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CHAPTER 5

Development of a Cloning System for *Streptomyces*

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Two potentially useful plasmid cloning vectors for *Streptomyces* are described. SCP2 and its high fertility variant, SCP2*, are self-transmissible plasmids which are capable of promoting chromosomal recombination. Although genetically distinguishable, no physical differences between SCP2 and SCP2* have been detected. Velocity sedimentation analyses indicate the plasmids to be of molecular weight 18–20 × 10^6 and restriction endonuclease cleavage maps of both plasmids indicate the presence of many restriction sites which ought to be amenable to the insertion of DNA without damaging functions essential for plasmid replication and maintenance. Although first isolated from *Streptomyces coelicolor* A3(2), SCP2* has been transferred by mating to *S. parvulus* ATCC 12434 and *S. lividans* JI1326 whereupon stable replication was observed.

A series of self-transmissible plasmids, SLP1.1–6, have been isolated from *S. lividans* JI1326 which range in molecular weight from 6.25 to 8.2 × 10^6. Some of these plasmids contain unique restriction sites which are known to be present within regions of the plasmid genome that are not essential for plasmid replication or maintenance, a useful characteristic for any potential cloning vehicle.

Essential to the development of a cloning system is a means of introducing the recombinant DNA into the host organism. SCP2, SCP2,* and all of the SLP1 series of plasmids exhibit a property termed "lethal zygosis," which allows for the recognition of plasmid-containing spores at very high resolution. A procedure has been developed which involves the uptake of covalently closed circular plasmid DNA by protoplasts in the presence of polyethylene glycol and the visual detection of transformants after regeneration of the protoplasts using the plasmid determined phenotype of lethal zygosis. Frequencies of transformation of up to 85% of the regenerated protoplasts are obtained.

INTRODUCTION

The development of a DNA cloning system for *Streptomyces* would be of tremendous value to both the applied and academic studies of these industrially important organisms. It would, for example, enable detailed genetical analyses of such processes as antibiotic production and of the mechanisms involved in the differentiation of these morphologically complex bacteria. Since over 60% of known antibiotics are produced by *Streptomyces* species (Bérdy 1974) including many substances with valuable clinical and other applications, these organisms assume a position of considerable medical, biological, and commercial importance. The ability to bring together new combinations of DNA sequences from perhaps evolutionary diverse organisms could have profoundly beneficial effects on both the range of chemical structure and the quantity of antibiotics produced by actinomycetes. Although the process of gene exchange mediated by conjugation within actinomycetes is apparently widespread (Hopwood and Merrick 1977), the transfer of genetic material between individuals by such sexual means is, by definition, predominantly restricted to members of the same species. Even though the

recent development of protoplast fusion in *Streptomyces* (Hopwood et al. 1977) represents a major breakthrough in the ability to use recombination as a practical tool in the field of strain improvement, the production of recombinants between species which are not closely related will still be limited by a lack of extensive DNA sequence homology. In contrast, recent developments in in vitro recombinant DNA technology (Timmis et al. 1978) in theory allow for the insertion of a DNA sequence of any origin into a desirable host strain provided: (1) a suitable vector system is available to maintain the cloned DNA; and (2) a means of introducing the vector, for example, a plasmid or bacteriophage derivative, and its inserted DNA into the host organism has been developed. Since the maintenance of the foreign DNA does not require recombination of homologous DNA sequences, there ought to be few restrictions on the origin of the genetic material introduced into the host organism. This paper describes the properties of two potentially useful cloning vehicles for *Streptomyces* and reports the development of a plasmid transformation system which should allow the cloning of any DNA sequence into these organisms and which, potentially, provides a means of directly manipulating the pathways of antibiotic production. The system involves the uptake of covalently closed circular (CCC) DNA by protoplasts in the presence of polyethylene glycol (PEG) and the visual detection of transformants at high resolution after regeneration of the protoplasts.

Materials and Methods

Genetic analysis. Standard methods of crossing, culture conditions, and media were as described by Hopwood (1967) and Hopwood et al. (1973).

Isolation of covalently closed circular DNA. As described by Bibb et al. (1977).

Neutral and alkaline sucrose centrifugation. As described by Bibb et al. (1977).

Restriction endonuclease mapping. As described by Bibb et al. (1977).

Preparation of protoplasts. As described by Hopwood et al. (1977).

