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Cloning of Antibiotic Resistance and Nutritional Genes in Streptomycetes

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Methodology which allows consistent shotgun cloning of streptomycete genes is presented. Parameters that increase transformation efficiency of *Streptomyces lividans* 66 were adjusted to generate reproducibly a population of cloned genes likely to represent the entire genome. Factors which influence the recovery of viable transformants include: growth phase of the mycelium, ionic and osmotic characteristics of the medium during protoplast formation and transformation, and moisture content and protoplast density during regeneration. A modified transformation procedure was devised which increased transformation frequency more than 20-fold (allowing up to 10^7 primary transformants per μg of SLP1.2 covalently closed circular DNA) and greatly facilitated the cloning of drug resistance genes and biosynthetic genes, using one of two plasmid vectors. Viomycin resistance genes on *Bam*HI or *Pst*I fragments were cloned from *S. vinaceus* genomic DNA into *S. lividans*, using the SLP1.2 vector. At least three different *S. vinaceus Bam*HI fragments (1.9, 5.8, or 8.5 kilobases) confer viomycin resistance; only one *Pst*I fragment (4.3 kilobases) was found. Recombinant plasmids were all able to produce lethal zygosis and to be transferred by conjugation within *S. lividans*. SCP2* was used to clone *S. coelicolor* A3(2) genes that "complemented" the auxotrophic mutation *hisD3*, *argA1*, or *guaA1*. Recombinant DNA technology can now be applied to economically and academically interesting problems unique to streptomycete molecular biology.

Although there has been great progress in the development of recombinant DNA technology in *Escherichia coli* host-vector systems, there is little doubt that alternative host organisms will be more suitably applied to certain problems. Interest in both commercial and biological problems associated with the regulation of streptomycete gene products encouraged us to consider these organisms as potential hosts. Our laboratory has had a long-term interest in streptomycete genetics in relation particularly to plasmid function, antibiotic production, and differentiation. Such processes can be analyzed only in a host which contains the complex background allowing normal expression of wild-type streptomycete genes. Previous work not only suggested the usefulness of recombinant DNA technology, but also made available several plasmids as potential vectors.

SCP2 was the first *Streptomyces* plasmid to be isolated as covalently closed circular (CCC) DNA (2, 20, 21). It has a molecular size, derived from its restriction endonuclease cleavage map (Fig. 1, taken from reference 4), of about 31 kilobases (kb). SCP2 is self-transmissible at high frequency within *Streptomyces coelicolor* and enhances chromosomal recombination ("fertility") in matings (2, 3). Much of its coding capacity remains cryptic. Spontaneous plasmid mutants (SCP2*) can be isolated which further increase chromosomal recombination and display a more pronounced lethal zygosis (Ltz^+) phenotype, observed as an inhibition of mycelial development as the plasmid is transferred into an initially plasmid-free culture (2). When a spore containing SCP2* is plated on a plasmid-free lawn, it can be visualized as a small area of growth inhibition, known as a "pock" (3, 5). Lethal zygosis is also shown by SCP1, the first *S. coelicolor* sex plasmid to be investigated (2).

The Ltz^+ phenotype led to the discovery of a series of other plasmid vectors, the SLP1 family. When an *S. coelicolor* A3(2) culture lacking SCP1 and SCP2, and showing no physical evidence of CCC DNA, is grown with *S. lividans* 66, a proportion of the *S. lividans* spores derived from the cross gives rise to pocks when plated in a lawn of *S. lividans*. Isolates purified from some such pocks each contain a member of the SLP1 family (SLP1.1, SLP1.2, etc.). These self-transmissible plasmids, which appear to arise by excision and circularization of DNA sequences from the chromosome of *S. coelicolor* A3(2) (7),

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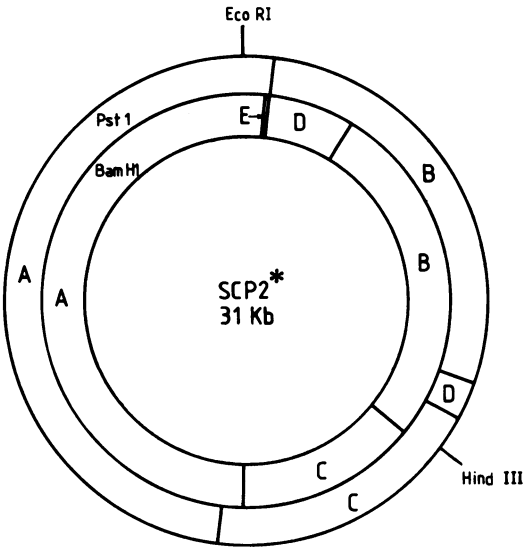

FIG. 1. Restriction endonuclease cleavage map of SCP2 (redrawn from reference 4).

are variable in size, but each contains a common region (the entire sequence of the smallest member of the family) together with a variable segment, presumably derived from the *S. coelicolor* chromosome. In other words, the largest plasmid (SLP1.2) contains all of the information required for plasmid maintenance and transfer, together with a block of dispensable DNA. The only *Bam*HI, *Pst*I, *Xho*I, *Kpn*I, and *Sph*I recognition sequences in SLP1.2 are located in this region of the replicon (Fig. 2). It should be possible, therefore, to insert DNA into these sites without disturbing any essential plasmid functions.

Plasmid DNA can be introduced into a streptomycete host by transformation with relatively high efficiency. This technique calls for removal of the gram-positive cell wall with lysozyme to generate protoplasts. In the presence of polyethylene glycol, DNA enters the protoplasts by an unknown mechanism. This was initially demonstrated for SCP2* and SLP1 by the Ltz^+ phenotype (5, 6).

