Plasmid transformation of *Streptomyces venezuelae*: modified procedures used to introduce the gene(s) for *p*-aminobenzoate synthase

DAVID A. AIDOO, KATHERINE BARRETT and LEO C. VINING

Biology Department, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J1

(Received 24 May 1989; revised 22 December 1989; accepted 8 January 1990)

Sucrose was unsuitable as an osmotic stabilizer in buffer solutions and media used for transformation of *Streptomyces venezuelae* ISP5230. Its replacement with NaCl, together with other modifications in the procedure, allowed efficient formation and regeneration of protoplasts but did not support transformation of *S. venezuelae* ISP5230 by vectors pIJ41 and pIJ941. With pIJ702, transformants with a low plasmid-copy-number and altered growth characteristics were obtained. Both pIJ702 and pIJ941, but not pIJ41, transformed *S. venezuelae* 13s; when pIJ941 was used, the plasmid in 18 of 20 transformants contained a deletion in the region reported to code for replication and transfer. The modified plasmid transformed *S. venezuelae* ISP5230 efficiently and was used to introduce a fragment of DNA from the *pab* locus of the wild-type into a *Cml*-1 mutant of ISP5230 blocked in chloramphenicol formation. Transformants that overproduced *p*-aminobenzoic acid were obtained but they remained blocked in chloramphenicol production; thus, the cloned *pab* fragment did not contain genes able to complement the *cml*-1 mutation. The results also suggest that the *Cml*-1 phenotype is not due to a defective reaction common to the biosynthesis of *p*-aminobenzoic acid and chloramphenicol.

Introduction

Streptomyces are Gram-positive filamentous bacteria that produce a diverse array of secondary metabolites, many of which have found application as antibiotics. Development of economic methods for producing these compounds has exploited the ease with which antibiotic activity can be quantitatively assessed. Rapid assay procedures have been used to screen empirically for strains with higher productivity and other desirable characteristics, usually after genetic change introduced by mutagenic treatment. However, the development of host-vector systems for gene cloning in these organisms (Bibb *et al.*, 1980, 1983; Hunter, 1985; Hopwood *et al.*, 1985) has introduced new opportunities for manipulating the secondary metabolic pathways and regulatory mechanisms responsible for antibiotic production.

Chloramphenicol is produced by *Streptomyces venezuelae* and several related species. The pathway by which the antibiotic is synthesized branches from that used for biosynthesis of protein aromatic amino acids at chorismic acid (Fig. 1), and much is now known about the biochemical reactions leading to chloramphenicol (Vining & Westlake, 1984). The organization of the *cml* genes that specify these reactions has also been investigated (Doull *et al.*, 1986; Vats *et al.*, 1987). Analysis of

recombinants obtained by conjugation and transduction has established that the *cml* genes are clustered on the chromosome of *S. venezuelae* ISP5230, and has indicated their probable arrangement.

These studies have made available a number of mutants blocked in formation of chloramphenicol. Transformation of such strains with plasmid vectors carrying wild-type genomic DNA, followed by selection of transformants complemented either for antibiotic production or for an auxotrophic requirement that maps near the *cml* genes, presents an attractive strategy for cloning the complete set of genes involved in chloramphenicol biosynthesis. This paper reports the development of procedures for efficient preparation and regeneration of *S. venezuelae* ISP5230 protoplasts, and for their transformation. It describes the use of these procedures to introduce a DNA fragment conferring the ability to overproduce *p*-aminobenzoic acid into an *S. venezuelae* strain blocked at an early step in chloramphenicol production.

