

GENE 1537

Cloning and amplified expression in *Streptomyces lividans* of a gene encoding extracellular β -lactamase from *Streptomyces albus* G

(Actinomycetes; recombinant DNA; plasmids; DD-peptidases; branched pathway; ampicillin resistance)

Philippe Dehottay^{}, Jean Dusart^{***}, Colette Duez^a, Mauro V. Lenzini^a, Joseph A. Martial^b, Jean-Marie Frère^a, Jean-Marie Ghuysen^a and Tobias Kieser^c**

^a Service de Microbiologie and ^b Laboratoire Central de Génie génétique, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman, Liège (Belgium) Tel. (41)56.13.96/99, and ^c John Innes Institute, Colney Lane, Norwich NR4 7UH (U.K.) Tel. 0603-52571

(Received July 12th, 1985)

(Revision received October 23rd, 1985)

(Accepted May 12th, 1985)

SUMMARY

A 4.9-kb DNA fragment containing the *bla* gene for the extracellular β -lactamase (BLA) of *Streptomyces albus* G was cloned in *Streptomyces lividans* using the conjugative, low-copy-number plasmid pIJ61 as vector. No expression of *bla* was observed when this DNA fragment was introduced into *Escherichia coli* HB101 on a plasmid vector. A 1.5-kb *Pst*I-*Sst*I fragment containing the *bla* gene was cloned in *S. lividans* on the non-conjugative, high-copy-number plasmid pIJ702. A tenfold higher yield of BLA was obtained from *S. lividans* carrying this plasmid than from *S. albus* G grown under optimal production conditions. The BLA from the clone reacts with β -iodopenicillanate according to a branched pathway which is characteristic of the original *S. albus* G BLA enzyme.

INTRODUCTION

Streptomyces spp. secrete during growth DD-peptidases of varying sensitivity to β -lactam compounds (Leyh-Bouille et al., 1971; 1972) and β -lactamases (BLA) of varying specificity profile (Johnson et al., 1973; Duez et al., 1981; Ogawara et al., 1981).

These enzymes, which recognize the β -lactam fused ring system, are valuable models to study the mode of action of β -lactam antibiotics at the molecular level (Ghuysen et al., 1984). In particular the Zn²⁺ DD-peptidase of *S. albus* G (of known sequence; Joris et al., 1983) and the serine DD-peptidase of *Streptomyces* R61 have been crystallized and the

* This paper is part of a dissertation of P.D. presented as partial fulfilment for a Ph.D. thesis, at the University of Liège.

** To whom correspondence and reprint requests should be addressed.

Abbreviations: Ap, ampicillin; BLA, β -lactamase; *bla*, gene coding for BLA; bp, base pair(s); branched pathway, see

RESULTS AND DISCUSSION, section c and Frère et al. (1982); DD-peptidase, D-alanyl-D-alanine-cleaving carboxypeptidase; kb, 1000 bp; MM, YEME, see MATERIALS AND METHODS, section b; Nm, neomycin; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^R, resistant; R2YE, protoplast regeneration medium; ^S, sensitive; SDS, sodium dodecyl sulfate; Tc, tetracycline; Thio, thiostrepton.

unravelling of their three-dimensional structure by X-ray diffraction at high resolution is in progress (Dideberg et al., 1982; Kelly et al., 1985). A practical limitation of these studies, however, is the low level at which these extracellular enzymes are produced. Thus, for example, the yields of purified protein per litre of culture filtrate are 67 μg of *S. R61* DD-peptidase (Fossati et al., 1978) and 3 μg of *S. albus* G BLA (Duez et al., 1981), starting from 15 000 and 500 litres of culture, respectively. This paper describes the cloning and amplified expression of the *S. albus* G bla gene in a derivative of *S. lividans* via the high-copy-number plasmid pIJ702.

