Cloning of a Thermostable α -Amylase Gene from *Thermomonospora curvata* and Its Expression in *Streptomyces lividans*

By MIROSLAV PETŘÍČEK, KAREL STAJNER AND PAVEL TICHÝ*

Department of General Microbiology, Institute of Microbiology, Czechoslovak Academy of Sciences, Václavská 1083, 142 20 Prague 4, Czechoslovakia

(Received 17 April 1989; revised 24 July 1989; accepted 6 September 1989)

The gene from *Thermomonospora curvata* CCM 3312 coding for thermostable α -amylase (*tam*) has been cloned in *Streptomyces lividans* TK 24 and localized to a 2.6 kb *Hind*III–*Bam*HI fragment of DNA. The data presented here show that the *tam* gene is expressed at a high level in *S. lividans* and that the protein is efficiently excreted.

INTRODUCTION

Interspecific cloning of *Streptomyces* genes coding for polysaccharide-degrading enzymes has been reported in several papers. Using streptomycete plasmid vectors, genes coding for endoglycosidase H from *S. plicatus* (Robbins *et al.*, 1981), agarase from *S. coelicolor* (Kendall & Cullum, 1984; Bibb *et al.*, 1987; Buttner *et al.*, 1987), α -amylase from *S. hygroscopicus* (McKillop *et al.*, 1986; Hoshiko *et al.*, 1987) and *S. limosus* (Long *et al.*, 1987, Virolle & Bibb, 1988), and endoglucanase from *Streptomyces* no. 36a (Iwasaki *et al.*, 1987) and *S. lividans* (Shareck *et al.*, 1987) have been cloned and expressed in streptomycetes. There have been two reports concerning the successful intergeneric transfer of genes coding for polysaccharide-degrading enzymes from *Thermomonospora fusca* in *S. lividans*. These are the genes coding for endocellulase E₂ (Ghangas & Wilson, 1988) and endocellulase E₅ (Ghangas & Wilson, 1987). The transfer of genetic material between *Thermomonospora* and *Streptomyces* in the opposite direction was demonstrated by transfer of the *Streptomyces* plasmid pIJ702 from *S. lividans* to *T. fusca* protoplasts (Pidcock *et al.*, 1985).

Members of the genus *Thermomonospora*, facultatively thermophilic actinomycetes, play an important role in decomposition of organic compounds in composting plant material (Goodfellow & Cross, 1984). This genus includes sugar-degrading actinomycetes which can utilize a wide variety of plant sugars and polymeric carbohydrates as carbon and energy sources (Crawford *et al.*, 1973; Bellamy, 1974, 1977; Hägerdal *et al.*, 1980). The family *Thermomonosporaceae* shows a specific, but not close relationship to the *Streptomycetaceae* (Stackenbrandt & Schleifer, 1984). With respect to genetic relationship and similarities in its secretion of extracellular hydrolytic enzymes, it appears to be an appropriate source of genetic material for cloning in streptomycetes.

Another example of intergeneric transfer of genetic material between *Thermomonospora* and *Streptomyces* species is described in the present work. The gene coding for thermostable α -amylase in *T. curvata*, an enzyme which has already been purified and characterized (Stutzenberger & Carnell, 1977; Glymph & Stutzenberger, 1977), was cloned in streptomycete plasmid vectors and expressed in *S. lividans*.

METHODS

Bacteria and plasmids. These are listed in Table 1. With the exception of *T. curvata*, the manual of Hopwood *et al.* (1985) contains details of conditions for growth of bacterial strains, as well as methods for plasmid and chromosomal DNA isolation and their use in transformation.

Abbreviation: CIAP, calf intestinal alkaline phosphatase.