Transformation. As described by Bibb et al. (1978) with the following modifications. After treating the protoplast-DNA mixture with 0.5 ml 20% (w/v) PEG for 1 min, 0.25 ml medium P was added, followed 1 min later by 3.25 ml medium P. The protoplasts were then pelleted as described.

Results

Potential Cloning Vehicles

*SCP2 and SCP2**. SCP2, together with its high fertility variant, SCP2*, represents the first example of a physically and genetically characterized streptomycete plasmid. Both plasmids were first discovered in *Streptomyces coelicolor* A3(2) (Schrempf et al. 1975; Bibb et al. 1977; Schrempf and Goebel 1977) and they exist as autonomously replicating, extrachromosomal CCC DNA molecules of molecular weight $18-20 \times 10^6$. So far no physical difference between the two plasmids has been detected. Both plasmids are

self-transmissible, undergoing a process analogous to conjugation in the well-characterized eubacteria, and they both promote chromosomal recombination without exhibiting any preferred regions of transfer of the donor chromosome. SCP2 was initially detected through the occurrence of a high fertility variant of the sex factor, designated SCP2*, and both plasmids have since been subjected to genetic and physical analysis.

The genetic properties of SCP2 and SCP2* were determined by carrying out a series of matings between strains of different plasmid status. Several interesting properties emerged.

The transfer frequency of SCP2* or SCP2 into an $SCP2^-$ isolate always approached 100%, whereas the transfer of SCP2* into an $SCP2^+$ strain occurred at a frequency of approx. 5%. The enhanced recipient characteristics of $SCP2^-$ isolates towards SCP2* (and presumably SCP2) in comparison with normal $SCP2^+$ strains also was indicated by increased levels of chromosomal recombination and probably reflects the absence of an SCP2-determined entry disadvantage system, a property analogous to the surface exclusion, and possibly incompatibility properties of plasmids found in many gram-negative genera, e.g. *Escherichia coli*.

A phenomenon analogous to the High Frequency Transfer (HFT) state of other sex factors such as Col I has also been recognized, indicating that the transfer functions of SCP2 are normally repressed. Genetic evidence suggests that SCP2* arose through a mutation which produced, at least, a partial derepression of the transfer genes of SCP2. Further examples of high fertility variants of SCP2 which appear identical to the original SCP2* isolate can be obtained by screening recombinants produced in $SCP2^+ \times SCP2^+$ matings for either enhanced fertility properties or for their ability to elicit the phenomenon of lethal zygosis which is described below (Bibb et al. 1977).

When grown in contact with a confluent culture of an $SCP2^-$ strain, isolates carrying SCP2* and, to a lesser extent, SCP2 are surrounded by a narrow zone in which the development of the $SCP2^-$ culture is inhibited. This inhibition requires hyphal contact leading to conjugation between the two types of strain and may have features in common with lethal zygosis in *E. coli*, in which cells lacking the F plasmid are often killed when involved in multiple mating reactions with strains carrying the F factor in the integrated (Hfr) state (Skurray and Reeves 1973). Cultures containing either SCP2* or SCP2 are resistant to this inhibition, presumably due to the expression of the plasmid-determined entry disadvantage system described above. The greater intensity of the SCP2*-determined lethal zygosis when compared with that produced by SCP2 when replicated onto an $SCP2^-$ lawn presumably reflects the difference in the efficiency of transfer of the two plasmids. Reconstruction experiments indicated that this phenotype allowed for the recognition of SCP2* or SCP2-containing spores in an $SCP2^-$ population at a frequency of $<10^{-9}$ and thus should provide a powerful system for the detection of cells which have received the plasmids either by conjugation or transformation. Using this property of lethal zygosis, it is possible to detect the transfer, by conjugation, of SCP2* from *S. coelicolor* A3(2) to *S. parvulus* ATCC 12434 and to *S. lividans* JI 1326. The lethal zygosis phenomenon is shown by the resulting SCP2* *S. parvulus* and SCP2* *S. lividans* strains against their parental $SCP2^-$ strains. Using a procedure identical to that employed to isolate SCP2* from *S. coelicolor* A3(2), the SCP2* *S. parvulus* and SCP2* *S. lividans* isolates yield CCC DNA which proved to be indistinguishable upon restriction enzyme analysis from SCP2*.

Neutral and alkaline sucrose gradient analysis shows both SCP2* and SCP2 to be CCC DNA molecules of molecular weight 18–20 × 10^6.

