allowed us to determine the orientation of the two component plasmids of the construct; the Cm resistance gene on pACYC184 is transcribed counterclockwise on the plasmid restriction map when the *Hin*dIII site is clockwise to the *Eco*RI site, as shown in reference 20. We therefore conclude that on hybrid plasmid pSLP120, the Cm resistance gene is transcribed in a counterclockwise direction, as indicated in Fig. 1.

pACYC177 contains a single cleavage site for endonuclease *Pst*I, whereas pSLP111 contains two cleavage sites for this enzyme. Since we wanted to retain on pSLP111 the *S. coelicolor*-derived *Pst*I fragment containing the Mm^r marker, plasmid pSLP111 was digested partially with *Pst*I and pACYC177 was digested completely. The enzyme-treated samples were mixed, ligated, and introduced into *E. coli* C600 by transformation. Insertion of DNA into the *Pst*I site of pACYC177 inactivates the Ap resistance gene (9); therefore, *E. coli* transformants harboring the hybrid plasmid (designated pSLP125) were detected by selection for Km^r colonies and screening for Ap sensitivity. Plasmid DNA was extracted from several candidates; digestion with *Pst*I and subsequent analysis on agarose gels revealed *Pst*I fragments that corresponded in length to the two *Pst*I fragments of pSLP111 and the linear pACYC177. Digestion of the hybrid plasmid with endonuclease *Bam*HI indicated which *Pst*I site of pSLP111 the pACYC177 plasmid had been inserted into; this is shown in Fig. 1.

Not all of the candidates having a correct phenotype (Cm^r Tc^s or Km^r Ap^s) contained complete hybrid plasmids; analysis of the plasmid DNA in 6 of the 12 candidates examined indicated the occurrence of deletions that appeared to remove part of the pSLP111 component. We have not studied this phenomenon in more detail to determine whether, for example, the end points of the deletions exhibit any site-specificity within pSLP111.

Introduction of hybrid plasmids into *S. lividans* by transformation and expression of Cm and Km resistance phenotypes. The selection of *S. lividans* transformants harboring plasmid pSLP120 or pSLP125 was done in two ways. Since we could not predict the level of Cm or Km resistance, if any, in *S. lividans* because of previous reports indicating a lack of expression of *E. coli* genes in gram-positive bacteria (13), we initially selected for Mm^r colonies. The occurrence of such transformants would suggest that the hybrid plasmid had been taken up and was being maintained in the cell. We could then screen for any instances of resistance to Cm or Km.

Mm^r transformants were selected by plating the spores from regeneration plates onto complete medium and adding patches of a Mm-producing strain (18); Mm^r colonies appeared in the zones of growth inhibition caused by the diffusion of Mm from the producing strain. Alternatively, the lethal zygosis phenotype elicited by plasmid pSLP111 was used for selection; a plasmid-containing isolate produces a "pock" on a lawn of plasmid-less bacteria (17), and spores picked from the center of such pocks contain the plasmid. Therefore, pocks observed on the original regeneration plates or on confluent lawns of M252 or 1326 to which spores from the regeneration mixtures had been added were picked and tested for Mm resistance.

In one experiment, the plasmid DNAs from one Mm^r clone isolated from *S. lividans* after transformation with pSLP120 and from two Mm^r candidates that resulted from transformation with pSLP125 were analyzed by agarose gel electrophoresis. In all three cases, the pSLP111 segment of the plasmid appeared to remain intact, whereas the segment derived from the *E. coli* plasmid had undergone deletion. In the case of pSLP120, the resulting length of the *E. coli*-derived segment of the hybrid plasmid was less than half the size of the original pACYC184 plasmid. None of the three clones was resistant to either Cm (pSLP120) or Km (pSLP125), nor did the plasmid DNA obtained from the isolates transform *S. lividans* to resistance to these antibiotics. These results suggested either that the deletions had removed DNA sequences necessary for resistance to Cm or to Km or that if the genes were intact, they were not expressed in *S. lividans*.

