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**

*Biochemistry Department, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J1*

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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
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(pabF) 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 SLPl2 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* (Lydiate 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) glucose (YEME/glucose 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 0.41% 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/glucose medium by digesting the cell walls with lysozyme (2 mg ml⁻¹) for 1 h. For transformation of S. venezuelae, protoplasts (4 × 10⁸) 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 2.5% 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 R₅ values of p-aminobenzoic acid and chloramphenicol in this system were 0.60 and 0.27, respectively.

Fig. 1. Conversion of chorismic acid to 4-amino-4-deoxypchorismic 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 I and II, respectively; pabX is used to designate enzyme X (Nichols et al., 1989).
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 $10^6$–$10^7$ (μg 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 (μg 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 (μg 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 (μg 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 $10^6$ (μg 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 (μg 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 $2 \times 10^2$ (μg DNA)$^{-1}$. The frequency increased to $10^5$ (μg 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 (μg 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 250 kb parent plasmid (Fig. 2). The deletion was
DNA fragment carrying the pab gene might also contain of chorismic acid to ISP5230 blocked in chloramphenicol biosynthesis at a Cml- mutation affects a step after the pathways synthase are present in cml-1.

(Gil et al., 1985). Doull et al. (1985) isolated a mutant of the genes for chloramphenicol biosynthesis (Vats et al., 1987), a sufficiently large clipping that gives pDQ101 is indicated by the dashed line.

The initial reaction in the formation of p-aminobenzoic acid and p-aminophenylalanine (Fig. 1) is the conversion of chorismic acid to 4-amino-4-deoxychorismic acid (Teng et al., 1985). Doull et al. (1985) isolated a mutant of ISP5230 blocked in choramphenicol biosynthesis at a step between chorismic acid and p-aminophenylalanine. This Cml-1 mutant is not auxotrophic for p-aminobenzoic acid so, unless the genes for p-aminobenzoate synthase are present in S. venezuelae ISP5230 as separate alleles for primary and secondary metabolic pathways, the Cml- mutation affects a step after the pathways diverge. Since the genes for choramphenicol biosynthesis are clustered (Vats et al., 1987), a sufficiently large DNA fragment carrying the pab gene might also contain cml-1. By complementing the defective gene, such a fragment should restore choramphenicol synthesis when introduced into the mutant. The vector pDQ101 was used to carry out the transformation.

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 PstI fragment that included the insert and approximately 0.8 kb of adjacent vector DNA (Fig. 3) was ligated to PstI-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 PstI 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 PstI 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 choramphenicol production (Doull et al., 1985). The yields of mixed product were 63, 30 and 3 µg ml^{-1}, respectively. By TLC of extracts from the filtered broth, p-aminobenzoic acid was identified as a major product of cultures 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 choramphenicol production medium did not yield any

Fig. 2. Recognition sites for restriction enzymes on pIJ941 (redrawn from Lydiate et al., 1985). The approximate location of the 5-3 kb deletion that gives pDQ101 is indicated by the dashed line.

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 PstI fragment subcloned in pDQ102 and pDQ103.

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Fig. 1. Diagram showing the initial reaction in the formation of p-aminobenzoic acid and p-aminophenylalanine. The initial reaction is the conversion of chorismic acid to 4-amino-4-deoxychorismic acid. The genes for choramphenicol biosynthesis are clustered, with the Cml-1 mutation affecting a step after the pathways diverge. The Cml-1 mutation is not auxotrophic for p-aminobenzoic acid.

The DNA fragment carrying the pab gene was ligated to PstI-digested pDQ101, and the resulting plasmids were used to transform S. venezuelae Cml-1. Putative transformants were screened for the presence of the recombinant plasmid. The plasmids pDQ102 and pDQ103, which contain oppositely oriented PstI inserts, were shown to complement the defective pab gene.

When thiostrepton-resistant colonies were obtained after transformation with pDQ102, they were assayed for production of inhibitory substances using Micrococcus luteus. Some colonies (VS607 and VS612) showed 5–8 mm zones of inhibition, whereas others showed no zones. In contrast, wild-type S. venezuelae ISP5230 colonies gave 18 mm inhibition zones.
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 streptomyces 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; Matsu-shima 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 (Lydiate 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− 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-1 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.

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References