A restriction endonuclease cleavage map of SCP2 (SCP2* is identical in this respect) has been constructed using the enzymes *Eco*RI, *Hin*dIII, *Sal*PI (an isoschizomer of *Pst*I), and *Bam*HI (Fig. 1). The relative positions of each of the enzyme recognition sites were determined by agarose gel electrophoresis of the reaction products obtained

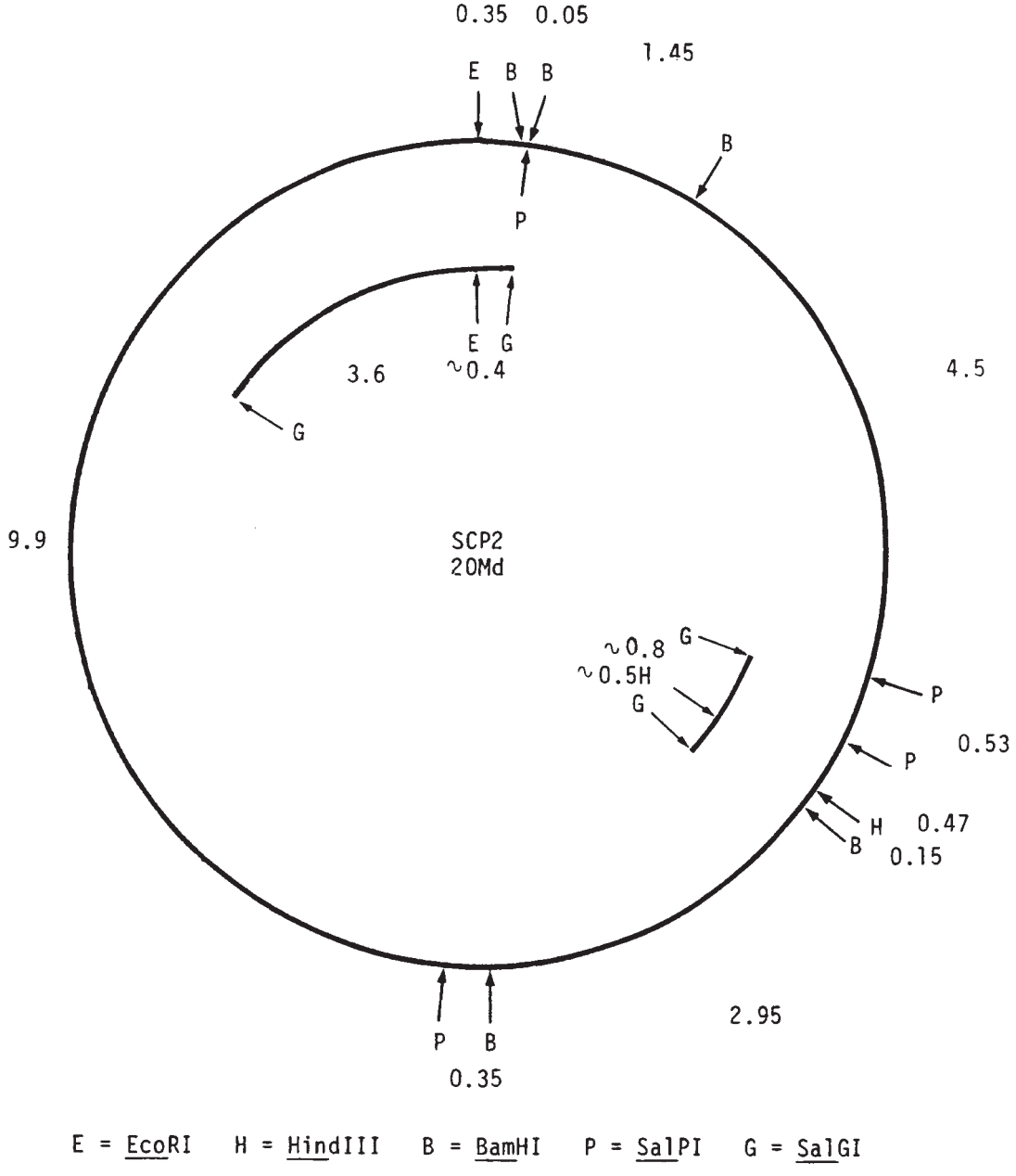


FIG. 1. Restriction endonuclease cleavage map of SCP2. The numbers indicate the sizes of the fragments (in M daltons) between adjacent arrows.

from single, double, triple, and partial digests of SCP2 with various of the enzymes. Each of the four enzymes produce overlapping termini upon DNA cleavage and hence could be used in any subsequent attempts to use SCP2, SCP2*, or their derivatives as cloning vectors within actinomycetes.

