Cloning and amplified expression in *Streptomyces lividans* of the gene encoding the extracellular *β*-lactamase of *Actinomadura* R39

Claudine PIRON-FRAIPONT, Colette DUEZ, André MATAGNE, Chantal MOLITOR,* Jean DUSART,† Jean-Marie FRÈRE and Jean-Marie GHUYSEN
Département de Microbiologie, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman (Liège 1), Belgium

By using the promoter-probe plasmid pIJ424, genomic DNA fragments of *Actinomadura* R39 were shown to have promoter activity in *Streptomyces lividans*. The same 100–200-copy-number plasmid was used to clone in *S. lividans* TK24, the gene that encodes the *Actinomadura* R39 *β*-lactamase. Gene cloning resulted in an amplified expression of the *β*-lactamase when compared with the amounts of enzyme produced by the original strain (1 mg versus 0.008 mg·litre of culture^{-1}).

INTRODUCTION

Actinomadura R39, isolated from an African soil and initially designated '*Streptomyces* R39', has a wall peptidoglycan of the *meso*-diaminopimelic acid chemotype I (Ghuysen *et al.*, 1973). It secretes during growth a hypersensitive DD-peptidase/penicillin-binding protein (Leyh-Bouille *et al.*, 1972; Ghuysen *et al.*, 1974; Frère *et al.*, 1974, 1980; Duez *et al.*, 1981*a,b*) and very low amounts of a *β*-lactamase (Johnson *et al.*, 1973; Duez *et al.*, 1982). *Actinomadura* spp. belong to the Order Actinomycetales and possess a DNA G+C content of 60–72 mol% (McClung, 1974), which is similar to that of the genus *Streptomyces*. Given this similarity and the fact that several genes encoding *Streptomyces* enzymes have been cloned in a *Streptomyces* high-copy-number vector–host system with amplification of the expressed proteins (Dehottay *et al.*, 1986; Duez *et al.*, 1987; Lenzini *et al.*, 1987), the same strategy was used to overproduce the *Actinomadura* R39 *β*-lactamase.

MATERIALS AND METHODS

Bacterial strains and plasmids

Actinomadura R39 was from the local collection. *S. lividans* TK24 (Hopwood *et al.*, 1983) and *S. lividans* M336, i.e. *S. lividans* TK24 bearing the promoter–probe plasmid pIJ424 (Ward *et al.*, 1986), were from the John Innes Institute, Norwich, U.K.

Media and growth conditions

Cultures were grown at 28 °C, with vigorous orbital shaking, in five different media: Difco brain–heart broth, Merck peptone broth [as described by Leyh-Bouille *et al.* (1971), except that peptone Merck 7213 was used], Lennox broth (Lennox, 1955), L broth (Miller, 1972) and E9 broth (Dehottay *et al.*, 1986). R2YE agar (Hopwood *et al.*, 1985) was used for plating. Kanamycin gradient plates were made with MMT medium (Katz *et al.*, 1983) as described (Ward *et al.*, 1986). Spore suspensions were prepared as described by Hopwood *et al.* (1985).

Recombinant DNA techniques

Chromosomal and plasmid DNAs were prepared and *Streptomyces* protoplasts were transformed as described by Hopwood *et al.* (1985). DNA minipreparations were made using the alkaline-lysis procedure of Birnboim & Doly (1979) as modified by Kieser (1984). Treatments with restriction endonucleases (from various commercial sources), bacterial alkaline phosphatase (The Radiochemical Centre, Amersham, Bucks., U.K.) and phage-T4 DNA ligase (Boehringer, Mannheim, Germany), separation of digested DNAs by agarose-gel electrophoresis and elution of DNA fragments from the gels were performed essentially as described in Maniatis *et al.* (1982).

β-Lactam compounds and other antibiotics

Nitrocefin was purchased from Oxoid Ltd., Basingstoke, Hants., U.K., and kanamycin from Sigma, St. Louis, MO, U.S.A. The following compounds were gifts: thiostrepton (from S. J. Lucania, E. R. Squibb and Sons, New Brunswick, NJ, U.S.A.); phenoxymethylpenicillin (from Dr. J. Vanderhaeghe, Rega Institute, Leuven, Belgium); *N*-formimidoylthienamycin and cefoxitin (from the Merck Institute for Therapeutic Research, Rahway, NJ, U.S.A.); and cephaloridine (from E. Lilly and Co., Indianapolis, IN, U.S.A.).

