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J. Magdalena · C. Gérard · B. Joris · M. Forsman
J. Dusart

The two β-lactamase genes of *Streptomyces cacaoi*, *blaL* and *blaU*, are under the control of the same regulatory system

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Abstract The production of β-lactamase in *Streptomyces cacaoi*, which contains two β-lactamase-encoding genes, *blaL* and *blaU*, is inducible by β-lactam compounds. The two genes have been cloned independently in *S. lividans* TK24, a β-lactamase-negative species. The *blaU* clone did not respond to the presence of β-lactams, whereas the *blaL* clone appeared to be inducible in *S. lividans*. The latter clone contains two open reading frames, *blaA* and *blaB*, located just upstream of but transcribed divergently from *blaL*, which were shown to be required for the production as well as the induction of BlaL. The deduced BlaA protein belongs to the LysR family of transcription regulators. In order to examine the role of BlaA in regulation, we here report on overexpression of a GST-BlaA fusion protein in *Escherichia coli* and its use for antibody preparation. The GST-BlaA fusion protein was partially purified and bandshift assays showed that it bound the 197-bp *blaL*-*blaA* intergenic region. The BlaA DNA binding-site was further restricted to a 30-bp sequence containing a T-N_{11}-A motif, a characteristic of LysR-type promoters. Another T-N_{11}-A motif upstream of the *blaU* gene was also shown to bind BlaA. The affinities of these two T-N_{11}-A motifs in BlaA binding were comparable. A plasmid bearing the *blaU* structural gene and the *blaA*-*blaB* regulatory region was constructed and shown to confer on an *S. lividans* host the capacity to produce inducible β-lactamase. It can thus be concluded that the *S. cacaoi* *blaL* and *blaU* genes are controlled by the same regulatory system.

Key words Bandshift assay · GST-fusion protein · β-Lactamase regulation · LysR-type activator · *Streptomyces*

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J. Magdalena[1] · C. Gérard · B. Joris · J. Dusart (✉)
Centre d'Ingénierie des Protéines, Université de Liège,
Institut de Chimie, B6,
B-4000 Sart-Tilman (Liège 1), Belgium

M. Forsman
Department of Cell- and Microbiology,
National Defence Research Establishment, Umeå, Sweden

Present address:
[1]Laboratoire des mécanismes moléculaires de la pathogénie bactérienne, Institut Pasteur, 1, rue Calmette, B.P. 245, F-59019 LILLE cédex, France

Introduction

The ability of bacteria to produce β-lactamase is causing increasing problems in the clinical treatment of many infectious diseases (Neu 1992). In some organisms, this production is regulated at the level of gene expression, in response to the presence of β-lactam compounds in the environment. Two different modes of control of the β-lactamase genes have been studied for many years, one in enterobacteria, the second in *Bacillus* and *Staphylococcus* (see Bennett and Chopra 1993, for a mini-review). Regulation of β-lactamase was also reported in *Streptomyces cacaoi* (Forsman et al. 1989) and the regulatory region responsible has been identified (Lenzini et al. 1992) and shown to differ in some aspects from those previously described.

S. cacaoi possesses two different unlinked β-lactamase genes (Magdalena et al. 1992): *blaL* (Lenzini et al. 1987, 1988) and *blaU* (Jaurin et al. 1988; Forsman et al. 1990), the products of which are 49% identical. Both genes are inducible by β-lactam compounds (Forsman et al. 1989; Forsman 1991). However, when *blaL* and *blaU* were cloned in *S. lividans*, only *blaL* responded to β-lactam inducers (Lenzini et al. 1992).

Sequence analysis and genetic studies of the *blaL* regulatory region in the *S. lividans* clone demonstrated that two adjacent genes, *blaA* and *blaB*, were required, not only for induction, but also for basal expression of *blaL* (Lenzini et al. 1992). It has been shown that BlaB is attached to the internal face of the cytoplasmic membrane. Some possible functions for this protein have been tested (β-lactamase, penicillin-binding and

D-aminopeptidase activities, target of phosphorylation), but its activity remains unknown (Magdalena et al. 1995).