SLP1. Streptomyces lividans JI 1326 does not yield plasmid DNA when subjected to the normal procedure which is used in the isolation of SCP2 and SCP2* from *S. coelicolor* A3(2). This species does not exhibit any phenotypic or genetic properties which one might associate with the presence of plasmids. However, when sufficient spores of 1326 are spread on an agar surface to produce confluent growth, centers of inhibition can be observed within the lawn occurring at a frequency of 10^{-6}–10^{-7}. These centers of inhibition are morphologically similar to those produced by SCP2* spores in a background lawn of an SCP2$^-$ strain, the latter being known to arise through SCP2*-determined lethal zygosis upon plasmid transfer. All of the isolates obtained from *S. lividans* 1326 which elicit this phenomenon are also resistant to the effect.

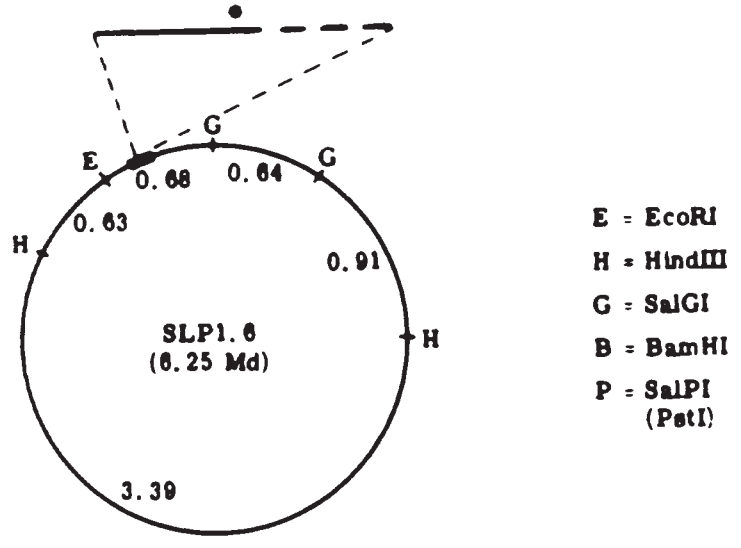
Attempts therefore were made to isolate CCC DNA from a number of these new variants. From a total of 30 isolates examined, CCC DNA was obtained from six of them. Analysis of these CCC DNA species by restriction endonuclease mapping using *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*GI, and *Pst*I (an isoschizomer of *Sal*PI) produced the restriction maps shown in Fig. 2. The maps indicate that although sharing a large degree of homology, each of the six plasmid preparations differ in a single region of their genomes and that the DNA sequences present within the smaller species were also present in the larger variants. The plasmids were designated SLP1.1 to SLP1.6.

Since no CCC DNA can be detected within the original parental strain 1326, then, in those instances where CCC DNA can be isolated from the new variants, the expression of this phenotype involves some event which leads to the formation of an isolable CCC DNA molecule. Since there is no evidence to suggest the existence of other plasmids within *S. lividans* 1326, it would appear likely that the SLP1 series of plasmids originate from the chromosome itself. If this assumption is correct, then the restriction endonuclease cleavage maps indicate that on excision from the host genome sequences of variable length are picked up by the plasmid, but extending in one direction only from the site of plasmid integration. Excision could be accounted for by some sort of site-specific recombinational event.

Since the inhibition of the parental strain 1326 by these new isolates is believed to be due to lethal zygosis, this indicates that the SLP1 series of plasmids are self-transmissible elements. Attempts therefore were made to transfer SLP1.1 between genetically distinguishable derivatives of *S. lividans* 1326, using the ability to express lethal zygosis as a detectable plasmid phenotype. This property was found to be transferred to 36–70% of the recipient population, frequencies at least a thousandfold greater than the occurrence of possible recombinants between the two parental strains. These results support the suggestion that SLP1.1, and by implication SLP1.2–SLP1.6, are self-transmissible plasmids.

From the yields of CCC DNA obtained from SLP1$^+$ cultures, the plasmids would appear to be present at 3–4 copies per genome (Bibb et al. 1977).