After the theoretical requirements for a successful shotgun cloning system in streptomycetes had been satisfied, its general efficacy was demonstrated (4, 23). Bibb et al. (4) cloned a methylenomycin resistance gene from *S. coelicolor* genomic DNA into SCP2* and SLP1.2; we (23) reported the cloning of neomycin resistance determinants (involving either neomycin phosphotransferase or neomycin acetyltransferase) from *S. fradiae* and a thiostrepton resistance determinant (encoding a 23S rRNA methylase) from *S. azureus* into SLP1.2.

The refinements in the cloning technology reported here, including a modified version of the original transformation technique yielding up to 10^7 transformants per μg of SLP1.2 DNA, have allowed the consistent shotgun cloning of selectable biosynthetic and antibiotic resistance genes. In addition, this work further explores the characteristics of two vector-host systems, SLP1.2-*S. lividans* and SCP2*-*S. coelicolor*.

MATERIALS AND METHODS

Media and buffers. Most of the media and buffers have been described previously: minimal medium (MM [12]), yeast extract-malt extract medium plus 34% sucrose (YEME [2]), R2 medium (19), and P medium (19). R2YE consists of R2 with 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) (23). R2YE or MM was supplemented as indicated with 50 μg of arginine, 10 μg of guanine, or 75 μg of histidine per ml. Tryptone soya broth was obtained from Oxoid Ltd., London, U.K. P medium was modified for more efficient use in lysozyme digestion of mycelium (L medium) and transformation (T medium). L medium has qualitatively the same composition as P medium but different concentrations of some components {10% sucrose; 25 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid] buffer, pH 7.2; 1.4 mM K_2SO_4; 0.4 mM K_2HPO_4; 2.5 mM $MgCl_2$; 2.5 mM Ca Cl_2) and contains 1 mg of lysozyme per ml [Sigma Chemical Co., St. Louis, Mo.]}. T medium differs from P medium in the concentrations of sucrose (2.5%) and $CaCl_2$ (0.1 M); it has 50 mM Tris-maleic acid (pH 8)

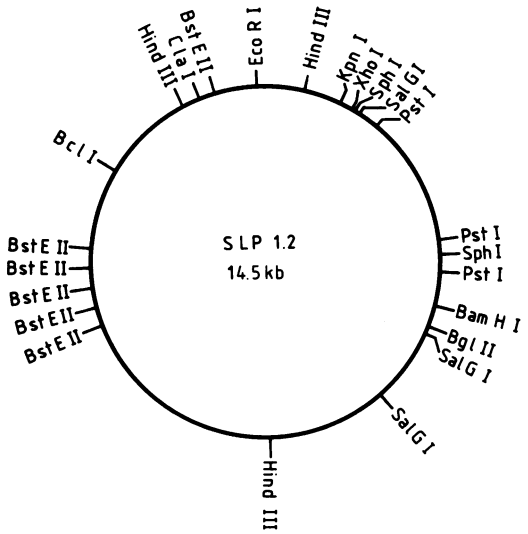

FIG. 2. Restriction endonuclease cleavage map of SLP1.2 (Thompson et al., manuscript in preparation). The map was derived independently from that of Bibb et al. (7) and is based on a different set of molecular size standards, hence the slight differences in estimates of map interval lengths. In addition to the cleavage sites originally reported (6) for the restriction endonucleases *Hin*dIII, *Sal*GI, *Eco*RI, *Pst*I (an extra site was subsequently detected), and *Bam*HI, this map includes the recognition sites for *Bcl*I, *Cla*I, and *Bgl*II also used by Bibb et al. (7), as well as those for *Kpn*I, *Xho*I, *Sph*I, and *Bst*EII.

instead of TES buffer and contains 25% (wt/vol) polyethylene glycol 1000 (Koch-Light, Colnbrook, Buckinghamshire, U.K.).

For the selection of viomycin-resistant strains, MM was supplemented with 30 μg of viomycin per ml (obtained from Henry Dion, Parke Davis and Co., Detroit, Mich., or Pfizer, Central Research, Sandwich, Kent, U.K.) or 30 μg of capreomycin IA per ml (a substrate analog of viomycin, purified from a mixture of capreomycins [22]).

Bacterial strains. The recipient organisms used as hosts for recombinant plasmids were *S. lividans* 66 (16; JI strain no. 1326) and *S. coelicolor* A3(2) strain B341 (*argA1 hisD3 guaA1* SCP1$^-$ SCP2$^-$). CT2 was a streptomycin-resistant derivative of JI1326. Donor organisms were *S. vinaceus* NCIB 8852 and *S. coelicolor* A3(2).

DNA preparation. Chromosomal DNA was isolated from mycelium by using the technique detailed in reference 9. Briefly, the mycelium was treated with lysozyme and then with EDTA-pronase. The material was then solubilized in sodium dodecyl sulfate, the nucleic acids were extracted with phenol, and the RNA was destroyed by RNase digestion. Large-scale plasmid isolation was by the method of Bibb et al. (2).

Plasmid minipreparations. Plasmid DNA was prepared on a small scale, using a procedure modified by T. Kieser (personal communication) from Birnboim and Doly (8). Spores scraped from a single colony were inoculated into 5 ml of tryptone soya broth. After 20 h of growth at 30°C, the mycelium was transferred to a 1.5-ml Eppendorf centrifuge tube and washed in 10.3% sucrose. The mycelium was digested for 1 h at 30°C in 1 ml of a solution which contained 10% sucrose, 1 mg of lysozyme per ml, 100 mM glucose, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.4 mM KH_2PO_4, trace elements (19), 1.4 mM K_2SO_4, and 25 mM Tris buffer (pH 7.2). The mixture was centrifuged for 1 min, and the pellet was suspended in 0.1 ml of the same solution lacking lysozyme. An alkaline lytic solution (0.2 ml containing 1% sodium dodecyl sulfate, 0.2 N NaOH, and 25 mM EDTA) was added and mixed. After 5 min at 4°C, 0.2 ml of 3 M sodium acetate (pH 4.8) was added to the samples, which were chilled for 60 min at 4°C and centrifuged for 5 min. Plasmid DNA contained in 0.4 ml of the supernatant solution was precipitated by the addition of 1 ml of ethanol (−20°C) at −20°C for 30 min and pelleted by 2 min of centrifugation. Samples were dissolved in 0.1 ml of a solution which contained 0.3 M sodium acetate, 10 mM, Tris (pH 8.0), and 1 mM EDTA and reprecipitated as above by the addition of 2.5 volumes of cold ethanol. After centrifugation, the pellets were dried under vacuum, dissolved in 0.1 ml of sterile TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and stored at −20°C. This solution (0.01 ml) was used to transform protoplasts.