MATERIALS AND METHODS

(a) Bacterial strains and plasmids

E. coli HB101 was used as host. *S. albus* G and *S. R61* were from the Microbiology Department of the University of Liège and *Streptomyces coelicolor* A3(2) and *S. lividans* TK24 (*str-6*) and TK23 (*spe-1*), both cured of the natural plasmids SLP2 and SLP3 (Hopwood et al., 1983), were from the John Innes Institute collection. *Streptomyces cacaoi* KCC-SO352 was a gift from Dr. A. Seino, Kaken Chemical Company Ltd., Tokyo (Japan).

Streptomyces plasmid vectors pIJ702 and pIJ61 were described by Katz et al. (1983) and Thompson et al. (1982), respectively.

(b) Growth conditions and media

Growth of *Streptomyces* cultures at 28°C was in Oxoid tryptone soya broth or yeast extract-malt extract (YEME) medium (Chater et al., 1982) with vigorous orbital shaking. The following liquid media were also used for special purposes: (i) P medium (Okanishi et al., 1974) supplemented with 0.5% yeast extract; (ii) Difco brain-heart broth; (iii) Oxoid Mueller-Hinton broth; (iv) Merck peptone medium [as described by Leyh-Bouille et al. (1971), except that Merck peptone No. 7213 was used]; (v) Lennox (1955) broth, and (vi) E9 medium (containing per litre, NaNO₃ 4 g; K₂HPO₄ 2 g; MgSO₄ 0.5 g; KCl 0.3 g; CaCO₃ 1 g; glycerol 10 g; yeast extract 10 g; pancreatic peptone 10 g). Agar R2YE medium and agar minimal medium (MM) (Chater et al., 1982;

Hopwood, 1967) were also used. *E. coli* HB101 was grown at 37°C in Luria-Bertani or M9 media (Maniatis et al., 1982) with vigorous shaking.

(c) Enzymes

Restriction endonucleases were from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.), New England Biolabs (Beverly, MA, U.S.A.), Boehringer (Mannheim, F.R.G.) or The Radiochemical Centre (Amersham, U.K.). PolIk, calf intestinal alkaline phosphatase and T4 DNA ligase were from Boehringer (Mannheim, F.R.G.). Bacterial alkaline phosphatase was from The Radiochemical Centre (Amersham, U.K.) and lysozyme was from Sigma (St. Louis, MO, U.S.A.).

(d) β -Lactam compounds and other antibiotics

The chromogenic β -lactam nitrocefin was from BBL Microbiology Systems (Cockeysville, MD, U.S.A.). β -Iodopenicillanate was a gift from Dr. J. Kemp (Pfizer Central Research, Sandwich, Kent, U.K.). Ap was from Bristol Benelux (Brussels, Belgium); Nm, spectinomycin, streptomycin and Tc were from Sigma (St. Louis, MO, U.S.A.). Thio was a gift from Dr. R.B. Sykes (E.R. Squibb and Sons, New Brunswick, NJ, U.S.A.).

(e) BLA activity

Nitrocefin was used as substrate. (i) The BLA activity of *Streptomyces* liquid culture supernatants or in cell-free extracts of washed and sonicated mycelium was estimated by the method of O'Callaghan et al. (1972). (ii) BLA-producing *Streptomyces* colonies grown on R2YE agar plates and overlaid with 200 μM nitrocefin in 50 mM sodium phosphate buffer pH 7.0 developed a red halo within a few minutes at room temperature. (iii) When applied to BLA-producing *E. coli* colonies grown on nitrocellulose membranes, the same procedure resulted in the development of a pink coloration of the colonies themselves.

(f) Recombinant DNA techniques

Preparation of *Streptomyces* chromosomal and plasmid DNA and transformation of *Streptomyces*

protoplasts were according to Chater et al. (1982). Digestion with the restriction endonucleases or alkaline phosphatases, treatment with PolIk and ligation experiments were carried out using standard procedures (Maniatis et al., 1982). The digested products were separated by agarose gel electrophoresis (Maniatis et al., 1982) and DNA fragments were eluted from the gels as described in Dretzen et al. (1981). Plasmid DNA was radioactively ^{32}P -labelled by nick translation (Maniatis et al., 1975). Hybridization was performed on digested DNAs after transfer to nitrocellulose filters (Southern, 1975).