Since SLP1.6, the smallest of the SLP1 series of plasmids, contains all the information essential for plasmid replication, then it follows that any additional DNA sequences present within the larger variants are not necessary for plasmid maintenance. This



Plasmid	Size (Md)	Restriction Sites E	H	G	B	P	Extra segment*
SLP1.6	6.25	1	2	2	0	0	None
SLP1.4	6.4	1	3	2	0	0	H
SLP1.3	6.88	1	3	3	0	0	H G
SLP1.1	7.13	1	3	3	0	1	H G P
SLP1.5	7.28	1	3	3	0	1	H G P
SLP1.2	8.23	1	3	3	1	2	H G P P B

FIG. 2. Restriction endonuclease cleavage maps of SLP1.1-6. The numbers indicate the sizes of the fragments (in M daltons) between adjacent restriction enzyme sites.

means that any restriction enzyme sites within this nonessential DNA segment ought to be amenable to the insertion of foreign DNA without damaging functions essential for plasmid replication and maintenance. The occurrence of *Bam*HI and *Pst*I sites within the nonessential segments of SLP1.1, .2, or .5 (Fig. 2) are particularly attractive sites for the insertion of cloned DNA, since there are no other recognition sites for these enzymes in the remainder of the plasmid molecule. Thus, in the case of SLP1, potential cloning vehicles are available with restriction enzyme sites in regions of the plasmid which are known not to be essential for plasmid replication and maintenance, an important step toward the development of a cloning system for *Streptomyces*.

Transformation. Apart from the availability of suitable vectors, the development of any cloning system requires a method of introducing the recombinant DNA into the desired host organism. The infection of protoplasts of *S. kanamyceticus* with actinophage DNA (Okanishi et al. 1968) and the enhancing effect of polyethylene glycol (PEG) on the transformation of *E. coli* spheroplasts with DNA of the bacteriophage ϕX174 (Morita et al. 1977) have been reported.

Attempts therefore were made to transform PEG-treated protoplasts of derivatives of *S. coelicolor* A3(2), *S. parvulus* ATCC 12434, and *S. lividans* JI 1326 with SCP2*, SLP1.1, or SLP1.2 DNA and to detect the transformants by regenerating the protoplasts and assaying spores from the resulting culture for their ability to show plasmid-determined lethal zygosis within a confluent plasmid minus lawn.

Covalently closed circular SCP2*, SLP1.1, and SLP1.2 DNAs were prepared by the large-scale method and then used in attempts to transform protoplasts of *S. coelicolor* A3(2), *S. parvulus* ATCC 12434, or *S. lividans* JI 1326 as referred to above. Serial dilutions of the transformation mixtures were made onto appropriately supplemented R2 regeneration media (Hopwood et al. 1977) to obtain accurate estimates of the frequency of DNA uptake.

After incubation of the regeneration plates for up to 10 days at 30 C,. spores were collected from the confluent cultures and serial dilutions of the spore suspensions were spread on to appropriately supplemented plates of R2 media which had also received enough spores of an SCP2$^-$ isolate of the same species to produce a confluent lawn. In this way, plasmid-containing spores could be readily detected by their ability to elict lethal zygosis (for example, see Fig. 3). Where serial dilutions of the transformation mixture gave rise to single colonies, these were picked to patch plates and assayed for their ability to produce SCP2* or SLP1-determined lethal zygosis by later replicating the patches to plasmid minus lawns spread on appropriately supplemented R2 media plates. Several presumptive transformants were purified and attempts made to isolate CCC DNA from them. All yielded CCC DNA which proved to be indistinguishable upon restriction enzyme analysis from the plasmid DNA used initially in the transformation procedure. These results demonstrated unambiguously that transformation by plasmid DNA had occurred.

The transformation system was further characterized as described below. The effect of PEG concn on the frequency of transformation of *S. coelicolor* A3(2) by SCP2* DNA is shown in Fig. 4. A concn of 20% (w/v) seems to be optimal although only slightly lower levels are obtained at higher concn; no reproducible transformation is obtained in the absence of PEG. All subsequent transformations have been carried out with 20% PEG.

The effect of DNA concn on the frequency of transformation of *S. coelicolor* A3(2) is shown in Fig. 5. The yield of transformants is apparently proportional to a wide range of SCP2* DNA concn up to at least 2×10^{11} SCP2* molecules ml^{-1} (5.6 μg ml^{-1}), which represents a plasmid molecule: protoplast ratio of 5,000. Transformation has not been detected at DNA concn of less than 10^5 molecules ml^{-1}.