Restriction enzyme mapping of plasmids. Details of digestions and agarose gel electrophoresis of DNA fragments are described elsewhere (C. J. Thompson, T. Kieser, J. M. Ward, and D. A. Hopwood, manuscript in preparation). Molecular weight standards were *Hin*dIII fragments of bacteriophage lambda (23.52, 9.59, 6.76, 4.45, 2.29, 1.95, and 0.58 kb) estimated by Daniels et al. (10) from standards having a known sequence, using a least-squares analysis of multiple restriction endonuclease digestions.

Preparation and transformation of streptomycete protoplasts. The original procedure for the preparation and transformation of streptomycete protoplasts (5) was taken as a starting point for modifications. Mycelium grown in 25 ml of S medium (in 250-ml flasks) containing 0.5% glycine and 5 mM $MgCl_2$ was pelleted and washed twice in 10.3% sucrose. Protoplasts were generated by cell wall digestion with lysozyme (1 mg/ml) in P medium for 1 h at 32°C. Most of the nonprotoplasted mycelial material was removed by filtration through a cotton plug (<0.1% of the viable count were osmotically stable, nonprotoplasted units), and protoplasts were collected by centrifugation (800 × *g*, 7 min). The protoplasts were counted in a hemocytometer and washed, and the pellet (containing 4 × 10^9 protoplasts) was suspended in the small volume of P medium remaining after the supernatant from the second wash was decanted. Plasmid DNA was added in less than 20 μl of TE buffer. Transformation was achieved by the addition of 0.5 ml of 20% polyethylene glycol 1000 dissolved in P medium.

The following modifications were introduced to increase transformation efficiencies. *S. lividans* mycelium was grown in YEME medium containing 34% sucrose, 5 mM $MgCl_2$, and 0.5% glycine for 30 to 36 h at 32°C (*S. coelicolor* mycelium required 40 to 60 h for growth) in a 250-ml flask containing a coiled spring for dispersal of mycelial clumps. After two washes in 10.3% sucrose, the mycelium was suspended for lysozyme digestion in L medium (4 ml per original 25 ml of culture) and incubated for 1 h at 32°C. All subsequent steps were carried out at room temperature. After the addition of 5 ml of P medium, protoplasts were filtered through a cotton plug, pelleted (800 × *g*, 7 min), washed once in P medium, and counted in a hemocytometer. Samples of 4 × 10^9 protoplasts were washed again in P medium, pelleted, and suspended in the small volume of P medium remaining after the supernatant was poured off. Plasmid DNA suspended in less than 20 μl of equal volumes of TE buffer and 10.3% sucrose was mixed with the protoplasts. T medium (0.5 ml) was added, and within 30 s transformation was terminated by the addition of 5 ml of P medium. Protoplasts were pelleted, suspended in P medium, and spread on plates of R2YE agar medium (which had been dried in a laminar-flow cabinet for about 4 h to remove about 23% of the weight of the medium).

Shotgun cloning experiments. Restriction enzyme-digested total DNA of *S. fradiae*, *S. azureus*, or *S. vinaceus* was ligated with *Bam*HI- or *Pst*I-cleaved SLP1.2, using conventional recombinant DNA techniques (digestions and ligations were monitored by agarose gel electrophoresis). The construction of *S. fradiae* and *S. azureus* gene libraries was described previously (23). For *S. vinaceus*, total DNA was cleaved with either *Pst*I or *Bam*HI and ligated with SLP1.2 cleaved with the same enzyme. *S. coelicolor* A3(2) DNA was partially cleaved with *Mbo*I into fragments of 2 to 20 kb in length and ligated to SCP2* *Bam*HI fragments. (*Mbo*I and *Bam*HI generate the same 5′ four-base-pair, single-strand complementary ends.) Vector (0.5 μg) and genomic (2.5 μg) fragments were mixed and heated to 75°C for 10 min, precipitated in 2.5 volumes of ethanol, suspended at a DNA concentration of 40 μg/ml in ligation salts (75 μl of 66 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA, 10 mM $MgCl_2$, 10 mM mercaptoethanol, 0.1 mM ATP), and ligated with T4 DNA ligase (prepared by induction of a lysogen containing a recombinant lambda phage which directs the synthesis of a T4 DNA ligase-cloned frag-

ment [18]). The ligation reaction was cooled over a 24-h period from 18 to 4°C and maintained at 4°C for 48 to 96 h. Under these conditions the amount of enzyme used to ligate the mixture was sufficient to circularize 3.0 μg of SLP1.2 DNA cleaved with *Bam*HI.

Ligated DNA was introduced into 4×10^9 protoplasts, which were allowed to regenerate on 10 R2YE plates (supplemented with histidine, arginine, and guanine in the case of B341) and replicated to the appropriate selective medium. *S. lividans* 1326, the recipient for interspecific drug resistance transfers, was replicated to MM containing viomycin. Biosynthetic genes cloned intraspecifically in *S. coelicolor* B341 (*hisD3 argA1 guaA1*) were selected by replication to MM containing histidine plus arginine, arginine plus guanine, or guanine plus histidine.

Matings. Matings were made (13) by mixing donor and recipient spore suspensions on R2YE slants. After 6 to 10 days (*S. lividans*) or 14 to 20 days (*S. coelicolor*), spores were scraped from the slant, filtered through cotton plugs, and plated under selective conditions on appropriately supplemented MM.

