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Induction of a *Streptomyces cacaoi* β-lactamase gene cloned in *S. lividans*

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Summary. The previously cloned class A β-lactamase gene (*bla*) of *Streptomyces cacaoi* was shown to be inducible by β-lactam compounds in the host organism *S. lividans*. A regulatory region of 2.75 kb was identified and the nucleotide sequence determined. It contained four open reading frames (ORFs) of which only two were complete and required for induction. ORF1–ORF2 exerted a positive regulatory effect on the expression of *bla*. Inactivation of ORF1 or of ORF2 resulted not only in the loss of induction, but also in a 30- to 60-fold decrease in the basal (non-induced) level of β-lactamase production. ORF1 codes for a DNA-binding protein related to the AmpR repressor/activator, which controls the expression of *ampC* (class C β-lactamase) genes in several Enterobacteria.

Key words: *amp*R gene – Gene regulation – β-Lactamase – *Streptomyces*

Introduction

β-Lactamases (EC 3.5.2.6) are widely distributed in the bacterial world, and can be divided into four classes on the basis of their primary structures (Joris et al. 1988). Enzymes of classes A, C and D have serine in the active site, while class B contains a few metalloenzymes (Waley 1988). X-ray crystallographic studies (Kelly et al. 1986; Samraoui et al. 1986; Herzberg and Moult 1987; Oefner et al. 1990) have shown that proteins of class A and class C proteins share the same three-dimensional architecture. On this basis, it has been suggested that they could derive from the same ancestral gene.

The regulation of β-lactamase production has been studied in the gram-positive *Bacillus licheniformis*, *B. cereus* (Lampen et al. 1988) and *Staphylococcus aureus* (Rowland and Dyke 1989) and in several Enterobacteria (Lindberg et al. 1988). In *B. licheniformis* and *S. aureus*, a class A β-lactamase gene is under the control of a repressor (BlaI) and of a sensor (BLaR). In *Enterobacter cloacae*, a class C β-lactamase gene is regulated by an AmpR activator/repressor and several other proteins (AmpD, AmpE) of still undefined function. In *Escherichia coli*, the role of the sensor could be played by the penicillin-binding protein 2 (Oliva et al. 1989). In every case, the structural and regulatory genes are transcribed divergently and their respective promoters are very close to each other. Genes *blaI* and *blaR* of *B. licheniformis* are very probably cotranscribed (Kobayashi et al. 1987; Lampen et al. 1988) whereas in the Enterobacteria, only *ampR* is linked to the structural *ampC* gene (Honoré et al. 1986; Lindberg et al. 1985), a situation that has also been reported in *Pseudomonas aeruginosa* (Lodge et al. 1990). An *ampR* gene has also been found in a member of the Rhodospirillaceae, *Rhodopseudomonas capsulata* (Campbell et al. 1989), and is also transcribed divergently from the structural gene it controls; however, in this case, the β-lactamase is not a class C, but a class A enzyme (*bla* gene).

A *Streptomyces cacaoi* β-lactamase, previously cloned in *S. lividans* (Lenzini et al. 1987), has been shown to belong to class A (Lenzini et al. 1988; Urabe et al. 1990). On the other hand, Forsman et al. (1989) have reported that β-lactamase production by the *S. cacaoi* strain is inducible by a β-lactam molecule, such as 6-aminopenicillanic acid (6-APA). Various β-lactamase-producing *S. lividans* clones are available which differ in the length of the *S. cacaoi* DNA fragment inserted in the vector. These clones have been examined for induction by β-lactam. We report here the sequence of a *S. cacaoi* DNA fragment, which renders the production of the cloned *S. lividans* β-lactamase inducible by β-lactams. A preliminary report of this work has been presented previously (Lenzini et al. 1991).

Materials and methods

Strains, plasmids and media: *S. cacaoi* KCC-S0352 and *S. lividans* TK24 (Hopwood et al. 1983) were gifts from

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the Kaken Chemical Co. (Tokyo, Japan) and the John Innes Institute (Norwich, UK), respectively. *Streptomyces* plasmids pDML51, pDML52, pMCP38, pMCP39 and *E. coli* plasmid pDML72 were previously described (Lenzini et al. 1987). All of them were derived from pIJ702 (Katz et al. 1983) and contained a β-lactamase structural gene (*bla*) from *S. cacaoi*. pIJ2925 graciously provided by the John Innes Institute, was derived from pUC18 by insertion of the polylinker of pIJ486 (Ward et al. 1986), slightly modified. pACYC184 (Chang and Cohen 1978) was also used. The *E. coli* host strain was HB101 (Boyer and Roulland-Dussoix 1969). Liquid cultures were maintained at 28° C in E9 (Dehottay et al. 1986) or YEME medium (Hopwood et al. 1985) with vigorous orbital shaking. R2YE agar was used for plating.

Recombinant DNA techniques and DNA sequencing. The procedures applied were essentially those described by Maniatis et al. (1982) and Hopwood et al. (1985). Nucleotide sequence determination was performed by the M13 dideoxynucleotide method (Sanger et al. 1977, 1980), using [α-^{35}S]dATP or fluorescent primer. In this last case, the experiments were performed on the EMBL automated fluorescent DNA sequencer (Ansorge et al. 1987). Deletions were obtained by using the Double-stranded Nested Deletion Kit of Pharmacia (Uppsala, Sweden).

Computer analysis. Codon usage was assessed using Staden's programs (Staden and McLachlan 1982; Staden 1984). Screening of the gene databanks was carried out with the FASTA peptide program (Devereux et al. 1984). The SEQDP program of Goad and Kanehisa (1982) was also used.

β-Lactamase activity. The β-lactamase activity was estimated by the method of O'Callaghan et al. (1972) using nitrocefin as substrate.