Table 1. *Bacterial strains and plasmids*

Strains and plasmids	Relevant characteristics	Source/reference
<i>Thermomonospora curvata</i> CCM 312	Thermostable α -amylase (Tam ⁺) producer	Czechoslovak Collection of Micro-organisms
<i>Streptomyces lavendulae</i> 1080		Institute of Microbiology, Moscow
<i>Streptomyces lividans</i> TK 24	Sm ^r SLP2 ⁻ SLP3 ⁻	John Innes Institute (Hopwood <i>et al.</i> , 1985)
pIJ61	Ts ^r Nm ^r	John Innes Institute (Thompson <i>et al.</i> , 1982)
pIJ487	Ts ^r , promoterless <i>aph</i> gene (promoter-probe plasmid)	John Innes Institute (Ward <i>et al.</i> , 1986)
pIJ622	Ts ^r Mel ⁺ ; pIJ702 derivative	John Innes Institute (T. Kieser, unpublished)
pRS410	Plasmid vector; Nm ^r Ts ^r (pSLG33 replicon)	M. Petříček (unpublished)
pRS416	Recombinant plasmid Tam ⁺ Ts ^r (pIJ61 replicon)	This work
pRS418	Tam ⁺ Ts ^r } Deletion derivatives of pRS416	This work
pRS419		
pRS420		
pRS421	Recombinant plasmid Tam ⁺ Ts ^r (pIJ487 replicon)	This work
pRS417	Recombinant plasmid Tam ⁺ Ts ^r (pIJ622 replicon)	This work
pRS422	Recombinant plasmid Tam ⁺ Ts ^r (pRS410 replicon)	This work

Media. *T. curvata* CCM 3312 was grown in liquid medium with the following composition: 0.2% (w/v) (NH₄)₂SO₄, 0.02% MgCl₂, 1 mM-CaCl₂, 10 mM-K₂HPO₄, 0.1% yeast extract (Oxoid), 1% soluble starch (Lachema); pH 8.0 at 53 °C.

The medium used for sporulation of *T. curvata* (SPTC) contained: 0.05% MgSO₄·7H₂O, 8 mM-K₂HPO₄, 0.05% glucose, 0.5% yeast extract (Oxoid), 1% crystalline cellulose, 1.7% agar and 0.2 ml trace element solution (Hopwood *et al.*, 1985) per 100 ml SPTC; pH 8.0.

BA agar medium was used to screen for α -amylase-producing colonies: 0.05 M-potassium phosphate buffer pH 7.2, 0.02% MgSO₄·7H₂O, 0.1% yeast extract (Oxoid), 1% soluble starch (Lachema), 1.5% agar, 0.2 ml trace element solution (Hopwood *et al.*, 1985) per 100 ml BA and 0.15% highly cross-linked Remazol-blue-conjugated starch.

Preparation of T. curvata chromosomal DNA. The chromosomal DNA of *T. curvata* was isolated from fresh mycelium collected from exponential-phase cultures (30 h) by the method described by Hopwood *et al.* (1985) for isolation of total DNA from streptomycetes. The relative insensitivity of *T. curvata* to digestion by lysozyme prompted us to increase the starting amount of mycelium to 29 g (wet weight) and proportionally to scale up the volumes of solutions used in particular steps of DNA isolation. The mycelium was digested in buffer containing 10 mM-Tris/HCl pH 8.0, 0.05 M-EDTA and 10.3% (w/v) sucrose. DNA was precipitated by addition of PEG 6000 (10%, w/v, final concentration) and after overnight incubation it was collected by gentle centrifugation (4000 g, 6 min).

Cloning manipulation. Linearized *Bam*HI-digested pIJ61 and pIJ487 plasmid vectors were prevented from recircularization by incubation with CIAP (0.1 unit per μ g plasmid DNA) in 50 mM-Tris/HCl buffer pH 8.0 at 30 °C for 30 min. The reaction was terminated by heating to 68 °C for 10 min followed by extraction with neutral phenol/chloroform. Chromosomal DNA of *T. curvata* was partially digested with *Sau*3AI and 6–12 kb fragments were collected from a 10–30% sucrose gradient.

The ligation of CIAP-treated vector DNA (1 μ g) with *Sau*3AI fragments of chromosomal DNA (5 μ g) was performed in ligation buffer as described by Hopwood *et al.* (1985) at a DNA concentration of 50 μ g ml⁻¹ for 20 h at 14 °C.

Protoplasting and transformation of *S. lividans* TK 24 (the host strain) was performed as described by Hopwood *et al.* (1985). After 18–20 h, thiostrepton was added to a final concentration of 30 μ g ml⁻¹ to select plasmid-containing transformants. For improved regeneration of *S. lividans* TK 24 protoplasts, 0.5% gelatin was added to the R2YE medium.