Since deletions within the *E. coli*-derived segment of the hybrid plasmid occurred at high frequency when transformants receiving hybrid plasmids were initially selected as Mm^r, we attempted direct selection for resistance to Cm or Km. Spores harvested from the regeneration plates were spread either onto R2 plates containing Cm at 5 μg/ml or onto minimal medium plates containing Km at 0.5 or 1 μg/ml for the selection of transformants carrying pSLP120 or pSLP125, respectively. Spores from Cm^r or Km^r colonies were restreaked and tested for their ability to elicit the lethal zygosis phenotype (which would indicate the presence of plasmid pSLP111) and for their ability to form colonies on Cm- or Km-containing medium. Spores were harvested from those candidates that were lethal zygosis positive/Cm^r or lethal zygosis positive/Km^r, and the plasmid DNA was isolated. Subsequent restriction endonuclease cleavage analysis confirmed the presence of intact hybrid plasmids. The lengths of the two *Bam*HI fragments of the plasmid isolated from transformants containing pSLP120 were identical to those of the original plasmid. Similarly, the three *Pst*I fragments obtained after digestion of plasmid DNA isolated from transformants containing pSLP125 were equal in length to the three *Pst*I fragments of pSLP125 (data not shown).

To demonstrate that the Cm^r and Km^r phenotypes were due to the introduction of the hybrid plasmids and not, for example, to spontaneous mutations, the plasmid DNAs isolated from the *S. lividans* antibiotic-resistant clones were used for retransformation into *S. lividans* M252. Cm^r or Km^r transformants were obtained after nonselective regeneration (6) at frequencies 10^3-fold higher than the spontaneous reversion frequency of the Cm^s host to Cm^r. Moreover, the initial frequency of transformation of the hybrid plasmids from *E. coli* to *S. lividans*, which was similar to the spontaneous reversion frequency, was estimated to be about 10^3- to 10^4-fold lower than comparable estimates of the frequencies of retransformation. This finding suggests that restriction may have drastically reduced the frequency of transformation when the hybrid plasmids were initially introduced into *S. lividans* from *E. coli*; having survived restriction and presumably having undergone modification by passage through *S. lividans*, the hybrid plasmids isolated from *S. lividans* retransformed the same host at a three to four orders of magnitude higher frequency.

The hybrid plasmid DNAs isolated from *S. lividans* were also reintroduced by transformation into *E. coli*. Cm^r transformants (for pSLP120) and Km^r transformants (for pSLP125) were selected, and the plasmid DNA was characterized by restriction enzyme analysis and electrophoresis on agarose gels. The plasmid DNA from all four candidates selected from each transformation mixture was found to be identical to the original hybrid plasmid (data not shown). There was no evidence of deletions, and the frequency of transformation of *E. coli* C600 with these plasmids was about 10^5 transformants per μg of DNA, which was only about 10-fold lower than one might expect under optimal conditions with either pACYC177 or pACYC184 DNA.

Insertion of the *Sau*3A fragment encoding pACYC184-derived Cm acetyltransferase into pSLP111 and pSLP101. To reduce the size of the *E. coli*-derived DNA fragment and thereby hopefully decrease the number of presumed restriction targets on the transforming DNA, we inserted into pSLP111 just that part of pACYC184 that contained the Cm resistance gene rather than the entire pACYC184 plasmid. The largest *Sau*3A fragment of pACYC184 encodes the structural gene for the Cm acetyltransferase; this fragment is about 1 kilobase (kb) in length and does not contain the DNA sequence that functions as the promoter for the Cm resistance gene in *E. coli* (J. L. Schottel, J. J. Sninsky, and S. N. Cohen, manuscript in preparation). The nucleotide sequence of the Cm resistance gene of Tn*9* (1) and the length of mRNA encoding Cm acetyltransferase (20) make it highly likely that the promoter sequence for the Cm resistance gene is contained on the 43-base pair *Sau*3A fragment that is immediately 5′ to the 1-kb *Sau*3A fragment encoding the Cm acetyltransferase protein itself.

Since the promoter used in *E. coli* for transcription of the Cm acetyltransferase gene might also be necessary for expression of Cm resistance

in *S. lividans*, we partially digested pACYC184 DNA with *Sau*3A endonuclease, mixed this partial digest with pSLP111 DNA completely digested with *Bam*HI, and then ligated the DNA fragments by using T4 DNA ligase. After transformation of *S. lividans* M252 and protoplast regeneration, transformants were selected for resistance to Cm. Analysis of plasmid DNA isolated from 12 of these candidates revealed that whereas a variety of different sized *Sau*3A fragments had been inserted into the *Bam*HI site, all 12 Cm^r transformants had received at least the 1-kb *Sau*3A fragment that encodes the Cm acetyltransferase structural gene. However, these gels could not preclude the presence of an additional small *Sau*3A fragment.