Enzymes and antibiotics. Enzymes for recombinant DNA experiments were purchased from Boehringer (Mannheim, FRG), Bethesda Research Laboratories (Gaithersburg, Md., USA) or Biolabs (Beverley, Mass., USA) and nitrocefin was from Oxoid Ltd. (Basingstoke, Hampshire, UK). Temocillin was from Beecham (Brentford, Middlesex, UK), imipenem from Merck, Sharp and Dohme (Rahway, N.J., USA) and 7-aminodesacetoxycephalosporanic acid (7-ADCA) from Gist-Brocades (Delft, The Netherlands). These latter compounds were kindly donated by the various companies. Sodium penicillanate and thiostrepton were gifts of Professors Claes and Vanderhaeghe (University of Leuven, Belgium) and by S.J. Lucania (Squibb, New Brunswick, N.J., USA) respectively.

Sequence accession number. The nucleotide sequence data in this paper have been submitted to the EMBL Data Library and have been assigned the following accession number: X63780 *S. cacaoi* DNA (ORF1, ORF2 and ORF3).

Table 1. Induction of *Streptomyces cacaoi* KCC-S0352 by various β-lactam compounds

Inducer	Amplification factor
None	1
Sodium penicillanate	7
Temocillin	10
Imipenem	24
7-ADCA	38

7-ADCA, 7-aminodesacetoxycephalosporanic acid
The inducers were added at a concentration of 10 µg/ml of culture medium, to 12 h old cultures in E9 medium, and β-lactamase activity measured 96 h later. Results are expressed relative to the β-lactamase activity of a non-induced culture

Table 2. Amplification factors of β-lactamase production by *S. lividans*/pMCP38 induced with 7-ADCA

7-ADCA concentrations (µg/ml)	Time after the addition of 7-ADCA (h)			
	20	26	50	100
0	(1)	(1)	(1)	(1)
10	9	15	39	33
25	19	26	79	60
100	26	30	76	74
200	26	28	90	79

The inducer was added at the indicated concentration to 12 h old cultures in YEME medium. The β-lactamase activity was measured at various times after 7-ADCA addition

Results

Inducibility of S. lividans *β-lactamase producing clones*

Poor substrates for the *S. cacaoi* β-lactamase (Matagne et al. 1990) were tested for inducing activity (Table 1). 7-ADCA was the most efficient compound and was used in further experiments. Four previously constructed *S. lividans* clones were studied, which harboured a pIJ702 derivative containing the *S. cacaoi* β-lactamase gene and various lengths (from 15 to 0.35 kb) of the DNA region that is located upstream of the *bla* promoter. Induction by 7-ADCA was observed in those *S. lividans* clones in which the 2.75 kb *Sph*I-*Nco*I segment upstream of the *bla* gene was present (Fig. 1 A). The conditions for maximal induction were determined with the *S. lividans*/pMCP38 clone (see Table 2). The highest levels of amplification were obtained with inducer concentrations of 25–200 µg/ml and after 50 h of induction.

Nucleotide sequence and ORF analysis of the 2.75 kb Sph*I*-Nco*I fragment*

The β-lactamase (*bla*)-encoding segment that was previously sequenced spans a region of 1234 bp, located downstream of the *Nco*I site, transcriptional and translational signals for *bla* being located 111–113 and 201 bp