RESULTS AND DISCUSSION

(a) Shotgun cloning of the *S. albus* G *bla* gene

Attempts to clone the *S. albus* G *bla* gene using the *Bgl*II, *Sph*I or *Sst*I sites of the high-copy-number *Streptomyces* plasmid pIJ702 did not succeed. However, six BLA^+ colonies were found among 5000 Nm^S Thio R *S. lividans* TK24 transformants obtained in a *Pst*I-mediated cloning experiment with pIJ61 as vector. Five of the clones were lost after the first subculture, but one survived.

(b) Characterization of plasmid pDML1

After retransformation of *S. lividans* TK24 with plasmid DNA of the single remaining Bla^+ clone or conjugal transfer of the plasmid to *S. lividans* TK23, 60% or 30%, respectively, of the resulting Thio R colonies were Bla^+ . This demonstrated that the BLA activity was plasmid-dependent but the clone was either mixed or unstable. The relevant plasmid was designated pDML1 and the initial strain bearing it was called *S. lividans* PD1. When PD1 was propagated on Thio-containing agar plates, the resulting spore population contained only 10–70% Bla^+ colonies. When grown in flasks (50 ml cultures) containing the same Thio-supplemented YEME medium, 4 out of 5 culture supernatants were Bla^- . Plasmids isolated from the non-producing cultures lacked *Pst*I sites and were smaller than pIJ61. The level of enzyme production in the single Bla^+ culture was about four times that obtained with *S. albus* G grown under identical conditions.

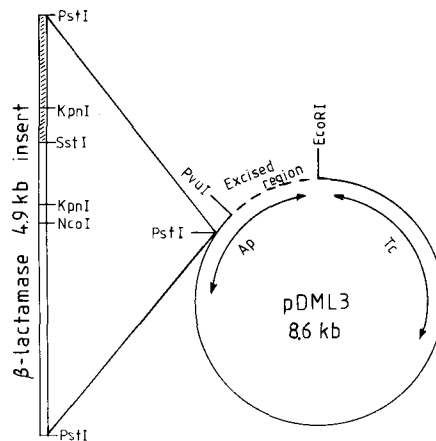


Fig. 1. Construction of plasmid pDML3. pDML3 is a derivative of pBR322. First the pBR322 *bla* gene (Ap^R) was inactivated by deleting the *Pvu*I + *Eco*RI fragment (pBR322 was restricted by *Pvu*I + *Eco*RI and the large fragment was recovered by elution from agarose gel electrophoresis, filtered through a small Sephadex G-50 column in 20 mM Tris buffer pH 9 containing 1 mM EDTA and 100 mM NaCl, precipitated with ethanol and redissolved. The extremities were transformed into blunt ends by treatment with PolIk and religated). The resulting plasmid, pDML2, was then digested with *Pst*I, treated with bacterial alkaline phosphatase and ligated to the 4.9-kb *Pst*I fragment from pDML1. Tc^R transformants of *E. coli* strain HB101 were selected and screened for the presence of plasmids containing a 4.9-kb insert. The insert has no sites for *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hind*III, *Hpa*I, *Nde*I, *Sph*I and *Xba*I, and unmapped sites for *Apa*I, *Bst*EII and *Sma*I. The *S. albus* G *bla* gene was located in the hatched segment of the insert.

