Regulation of the β -lactamase BlaL of *Streptomyces cacaoi*: the product of the *blaB* regulatory gene is an internal membrane-bound protein

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The β -lactamase-encoding gene *blaL*, cloned from *Streptomyces cacaoi* in *Streptomyces lividans*, is inducible by β -lactam compounds. This regulation has been shown to depend on the products of two open reading frames, ORF1 (*blaA*) and ORF2 (*blaB*) [Lenzini, Magdalena, Fraipont, Joris, Matagne and Dusart (1992) Mol. Gen. Genet. 235, 41–48]. *BlaA* belongs to the LysR family of transcription activators, whereas *BlaB* shares some features with the penicillin-recognizing proteins. *BlaB* has now

been overexpressed in *Escherichia coli*, purified and used for antibody preparation. Immunoblotting of cell-fractionated materials from *S. cacaoi* showed that *BlaB* is attached to the internal face of the cytoplasmic membrane. It could not be released by high salt concentrations or EDTA, but only by protease treatment. Under the assay conditions, *BlaB* did not act as a penicillin-binding protein, a β -lactamase, a D-amino-peptidase or a target in a phosphorylation step.

INTRODUCTION

The bacterial strain *Streptomyces cacaoi* possesses two different β -lactamase genes [1]: *blaL* [2] and *blaU* [3]. In the original strain, the β -lactamase activity was shown to be inducible by β -lactam compounds [4] and it seems that both genes are induced, although by two different mechanisms [5]. When cloned in *Streptomyces lividans*, however, only *blaL* remained inducible [6]. Analysis of the *blaL* regulatory region in the *S. lividans* clone revealed the organization shown in Figure 1, and genetic study demonstrated that two genes, *blaA* and *blaB*, were required, not only for induction, but also for basal expression of *blaL* [6]. From the nucleotide sequences of these two regulatory genes, it was deduced that the product of *blaA* was a DNA-binding protein, belonging to the LysR family. It is thus supposed to bind to the intercistronic region separating *blaL* and *blaA*, as suggested by gel-shift experiments carried out with crude cell extracts [7]. *blaB* coded for a molecule that possesses most of the conserved motifs of the penicillin-recognizing proteins. Differently from all the penicillin-binding enzymes and β -lactamases, *blaB* is devoid of any signal peptide. In the present paper, the product of the *blaB* gene was overproduced, purified and localized at the internal face of the plasma membrane. Some possible functions of the protein have also been explored.

MATERIALS AND METHODS

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 TS broth or YEME medium [10]. *E. coli* was cultivated at 37 °C in Luria–Bertani broth or minimal medium M9; *E. coli* plating medium was Luria–Bertani agar [11]. β -Lactamase production was induced by the addition of the β -lactam 7-amino-deacetoxycephalosporanic acid (7-ADCA; 40 μ g/ml) in 11 h cultures. Selective pressure was maintained during the growth of strains ML2 or MJ12 by the presence of thiostrepton (50 μ g/ml).

Streptomyces protoplasts were prepared in P buffer, following the method of Hopwood et al. [10].

Protein analysis and enzyme assays

Protein concentrations were determined by the method of Bradford [12]. N-terminal sequence was determined by automated microsequencing on a 477A pulsed liquid sequenator, using a 120A Applied Biosystems analyser (Foster City, CA, U.S.A.). Western blotting was as described in Sambrook et al. [11] and penicillin-binding protein (PBP) assays were carried out as in Fraipont et al. [13]. Calculation of the theoretical pI of *BlaB* was carried out with the ISOELECTRIC program [14], and the experimental value was determined by using the protocol of Hoefer Scientific Instruments (San Francisco, CA, U.S.A.). β -Lactamase, D-aminopeptidase and phosphorylation assays were performed as described by O'Callaghan et al. [15], Asano et al. [16] and Soll and Buchanan [17] respectively.

DNA manipulation and analysis

The DNA recombinant procedures applied were essentially those described by Sambrook et al. [11] and Hopwood et al. [10]. PCR amplification was carried out as in Saiki et al. [18]. The gene-sequencing method of Sanger et al. [19,20] was used with

Figure 1 Schematic organization of the *S. cacaoi* *blaL* locus

The arrows give the relative lengths of the genes and indicate the sense of transcription. The PCR-synthesized *blaB* started with an *Nde*I site at the ATG of the *blaB* open reading frame and ended with a *Bam*HI site, five triplets over the stop codon.