β-Lactamase activity and enzyme units

Unless otherwise stated, nitrocefin (O'Callaghan *et al.*, 1972) was used as substrate and the tests were performed in 50 mM-sodium phosphate, pH 7.0, containing 0.1 mg of bovine serum albumin·ml^{-1}. Routinely, 5–10 μl samples of enzyme were added to 450 μl of 100 μM-nitrocefin in buffer and incubated at 30 °C. One unit is defined as the amount of enzyme hydrolysing 1 μmol of substrate/min at maximal velocity.

Kinetic parameters

Enzyme samples and various concentrations of *β*-lactam substrates were incubated at 30 °C in 50 mM-sodium phosphate, pH 7.0, and the absorbances of the

* Present address: Smith Kline RIT, Service de Génétique, rue de l'Institut 89, B-1330 Rixensart, Belgium.
† To whom correspondence and requests for reprints should be addressed.

solutions were monitored until stabilization, at 482 nm for nitrocefin, 260 nm for cephaloridine and 235 nm for phenoxymethylpenicillin. The reading frequency was $2\ s^{-1}$ and the kinetic parameters were estimated as described in De Meester *et al.* (1987). The enzyme concentrations were such that completion of the reactions was achieved in less than 5 min. Inactivation of the β-lactamases by cefoxitin and *N*-formimidoylthienamycin was analysed on the basis of the simple model:

$$E + I \underset{}{\overset{K}{\rightleftharpoons}} E \cdot I \xrightarrow{k_{+2}} E\text{–}I^*$$

where E is the enzyme, I is the β-lactam inactivator, K is the dissociation constant, k_{+2} is the first-order rate constant and E–I* is the acyl-enzyme. Two methods were used. With cefoxitin, the enzyme and the inactivator were incubated at 30 °C in buffer, and the residual enzyme activity was measured, by using nitrocefin as substrate, in samples removed after increasing incubation times. With *N*-formimidoylthienamycin, the reporter-substrate method (De Meester *et al.*, 1987) was used. The progressive decrease of the rate of hydrolysis of 100 μM-nitrocefin was measured on ternary mixtures containing the enzyme, the inactivator and nitrocefin.

Enzyme purification

Actinomadura R39 was grown for 65 h at 28 °C and with strong aeration in a tank containing 200 litres of the Merck peptone medium, and *S. lividans* CM3 was grown for 96 h at 28 °C, in 60 1-litre flasks containing 500 ml of the same peptone medium. After elimination of the mycelium by centrifugation, the supernatant fluids were stirred with DEAE-cellulose (10 g/litre) previously equilibrated against 50 mM-sodium phosphate, pH 7.0, until disappearance of the enzyme activity. The DEAE-cellulose was washed exhaustively with 100 mM-Tris/HCl, pH 7.2, containing 0.2 M-NaCl, until the absorbance of the buffer at 280 nm was less than 0.2, and packed in 5 cm × 30 cm columns (for 350 g samples of ion-exchanger). The enzymes were eluted with an NaCl gradient made in 100 mM-Tris/HCl, pH 7.2, by dropwise addition, at constant volume, of 1350 ml of 0.5 M-NaCl in buffer to 600 ml of 0.2 M-NaCl in buffer. The active fractions (total volume 800 ml) were concentrated to 32 ml by ultrafiltration on a Diaflo UM10 membrane (Amicon Corp., Danvers, MA, U.S.A.). The β-lactamase of *S. lividans* CM3 was further purified by three successive runs on a 2.2 cm × 25 cm column of phenylboronic acid–agarose type B (Cartwright & Waley, 1984). Elution was carried out with 100 mM-Tris/HCl (pH 7.2)/0.5 M-NaCl at a fow rate of $50\ ml \cdot h^{-1}$ (Fig. 1). With the *Actinomadura* R39 β-lactamase the third run was omitted and replaced by an f.p.l.c. step on MonoQ HR5/5 (Pharmacia, Uppsala, Sweden).