Methods

Cultures. *Streptomyces venezuelae* strain ISP5230 (also listed as ATCC 10712) is the type strain for the species and was provided by C. Stuttard (Stuttard, 1982). *Streptomyces venezuelae* strain 13s is a natural

Fig. 1. Conversion of chorismic acid to 4-amino-4-deoxychorismic acid and the branching reactions leading to *p*-aminobenzoic acid or, via *p*-aminophenylalanine, to chloramphenicol. The *pabA* and *pabB* genes encode *p*-aminobenzoate synthase enzymes II and I, respectively; *pabX* is used to designate enzyme X (Nichols *et al.*, 1989).

variant of *Streptomyces* sp. 3022a selected for high chloramphenicol production (Malik & Vining, 1970); it has been identified as *S. venezuelae* (see Ahmed & Vining, 1983). The derivation of *S. venezuelae* strain Cml-1 has been described (Doull *et al.*, 1985), as have methods for culturing the above strains (Malik & Vining, 1970; Doull *et al.*, 1986). *Streptomyces lividans* strains 66 and JG10 (*pab*⁻) were provided by Drs D. A. Hopwood, John Innes Institute, Norwich, UK, and J. A. Gil, Oviedo University, Spain, respectively. The plasmid vectors used were: pIJ702, a high-copy-number vector derived from the *S. lividans* ISP5434 plasmid pIJ101 (Katz *et al.*, 1983); pIJ41, a low-copy-number vector derived from the SLP1.2 replicon that originates in *Streptomyces coelicolor* A3(2) (Thompson *et al.*, 1982a); and pIJ941, a low-copy-number vector derived from the *S. coelicolor* A3(2) plasmid SCP2* (Lydiat *et al.*, 1985). The vectors were also gifts from Dr Hopwood.

Media. For *S. lividans*, YEME and R2YE media were used as

described by Hopwood *et al.* (1985) except that the concentration of sucrose in YEME medium was reduced to 10.3% (w/v) to promote sedimentation of the mycelium when cells were harvested by centrifugation. Clumping of the mycelium in cultures at the lower sucrose concentration was overcome by inoculating with spores from confluent growth on a Petri dish. Cultures of *S. venezuelae* were grown on MYM agar (Stuttard, 1982), or in YEME medium supplemented with 1.0% (w/v) glycine (YEME/glycine medium). Protoplasts of *S. venezuelae* were regenerated on R5N agar. This was a modification of R2 agar (Okanishi *et al.*, 1974) in which the yeast extract content was increased to 0.5% (Thompson *et al.*, 1980), glucose was replaced by maltose, and 0.3 M-NaCl was substituted for sucrose. Thiostrepton (20 µg ml⁻¹) was added to media when necessary.

Chemicals and biochemicals. Thiostrepton was generously provided by S. J. Lucania, E. R. Squibb & Sons, Princeton, NJ, USA. Restriction enzymes were purchased from BRL. Polyethylene glycol (PEG) 1000 was from Koch-Light. Bactopeptone, malt extract, Casamino acids and yeast extract were from Difco.

Plasmid isolation. Except where noted, plasmid DNA was isolated by the alkaline lysis procedure described by Kieser (1984) and purified by centrifugation in a CsCl gradient containing ethidium bromide (Maniatis *et al.*, 1982). In some experiments, transformants were screened for the presence of plasmids by removing mycelium from cultures growing on an agar surface and suspending the cells in 0.5 ml of the lysozyme solution before alkaline lysis.

Transformation. The general procedures used to prepare, regenerate and transform protoplasts were those described by Hopwood *et al.* (1985). These procedures were followed without modification for *S. lividans*. Efficiency of protoplast formation was estimated by plating samples on regeneration medium before and after treatment with 0.01% SDS in P buffer (Hopwood *et al.*, 1985). The number of c.f.u. ml⁻¹ obtained from the detergent-treated sample was subtracted from that given by the untreated sample and the difference was expressed as a percentage of the latter. In experiments with *S. venezuelae* ISP5230, the sucrose included in P-buffer for osmotic protection was replaced with 0.3 M-NaCl. Protoplasts of *S. venezuelae* were prepared from the mycelium of cultures grown for 24 h in YEME/glycine medium by digesting the cell walls with lysozyme (2 mg ml⁻¹) for 1 h. For transformation of *S. venezuelae*, protoplasts (4 × 10⁹) prepared in modified L buffer and transferred to modified P buffer were collected by centrifugation. The pellet was mixed with plasmid DNA (1–2 µg) in not more than 20 µl of TE buffer (1.0 mM-EDTA, 10 mM-Tris/HCl, pH 8.0), then immediately diluted with 0.5 ml of modified T buffer containing 25% PEG 1000. After 3 min, 5 ml of modified P buffer was added; the transformed protoplasts were collected by centrifugation, resuspended in modified P buffer and regenerated on R5N agar. The compositions of L, P, and T buffers (Thompson *et al.*, 1982b; Hopwood *et al.*, 1985) were modified by replacing sucrose with 0.3 M-NaCl.

