Cloning of a DNA Fragment from *Streptomyces griseus* which Directs Streptomycin Phosphotransferase Activity

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DNA from *Streptomyces griseus* ATCC 12475 was partially digested with Sau3A and fragments were ligated into BglII-cleaved pIJ702. When the ligation mixture was used to transform protoplasts of *Streptomyces lividans* TK54, two transformants resistant to both thiostrepton and streptomycin were isolated. The hybrid plasmids pBV3 and pBV4 which they contained, carrying inserts of sizes 4.45 and 11.55 kbp respectively, each retransformed *S. lividans* to streptomycin resistance at high efficiency. Both plasmids hybridized to restriction digests of *S. griseus* chromosomal DNA in Southern blot experiments. In vitro deletion and sub-cloning experiments showed the sequence conferring streptomycin resistance to lie within a segment of 1.95 kbp. Extracts of TK54(pBV3) and TK54(pBV4) contained a streptomycin phosphotransferase similar to that in extracts of *S. griseus*. Streptomycin phosphotransferase activity appeared in extracts of *S. griseus*, TK54(pBV3) and TK54(pBV4) within 2 d of inoculation. When pBV3 and pBV4 were retransformed into *S. griseus* with selection for thiostrepton resistance, plasmid DNA of sizes corresponding to the incoming plasmids was found in the transformants. In these transformants the phosphotransferase appeared at 1.5 rather than 2 d, and reached a level over twice that of the original *S. griseus* strain.

INTRODUCTION

The evolutionary origin and modes of regulation of genes encoding enzymes of 'secondary metabolism' represent two of the most interesting unsolved problems in microbiology. Antibiotics are the most important secondary metabolites, from the industrial and medical viewpoints; their secondary metabolic systems include not only the biosynthetic pathways but also antibiotic-modifying enzymes which may play a role in resistance and/or production. As yet, relatively little is known of the control mechanisms which regulate expression of genes of antibiotic synthesis or modification (Martin & Demain, 1980). Recently a number of genes encoding enzymes of antibiotic biosynthesis (Feitelson & Hopwood, 1983; Gil & Hopwood, 1983; Martin & Gil, 1984; Malpartida & Hopwood, 1984) and many involved in their modification (for references see Martin & Gil, 1984; also Malpartida et al., 1983; Murakami et al., 1983; Nakano et al., 1984) have been cloned. In some cases it appears that an antibiotic resistance gene is part of a cluster containing at least some of the corresponding biosynthesis genes (Rhodes et al., 1984; I. S. Hunter, personal communication).

We decided to use as a model system the production of streptomycin in *Streptomyces griseus*. The biochemistry of synthesis of the streptidine moiety of this molecule is well established (Walker, 1975) and there is a good deal of information on the roles of streptomycin phosphotransferase (referred to as the 6-kinase) and of streptomycin 6-phosphatase in this organism: it seems likely that the phosphotransferase operates both to channel intermediates towards streptomycin synthesis and to circumvent its toxic effects, while the phosphatase permits production of the antibiotic presumably by acting during or after transport across the membrane (Nimi et al., 1971a, b; Sugiyama et al., 1981a, b, 1982; Ono et al., 1983; Cundliffe, 1984; Phillips & Shannon, 1984). In addition, a very interesting pleiotropic system affecting streptomycin biosynthesis has been revealed; this involves a substance termed A-factor...
described by Chater et al. (1967; Kleiner et al., 1977; Hara & Beppu, 1982). The sequences involved in the formation of this A-factor have recently been cloned (Horinouchi et al., 1983, 1984). We started by attempting to clone a gene for a streptomycin phosphotransferase such as the 6-kinase. This would be of interest for two reasons: first, expression of the 6-kinase shows the type of dependence on stage of the growth cycle that is typical of enzymes of secondary metabolism (Miller & Walker, 1969; Nimi et al., 1971b), so that it could on its own provide a suitable model for regulation of expression of genes encoding such enzymes; and second, there seems a reasonable chance from the above precedents that it might be linked to genes specifying enzymes of streptomycin biosynthesis. This paper reports the cloning of a streptomycin phosphotransferase gene from a streptomycin-producing S. griseus in the non-producer S. lividans and its reintroduction into the original strain.