Protein content and amino acid composition

The proteins were estimated by measuring the amounts of amino groups available for dinitrophenylation after hydrolysis by 6 M-HCl, at 110 °C (Duez *et al.*, 1978). The amino acid composition was established as described by Joris *et al.* (1983).

Polyacrylamide-gel electrophoresis

Separation gel electrophoresis [7%, (w/v) acrylamide, pH 8.4] was run using 0.7 cm × 9.5 cm cylindrical gels and a current of 5 mA/gel. After an overnight pre-run, the samples were loaded and allowed to migrate until the Bromophenol Blue marker reached the bottom of the tube. Gel electrophoresis in the presence of SDS was carried out as described by Laemmli & Favre (1973).

RESULTS AND DISCUSSION

Transcriptional efficacy of *Actinomadura* R39 promoters in *S. lividans*

A portion (2 μg) of genomic DNA of *Actinomadura* R39 was cleaved with restriction endonucleases *Bam*HI, *Bcl*I and *Bgl*II respectively, and each digest was separately ligated to 1 μg of promoter–probe plasmid pIJ424 (Fig. 2) previously cleaved with *Bgl*II and treated with bacterial alkaline phosphatase. The resulting ligation mixtures were used to transform *S. lividans* TK24 protoplasts, and the transformants were selected on R2YE agar plates containing 50 μg of thiostrepton $\cdot$ ml^{-1} (Hopwood

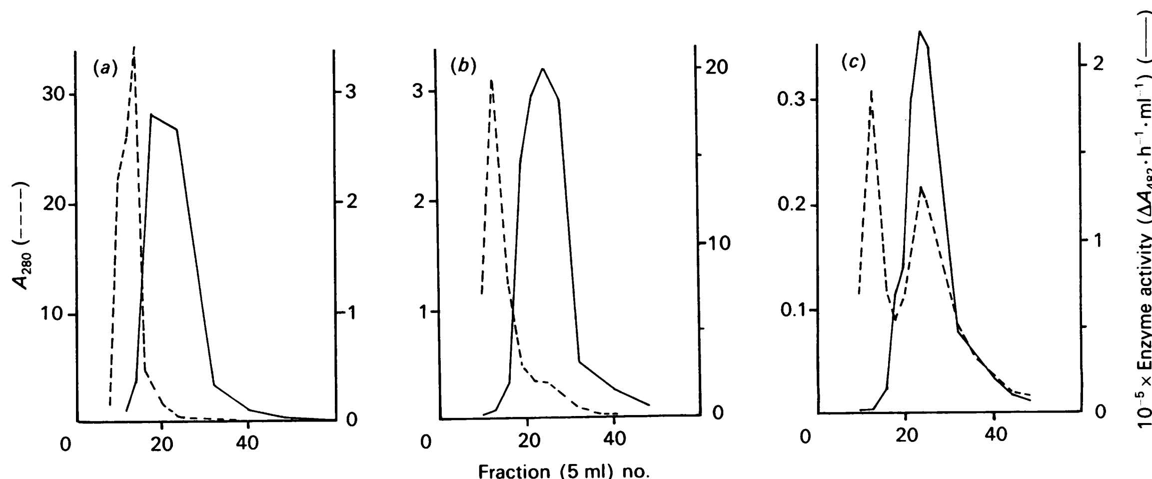

Fig. 1. Purification of the *S. lividans* CM3 β-lactamase on a phenylboronic acid–agarose affinity column

Three successive runs, (*a*), (*b*) and (*c*), were performed. For details, see the Materials and methods section. Enzyme activity is expressed in arbitrary units corresponding to an absorbance increase of 1.0 at 482 nm after 1 h of incubation. Such arbitrary unit is equivalent to 0.71×10^{-6} enzyme unit as defined in the Materials and methods section, nitrocefin being the substrate.