For unknown reasons, pDML1 gained stability on conjugal transfer to *S. lividans* TK23 (10% loss only after one round of sporulation). After extraction from TK23, pDML1 was found to contain a 4.9-kb *Pst*I fragment which presumably contained the *S. albus* G *bla* gene. This fragment was inserted into the *Pst*I site of pDML2, a derivative of pBR322 lacking the *Pvu*I-*Eco*RI sequence (Fig. 1, legend). The resulting plasmid, pDML3, shown in Fig. 1, gave no detectable (nitrocefin test) BLA activity in *E. coli* HB101, consistent with the general lack of expression of *Streptomyces* genes subcloned into *E. coli* vectors (Bibb and Cohen, 1982; Horinouchi et al., 1980; Schupp et al., 1983; Gil and Hopwood, 1983).

pDML3 was ^{32}P -labelled by nick translation and used as probe in hybridization experiments involving *Pst*I digests of pDML1 and chromosomal DNAs from the Bla^+ strains *S. albus* G, *S. cacaoi* and *S. coelicolor* A3(2) and the non- or very low-pro-

ducing strains *S. lividans* TK24 and *S. R61*. Hybridization occurred only with the 4.9-kb insert of pDML1 and a fragment of the restricted *S. albus* G chromosomal DNA. The hybridizing *S. albus* G *Pst*I fragment (11 kb) was larger than the *Pst*I fragment cloned in pDML1 and pDML3 suggesting that the cloned fragment contained a deletion. The occurrence of this deletion, the failure to clone *bla*⁺ in the high-copy-number vector and the loss of five out of six primary clones in pIJ61 all suggest the occurrence of a sequence close to gene *bla* that interferes with growth of the clones in *S. lividans*.

The 4.9-kb fragment of pDML3 and the 3.4-kb and 1.5-kb subfragments derived from it by *Sst*I digestion were separately ligated to appropriately cleaved pIJ702 and used to transform *S. lividans* TK24. BLA production was found in Thio^R colonies containing the 4.9-kb fragment (pDML4) or the 1.5-kb subfragment (pDML6). pDML6 was 100% stable in *S. lividans* TK24 and as shown below, the resulting strain, PD6, was an excellent producer of extracellular BLA.

(c) Properties of the extracellular BLA of *S. lividans* PD6

S. lividans PD6 and *S. albus* G were grown at 28°C in various liquid media and the BLA activity in the culture filtrates was estimated as a function of time (Table I; Fig. 2). Comparable, low levels of enzyme excretion by the two strains was observed in YEME medium, with 22% of the PD6 activity being intracellular. In contrast, in both brain-heart and

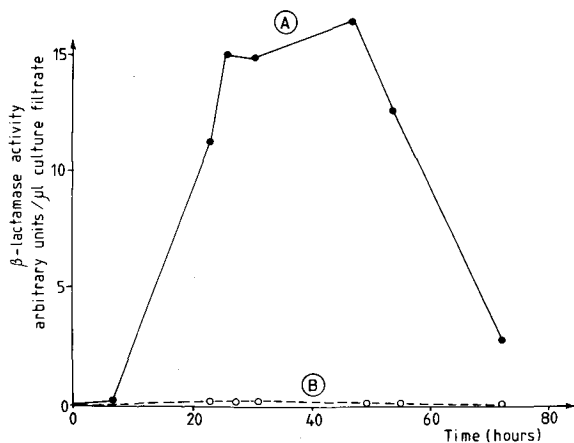


Fig. 2. Time course of BLA excretion by *S. lividans* PD6 (A) and *S. albus* G (B) grown in Lennox medium at 28°C. For definition of unit, see Table I, footnote a.

TABLE I

Maximal level of BLA in the medium supernatant of cultures of *S. lividans* PD6 and *S. albus* G grown at 28°C in various liquid media

Media ^b	BLA arbitrary units ^a produced by:		
	<i>S. lividans</i> PD6		<i>S. albus</i> G
	Extracellular	Intracellular	Extracellular
YEME	2	0.7	1.5
P + 0.5% YE	1.4	n.t.	n.t.
Brain-heart	13.4	n.t.	0.3
Mueller-Hinton	2.3	n.t.	n.t.
Lennox ^c	16.5	0.15	0.2
Peptone 7213	1.2	n.t.	n.t.
E9	2	n.t.	n.t.