RESULTS

Modified transformation conditions. To increase the reproducibility and efficiency of the original transformation procedure (5), we investigated the effect of changing a number of parameters.

The growth phase of the mycelium was found to have a dramatic effect on protoplast transformability (Fig. 3). Protoplasts generated from cultures which had been growing for 24 to 60 h could be most efficiently transformed. The earliest logarithmic cultures tested (18 h) yielded few protoplasts, whereas older cultures (more than 60 h) yielded fewer viable protoplasts (data not shown). Logarithmically growing mycelium (24 h) yielded protoplasts of low viability (plating efficiency, 0.11%), which in turn generated relatively few viable transformants (6.1×10^5 per μg of SLP1.2 DNA). The transition period between exponential and stationary phases (30 h) yielded the most efficient transformation (4.6×10^6/μg) and regeneration (0.7%) frequencies. As the growth rate of the culture decreased in later stationary phase, a decrease in the regeneration frequency coincided with a decrease in transformation frequency. Competition between viable and nonviable protoplasts for plasmid uptake could explain most of the observed changes in transformation frequency.

P medium was used for both protoplast formation and transformation in the original procedure. Separate modified solutions (L and T) were found to allow for the higher transformation efficiency shown in Table 1. In contrast to the effect of growth phase on the yield of viable transformants, the increases resulting from the use of the modified solutions were independent of regeneration efficiency. Under both conditions, in two different experiments, similar numbers of protoplasts were able to regenerate (averaging 1.2% with the original procedure versus 1.6% with the modified procedure); however, 24 times more transformants resulted from the modified procedure (averaging 1.9×10^6 per μg of SLP1.2 DNA) than from the original procedure (averaging 8×10^4 per μg of SLP1.2 DNA). These new conditions, therefore, generate a more competent population of protoplasts from mycelium grown in YEME. We cannot exclude the possibility that mycelium grown in other media (such as the S medium used in previously reported experiments) might respond differently to these changes in the transformation procedure.

The density of protoplasts on regeneration plates also had a dramatic effect on the recovery of transformants. This was determined by plating out various numbers of *S. lividans* proto-

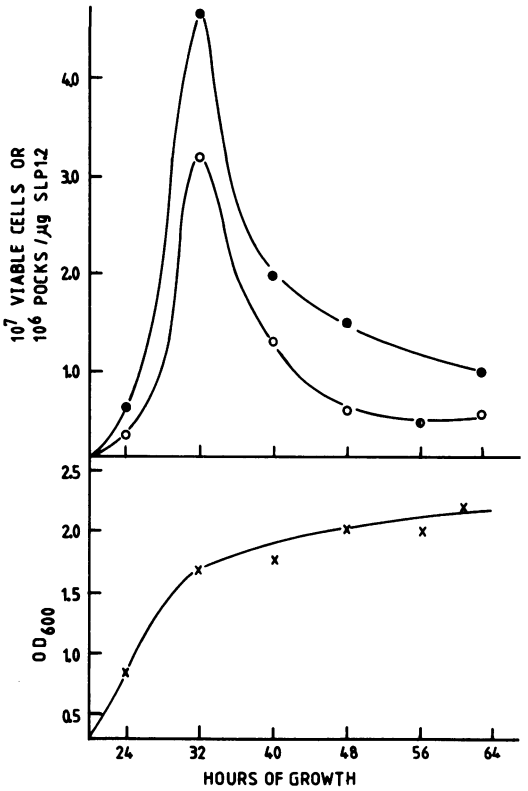

FIG. 3. Effect of mycelial growth phase on protoplast transformability. *S. lividans* JI1326 was grown in YEME medium at 30°C for 24, 32, 40, 48, 56, and 62 h. After the optical density at 600 mm (OD_{600}) was recorded, protoplasts were prepared from the mycelium, using the modified procedure described in the text. Identical numbers of protoplasts (4×10^9) for each time point were then transformed with 0.01 μg of SLP1.2. Protoplasts were regenerated on R2YE plates to quantify the number of viable protoplasts (O) or the number of transformants (●; i.e., the number of pocks).

TABLE 1. Alterations in transformation procedure improves yield of pocks on regeneration plates[a]

Transformation procedure	Expt 1		Expt 2		Expt 3	
	% Regeneration[b]	Transformation frequency[c]	% Regeneration	Transformation frequency	% Regeneration	Transformation frequency
Original[d]	ND	3.0×10^4	1.7	1.6×10^5	0.7	5.0×10^4
Modified[e]	ND	1.9×10^6	1.8	2.0×10^6	1.4	1.9×10^6

[a] Protoplasts (4×10^9 by hemocytometer count) were prepared from *S. lividans* 1326 mycelium and transformed with SLP1.2 DNA. ND, Not determined.
[b] Percent regeneration is colony count on regeneration plates/hemocytometer count of protoplasts × 100.
[c] Transformation frequencies (pocks per microgram of SLP1.2) were determined from the number of pocks observed on regeneration plates.
[d] The procedure of Bibb et al. (5) was used.
[e] The modified procedure described in the text was used.

plasts containing a small population transformed with SLP1.2 and counting the number of pocks (transformants) per plate after regeneration. Above about 4×10^8 protoplasts (as determined by hemocytometer counts) per plate the number of transformants recorded fell. Therefore, the ligation products in a typical shotgun cloning experiment were introduced into a suspension containing 4×10^9 protoplasts, which were then spread on 10 plates. (Since these experiments were done, we discovered [see below] that plate moisture content has a more dramatic influence than was previously recognized on the regeneration of transformants [pocks] on plates containing 4×10^8 protoplasts. This may allow for the use of larger numbers of protoplasts in future shotgun cloning experiments.)