To determine whether expression of Cm resistance required only the 1-kb *Sau*3A fragment in *S. lividans* and whether it was influenced by the site of insertion of the fragment into the vector, the purified 1-kb fragment, which lacks the sequence that functions as the promoter for the Cm resistance gene in *E. coli*, was introduced into different sites on plasmids pSLP101 and pSLP111; those clones that expressed high-level resistance to Cm were identified by direct selection on medium containing the antibiotic. Clones from 10 separate transformations with pSLP111 plasmids containing the *Sau*3A fragment inserted into the *Bam*HI site showed insertion in only one orientation (depicted by plasmid pSLP118 [Fig. 2]). Insertion of the *Sau*3A fragment into the analogous *Bam*HI cleavage site of pSLP101 DNA, which lacks the Mm resistance fragment but is otherwise identical to pSLP111, showed preference for the opposite orientation when selection was carried out directly for Cm resistance (depicted by pSLP109 [Fig. 2]). However, Cm resistance was observed when derivatives of the fragment were inserted in either orientation between the *Bam*HI and *Pst*I sites of pSLP101 and when transformants selected initially by the lethal zygosis phenotype were subsequently tested for Cm acetyltransferase gene expression.

Insertion of the *Sau*3A fragment into the *Bcl*I

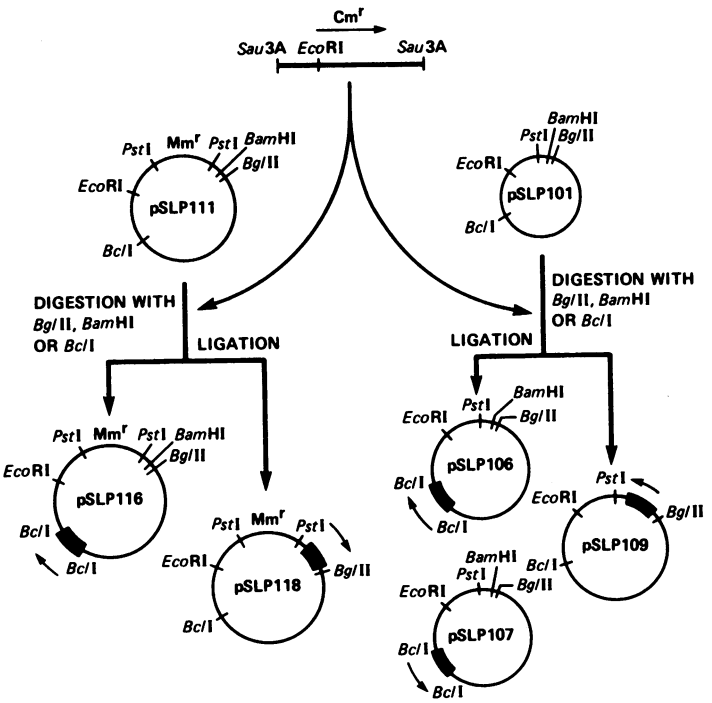

FIG. 2. *Insertion of the purified Sau3A fragment encoding the Cm acetyltransferase from pACYC184 into pSLP111 and pSLP101. The purified 1-kb Sau3A fragment from pACYC184, which encodes Cm acetyltransferase, was ligated to either pSLP111 or pSLP101 DNA that had been digested with either BamHI, BglII, or BclI. Analysis of the plasmid DNA from Cm^r transformants revealed the different structures shown in the diagram. The thin lines represent the pSLP111 or pSLP101 DNA, and the thick lines indicate the position of the inserted E. coli-derived Sau3A fragment. The arrows illustrate the direction of transcription of the Cm resistance gene. No insertions into the BglII site of either plasmid were found, and only one orientation of the fragment into the BamHI site of pSLP111 and pSLP101 was observed.*

site of pSLP101 showed no orientation preference, and plasmid DNAs from 11 independent Cm^r transformants were identical in structure to that of either pSLP106 (four transformants, Fig. 2) or pSLP107 (seven transformants; Fig. 2). Four separate Cm^r transformants resulting from introduction of the *Sau*3A fragment into the *BclI* site of pSLP111 revealed structures identical to that of pSLP116. Plasmids with insertions in the opposite orientation were not observed in this study. However, plasmids having *Sau*3A fragment insertions in both orientations in the *BclI* site of pSLP111 should be obtainable, since pSLP111 and pSLP101 are identical around their *BclI* sites.