METHODS

Bacterial strains and plasmids. These are listed in Table 1.

Media and growth conditions. YEWE for routine growth of cultures and for plasmid DNA preparation was as described by Chater et al. (1982). Minimal medium (MM) was a variant of that of Hopwood (1967), and contained (per l): K₂HPO₄, 0·5 g; KOH, 0·5 g; asparagine, 0·5 g; MgSO₄, 0·2 g; FeSO₄, 7H₂O, 0·01 g; glucose (autoclaved separately as 50% solution), 10 g. For solid media the asparagine was replaced by NH₄NO₃ (1 g l⁻¹) and the medium was buffered by the addition of 100 mm-KH₂PO₄/Na₂HPO₄ pH 6·8 at 150 ml l⁻¹; Lab M Bacto-agar was added at 15 g l⁻¹. Histidine and leucine were added at 70 and 50 mg l⁻¹ respectively. R5 (a modification of R2YE) sporulation medium, used for production of spores of all strains, contained (per l): yeast extract (Oxoid), 10 g; peptone (Oxoid), 10 g; tryptone (Oxoid), 10 g; yeast extract (Oxoid), 2 g; K₂HPO₄, 0·3 g and 20% L-proline per 100 ml medium. P and R2YE media were as described by Thompson et al. (1981). PWP and modified R3 media were as described by Shirahama et al. (1981). Media for melanin production were YEWE, MM or R5 with the addition of 50 ml tyrosine l⁻¹ (750 µg ml⁻¹). SP medium for growth of cultures to be tested for streptomycin phosphotransferase activity (also used for tests on streptomycin production, hence the initials) contained (per l): peptone (Oxoid), 10 g; tryptone (Oxoid), 10 g; yeast extract (Oxoid), 2 g; K₂HPO₄, 0·3 g and glucose, 0·5 g. Ono et al. (1983) recommend that omission of the K₂HPO₄ from this medium is likely to allow better production of streptomycin, but in our experiments its inclusion did not affect streptomycin production or phosphotransferase synthesis. Strains TK54(pIJ702) and TK54(pBV3) grew very poorly in this medium and were therefore grown for the corresponding purposes in YEWE containing 0·03% K₂HPO₄, 5 mm-MgCl₂ and 34% sucrose. Thioestrepton (kindly provided by Mr S. J. Lucania of E. J. Squibb & Sons, New Brunswick, NJ, USA) and streptomycin sulphate (Sigma) were present in media at 50 and 60 µg ml⁻¹ respectively, unless otherwise stated. All cultures were grown (on both solid and liquid media) at 30 °C.

Growth, harvesting and preparation of extracts of cultures for investigation of streptomycin phosphotransferase activity. Spore suspensions (prepared from cultures on R5 medium) were inoculated, to give initial densities of about 10⁷ ml⁻¹, into a number (equal to the number of samples to be taken) of 250 ml conical flasks containing 50 ml of the appropriate medium together with a short length of stainless steel spring to shred the mycelium. The flasks were incubated on an orbital incubator at 200 r.p.m. At appropriate intervals, the contents of one or more flasks were harvested by filtration in a Büchner funnel, washed with water and the mycelium weighed, suspended in 5 ml 0·1 M-potassium phosphate buffer pH 7 and disrupted by ultrasonic disintegration (MSE 100 W ultrasonic disintegrator at maximum amplitude). The extract was clarified by centrifugation at 30000 g for 15 min.

Tests for streptomycin phosphotransferase activity. Two methods were used. (a) This was essentially the protocol of Ono et al. (1983). The reaction mixture contained in a total volume of 0·5 ml: 488 nmol Tris/HCl pH 7·8; 195 nmol MgCl₂; 72·5 nmol ATP; 25 nmol dithiothreitol; 39 nmol streptomycin sulphate; and 0·25 ml mycelial extract. Controls were run without streptomycin or ATP. The combined mixtures were incubated for 2 h at 30 °C. To show that phosphorylation of streptomycin had occurred, the mixture was heated at 100 °C for 5 min, and on cooling to 30 °C alkaline phosphatase (BRL) was added (2 units ml⁻¹). Incubation was continued for 5 min and then 0·05 ml samples of the final mixture were placed in wells cut with a sterile cork borer in an NA agar plate overlaid with Escherichia coli C600 in soft agar. (b) A quantitative spectrophotometric assay was based on the method of Hintermann (1983). The reaction mixture contained in a total volume of 2 ml: 100 µmol triethanolamine/HCl pH 7·4; 20 µmol MgCl₂; 4 µmol ATP; 0·5 µmol NADH; 3·2 units pyruvate kinase (Boehringer-Mannheim); 11 units lactate dehydrogenase (Boehringer-Mannheim); and 50 µl mycelial extract. Incubation was at 25 °C. Two parallel cuvettes were set up with each extract to serve as reference and assay samples; the reaction was started by addition of 10 µl 0·5 M-streptomycin sulphate to the assay cuvette, and the OD difference at 340 nm between the
**RESULTS AND DISCUSSION**

