The EMBO Journal vol.12 no.2 pp.631-639, 1993

Genes for a β-lactamase, a penicillin-binding protein and a transmembrane protein are clustered with the cephamycin biosynthetic genes in *Nocardia lactamdurans*

Juan José R.Coque, Paloma Liras and Juan F.Martín[1]

Section of Microbiology, Department of Ecology, Genetics and Microbiology, Faculty of Biology, University of León, 24071 León, Spain

[1]Corresponding author

Communicated by D.A.Hopwood

Three genes encoding a typical β-lactamase, a penicillin-binding protein (PBP4) and a transmembrane protein are located in the cluster of cephamycin biosynthetic genes in *Nocardia lactamdurans*. The similarity of the *N.lactamdurans* β-lactamase to class A β-lactamases from clinical isolates supports the hypothesis that antibiotic resistance genes in pathogenic bacteria are derived from antibiotic-producing organisms. The β-lactamase is secreted and is active against penicillins (including the biosynthetic intermediates penicillin N and isopenicillin N), but not against cephamycin C. The β-lactamase is synthesized during the active growth phase, prior to the formation of three cephamycin biosynthetic enzymes. The PBP of *N.lactamdurans* is a low-M_r protein that is very similar to DD-carboxypeptidases of *Streptomyces* and *Actinomadura*. The *pbp* gene product expressed in *Streptomyces lividans* accumulates in the membrane fraction. By disruption of *N.lactamdurans* protoplasts, the PBP4 was shown to be located in the plasma membrane. Eight PBPs were found in the membranes of *N.lactamdurans*, none of which bind cephamycin C, which explains the resistance of this strain to its own antibiotic. A transmembrane protein encoded by the *cmc*T gene of the cluster also accumulates in the membrane fraction and is probably related to the control of synthesis and secretion of the antibiotic. A balanced synthesis of β-lactam antibiotics, β-lactamase and PBP is postulated to be critical for the survival of β-lactam-producing actinomycetes.

Key words: cephamycin/β-lactamase/PBP/transmembrane protein

Introduction

Genes involved in the biosynthesis of antibiotics are often clustered together with genes conferring resistance to antibiotics (Hopwood *et al.*, 1986; Martín and Liras, 1989b) and sometimes also with genes that encode enzymes which convert primary metabolites into precursors of the antibiotic molecules (Coque *et al.*, 1991b; Martín, 1992), Cephamycin C is a β-lactam antibiotic produced by *Nocardia lactamdurans, Streptomyces clavuligerus* and several other actinomycetes (Martín and Liras, 1989a; Aharonowitz *et al.*, 1992). Genes encoding enzymes that carry out some of the steps of the cephamycin pathway have been isolated from both *N.lactamdurans* and *S.clavuligerus*, but knowlege of the organization of the entire clusters is still limited.

Cephamycin C is synthesized from three precursor amino acids: L-α-aminoadipic acid, L-cysteine and L-valine (Figure 1). The three amino acids are condensed to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine) (ACV) which is later cyclized to form isopenicillin N, an intermediate which shows some antibiotic activity. After epimerization to form penicillin N, the five-membered thiazolidine ring of this intermediate is expanded to the six-membered dihydrothiazine ring present in deacetoxycephalosporin C. The latter compound is converted to cephamycin C by a series of late transformation reactions [reviewed by Martín and Liras (1989b)].

Chen *et al.* (1988) proposed that the entire cephamycin pathway of *Streptomyces cattleya* was encoded by a set of adjacent genes. Recently, we have found that all genes for the cephamycin pathway are clustered together in a 30 kb region of *N.lactamdurans* DNA. The genes *lat*, encoding lysine-6-aminotransferase, which forms α-aminoadipic acid (a precursor of β-lactam antibiotics) from lysine (Coque *et al.*, 1991b; Tobin *et al.*, 1991), *pcb*AB encoding an unusually large non-ribosomal peptide synthetase that assembles the ACV tripeptide, and *pcb*C which encodes the ring-forming isopenicillin N synthase have been fully characterized (Coque *et al.*, 1991a). Two other genes, *cef*D and *cef*E, which encode isopenicillin N epimerase and the ring-expanding enzyme deacetoxycephalosporin C synthase, have also been studied (Coque *et al.*, 1992). Four genes (*orf*7−*orf*10 in Figure 1) are involved in the conversion of deacetoxycephalosporin C to cephamycin C (called 'late' steps of the cephamycin pathway) (J.J.R.Coque, R.E.Cardoza and P.Liras, unpublished).

A striking observation was the finding of three other genes (*bla*, *pbp* and *cmc*T in Figure 1) which encode, respectively, a typical β-lactamase, a penicillin-binding protein (PBP) and a transmembrane protein, in the cephamycin cluster. Here, we describe the characterization of these genes and discuss their possible role in the modulation of β-lactam production and resistance.

Results

A typical β-lactamase is encoded by the bla gene of the cephamycin cluster

A 3.6 kb *Bam*HI fragment of the cephamycin gene cluster cloned in phage λEMBL-C11 was subcloned and sequenced on both strands. An open reading frame (ORF11 in Figure 1) of 909 nt was found (Figure 2) with a G+C content of 74.47%, which encodes a typical β-lactamase precursor of 302 amino acids with an M_r of 32 054. The beginning of the ORF was confirmed with the GENEPLOT Program (DNASTAR) using data on codon usage in *Streptomyces* as reference. The protein has a predicted pI of 5.02 and a charge