Three attempts to insert the 1-kb *Sau*3A fragment into the *BglII* site of pSLP111 and one attempt to insert this gene into the *BglII* site of pSLP101 were unsuccessful. These results suggest that cloning of DNA fragments into the *BglII* site interrupts a vital function necessary for plasmid replication or maintenance. This view is consistent with the inability to obtain deletions within pSLP101 of sequences containing the *BglII* site of this plasmid (M. Bibb, unpublished data).

The frequency of transformation with the ligated mixture of pSLP111 and the *Sau*3A fragment was much higher than was the transformation frequency observed with a hybrid plasmid isolated from *E. coli* and containing the entire pACYC184 replicon (i.e., pSLP120). Assuming 100% ligation (which yields the lowest estimate for transformation frequency), we obtained after nonselective regeneration about 2×10^6 Cm^r colonies per μg of DNA with the *Sau*3A fragment constructs, compared with 15 Cm^r colonies per μg during an initial transformation with the *E. coli*-derived pSLP120 plasmid. On retransformation of *S. lividans* with plasmid pSLP116 or pSLP118, we obtained 1.1×10^7 and 2.4×10^6 antibiotic-resistant colonies per μg of DNA, respectively.

Production of Cm acetyltransferase by Cm^r *S. lividans* transformants harboring pSLP120, pSLP116, or pSLP118. Cm resistance encoded by plasmid pACYC184 is due to the production of the Cm acetyltransferase enzyme, which acetylates Cm and thus renders it inactive (26). *S. lividans* does not produce Cm acetyltransferase (27). If the observed resistance to Cm of *S. lividans* is due to expression of the Cm resistance gene present on the constructed plasmids, the inactivating enzyme should be detectable in the host cells. Cell extracts of *S. lividans* M252 harboring pSLP111 (as a control) or harboring recombinant plasmid pSLP120, pSLP116, or pSLP118 were tested for the presence of Cm acetyltransferase by measuring the acetylation of [^{14}C]Cm (27).

Extracts of *S. lividans* M252 harboring the recombinant plasmids exhibited Cm acetylation, whereas the strain harboring plasmid pSLP111 had no detectable levels of acetyltransferase (Fig. 3). A lower level of acetylation was detected in extracts of M252 cells that contained plasmids having the Cm gene insert when compared to the level of acetylation produced by extracts of C600(pACYC184). Possible explanations include the differences in copy number of the plasmids in the two bacterial species (pACYC184 is present at about 20 copies per chromosome in *E. coli* [9], whereas SLP1.2, the parent replicon of pSLP111 and of the recombinant plasmids, has a copy number of 4 to 5 in *S. lividans* (Bibb, unpublished data), in the efficiency of expression of the *E. coli* Cm resistance gene between the two species, or in the efficiency of extraction of the enzyme from the two bacteria.

Correlation of the lethal zygosis phenotype with Cm resistance. To further confirm the cloning and expression of the *E. coli* Cm resistance gene within *S. lividans*, we undertook to show a direct correlation between the presence of the hybrid plasmid and the Cm resistance phenotype. The Cm resistance phenotype could be tested directly, and the presence of the plasmid could be confirmed by the ability of the

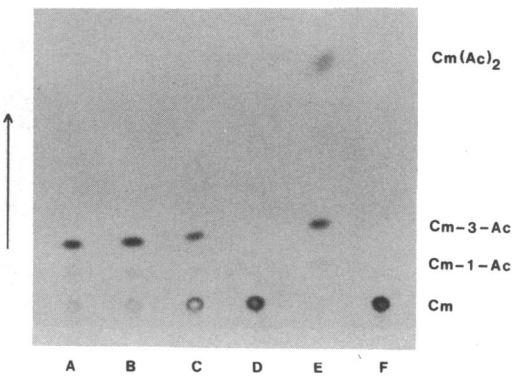

FIG. 3. *Cm acetyltransferase assays of extracts from S. lividans carrying recombinant plasmid pSLP116, pSLP118, or pSLP120. Thin-layer silica gel chromatography of [^{14}C]Cm and its acetylated derivatives was performed as described by Shaw and Hopwood (27). The direction of migration is indicated by the arrow on the left. Unacetylated Cm (F) remains at the origin; the positions of the acetylated derivatives are indicated at the right [Cm-1-Ac, chloramphenicol 1-acetate; Cm-3-Ac, chloramphenicol 3-acetate; Cm(Ac)$_2$, chloramphenicol 1,3-diacetate]. Extracts of the following strains were tested: (A) M252(pSLP120), (B) M252(pSLP118), (C) M252-(pSLP116), (D) M252(pSLP111), (E) C600(pACYC184).*

cells to elicit the lethal zygosis phenotype. Such an experiment is shown in Fig. 4. The results indicated that whenever the recombinant plasmid was lost (as shown by the absence of lethal zygosis), resistance to Cm was also lost; conversely, whenever Cm resistance was absent, the lethal zygosis phenotype was no longer expressed.