The primary structure of BlaA, as deduced from the nucleotide sequence of the gene, indicated that this protein is a member of the LysR family of regulator proteins (Lenzini et al. 1992; Urabe and Ogawara 1992). Urabe and Ogawara (1992) showed that a 173-bp DNA fragment – located between the *blaL* and *blaA* translation initiation codons – can bind (a) protein(s) present in total protein extracts from an *S. lividans* clone containing an intact *blaA* gene. In the present work, we constructed a GST-BlaA fusion protein and obtained antibody directed against it. This allowed the identification of the BlaA binding site in the *blaL* promoter region. It corresponds to a T-N_{11}-A motif like that described in Goethals et al. (1992). The same motif was discovered in the *blaU* promoter region and it was shown that *blaU* also can be regulated by BlaA.

Materials and methods

Enzymes, antibiotics and chemicals

Restriction endonucleases were obtained from Life Technologies (Merelbeke, Belgium), Boehringer Mannheim (Brussels, Belgium), Eurogentec (Seraing, Belgium) or Pharmacia Biotech. (Rosendaal, The Netherlands) and the Goldstar DNA polymerase was from Eurogentec (Seraing, Belgium). Thiostrepton was a gift of S. J. Lucania (Squibb and Sons, Princeton, N.J., USA) and ampicillin was obtained from Sigma Chemie (Bornem, Belgium). [α-^{35}S]dATP (1000 Ci/mmol, 10 μM) was purchased from Amersham Belgium (Gent, Belgium). Nitrocefin was from Unipath (Gent, Belgium).

Strains, plasmids and growth conditions

Strains and plasmids are described in Table 1. *Streptomyces* liquid cultures were grown with vigorous orbital shaking, at 28°C in YEME medium (Hopwood et al. 1985). Selective pressure was maintained during the growth of the strains *S. lividans* ML2 (*blaA*$^+$), *S. lividans* MJ13 (*blaA*$^-$), and *S. lividans* CG1 (*blaU*$^+$ *blaA-blaB*) by the addition of thiostrepton (50 μg/ml). *Escherichia coli* was cultivated at 25°C in LB broth. The *E. coli* plating medium was LB agar (Sambrook et al. 1989).

DNA and protein methods

The DNA recombinant procedures applied were essentially those described in Sambrook et al. (1989) and in Hopwood et al. (1985). PCR amplification was carried out as in Saiki et al. (1988). The gene sequencing method of Sanger et al. (Sanger et al. 1970, 1977) was used with fluorescent primer. The analyses were performed on an EMBL automated DNA sequencer (Ansorge et al. 1987). Protein concentration was estimated by the method of Bradford (1976). SDS-PAGE and Western blotting were as described in Laemmli and Favre (1973) and Sambrook et al. (1989), respectively. The β-lactamase activity was estimated by the method of O'Callaghan et al. (1972) using nitrocefin as substrate.

S. lividans ML2 (*blaA*$^+$) protoplasts, obtained as in Hopwood et al. (1985), were lysed by high speed centrifugation ($37\,000 \times g$, 30 min) to separate membrane proteins (pellet) from cytoplasmic proteins (supernatant) for Western blotting. *S. lividans* ML2 (*blaA*$^+$) and MJ13 (*blaA*$^-$) cytoplasmic proteins were purified as described by Lindquist et al. (1989), for use in bandshift assays.

GST-BlaA and GST overproduction in *E. coli*

The PCR amplified *blaA* gene was cloned into the *E. coli* expression vector pGEX5X1, and the recombinant plasmid was named pDML681. *E. coli* DH5α containing pDML681 (*E. coli* MJ1) and *E. coli* DH5α harbouring the original pGEX5X1 (*E. coli* MJ2) were grown at 25°C to OD_{600} = 1, and induced with IPTG (100 μM final concentration). *E. coli* MJ2 produced about 70 μg of GST-BlaA (60 kDa) per ml of culture, 10 μg of which were soluble and *E. coli* MJ1 yielded 200 μg of GST (26 kDa), 100% of which was soluble.

The GST-BlaA protein was partially purified from the soluble fraction of MJ2 (GST detection and GST purification module kits; Pharmacia, Rosendaal, The Netherlands). About 1 μg of purified