**Isolation and characterization of plasmids pBV3 and pBV4**

*S. griseus* SG1 DNA was partially digested with Sau3A, fragments in the size range 3–23 kbp were ligated to *BglIII*-cleaved pIJ702, and the ligation mixture was used to transform *S. lividans* TK54, as described in Methods. The resulting semi-confluent lawns of thiostrepton-resistant transformants were replica plated on to MM containing streptomycin sulphate at 60 µg ml⁻¹ (the use of MM reduced background growth). Four colonies resulted; plasmid DNA was prepared from cultures of these isolates, and two of these (termed pBV3 and pBV4) were isolated by the method of Chater *et al.* (1982) and Shirahama *et al.* (1981) respectively. Transformation and regeneration of protoplasts of both species were carried out by the methods of Thompson *et al.* (1980), except that in the case of *S. griseus* P and R2YE media were replaced by PWP and modified R3 media respectively (Shirahama *et al.*, 1981). Plasmid probes were 32P-labelled by nick translation (Rigby *et al.*, 1977) and incorporated label was separated from unincorporated label by selectively precipitating the former with spermine (Hoopes & McClure, 1981). Blot hybridization was done by the method of Southern (1975) with Schleicher & Schüll nitrocellulose sheets (Anderman & Co., East Molesey, Surrey, UK). Autoradiography was carried out with intensifying screens at −70 °C on Fuji RX films.
plasmid has the same size, suggesting that some DNA is common to the two inserts. Other experiments (not shown) indicated that (a) pBV3 and pBV4 are stably maintained in TK54 during growth on solid YEME or R5 media in the absence of either thiostrepton or streptomycin (though a low segregation frequency could not be ruled out), and (b) the intensities of the unrestricted DNA bands for pIJ702, pBV3 and pBV4 in comparable preparations were similar, suggesting that the latter two plasmids were present, like pIJ702, in relatively high copy number (Katz et al., 1983).

The published restriction map of pIJ702 shows single sites for BamHI, BglII, KpnI, PstI, SphI and SstI (Katz et al., 1983). Single and double digests of pBV3 and pBV4 with these enzymes were therefore made, and restriction maps consistent with the sizes of the resulting fragments are presented in Fig. 2. It is noteworthy that the set of six restriction sites for these enzymes within the insert in pBV3 appear in the same order and with the same spacing at the right-hand end of the insert in pBV4. It therefore seems likely that the insert in pBV3 is almost wholly contained within that in pBV4; the only part not contained therein is presumably the rightmost 0.8-kbp, since site BamHI(5') in pBV3 lies 2.1 kbp from the BglII/Sau3A(l) junction site, whereas the corresponding site BamHI(5) in pBV4 lies only 1.3 kbp from that site. The three BamHI sites towards the right-hand end of the insert are equally spaced, so that BamHI digestion of pBV3 and pBV4 apparently yields three and four (as noted also from Fig. 1), rather than four and five, fragments, the smallest in each case being a doublet of size 0.65 kbp.