^a One arbitrary unit was defined as the quantity of enzyme (BLA) which resulted in an increase of 1 absorbance unit at 482 nm after 1 h of incubation at 30°C in 500 μl of 50 mM sodium phosphate buffer pH 7.0 containing 100 μM nitrocefin (O'Callaghan et al., 1972). This unit is equivalent to 1/900 international unit (μmol of hydrolysed nitrocefin × min⁻¹). n.t., not tested.

^b See MATERIALS AND METHODS, section b.

^c See Fig. 2 for time course.

Lennox media, *S. lividans* PD6 produced 45- to 80-fold more extracellular BLA than *S. albus* G in the same medium, with about 1% being intracellular. The BLA of *S. lividans* PD6, grown in Lennox broth, was purified to protein homogeneity using essentially the procedure described previously for the *S. albus* G BLA (Duez et al., 1981). The behaviour of the enzyme at each step of the purification (which included various chromatographies and filtrations on DEAE cellulose, Sephadex G-100, DEAE Sephadex and DEAE Sephacel) and the *M_r* of the purified enzyme (30 000 as revealed by SDS-polyacrylamide gel electrophoresis) were those expected from the known properties of the original *S. albus* G BLA. The yield was 1.35 mg of pure enzyme per litre of culture filtrate, starting from a volume of 40 litres. This yield per litre, 450 times higher than that obtained by Duez et al. (1981), is attributable not only to overproduction by the strain carrying the cloned gene but also to the choice of the most suitable medium (Lennox broth) and a better percentage of recovery [11% instead of 3% in Duez et al. (1981)].

TABLE II

Inactivation of the BLA of *S. lividans* PD6 and *S. albus* G by increasing concentrations of β -iodopenicillanate^a

[β -lactam] (μ M)	[β -lactam]/ [enzyme] (molar ratio)	Enzyme (BLA) activity (%)	
		<i>S. lividans</i> PD6	<i>S. albus</i> G
0	0	(100)	(100)
10	133	74	71
20	266	35	26
40	532	10	6
80	1064	0	0
160	2130	0	0

^a The extracellular BLA produced by *S. lividans* PD6 was compared to the *S. albus* G enzyme by incubating each enzyme (75 nM) in 50 μ l (final volume) of 50 mM sodium phosphate buffer pH 7.0 containing β -iodopenicillanate (specified as β -lactam in Table II) at concentrations ranging from 0–160 μ M (so that the molar ratios of β -lactam to enzyme varied from 0–2130). After 10 min of incubation at 22°C, the reaction mixtures were diluted ten-fold with the buffer and the BLA activity was determined on 10- μ l samples.

Previous studies (Frère et al., 1982) had shown that in the reaction of the *S. albus* G BLA with β -iodopenicillanate turnover of this β -lactam compound and inactivation of the enzyme occurred concomitantly. This behaviour has been explained on the basis of a branched pathway, in which the acylenzyme intermediate can either rearrange into irreversibly inactivated enzyme or regenerate free enzyme through the usual hydrolytic reaction. The number of hydrolytic turnovers before enzyme inactivation was about 500 and consequently complete enzyme inactivation required a molar ratio of β -iodopenicillanate to enzyme of at least 500, characteristic of the considered enzyme. As shown in Table II, a molar ratio of β -lactam to enzyme slightly above 500 was necessary to achieve complete inactivation of both enzymes. On incubation of 1 μ M *S. lividans* PD6 BLA with 100 μ M β -iodopenicillanate (in 250 μ l, final volume), the β -lactam was quantitatively hydrolyzed, yielding a compound whose absorption spectrum in the near UV was identical to that of 2,3-dihydro-2,2-dimethyl-1,4-thiazine-3,4-dicarboxylate, a compound which was obtained under the same conditions using the *S. albus* G BLA (Frère et al., 1982).