Table 1 Strains and plasmids used

Strains Designation	Properties	Source	Reference
S. cacaoi KCC-S0352	Wild type	Kaken Chemical Co (Tokyo, Japan)	
S. lividans CG1	From TK24, harbours pDML1158	University of Liège (CIP)	This paper
S. lividans ML2	From TK24, harbours pDML52	University of Liège (CIP)	Lenzini et al. (1988)
S. lividans MJ13	From TK24, harbours pDML653	University of Liège (CIP)	Lenzini et al. (1992)
S. lividans TK24	From *S. lividans* 66 str-6, cured of natural plasmids SLP1 and SLP2	John Innes Centre (Norwich, U.K.)	Hopwood et al. (1983)
E. coli DH5α		Life Technologies (Merelbeke, Belgium)	
E. coli MJ1	Harbours PGEX5X1	University of Liège (CIP)	This paper
E. coli MJ2	Harbours pDML681	University of Liège (CIP)	This paper
Plasmids			
pβC	Plasmid containing the *blaU* primary clone	University of Umeå (Umeå, Sweden)	Jaurin et al. (1988)
pDML52	pIJ702 + *bla*(*L-ABC*)	University of Liège (CIP)	Lenzini et al. (1988)
pDML72	pBR322 + *bla*(*L-ABC*)	University of Liège (CIP)	Lenzini et al. (1987)
pDML653	pDML52 with *blaA*$^-$	University of Liège (CIP)	Lenzini et al. (1992)
pDML681	pGEX5X1 containing the PCR-amplified *blaA* gene	University of Liège (CIP)	This paper
pDML1158	pIJ702 + *blaU*, *blaAB*	University of Liège (CIP)	This paper
PGEX5X1	Expression plasmid for proteins fused to GST	Pharmacia (Rosendaal The Netherlands)	
pIJ702	*Streptomyces* vector	John Innes Centre, (Norwich, U.K.)	Katz et al. (1983)

material was obtained per ml of culture (10% yield). The same purification process yielded 100% (200 µg per ml of culture) of GST from the soluble protein fraction of MJ1.

Polyclonal antibodies: generation and use

The GST-BlaA preparation used was extracted from an SDS-PAGE gel, by the procedure of Bikel et al. (1983). Rabbit anti-GST-BlaA antibodies were made by Gamma S.A. (Liège, Belgium), by injecting four 300-µg samples of protein, at 15-day intervals. The preimmune serum, taken before immunization, was also made available. Secondary antibodies were alkaline phosphatase conjugates directed against rabbit IgG (Sigma Chemie, Bornem, Belgium). The primary and secondary antibodies were diluted 1000- and 5000-fold, respectively.

Bandshift assay

For radiolabelling DNA, PCR-amplified DNA fragments (1 µl) were purified and diluted 10-fold, mixed for the second time with the same primers (900 ng of each primers; Fig. 1), 10 mM dTTP, dGTP, dCTP mixture (2 µl), [α-^{35}S]dATP (10 µl), 10 × Goldstar PCR buffer (10 µl), 2 mM $MgCl_2$ (6 µl), DMSO (10 µl), Goldstar DNA polymerase (1 U), and H_2O to 100 µl, then covered with mineral oil and submitted to 15 amplification cycles (94° C, 1 min; 65° C, 1 min; 72° C, 10 s) in a Biometra apparatus (Eurogentec, Seraing, Belgium). Then 2 µl of 10 mM unlabelled dATP was added and a polymerization step was performed for 10 min. The labelled DNA fragments were purified using the Qiaquick Spin PCR Purification kit (Qiagen, Westburg b.v., Leusden, The Netherlands).

The 30-bp DNA fragments used in the competition bandshift assays were obtained by incubating for 10 min at 72° C the two complementary oligonucleotides: nos. 12 and 13 for the fragment including the T-N_{11}-A motif upstream of *blaL*, nos. 14 and 15 in the case of *blaU* (Fig. 1).

The incubation mixture for protein-DNA binding contained 50% (w/v) glycerol (2 µl), 1 M NaCl (2 µl), buffer B10× (250 mM HEPES pH 7.5, 1 mM EDTA, 50 mM dithiotreitol and 500 mM KCl) (1 µl), poly(dI.dC)(dI.dC) (Pharmacia, Rosendaal, The Netherlands) (2 µg), protein sample (11 µl maximum), and ^{35}S-labelled DNA fragment (2 µl). After 20 min at 30° C, the incubation was stopped by adding to each sample 2 µl of buffer C10× (250 mM Tris-HCl pH 7, 0.2% w/v bromophenol blue, 0.2% v/v xylene cyanol and 40% w/v glycerol).

The samples were analysed by electrophoresis on a 5% polyacrylamide gel prepared in TGE buffer (40 mM TRIS-Hcl pH 8.5, 0.4 M glycine, 2 mM EDTA). After electrophoresis the gel was vacuum dried at 80° C for 30 min, and then exposed to X-ray film for 24 or 48 h.

