

Cloning and Amplified Expression in *Streptomyces lividans* of the Gene Encoding the Extracellular β -Lactamase from *Streptomyces cacaoi*

By MAURO V. LENZINI,¹ SHIGEO NOJIMA,² JEAN DUSART,^{1*} HIROSHI OGAWARA,² PHILIPPE DEHOTTAY,¹ JEAN-MARIE FRERE¹ AND JEAN-MARIE GHUYSEN¹

¹Service de Microbiologie, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman, Liège, Belgium

²Department of Biochemistry, Meiji College of Pharmacy, Nozawa-1, Setagaya-ku, Tokyo 154, Japan

(Received 15 April 1987; revised 10 June 1987)

A 19 kb *SphI* DNA fragment containing the gene for the extracellular active-site serine β -lactamase of *Streptomyces cacaoi* KCC-SO352 was cloned in *Streptomyces lividans* TK24 using the high-copy-number plasmid pIJ702 as vector. A 30-fold higher yield of β -lactamase was obtained from *S. lividans* strain ML1, carrying the recombinant plasmid pDML51, than from *S. cacaoi* grown under optimal production conditions. In all respects (molecular mass, isoelectric point, kinetics of inhibition by β -iodopenicillanate) the overproduced *S. lividans* ML1 β -lactamase was identical to the original *S. cacaoi* enzyme. A considerable reduction of β -lactamase production was caused by elimination of a 12.8 kb portion of the 19 kb DNA fragment by cleavage at an internal *SphI* site located more than 3 kb upstream of the β -lactamase structural gene. The β -lactamase gene was located within a 1.8 *NcoI*–*BclI* fragment but when this fragment was cloned in *S. lividans* pIJ702, the resulting strain produced hardly any more β -lactamase than the original *S. cacaoi*.

INTRODUCTION

Active-site serine β -lactamases (EC 3.5.2.6) and active-site serine DD-peptidases (EC number not attributed) react with β -lactam antibiotics by an acyl enzyme mechanism that involves formation of a serine ester-linked penicilloyl (cephalosporoyl, etc.) enzyme derivative. In most cases, the acyl enzyme formed with the β -lactamases is very short-lived so that the reaction flux rapidly proceeds to product. With the DD-peptidases, the acyl enzyme is very long-lived so that the DD-peptidases become immobilized in an inactive form for a long time; they therefore behave as penicillin-binding proteins (Ghuysen *et al.*, 1984). Questions about the structures and functions of these two classes of β -lactam antibiotic-recognizing enzymes are being investigated by using a battery of techniques such as X-ray crystallography, kinetic and spectroscopic studies, amino acid sequencing, gene analysis and site-directed mutagenesis. The extracellular active-site serine β -lactamases produced by *Bacillus licheniformis*, *Bacillus cereus* and *Streptomyces albus* G, and the extracellular active-site serine DD-peptidase of *Streptomyces* R61, are unrelated in their primary structures but, nevertheless, show striking similarities in the spatial disposition of their secondary structure elements (α -helices and β -strands), suggesting a close evolutionary relationship (Kelly *et al.*, 1986; Samraoui *et al.*, 1986; O. Dideberg and co-workers, unpublished results). The *Streptomyces* R61 DD-peptidase gene was cloned and amplified in *Streptomyces lividans* on plasmid pIJ702 (Duez *et al.*, 1987). In parallel to this, the same host-vector system was used to study *Streptomyces* β -lactamase genes. Following previous reports on the β -lactamase gene of *Streptomyces albus* G (Dehottay *et al.*, 1986, 1987) this paper

describes the cloning of the gene that codes for the β -lactamase of *Streptomyces cacaoi*. The biochemical and kinetic properties of this β -lactamase were described by Ogawara (1975), Ogawara *et al.* (1981) and Lenzini & Frère (1985).