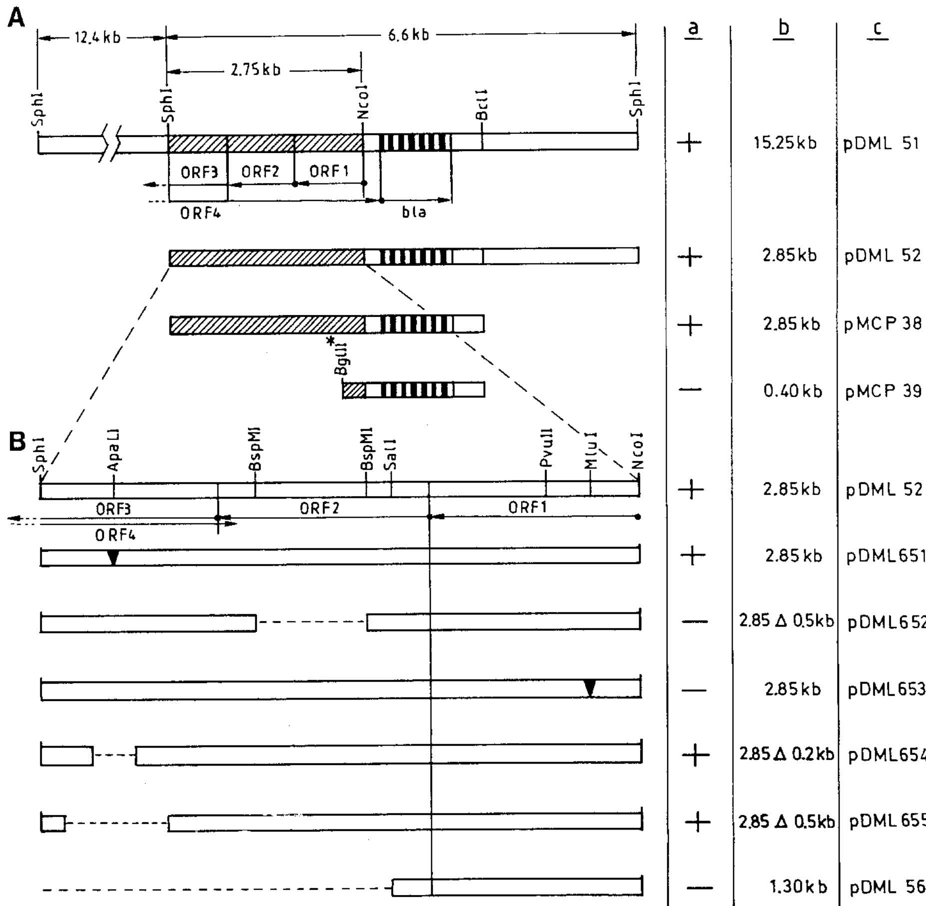

Fig. 1A and B. *Streptomyces cacaoi* DNA inserts of various *S. lividans* β-lactamase producing clones and their inducibility. Inducible clones are indicated by + in column a. Cultures at 12 h were divided in two parts, to one of which was added 25 μg/ml 7-ADCA (7-aminodesacetoxycephalosporanic acid). β-Lactamase activity was measured 50 h later in induced and non-induced cultures. The size of the *S. cacaoi* DNA segment upstream of *bla* is given in b and the designation of the clones in c. **A** Previously constructed clones. The *vertically hatched* area corresponds to the β-lactamase structural gene; the *diagonally hatched* area indicates the segment sequenced in this work. The vector is pIJ702 in every case. The site designated *Bgl*II* was added as a linker at a *Pvu*II end (Lenzini et al. 1987). **B** Insertional and deletional derivatives of pDML52. Insertions (synthetic oligonucleotides containing a *Hin*dIII site) are shown as *black arrowheads*. *Dotted lines* represent deleted segments.