These results confirm that the *S. albus* G gene for extracellular BLA was contained in the 1.5-kb sub-fragment cloned in pDML6.

ACKNOWLEDGEMENTS

This work was supported by the Fonds de la Recherche Scientifique Médicale, Brussels (Belgium) (contract No. 3.4507.83), the Actions Concertées with the Belgian Government (conventions 79/84-II and 81/86) and a Convention tripartite between the Région wallonne, Continental Pharma and the University of Liège. JD is indebted to the European Molecular Biology Organization for a short-term fellowship to enable him to visit the John Innes Institute. PD and MVL are indebted to the Ministère de l'Emploi et du Travail for a Cadre Spécial Temporaire. JD is Research Associate to the National Fund for Scientific Research (Belgium).

We thank D.A. Hopwood for his encouragement during the work and for his many useful comments on the manuscript, M.R. Rodicio and J.M. Piret for their interest.

REFERENCES

- Bibb, M.J. and Cohen, S.N.: Gene expression in *Streptomyces*: construction and application of promoter-probe plasmid vectors in *Streptomyces lividans*. *Mol. Gen. Genet.* 187 (1982) 265–277.
- Chater, K.F., Hopwood, D.A., Kieser, T. and Thompson, C.J.: Gene cloning in *Streptomyces*. *Curr. Topics Microbiol. Immunol.* 96 (1982) 69–95.
- Dideberg, O., Charlier, P., Dive, G., Joris, B., Frère, J.M. and Ghuysen, J.M.: Structure of a Zn²⁺-containing D-alanyl-D-alanine cleaving carboxypeptidase at 2.5Å resolution. *Nature* 299 (1982) 469–470.
- Dretzen, G., Bellard, M., Sassone-Corsi, P. and Chambon, P.: A reliable method for recovery of DNA fragments from agarose and acrylamide gels. *Anal. Biochem.* 112 (1981) 295–298.
- Duez, C., Frère, J.M., Klein, D., Noël, M., Ghuysen, J.M., Delcambe, L. and Dierickx, L.: The exocellular β -lactamase of *Streptomyces albus* G. Purification, properties and comparison with the exocellular DD-carboxypeptidase. *Biochem. J.* 193 (1981) 75–82.
- Fossati, P., Saint-Ghislain, M., Sicard, P.J., Frère, J.M., Dusart, J., Klein, D. and Ghuysen, J.M.: Large-scale preparation of purified exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* strain R61. *Biotechnol. Bioeng.* 20 (1978) 577–587.
- Frère, J.M., Dormans, C., Duyckaerts, C. and De Graeve, J.: Interaction of β -iodopenicillanate with the β -lactamase of *Streptomyces albus* G and *Actinomadura* R39. *Biochem. J.* 207 (1982) 437–444.
- Ghuysen, J.M., Frère, J.M., Leyh-Bouille, M., Nguyen-Distèche, M., Coyette, J., Dusart, J., Joris, B., Duez, C., Dideberg, O.,