Restriction endonuclease mapping. *Bam*HI, *Bcl*I, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Pvu*II, *Sau*I, *Sph*I, *Xba*I and *Xho*I endonucleases were used to construct the physical map of the cloned fragment and to prepare its

deletion derivatives. Single and double digestions were performed to establish unambiguous maps of each plasmid. Horizontal agarose gel electrophoresis in TAE and TBE buffers with agarose concentrations of 0.8–1.1% was as described by Maniatis *et al.* (1982). λ DNA digested with *Hind*III and *Pst*I was used as a size marker.

Deletion derivatives of plasmid pRS416 were prepared by full or partial cleavage of 1 μ g plasmid DNA with restriction endonucleases and by ligation with T4 DNA ligase for 16 h at 14 °C, using a low DNA concentration (5 μ g ml⁻¹), which favoured recircularization.

Quantitative assay of thermostable α -amylase activity. A single sporulating colony of each transformant was inoculated into 80 ml YEME medium (Hopwood *et al.*, 1985) in which sucrose was replaced by 2% starch or 2% maltose. After 5–7 d, the culture medium was centrifuged in Eppendorf tubes and 0.1 ml supernatant was made up to 1.0 ml with 10 mM-potassium phosphate buffer pH 6.5. Each sample was preincubated for 10 min at 67 °C and, after addition of α -amylase assay tablets (Spofa, Czechoslovakia), each sample was incubated for a further 15 min at the same temperature. The Spofa ALFA-AMYLASE TEST is highly specific towards α -amylase so that the presence of glucoamylase and/or other amylolytic enzymes does not interfere with the assay. As a result of the hydrolytic action of α -amylase upon the chromogenic starch, water-soluble, dyed maltose-type oligosaccharide fragments were liberated. The reaction was terminated by adding 4 ml stop solution (10 g calcium carbonate, 100 ml acetone, 900 ml H₂O). The intensity of the released blue colour was measured at 620 nm. The enzyme activity was estimated according to the instructions provided by the supplier.

SDS-PAGE of supernatant proteins. The mycelium was removed by centrifugation and 20 ml portions of the supernatant were treated with ammonium sulphate at 80% saturation. Precipitated fractions were centrifuged for 10 min at 6000 r.p.m. in a Sorvall RT 6000B (A-500 rotor) and dialysed overnight against 20 vols PBS. The samples were analysed by SDS-PAGE (Laemmli, 1970) using a 5% (w/v) stacking gel and a 15% separation gel under non-reducing conditions. Protein bands were visualized by staining with Coomassie Blue R-250.

RESULTS AND DISCUSSION

Cloning and identification of the tam gene

To clone the α -amylase gene, *Sau*3AI fragments, approximately 6–12 kb in size, of chromosomal DNA of *T. curvata* were inserted into *Bam*HI-linearized pIJ61 vector that had been dephosphorylated with CIAP. About 15 000 thiostrepton-resistant colonies were obtained after transformation of *S. lividans* TK 24 protoplasts with the ligation mixture. More than 95% of them were neomycin-sensitive, which indicated a high frequency of insertion in the *Bam*HI site of pIJ61 vector. The sporulating colonies were replica-plated onto BA plates containing Remazol-blue-conjugated starch. One transformant produced a colourless zone around the colony, indicating that it had α -amylase activity. Retransformation of the plasmid, termed pRS416, isolated from this transformant confirmed that the thermostable α -amylase coding gene, *tam*, was carried on the plasmid. Thermostability of the α -amylase produced by the transformant was tested by transfer to 65 °C of BA agar plates with 48 h colonies grown at 30 °C. At the higher temperature the presecreted thermostable α -amylase cleared and enlarged the originally faint zone around the producer colonies, although the *Streptomyces* transformants were killed at this temperature.

Restriction analysis of isolated plasmid pRS416 revealed an 8.3 kb DNA insert in the *Bam*HI site of pIJ61 and allowed construction of the restriction map (Fig. 1).