METHODS

Bacterial strains and plasmids. *Streptomyces lividans* TK24 (*str*-6) (Hopwood *et al.*, 1983), *Streptomyces coelicolor* A3(2) and *Streptomyces* plasmid vector pIJ702 (Katz *et al.*, 1983) were gifts from D. A. Hopwood and T. Kieser of the John Innes Institute, Norwich, UK. *Streptomyces cacaoi* KCC-SO352 was obtained from A. Seino, Kaken Chemical Co. Ltd, Tokyo, Japan. *Streptomyces albus* G, *Streptomyces* R61 and *Actinomadura* R39 were from the Microbiology Department of the University of Liège. *Escherichia coli* HB101 (Boyer & Roulland-Dussoix, 1969) and plasmid vector pBR322 (Bolivar *et al.*, 1977) were also used.

Media and growth conditions. Growth of *Streptomyces* cultures was at 28 °C in YEME medium (Hopwood *et al.*, 1985) with vigorous orbital shaking (New Brunswick G25 shaker, 300 r.p.m.). The following liquid media were also used: (i) P medium (Okaniishi *et al.*, 1974) supplemented with 0.5% yeast extract; (ii) Oxoid Mueller-Hinton broth; (iii) Lennox broth (Lennox, 1955); (iv) Merck peptone broth; and (v) E9 broth (Dehottay *et al.*, 1986). R2YE agar (Hopwood *et al.*, 1985) was used for maintenance and plating of *Streptomyces* strains. *E. coli* was grown at 37 °C in Luria-Bertani or M9 medium (Maniatis *et al.*, 1982) with vigorous orbital shaking (see above).

Recombinant DNA techniques. Preparation of *Streptomyces* chromosomal and plasmid DNA and transformation of *Streptomyces* protoplasts were done as described by Hopwood *et al.* (1985). Treatment with restriction endonucleases or Klenow fragment, separation of the digested products by agarose gel electrophoresis, treatment with alkaline phosphatase, and ligation experiments were performed as described by Maniatis *et al.* (1982). Elution of the DNA fragments from the gels (Dretzen *et al.*, 1981). ³²P-labelling by nick translation (Maniatis *et al.*, 1975), hybridization experiments (Southern, 1975), addition of *Bgl*II linkers (Maniatis *et al.*, 1982), and isoelectric focusing (Righetti & Drysdale, 1971) were done as described in the relevant references.

Enzymes and antibiotics. The enzymes used in recombinant DNA techniques were from Bethesda Research Laboratories, Boehringer Mannheim, Amersham or Sigma. The antibiotics were from Sigma except for nitrocefin (BBL Microbiology Systems), thiostrepton (a gift from S. J. Lucania, Squibb & Sons, New Brunswick, NJ, USA) and β -iodopenicillanate (a gift from J. Kemp, Pfizer Central Research, Sandwich, UK).

β -Lactamase activity. Nitrocefin was used as substrate. The β -lactamase activity of *Streptomyces* liquid culture supernatants was estimated (O'Callaghan *et al.*, 1972) by incubating samples for 1 h at 30 °C with 100 μ M-nitrocefin (500 μ l final volume) in 50 mM-sodium phosphate pH 7.0. An increase of one absorbance unit at 488 nm corresponded to 4.5 ng enzyme (Lenzini & Frère, 1985; and unpublished results). β -Lactamase-producing *Streptomyces* colonies grown on R2YE agar plates were detected on the basis of the red halo that they developed on exposure to nitrocefin (Dehottay *et al.*, 1986).

RESULTS AND DISCUSSION

Cloning of the β -lactamase gene from S. cacaoi

Fig. 1 shows the restriction maps of the *S. cacaoi* chromosomal DNA fragments used in the experiments described below, and the various derivatives of pIJ702 in which these fragments were inserted. pIJ702 carries a thiostrepton-resistance marker (*tsr*) and a tyrosinase-encoding gene (*mel*) which offers three unique restriction sites for insertional inactivation.