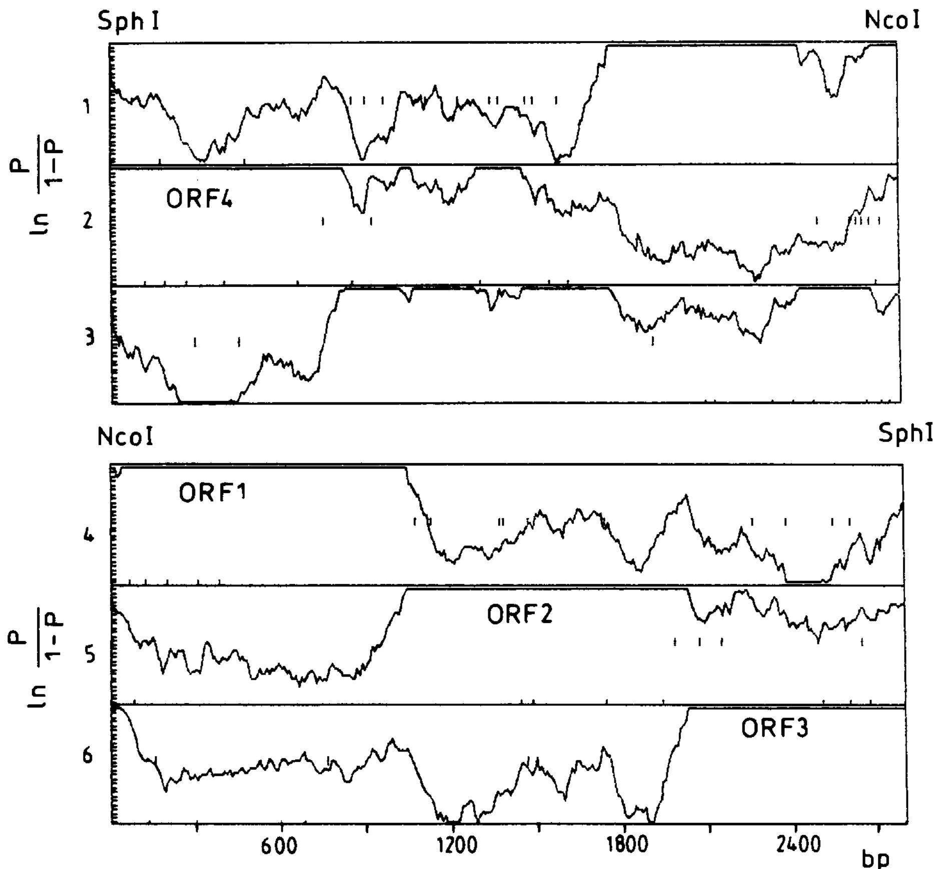

Fig. 2. Codon usage analysis of the nucleotide sequence of the *Sph*I-*Nco*I fragment. *P* expresses the probability of coding. Phases 1 to 3 are on the same strand as *bla* (transcriptional orientation *Sph*I-*Nco*I), phases 4 to 6 on the opposite strand (orientation *Nco*I-*Sph*I)

```
1                                                                                         90
GCCGTGAGCTGCGAGAACACTCTCGTCCCGGTGCGGACATACGGTCGAAGACCCGAGTGCGCCGGTTCATGCGGAAACGGAATACATGCC
          ***     -10                     -35

                              NcoI                                                       180
TGCTGGGAGCGGCTCCGCTTACAGTCGGGGCCATGGACGTGGTCAATGCCTGCCGCGCGTTCGTCAAGGTGAGCGAGCGGGGCAGCTTCA
                                M  D  V  V  N  A  C  R  A  F  V  K  V  S  E  R  G  S  F  T
                              ORF1 -->
                                                                                         270
CGGTGGGTGCGGCGGCGGCGCAGATGTCGCAGTCGGTCGCGAGCCGGCGGGTGGCGGCGCTGGAGAAGCACTTCGGCGAGCGGCTGTTCG
 V  G  A  A  A  A  Q  M  S  Q  S  V  A  S  R  R  V  A  A  L  E  K  H  F  G  E  R  L  F  D
                                                                          MluI           360
ACAGGGCGTCCCGGCGGCCCTCCCTCACCCCCTTCGGCCGGGACATGCTCCCGGCCGCCCGCCGGCTGGTACGCGTCGCCGACGTACTGG
 R  A  S  R  R  P  S  L  T  P  F  G  R  D  M  L  P  A  A  R  R  L  V  R  V  A  D  V  L  E
                                                     PvuII                               450
AGGACGAGGCCCGGGCGGCCCGCAGCCGGCCCATGCGGCTCGCGGTGCCCGCCAGCTGCACGACCGCCGAACTCGCGCGCCTGGTCGCCG
 D  E  A  R  A  A  R  S  R  P  M  R  L  A  V  P  A  S  C  T  T  A  E  L  A  R  L  V  A  D
                                                                                         540
ACTCCCGCGAACGCGACATCCGGCTCGACGTGCGGACCGCCGGCCCGGAACAGCGCGCCGAACTCGTCCGCACCCAGGAGGTGCGGGCCG
 S  R  E  R  D  I  R  L  D  V  R  T  A  G  P  E  Q  R  A  E  L  V  R  T  Q  E  V  R  A  G
                                                                                         630
GCCTGCTCGCCGTACCGCCCGACCAGGCGCTCTGGGCCGTCCCCCTCGGGCTCGCCGGGGCGGACGAGCCGCAGACCCGGCGTGTGTTCG
 L  L  A  V  P  P  D  Q  A  L  W  A  V  P  L  G  L  A  G  A  D  E  P  Q  T  R  R  V  F  V
                                                                                         720
TGGAGTCGCTGCGCCTGCGCCGCGGCGAGCCGGGCCCCGCCCGCTGCATCCGGGTCCAGCCGGAGGACGATGTTCCGCACATCCAGGGCC
 E  S  L  R  L  R  R  G  E  P  G  P  A  R  C  I  R  V  Q  P  E  D  D  V  P  H  I  Q  G  R
                                                                                         810
GGCTGGCGCGGCTGCGCGACGCCGTCGGGCTGCGCCCCGCCCAGCTGTCCGTCGCACCGGACCTGACCTCCGCCACGGCCGACGTGCTCT
 L  A  R  L  R  D  A  V  G  L  R  P  A  Q  L  S  V  A  P  D  L  T  S  A  T  A  D  V  L  S
                                                                                         900
CCTCCGACGACCTCCTGCTGTGCTCCCCCGCGCAGGCCGCCGAACTGGGCCTGTACTGGCGGCCCGTCGGCGAACTCCGGCTCGCCCGCG
 S  D  D  L  L  L  C  S  P  A  Q  A  A  E  L  G  L  Y  W  R  P  V  G  E  L  R  L  A  R  G
                                                                                         990
GCTACGTCCTGGACGCCGCGGTCGAGGCCGACGCCGAACGTCTGCGCGGGCGGCTGGCGCCGTACCTCGCGCGGGCCCTCGGCGCCACGG
 Y  V  L  D  A  A  V  E  A  D  A  E  R  L  R  G  R  L  A  P  Y  L  A  R  A  L  G  A  T  A
                                                                                        1080
CCGACCACGGGCCCGATCCCGCGACCGGAGCCGGGCCCGGGGCCGACGCCGGGACCGAGCCCGGCGCCCGTGCCGAGCCCGGCGCCCCGG
 D  H  G  P  D  P  A  T  G  A  G  P  G  A  D  A  G  T  E  P  G  A  R  A  E  P  G  A  P  E
                                                                                        1170
AAGAGGGGGCGCAGGCGTGCTGAACTCCGAGAGTCTGCTGCGCGAACTGCGCGACGCGCTGCACGAGGGCGGTCTGACCGGCTCGTTCCT
 E  G  A  Q  A  C  A
                   M  L  N  S  E  S  L  L  R  E  L  R  D  A  L  H  E  G  G  L  T  G  S  F  L
      ORF2 -->                                                                          1260
CGTCCGCGACCTGTACACCGGCGAGGAACTCGGCATCGACCCGGACACCGAACTGCCCACCGCCTCCCTGGTCAAGCTGCCCCTCGCCCT
 V  R  D  L  Y  T  G  E  E  L  G  I  D  P  D  T  E  L  P  T  A [S  L  V  K] L  P  L  A  L
                              SalI                                                      1350
CGCCACGCTGGAACGCATCCGGCTCGGGGAGGTCGACGGCGCCCAGCAGATCGAGGTCGCGCCCGGCCGGATCACCACCCCCGGCCCGAC
 A  T  L  E  R  I  R  L  G  E  V  D  G  A  Q  Q  I  E  V  A  P  G  R  I  T  T  P  G  P  T
                                           BspMI                                        1440