Results

Immunodetection of BlaA in the cytoplasm of *blaA*$^+$ cells

GST-BlaA was detected in total protein extracts from IPTG-induced *E. coli* MJ2 by anti-GST-BlaA antibody, but not by the preimmune serum. GST was detected by both sera. Moreover, GST-BlaA was also present in the non-induced *E. coli* MJ2 protein sample.

Total protein extract, membrane and cytoplasmic protein fractions of the *S. lividans* ML2 strain (*blaA*$^+$) were analysed by Western blotting with the anti-GST-BlaA antibody. A reacting protein was found in the total extract and in the cytoplasmic fraction. The protein had a molecular weight of about 34 kDa in agreement with the calculated size of the protein encoded by *blaA*. No reaction was observed with the membrane fraction (data not shown).

Binding of purified GST-BlaA to DNA

The binding of BlaA to DNA was assayed by bandshift experiment, using a 179-bp PCR-generated radiolabelled fragment of the *blaL-blaA* intergenic region (Fig. 1). A shift was observed with the *S. lividans* ML2 (*blaA*$^+$) protein extract. The intensity of the shifted band increased with the amount of extract added. The *S. lividans* MJ13 (*blaA*$^-$) total protein extract did not cause any shift (data not shown). Increasing quantities (20, 200 or 600 ng) of the partially purified GST-BlaA protein and the partially purified GST (5 µg) were also assayed. No shift was observed with the GST sample and the lowest concentration of the GST-BlaA extract. The shift appeared when the 200-ng GST-BlaA sample was used and became more intense with the 600-ng sample (Fig. 2). When the GST-BlaA sample was incubated for 20 min at 30° C with the unlabelled 50-bp fragment (Fig. 1) prior to the incubation with the labelled 80-bp fragment at a fragment ratio (unlabelled/labelled) of 40/1 the amount of the shifted fragment decreased and no shift was seen at a ratio of 80/1 (data not shown). Given that non-specific competitor [2 µg of poly(dI.dC)(dI.dC)] was included in each reaction mixture, and that BlaA$^-$ protein extract was used as negative control, the competition described is clearly specific.

Localization of the BlaA-binding DNA segment

In order to localize the BlaA binding site within the intergenic region, several fragments of different sizes (Fig. 1A) were amplified by PCR. Their respective DNA sequences were verified and they were used in bandshift assays, either as radiolabelled probes or as unlabelled competitor of a labelled probe. The labelled 126-bp fragment was retarded to the same extent as the 179-bp fragment, by the *S. lividans* ML2 (*blaA*$^+$) total protein extract, but not by the *S. lividans* MJ13 (*blaA*$^-$) extract (data not shown).

With the 56-, 71- and 124-bp fragments, no specific bandshift was observed with the ML2 extract. The portion of DNA which binds specifically BlaA was thus located partly on the 71-bp and partly on the 56-bp fragment. In agreement with this conclusion, the labelled 80-, 65-, 65′- and 50-bp fragments were shifted by the *S. lividans* ML2 (*blaA*$^+$) extract and not by the *S. lividans* MJ13 (*blaA*$^-$) extract. The intensities of the shifted bands obtained with the 50- and the 65′-bp fragments were weaker.

All these fragments (80, 65, 65′ and 50 bp) contain a T-N_{11}-A motif like that described in Goethals et al. (1992). This motif is situated 55 bp upstream of the transcription start site of the *blaL* gene and 40 bp