Stability of the recombinant plasmids. The stabilities of plasmids pSLP118, pSLP120, and pSLP125 in *S. lividans* were determined (data not shown) by first isolating spores from single colonies that showed the lethal zygosis phenotype. The spores were diluted and plated to obtain single colonies on nonselective R2 medium. The single colonies were tested for resistance to either Cm or Km and for the lethal zygosis phenotype; therefore, the estimates of stability are based on one life cycle of nonselective growth. pSLP118, pSLP120, and pSLP125 had stabilities of 76, 54, and 45%, respectively. Moreover, there was no difference in the frequency of expression of the antibiotic resistance phenotype versus the lethal zygosis phenotype, indicating that deletions of the *E. coli*-derived segment did not occur at high frequency once initial propagation of the recombinant plasmid in *S. lividans* had been accomplished.

The stability of plasmid pSLP111 was not tested in *S. lividans* M252, but the family of SLP1 plasmids from *S. lividans* 1326 (17) range in stability from 25 to 98% (Bibb, unpublished data). Therefore, introduction of the *Sau*3A fragment encoding the Cm resistance gene into plasmid pSLP111 or construction of hybrid plasmids containing pSLP111 and pACYC184 or pACYC177 does not significantly alter the stability of the resulting plasmid.

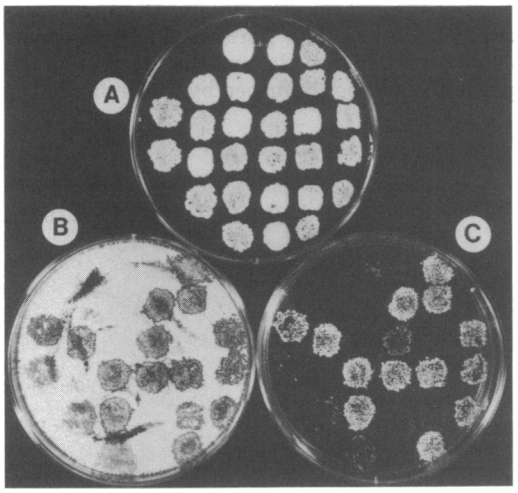

FIG. 4. *Correlation of the lethal zygosis phenotype with Cm resistance. A spore preparation resulting from a purified Cm^r transformant harboring plasmid pSLP118 was plated to yield single colonies on R2 medium containing 0.1% yeast extract; single colonies were then patched onto the same medium (plate A) and allowed to sporulate. The patches were replica plated to R2 medium that had been spread with a lawn of S. lividans 1326 to test for the lethal zygosis (Lz) phenotype (plate B) and to minimal medium containing Cm at 5 μg/ml (plate C). After incubation at 30°C for 3 days, the colonies were scored as either Cm^r/Lz$^+$ or Cm^s/Lz$^-$. Patches that showed partial resistance to Cm also exhibited comparable segregation of the Lz phenotype, presumbaly due to instability of the recombinant plasmid.*

DISCUSSION

The findings presented here indicate that genes derived from *E. coli* and conferring resistance to Cm or Km are expressed phenotypically in *S. lividans*. The direct expression within *S. lividans* of the Cm and Km resistance genes derived originally from *E. coli* plasmid R6-5 (31) via pACYC184 and pACYC177 was surprising in the light of earlier results involving genes transplanted from gram-negative to gram-positive bacterial species (12, 13). Recently, however, Rubin et al. (25) have reported the expression in *Bacillus subtilis* of the *E. coli* gene encoding thymidylate synthetase. Their evidence indicates that after transformation into *B. subtilis*, the plasmid is not maintained extrachromosomally but is integrated into the chromosome.