GGGCCTCAGCCGCTTCCGGCACCCGGCCCGCGTCGCCGTCGACGACCTGCTGTACCTGAGCACCTCGGTGAGCGACGGCACCGCCTCCGA
 G  L  S  R  F  R  H  P  A  R  V  A  V  D  D  L  L  Y  L  S  T  S  V  S  D  G  T  A  S  D
                                                                                        1530
CGCGCTCTTCGAGATCACACCGCCCGCCCAGGTCGAACAGATGGTCAGGGAGTGGGGGTTCCGTGATCTGACCGTCCGCCACTCCATGCG
 A  L  F  E  I  T  P  P  A  Q  V  E  Q  M  V  R  E  W  G  F  R  D  L  T  V  R  H  S  M  R
                                                                                        1620
GGAACTGTCCGAGACACCCGCCGAACGGTTCGAGAGCGCCGACGCCCACCTCGCACACGCCCTCGCGATCAGCGCCGGCACCAGCGGACG
 E  L  S  E  T  P  A  E  R  F  E  S  A  D  A  H  L  A  H  A  L  A  I  S  A  G  T  S  G  R
                                                                                        1710
CGGCCACCGCGTCCCCCAACTGGACGTCGCGCGCGCCAACACCGGCACCGCGCGCGCCTTCGTCGACCTGCTCGAAGCGCTGTGGGCGCC
 G  H  R  V  P  Q  L  D  V  A  R  A  N  T  G  T  A  R  A  F  V  D  L  L  E  A  L  W  A  P
                                                                                        1800
CGTCCTCACCGGACCCCGGCCCGGCAGAACGAGCCGCGCCCTCCCGCCGAAACCCGCCGCGCGGCTCCGCGAACTGATGGCCGCCAACCT
 V  L  T  G  P  R  P  G  R  T  S  R  A  L  P  P  K  P  A  A  R  L  R  E  L  M  A  A  N  L
                                                                            BspMI       1890
GCTGCGCCACCGTCTCGCCCCGGACTTCGCCTCCGACGCCGCGACCTGGTCGTCGAAGACCGGCACCCTGCTCAACCTGCGGCACGAGGT
 L  R  H  R  L  A  P  D  F  A  S  D  A  A  T  W  S  S [K  T  G] T  L  L  N  L  R  H  E  V
                                                                                        1980
GGGCGTCGTCGAGCACGCGGACGGTCAGGTGTTCGCCGTCGCCGTGCTCACCGAGTCCCAGGTGCCGGCCGACAGCCAGCCCGGCGCCGA
 G  V  V  E  H  A  D  G  Q  V  F  A  V  A  V  L  T  E  S  Q  V  P  A  D  S  Q  P  G  A  E
                                                                                        2070
GGCCCTCATGGCCCAGGTGGCCCGCCGCCTCCGCGACCGCCTCCGGGAGTGGTGACCCGCCAGCCCTGCCGAAGGGGGCCGGTCCTCGGC
 A  L  M  A  Q  V  A  R  R  L  R  D  R  L  R  E  W  *
                                                    M  T  R  Q  P  C  R  R  G  P  V  L  G
                                                  ORF3 -->                              2160
CCCGGCGTACCGTCCGCCTCAGCCCTCCGCCGCGCGGATCGCGTCCCGGTAGACGTGGGCAGCGGCGCGCAGCGCGGACTCCGGATCGAC
 P  G  V  P  S  A  S  A  L  R  R  A  D  R  V  P  V  D  V  G  S  G  A  Q  R  G  L  R  I  D
                                                                                        2250
GCCGTCCGCCTCCGCGCCGACCGCCATCTCCAGCAGCCGGTAGCCGACACCCGTCCCGCACTCCTCCGGCCGCACCGCGATCCCCGCCGC
 A  V  R  L  R  A  D  R  H  L  Q  Q  P  V  A  D  T  R  P  A  L  L  R  P  H  R  D  P  R  R
                                                                                        2340
GCGGGCGCGCGAGGCCAGCTTCGCGGCGAGGGCGAGAGCCGGCTGCCCGAGCGGAACCCCCTCCGTGACGGAGGTGCGCTCGTGCTTCTC
 A  G  A  R  G  Q  L  R  G  E  G  E  S  R  L  P  E  R  N  P  L  R  D  G  G  A  L  V  L  L
                                                                               ApaLI    2430
CTCCGCCTTGCGGCGGCGCCAGTGCGCCTCGACATCGGCGGCGGTCTCGGCCACGTCGTCACCGAAGACGTGCGGGTGCCGGTGCACCAG
 L  R  L  A  A  A  P  V  R  L  D  I  G  G  G  L  G  H  V  V  T  E  D  V  R  V  P  V  H  Q
                                                                                        2520
CTTCGTGACGAGCGTGCCGGCCATATCGTCGATCGCGAACGGCTCGTCCGCGTCGTCCTGGGCGATACGGGCGTGGAAGACGACCTGGAG
 L  R  D  E  R  A  G  H  I  V  D  R  E  R  L  V  R  V  V  L  G  D  T  G  V  E  D  D  L  E
                                                                                        2610
CAGGACGTCGCCCAACTCCTCGCGCAGCTCCCCGCGGTCACCCGCCTCGATGGCGTCGACCAGCTCGTACATCTCCTCCACGCCGTACTT
 Q  D  V  A  Q  L  L  A  Q  L  P  A  V  T  R  L  D  G  V  D  Q  L  V  H  L  L  H  A  V  L
                                                                                        2700
GACCAGATCGTGGTGGGTGCGCCGGGCGCTCCACGGGCACGCGAGGCGGATACGGTCCATGACCTCGACGACATCCAGCAGGCGTGCCCC
 D  Q  I  V  V  G  A  P  G  A  P  R  A  R  E  A  D  T  V  H  D  L  D  D  I  Q  Q  A  C  P
                                                                                        2790
GGGCAGATCGTAGGAACCCGGCAGCAGTTCGAGGTCCGGCATGTCCGCCCGCCCGGAGCCGGCGAGCCGGGCCAGCGCGTCGGTCAGCCC
 G  Q  I  V  G  T  R  Q  Q  F  E  V  R  H  V  R  P  P  G  A  G  E  P  G  Q  R  V  G  Q  P
                                                                  2860 SphI
CGGCTCCCCCTCGGCGGTCGCCACCACGACGACGGTGCGCCCCCCGTCACGCGCACACTCGGCGACCAGCGCATGC
 R  L  P  L  G  G  R  H  H  D  D  G  A  P  P  V  T  R  T  L  G  D  Q  R  M
```