- Charlier, P., Dive, G. and Lamotte-Brasseur, J.: Bacterial wall peptidoglycan, DD-peptidases and β -lactam antibiotics. *Scand. J. Infect. Dis., Suppl.* 42 (1984) 17–37.
- Gil, J.A. and Hopwood, D.A.: Cloning and expression of a *p*-aminobenzoic acid synthetase gene of the candidin-producing *Streptomyces griseus*. *Gene* 25 (1983) 119–132.
- Hopwood, D.A.: Genetic analysis and genome structure in *Streptomyces coelicolor*. *Bacteriol. Rev.* 31 (1967) 373–403.
- Hopwood, D.A.: Kieser, T., Wright, H.M. and Bibb, M.J.: Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. *J. Gen. Microbiol.* 129 (1983b) 2257–2269.
- Horinouchi, S., Uozumi, T. and Beppu, T.: Cloning of *Streptomyces* DNA into *Escherichia coli*: absence of hetero-specific gene expression of *Streptomyces* genes in *E. coli*. *Agric. Biol. Chem.* 44 (1980) 367–381.
- Johnson, K., Dusart, J., Campbell, J.N. and Ghuysen, J.M.: Exocellular β -lactamases of *Streptomyces albus* G, and strains R39 and K11. *Antimicrob. Agents Chemother.* 3 (1973) 289–298.
- Joris, B., Van Beeumen, J., Casagrande, F., Gerday, C., Frère, J.M. and Ghuysen, J.M.: The complete amino acid sequence of the Zn²⁺-containing D-alanyl-D-alanine-cleaving carboxypeptidase of *Streptomyces albus* G. *Eur. J. Biochem.* 130 (1983) 53–69.
- Katz, E., Thompson, C.J. and Hopwood, D.A.: Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *J. Gen. Microbiol.* 129 (1983) 2703–2714.
- Kelly, J.A., Knox, J.R., Moews, P.C., Hite, G.J., Bartolone, J.B., Zhao, H., Joris, B., Frère, J.M. and Ghuysen, J.M.: 2.8 Å structure of penicillin-sensitive D-alanyl-carboxypeptidase-transpeptidase from *Streptomyces* R61 and complexes with β -lactams. *J. Biol. Chem.* 260 (1985) 6449–6458.
- Lennox, E.S.: Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* 1 (1955) 190–206.
- Leyh-Bouille, M., Coyette, J., Ghuysen, J.M., Idczak, J., Perkins, H.R. and Nieto, M.: Penicillin-sensitive DD-carboxypeptidase from *Streptomyces* strain R61. *Biochemistry* 10 (1971) 2163–2170.
- Leyh-Bouille, M., Nakel, M., Frère, J.M., Johnson, K., Ghuysen, J.M., Nieto, M. and Perkins, H.R.: Penicillin-sensitive DD-carboxypeptidase from *Streptomyces* strains R39 and K11. *Biochemistry* 11 (1972) 1290–1298.
- Maniatis, T., Jeffrey, A. and Kleid, D.G.: Nucleotide sequence of the rightward operator of phage λ . *Proc. Natl. Acad. Sci.* 72 (1975) 1184–1188.
- Maniatis, T., Fritsch, E.F. and Sambrook, J.: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
- O'Callaghan, C.H., Morris, A., Kirby, M. and Singer, A.H.: Novel method for detection of β -lactamase by using a chromogenic cephalosporin substrate. *Antimicrob. Agents Chemother.* 1 (1972) 283–288.
- Uogawara, H., Mantoku, A. and Shimada, S.: β -Lactamase from *Streptomyces cacaoi*: purification and properties. *J. Biol. Chem.* 256 (1981) 2649–2655.
- Okanishi, M., Suzuki, K. and Umezawa, H.: Formation and reversion of streptomycete protoplasts: cultural conditions and morphological study. *J. Gen. Microbiol.* 80 (1974) 389–400.
- Robbins, W., Trimble, R.B., Wirth, D.F., Hering, C., Maley, F., Maley, G.F., Das, R., Gibson, B.W., Royal, N. and Biemann, K.: Primary structure of the *Streptomyces* enzyme endo- β -N-acetylglucosaminidase H. *J. Biol. Chem.* 259 (1984) 7577–7583.
- Schupp, T., Toupet, G., Stålhammar-Carlemalm, M. and Meyer, J.: Expression of a neomycin phosphotransferase gene from *Streptomyces fradiae* in *E. coli* after interplasmidic recombination. *Mol. Gen. Genet.* 189 (1983) 27–33.
- Southern, E.M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98 (1975) 503–517.
- Thompson, C.J., Kieser, T., Ward, J.M. and Hopwood, D.A.: Physical analysis of antibiotic-resistance genes and their use in vector construction. *Gene* 20 (1982) 51–62.

Communicated by K.F. Chater.