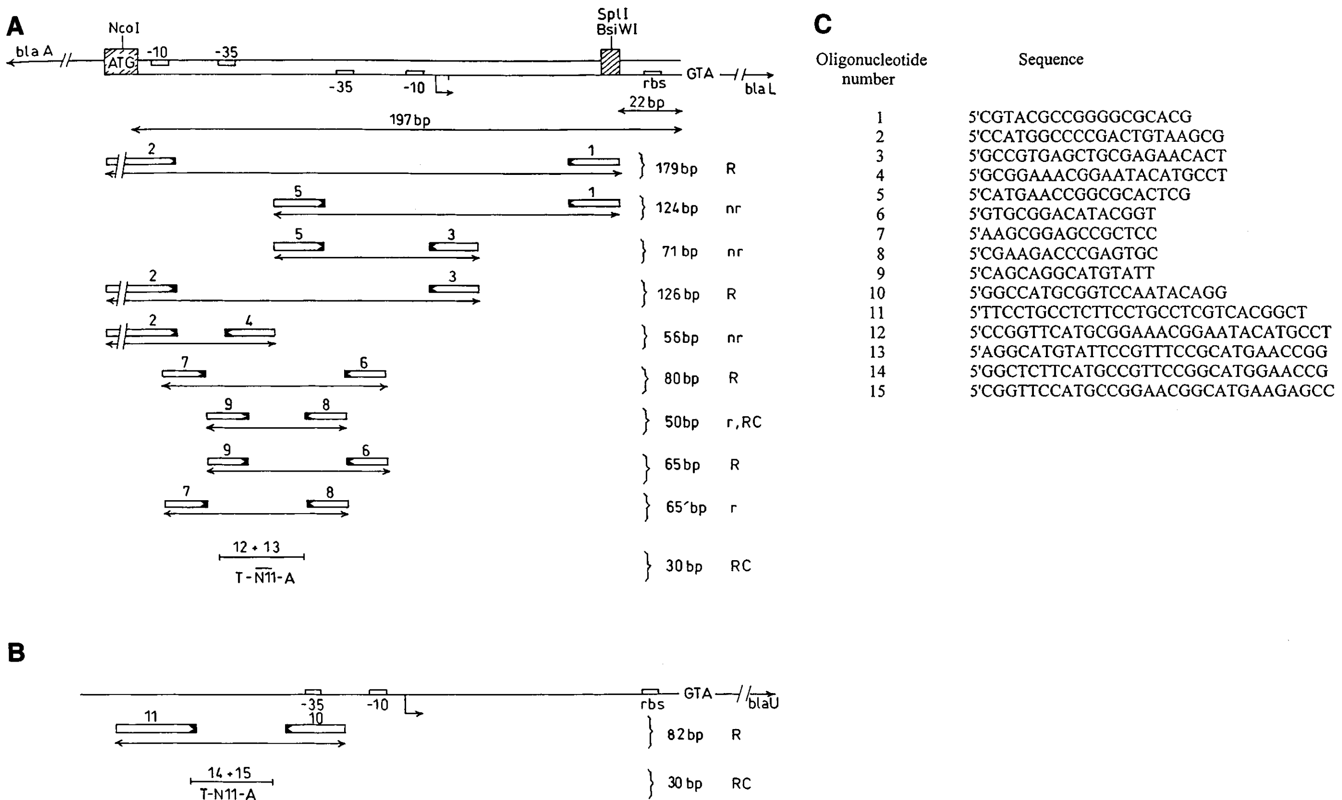

Fig. 1A, B, C Schematic representation of the 197-bp DNA fragment separating the translation initiation codons of the diverging *blaL* and *blaA* genes (**A**) and of the blaU upstream region (**B**). **A**, **B** The *open bars* numbered from 1 to 11 indicate the localization of the pairs of oligonucleotides used in the PCR reactions. The amplified fragments correspond to the *arrowed lines*, their sizes are given on the right. 'R' indicates BlaA-retarded fragments, 'r' indicates that the retarded band is less intense. 'nr' corresponds to non-retarded fragments and 'RC' indicates fragments that compete with the 80-bp fragment for BlaA binding. The two 30 bp-DNA fragments were synthesized by hybridization of two complementary oligonucleotides: nos. 12 and 13 for the *blaL* upstream region, and nos. 14 and 15 for the *blaU* upstream region. The sequences of the oligonucleotides 1 to 15 are given in **C**. The −10 and −35 promoter regions of *blaL* (Urabe et al. 1990), *blaA* (Urabe and Ogawara 1992) and *blaU* (Forsman 1991) genes are shown, as well as the transcription starts and the ribosome-binding sites of *blaL* and *blaU* (rbs). For BlaA, the transcription start coincides with the translation start (Urabe and Ogawara 1992)