© Oxford University Press

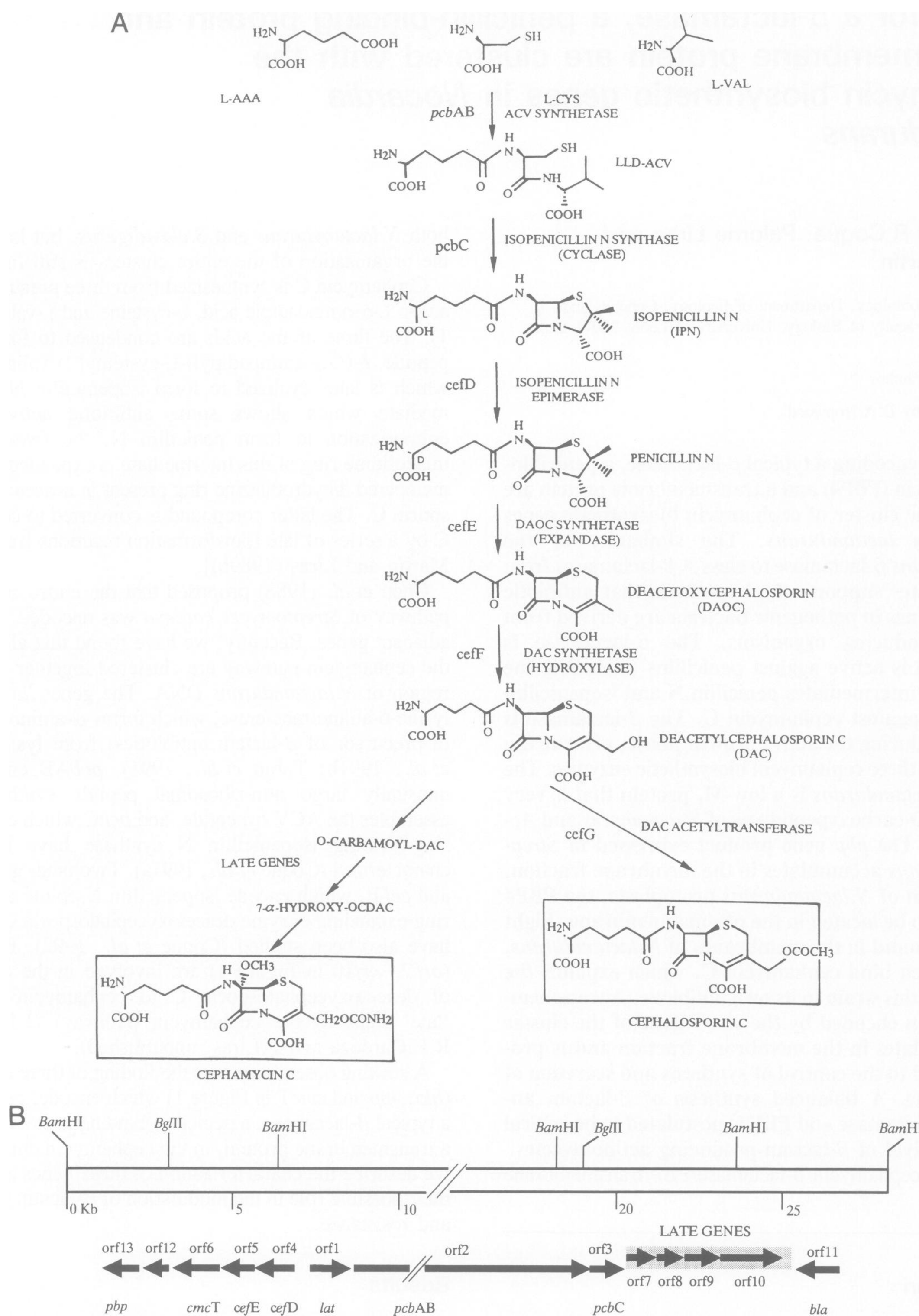

Fig. 1. (**A**) Biosynthetic pathway of cephamycin C in *N.lactamdurans*. (**B**) Physical map of a 30 kb region of *N.lactamdurans* DNA carrying 13 genes related to cephamycin biosynthesis and resistance. *lat*, lysine-6-aminotransferase; *pcb*AB, unusually large gene encoding α-aminoadipyl-cysteinyl-valine synthetase; *pcb*C, isopenicillin N synthase; *cef*D, isopenicillin N epimerase; *cef*E, deacetoxycephalosporin C synthase (expandase); *cmc*T, transmembrane protein; ORF7−ORF10, late genes involved in the conversion of deacetoxycephalosporin C to cephamycin C; *bla*, β-lactamase; *pbp*, penicillin-binding protein. ORF12, short gene of unknown function. Gene designations are given according to Martín *et al.* (1991) and Aharonowitz *et al.* (1992).

at pH 7.0 of −7.41. The N-terminal end of the β-lactamase contains a 29 amino acid sequence extending from the initial codon to ala-ala-ala[29], which resembles the leader peptide of extracellular enzymes of *Streptomyces* (García-González *et al.*, 1991). The sequence of ala-ala-ala agrees with the consensus ala-X-ala motif recognized by the signal peptidase of *Streptomyces* (García-González *et al.*, 1991) and other organisms (Watson, 1984).

The enzyme encoded by the *bla* gene of *N.lactamdurans* is very similar to the β-lactamases of *Streptomyces badius*

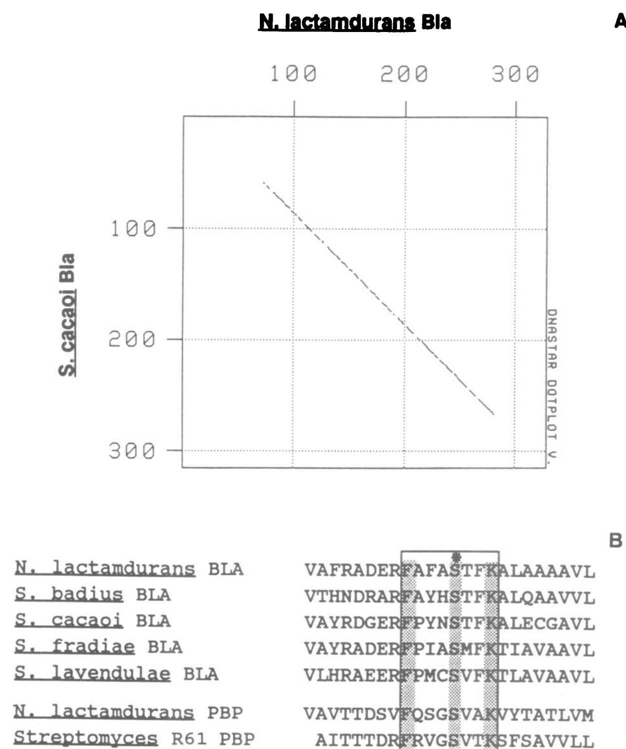

Fig. 2. (**A**) DOTPLOT analysis of the *N.lactamdurans* β-lactamase as compared to the *S.cacaoi* enzyme. Comparisons were made with the DOTPLOT program (DNASTAR) using a percent match of 50 for the β-lactamases. (**B**) Alignment of the active centres of the *N.lactamdurans* β-lactamase with the β-lactamases of *S.badius*, *S.cacaoi*, *S.fradiae* and *S.lavendulae*. The motif FXXXSXXK is boxed and the conserved F, S and K residues are shaded. The active centres of the *N.lactamdurans* and the *Streptomyces* R61 PBPs are also shown. β-Lactamases and PBPs have a similar penicillin-binding active centre, but differ in the rest of the protein. Both types of enzymes catalyse the transfer of the penicilloyl residue to the serine in the active centre (indicated with an asterisk) and the hydrolysis of the lactam bond. The penicilloic acid remains enzyme bound in the PBPs, whereas it is rapidly released in the β-lactamases.

(55% identical amino acid residues), *S.cacaoi* (56.3%), *S.fradiae* (45.8%) and *S.lavendulae* (45.6%); all of them contain the consensus sequence FXXXSXXK in the active site (Figure 2) found in class A β-lactamase (see Discussion) from other bacteria (Ghuysen, 1991).