Conjugative plasmids. As an indication of the ability of plasmids to promote conjugation in *S. lividans*, cultures were tested for pock formation as described by Hopwood *et al.* (1985).

Analyses. Assays for antibiotic activity were done as described by Doull *et al.* (1985). Total aromatic amino and nitro compounds in culture broths were determined colorimetrically by the procedure (Levine & Fischbach, 1951) used to measure chloramphenicol. Samples for thin-layer chromatography (TLC) were extracted from filtered broth at neutral pH with ethyl acetate and were applied to silica gel (Sil 60F₂₅₄). Chromatograms were developed with benzene/acetic acid/water (42:24:1, by vol.), examined under light of 254 nm and sprayed with dimethylaminobenzaldehyde. The *R_F* values of *p*-aminobenzoic acid and chloramphenicol in this system were 0.50 and 0.27, respectively.

Results

Sucrose-stabilized media

Attempts to form protoplasts from the mycelium of *S. venezuelae* ISP5230 grown in YEME/glycine medium and digested at 30 °C for 1–3 h with lysozyme in L buffer, as described by Hopwood *et al.* (1985) for *S. lividans*, consistently gave yields below 70%. Under these conditions, *S. lividans* mycelium gave the expected high yield of protoplasts, and then could be transformed to thiostrepton resistance by pIJ702, pIJ41 and pIJ941 isolated from *S. lividans* with efficiencies of 10^6 – 10^7 ($\mu\text{g DNA}$)⁻¹. In contrast, transformation of the protoplasts prepared from *S. venezuelae* with these same vectors gave, under comparable conditions, no thiostrepton-resistant colonies.

Replacement of sucrose

Mycelium of *S. venezuelae* readily formed protoplasts when incubated with lysozyme in the TE buffer used for genomic DNA isolation. This buffer lacks sucrose, and addition of 0.3 M-sucrose to it caused a severe reduction in protoplast formation. Omission of sucrose from the L buffer normally used for preparing protoplasts gave improved yields, but the osmotic fragility of protoplasts prepared in such unsupplemented buffers prevented their successful transformation. Substitution of 20% (w/v) glycerol or 0.3 M-NaCl for the sucrose in L buffer stabilized the protoplasts without reducing the 95–99% yield from digestion of the cell walls with lysozyme. Media stabilized osmotically with glycerol did not support regeneration, but on R2YE or R5N agar, 90% of the protoplasts regenerated.

Protoplasts of *S. venezuelae* ISP5230 prepared by the modified procedure could not be transformed by pIJ702, pIJ41 or pIJ941 when they were regenerated on R2YE agar. However, transformation with pIJ702 succeeded when protoplasts prepared in NaCl-stabilized modified L buffer were regenerated on R5N agar, in which sucrose is replaced with NaCl. Similar attempts with pIJ41 or pIJ941 were not successful. Protoplasts transformed with pIJ702 isolated from *S. lividans* gave 100 thiostrepton-resistant colonies ($\mu\text{g DNA}$)⁻¹, but growth of the colonies was slow, both on R5N agar and after subsequent transfer to MYM agar. The plasmid isolated from transformants was indistinguishable from pIJ702 in its mobility during agarose gel electrophoresis and fragmentation pattern with restriction enzymes, but absorbance measurements at 260 nm showed that its concentration was only about 1% of that in *S. lividans* transformants. Transformation of *S. venezuelae* with plasmid DNA isolated from the primary transformants