Chromosomal DNA from *S. cacaoi* was fully digested with *Bgl*II, *Sph*I or *Sst*I and the unfractionated digests were used in shotgun cloning experiments. Shotgun cloning using the *Sph*I site of the high-copy-number *Streptomyces* plasmid pIJ702 led to the isolation of one β -lactamase-producing colony, among 3000 melanin-negative, thiostrepton-resistant *S. lividans* TK24 transformants. The resulting plasmid contained a 19 kb insert; it was called pDML51 and the strain bearing it was called *S. lividans* ML1. *Sph*I digestion of pDML51 not only regenerated the 5.8 kb pIJ702 and the 19 kb insert but also produced two other fragments of 6.6 and 12.4 kb. Time-course experiments showed that these fragments originated by cleavage of an additional *Sph*I site of relatively low susceptibility in the 19 kb insert. Each fragment was recloned into pIJ702 using the *Sph*I sites as above. β -Lactamase activity was associated only with the 6.6 kb fragment. The plasmid with this 6.6 kb insert was called pDML52 and the strain bearing it was called *S. lividans* ML2.

The 6.6 kb fragment containing the β -lactamase gene was inserted into the *Sph*I site of pBR322 and the recombinant plasmid pDML72 was isolated from ampicillin-resistant,

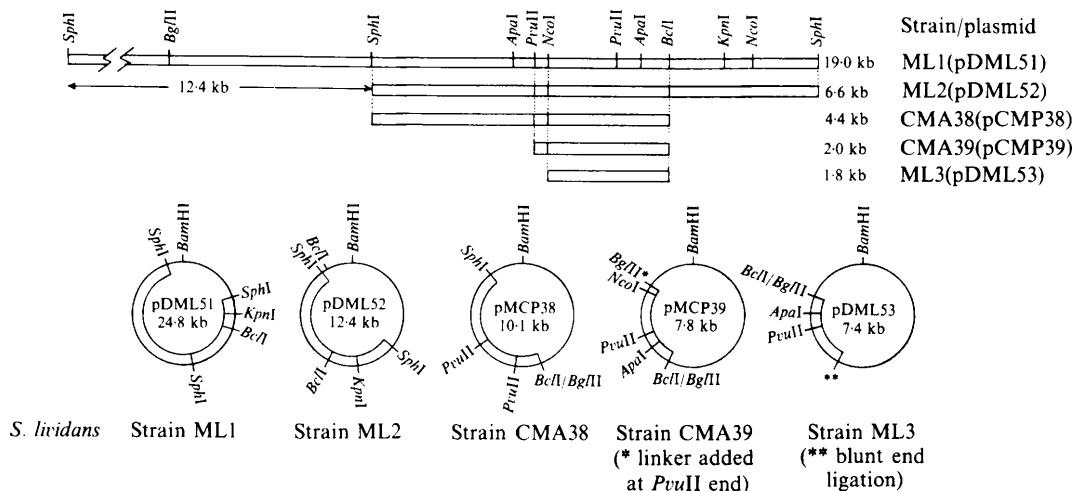


Fig. 1. Restriction maps and cloning in pIJ702 of chromosomal DNA fragments carrying the *S. cacaoi* β -lactamase gene. (Plasmids are not drawn to scale.) The restriction map was derived from pMCP38 and pBR322-derived pDML72 (not shown; see text).

tetracycline-sensitive *E. coli* HB101 transformants. pDML72 was ^{32}P -labelled by nick-translation and used as a probe in hybridization experiments against *Sph*I digests of chromosomal DNAs from the β -lactamase-producing *Actinomadura* R39, *S. coelicolor*, *S. albus* G and *S. cacaoi* and the β -lactamase-non-producing or very poorly producing *Streptomyces* R61 and *S. lividans* TK24. Hybridization occurred only with the *S. cacaoi* 6.6 kb fragment and the pDML51 and pDML52 digests (Fig. 2). With the *S. cacaoi* DNA *Sph*I digest used for the shotgun cloning, two hybridization bands (6.6 and 19 kb) were observed, indicating incomplete digestion (not shown).