The β-lactamase of N.lactamdurans remains largely cell wall bound

A weak β-lactamase activity was found in the culture supernatant of *N.lactamdurans* using the chromogenic substrate PADAC, but only after 48 h of growth, reaching a peak of activity at 60−72 h (Figure 3). Most activity remained cell bound. To study whether the β-lactamase activity was located in the cytoplasm, in the membrane or externally to the membrane, protoplasts of *N.lactamdurans* were obtained and the β-lactamase activity was assayed in the isotonic solution used to prepare the protoplasts (Sp) and in the two fractions obtained after disruption of the protoplasts by osmotic shock, namely the membrane fraction (M_{100}) and the intracellular content of the protoplasts (S_{100}). All β-lactamase activity was released in the protoplast preparation solution and no activity was detected in the membrane fraction or in the S_{100} fraction. These results indicate that the β-lactamase is secreted, but remains largely cell bound, trapped by the cell wall. This may be due to the well known presence of mycolic acids in the cell wall of *Nocardia*, which create a 'periplasmic-like' space.

Time-course studies of *N.lactamdurans* β-lactamase formation (Figure 3) showed that the cell-bound enzyme is formed from the beginning of the culture, i.e. the *bla* is an 'early' gene which is expressed in a constitutive form (Figure 3), as reported for the β-lactamases of other species of *Streptomyces* (Ogawara, 1981), and its expression precedes those of the cephamycin biosynthetic genes encoding isopenicillin N synthase, isopenicillin N epimerase and deacetoxycephalosporin C synthase (Figure 3). The *lat* gene, encoding lysine-6-amino transferase, an enzyme involved in precursor formation, is expressed early.

The finding of considerable cell-bound β-lactamase suggests that the low extracellular activity is probably due to the release of enzyme from the cell wall.

The β-lactamase of N.lactamdurans is active on penicillins but not on cephalosporins

The β-lactamase encoded by the *bla* gene is very active on penicillin G and ampicillin (50 μg of penicillin/ml are fully degraded in 180 min), using the *in vivo* assay, whereas the enzyme shows only ~20% of the activity using isopenicillin N or penicillin N as substrates. This β-lactamase had no effect on cephalosporin C, deacetoxycephalosporin C, cephamycin C and its semisynthetic derivative cefoxitin.

These results indicate that the β-lactamase of *N.lactamdurans* has a typical spectrum of class A β-lactamases and does not inactivate the cephamycin C produced by the same strain (see Discussion).

The penicillin-binding protein encoded by the pbp gene is similar to DD-carboxypeptidases

An ORF of 1146 nt with a G+C content of 74.3%, located in a 6.3 kb *Bam*HI fragment subcloned in pULC203 (Coque *et al.*, 1992) at the other end of the cephamycin cluster (ORF13 in Figure 1), was found to encode a protein of 381 amino acids with an M_r of 40 492; this protein was very similar to the PBP (D-alanyl-D-alanyl carboxypeptidase) of *Streptomyces* R61 (24.7% identical amino acids) (Figure 4) and also to the low M_r PBPs of other actinomycetes (Duez *et al.*, 1987; Ghuysen, 1991). The amino acid sequence of the PBP of *N.lactamdurans* contained regions similar to three of the secondary structural elements proposed for all the penicillin-recognizing enzymes: SVAK (which includes the serine common to all penicillin-interacting enzymes), YCST and GHDG (Joris *et al.*, 1991) (Figure 4). The similarity is good for element 1 (^{62}SVTK in *Streptomyces* R61, using the amino acid numbers corresponding to the processed R61 PBP protein, and ^{60}SVAK in *N.lactamdurans*), element 2 (^{159}YSNT and ^{152}YCST, respectively) and element 4 (^{297}GHTG and ^{301}GHDG, respectively). Interestingly, element 4 is located between two putative hydrophobic membrane-spanning domains and is part of an NAD(H) binding motif, GYGHDGASGG, which fits perfectly with the consensus GXGXXGXXXG motif of NAD(H) binding sequences (Scrutton *et al.*, 1990).

The *N.lactamdurans* PBP binds labelled penicillin, as shown by the classical binding assay (Figure 5). The penicillin-interactive proteins fall into three groups: β-lactamases, low-M_r PBPs and high-M_r PBPs, all of which possess a serine residue in their active centre (Ghuysen,

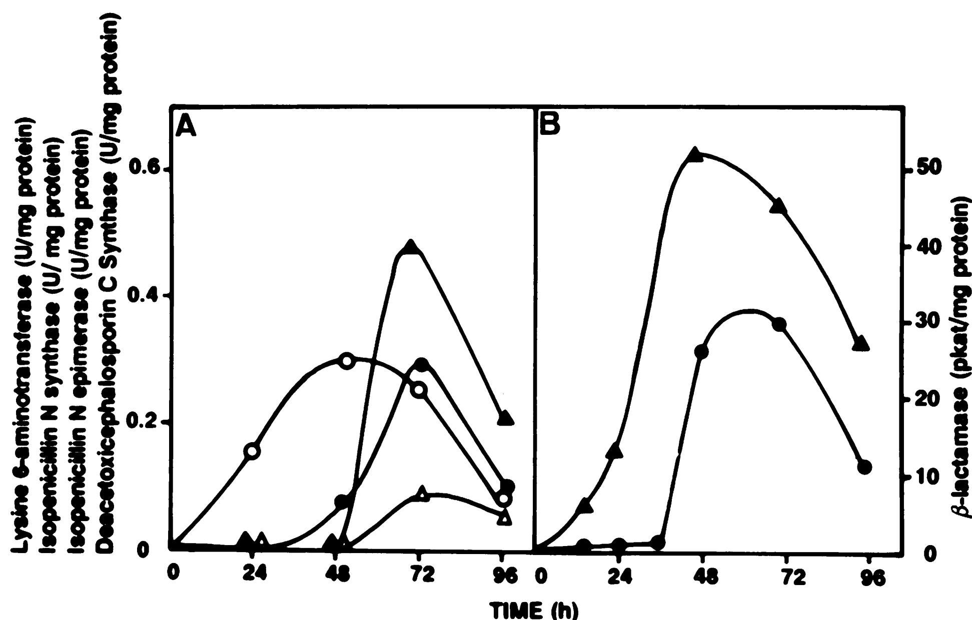

Fig. 3. (A) Time course of four different enzyme activities involved in cephamycin biosynthesis: lysine-6-aminotransferase (○); isopenicillin N synthase (●); isopenicillin N epimerase (▲); deacetoxycephalosporin C synthase (△). (B) Cell wall-bound (▲) and extracellular (●) β-lactamase.

1991). The β-lactamases and the low-M_r PBPs are monofunctional enzymes in which the active site serine is located close to the N-terminus of the protein; the high-M_r PBPs possess a large domain (several hundred amino acids) fused to the N-terminus of the authentic penicillin-binding domain. The active site serine of the protein encoded by the *pbp* gene of *N.lactamdurans* is located at residue 60. A stretch of eight amino acids, FQSGSVAK, flanking the Ser-60 active site, which corresponds to the consensus FXXXSXXK motif of β-lactamases and PBPs (Figure 2), is extremely similar to the active site of the PBP of *Streptomyces* R61, *Streptomyces* K15 and *Actinomadura* R39 (Ghuysen, 1991; Granier *et al.*, 1992).

No homology was found with the N-terminal region of the *Streptomyces* R61 PBP (which is known to be secreted) and no amino acid sequences similar to the leader peptide could be identified in the *N.lactamdurans* PBP, suggesting that this protein is not secreted.