downstream of the *Nco*I site, respectively. The upstream *Sph*I-*Nco*I segment of 2.75 kb (see Fig. 1A) was completely sequenced on both strands. Analysis of the nucleotide sequence by Staden's (1984) program using the codon usage table for the *S. cacaoi bla* gene as a reference revealed the presence of four putative open reading frames (Fig. 2). ORF1, 2 and 3 are oriented divergently with respect to *bla*, whereas ORF4 is located on the same strand as *bla* (see Fig. 1A). ORF1 begins at the *Nco*I site; ORF3 and ORF4 are only partially present, being interrupted by the *Sph*I site.

The nucleotide and deduced amino acid sequences of ORFs 1–3 are given in Fig. 3. ORF1, 2 and 3 appear to be disposed end-to-end with overlapping of terminator and initiator codons of adjacent genes. The complete sequence of the *Sph*I-*Nco*I fragment comprises 2746 bp, with 74.8% GC pairs. The respective lengths of ORF1, ORF2 and ORF3 (incomplete) are 981, 939 and 835 bp.

Only ORF1 and 2 are needed for induction

In order to investigate the involvement of ORFs 1–3 gene products in the induction phenomenon, constructs were prepared in which each one of the ORFs was disrupted either by insertion or by deletion. These constructions, shown in Fig. 1B, were derived from the 2.75 kb *Sph*I-*Nco*I fragment of pDML52, inserted into the *E. coli* vectors pIJ2925 or pACYC184. The modified *Sph*I-*Nco*I segments were isolated and religated with the *bla*-containing *Nco*I-*Sph*I segment also inserted in pIJ2925. A further transfer into the *Streptomyces* pIJ702 vector allowed expression in *S. lividans*. Advantage was taken of the presence of unique restriction sites *Apa*LI and *Mlu*I, in ORF3 and ORF1, respectively, to insert a 10 bp oligonucleotide containing a *Hin*dIII site, thus obtaining pDML651 and pDML653 (Fig. 1B). A segment of 480 bp flanked by two *Bsp*MI sites was removed to inactivate ORF2 (see Fig. 1B, pDML652). The final constructs were characterized by restriction analysis and used to transform *S. lividans*.

Tests for β-lactamase induction in the transformants showed that inactivation of ORF1 or of ORF2 products resulted in the loss of inducibility of *bla*, whereas inactivation of ORF3 did not influence the phenomenon. To be sure that this last observation was not due to any residual activity of the remaining N-terminal part of the ORF3 product, this N-terminal part was shortened by carrying out progressive bidirectional deletions from the *Hin*dIII site introduced into pDML651. Constructs pDML654 (200 bp deletion) and pDML655 (500 bp deletion) confirmed that ORF3 is dispensable for *bla* induction. Finally, a deletion made by a different strategy using the *Sal*I site in ORF2 (data not shown), also led to the loss of *bla* induction (see Fig. 1B, pDML56).

Moreover, inactivation of ORF1 or of ORF2 (pDML652, pDML653 and pDML56) also influences the basal (non-induced) level of β-lactamase production, which is reduced by a factor of 30 to 60 relative to the wild type. For example, under non-induced conditions, *S. lividans*/ pDML52 (ORF1^{+}, ORF2^{+}) produced