gave 10^3 thiostrepton-resistant colonies ($\mu\text{g DNA}$)⁻¹. These transformants exhibited the same slow-growth characteristics and low plasmid content as those from the initial transformation. When *S. lividans* JG10 was transformed under the conditions described by Hopwood *et al.* (1985) with pIJ702 isolated from the *S. venezuelae* transformants, 10^6 – 10^7 thiostrepton-resistant colonies ($\mu\text{g DNA}$)⁻¹ were obtained. These grew at the normal rate, and the plasmid isolated from them could not be distinguished by restriction endonuclease analysis from the original pIJ702.

Modified vector

To determine whether another strain of *S. venezuelae* might be more amenable than ISP5230 to transformation with pIJ702, *S. venezuelae* 13s protoplasts were transformed with vector DNA isolated from *S. lividans* 66. Regardless of whether the transformation conditions used were those developed for *S. lividans* or for *S. venezuelae* ISP5230, thiostrepton-resistant colonies were obtained at frequencies of 10^6 ($\mu\text{g DNA}$)⁻¹. Similar results were obtained with pIJ941, whereas no transformants were obtained with pIJ41. Transformation of ISP5230, under conditions optimized for this strain, with pIJ702 DNA recovered from 13s transformants gave approximately 100 slow growing colonies ($\mu\text{g DNA}$)⁻¹, as found with pIJ702 DNA isolated directly from *S. lividans* 66.

Thiostrepton-resistant colonies from *S. venezuelae* 13s protoplasts transformed with pIJ941 contained one of two types of plasmid. Gel electrophoresis of plasmid DNA extracted from 20 of the colonies showed only two samples that corresponded in mobility with pIJ941; the remaining 18 colonies contained a smaller plasmid, designated pDQ101. Whereas efforts to transform strain ISP5230 with pIJ941 isolated from the appropriate *S. venezuelae* 13s transformants failed, comparable experiments with pDQ101 yielded thiostrepton-resistant colonies at a frequency of 2×10^2 ($\mu\text{g DNA}$)⁻¹. The frequency increased to 10^5 ($\mu\text{g DNA}$)⁻¹ when ISP5230 was subsequently transformed with pDQ101 isolated from ISP5230. Both pIJ941 isolated from strain 13s and pDQ101 isolated from strain ISP5230 transformed *S. lividans* JG10 efficiently, yielding thiostrepton-resistant colonies at frequencies of 10^6 transformants ($\mu\text{g DNA}$)⁻¹.

Characterization of pDQ101

Restriction enzyme analysis of pIJ941 and pDQ101 showed that in pDQ101 a 5.3 kb fragment had been deleted from the 25.0 kb parent plasmid (Fig. 2). The deletion was

Fig. 3. Restriction map of the *S. venezuelae* ISP5230 DNA insert and adjacent DNA in pIJ41 that complements the *pab* mutation in *S. lividans* JG10. The dashed line below shows the *Pst*I fragment subcloned in pDQ102 and pDQ103.

approximately 0.8 kb of adjacent vector DNA (Fig. 3) was ligated to *Pst*I-digested pDQ101. The ligation mixture was used to transform *S. venezuelae* strain 13s and thiostrepton-resistant colonies [10^5 (μg DNA) $^{-1}$] were isolated. Six of 50 transformants, screened by gel electrophoresis of extracted plasmid DNA, contained pDQ101 with the *Pst*I fragment insert. Restriction enzyme analysis of the recombinant plasmids from these strains showed the insert to be present in both orientations. Plasmids pDQ102 and pDQ103, recombinants with oppositely oriented *Pst*I inserts, both complemented the defective *pab* gene when introduced into *S. lividans* JG10 by transformation.