Computer analysis with the RAOARGOS Program of PC/GENE (using a peak minimal value of 1.13 and a baseline value of 1.05) which predicts integral membrane protein helices, revealed the existence in the PBP of two hypothetical transmembrane helices very near the carboxyl terminal end of the protein [amino acids 271–291 (peak value 1.14) and 315–340 (peak value 1.22)] (Figure 4); these observations suggested that the PBP could be anchored in the plasma membrane by its carboxyl end. To test this possibility, the PBP gene was subcloned in pIJ702 in a 3.0 kb *BglII–BamHI* fragment downstream from the *mel* promoter, in the same orientation as the *mel* gene, to increase its expression (construction named pULPBPa). SDS–PAGE of proteins in plasma membranes isolated from *S.lividans* transformants containing pULBPBa shows that the PBP protein of *N.lactamdurans* had accumulated in the plasma membrane of *S.lividans* (Figure 5), although it was found also in the supernatant of the membrane fraction (S_{100}), probably due to its release during the cell disruption process. At least eight proteins able to bind penicillin were observed in *S.lividans* (S.l.PBP1–S.l.PBP8 in Figure 5). The cloned *N.lactamdurans* PBP (named PBP4, see below) migrates on electrophoresis between *S.lividans* PBP5 and PBP6.

Protoplast disruption indicates that PBP4 is located in plasma membranes

Penicillin-binding studies of *S.lividans* [pULPBPa] cells disrupted in the French press showed that the PBP was located both in the plasma membrane and in the supernatants (S_{100}) obtained after centrifugation. Since French press disruption is a drastic treatment which may release membrane-bound proteins, PBP was assayed in extracts and membrane fractions obtained by osmotic lysis of protoplasts of *N.lactamdurans* (the experiments could not be carried out in parallel with *S.lividans* since the *pbp* gene was not expressed in culture media that supported good growth to obtain protoplasts). At least eight PBPs (N.l.PBP1–N.l.PBP8 in Figure 5) were observed in the plasma membranes of *N.lactamdurans*, a number similar to those found in *S.lividans* (Figure 5). Those with high mol. wt (N.l.PBP1 and PBP2) were poorly labelled with penicillin G.

The protein encoded by the *pbp* gene of the cephamycin cluster (PBP4 in Figure 5; 40.5 kDa) was located in the membrane fraction (M_{100}) obtained by osmotic lysis of protoplasts prepared from cultures of 24 and 48 h, but it was not detected in the corresponding intracellular extracts (S_{100}) obtained after protoplast lysis, or in the culture medium used to obtain protoplasts (not shown). This result confirms that PBP4 is located in the plasma membranes of *N.lactamdurans*.

PBP4 shows no affinity for cephamycin C

Since no labelled cephamycin C is available, competition experiments were run in which 50 μg of membrane protein (M_{100}) of *N.lactamdurans* were preincubated with increasing amounts of cephamycin C for 15 min at 30°C, followed by addition of labelled penicillin G (40 μg/ml) and

A

```
N. lactamdurans PBP                              MPFDHAHWQERFDALRTEHHVPGAALAVFVDGDLHELASGVLHRG    45
                                                 :P  :.   :       .: .PGA : V  :G.:H:L:.GV :R:
Streptomyces R61 PBP  MVSGTVGRGTALGAVLLALLAVPAQAGTAAAADLPAPDDTGLQAVLHTALSQGAPGAMVRVDDNGTIHQLSEGVADRA    78

                 1
TGVAVTTDSVFQSGSVAKVYTATLVMQLVDAGELRLDTRVADVLPGFAVADAEVARTVTIGR--LLSHTSGI--AGDFTLDTGRGDD-CLARFVDACADV   140
TG A:TT.. F: GSV:K ::A.:::QLVD.G.L LD:.V.. LPG:  .D  ..R V   R  L ..T:::  ..  .::: R..  . . ::. . .
TGRAITTTDRFRVGSVTKSFSAVVLLQLVDEGKLDLDASVNTYLPGLLPDDRITVRQVMSHRSGLYDYTNDMFAQTVPGFESVRNKVFSYQDLITLSLKH   178

              2                                                                           3
GQDCPPDTVISYCSTGYAILGRIVEVLTGQSWDDALRDRLFTPLGLHQSMTLPEEALRFRVAMSHLGELGTDPEPAPVWDMLPRSAGPYGRVLITAADVV   240
G . :P::. SY.:T.:.: G.::E LTG:S ... ::R:FTPL.L ::. :. :::   :: :H ..  T  E:: .  :: .:. . ::.  T:A.V:
GVTNAPGAAYSYSNTNFVVAGMLIEKLTGHSVATEYQNRIFTPLNLTDTFYVHPDTV---IPGTHANGYLTPDEAGGA--LVDSTEQTVSWAQ-TGAAVI   272

                                                                  4
RFARMHLDDGVAPDGTRVLSAASAALMRQQVAGCLDTWSFMATGWGHGWALYDWD---GVPGYGHDGASGGQFSYLRVVPGSGVVAALLTNGGVATSFFT   337
        .:..  :T   :  S:.LM...  : :: W: :.:. G.G :L  :D   G:: YGH.G:  G ::Y  .  :: . .: L:N.: ..: :.
--------SSTQDLDTFFSALMSGQLMSAAQLAQMQQWTTVNSTQGYGLGLRRRDLSCGISVYGHTGTVQGYYTYAFASKDGQAHVTALANTSNNVNVLN   364

DLFRELLGELAGVRMPEVFAPPAEPRPIDVAPLAGTYEREGGAP                                                         381
.: R.L :.:    . ..  A...       AP . : . . :
TMARTLESAFCGKPTTGEAAQRDLLGDHRGAPEDIAPGIARD                                                           406
```

B

Buried helix parameter (units)

1.4
1.2
1
.8
.6
.4
.2
0

1 70 140 210 280 350

Amino Acids

C

N. lactamdurans low-Mr PBP

100 200 300

Streptomyces R61 low-Mr PBP

100
200
300
400

Fig. 4. (**A**) Computer alignment of the PBP of the cephamycin cluster with the *Streptomyces* R61 PBP (D-alanyl-D-alanyl-carboxypeptidase). The amino acid sequences of the two transmembrane buried helices are boxed. The secondary structural elements 1, 2, 3 and 4 involved in the active centre of PBPs are also boxed. The putative NAD(H)-binding motif GYGHDGASGG is underlined. (**B**) Buried transmembrane helices in the amino acid sequence of the *N.lactamdurans* PBP according to the RAOARGOS Program of PC/GENE (amino acids 271–291 and 315–340) using a minimal length of transmembrane helix of 17 amino acids. (**C**) Dot-plot comparison between *N.lactamdurans* PBP and *Streptomyces* R61 D-alanyl-carboxypeptidase.

incubation for another 15 min at the same temperature. As shown in Figure 6, cephamycin has no affinity for PBP4 of *N.lactamdurans*. Similarly, no significant competition by cephamcyin C with penicillin binding by the other seven PBPs of *N.lactamdurans* was observed. This result may explain the resistance of *N.lactamdurans* to the cephamycin that it produces.