More efficient conditions for regeneration of the transformed population were discovered by examining the effect of plate drying on pock formation (Table 2). *S. lividans* protoplasts were transformed with SLP1.2, using the modified transformation procedure, and identical samples were regenerated on R2YE plates which had been dried in a laminar-flow cabinet for various periods of time ranging from 15 to 240 min. After 240 min water loss amounted to 22.8% of the initial weight of the medium. In the absence of drying, incomplete lawns were generated. A progressive 60-fold increase in the number of pocks was observed as the drying time was increased from 15 to 240 min. Over the same time interval plating efficiency of the protoplasts diluted by 10^6 also increased slightly, from 0.2 to 0.8%. We conclude that the increased yield of viable transformants in confluent lawns is mostly due to an increase in the plating efficiency of transformants under the adverse regeneration conditions which exist on heavily inoculated plates and, in smaller measure, to an increase in viability of protoplasts plated individually after dilution.

The increased transformation frequencies which resulted from these modifications greatly facilitated the isolation of drug resistance and biosynthetic genes with several different plasmid vectors.

Insertion of *S. vinaceus* DNA into SLP1.2. In theory, it should be possible to insert DNA into any site within the dispensable region without destroying the ability of SLP1.2 to replicate or transfer. However, in this work we used only the unique *Bam*HI site and the *Pst*I sites, three of which occur in the dispensable region (Fig. 2).

A typical experiment in which *S. vinaceus* genomic DNA was inserted into the *Bam*HI site of SLP1.2 is shown in Fig. 4. *Bam*HI generated a single linearized plasmid band from SLP1.2. High-molecular-weight *S. vinaceus* total DNA was cleaved by *Bam*HI into a "striated smear" of bands ranging from at least 25 kb down to <1 kb. Successful ligation of a mixture of vector and donor DNA was apparent from the decrease in the migration rate of chromosomal bands as circular and long linear forms were generated. A decrease in the intensity of the band representing SLP1.2-linearized DNA coincided with the appearance of a more slowly migrating band corresponding to the open circular (ligated) form.

Viomycin-resistant clones. A portion of the ligation mixture containing 0.5 μg of SLP1.2 and 2.5 μg of *S. vinaceus* DNA was introduced to *S. lividans* protoplasts, using the modified transformation procedure described in Materials and Methods. The pocks seen on regeneration plates revealed about 10^5 primary transformants. Replication of these lawns to MM containing viomy-

TABLE 2. Effect of plate moisture content on transformant recovery[a]

Drying time (min)	Medium wt loss (%)	% Regeneration[b]	Transformation frequency[b]
0	0.0	0.20	—[c]
15	3.9 ± 0.1	0.20	$7.4 \pm 1.6 \times 10^4$
30	6.2 ± 0.2	0.26	$7.2 \pm 2.5 \times 10^5$
60	9.5 ± 0.3	0.14	$1.5 \pm 0.5 \times 10^6$
120	13.9 ± 0.5	0.46	$2.1 \pm 0.1 \times 10^6$
240	22.8 ± 0.5	0.76	4.4×10^6[d]

[a] Identical portions (4×10^8 protoplasts) of a population of protoplasts transformed with SLP1.2 DNA and therefore containing a small minority of transformants were plated on regeneration medium.
[b] Determined as in Table 1, footnotes *b* and *c*.
[c] —, Nonconfluent lawns.
[d] Estimated from plate containing too many pocks to count accurately.

cin yielded 13 viomycin-resistant isolates. Cleared minilysates of these strains were used to retransform *S. lividans*. The pocks generated by all but one of the minilysates were >98% viomycin resistant. The remaining lysate generated a mixture of Vio^r and Vio^s pocks. A Vio^r transformant was purified, lysed, and used to retransform *S. lividans*. The resulting unmixed population of Vio^r pocks implied that this unusual isolate originally contained two SLP1.2-derived plasmids (Vio^r plasmid designated pIJ16) obtained either by cotransformation or during regeneration.

Restriction endonuclease cleavage of 11 of the 13 isolates demonstrated the presence of fragments (in addition to the linearized vector) which could be excised with *Bam*HI. (Nine representative *Bam*HI-cleaved plasmids are shown in Fig. 5A.) Four plasmids contained one *Bam*HI fragment, four contained two, and two contained three or more. The single inserts had sizes of 1.9 (pIJ13), 5.8 (pIJ17), and 8.5 (pIJ11, pIJ15) kb (Fig. 4; Table 3). Each of the plasmids contained one (and only one) of these three fragments. (Although pIJ9 contained four inserted *Bam*HI fragments, including two which comigrated with the 5.8- and 1.9-kb fragments, restriction endonuclease analyses showed that the 5.8-kb fragment corresponds to the single insert in pIJ17 and that the 1.9-kb fragment does not correspond to the single insert in pIJ13 [data not shown].) Since no other fragments were common to two or more recombinant plasmids, we conclude that the multiple inserts probably resulted from the ligation of random, rather than partially digested, chromosomal sequences into the vector. In total, the 1.9-kb fragment was represented in one clone, the 5.8-kb fragment was represented in seven clones, and the 8.5-kb fragment was represented in three clones.

All clones whose resistance was determined by the 1.9- or 5.8-kb fragments were biologically and biochemically similar to each other but different from those strains which contained the 8.5-kb fragment. The relationship of these fragments to each other was first determined by the antibiotic resistance levels conferred by each plasmid. Sporulated patches of strains containing the plasmids were replica plated to MM containing viomycin and also to the closely related tuberactinomycin capreomycin 1A. Whereas all clones containing the 1.9- or 5.8-kb fragment made *S. lividans* resistant to 400 μg of viomycin per ml and at least 250 μg of capreomycin 1A, the large fragment of 8.5 kb conferred lower levels of resistance to viomycin (5 μg/ml) and no detectable resistance to capreomycin 1A (<2 μg/ml). Functional independence of the 1.9- and 5.8-kb fragments from the 8.5-kb fragment was confirmed by the observation that clones carrying the 1.9- or 5.8-kb fragment contain viomycin phosphotransferase activity, which was not found in those carrying the 8.5-kb fragment (22). Hybridization experiments as