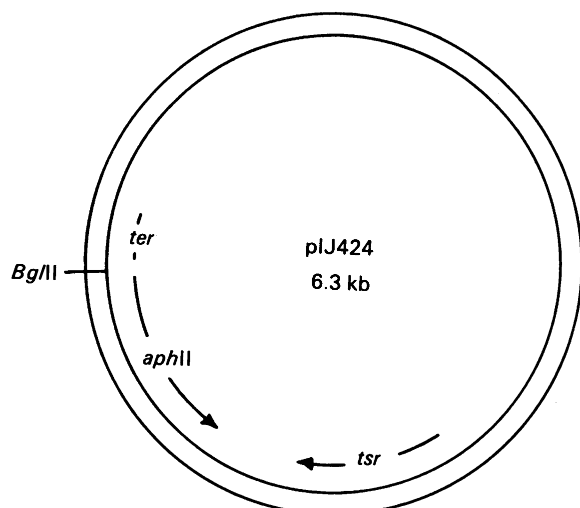

Fig. 2. Promoter–probe plasmid vector pIJ424 (Ward *et al.*, 1986)

pIJ424 uses the promoterless aminoglycoside phosphotransferase gene (*aph*II) of transposon Tn*5* as the indicator for promoter activity. There is a unique *Bgl*II site located downstream of the major terminator (*ter*) of the *Escherichia coli* phage fd (so that readthrough from upstream vector promoters is prevented) and upstream of a ribosome-binding site, an in-frame stop codon and the start codon of the *aph*II gene. Insertion of a promoter-containing DNA fragment in the *Bgl*II site results in the expression of kanamycin resistance in a suitable host such as *S. lividans* TK24. pIJ424 is a derivative of the pIJ101 replicon (Kieser *et al.*, 1982); it occurs in a 100–200 copy number per chromosome (Ward *et al.*, 1986) and also contains the thiostrepton-resistance marker (*tsr*).

et al., 1985). Transformation rates (numbers of transformants·μg of plasmid^{-1}) ranged from 1.4 to 3.0×10^4.

Spore suspensions of thiostrepton-resistant transformants were streaked on kanamycin gradient (from 0 to 250 μg·ml^{-1}) plates. Growth was frequently observed up to half way on the gradient (Fig. 3). *S. lividans* M336, which contained the original pIJ424, served as control; few colonies grew only at the origin of the gradient. The plasmid DNAs of 30 kanamycin-resistant clones were analysed by agarose-gel electrophoresis. They all had an increased molecular mass when compared with that of pIJ424. In all likelihood these plasmids had acquired *Actinomadura* R39 genomic DNA fragments that contained promoter regions, thus permitting expression of the promoterless *aph*II gene of pIJ424.

β-Lactamase-producing *S. lividans* CM3

Since *Actinomadura* R39 promoters were recognized and effectively utilized by *S. lividans*, thiostrepton-resistant transformants having the capacity of producing an extracellular β-lactamase were selected by nitrocefin test on R2YE agar plates (Dehottay *et al.*, 1986). Nine of them produced the desired activity; seven originated from the *Bcl*I library, two from the *Bam*HI library and none from the *Bgl*II library. Their β-lactamase-producing capacity was tested in five different growth media (see the Materials and methods section). *S. lividans* CM3, from the *Bam*HI library, was the best β-lactamase producer. Its 8.1 kb plasmid pDML150 had no *Bgl*II site. The occurrence of hybrid *Bgl*II/*Bam*HI sites prevented the 1.8 kb insert from being isolated.

Maximal level of β-lactamase secretion by *S. lividans* CM3, i.e. ≈ 1 mg of enzyme·litre of culture^{-1}, occurred in the Merck peptone broth after 96 h of growth. This