The cmcT product is a protein with several transmembrane domains

An additional open reading frame (ORF6) of 1461 nt was found when the 6.3 kb *Bam*HI fragment of the cephamycin cluster was sequenced. It is tightly linked to the *cef*E gene, encoding deacetoxycephalosporin C synthase, as the termination codon of *cef*E overlaps the initiation triplet of ORF6. This ORF shows a G+C content of 74% and encodes a polypeptide of 486 amino acids with a molecular mass of 49 300 Da. This protein is highly hydrophobic, containing 250 hydrophobic residues (51.4% of the total), and includes 11 transmembrane domains separated by less hydrophobic stretches (Figure 7); it shows no consensus N-terminal signal sequence (leader peptide) characteristic of *Streptomyces* extracellular proteins (Watson, 1984; García-González *et al.*, 1991). Expression of this gene (named *cmc*T) in *S.lividans* results in a high content of this protein in plasma membrane fractions (Figure 7). The amino acid sequence showed structural motifs which are characteristic of several integral membrane proteins that are believed to transport solutes (Maiden *et al.*, 1987). The best match of the amino acid sequence was to proteins found in the clusters of bialaphos (Raibaud *et al.*, 1991) (24.2% identical amino acids), methylenomycin (28.5%) (Neal and Chater, 1987) and actinorhodin (31.7%) (Fernández-Moreno *et al.*, 1991) biosynthetic genes. Some similarity was also seen with two glucose transporters of *Synechocystis* sp. (21.9 and 22.1% identity, respectively), an arabinose transport protein, *ara*E, (23.4%) from *Escherichia coli* and a membrane protein involved in arginine catabolism (22.0%) in *Pseudomonas aeruginosa*. The *cmc*T-encoded protein is also similar to a transmembrane protein of *Saccharomyces cerevisiae* required for aminotriazole resistance (23.3% identical residues) (Kanazawa *et al.*, 1988) and to a tetracycline resistance protein required for efflux of this antibiotic (~24.5% identity). These similarities, and the linkage to the cephamycin biosynthetic genes, suggest that the protein encoded by the *cmc*T gene might be directly or indirectly related to the export of cephamycin, as proposed for the methylenomycin, bialaphos and actinorhodin antibiotics.

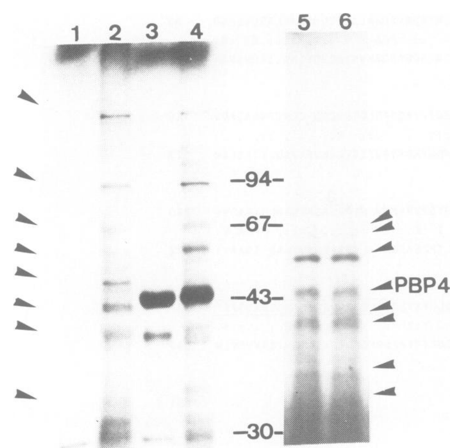

Fig. 5. Autoradiography of PBPs resolved by SDS–PAGE (10% polyacrylamide) using ~70 μg of protein from the binding mixture (see Materials and methods). **Lanes 1**, S_{100} extract of *S.lividans* [pIJ702; **2**, P_{100} membrane preparation of *S.lividans* [pIJ702]; 3, S_{100} extract of *S.lividans* [pULPBPa]; 4, P_{100} membrane preparation of *S.lividans* [pULPBPa]. The protein encoded by the *pbp* gene (PBP4) is indicated by an arrow. The eight major PBPs of *S.lividans* are indicated by arrowheads on the left. **Lanes 5** and **6**, M_{100} membrane extracts of *N.lactamdurans* protoplasts obtained from cultures of 24 and 48 h, respectively. The *N.lactamdurans* PBP1–PBP8 are shown on the right. Protein size markers in kDa are indicated between lanes 4 and 5.

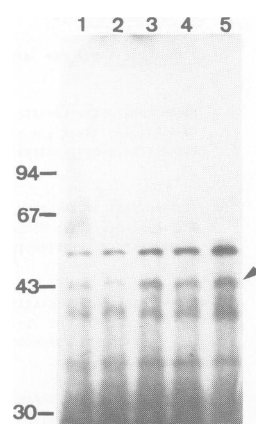

Fig. 6. Lack of competition of cephamycin C with penicillin binding by PBP4 of *N.lactamdurans*. 50 μg of M_{100} membrane preparations were incubated with increasing amounts of cephamycin C (0, 25, 50, 100 and 200 μg/ml, respectively, in lanes 1–5) prior to incubation with labelled penicillin G (see Materials and methods). PBP4 encoded by the *pbp* of the cephamycin C cluster is indicated by an arrow.

Discussion

Synthesis of β-lactam antibiotics, β-lactamase and PBP

Plasmid-encoded or chromosome-determined β-lactamases have been found in many Gram-positive and Gram-negative bacteria. They catalyse the transfer of the penicilloyl moiety to an essential serine residue in the active site of the enzyme and the hydrolysis of the lactam amide bond. Four distinct β-lactamase groups (classes A, B, C and D) have been described. Class A includes enzymes from *Staphylococcus aureus* and *Bacillus licheniformis*. The Zn^{2+}-requiring *Bacillus cereus* type II enzyme belongs to class B. Class C includes the chromosomally determined cephalosporinases from *E.coli* and other Gram-negative bacteria. Class D is represented by a group of oxacillin-hydrolysing β-lactamases (Forsman *et al.*, 1990). Several *Streptomyces* strains produce extracellular β-lactamases, all of those studied belonging to class A (Ogawara and Horikawa, 1980).

The existence of bacterial β-lactamases preceded the relatively recent widespread clinical use of β-lactam antibiotics (Ogawara *et al.*, 1978). In this article, we provide the first evidence that a β-lactamase gene is associated with the cluster of β-lactam biosynthetic genes in a β-lactam producer organism. The high degree of similarity between the β-lactamase encoded by the *bla* gene of *N.lactamdurans* and β-lactamases which occur at present in clinical isolates, strongly supports the hypothesis of Benveniste and Davies (1973) who proposed that antibiotic resistance genes in clinical isolates were derived from antibiotic-producing microorganisms (Piepersberg *et al.*, 1988; Kirby, 1991). It is well known that several actinomycetes, including species of the genera *Streptomyces* (Ogawara and Horikawa, 1980; Forsman *et al.*, 1990) and *Actinomadura* (Ghuysen, 1991), possess β-lactamases, whereas others do not. Studies are in progress in our laboratory to establish if the synthesis of β-lactamase in actinomycetes is always associated with the presence of a β-lactam biosynthetic cluster, or with silent β-lactam biosynthetic genes.