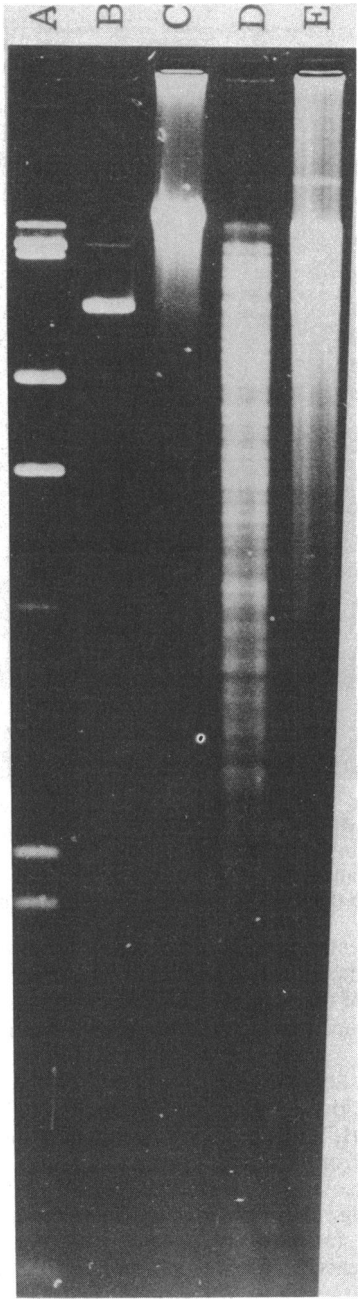

FIG. 4. Shotgun cloning of *S. vinaceus* DNA, using SLP1.2. Agarose gel electrophoretic separations of: (A) bacteriophage lambda DNA cut with *Hin*dIII; (B) SLP1.2 digested with *Bam*HI; (C) *S. vinaceus* genomic DNA undigested; (D) *S. vinaceus* genomic DNA digested with *Bam*HI; (E) a mixture of the SLP1.2 and *S. vinaceus* *Bam*HI fragments after ligation.

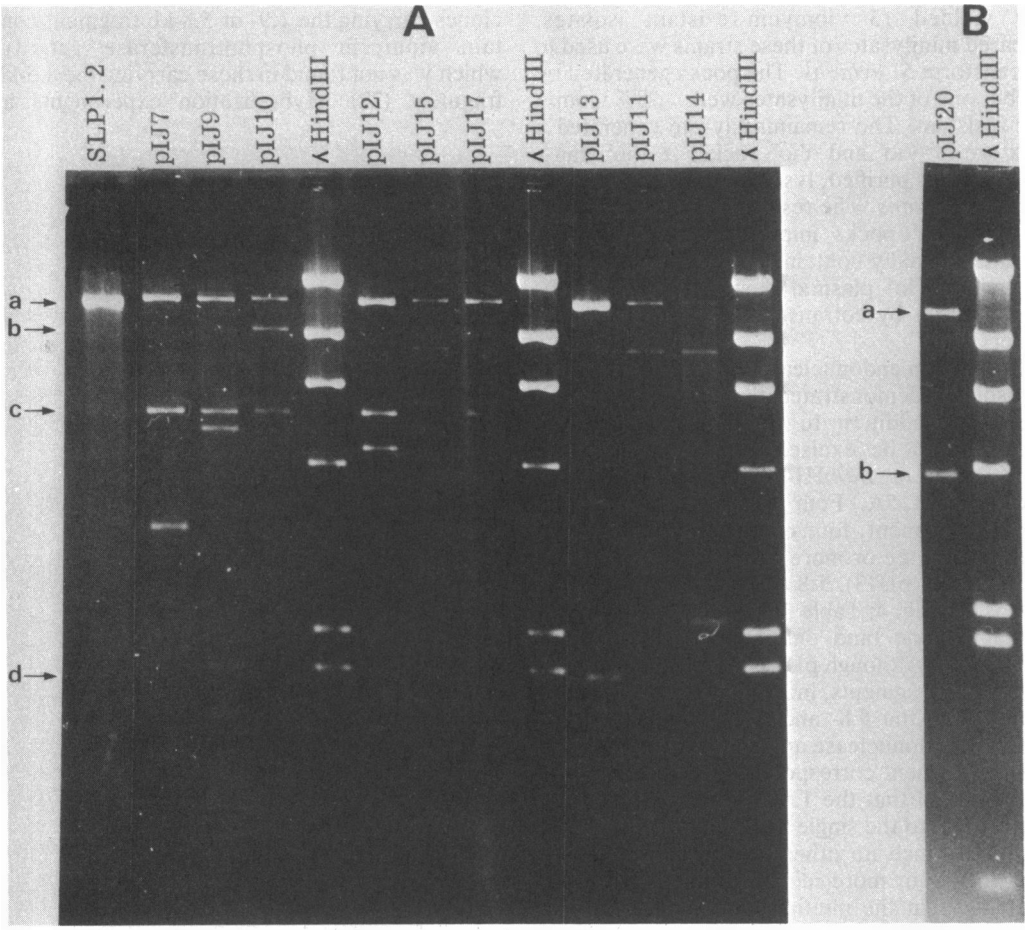

FIG. 5. Viomycin resistance plasmids. Independently isolated viomycin resistance plasmids were isolated by CsCl centrifugation (2). After cleavage with *Bam*HI (gel A) or *Pst*I (gel B); the fragments were electrophoretically separated on a 1% agarose gel. Each *Bam*HI-generated plasmid (gel A) contained a 14.5-kb (a) SLP1.2 fragment and at least one insert of 8.5 (b), 5.8 (c), or 1.9 (d) kb. *Pst*I-generated plasmids contained *Pst* fragments of 11.7 kb (largest vector *Pst*I fragment) (a) and 4.3 kb (b).

well as restriction endonuclease analysis indicated that the 1.9-kb fragment is homologous to a portion of the 5.8-kb fragment (G. Hombrecher, J. M. Ward, and C. J. Thompson, unpublished data).