Reconstruction experiments indicate that an enrichment for the self-transmissible plasmids of up to 10^4 occurs when plasmid-containing protoplasts are regenerated and grown in a predominantly plasmid-minus protoplast lawn. Because of this enrichment factor, and the possibility of a variable loss of viability of protoplasts during the transformation procedure, the proportion of transformed protoplasts can be determined in

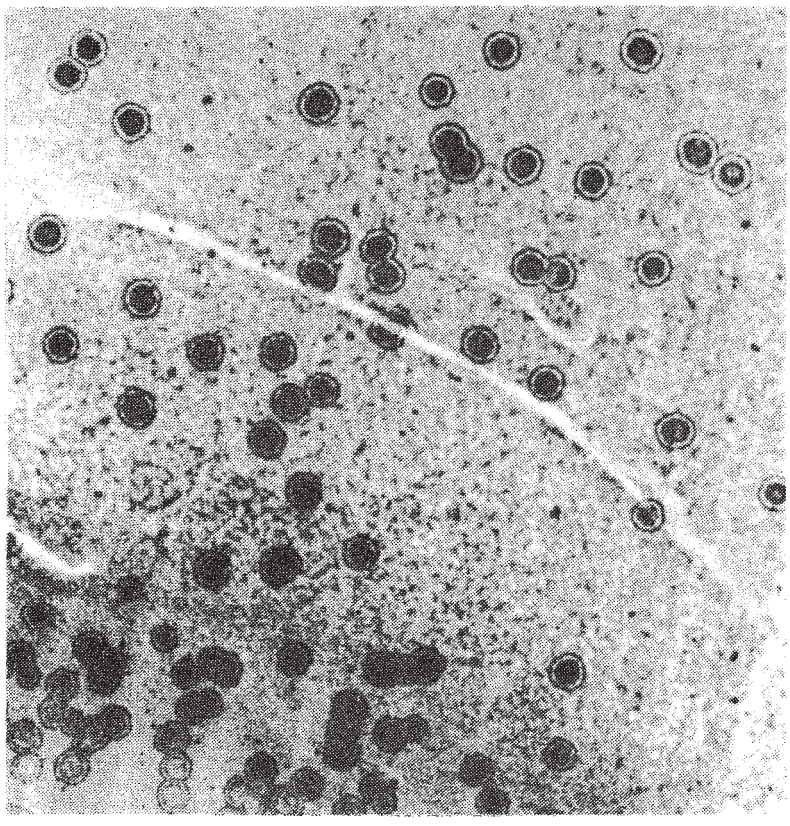


FIG. 3. SCP2* determined lethal zygosis in *S. coelicolor*. Centers of lethal zygosis resulting from outgrowth of single SCP2*-containing spores in a confluent lawn of an SCP2$^-$ strain. Magnification 4X.

experiments in which either individual colonies derived from single regenerated protoplasts or spores from confluent lawns of dilutions of the transformation mixtures which originate from a known number of treated viable protoplasts are assayed for their ability to elicit lethal zygosis. The results obtained using DNA concn of 2×10^{11} molecules ml^{-1} of SCP2* indicate remarkably high transformation frequencies: 4–20% for *S. coelicolor* A3(2) and >0.1% for *S. parvulus* and *S. lividans* (a proportion of the spores collected from lawns of $<10^3$ regenerated protoplasts of *S. parvulus* or *S. lividans* had acquired SCP2*, indicating a transformation frequency of at least 1 in 10^3 protoplasts). For SLP1.1 and SLP1.2, transformation frequencies into *S. lividans* 1326 of up to 85% have been obtained using similar plasmid DNA concn.

Discussion

The results and information presented here describe the essential components required for the development of a cloning system for *Streptomyces*. With judicious application there is good reason to believe that such a system could have a tremendously beneficial effect on the ability to manipulate the antibiotic production pathways of these organisms. The production of modified or hybrid antibiotics with new or improved antimicrobial