The β-lactamases may be considered to be defensive enzymes synthesized to protect the bacterial DD-carboxypeptidases from the deleterious effect of β-lactams. The linkage of a β-lactamase gene with the cluster of cephamycin biosynthetic genes is intriguing. The β-lactamase of *N.lactamdurans* is extracellular, although it remains largely cell bound. It might protect DD-carboxypeptidases from inhibition by endogenously synthesized penicillins (penicillin N and isopenicillin N) if they are secreted or released by lysis of old cells, or from exogenous penicillin produced by other soil microorganisms. The β-lactamase is synthesized prior to three of the cephamycin biosynthetic enzymes, which suggests that a carefully controlled timing of the synthesis of the β-lactamase and the antibiotic is important for the producer cell. The end product of the biosynthetic pathway, cephamycin, which is secreted after being synthesized, is not destroyed by the *N.lactamdurans* β-lactamase.

An interesting question is how the cephamycin-producing strains protect endogenous penicillin N and isopenicillin N intermediates which are sensitive to the β-lactamase after cell disruption (J.Cortés and P.Liras, unpublished). We postulate that synthesis of the inactive β-lactamase precursor and its membrane translocation are concomitant events followed by the extracellular release of the mature enzyme and, therefore, the intracellular β-lactam intermediates are protected (i.e. physiological compartmentalization). Additionally, a β-lactamase inhibitor has been reported in *S.clavuligerus* (Doran *et al.*, 1990).

The presence, in the cluster, of a gene (*pbp*) encoding a protein similar to the D-alanyl-D-alanyl carboxypeptidase of *Streptomyces* R61 (Duez *et al.*, 1987), a typical PBP (Figure 4), was also unexpected. Low-M_r PBPs and high-M_r PBPs are involved in one way or another in peptidoglycan (cell wall) synthesis. Some low-M_r PBPs are DD-carboxypeptidases that catalyse acyltransfer reactions from peptides ending in D-alanyl-D-alanine. These cross-linking enzymes are the targets of β-lactam antibiotics. The product of the *N.lactamdurans pbp* gene, when expressed

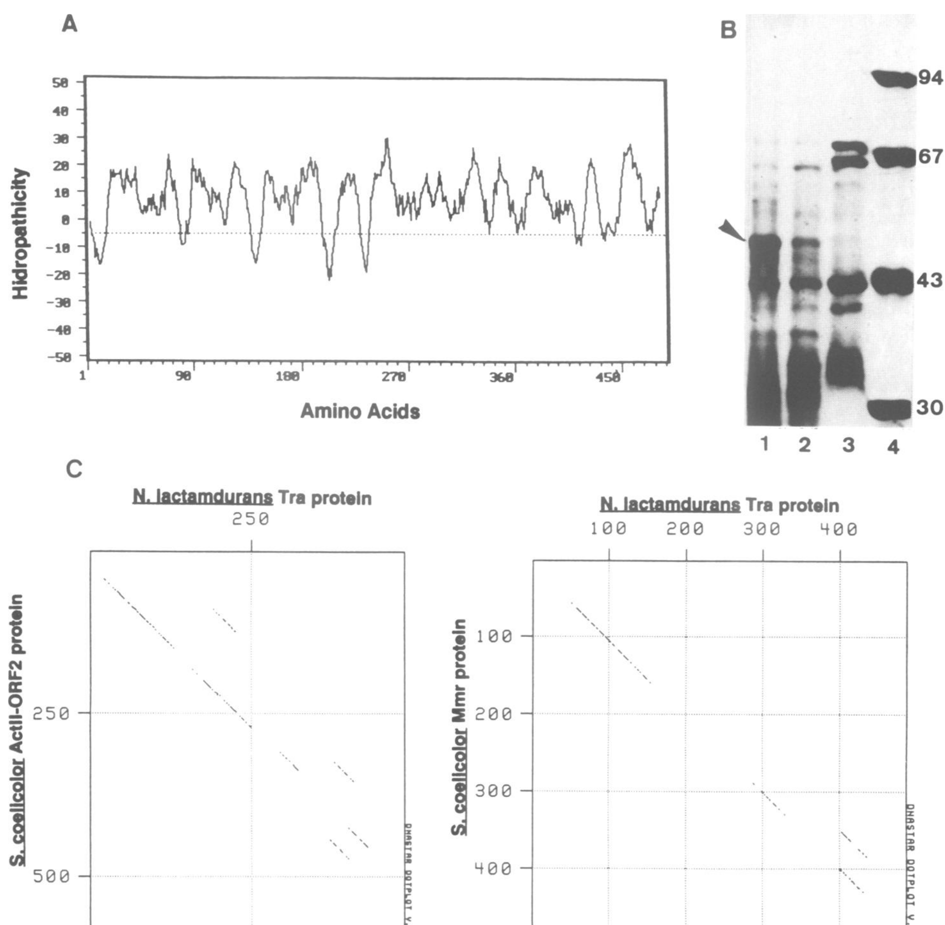

Fig. 7. (A) Hydropathic index plot of the *N.lactamdurans* transmembrane protein from amino acids 1–486 (GRAVY 8.47) generated with the SOAP program (PC/GENE). (B) SDS–PAGE of *S.lividans* P_{100} membrane fraction from *S.lividans* [pUL702-202a] (lane 1) and *S.lividans* [pUL702-202b] (lane 2), carrying in pIJ702, in opposite orientations, a 4.3 kb *Bgl*II–*Eco*RI DNA fragment containing the *cmc*T gene. Lane 3, *S.lividans* [pIJ702] without insert. Lane 4, size markers (Pharmacia; in kDa at the right). The transmembrane protein encoded by the *cmc*T is indicated by an arrow. (C) DOTPLOT analysis of the *N.lactamdurans* transmembrane protein as compared to the *S.coelicolor* ActII-ORF2 protein (left) and to the *S.coelicolor* Mmr (methylenomycin resistance) protein (right). Comparisons were made with the DOTPLOT (DNASTAR) using a percent match of 40.

in *S.lividans*, accumulates in the cytoplasmic membrane (Figure 5) and is not released by extensive washing of the membranes. This location is consistent with its involvement in cell wall biosynthesis, since assembly of the peptidoglycan structure in bacteria takes place on the outer surface of the cytoplasmic membrane (Ghuysen, 1991). Efflux of the antibiotic is one of the known antibiotic resistance mechanisms (Cundliffe, 1989). However, peptidoglycan cross-linking might still be sensitive to cephamycin. Our results indicate that PBP4 and other *N.lactamdurans* PBPs do not bind cephamycin, which most likely contributes to the resistance of *N.lactamdurans* to its own antibiotic. Similar results were reported by Ogawara and Horikawa (1980) in *S.clavuligerus*, another cephamycin-producing strain.

Another possible role of *N.lactamdurans* PBP4 is to act as a sensor of external penicillins, as occurs in *Bacillus licheniformis* where expression of the β-lactamase is regulated by a repressor protein and an anti-repressor which is a PBP (Imanaka *et al.*, 1987; Kobayashi *et al.*, 1987). It is tempting to speculate that the PBP of *N.lactamdurans* may act as a sensor of extracellular penicillins, which would trigger β-lactamase synthesis such as occurs in *B.licheniformis*.