In parallel to the experiment described above, we also cloned viomycin resistance into SLP1.2, using *Pst*I. At least 10 independent clones were isolated from about 10^5 pocks. Analyses of four random isolates revealed the present of a 4.3-kb insert (Fig. 5B). These strains were resistant to viomycin (at least 100 μg/ml) and capreomycin 1A (at least 5 μg/ml) and contained viomycin phosphotransferase activity.

Transfer and lethal zygosis properties of the antibiotic resistance plasmids. The recombinant plasmids all generated Ltz phenotypes which were broadly similar. The pock phenotypes of the various recombinant plasmids did, however, vary slightly from the SLP1.2 parent and from each other in both size and amount of sporulation. Thus, although we do not understand the factors which control this subtle plasmid-specific variability, it cannot be attributed exclusively to the site of inserted DNA.

Since the ability to produce pocks was always associated with plasmid transfer in earlier studies of SLP1 and other plasmids (2, 3, 5, 6), it was not surprising to find that these plasmids showed the high levels of transfer shown in Table 3. Apparent transfer frequencies varied from about 18 to 82% (an insignificant difference in practical terms). Transfer of the Ltz phenotype was associated (>95%) with antibiotic resistance (data not shown).

Intraspecific shotgun cloning of *S. coelicolor* biosynthetic genes, using SCP2* as a vector. SCP2* was used for intraspecific cloning of biosynthetic genes which suppressed ("complemented") the auxotrophic mutation *hisD3*,

argA1, or *guaA1* in *S. coelicolor* B341. We chose to cleave SCP2* at the *Bam*HI sites shown in Fig. 1 since fragment A can exist independently as a replicon (4). Chromosomal DNA extracted from prototrophic strain A3(2) was partially cleaved with *Mbo*I into fragments averaging 8 kb. Chromosomal and vector fragments were ligated and used to transform strain B341. The regeneration plates contained a total of about 40,000 pocks. After replication to media lacking arginine, three Arg^+ strains (TC4, TC5, and TC6) were isolated; selection for His^+ yielded one isolate (TC3) and there were two Gua^+ isolates (TC1 and TC2). To demonstrate that these strains contained biosynthetic genes carried by SCP2* and were not merely chromosomal revertants, a miniplasmid lysate from each strain was tested for sterility and used to retransform B341. After nonselective regeneration the lawns containing visible pocks were replicated to the appropriate selective medium. For each presumptive clone, all pocks corresponded to areas of growth and vice versa. Hence, the Ltz character of SCP2* was always linked to suppression of the auxotrophic marker in question; successful cloning was demonstrated.

All of these strains had similar unstable properties. Even after growth on selective media, only 30 to 40% of the spores were phenotypically prototrophic for the marker in question. Lysates of the clones showed low yields of plasmid DNA, presumably reflecting this instability. For this reason we have not pursued the physical characterization of these plasmids. The molecular weights of two of these plasmids, however, were determined from their mobility in agarose gels. One Arg^+ clone (pIJ47) had a molecular size of 23.3 kb, whereas the His^+ (pIJ46) clone was estimated as 25.0 kb.

DISCUSSION

The experiments described here, together with preliminary results published earlier (23), demonstrate that interspecific shotgun cloning in certain streptomycetes can be carried out reproducibly. In contrast, several laboratories have encountered problems in attempts to utilize the primary alternative gram-positive host-vector system, *Bacillus subtilis*, for shotgun cloning (11, 14). The streptomycete cloning capability can now be applied both to academic studies of streptomycete molecular biology and to economically relevant problems.

At the outset of this work, the application of previous transformation techniques did not allow us to isolate clones from interspecific shotgun cloning experiments, apparently because too few plasmids containing inserts were being detected by transformation. This called for a definition of the conditions which affect transformation frequency.

At that time, conditions allowing visualization of transformants on regeneration plates had not been defined and a precise estimate of the number of primary transformants could not be made. Previous demonstrations of transformation had been made by harvesting spores after regeneration of protoplasts, replating at various dilutions on a lawn of a plasmid-free strain, and counting the numbers of pocks. Transfer of the plasmid during regeneration can amplify the number of plasmid-containing spores by a factor of 10^4 (5). Precise estimates of the original number of viable transformants was possible only by using near-saturating amounts of plasmid DNA, replica plating regenerated single colonies to plasmid-free lawns, and scoring for the Ltz phenotype. Replication of large enough samples of single colonies to a second lawn was tedious, and the large amount of plasmid DNA required for each transformation (about 10 μg) was prohibitive. Adjustments in agar moisture content and protoplast density during regeneration allowed visualization of pocks on primary regeneration plates, using 10^{-5} μg or less of plasmid DNA.

This ability to quantify primary transformation events provided a tool to increase the

TABLE 3. Summary of physical and genetic data on representative viomycin resistance plasmids

Strain	Plasmid	Cloning enzyme	Size of inserts (kb)	Stability of plasmid-bearing parent (%)[a]	Transfer frequency[a] (% recipient parent)	
					Direct Selection	Selective replicate of progeny
M180	SLP1.2	None	None	97		97
TC19	pIJ11	*Bam*HI	8.5	99	74	76
TC21	pIJ13	*Bam*HI	1.9	100	79	82
TC25	pIJ17	*Bam*HI	5.8	99	37	39
TC28	pIJ20	*Pst*I	4.3	85	33	18

[a] Viomycin-resistant strains were grown individually or in a mixed lawn containing similar amounts of CT2 (a streptomycin-resistant derivative of *S. lividans* strain 1326). Spores were harvested and plated selectively on MM which contained either viomycin and streptomycin or streptomycin alone. Streptomycin-resistant colonies were replicated to viomycin-containing media to determine the stability of the plasmid-bearing parent or to determine transfer frequency. Transfer of SLP1.2 from M180 into CT2 was assayed by replicating streptomycin-resistant progeny to a lawn of 1326 and scoring for the Ltz^+ phenotype. Spores from matings were also plated out on MM containing both viomycin and streptomycin to determine the percentage of streptomycin-resistant recipients which had received the SLP1.2-derived viomycin resistance plasmid.

efficiency and reproducibility of the transformation procedure. The parameters which influence the recovery of viable transformants include growth phase of the mycelium, concentration of $CaCl_2$ and $MgCl_2$ both during lysozyme treatment and at the time of transformation, sucrose concentration at the time of transformation, and moisture content and protoplast density during regeneration. Alterations of these conditions increases the efficiency of the procedure by more than 20-fold and allows the recovery of 5×10^5 to 1×10^7 transformants per μg of SLP1.2 CCC DNA.