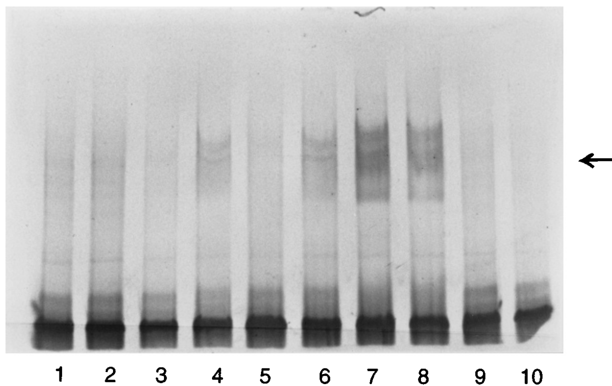

Fig. 2 Bandshift assay with the GST-BlaA protein. The 179-bp DNA segment separating *blaL* from *blaA* (Fig. 1) was synthesized by PCR and labelled with [α-^{35}S]dATP. The protein samples tested were partially purified GST-BlaA: various amounts of 20 ng (lanes 3, 5); 200 ng (lanes 4, 6) and 600 ng (lanes 7, 8). Controls are in lanes 1 and 2 (no protein) and lanes 9 and 10 (5 μg of GST). Incubations were carried out in the absence (lanes 1, 3, 4, 7, 9) or presence (lanes 2, 5, 6, 8, 10) of 100 mM NaCl. The *arrow* indicates the DNA-GST-BlaA complex

upstream of the transcription-translation start site of *blaA*. A 30-bp fragment, centred on this T-N_{11}-A motif, was used in excess in a competition assay with the labelled 80-bp fragment. This 30-bp segment competed for binding, causing the disappearance of the shifted band (Fig. 3, lanes 6 and 7).

A T-N_{11}-A motif is also present upstream of *blaU*

A T-N_{11}-A motif was also discovered upstream of the *blaU* gene (56 bp from the transcription start site). It shares 80% sequence identity with the T-N_{11}-A motif present upstream of *blaL* (Fig. 6). A second 30-bp fragment, centred on the T-N_{11}-A found upstream of *blaU* and 60% identical to the *blaL* 30-bp fragment, was used in competition bandshift assay. The *blaU* 30-bp fragment also competed with the labelled 80-bp DNA fragment (Fig. 3, lanes 8 and 9). The binding of BlaA to the *blaU* upstream region has been confirmed using as a probe an 82-bp segment located upstream of *blaU* (Fig. 1B). This probe was retarded by a $BlaA^+$ protein extract, while the $BlaA^-$ protein extract was without

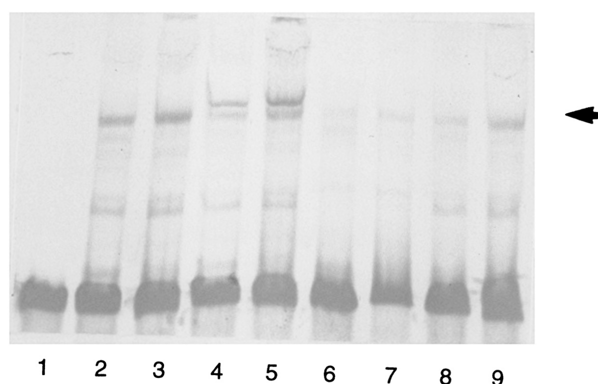

Fig. 3 Competition-bandshift assay with two 30-bp fragments centred on T-N_{11}-A motifs found upstream of *blaL* and *blaU*. The 80-bp fragment (see Fig. 1A) was synthesized by PCR and labelled with [α-^{35}S]dATP. Unlabelled 30-bp fragments, corresponding to *blaL* or *blaU* promoter regions, were obtained as described in the legend to Fig. 1. Total protein extracts were preincubated with a 30-bp fragment before mixing with the labelled probe. The ratio of 30-bp fragment to 80-bp probe was 60. The control is in lane 1 (no protein extract, no 30-bp competitor). Extracts from *S. lividans* MJ13 (*blaA*$^-$) are in lanes 2 (2 μl) and 3 (6 μl) and show the level of unspecific labelling. Extracts from *S. lividans* ML2 (*blaA*$^+$) are in lanes 4, 6, 8 (2 μl) and 5, 7, 9 (6 μl). The total protein concentration of extracts was about 10 μg/μl. Competition with the *blaL* 30-bp fragment is shown in lanes 6 and 7, and with the *blaU* 30-bp fragment in lanes 8 and 9. The specific labelling appears in lanes 4 and 5, and is absent from lanes 6–9. The *arrow* indicates the level of the DNA-BlaA complex

retarding effect. Moreover, it was found that the affinity of the *blaU* T-N_{11}-A motif for BlaA was of the same order of magnitude (1.5 to 2 times lower) as the affinity of the *blaL* T-N_{11}-A motif (data not shown).

blaU is inducible by β-lactam when cloned together with the *blaA-blaB* region

The *blaU* gene and the *blaA-blaB* regulatory region (*blaL* being inactivated) were cloned on the same