The association of genes encoding transmembrane proteins with the clusters of several antibiotic biosynthetic genes is of interest. It has been proposed that transmembrane proteins encoded by genes associated with antibiotic biosynthetic clusters are involved in the export of antibiotic from the producer cells (Neal and Chater, 1987; Fernández-Moreno *et al.*, 1991; Raibaud *et al.*, 1991). Antibiotic synthesis and secretion seem to be coupled to a stress-sensing system. It is well known that no secretion (and no synthesis) of most antibiotics takes place in the presence of high glucose, phosphate or ammonium concentrations, whereas expression of antibiotic synthesis is triggered by stress conditions induced by high osmolarity or by nutrient limitation (Martín and Liras, 1989b; Asturias *et al.*, 1990). The transmembrane protein may have been derived from an ancestor of the consensus bacterial and mammalian sugar transport proteins (Maiden *et al.*, 1987) and adapted as a sensor of extracellular factors that control antibiotic synthesis and secretion.

A balanced synthesis of β-lactam antibiotics, β-lactamases and DD-carboxypeptidases is probably critical for the survival of the producer organisms.

Nucleotide sequences

The nucleotide sequences of the *bla*, *pbp* and *cmc*T genes of *N.lactamdurans* have been deposited in the EMBL Data

Library under the accession numbers Z13971, Z13972 and Z13973, respectively.

Materials and methods

Microorganisms and vectors used

Nocardia lactamdurans LC411, an improved cephamycin-producing strain (Coque *et al.*, 1991a), was used as the source of DNA. *Streptomyces lividans* JI1326, an actinomycete which does not produce β-lactam antibiotics (García-Domínguez *et al.*, 1991), was used as host strain for expression experiments with the *N.lactamdurans* genes. *Escherichia coli* DH5α was utilized for high-frequency plasmid transformation; *E.coli* WK6 and the helper bacteriophage M13K07 were used to obtain single-stranded DNA for sequencing.

Genes of the cephamycin biosynthetic cluster were isolated from a previously constructed gene library of *N.lactamdurans* DNA in vector λEMBL3 (Coque *et al.*, 1991a,b). pBluescript KS(+), and pUC118 and pUC119 were used to subclone DNA fragments for sequencing. Plasmids pIJ702 (Katz *et al.*, 1983) and pIJ699 (Kieser and Melton, 1988) were used to express the *bla*, *pbp* and *cmcT* genes of *N.lactamdurans* in *S.lividans* 1326.

DNA isolation and sequencing procedures

Fragments carrying the genes of interest were subcloned from phage λEMBL-C8 (Coque *et al.*, 1991a) into pBluescript KS(+) in both orientations. Ordered sets of nested DNA fragments were generated by sequential deletions using the Erase-a-base system (Promega, Madison, WI) as described previously (Coque *et al.*, 1991b). The DNA fragments were sequenced in both orientations by the dideoxynucleotide method (Sanger *et al.*, 1977) using Taq DNA polymerase (Promega) and 7-deaza-dGTP to avoid compressions.

Isolation of plasmid DNA, digestion with restriction endonucleases, labelling of DNA fragments and Southern hybridizations were carried out according to standard procedures (Sambrook *et al.*, 1989).

Isolation of cytoplasmic membranes and SDS–PAGE of proteins

Streptomyces lividans [pIJ702], *S.lividans* [pULPBP] and *S.lividans* [pUL702-202a or b] were grown for 72 h in minimal medium supplemented with 15 mM each of lysine and asparagine (Madduri *et al.*, 1991). The cells were collected, washed twice with saline solution, suspended in 10 mM phosphate buffer (pH 7.3) and disrupted in an Aminco French Press. The cell-free extract was centrifuged first at 10 000 *g* for 20 min and the supernatant (S_{20}) ultracentrifuged later at 100 000 *g* for 30 min. The membrane pellet (P_{100}), washed three times in the same buffer, and the supernatant (S_{100}) were used for PBP assays.

SDS–PAGE of proteins was carried out as reported previously (Láiz *et al.*, 1990). Proteins were quantified by the Bradford method.

Assay of penicillin-binding proteins

PBPs were assayed in membrane-rich pellets obtained by French press disruption (P_{100}) and in protoplast membranes (M_{100}), as well as in supernatants after removal of the membranes by ultracentrifugation at 100 000 *g* (S_{100}). The binding mixture, containing 50 μg of protein and 40 μg/ml of [benzyl-4-H^3]penicillin G (21.4 Ci/mmol) in a total volume of 15 μl, was incubated for 10 min at 30°C. The reactions were stopped by the addition of lauryl-sarcosine (1% final concentration) and incubation at room temperature to disaggregate the membranes and release the proteins. The binding mixtures were loaded on SDS–polyacrylamide (10%) gels. Penicillin binding was detected by autoradiography of the gel after soaking it in Hyperfilm solution (Amersham), to enhance the signal, and drying the gel.

Competition assays by different unlabelled β-lactam antibiotics were done by preincubating the PBP-containing membrane fraction with increasing concentrations of the unlabelled antibiotic for 15 min at 30°C prior to addition of the radioactive penicillin at the same final concentration as above. Protoplasts of *N.lactamdurans* LC411 cells (grown in NYG medium supplemented with 20 mM $MgCl_2$) were obtained by incubating the washed cells in the protoplasting solution containing 0.3 M sucrose, 25 mM Tris (pH 8.0), 25 mM EDTA and lysozyme (10–15 mg/ml) for 20 min at 30°C.

To obtain membranes, protoplasts were filtered and disrupted by osmotic shock in 10 mM phosphate buffer (pH 7.3). The residual cellular debris was removed by centrifugation at 10 000 *g* for 20 min at 4°C, and the membrane fraction was collected by ultracentrifugation at 100 000 *g* for 20 min at 4°C. The membrane pellet (M_{100}) was washed three times with 10 ml of 10 mM phosphate buffer and resuspended in the same buffer at a final concentration of 10 mg/ml. The membrane fraction (M_{100}), the supernatant of the ultracentrifugation (S_{100}) and the protoplasting solution, after removal of the protoplasts, were assayed for penicillin-binding activity.

Assay of β-lactamase activity

Two different procedures were used to determine β-lactamase activity in *N.lactamdurans*. The first assay (*in vitro*) was carried out using PADAC, a chromogenic cephalosporin, as substrate (25 mM) (Kobayashi *et al.*, 1978). Reactions were carried out in a final volume of 800 μl of 50 mM phosphate buffer (pH 7.3) using 100 μl of protein. Samples containing β-lactamase activity (according to the PADAC assay) were incubated at 30°C for 1 h with known concentrations of different β-lactam antibiotics in 10 mM Tris (pH 7.3) buffer, and the inactivation of the β-lactam antibiotic was tested by bioassay.

Since most β-lactamase remains cell bound, a second assay was carried out *in vivo* using entire undisrupted cells of 48-h-old *N.lactamdurans* cultures grown in $MgCl_2$-supplemented NYG as described above. Cells were collected and washed twice with 1 vol of 10 mM Tris (pH 7.3). Washed cells were resuspended in the same medium and mixed with known concentrations of different β-lactam antibiotics, and the rate of antibiotic degradation was quantified by bioassay.