The presence of S. griseus DNA in pBV3, and the partial homology of the inserts in pBV3 and pBV4, were demonstrated by Southern blot transfer. Fig. 3(a) shows results of gel electrophoresis of SG1 DNA digested with PstI or BamHI, and of pBV4 digested with PstI + SphI, and Fig. 3(b) shows the results of probing these with 32P-labelled pBV3 DNA. (When pIJ702 was used as probe, no hybridization with chromosomal sequences was detected; results not shown.) BamHI-cleaved SG1 DNA would be expected to show three hybridizing bands, one corresponding to the 1.1 kbp BamHI(2)/BamHI(3) fragment of pBV4, another corresponding to
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Fig. 2. Restriction maps of pBV3 and pBV4. Vector DNA, the inserts of the two plasmids, restriction sites, and the approximate location of the region determining streptomycin resistance (inferred from data presented in Fig. 4 and described in the text) are aligned and to scale. Co-ordinates (in kbp), taking the left-hand BglII/Sau3A hybrid site as 0.0, are as follows (figures for pIJ702 are taken from Katz et al., 1983). pBV3 (total size 10.1 kbp): SphI(l), 0.16; PstI(l), 0.53; KpnI(l), 0.95; BamHI(l), 1.33; SstI(l), 5.38; BglII/Sau3A(2), 5.65; SphI(3'), 6.0; SstI(4'), 6.4; BamHI(3'), 6.7; BamHI(4'), 7.35; PstI(3'), 7.9; BamHI(5'), 8.0. pBV4 (total size 17.2 kbp): SphI(l), 0.16; PstI(l), 0.53; KpnI(l), 0.95; BamHI(l), 1.33; SstI(l), 5.38; BglII/Sau3A(2), 5.65; PstI(2), 6.05; KpnI(2), 6.7; SstI(2), 8.45; BglII(l), 12.1; SstI(3), 12.3; KpnI(3), 13.15; SphI(2), 13.35; BamHI(2), 13.5; SphI(3), 13.9; SstI(4), 14.3; BamHI(3), 14.6; BamHI(4), 15.25; PstI(3), 15.8; BamHI(5), 15.9. The five restriction sites in the insert of pBV3 also appear in the same order and with the same spacings at the right-hand end of the insert of pBV4. It has been assumed (see also text) that they represent the same sites. They are therefore given the same numbers in both plasmids, except that for pBV3 a prime has been added. These co-ordinates provide maximum consistency in harmonizing results from a large number of single and double digests. They may therefore not accord totally with fragment sizes from individual experiments as noted for instance in Figs 3 and 4.

the 0.65 kbp doublet, and the third of unpredictable length: this is in fact what is seen. PstI-cleaved SG1 DNA would be expected to show two hybridizing bands of unpredictable length: only one is apparent, which may be a doublet. Finally, the hypothesis that the insert in pBV3 comprises the right-hand end (with the orientation shown in Fig. 2) of the insert in pBV4 would suggest that the PstI + SphI double digest of pBV4 should yield five hybridizing bands, of sizes 5.52, 1.9, 1.56, 0.55 and 0.37 kbp [corresponding to fragments bounded respectively by PstI(1)/PstI(2), SphI(3)/PstI(3), PstI(3)/SphI(1), SphI(2)/SphI(3), and SphI(1)/PstI(1)]. Track 7 of Fig. 3(b) shows the five major bands corresponding to the five largest of these; that no band corresponding to the 0.37 kbp fragment can be seen may reflect only the small amount of hybridization expected for this low molecular size.

Localization of the streptomycin resistance determinant within the inserts

Further experiments were done to define the position of the streptomycin resistance determinant within the cloned inserts. First, pBV3 was completely digested in separate experiments with two restriction endonucleases, PstI and SstI, which each cut pBV3 twice. The products were allowed to recircularize under conditions (see Methods) favouring intramolecular recircularization; this would be expected to yield products lacking a portion of the insert (and
Fig. 3. Blot hybridizations with pBV3 as probe. (a) Agarose (0.8%) gel electrophoretogram of: track 1, \(\text{PstI}\)-digested \textit{S. griseus} chromosomal DNA; track 2, \textit{BamHI}\-digested \textit{S. griseus} chromosomal DNA; track 3, pBV4 digested with \(\text{PstI} + \text{SphI}\); track 4, size markers (\(\lambda\ \text{HindIII}\) fragments). (b) Autoradiogram of tracks 1, 2 and 3 of (a) following Southern blot hybridization with pBV3 DNA \(^{32}\text{P}\)-labelled by nick translation as probe (see Methods); track 5 corresponds to track 1, track 6 to track 2, and track 7 to track 3. Minor bands (especially noticeable in track 7) are assumed to correspond to partially digested fragments.