Although we have not specified the contributing factors, with the same procedure our yield of transformants from ligations containing both the SLP1.2 vector and chromosomal DNA is 10 to 100 times lower than the SLP1.2 CCC frequencies. This is not surprising when one imagines the topological problems involved with introducing a relaxed circular molecule the size of SLP1.2 (about 5 μm) into a spherical protoplast (about 1 μm in diameter); inefficiencies in recircularization of plasmid during ligation (linear plasmid DNA being 10 to 100 less efficient in transformation than religated circular DNA [4]) and possible competition for uptake between chromosomal and plasmid DNA are also suspected.

In spite of these factors, the yield of transformants in a typical shotgun cloning experiment, 5×10^3 to 1×10^5, should contain a statistically representative population of an entire streptomycete genome. The genome of 9×10^3 kb (1) is composed of about 1,100 *Bam*HI fragments (averaging 8 kb in length). Reconstruction experiments indicate that, under the ligation conditions used, after transformation at least 10% of the vector molecules contain inserts (data not shown). It follows that we should find one clone in about 10^4 transformants. Our observation of one clone in 2×10^3 to 9×10^3 transformants in one intraspecific (*S. coelicolor*) and five interspecific (*S. fradiae*, *S. azureus*, *S. vinaceus*, *S. antibioticus*, or *S. erythreus* DNA cloned in the SLP1.2-*S. lividans* system [unpublished data]) cloning experiments bears out this prediction and indicates that transformation efficiency is no longer a serious problem.

Restriction-modification systems, which might limit interspecific transfer of DNA, are commonly found among the streptomycetes (15). That the frequency at which clones appeared is in good agreement with the predicted value indicates that any restriction of heterospecific DNA by *S. lividans* does not occur to any significant extent, at least with the particular donors used here.

SLP1.2 is unusually well suited for the insertion of various restriction fragments. Five restriction endonucleases (*Bam*HI, *Pst*I, *Xho*I, *Sph*I, and *Kpn*I) cleave SLP1.2 exclusively within dispensable DNA; insertion of DNA into the *Pst*I and *Bam*HI sites has so far been demonstrated. Other potentially useful cloning enzymes having unique recognition sites on SLP1.2 are *Eco*RI, *Cla*I, *Bgl*II, and *Bcl*I. The isolation of insertions at the *Bam*HI and *Pst*I sites ranging from 1.9 (pIJ36) to 13.0 (pIJ1) kb indicates that a broad size range of hybrid plasmids can be stably maintained by the SLP1.2 replicon (23).

Another potential stability problem found in the *B. subtilis* system (17), deletion of cloned sequences by the host during either transformation or maintenance, is not troublesome. All of the *Bam*HI or *Pst*I terminal sites of the clones we have mapped have been represented. Occasional plasmid variants (about 1%) do arise in a clonal population which are able to give pocks but do not express the appropriate drug resistance markers after retransformation. Although we have not investigated this phenomenon with respect to the viomycin-resistant clones, one similarly constructed SLP1.2 replicon carrying neomycin resistance (pIJ1) also generated antibiotic-sensitive variants. One of these contained a spontaneous deletion which eliminated both host and inserted restriction endonuclease cleavage sites (unpublished data). This phenomenon could be exploited for deletion mapping of cloned fragments.

The distribution of known restriction endonuclease cleavage sites in the SCP2* replicon presently makes it a less desirable general cloning vehicle. Bibb et al. (4) have reported that replicons can be formed from the largest *Bam*HI and *Pst*I fragments of SCP2*; the *Pst*I fragment was used as a cloning vehicle for the methylenomycin resistance gene. These replicons are unstable; restoration of stability requires a function found on the second largest *Pst*I fragment. Our Gua^+, His^+, and Arg^+ clones isolated from a shotgun cloning experiment using *Mbo*I chromosomal fragments ligated to the *Bam*HI-generated fragments of SCP2* were similarly unstable. This instability probably explains the low yields of recombinant CCC DNA in cleared lysates and makes the physical analysis of the plasmids tedious. The hybrid *Bam*HI-*Mbo*I recognition sites created at the insert-vector junction further complicate the physical analysis of these clones. Only preliminary CCC molecular weight data were obtained for two clones. If the SCP2* *Bam*HI fragment (15.8 kb) serves as the replicon, as would be predicted by the work of Bibb et al. (4), inserts of approximately 7.5 and 9.3 kb contain biosynthetic genes which suppress the *argA1* and *hisD3* mutations. Perhaps the most significant conclusion to be drawn from these

data is that it is possible to construct a gene bank containing a representative sample of conventional biosynthetic streptomycete genes.

We believe that the technology presented here and elsewhere (9) makes the streptomycete gene cloning system reliable and potentially very useful. Such a consistent plasmid shotgun cloning capability has yet to be demonstrated in other gram-positive systems currently under development. Its application to studies of streptomycete molecular biology is currently under way. The economic relevance of endogenous streptomycete products such as antibiotics is well known. The introduction of foreign (including eucaryotic) genes into these industrially well-characterized organisms for possible production purposes is foreseen.

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