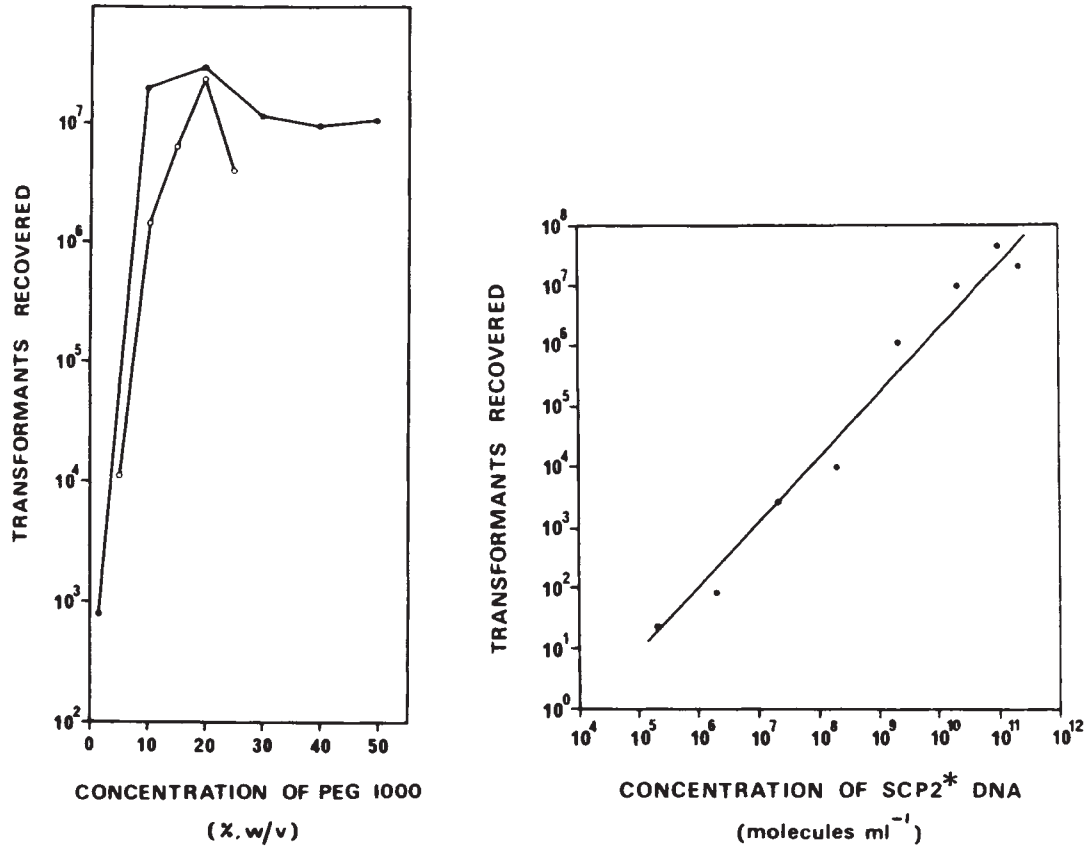


FIG. 4. Effect of PEG concn on transformation of *S. coelicolor* A3(2) with SCP2* DNA. SCP2* DNA was added to 10^7 protoplasts of a SCP2$^-$ strain to a final concn of 2×10^{11} molecules ml^{-1} at various PEG 1000 concn. The regenerated protoplasts were assayed for the presence of SCP2* as described in the text. The results are expressed as the number of SCP2*-containing spores recovered after regeneration and enrichment per 10^7 original viable protoplasts. • Mean results obtained from three experiments using PEG 1000 concn of 1.5–50%. o Mean results obtained from two experiments using PEG 1000 concn of 5–25%.

FIG. 5. Effect of DNA concn on transformation. 2×10^7 protoplasts of a SCP2$^-$ derivative of *S. coelicolor* were treated with various concn of SCP2* DNA in the presence of 20% PEG. Regenerated protoplasts were assayed for the presence of SCP2* as described in the text. The results are expressed as the number of SCP2*-containing spores recovered after regeneration and enrichment per 2×10^7 original viable protoplasts.

activities by the introduction of new enzymatic systems into well-characterized antibiotic producers is an obvious goal. It will not always be necessary to insert foreign DNA sequences into an organism to increase its usefulness as an antibiotic producer. Instead, assuming that a gene-dosage effect occurs, an increase in the copy number of certain of the genes involved in the antibiotic production pathways may have a significant effect on the final yield of antibiotic produced. Since plasmids and bacteriophages are capable of existing and replicating separately from the bacterial chromosome, the possibility exists for the selective amplification of the genes cloned into the vector and therefore of their products.

The additional application of techniques such as DNA cloning and protoplast fusion to the well-established procedures of mutation, recombination, and selection should add another dimension to the possibilities available for strain improvement within these industrially important organisms.

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