Fig. 4. Agarose (0.8\%) gel electrophoretogram of pBV4 and six subclones, obtained by partial \textit{Sau3A} digestion and religation into pIJ702, that still confer streptomycin resistance. Tracks 1 and 16, size markers (\(\lambda\ \text{HindIII}\) fragments); tracks 2 and 3, pBV4 digested with \textit{BamHI} and \textit{PstI} respectively; tracks 4, 6, 8, 10, 12 and 14, subclones digested with \textit{BamHI}; tracks 5, 7, 9, 11, 13 and 15, subclones digested with \textit{PstI} (4 and 5 refer to subclone 1, 6 and 7 to subclone 2, etc.).
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also a dispensable portion of the vector). The final ligation mixtures were used to transform TK54 to thiostrepton resistance, and 16 such transformants from each ligation were tested for streptomycin resistance. All the PsII-produced transformants were streptomycin-sensitive, while all but two of the SstI-produced transformants were streptomycin-resistant. Mini-preparations of DNA from the 32 transformants were analysed: the results (not shown) indicated that the two SstI-derived plasmids that failed to confer streptomycin resistance had suffered deletions spanning the entire insert, while the other 30 plasmids had lost only the fragment expected in each case. This indicates that the streptomycin resistance determinant includes sequences within the smaller PsII fragment of pBV3, but not within its smaller SstI fragment.

Next, pBV4 was partially digested with Sau3A and the digest ligated with BgII-cleaved pIJ702: the ligation mixture was used to transform TK54 to resistance to streptomycin (100 μg ml⁻¹) and thiostrepton. Six isolates were obtained that contained plasmids smaller than pBV4. These were digested with BamHI or PsII (Fig. 4, tracks 4–15). The only fragments present in all the isolates are (refer to the restriction map of pBV4 in Fig. 2) the fragments bounded by BamH(S)/BamHI(1) and by PsII(3)/PsII(1). The simplest interpretation is that these smaller plasmids comprise regions of pBV4 as shown in Fig. 5, and derive from it either by the ligation of Sau3A-generated fragments into pIJ702 (with loss by intramolecular recombination of any duplicated vector sequences thus produced) or by in vitro deletion of Sau3A fragments from pBV4.

These data locate the streptomycin resistance determinant between BamH(1) and BgII/Sau3A(1) in pBV4, a region 1-95 kbp in extent, as indicated in Fig. 2.

pBV3 determines the appearance of a streptomycin phosphotransferase activity

Nimi et al. (1971 a, b) and Sugiyama et al. (1981 a, b) have demonstrated the presence in S. griseus of a streptomycin phosphotransferase which inactivates the antibiotic effect of streptomycin. The presence of this enzyme activity in S. lividans TK54 (which does not produce such an enzyme) containing pBV3 was therefore investigated. Strains SG1, TK54 and TK54(pBV3) were grown up, cell extracts produced by ultrasonic disintegration, and the extracts incubated with streptomycin in the presence of ATP as described in Methods (a control was included without any extract). Control incubations were: streptomycin omitted; ATP omitted; and incubation mixtures heated to 100°C (to inactivate the phosphotransferase) followed by further incubation with alkaline phosphatase. Samples from the incubation mixtures were applied to wells in agar plates overlaid with Escherichia coli C600 (see Methods).
Fig. 6. Plate test for streptomycin phosphotransferase activity. In all cases the products of the appropriate reaction mixture were placed in wells cut in a nutrient overlaid with a streptomycin-sensitive E. coli strain (see Methods). (a, b, c) Demonstration of an ATP-dependent streptomycin-inactivating activity. (a) Complete reaction mixture, with the following cell extracts: (i) S. lividans TK54, (ii) S. griseus, (iii) TK54(pBV3) and (iv) no extract. (b) (i)–(iv) Same extracts as in (a) together with the reaction mixture from which ATP had been omitted. (c) (i)–(iv) Same extracts as in (a) together with the reaction mixture from which streptomycin had been omitted. (d, e) Demonstration that the inactivation of streptomycin involves phosphorylation. (d) (i)–(iv) As (a). (e) (i)–(iv) As (a) but with the reaction mixtures being finally incubated with alkaline phosphatase.