Localization of tam gene by subcloning and deletion procedures

Two DNA regions, 5.22 kb and 6.65 kb, located in the left part of the inserted fragment and partially in the adjoining vector molecule were deleted from pRS416 using *Xho*I as described in Methods. The resulting plasmids, pRS418 and pRS419, having inserts of only 4.31 and 2.87 kb, respectively, could still encode the synthesis of thermostable α -amylase (Fig. 1).

The ability to encode production of thermostable α -amylase was preserved when fragments of various lengths obtained after digestion of pRS418 and pRS419 with different restriction endonuclease were subcloned in plasmid vectors pIJ622, pRS487 and pRS410. Plasmid pRS417 was constructed by ligation of a 3.0 kb *Sst*I fragment of pRS419 in the *Sst*I target site of pIJ622 vector. Plasmid pRS421 was obtained by the insertion of a *Bam*HI–*Hind*III fragment of pRS418 in the polylinker region of pIJ487, and plasmid pRS422 was obtained by replacing the *Bam*HI–*Xba*I fragment of the *aph* gene of pRS410 with the *Bam*HI–*Xba*I fragment of pRS418. Plasmid pRS420 was prepared by a double digestion of pRS418 with *Sau*I and *Bam*HI, followed by

Fig. 1. Comparative restriction maps of recombinant plasmids pRS416-pRS422 bearing *T. curvata* CCM 3312 DNA restriction fragments coding for thermostable α -amylase. Hatched areas indicate *T. curvata* DNA inserts.

Fig. 2. SDS-PAGE of extracellular proteins from: lane B, *T. curvata* CCM 3325 – amount of applied extracellular proteins 6 μ g; lane C, *S. lividans* TK 24(pRS416) – 7.5 μ g; lane D, *S. lividans* TK 24(pIJ61) – 8.5 μ g; lane E, *S. lividans* TK 24(pRS420) – 12.5 μ g; lane F, *S. lividans* TK 24(pRS418) – 7.5 μ g. Lane A, Low Molecular Weight Calibration Kit (Pharmacia-LKB): phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α -lactalbumin, 14.4 kDa. The position of α -amylase protein is indicated by the arrow.

filling cohesive ends of the plasmid molecule with the aid of dNTPs and Klenow fragment of DNA polymerase and religation of the resulting blunt ends of the plasmid molecule. No thermostable α -amylase activity was detected in cells transformed by this plasmid.

Although several taxonomic studies on *Thermomonospora* have been published, surprisingly none of them has, to our knowledge, included any data about the G+C content of *Thermomonospora* chromosomal DNA. The percentage of G+C in *T. curvata* chromosomal DNA was therefore calculated in this laboratory on the basis of melting temperature of chromosomal DNA in $0.01 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M-NaCl , $0.015 \text{ M-trisodium citrate}$, pH 7). The calculation was performed according to Stackenbrandt & Kandler (1979); the melting temperatures obtained in two determinations were 82.28°C and 81.88°C . The G+C content of *Thermomonospora* chromosomal DNA thus calculated, 67 mol%, is quite close to that of streptomycete DNA, 73% (Benigni *et al.*, 1975) and it is likely that the codon use in both bacteria will be very similar.

The α -amylase protein excreted by *S. lividans* TK 24 bearing the *tam* gene appeared as a 62 kDa band on SDS-PAGE. This size of protein is in good agreement with the molecular mass of the thermostable α -amylase protein secreted by *T. curvata* (Glymph & Stutzenberger, 1977). It is very likely that the signal peptide of proamylase coded by *T. curvata tam* gene is correctly recognized by the *S. lividans* TK 24 host (Fig. 2).

The α -amylase activity in the culture medium reached $60 \mu\text{kat l}^{-1}$, when *S. lividans* TK 24 strains bearing low-copy-number recombinant plasmids pRS416, pRS418 and pRS419 were cultivated in YEME medium (Hopwood *et al.*, 1985) in which sucrose was replaced with 2% starch or 2% maltose. However, contrary to expectation, *S. lividans* TK 24 strains bearing the *tam* gene subcloned in multicopy plasmids pIJ622, pIJ487 or pRS410 secreted less α -amylase in the same medium than did the transformants carrying low-copy-number recombinant plasmids.