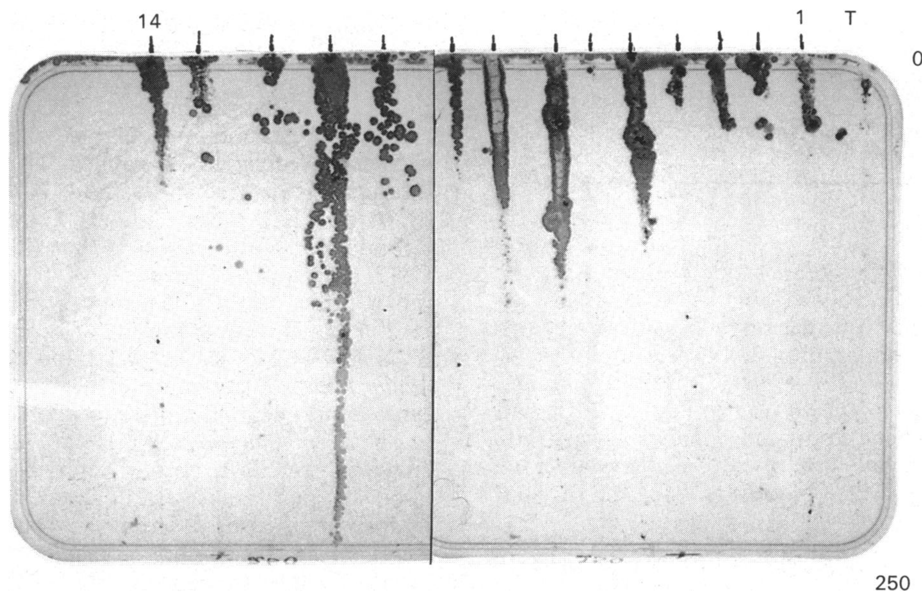

Fig. 3. Growth of *S. lividans* transformants on kanamycin gradient (0–250 μg·ml^{-1}) plates

T, *S. lividans* M336 (TK24+pIJ424). 1–14, *S. lividans* strains transformed with the ligation mixtures described in the text.

Table 1. Purification of the extracellular β-lactamase produced by *Actinomadura* R39 and *S. lividans* CM3

Strain	Purification step	Enzyme units (μmol·min^{-1})	Total protein (mg)	Specific activity (units·mg of protein^{-1})	Recovery (%)	Enrichment (fold)
R39	Culture supernatant (200 l)	1875	96400	0.019	(100)	–
	DEAE-cellulose	2280	750	3	121	158
	Phenylboronic acid–agarose (two steps)	980	3.2	307	52	16150
	F.p.l.c. MonoQ	980	0.80	1245	52	65500
CM3	Culture supernatant (30 l)	31300	182350	0.17	(100)	–
	DEAE-cellulose	29400	1660	17.7	94	104
	Phenylboronic acid–agarose (three steps)	12400	10	1230	40	7230

Table 2. Amino acid composition of the *Actinomadura* R39 and *S. lividans* CM3 β-lactamases

Residue	Composition (mol/100 mol)	
	R39 β-lactamase*	CM3 β-lactamase
Lys	1.6	1.6
His	1.6	1.4
Arg	5.6	3.0
Trp	3.2	2.0
Asx	11.2	13.8
Thr	6.4	7.8
Ser	4.0	4.6
Glx	14.4	15.8
Pro	4.8	5.6
Gly	9.6	11.0
Ala	10.4	7.8
½-Cys	1.6	0.6
Val	8.0	10.0
Met	1.6	0.8
Ile	2.4	2.4
Leu	8.8	8.4
Tyr	1.6	1.0
Phe	3.2	2.6

* Data from Duez *et al.* (1982).

level was 100-fold higher than that observed with the original *Actinomadura* R39 strain (≈ 0.008 mg of enzyme·litre of culture^{-1}) grown under identical and optimal conditions. A similar amplified expression of the β-lactamases of *Streptomyces albus* G (Dehottay *et al.*, 1986) and *Streptomyces cacaoi* (Lenzini *et al.*, 1987) had been observed after gene cloning in *S. lividans* via pIJ702 (30 and 40 mg of enzyme·litre of culture^{-1} as against 0.5 and 1.5 mg of enzyme·litre of culture^{-1} for the original strains).

Actinomadura R39 and *S. lividans* CM3 β-lactamases

Actinomadura R39 and *S. lividans* CM3 were grown, and the secreted β-lactamases were purified (see the Materials and methods section). The last purification step yielded fractions which, irrespective of the producing strain, had a constant specific enzyme activity of about 1200 units·mg of protein^{-1}, using nitrocefin as substrate. Table 1 gives the enzyme recoveries and enrichments.