<———————————————

Fig. 3. Nucleotide sequence of the *Sph*I-*Nco*I fragment. The sequence is given in the *Nco*I to *Sph*I direction, thus showing the strand coding for ORF1, 2 and 3. It starts 120 nucleotides before the *Nco*I site and shows the transcription signals for the *bla* gene (*double underlining* and *asterisks*). Restriction sites used in the present work are also indicated. Amino acid translation delineates the identified open reading frames (ORFs). *Boxes* in ORF2 show the SXXK and KTG motifs

```
A
              1                                                    50
AMPR E.cl     MTRSYLPLNS LRAFEAAARH LSFTHAAIEL NVTHSAISQH VKTLEQHLNC
TRPBA Ps.a.   MSRDLPSLNA LRAFEAAARL HSISLAAEEL HVTHGAVSRQ VRLLEEDLGV
AMPR S.ca.    MDV....VNA CRAFVKVSER GSFTVGAAAA QMSQSVASRR VAALEKHFGE
ILVY E.c.     MDL.....RD LKTFLHLAES RHFGRSARAM HVSPSTLSRQ IQRLEEDLGQ
NODD2 R.m.    MRFRGLDLNL LVALDALMTE RKLTAAARRV KLSQPAMSAA IARLRTYFGD
 Consensus    M------LN- LRAF-A-A-- -SFT-AA--- -VS-SA-SR- V--LE--LG-

              51                                                  100
AMPR E.cl     QLFVRVSRGL MLTTEGENLL PVLNDSFDRI A.GMLDRFAN HRAQEKLKIG
TRPBA Ps.a.   ALFGRDGRGV KLTDSGVRLR DACGDAFERL R.GVCAELRR QTAEAPFVLG
AMPR S.ca.    RLFDRASRRP SLTPFGRDML PA.ARRLVRV A.DVLEDEAR AARSRPMRLA
ILVY E.c.     PLFVRDNRTV TLTEAGEELR VFAQQTLLQY Q.QLRHTIDQ QGPSLSGELH
NODD2 R.m.    ELFSMQGREL IPTPRAEALA PAVRDALLHI QLSVIAWEPI NPAQSDRRFR
 Consensus    -LF-R--R-- -LT--GE-L- PA--D-L-R- ---V------ --A-----L-

B
              1                                                    50
S.ca.         MDV....VNA CRAFVKVSER GSFTVGAAAA QMSQSVASRR VAALEKHFGE
E.cl.         MTRSYLPLNS LRAFEAAARH LSFTHAAIEL NVTHSAISQH VKTLEQHLNC
C.f.          MTRSYIPLNS LRAFEAAARH LSFTRAAIEL NVTHSAISQH VKSLEQQLNC
R.c.          MDRPDLPLNA LRVFEVAMRQ GSFTKAAIEL RVTQAAVSHQ VARLEDLLGT
Consensus     M-------N- -R-F------ -SFT--A--- -------S-- V--LE--L--

              51                                                  100
S.ca.         RLFDRASRRP SLTPFGRDML PA.ARRLVRV ADVLEDEARA ARSRPMRLAV
E.cl.         QLFVRVSRGL MLTTEGENLL PVLNDSFDRI AGMLDRFANH RAQEKLKIGV
C.f.          QLFVRGSRGL MLTTEGESLL PVLNDSFDRM AGMLDRFATK QTQEKLKIGV
R.c.          ALFLRTSQGL IPTDEGRLLF PVLEHGFDAM SRVLDRLGGR RDIEVLKVGV
Consensus     -LF-R-S--- --T--G--LL P-----F--- ---LD----- -----L---V

              101                                                 150
S.ca.         PASCTTAELA RLVADSRERD IRLDVRTAGP EQRAELVRTQ ...EVR..AG
E.cl.         VGTFATGVLF SQLEDFRRGY PHIDLQLSTH NNRVDPAAEG LDYTIRYGGG
C.f.          VGTFAIGCLF PLLSDFKRSY PHIDLHISTH NNRVDPAAEG LDYTIRYGGG
R.c.          NTTFAMCWLM PRLEAFRQAH PQIDLRISTN NNRVEILREG LDMAIRFGTG
Consensus     --------L- ---------- ---D------ --R------- ----IR---G

              151                                                 200
S.ca.         LLAVPPDQAL WAVPLG.LAG ADEPQTRRVF VESLR..LRR GEPGPARCIR
E.cl.         AWHGTEAEFL CHAPLAPLCT PDIAASLHSP ADILRFTLLR SYRRDEWTAW
C.f.          AWHDTDAQYL CSALMSPLCS PTLASQIQTP ADILKFPLLR SYRRDEWALW
R.c.          GWTGHDAIPL AEAPMAPLCA PGLASRLLHP SDLGQVTLLR SYRSAEWPGW
Consensus     ---------L -------L-- ---------- -D-----L-R ------W--W

              201                                                 250
S.ca.         VQPEDDVPHI QG.RLARLRD AVGL.RPAQL SVAPDLTSAT ..ADVLSSDD
E.cl.         MQAAGEHPPS PTHRVMVFDS SVTMLEAAQA GVGIAIAPVD MFTHLLASER
C.f.          MQAAGEAPPS PTHNVMVFDS SVTMLEAAQA GMGVAIAPVR MFTHLLSSER
R.c.          FEAAGVPCPP VTGPVFDSSV ALAELATSGA GV..ALLPIS MFESYIAQGR
Consensus     ---------- ---------- ---------- ---------- ----------

              251                                                 300
S.ca.         LLLCSPAQAA ELGLYWRPVG ELRLARGYVL DAAVEADAER LRGRLAPYLA
E.cl.         IVQPFATQ.I ELGSYWLTRL QSRAETPAMR EFSRWL.VEK MKK.......
C.f.          IVQPFLTQ.I DLGSYWITRL QSRPETPAMR EFSRWL.TGV LHK.......
R.c.          LAQPFGVT.V SVGRYYLAWP SDRPATSAMS TFSRWL.TGQ SAE.......
Consensus     ---------- --G-YW---- --R------- ---------- ----------

              301                                         343
S.ca.         RALGATADHG PDPATGAGPG ADAGTEPGAR AEPGAPEEGA QAC
E.cl.         .......... .......... .......... .......... ...
C.f.          .......... .......... .......... .......... ...
R.c.          .......... .......... .......... .......... ...
Consensus     ---------- ---------- ---------- ---------- ---
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Fig. 4. A Alignment of the N-terminal parts of the ORF1 product and of several regulatory proteins. The proteins aligned here do not constitute an exhaustive list. AmpR (Honoré et al. 1986), IlvY (Wek and Hatfield 1986), TrpBA (Chang et al. 1988) and NodD2 (Egelhoff et al. 1985) are some examples among numerous well-known DNA-binding proteins. The helix-turn-helix motif as delimited by Henikoff et al. (1988) is *boxed*. The consensus is derived from positions in which the *S. cacaoi* residue is identical to that in at least two other sequences. E.cl., *Enterobacter cloacae*; Ps.a., *Pseudomonas aeruginosa*; S.ca., *S. cacaoi*; E.c., *Escherichia coli*; R.m., *Rhizobium meliloti*. **B** Alignment of the entire ORF1 product with the AmpR activator/repressor of Enterobacteria. E.cl., *E. cloacae* (Honoré et al. 1986); C.f., *Citrobacter freundii* (Lindquist et al. 1989); R.c., *Rhodopseudomonas capsulata* (Campbell et al. 1989). The consensus was derived as in **B**

3 µg of β-lactamase/ml of culture, *S. lividans*/pDML653 ($ORF1^-$, $ORF2^+$) produced 0.05 µg/ml and *S. lividans*/pDML652 ($ORF1^+$, $ORF2^-$) 0.07 µg/ml. This means that ORF1 and ORF2 products are both needed to exert a positive regulatory effect on *bla*, even in the absence of inducer.

ORF1 product is a DNA-binding protein related to the AmpR regulatory protein of Enterobacteria

All four identified ORFs were used to screen the EMBL database (Version 27) with the FASTA peptide program. No significant similarity with any known proteins was detected for the ORF3 and ORF4 products. The polypeptide encoded by ORF1 is 326 amino acids long and presents features of a DNA-binding protein of the helix-turn-helix type. The N-terminal part is related to the corresponding region in numerous regulatory proteins of this type (Fig. 4A) and this relationship holds for the entire sequence of the AmpR regulatory protein of several Enterobacteria (Fig. 4B). The degree of strict identity between ORF1 and AmpR activator/repressors reaches levels of 22, 20 and 18% in comparisons with the *E. cloacae, Citrobacter freundii* and *Rhodopseudomonas capsulata* sequences, respectively. This is far below the scores found for comparisons between the enterobacterial proteins themselves (81% strict identity between *E. cloacae* and *C. freundii* AmpR proteins) or between the enterobacterial and the *R. capsulata* proteins (46%).