pDML52 was also used to prepare smaller DNA fragments containing the β -lactamase gene (Fig. 1). Three constructions were made. (i) pDML52 was cut with *Bcl*I and *Sph*I and the 4.4 kb fragment thus produced was isolated and ligated to pIJ702 restricted with *Bgl*II and *Sph*I to produce pMCP38 (in *S. lividans* strain CMA38). (ii) pDML52 was cut with *Bcl*I and the 4.7 kb fragment thus produced was in turn partially digested with *Pvu*II with generation of a 2 kb fragment. After addition of *Bgl*II linkers, this fragment was ligated to pIJ702 cut with *Bgl*II and treated with bacterial alkaline phosphatase to produce pMCP39 (in *S. lividans* strain CMA39). (iii) Cleavage of pDML52 with *Bcl*I and *Nco*I generated a 1.8 kb fragment and cleavage of pIJ702 with *Bgl*II and *Sst*I generated a 5.5 kb fragment. These fragments were ligated to give rise to a 7.3 kb *Nco*I-*Sst*I linear fragment. After treatment of the *Nco*I and *Sst*I ends by the Klenow fragment of *E. coli* DNA polymerase I, blunt-end ligation yielded the 7.3 kb plasmid pDML53 (in *S. lividans* strain ML3).

Strains CMA38, CMA39 and ML3 gave, to varying extents, a positive nitrocefin test on R2YE agar. However, as shown below, the abilities of these strains to produce extracellular β -lactamase in liquid media were very different.

Expression and properties of the extracellular β -lactamase of S. lividans ML1

S. lividans ML1 was grown in various liquid media and the β -lactamase activity of the culture filtrates was estimated. The following data are mean values of two independent experiments, the observed variations being less than 10%. Maximal production, 38 mg enzyme l^{-1} , occurred in E9 broth after 3–4 d growth at 28 °C. The yields were 16–17 mg l^{-1} in P medium and Lennox broth, 12 mg l^{-1} in YEME, 9 mg l^{-1} in Merck peptone broth and 6 mg l^{-1} in Oxoid Mueller–Hinton broth. With *S. cacaoi* KCC-SO352, production was also maximal in E9 broth (1.4 mg l^{-1}).