Assay of lysine-6-aminotransferase, isopenicillin N synthase, isopenicillin N epimerase and deacetoxycephalosporin C synthase

The four cephamycin biosynthetic activities were assayed as described previously (Castro *et al.*, 1985; Cortés *et al.*, 1987; Láiz *et al.*, 1990, Coque *et al.*, 1991a).

Acknowledgements

We thank J.G.Calzada for communicating unpublished results and J.Ayala for valuable discussions. We acknowledge S.Mochales (Merck, Spain) for the gift of labelled penicillin, K.Roy and D.Hopwood for critical reading of the manuscript, and V.Kumar and M.I.Corrales for excellent technical assistance. This work was supported by grant BI090-0556 of the CICYT, Madrid.

References

Aharonowitz,Y., Cohen,G. and Martín,J.F. (1992) *Annu. Rev. Microbiol.*, **46**, 461–495.

Asturias,J.A., Liras,P. and Martín,J.F. (1990) *Gene*, **93**, 79–84.

Benveniste,R. and Davies,J. (1973) *Proc. Natl. Acad. Sci.*, **70**, 2276–2280.

Castro,J.M., Liras,P., Cortés,J. and Martín,J.F. (1985) *Appl. Microbiol. Biotechnol.*, **22**, 32–40.

Chen,C.W., Lin,H.-F., Kuo,C.L., Tsai,H.-L. and Tasai,J.F.-Y. (1988) *Bio/Technology*, **6**, 1222–1224.

Coque,J.J.R., Martín,J.F., Calzada,J.G. and Liras,P. (1991a) *Mol. Microbiol.*, **5**, 1125–1133.

Coque,J.J.R., Liras,P., Láiz,L. and Martín,J.F. (1991b) *J. Bacteriol.*, **173**, 6258–6264.

Coque,J.J.R., Martín,J.F. and Liras,P. (1992) *Mol. Gen. Genet.*, in press.

Cortés,J., Martín,J.F., Castro,J.M., Láiz,L. and Liras,P. (1987) *J. Gen. Microbiol.*, **133**, 3165–3174.

Cundliffe,E. (1989) *Annu. Rev. Microbiol.*, **43**, 207–233.

Doran,J.L., Leskiw,B.K., Aippersbach,S. and Jensen,S.E. (1990) *J. Bacteriol.*, **172**, 4909–4918.

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

Fernández-Moreno,M.A., Caballero,J.L., Hopwood,D.A. and Malpartida,F. (1991) *Cell*, **66**, 769–780.

Forsman,M., Häggström,B., Lindgren,L. and Jaurin,B. (1990) *J. Gen. Microbiol.*, **136**, 589–598.

García-Domínguez,M., Liras,P. and Martín,J.F. (1991) *Antimicrob. Agents Chemother.*, **35**, 44–52.

García-González,M.D., Martín,J.F., Vigal,T. and Liras,P. (1991) *J. Bacteriol.*, **173**, 2451–2458.

Ghuysen,J.-M. (1991) *Annu. Rev. Microbiol.*, **45**, 37–67.

Granier,B., Duez,C., Lepage,S., Englebert,S., Dusart,J., Dideberg,O., Van Beeumen,J., Frère,J.M. and Ghuysen,J.M. (1992) *Biochem. J.*, **282**, 781–788.

Hopwood,D.A., Bibb,M.J., Chater,K.F., Janssen,G.R., Malpartida,F. and Smith,C.P. (1986) In Booth,I.R. (ed.), *Regulation of Gene Expression—25 Years On*. Cambridge University Press, Cambridge, UK, pp. 251–276.

Imanaka,T., Himeno,T. and Aiba,S. (1987) *J. Bacteriol.*, **169**, 3867–3872.

Joris,B., Ledent,P., Dieberg,P., Fonzé,E., Lamotte-Brasseur,J., Kelly,J.A.,

Ghuysen,J.M. and Frère,J.M. (1991) *Antimicrob. Agents Chemother.*, **35**, 2294–2301.
Kanazawa,S., Driscoll,M. and Struhl,K. (1988) *Mol. Cell. Biol.*, **8**, 664–673.
Katz,E., Thompson,C.J. and Hopwood,D.A. (1983) *J. Gen. Microbiol.*, **129**, 2703–2714.
Kieser,T. and Melton,R.E. (1988) *Gene*, **65**, 83–91.
Kirby,R. (1991) *Abstracts of the International Symposium on Biology of Actinomycetes*. P. 3-013, Madison, WI.
Kobayashi,T., Zhu,Y.F., Nicholls,N.J. and Lampen,J.O. (1987) *J. Bacteriol.*, **169**, 3873–3878.
Láiz,L., Liras,P., Castro,J.M. and Martín,J.F. (1990) *J. Gen. Microbiol.*, **136**, 663–671.
Madduri,K., Shapiro,S., DeMarco,A.C., White,R.L., Stuttard,C. and Vining,L.C. (1991) *Appl. Microbiol. Biotechnol.*, **35**, 358–363.
Maiden,M.C.J., Davis,E.O., Baldwin,S.A., Moore,D.C.M. and Henderson,P.J.F. (1987) *Nature*, **325**, 641–643.
Martín,J.F. (1992) *J. Ind. Microbiol.*, **9**, 73–90.
Martín,J.F. and Liras,P. (1989a) In Fiechter,A. (ed.), *Advances in Biochemical Engineering/Biotechnology*. Springer-Verlag, Berlin, Heidelberg, Vol. 39, pp. 153–187.
Martín,J.F. and Liras,P. (1989b) *Annu. Rev. Microbiol.*, **43**, 173–206.
Martín,J.F., Ingolia,T.D. and Queener,S.W. (1991) In Leong,S.A. and Berka,R.M. (eds), *Molecular Industrial Mycology*. Marcel Dekker, New York, pp. 149–196.
Neal,R.L. and Chater,K.F. (1987) *Gene*, **58**, 229–241.
Ogawara,H. (1981) *Microbiol. Rev.*, **45**, 591–619.
Ogawara,H. and Horikawa,S. (1980) *Antimicrob. Agents Chemother.*, **17**, 1–7.
Ogawara,H., Horikawa,S., Shimada-Miyoshi,S. and Yasuzawa,K. (1978) *Antimicrob. Agents Chemother.*, **13**, 865–870.
Piepersberg,W., Distler,J., Heinzel,P. and Pérez-González,J.-A. (1988) *Actinomycetologica*, **2**, 83–98.
Raibaud,A., Zalacain,M., Holt,T.G., Tizard,R. and Thompson,C.J. (1991) *J. Bacteriol.*, **173**, 4454–4463.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanger,F. Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Scrutton,N.S., Berry,A. and Perham,R.N. (1990) *Nature*, **343**, 38–43.
Tobin,M.B., Kovacevic,S., Madduri,K., Hoskins,J.A., Skatrud,P.L., Vining,L.C., Stuttard,C. and Miller,J.R. (1991) *J. Bacteriol.*, **173**, 6223–6229.
Watson,E.E. (1984) *Nucleic Acids Res.*, **12**, 5145–5165.

Received on May 13, 1992