The most likely explanation for this is instability of *T. curvata* DNA inserts in high-copy-number plasmid vectors. This could be demonstrated by the loss of thermostable α -amylase production by many colonies originally bearing pRS417, pRS421 and pRS422 plasmids and still exhibiting thiostrepton resistance.

Although the amount of mRNA hybridizing with the *tam* gene, and the intracellular concentration of thermostable α -amylase in cells containing recombinant plasmids, have not been determined, it does not seem likely that double transcription of the *tam* gene from its own promoter and simultaneously from the promoter situated in the plasmid vector would be involved in the loss of α -amylase production. A similar situation occurs, for example, in pRS416, which includes cloned DNA at the *Bam*HI site of pIJ61 in the orientation which enables transcription of the *tam* gene from *aph* promoter.

The different orientation of inserts in recombinant plasmids demonstrates that the *tam* gene is transcribed from its own promoter region. This conclusion is also confirmed by the construction of pRS421, where the *tam* gene was inserted behind the strong transcription terminator, preventing an occasional transcription of the *tam* gene from a plasmid promoter. It follows from the cloning in the promoter-probe plasmid pIJ487 that the orientation of transcription of the *tam* gene is probably from the *Bam*HI to the *Hind*III site of the inserts. Nevertheless, the presence of an additional promoter, which can initiate transcription of the promoterless *aph* gene in the promoter-probe plasmid pIJ487 between the *Sau*I and *Hind*III sites cannot be excluded. It is hoped that sequencing of the *tam* gene and neighbouring regions, which is now in progress, will clarify this question.

We gratefully acknowledge the receipt of plasmids and *S. lividans* strains from D. A. Hopwood and M. J. Bibb, Norwich, UK. *T. curvata* CCM 3312 was from the Czechoslovak Culture Collection and thiostrepton was from S. J. Lucania, Squibb.

REFERENCES

- BELLAMY, W. D. (1974). Single cell proteins from cellulose wastes. *Biotechnology and Bioengineering* **16**, 869–880.
- BELLAMY, W. D. (1977). Cellulose and lignocellulose digestion by thermophilic actinomycetes for single-cell protein production. *Developments in Industrial Microbiology* **18**, 249–254.
- BENIGNI, R., ANTONOV, P. P. & CARERE, A. (1975). Estimate of the genome size by renaturation studies in *Streptomyces*. *Applied Microbiology* **30**, 324–336.
- BIBB, M. J., JONES, G. H., JOSEPH, R., BUTTNER, M. J. & WARD, J. M. (1987). The agarase gene of *Streptomyces coelicolor* A3(2): affinity purification and characterization of the cloned gene product. *Journal of General Microbiology* **133**, 2089–2096.
- BUTTNER, M. J., FEARNLEY, I. M. & BIBB, M. J. (1987). The agarase gene (*dag*) of *Streptomyces coelicolor* A3(2): nucleotide sequence and transcriptional analysis. *Molecular and General Genetics* **209**, 101–109.
- CRAWFORD, D. L., MCCOY, E., HARKIN, J. M. & JONES, P. (1973). Production of microbial protein from waste cellulose by *Thermomonospora fusca*, a thermophilic actinomycete. *Biotechnology and Bioengineering* **25**, 833–843.
- GHANGAS, G. S. & WILSON, D. B. (1987). Expression of a *Thermomonospora fusca* cellulase gene in *Streptomyces lividans* and *Bacillus subtilis*. *Applied and Environmental Microbiology* **53**, 1470–1475.
- GHANGAS, G. S. & WILSON, D. B. (1988). Cloning of the *Thermomonospora fusca* endonuclease E2 gene in *Streptomyces lividans*: affinity purification and functional domains of the cloned gene product. *Applied and Environmental Microbiology* **54**, 2521–2526.
- GLYPH, J. L. & STUZENBERGER, F. J. (1977). Production, purification and characterization of α -amylase from *Thermomonospora curvata*. *Applied and Environmental Microbiology* **34**, 391–397.
- GOODFELLOW, M. & CROSS, T. (1984). Classification in *The Biology of Actinomycetes*, pp. 8–164. Edited by M. Goodfellow, M. Mordarski & S. T. Williams. London: Academic Press.
- HÄGERDAL, B., FERCHAK, J. D. & PYE, E. K. (1980). Saccharification of cellulose by the cellulolytic enzyme system of *Thermomonospora* sp. 1. Stability of cellulolytic activities with respect to time, temperature and pH. *Biotechnology and Bioengineering* **22**, 1515–1526.
- HOPWOOD, D. A., BIBB, M. J., CHATER, K. F., KIESER, T., BRUTON, C. J., 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.
- HOSHIKO, S., MAKABE, O., NOJIRI, C., KATSUMATA, K., SATOH, E. & NAGAOKA, K. (1987). Molecular cloning and characterization of the *Streptomyces hygroscopicus* α -amylase. *Journal of Bacteriology* **169**, 1029–1036.
- IWASAKI, A., KISHIDA, H. & OKANISHI, M. (1987). Molecular cloning of a xylanase gene from *Streptomyces* no. 36a and its expression in *Streptomyces lividans* 66. *Journal of Antibiotics* **39**, 985–993.
- KENDALL, K. & CULLUM, J. (1984). Cloning and expression of an extracellular agarase gene from *Streptomyces coelicolor* A3(2) in *Streptomyces lividans* 66. *Gene* **29**, 315–321.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