Plasmid pDQ102 was used to transform *S. venezuelae* Cml-1. Putative transformants were selected on R5N regeneration medium containing thiostrepton and then screened for the presence of pDQ102. Only two out of 12 colonies examined contained the recombinant plasmid. One of the two (VS607) and one of the plasmidless colonies (VS612) were compared with the host strain for production of aromatic amines and nitro compounds during growth of cultures in a medium that supported chloramphenicol production (Doull *et al.*, 1985). The yields of mixed product were 63, 30 and 3 $\mu\text{g ml}^{-1}$, respectively. By TLC of extracts from the filtered broth, *p*-aminobenzoic acid was identified as a major product of strains VS607 and VS612, whereas it was absent from cultures of *S. venezuelae* Cml-1.

When thiostrepton-resistant colonies obtained after transformation of the Cml-1 mutant with pDQ102 were assayed for production of inhibitory substances by overlaying the surrounding agar surface with *Micrococcus luteus*, some (including VS607 and VS612) gave small (5–8 mm) zones of inhibition around their margins. In parallel experiments, colonies of *S. venezuelae* Cml-1 transformed with pDQ101 showed no inhibition zones and those of the wild-type chloramphenicol producer, *S. venezuelae* ISP5230, gave 18 mm inhibition zones. However, cultures of strains VS607 and VS612 grown in chloramphenicol production medium did not yield any

A 5.75 kb *S. venezuelae* ISP5230 DNA fragment complementing the defective *pab* gene in *S. lividans* JG10 (Gil & Hopwood, 1983) was available as an insert in pIJ41 (D. A. Aidoo, unpublished). From the recombinant vector, a *Pst*I fragment that included the insert and

compound with the solvent extractability and chromatographic properties of chloramphenicol.

Discussion

Streptomyces phaeochromogenes strain NRRL B3559, which produces chloramphenicol, contains a resident plasmid, pJV1 (Doull *et al.*, 1983), which has been developed into the cloning vector pWOR109 (Bailey *et al.*, 1986). In preliminary experiments, pJV1 modified by the insertion of antibiotic-resistance genes failed to transform *S. venezuelae* ISP5230. Similar results were obtained with a vector developed from the plasmid pUC3 which Malik & Reusser (1979) isolated from the chloramphenicol-producing *S. venezuelae* strain 13s. No successful attempts to transform chloramphenicol-producing strains have yet been reported.

Sucrose has been used as the osmotic stabilizer in most buffers devised for the preparation and regeneration of streptomycete protoplasts. Use of NaCl in regeneration media was reported (Shirahama *et al.*, 1981) to be ineffective, although its addition to P buffer for preparing protoplasts of the cephamycin producer *Streptomyces wadayamensis* has since been shown to be beneficial (Acebal *et al.*, 1986). With *S. venezuelae* ISP5230, use of sucrose severely reduced the yield of protoplasts. Although the conditions used to grow mycelium influenced the efficiency of protoplast formation, it was only after sucrose had been replaced with NaCl that reproducible high yields of protoplasts were obtained. Furthermore, the protoplasts, in contrast to those obtained with sucrose-containing buffers, regenerated well and could be transformed with cloning vectors derived from *S. coelicolor* and *S. lividans* plasmids. By substituting maltose for glucose in NaCl-containing protoplast regeneration medium, the regeneration frequency was increased by 10%, a result similar to that reported by Acebal *et al.* (1986).

Restriction-modification systems are common in streptomycetes (Lomovskaya *et al.*, 1977; Chater & Carter, 1978; Stuttard, 1979) but can be bypassed by obtaining the cloning vector from transformants that escaped the restriction barrier in the primary transformation (Hunter & Friend, 1984; Engel, 1987; Matsuhashima *et al.*, 1987). It is likely that restriction activity is present in *S. venezuelae* ISP5230, since higher transformation efficiencies were obtained when vector plasmids were reisolated from the homologous strain. Thus the number of thiostrepton-resistant colonies obtained was greater by 10 and 1000 times, respectively, when ISP5230 was transformed with pIJ702 and pDQ101 isolated from ISP5230 than when the plasmids were isolated from *S. lividans*. However, this was not the principal source of the

difficulty encountered in developing a transformation system for *S. venezuelae* ISP5230.