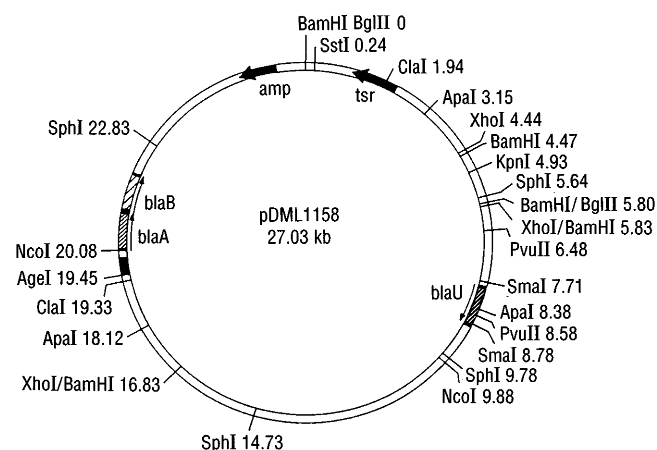

Fig. 4 Map of pDML1158. pDML1158 contains (a) the *blaA* and *blaB* regulatory genes, and the inactivated *blaL* structural gene from pDML72; (b) an 11-kb *Bam*HI fragment including *blaU*, obtained from pβC. The *Streptomyces* origin of replication is from pIJ702

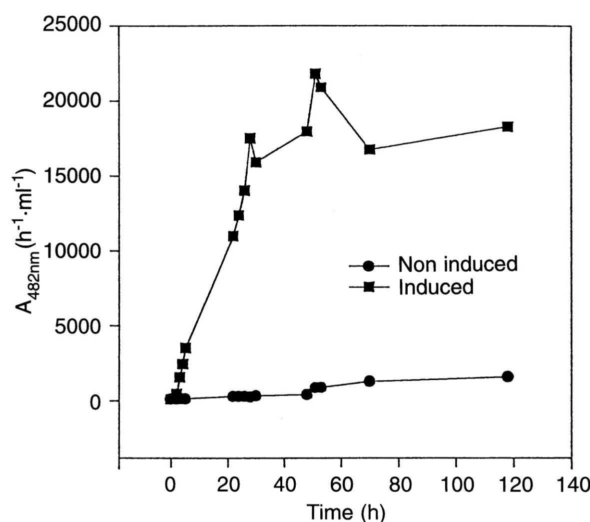

Fig. 5 β-Lactam induction of *blaU*. *S. lividans* TK24 bearing pDML1158 was cultivated in duplicate on YEME medium. After 12 h of growth, the inducer (7-ADCA, 25 μg/ml final) was added to one of the flasks and the β-lactamase activity was followed

plasmid, pDML1158 (Fig. 4). When *S. lividans* was transformed with pDML1158, it produced a β-lactamase which was inducible by 7-aminodesacetoxycephalosporanic acid (7-ADCA) (Fig. 5). The final concentration of 7-ADCA was of 25 μg/ml. It causes 100-fold induction, the maximal effect being reached after ca. 40 h.

Discussion

Fusion of DNA-binding proteins to GST with retention of binding activity has been described in several cases (Lassar et al. 1989; Blackwell et al. 1990; Braun et al. 1990; Chittendren et al. 1991). Indeed, bandshift experiments, using partially purified BlaA, as a GST-BlaA fusion protein, demonstrate the binding of BlaA to the *blaL-blaA* intergenic region, as previously suggested by Urabe and Ogawara (1992) based on assays carried out with BlaA$^+$ and BlaA$^-$ crude extracts. The difference in shift patterns observed between partially purified GST-BlaA (Fig. 2) and BlaA crude extract (Fig. 3) may be explained by differences between the GST-BlaA fusion protein (60 kDa) and the native BlaA (34 kDa) in the ability to bind the DNA. Indeed, it is known that AmpR, another LysR-type protein, binds the DNA as a tetramer (Normark et al. 1994). The BlaA and GST-BlaA proteins might therefore differ in the ability to form oligomers. Moreover, LysR-type protein, on binding to its DNA target, is able to bend the DNA giving rise to a more slowly migrating complex (Monroe et al. 1990; Hryniewicz and Kredich 1991; Wang et al. 1992; Bartowsky and Normark 1993; Normark et al. 1994), BlaA could have the same effect, whereas GST-BlaA may not, because of steric hindrance.