The ORF2 product shows some features of a penicillin-recognizing protein

ORF2 encodes a protein of 312 amino acids, which possesses an SXXK motif and a KTG triplet (see Fig. 3). These two motifs are characteristic of penicillin-recognizing enzymes (Joris et al. 1988). The comparison score (Goad and Kanehisa 1982) with this type of proteins however remains low. For penicillin-recognizing enzymes, the best score, obtained with the *E. coli* TEM-1 β-lactamase, is −104.0, more than 5 standard deviation units (SDU) from the mean score obtained for homologies occurring by chance. Hydrophobic cluster analysis (Gaboriaud et al. 1987; Henrissat et al. 1990) suggests that this polypeptide could share most of the secondary structures common to the penicillin-recognizing proteins (result not shown).

Discussion

The β-lactamase gene of *S. cacaoi* was cloned and expressed in *S. lividans* (Lenzini et al. 1987) and shown to be inducible by β-lactams provided the original upstream region of 2.75 kb (fragment *Sph*I-*Nco*I, Fig. 1A) was present in the clone. This fragment was shown to contain four ORFs. Three of them (ORF1, ORF2 and ORF3) cover the whole *Sph*I-*Nco*I segment and are transcribed divergently from the structural *bla* gene, whereas ORF4 is located on the same DNA strand as *bla*. The terminator codons of ORF1 and ORF2 overlap with the initiator codons of ORF2 and ORF3, respectively.

ORF3 and ORF4 are interrupted by the *Sph*I site and are thus incomplete in our clones. ORF4 has lost its initiation region and is very probably not transcribed. ORF3, which lacks its terminal part, could retain some activity, but deletions (pDML654, pDML655) or an insertion (pDML651) in these two ORFs do not influence the induction of *bla* (see Fig. 1B, column a). Their products are thus not required by the cell to respond to the presence of β-lactams.

The ORF1 product is a DNA-binding protein belonging to the LysR family (Henikoff et al. 1988) and is particularly related to AmpR, a protein controlling the expression of the *ampC* cephalosporinase gene in Enterobacteria. In the presence of an intact ORF2, the ORF1 product exerts a positive regulatory effect on *bla*. Its inactivation leads to a 30- to 60-fold decrease of the basal (non-induced) level of β-lactamase production and to the loss of *bla* inducibility. ORF1 product thus acts as an activator even in the absence of inducer. It can be supposed that this protein binds to the promoter region of the *bla* structural gene. The −35 box of this promoter is located only 73 bp upstream of the ATG initiator codon of ORF1 (see Fig. 3). There is a 197 bp stretch between the ATG initiator codon of ORF1 and *bla* (Lenzini et al. 1987). This situation is similar to those existing in *E. cloacae* or *C. freundii* and also in *B. licheniformis*. In the former, *ampR* and *ampC* genes are oriented divergently and separated by an intercistronic region of 131 bp (Honoré et al. 1986; Lindquist et al. 1989). In *B. licheniformis*, the corresponding distance between the structural *blaP*/*penP* gene and the regulatory *blaI*/*penI* gene is 364 bp (Himeno et al. 1986). Unlike the ORF1 product however, AmpR acts as a repressor in the absence and as an activator in the presence of β-lactams (Lindberg et al. 1985), whereas BlaI behaves as a purely negative regulatory element (Lampen et al. 1988).

The positive regulatory effect of the ORF1 product depends on the presence of an intact ORF2. The ORF1 protein alone (pDML652) is not sufficient either to promote the wild-type basal level of *bla*, or to allow induction by β-lactams. The polypeptide encoded by ORF2 presents some similarity with penicillin-recognizing proteins. The comparison score with TEM-1 β-lactamase (−104; significance in SDU, 7) is far below the value obtained between the true class A β-lactamases R-TEM from *E. coli* and BlaP from *B. licheniformis* (−462; SDU 27) but significantly better than the score found between TEM-1 and an unrelated subtilisin (−35; SDU 0.7). These data indicate a weak but significant relationship between the ORF2 product and the penicillin-recognizing enzymes. With respect to gene organization, ORF2 occupies exactly the position of the sensor *blaR* in *B. licheniformis* (Kobayashi et al. 1987). Like BlaR, the ORF2 product seems to share some secondary structure with penicillin-binding proteins, but this hydrophobic cluster analysis cannot be considered as compelling evi-

dence by itself. The ORF2 protein lacks the transmembrane segments present in BlaR (Zhu et al. 1990). Moreover, the N-terminus of the ORF2-encoded protein is clearly not a signal sequence and there is no indication that this protein might be exported and act as an external receptor. It is obvious, in fact, that the observed effect of the ORF2 product on the non-induced expression of *bla* is independent of this putative penicillin-recognizing capacity. An alternative hypothesis would suggest that the ORF1 DNA-binding protein is active only after interaction with, or modification by, the ORF2 product. This model is in agreement with a cytoplasmic localization and with the requirement of ORF2 for *bla* expression in non-induced cultures.

Further investigations are currently in progress in order to assess the exact role of each of these products in the induction phenomenon. However, it is already obvious that the regulation of the *bla* gene in the gram-positive *S. cacaoi* differs from the regulation in *Bacillus*, by involving an activator AmpR-like protein, and not a *BlaI*-like repressor. It also differs from enterobacterial *ampC* regulation, where the DNA-binding element AmpR is a repressor/activator protein. The gene pair *ampR-bla* present in *S. cacaoi* is also found in the gram-negative *Rhodospirillacea capsulata* (Campbell et al. 1989), indicating that dissemination of the β-lactamase encoding genes *ampC* or *bla* through the bacterial world occurred independently of the regulatory genes.

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