- LONG, C. M., VIROLLE, M. J., CHANG, S. Y., CHANG, S. & BIBB, M. J. (1987). α -amylase gene of *Streptomyces limosus*: nucleotide sequence, expression motifs, and amino acid sequence homology to mammalian and invertebrate α -amylase. *Journal of Bacteriology* **169**, 5745–5754.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- McKILLOP, C., ELVIN, P. & KENTEN, J. (1986). Cloning and expression of an extracellular α -amylase gene from *Streptomyces hygroscopicus* in *Streptomyces lividans* 66. *FEMS Microbiology Letters* **36**, 3–7.
- PIDCOCK, K. A., MONTENECOURT, B. S. & SANDS, J. A. (1985). Genetic recombination and transformation in protoplasts of *Thermomonospora fusca*. *Applied and Environmental Microbiology* **50**, 693–695.
- ROBBINS, P. W., WIRTH, D. F. & HERING, C. (1981). Expression of *Streptomyces* enzyme endoglycosidase H in *Escherichia coli*. *Journal of Biological Chemistry* **256**, 10640–10644.
- SHARECK, F., MONDOU, F., MOROSOLLI, R. & KLUEMPFEL, D. (1987). Cloning of DNA sequences involved in overproduction of endoglucanase activity in *Streptomyces lividans* 66. *Biotechnology Letters* **9**, 169–174.
- STACKEBRANDT, E. & KANDLER, O. (1979). Taxonomy of the genus *Cellulomonas* based on phenotypic characters and deoxyribonucleic acid–deoxyribonucleic acid homology, and proposal of seven neotype strains. *International Journal of Systematic Bacteriology* **29**, 273–282.
- STACKEBRANT, F. & SCHLEIFER, K.-H. (1984). Molecular systematics of Actinomycetes and related organisms. In *Biological, Biochemical and Medical Aspects of Actinomycetes*, pp. 485–504. Edited by Z. Ortiz-Ortiz, L. F. Bojalil & V. Yakoleff. Orlando: Academic Press.
- STUZENBERGER, F. & CARNELL, R. (1977). Amylase production by *Thermomonospora curvata*. *Applied and Environmental Microbiology* **34**, 234–236.
- THOMPSON, C. J., KIESER, T., WARD, J. M. & HOPWOOD, D. A. (1982). Physical analysis of antibiotic resistance genes from *Streptomyces* and their use in vector construction. *Gene* **20**, 51–62.
- VIROLLE, M.-J. & BIBB, M. J. (1988). Cloning, characterization and regulation of an α -amylase gene from *Streptomyces limosus*. *Molecular Microbiology* **2**, 197–208.
- WARD, J. M., JANSEN, G. R., KIESER, T., BIBB, M. J., BUTTNER, M. J. & BIBB, M. J. (1986). Construction and characterization of a series of multicopy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. *Molecular and General Genetics* **203**, 468–478.