Using various DNA fragments corresponding to portions of the *blaL-blaA* intergenic region, the BlaA

Fig. 6 T-N_{11}-A motif and adjacent sequence (towards the regulated gene) recognized by various LysR-type regulatory proteins. The inverted repeat centred on the T-N_{11}-A motif is indicated by *thin arrows*, the T and the A bases of the motif by crosses, and the second inverted repeat by *thick arrows*. The distance between the T-N_{11}-A motif and the transcriptional start site of the regulated gene is given. Sources: [a] Naas and Norman 1994; [b] Lenzini et al. 1992; [c] Forsman 1991

Strain	Regulatory gene	LysR-recognized sequence upstream of the regulated gene	Regulated gene
C. freundii OS60 [a]	*amp*R	5'CCTGTTAGAAAAACTTATATCTGCTGCTAAATTTAACC3' (59)	*amp*C
E. cloacae P99 [a]	*amp*R	5'TCCGTTAGAAAAATTAACAGCTAATGCTAAATTAACC3' (59)	*amp*C
Y. enterocolitica IP97 [a]	*amp*R	5'CTTGTTAGATTTTCTATTATCAAGTGCTAAAATATAATC3' (59)	*amp*C
S. cacaoi [b]	*bla*A	5'GTATTCCGTTTCCGCATGAACCGGCGCACTCGGGTCTTC3' (55)	*bla*L
S. cacaoi [c]	*bla*A	5'TCATGCCGTTCCGGCATGGAACCGCGCATACCTGTATTG3' (56)	*bla*U

binding site was localized precisely. By this way, a T-N_{11}-A motif was identified. Among the DNA fragments tested in bandshift assays, the fragments of 126, 80, 65, 65′ and 50 bp (Fig. 1A) were retarded on gel by BlaA: all these fragments contained the same T-N_{11}-A motif. Three DNA fragments of 124, 71 and 56 bp (Fig. 1A) which did not include this motif were not retarded by BlaA. Our experimental data are in perfect agreement with the model of Goethals et al. (1992) which proposed that the LysR-like regulatory proteins bind to T-N_{11}-A nucleotide sequences centred on an inverted repeat (Goethals et al. 1992; Naas and Nordmann 1994; Normark et al. 1994).

All the nucleotide sequences, that flank the T-N_{11}-A motifs recognized by several LysR-type regulatory proteins, contain two inverted repeat sequences: the T-N_{11}-A motif itself and a second inverted repeat not noted previously, situated 3′ to the first motif (Fig. 6). Moreover, with the 50- and 65′-bp DNA fragments which include the T-N_{11}-A motif present between *blaA* and *blaL* (see Fig. 1A), the BlaA-shifted DNA band was less intense than that obtained with the 179-, 126-, 80- and 65-bp DNA fragments, suggesting again the great importance of the 3′ flank of the T-N_{11}-A motif (relative to *bla*L, the regulated gene). The second inverted repeat – present at the 3′side of the T-N_{11}-A motif – could have something to do with the regulation and could be involved in the bending of the DNA. These features which are common to all the T-N_{11}-A motifs (Fig. 6) suggest that all the proteins belonging to the LysR family should behave in a similar way.

Interestingly, a T-N_{11}-A motif was also found, correctly positioned, upstream of the second *S. cacaoi* β-lactamase gene: *blaU*. An 82-bp DNA fragment including this motif was retarded on gel by the BlaA-containing protein extract, indicating that BlaA binds also to this region. In both *blaL* and *blaU*, the DNA region recognized by the BlaA protein was experimentally delimited to a 30-bp fragment including a T-N_{11}-A motif in an inverted repeat. The *blaL* and *blaU* T-N_{11}-A motifs bound BlaA with comparable affinities, the *blaL* motif being 1.5–2 times more efficient. Comparing the situation for *blaL* and *blaU*, it was noticed that the T-N_{11}-A motifs were located at essentially the same distance (55 and 56 bp, respectively) upstream of the transcriptional start site of the regulated gene. Moreover, the motifs share 80% identity. As BlaA is able to bind to the upstream region of *blaU*, the *blaA*-*blaB* regulatory region should play a role in *blaU* expression. This would explain why the *S. lividans blaU* clone, which lacks the *blaA*-*blaB* regulatory region, was not inducible by β-lactam compounds. This was demonstrated by constructing a plasmid harboring together both *blaU* and *blaA*-*blaB* region (Fig. 4). *S. lividans* transformed with this construct indeed responds to induction of β-lactamase (Fig. 5). Up to now nothing was known about the regulation of *blaU*; we have now demonstrated that *blaL* and *blaU* in *S. cacaoi* share a common regulation pathway.

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