Results following overnight incubation showed that the extracts of SG1 (Fig. 6a) and TK54(pBV3) (Fig. 6b), but not TK54 (Fig. 6c), convert streptomycin to a non-inhibitory derivative in an ATP-dependent reaction. Incubation with alkaline phosphatase (Fig. 6d, e) results in the reappearance of an inhibitory substance (presumably streptomycin), showing that the above conversion produces a streptomycin phosphate.

These results were extended to compare the time of appearance of the streptomycin phosphotransferase activity in the growth cycle of SG1 and TK54(pBV3), using the quantitative assay for the enzyme rather than the plate test for activity. Sets of 50 ml cultures of SG1,
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Table 2. Mycelial weight and streptomycin phosphotransferase specific activity in cultures of strains SG1, TK54(pIJ702) and TK54(pBV3) grown for varying numbers of days

50 ml cultures (six of SG1 and seven of the other two strains) were grown up in the appropriate media, harvested at half-day intervals, broken open by ultrasonic disintegration and the extracts assayed for streptomycin phosphotransferase (SP) activity, as described in Methods. Limits of detectability were estimated as the specific activity corresponding to a fall in OD of 0.002 in 6 min incubation of the reaction mixture after addition of substrate; ND, not determined.

TK54(pIJ702) and TK54(pBV3) were grown up and harvested, and cell extracts were prepared by ultrasonic disintegration and assayed by coupling to pyruvate kinase and lactate dehydrogenase (phosphorylation of streptomycin being finally monitored as oxidation of NADH to NAD\(^+\)) as described in Methods. Cultures were harvested after 1 d and thenceforth at half-day intervals. Results are given in Table 2. Streptomycin phosphotransferase activity appears in SG1 and TK54(pBV3) at around 2 d, by which time the most rapid phase of growth of both organisms is past. The interpretation of these data is complicated by the slower initial growth of TK54(pBV3) than of SG1, and by the high activity, thereafter falling by about 75\% in the TK54(pBV3) extract after 2 d. As expected, no activity could be detected in TK54(pIJ702).

These results suggest a similar timing of appearance of streptomycin phosphotransferase activity in the growth cycles (with the above provisos) of SG1 and TK54(pBV3).

The simplest explanation of the above is that pBV3 and pBV4 carry a structural gene for a streptomycin phosphotransferase (it has not been demonstrated that phosphorylation is at the 6-position in the streptidine moiety of the molecule), and that this gene is the streptomycin resistance determinant of these two plasmids. Other interpretations are however possible. For instance, it might be that pBV3 and pBV4 contain a regulatory sequence that activates a cryptic streptomycin phosphotransferase gene in *S. lividans* TK54. That this kind of phenomenon can occur has been shown by G. H. Jones & D. A. Hopwood (offered paper to the Genetics Group of the Society for General Microbiology, Sheffield, September 1984), who demonstrated that of three independent *SphI* DNA fragments of the actinomycin D producer *Streptomyces antibioticus* IMRU 3720 which when cloned into *S. lividans* on pIJ702 led to synthesis of phenoxazinone synthase, only one actually encoded this enzyme; the other two apparently carried sequences that activated a normally almost unexpressed structural gene of *S. lividans*. This interpretation is rendered unlikely by the recent isolation in our laboratory of hybrid plasmids conferring streptomycin resistance in *E. coli* in which fragments of pBV4 have been sub-cloned in the enterobacterial expression vector pRK9 (C.-K. Lim, unpublished results). Even if pBV3 and pBV4 do carry a streptomycin phosphotransferase structural gene, it might be that this is not the streptomycin resistance determinant, either in the original *S. griseus*, in *S. lividans* or in *E. coli*. Work is in progress to clarify this point.