Abbreviations used: 7-ADCA, 7-aminodeacetoxycephalosporanic acid; DTT, dithiothreitol; GdmCl, guanidinium chloride; IPTG, isopropyl β -D-thiogalactoside; PBP, penicillin-binding protein.

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Table 1 Strains and plasmids

IPTG, isopropyl thiogalactoside.

Designation	Properties	Source	Reference
Strains			
<i>S. cacaoi</i> KCC-S0352	Wild-type	Kaken Chemical Co. (Tokyo, Japan)	—
<i>Streptomyces coelicolor</i> A3 (2)	Wild-type	John Innes Centre (Norwich, U.K.)	—
<i>S. lividans</i> TK24	From <i>S. lividans</i> 66 <i>str-6</i> , cured of natural plasmids SLP1 and SLP2	John Innes Centre (Norwich, U.K.)	8
<i>S. lividans</i> ML2	From TK24, harbours pDML52	CIP (Liège, Belgium)	2
<i>S. lividans</i> MJ12	From TK24, harbours pDML652	CIP (Liège, Belgium)	6
<i>Escherichia coli</i> MV1190	<i>recA</i> [−]	Bio-Rad Laboratories (Brussels, Belgium)	—
<i>E. coli</i> BL21 (DE3)	Recommended host for pET derivatives expression	Novagen Inc. (Madison, WI, U.S.A.)	9
<i>E. coli</i> MJ4	From BL21 (DE3), harbours pDML682	CIP (Liège, Belgium)	This paper
Plasmids			
pDML52	<i>Streptomyces</i> vector pIJ702 + <i>blaB</i> , <i>blaA</i> and <i>blaL</i>	CIP (Liège, Belgium)	2
pDML652	<i>Streptomyces</i> vector pDML52 with <i>blaB</i> inactivated by deletion	CIP (Liège, Belgium)	6
pDML682	pET9a containing a PCR-synthesized <i>blaB</i> gene	CIP (Liège, Belgium)	This paper
pET9a	Expression plasmid based on the T7 RNA polymerase, under the control of an IPTG-inducible promoter	Novagen Inc. (Madison, WI, U.S.A.)	9
pLysE	Reduces the basal level of T7 RNA polymerase expression	Novagen Inc. (Madison, WI, U.S.A.)	9

fluorescent primer. The analyses were performed on an EMBL automated DNA sequencer [21].

Preparation of *Streptomyces* total protein extracts

Mycelium from 750 ml of a 3-day culture (YEME medium containing thiostrepton; 50 µg/ml) was pelleted, washed once in 0.9% NaCl, resuspended in 15 ml of Hepes buffer, and then broken in the French press.

Streptomyces cell fractionation

Mycelium was grown for 48 h in TS broth, pelleted (7500 g; 25 min) and lysed as described by Nguyen-Distèche et al. [22]. The lysate was centrifuged at 37000 g, 4 °C, for 1 h. The cytoplasmic fraction consisted of the supernatant dialysed for 24 h against 25 mM Tris/HCl, pH 7.5, and 10-fold concentrated by ultrafiltration. To obtain the membrane fraction, the lysis pellet was incubated for 1 h at room temperature in 50 mM Tris/HCl (pH 7.5)/5 mM MgCl₂ in the presence of DNase (100 µg/ml). Membranes were cleared of cell debris by centrifuging at 830 g for 10 min, resuspending the pellet in 50 mM Tris/HCl, pH 7.5, and centrifuging again under the same conditions. The two 830 g supernatants were pooled and centrifuged at 37000 g, for 25 min, giving a membrane pellet which was washed once in 50 mM Tris/HCl (pH 7.5)/5 mM MgCl₂ and finally resuspended in 10 mM phosphate buffer, pH 7.5.

E. coli cell fractionation

Cells were harvested by centrifugation, washed once in 0.9% NaCl, and resuspended in 20 mM Tris/HCl, pH 7.5, containing 1 mM EDTA and 1 mM dithiothreitol (DTT) (buffer/culture, 1:20, v/v). The cells, broken in the French press, were centrifuged (13000 g; 10 min) and both fractions were harvested. The supernatant was used as such and the pellet was washed in 50 mM Tris/HCl, pH 8, containing 100 mM NaCl, 100 mM EDTA and 0.5% Triton X-100.

Antibody production

Rabbit anti-BlaB antibodies were prepared by Gamma SA (Liège, Belgium). The BlaB protein preparation used was extracted from an SDS/polyacrylamide gel by the procedure of Bikel et al. [23]. A series of four injections of 300 µg of protein at 15-day intervals was applied. The harvested serum reacted positively in an ELISA against the purified BlaB antigen.