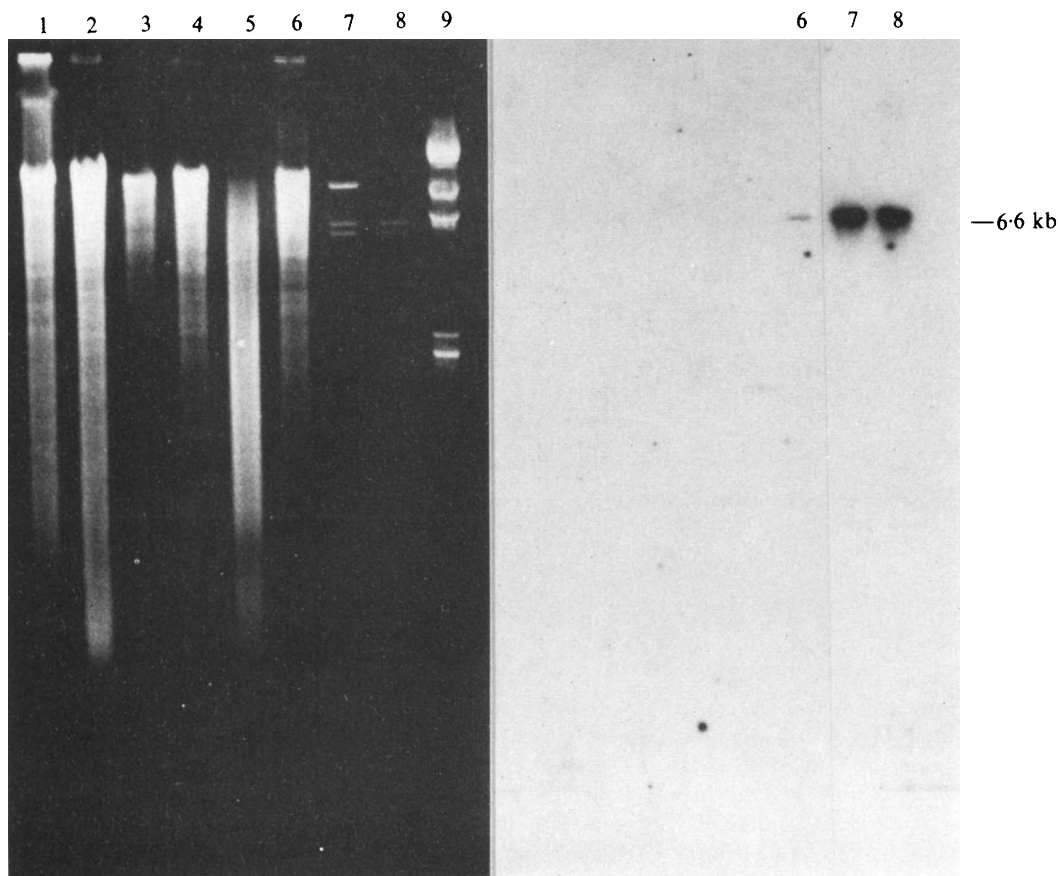


Fig. 2. Hybridization experiments using ^{32}P -labelled pDML72 as probe against: (i) *Sph*I-digested DNAs from *Actinomadura* R39 (lane 1), *S. albus* G (lane 2), *S. lividans* TK24 (lane 3), *S. coelicolor* (lane 4), *Streptomyces* R61 (lane 5) and *S. cacaoi* (lane 6); and (ii) *Sph*I-digested pDML51 (lane 7) and pDML52 (lane 8). Lane 9: *Hind*III-digested λ DNA.

Hence, *S. lividans* ML1 was a better β -lactamase producer than *S. cacaoi*, with a 30-fold increase in enzyme excretion observed under optimal conditions (though an effect of the medium on enzyme excretion could not be excluded; Dehottay *et al.*, 1986).

The extracellular β -lactamase of *S. lividans* ML1 grown in E9 medium was purified to protein homogeneity (specific activity $575 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$) using the procedure described for the *S. cacaoi* enzyme. Not only the behaviour of the *S. lividans* ML1 β -lactamase at each step of the purification but the molecular mass (34 kDa as revealed by SDS-polyacrylamide gel electrophoresis), isoelectric point (4.95), and kinetics of inhibition by β -iodopenicillanate of the purified enzyme were exactly those expected from the known properties of the *S. cacaoi* β -lactamase. Moreover, the two enzymes co-eluted by MonoQ FPLC chromatography.

Sub-cloning experiments (see Fig. 1) showed that the β -lactamase gene was associated with a 1.8 kb *Nco*I-*Bcl*I fragment (with the coding region starting close to the *Nco*I site; unpublished data). However, all these sub-clones resulted in *S. lividans* strains that had a much decreased capacity to produce the extracellular β -lactamase (Table 1, Fig. 3). In particular, cleavage of the original 19 kb DNA fragment at the internal *Sph*I site about 3 kb upstream of the β -lactamase structural gene (strain ML2) had a drastic effect, suggesting that the eliminated 12.4 kb segment had important information for β -lactamase gene expression (and/or enzyme secretion). It should be noted that the observed decreased yields in excreted enzyme by the sub-clones did not relate