In the present studies, hybrid plasmids that consisted of *S. lividans* plasmid pSLP111 plus either pACYC177 or pACYC184 were constructed. Although these plasmids could replicate and express their antibiotic resistance markers (Cm^r or Km^r) in either *E. coli* or *S. lividans*, there appeared to be a significant amount of restriction of the plasmids when they were introduced by transformation into *S. lividans* after having been propagated in *E. coli*; when the number of Mm^r colonies that resulted from transformation of *S. lividans* with pSLP111 was compared to the number of Mm^r colonies that resulted from transformation with one of the hybrid plasmids, the transformation efficiency was reduced by approximately 10^6 (data not shown). After surviving the apparent initial restriction barrier, however, hybrid plasmids subsequently transformed *S. lividans* at an efficiency 10^3- to 10^4-fold higher than initially.

It seems likely from the nucleotide sequence of the Cm resistance gene (1) that the 1-kb *Sau*3A fragment from pACYC184 does not contain the normal promoter for this gene. Supporting evidence for this view is the demon-

strated transcriptional regulation in *E. coli* of Cm acetyltransferase gene expression by a *lac* gene promoter introduced at a position 5′ to the structural gene on the 1-kb *Sau*3A fragment. The cloned 1-kb *Sau*3A fragment alone does not confer resistance to Cm in *E. coli* (J. S. Sninsky and J. L. Schottel, unpublished data). Our results therefore do not imply that an *E. coli* promoter is being recognized by the RNA polymerase of *Streptomyces* spp.

Two possibilities for the source of the transcriptional start signal exist: although no nucleotide sequence capable of functioning as a promoter in *E. coli* is present between the ATG translational start codon for the Cm acetyltransferase structural gene and the proximal *Sau*3A cleavage site, it is possible that a nucleotide sequence within this 59-base pair DNA segment is recognized by the *Streptomyces* RNA polymerase and can initiate RNA synthesis in *S. lividans*. Since promoters from *Streptomyces* spp. have yet to be analyzed, they may differ from the promoter sequences that have been identified and characterized on plasmids and phages (24) of *E. coli*. However, removal of most of the 59-base pair segment preceding the structural gene, except for the five base pairs immediately 5′ to the ATG translational start codon, does not abolish Cm acetyltransferase gene expression from the *Sau*3A fragment when it is inserted into the *Bam*HI site of pSLP101 in the counterclockwise orientation (our unpublished data), indicating that a promoter site within the *E. coli*-derived fragment is not required for initiation of RNA synthesis in at least one instance.

Alternatively, transcription may be initiated at a *Streptomyces* promoter sequence present on the pSLP101 and pSLP111 vectors. Readthrough into the *Sau*3A fragment from at least two *S. lividans* plasmid promoters would be necessary, since we have shown expression of the Cm resistance gene in two different orientations. Moreover, since the *Bam*HI and *Bcl*I sites of pSLP101 are located on opposite sides of the vector, this proposal would require the existence of either very long mRNA transcripts (at least 5 kb in length) or the coincidental presence of multiple promoters on each strand of the *Streptomyces* plasmid DNA. In any case, the nucleotide sequences of the recombinant plasmids that are functioning as promoters to accomplish expression of the Cm resistance gene in *S. lividans* are different from the normal promoter sequences that function in *E. coli*.

We were able to show expression of the Cm resistance gene when the *Sau*3A fragment was inserted into two different restriction sites (*Bam*HI and *Bcl*I) and in two different orientations at each site on the pSLP101 or pSLP111 plasmid, as well as expression of Cm acetyltransferase from a hybrid replicon containing the pSLP111 vector plus the entire pACYC184 plasmid. It seems unlikely that in all five of these separate constructs the phenotypically expressed enzyme was being made as part of a fused protein, which would require the Cm acetyltransferase sequence to be read in frame with another genetic sequence in each construct. Rather, these results strongly suggest that the ATG start codon for the Cm acetyltransferase structural gene is being used for initiation of translation. Whether the entire putative *E. coli* ribosomal binding site (TAAGGA) (28), which is located proximal to the structural gene on the 1-kb *Sau*3A fragment, is being recognized by a rRNA sequence in *S. lividans* or whether another nucleotide sequence on the 1-kb fragment serves to accomplish ribosomal binding in *S. lividans* is not known.

Further analysis of the plasmids containing the *Sau*3A Cm resistance gene fragment should provide additional information about the structure and function of transcriptional and translational regulatory signals in *Streptomyces* spp.

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