The finding that streptomycin phosphotransferase activity appeared in both SG1 and TK54(pBV3) after 2 d incubation is consistent – to the extent that the enzyme shows in its normal host environment ‘secondary metabolism’ type characteristics of appearance – with pBV3 carrying a structural gene together with information necessary for these characteristics, though it is not inconsistent with either of the alternative interpretations outlined above.
Effects of pBV3 and pBV4 following transformation into S. griseus SG1

It was of interest to see whether pBV3 and pBV4 could affect streptomycin phosphotransferase production, whether in quantity, timing or both, when introduced into S. griseus. Accordingly, SG1 was transformed to thiostrepton resistance with pIJ702 (control), pBV3 and pBV4 as described in Methods: transformation frequencies were $5 \times 10^3$, 7 and 4 per µg DNA respectively. Plasmid DNA was isolated from two of the pBV3 and three of the pBV4 transformants and digested with BamHI or PstI (Fig. 7). The patterns were the same as those for preparations from TK54(pBV3) and TK54(pBV4), indicating that some copies remain autonomous in spite of the homology between the inserts and the chromosome. The intensity of the bands suggests that the copy number is less than in TK54: whether there exist copies integrated into the chromosome is unknown.

Sets of 50 ml cultures of isolates of SG1(pBV3) and SG1(pJV4), as well as SG1 and SG1(pIJ702) controls, were grown up and harvested. Cell extracts were prepared and assayed quantitatively for streptomycin phosphotransferase activity as described in Methods. Results are given in Table 3. The timing of appearance of the enzyme in SG1 was much as before,
Table 3. Mycelial weight and streptomycin phosphotransferase specific activity in cultures of strains SG1, SG1(pIJ702), SG1(pBV3) and SG1(pBV4) grown for varying numbers of days.

50 ml cultures (five of each strain) were grown up in the appropriate media, harvested at half-day intervals, broken open by ultrasonic disintegration and the extracts assayed for streptomycin phosphotransferase (SP) activity, as described in Methods. Limits of detectability were estimated as the specific activity corresponding to a fall in OD of 0.002 in 6 min incubation after the addition of substrate.

<table>
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<th>No. of days growth</th>
<th>SG1</th>
<th>SG1(pIJ702)</th>
<th>SG1(pBV3)</th>
<th>SG1(pBV4)</th>
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<tr>
<td></td>
<td>Total mycelial weight (g)</td>
<td>SP activity (μmol min⁻¹ mg⁻¹)</td>
<td>Total mycelial weight (g)</td>
<td>SP activity (μmol min⁻¹ mg⁻¹)</td>
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although levels were initially lower than the data in Table 2. Remarkably, no activity was detectable in the SG1(pIJ702) extracts. Both SG1(pBV3) and SG1(pBV4) showed high levels of enzyme after 1.5 rather than 2 d; these levels (which eventually began to fall) were over twice those found in SG1 either in this or in the experiment summarized in Table 2. This experiment was done three times in all, in each case with essentially the same results.

The lack of streptomycin phosphotransferase activity in SG1(pIJ702) was unexpected. It is possible that the plasmid exerts an as yet undefined pleiotropic effect on expression of the (presumed chromosomal) gene. In accord with such speculations, anomalies have sometimes been observed (W. J. S. Vallins, unpublished results) in the production of streptomycin by SG1 carrying pIJ702, pBV3 or pBV4. It would be interesting to know whether the chromosomal gene is similarly silent in the presence of pBV3 or pBV4. It will also be desirable to find out why pBV3 and pBV4 lead to more rapid appearance of streptomycin phosphotransferase activity in SG1(pBV3) and SG1(pBV4) than in SG1 or TK54 carrying either of these plasmids. Finally, the observation that the maximal enzyme activity in SG1(pBV3) and SG1(pBV4) is over twice that for SG1 may have significant implications for possible improvement in antibiotic production if corresponding phenomena turn out to occur also with genes encoding enzymes of antibiotic biosynthesis.

We believe that priority in cloning a Streptomyces streptomycin phosphotransferase belongs to G. Hintermann (see Hintermann, 1983; Hintermann et al., 1984) who has cloned the gene from S. griseus. As this organism is not regarded, by standard taxonomic criteria, as being closely related to S. griseus (Buchanan & Gibbons, 1974), it will be interesting to compare the two genes, for instance in regard to the DNA sequence of coding and regulatory regions. Tohyama et al. (1984) have given a brief account of the cloning of such a gene from S. griseus. It is also understood that an S. griseus gene has been cloned by W. Piepersberg & J. Distler (programme of the Workshop 'Genetik antibiotikaproduzierender Mikroorganismen', University of Bielefeld, June 1984).

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