The *Actinomadura* R39 and *S. lividans* CM3 β-lactamases were co-eluted by f.p.l.c. on MonoQ HR5/5. Elution occurred at 0.51 M-NaCl. Upon polyacrylamide-gel electrophoresis under non-denaturing conditions, they migrated 7.8 cm towards the anode as a single protein band. Upon SDS/polyacrylamide-gel electrophoresis, they also migrated as a single protein band with an apparent relative molecular mass of 55–57 kDa. Upon filtration on Sephadex G-100 in 50 mM-sodium phosphate (pH 7.2)/0.5 M-NaCl, they exhibited an elution coefficient value which, by reference to standard proteins, indicated an apparent molecular mass of 38.5 kDa. Great variations in the estimated molecular mass of the *Actinomadura* R39 β-lactamase prepared as described by Duez *et al.* (1982) had been observed. That former preparation contained about 10% (w/w) of a polydeoxyribonucleotide material, apparently in the form of a stable complex. Though the enzyme preparations obtained in the course of the present study lacked this contaminating material, SDS/polyacrylamide-gel electrophoresis and molecular sieving failed to yield consistent molecular-mass values. The reason for these discrepancies is not understood. Nucleotide sequencing (S. Houba, S. Willem, C. Duez, C. Molitor & J. Dusart, unpublished work) revealed that the cloned *S. lividans* CM3 β-lactamase gene codes for a 304-amino-acid polypeptide, i.e. a 276-amino-acid mature protein of 29.27 kDa. Within the limits of experimental errors, the *S. lividans* CM3 β-lactamase and the original *Actinomadura* R39 β-lactamase (Duez *et al.*, 1982) have the same amino acid composition (Table 2).

The *Actinomadura* R39 β-lactamase is a wide-spectrum enzyme, penicillins and Δ^3-cephalosporins being either good or poor substrates. The kinetic parameters of hydrolysis of three different β-lactam antibiotics by the *Actinomadura* R39 and *S. lividans* CM3 β-lactamases prepared and purified as described above were redetermined (Table 3). Both enzymes behaved similarly. The two β-lactamase preparations also reacted similarly with two different β-lactamase inactivators (Table 4).

Table 3. Kinetic parameters of *Actinomadura* R39 and *S. lividans* CM3 β-lactamases

Each value represents the average (±S.D.) of at least three measurements.

Substrate	Concentration (μM)	Enzyme quantity (ng)	K_m (μM)		$V_{max.}$*	
			R39	CM3	R39	CM3
Nitrocefin	50–100	50–100	72±2	69±7	100±5	100±18
Cephaloridine	50–100	70–120	38±2	38±3	74±4	73±9
Phenoxymethylpenicillin	250–500	70–350	84±13	85±9	100±7	139±6

* $V_{max.}$ values are expressed as a percentage of that with nitrocefin (= 1200 μmol·min⁻¹·mg of protein⁻¹).

Table 4. Kinetic parameters of the inactivation of the *Actinomadura* R39 and *S. lividans* CM3 β-lactamases by β-lactam compounds

Each value represents the average (±S.D.) of at least three measurements.

Inhibitor	Kinetic parameter	Enzyme... Value R39	CM3
Cefoxitin	$10^{-3}\,k_{+2}$ (s^{-1})	1.23±0.03	1.12±0.04
N-Formimidoylthienamycin	K (μM)	0.23±0.01	0.22±0.01
	$10^{-3}\,k_{+2}$ (s^{-1})	1.85±0.15	2.1±0.15

With cefoxitin, the rate of inactivation did not vary between 20 and 60 mM, indicating a K value of much less than 20 mM. With *N*-formimidoylthienamycin, the values of k_i, where

$$k_i = \frac{k_{+2}[C]}{[C]+K}$$

were measured at concentrations ranging from 0.3 to 1.5 μM. The individual values of k_{+2} and K were derived from plots $1/k_i$ versus $1/[C]$, where [C] is the concentration of formimidoylthienamycin.

We thank Dr. D. A. Hopwood, Dr. T. Kieser and Dr. M. J. Bibb from the John Innes Institute (Norwich, U.K.) for generously providing strains and plasmids. This work was supported in part by the Fonds de la Recherche Scientifique Médicale (contract n° 3.4507.83), an Action concertée with the Belgian Government (convention 86/91-90, the Fonds de Recherche de la Faculté de Médicine ULg and a contract with the European Economic Community (BAP-0197-B). J.D. is Research Associate at the National Fund for Scientific Research (Belgium). A.M. is indebted to the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture for a predoctoral fellowship.