The event that contributed most to obtaining an efficient host-vector system proved to be the recovery of a deletion mutant of pIJ941 following transformation of *S. venezuelae* 13s. Unlike pIJ941, the derivative pDQ101 plasmid was stably maintained in strain ISP5230. The deletion that generated pDQ101 from pIJ941, so allowing efficient transformation of ISP5230, removed DNA from the region associated with replication of the parent SCP2* plasmid in *S. coelicolor* (Lydiat *et al.*, 1985). Within the replication region, three of four segments defined by subcloning experiments and identified as *pdlA*, *pdlB* and *pdlC*, influence plasmid copy-number (Larson & Hershberger, 1986). The fourth segment is essential for replication and is clearly retained in pDQ101 since the plasmid is maintained in *S. lividans*. The deletion in pDQ101 extends over *pdlA* and into the adjacent *tra* region involved in plasmid self-transmission. Most likely it is the removal of the negatively acting copy control element *pdlA* from pIJ941 that allows the modified plasmid pDQ101 to be maintained in *S. venezuelae* strains 13s and ISP5230.

The apparent reduction in copy number of pIJ702 when it is transferred to ISP5230 may be due to a similar control element that is more strongly expressed in the *S. venezuelae* strain than in *S. lividans*. The situation is analogous to that found with incompatible plasmids and suggests that a resident plasmid in strain ISP5230 might participate in the suppression of pIJ941. A search for extrachromosomal covalently closed circular DNA in ISP5230 failed to detect plasmids (Ahmed & Vining, 1983), despite evidence for the involvement of extrachromosomal elements in conjugative recombination (Doull *et al.*, 1986). However, a large linear plasmid has subsequently been discovered in this strain of *S. venezuelae* (Kinashi & Shimaji, 1987).

When pDQ102 was used to introduce a homologous gene for *p*-aminobenzoate synthase into a Cml^r mutant of *S. venezuelae* ISP5230, recombinant plasmids were detected in only a small fraction of thiostrepton-resistant transformants. The absence of an autonomous plasmid in most of the transformants can be accounted for by its integration into the bacterial chromosome, possibly as a result of recombination at the homologous *pab* regions. Evidence supporting this conclusion has been obtained by using the thiostrepton-resistance gene excised from pIJ702 as a hybridization probe; although transformant VS612 contained no detectable plasmid, a region in its genomic DNA hybridized with the thiostrepton resistance gene (D. A. Aidoo, unpublished).

The hope that transformants of *S. venezuelae* Cml^r into which a DNA fragment carrying *pab* genes from the wild-type were introduced might be restored for anti-

biotic production was not realized. Although these strains overproduced *p*-aminobenzoic acid to the extent that it accumulated in the culture broth, indicating that they may have acquired multiple copies of DNA promoting *p*-aminobenzoate synthesis, they did not acquire the ability to produce chloramphenicol. Since the common biosynthetic intermediate for both *p*-aminobenzoate and chloramphenicol must have been produced in substantial quantity, the mutant gene responsible for the inability to make *p*-aminophenylalanine in strain Cml-1 is not a defective secondary metabolic allele of *pabA* or *pabB*. Failure to restore chloramphenicol production presumably means also that the DNA introduced in pDQ102 does not contain a wild-type allele of the mutation in strain Cml-1.

This work was supported by the Natural Sciences and Engineering Research Council of Canada. We thank C. Stuttard, D. A. Hopwood and J. A. Gil for gifts of plasmids and cultures, and S. J. Lucania for providing thiostrepton. We also thank Dr Stuttard for many helpful discussions.