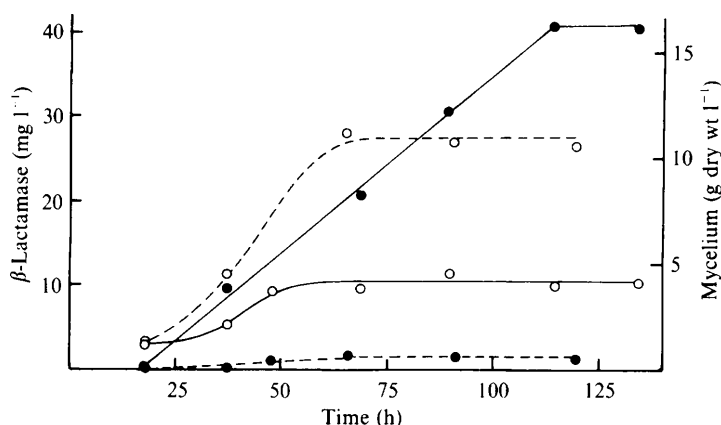


Fig. 3. Growth (○) and β -lactamase excretion (●) of *S. lividans* ML1 (—) and *S. cacaoi* (---) grown in medium E9 at 28 °C.

Table 1. Maximal levels of β -lactamase excretion in medium E9 by *S. lividans* carrying various recombinant plasmids and by *S. cacaoi* KCC-SO352

For strains and plasmids, see Fig. 1. All the strains – except *S. lividans* TK24 and *S. cacaoi* KCC-SO352 – were grown in medium E9 supplemented with 5 μ g thiostrepton ml⁻¹. The yields are those obtained during time-course experiments (Fig. 3). The results represent the mean of two independent experiments; individual variations were less than 10%.

Strain/plasmid	Size of <i>S. cacaoi</i> DNA insert (kb)	Mycelium (g dry wt l ⁻¹)	Extracellular β -lactamase (mg l ⁻¹)
<i>S. lividans</i> ML1/pDML51	19	4.6	41
ML2/pDML52	6.6	4.5	8.4
CMA38/pMCP38	4.4	6.6	4.8
CMA39/pMCP39	2.0	5.2	1.8
ML3/pDML53	1.8	4.6	0.14
TK24/—	—	4.4	ND
<i>S. cacaoi</i> KCC-SO352/—	—	11.2	1.6

ND, Not detectable.

to variation in the amounts of mycelium produced (Table 1) nor to instability and/or integrity of the plasmids (data not shown). Selection for the plasmids by thiostrepton was applied in all cases (except with *S. lividans* TK24 and *S. cacaoi* KCC-SO352) and the plasmids were found to have conserved their insert (at least after 3 d growth). However, the possible effects of plasmid copy number and growth rates (as opposed to growth yield) in strains containing the subclones have not been assessed; these factors cannot be entirely discounted, since they could potentially influence β -lactamase production.

We are grateful to Professor D. A. Hopwood for his helpful advice during the preparation of the manuscript. The work was supported by the Fonds de Recherche de la Faculté de Médecine, the Fonds de la Recherche Scientifique Médicale, Brussels (contract no. 3.4507.83), the Belgian Government (action concertée no. 86/91-90) and the Région wallonne (C2/C16/CONV.246/20428). J.D. is Chercheur qualifié of the Fonds National de la Recherche Scientifique (FNRS). M.V.L. and P.D. are indebted to the Ministère de l'Emploi et du Travail for a Cadre spécial temporaire.

This paper is part of a dissertation of M. V. L. presented as partial fulfilment for a PhD thesis at the University of Liège.