REFERENCES

Birnboim, H. C. & Doly, J. (1979) Nucleic Acids Res. **7**, 1513–1523

Cartwright, S. J. & Waley, S. G. (1984) Biochem. J. **221**, 505–512

Dehottay, P., Dusart, J., Duez, C., Lenzini, M. V., Martial, J. A, Frère, J. M., Ghuysen, J. M. & Kieser, T. (1986) Gene **42**, 31–36

De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frère, J. M. & Waley, S. G. (1987) Biochem. Pharmacol. **36**, 2393–2403

Duez, C., Frère, J. M., Geurts, F., Ghuysen, J. M., Dierickx, L. & Delcambe, L. (1978) Biochem. J. **175**, 793–800

Duez, C., Joris, B., Frère, J. M., Ghuysen, J. M. & Van Beeumen, J. (1981*a*) Biochem. J. **193**, 83–86

Duez, C., Frère, J. M., Ghuysen, J. M., Van Beeumen, J. & Vandekerckhove, J. (1981*b*) Biochim. Biophys. Acta **671**, 109–116

Duez, C., Frère, J. M., Ghuysen, J. M., Van Beeumen, J,. Delcambe, L. & Dierickx, L. (1982) Biochim. Biophys. Acta **700**, 24–32

Duez, C., Piron-Fraipont, C., Joris, B., Dusart, J., Urdea, M. S., Martial, J. A., Frère, J. M. & Ghuysen, J. M. (1987) Eur. J. Biochem. **162**, 509–518

Frère, J. M., Ghuysen, J. M., Reynolds, P. E., Moreno, R. & Perkins, H. R. (1974) Biochem. J. **143**, 241–249

Frère, J. M., Klein, D. & Ghuysen, J. M. (1980) Antimicrob. Agents Chemother. **18**, 506–510

Ghuysen, J. M., Leyh-Bouille, M., Campbell, J. N., Moreno, R., Frère, J. M., Duez, C., Nieto, M. & Perkins, H. R. (1973) Biochemistry **12**, 1243–1251

Ghuysen, J. M., Reynolds, P. E., Perkins, H. R., Frère, J. M. & Moreno, R. (1974) Biochemistry **13**, 2539–2547

Hopwood, D. A., Kieser, T., Wright, H. M. & Bibb, M. J. (1983) J. Gen. Microbiol. **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, The John Innes Foundation, Norwich

Johnson, K., Dusart, J., Campbell, J. N. & Ghuysen, J. M. (1973) Antimicrob. Agents Chemother. **3**, 284–298

Joris, B., Van Beeumen, J., Casagrande, F., Gerday, C., Frère, J. M. & Ghuysen, J. M. (1983) Eur. J. Biochem. **130**, 53–69

Katz, E., Thompson, C. J. & Hopwood, D. A. (1983) J. Gen. Microbiol. **129**, 2703–2714

Kieser, T. (1984) Plasmid **12**, 19–36

Kieser, T., Hopwood, D. A., Wright, H. M. & Thompson, C. J. (1982) Mol. Gen. Genet. **185**, 223–238

Laemmli, U. K. & Favre, M. (1973) J. Mol. Biol. **80**, 575–599

Lennox, E. S. (1955) Virology **1**, 190–206

Lenzini, M. V., Nojima, S., Dusart, J., Ogawara, H., Dehottay, P., Frère, J. M. & Ghuysen, J. M. (1987) J. Gen. Microbiol. **133**, 2915–2920

Leyh-Bouille, M., Coyette, J., Ghuysen, J. M., Idczak, J., Perkins, H. R. & Nieto, M. (1971) Biochemistry **10**, 2163–2170

Leyh-Bouille, M., Nakel, M., Frère, J. M., Johnson, K., Ghuysen, J. M., Nieto, M. & Perkins, H. R. (1972) **11**, 1290–1298

Maniatis, J., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

McClung, N. M. (1974) in Bergey's Manual of Determinative Bacteriology, 8th edn. (Buchanan, R. E. & Gibbons, N. E., eds.), pp. 726–747, Williams and Wilkins Co., Baltimore

Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

O'Callaghan, C. H., Morris, A., Kirby, S. M. & Shingler, A. H. (1972) Antimicrob. Agents Chemother. **1**, 283–288

Ward, J. M., Janssen, G. R., Kieser, T., Bibb, M. J., Buttner, M. J. & Bibb, M. J. (1986) Mol. Gen. Genet. **203**, 468–478

Received 6 February 1989/30 March 1989; accepted 11 April 1989