References

- ACEBAL, C., RUBIO, V. & MARQUEZ, G. (1986). A method to transform the β -lactam antibiotic producer, *Streptomyces wadayamensis*. *FEMS Microbiology Letters* **35**, 79–82.
- AHMED, Z. U. & VINING, L. C. (1983). Evidence for a chromosomal location of the genes coding for chloramphenicol production in *Streptomyces venezuelae*. *Journal of Bacteriology* **154**, 239–244.
- BAILEY, C. R., BRUTON, C. J., BUTLER, M. J., CHATER, K. F., HARRIS, J. E. & HOPWOOD, D. A. (1986). Properties of *in vitro* recombinant derivatives of pJV1, a multi-copy plasmid from *Streptomyces phaeochromogenes*. *Journal of General Microbiology* **132**, 2071–2078.
- BIBB, M. J., SCHOTTEL, J. L. & COHEN, S. N. (1980). A DNA cloning system for interspecies gene transfer in antibiotic-producing *Streptomyces*. *Nature, London* **274**, 398–400.
- BIBB, M. J., CHATER, K. F. & HOPWOOD, D. A. (1983). Developments in *Streptomyces* cloning. In *Experimental Manipulation of Gene Expression*, pp. 53–82. Edited by M. Inouye. New York: Academic Press.
- CHATER, K. F. & CARTER, A. T. (1978). Restriction of a bacteriophage in *Streptomyces albus* P (CMI 52766) by endonuclease *Sal*P1. *Journal of General Microbiology* **109**, 181–185.
- DOULL, J. L., VINING, L. C. & STUTTARD, C. (1983). A cryptic plasmid in the chloramphenicol-producing actinomycete, *Streptomyces phaeochromogenes*. *FEMS Microbiology Letters* **16**, 349–352.
- DOULL, J., AHMED, Z., STUTTARD, C. & VINING, L. C. (1985). Isolation and characterization of *Streptomyces venezuelae* mutants blocked in chloramphenicol biosynthesis. *Journal of General Microbiology* **131**, 97–104.
- DOULL, J. L., VATS, S., CHALICHOPOULOS, M., STUTTARD, C., WONG, K. & VINING, L. C. (1986). Conjugational fertility and location of chloramphenicol biosynthetic genes on the chromosome linkage map of *Streptomyces venezuelae*. *Journal of General Microbiology* **132**, 1327–1338.
- ENGEL, P. (1987). Plasmid transformation of *Streptomyces tendae* after heat attenuation of restriction. *Applied and Environmental Microbiology* **53**, 1–3.
- GIL, J. A. & HOPWOOD, D. A. (1983). Cloning and expression of a *p*-aminobenzoic acid synthetase gene of the candidicin-producing *Streptomyces griseus*. *Gene* **25**, 119–132.
- HOPWOOD, D. A., BIBB, M. J., CHATER, K. F., KIESER, T., BRUTON, C., KIESER, H. M., LYDIATE, D. J., SMITH, C. P., WARD, J. M. & SCHREMPF, H. (1985). *Genetic Manipulation of Streptomyces. A Laboratory Manual*. Norwich, UK: John Innes Foundation.
- HUNTER, I. S. (1985). Gene cloning in *Streptomyces*. In *DNA cloning*, Vol. 2, pp. 19–44. Edited by D. M. Glover. Oxford: IRL Press.
- HUNTER, I. S. & FRIEND, E. J. (1984). "Restriction-deficient" mutants of industrial *Streptomyces*. *Biochemical Society Transactions* **12**, 643–644.
- KATZ, E., THOMPSON, C. J. & HOPWOOD, D. A. (1983). Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *Journal of General Microbiology* **129**, 2703–2714.
- KIESER, T. (1984). Factors affecting the isolation of ccc DNA from *Streptomyces lividans* and *Escherichia coli*. *Plasmid* **12**, 19–36.
- KINASHI, H. & SHIMAJI, M. (1987). Detection of giant linear plasmids in antibiotic-producing strains of *Streptomyces* by the OFAGE technique. *Journal of Antibiotics* **40**, 913–916.