REFERENCES

- BOLIVAR, F., RODRIGUEZ, R. L., GREENE, P. J., BETLACH, M. C., HEYNEKER, H. L., BOYER, H. W., CROSA, J. H. & FALKOW, S. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95-113.
- BOYER, H. W. & ROULLAND-DUSSOIX, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *Journal of Molecular Biology* **41**, 459-472.
- DEHOTAY, P., DUSART, J., DUEZ, C., LENZINI, M. V., MARTIAL, J. A., FRERE, J. M., GHUYSEN, J. M. & KIESER, T. (1986). Cloning and amplified expression in *Streptomyces lividans* of a gene encoding extracellular β -lactamase from *Streptomyces albus* G. *Gene* **42**, 31-36.
- DEHOTAY, P., DUSART, J., DE MEESTER, F., JORIS, B., VAN BEEUMEN, J., ERPICUM, T., FRERE, J. M. & GHUYSEN, J. M. (1987). Nucleotide sequence of the gene encoding the *Streptomyces albus* G β -lactamase precursor. *European Journal of Biochemistry* **166**, 345-350.
- DRETZEN, G., BELLARD, M., SASSONE-CORSI, P. & CHAMON, P. (1981). A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. *Analytical Biochemistry* **112**, 295-298.
- DUEZ, C., PIRON-FRAIPONT, C., JORIS, B., DUSART, J., URDEA, M. S., MARTIAL, J. A., FRERE, J. M. & GHUYSEN, J. M. (1987). Primary structure of the *Streptomyces* R61 extracellular DD-peptidase. 1. Cloning into *Streptomyces lividans* and nucleotide sequence of the gene. *European Journal of Biochemistry* **162**, 509-518.
- GHUYSEN, J. M., FRERE, J. M., LEYH-BOUILLE, M., NGUYEN-DISTECHE, M., COYETTE, J., DUSART, J., JORIS, B., DUEZ, C., DIDEBERG, O., CHARLIER, P., DIVE, G. & LAMOTTE-BRASSEUR, J. (1984). Bacterial wall peptidoglycan, DD-peptidases and β -lactam antibiotics. *Scandinavian Journal of Infectious Diseases*, suppl. **42**, 17-37.
- HOPWOOD, D. A., KIESER, T., WRIGHT, H. M. & BIBB, M. J. (1983). Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. *Journal of General Microbiology* **129**, 2257-2269.
- 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.
- KATZ, E., THOMPSON, C. J. & HOPWOOD, D. A. (1983). Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *Journal of General Microbiology* **129**, 2703-2714.
- KELLY, J. A., DIDEBERG, O., CHARLIER, P., WERY, J. P., LIBERT, M., MOEWS, P. C., KNOX, J. R., DUEZ, C., FRAIPONT, C., JORIS, B., DUSART, J., FRERE, J. M. & GHUYSEN, J. M. (1986). On the origin of bacterial resistance to penicillin: comparison of a β -lactamase and a penicillin target. *Science* **231**, 1429-1431.
- LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190-206.
- LENZINI, M. V. & FRERE, J. M. (1985). The β -lactamase of *Streptomyces cacaoi*: interaction with cefoxitin and β -iodopenicillanate. *Journal of Enzyme Inhibition* **1**, 25-34.
- MANIATIS, J., JEFFREY, A. & KLEID, D. G. (1975). Nucleotide sequence of the rightward operator of phage λ . *Proceedings of the National Academy of Sciences of the United States of America* **72**, 1184-1188.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- O'CALLAGHAN, C. H., MORRIS, A., KIRBY, S. M. & SHINGLER, A. H. (1972). Novel method for detection of β -lactamases by using a chromogenic cephalosporin substrate. *Antimicrobial Agents and Chemotherapy* **1**, 283-288.
- OGAWARA, H. (1975). Production and properties of β -lactamases in *Streptomyces*. *Antimicrobial Agents and Chemotherapy* **8**, 402-408.
- OGAWARA, H., MANTOKU, A. & SHIMADA, S. (1981). β -Lactamase from *Streptomyces cacaoi*. Purification and properties. *Journal of Biological Chemistry* **256**, 2649-2655.
- OKANISHI, M., SUZUKI, K. & UMEZAWA, H. (1974). Formation and reversion of streptomycete protoplasts: cultural conditions and morphological study. *Journal of General Microbiology* **80**, 389-400.
- RIGHETTI, P. & DRYSDALE, J. W. (1971). Isoelectric focusing in polyacrylamide gels. *Biochimica et biophysica acta* **236**, 17-28.
- SAMRAOUI, B., SUTTON, B. J., TODD, R. J., ARTYMIUK, P. J., WALEY, S. G. & PHILLIPS, D. C. (1986). Tertiary structural similarity between a class A β -lactamase and a penicillin-sensitive D-alanyl-carboxypeptidase-transpeptidase. *Nature, London* **320**, 378-380.
- SOUTHERN, E. H. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-517.