- LARSON, J. L. & HERSHBERGER, C. L. (1986). The minimum replicon of a streptomycete plasmid produces an ultra-high level of plasmid DNA. *Plasmid* **15**, 199–209.
- LEVINE, J. & FISCHBACH, H. (1951). The chemical determination of chloramphenicol in biological materials. *Antibiotics and Chemotherapy* **1**, 59–62.
- LOMOVSKAYA, N. D., VOEYKOVA, T. A. & MKRTUMIAN, N. M. (1977). Construction and properties of hybrids obtained in interspecific crosses between *Streptomyces coelicolor* A3(2) and *Streptomyces griseus* Kr. 15. *Journal of General Microbiology* **98**, 187–198.
- LYDIATE, D. J., MALPARTIDA, F. & HOPWOOD, D. A. (1985). The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. *Gene* **35**, 223–235.
- MALIK, V. S. & REUSSER, F. (1979). Restriction enzyme map for streptomycete plasmid pUC3. *Plasmid* **2**, 627–631.
- MALIK, V. S. & VINING, L. C. (1970). Metabolism of chloramphenicol by the producing organism. *Canadian Journal of Microbiology* **16**, 173–180.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MATSUSHIMA, P., COX, K. L. & BALTZ, R. H. (1987). Highly transformable mutants of *Streptomyces fradiae* defective in several restriction systems. *Molecular and General Genetics* **206**, 393–400.
- NICHOLS, B. P., SEIBOLD, A. M. & DOKTOR, S. Z. (1989). *p*-Aminobenzoate synthesis from chorismate occurs in two steps. *Journal of Biological Chemistry* **264**, 8597–8601.
- OKANISHI, M., SUZUKI, K. & UMEZAWA, H. (1974). Formation and reversion of streptomycete protoplasts: cultural conditions and morphological study. *Journal of General Microbiology* **80**, 389–400.
- SHIRAHAMA, T., FURUMAI, T. & OKANISHI, M. (1981). A modified regeneration method for streptomycete protoplasts. *Agricultural and Biological Chemistry* **45**, 1271–1273.
- STUTTARD, C. (1979). Transduction of auxotrophic markers in a chloramphenicol-producing strain of *Streptomyces*. *Journal of General Microbiology* **110**, 479–482.
- STUTTARD, C. (1982). Temperate phages of *Streptomyces venezuelae*: lysogeny and host specificity shown by SV1 and SV2. *Journal of General Microbiology* **128**, 115–121.
- TENG, C.-Y. P., GANEM, B., DOKTOR, S. Z., NICHOLS, B. P., BHATNAGAR, R. K. & VINING, L. C. (1985). Total synthesis of ($\pm$) 4-amino-4-deoxychorismic acid: a key intermediate in the biosynthesis of *para*-aminobenzoic acid and *L*-*para*-aminophenylalanine. *Journal of the American Chemical Society* **107**, 5008–5009.
- THOMPSON, C. J., WARD, J. M. & HOPWOOD, D. A. (1980). DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species. *Nature, London* **286**, 525–527.
- THOMPSON, C. J., KIESER, T., WARD, J. M. & HOPWOOD, D. A. (1982a). Physical analysis of antibiotic resistance genes from *Streptomyces* and their use in vector construction. *Gene* **20**, 51–62.
- THOMPSON, C. J., WARD, J. M. & HOPWOOD, D. A. (1982b). Cloning of antibiotic resistance and nutritional genes in streptomycetes. *Journal of Bacteriology* **151**, 668–672.
- VATS, S., STUTTARD, C. & VINING, L. C. (1987). Transductional analysis and mutagenesis of chloramphenicol biosynthesis genes in *Streptomyces venezuelae*. *Journal of Bacteriology* **169**, 3809–3813.
- VINING, L. C. & WESTLAKE, D. W. S. (1984). Chloramphenicol: properties, biosynthesis and fermentation. In *Biotechnology of Industrial Antibiotics*, pp. 387–411. Edited by E. J. Vandamme. New York: Marcel Dekker.