Enzymes, antibiotics and chemicals

Trypsin (type IX, from porcine pancreas) was purchased from Sigma Chemie (Bornem, Belgium), and proteinase K from Merck (Darmstadt, Germany). Restriction endonucleases were from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.), Boehringer (Mannheim, Germany), Eurogentec (Seraing, Belgium) or Pharmacia Biotech. (Roosendaal, The Netherlands), and lysozyme was from Belovo (Bastogne, Belgium). Radioactive benzylpenicillin and [γ -³²P]ATP were obtained from Amersham International (Amersham, Bucks., U.K.). Trypsin inhibitor, chloramphenicol and kanamycin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Thiostrepton was a gift from S. J. Lucania (Squibb and Sons, Princeton, NJ, U.S.A.). 7-ADCA was kindly donated by Gist-Brocades (Delft, The Netherlands).

Buffers

The following buffers were used: FPLC buffer (Mono P column), 25 mM Tris/acetate (pH 8.3)/2 M urea or Polybuffer 74/HCl (pH 4)/2 M urea; Hepes buffer, 25 mM Hepes (pH 7.5)/5 mM MgCl₂/0.1 mM EDTA/5 mM 2-mercaptoethanol/10% glycerol/1 mM PMSF; P buffer, described in [10]; S buffer, 10 mM Tris/borate (pH 8)/10% glycerol/1 mM DTT. The pH scale used in the solubilization assays was composed of 50 mM formate (pH 4), 50 mM acetate/acetic acid (pH 5), 20 mM piperazine

(pH 6), 20 mM BisTrispropane (pH 7), 20 mM Tris/HCl (pH 8), 20 mM ethanolamine (pH 9) and 20 mM piperazine (pH 9.5). All these buffers contained 1 mM EDTA.

RESULTS

BlaB is not a PBP

By analogy with the system of *Bacillus licheniformis* and other Gram-positive bacteria [24], BlaB could play the role of external sensor, acting as a PBP. The PBP patterns were thus analysed in membrane preparations obtained from (i) the original strain, *S. cacaoi*, (ii) the host strain, *S. lividans* TK24, (iii) *S. lividans* containing intact *blaB* (strain ML2), (iv) *S. lividans* containing inactivated *blaB* (strain MJ12) and (v) *S. coelicolor* A3 (2). The same PBP pattern was found in all the three *S. lividans* strains (results not shown).

Overproduction of BlaB in *E. coli*

blaB was amplified by PCR. This step introduced an *NdeI* restriction site at the 5' end, and a *BamHI* site five codons downstream of the 3' end of the gene. The amplified material of the expected size was digested with *NdeI* and *BamHI* and ligated into the *E. coli* expression vector pET9a previously cleaved with the same enzymes. The recombinant pET9a was introduced into competent cells of the RecA⁻ *E. coli* strain MV1190. The first selection, carried out on Luria-Bertani agar plus kanamycin (50 μ g/ml), was confirmed in the corresponding liquid medium. Plasmid DNA from the transformants was prepared and analysed for the presence of the desired insert. This plasmid, called pDML682, was then used for BlaB production. *E. coli* strain BL21 (DE3) was co-transformed with pDML682 and pLysE. A derived colony (called strain MJ4) was cultivated in duplicate, in Luria-Bertani broth plus kanamycin (50 μ g/ml) plus chloramphenicol (30 μ g/ml). When A_{600} was close to 1, IPTG was added (final concn. 2 mM) to one of the cultures. Samples were withdrawn from both cultures, after 2.5 h, 4 h and overnight, and total protein extracts were analysed by SDS/PAGE (12% polyacrylamide). Coomassie Blue staining revealed a supernumerary protein of about 35 kDa in the induced compared with the uninduced samples; this 35 kDa band increased with the duration of induction.

The 1 kb nucleotide sequence of the PCR-amplified *blaB* has been completely verified. It corresponded exactly to the original sequence of *blaB* [6]. The overproduced protein (35 kDa band) was transferred from an SDS/polyacrylamide gel to an Immobilon membrane (Millipore Corp., Bedford, MA, U.S.A.) for N-terminus amino acid analysis. The sequence was determined until His-16 and corresponded precisely to the primary structure deduced from the nucleotide sequence. It was thus concluded that the 35 kDa band consisted of overproduced BlaB.

E. coli strain MJ4 was cultivated at 37 °C in the presence of inducer. At different times after induction, the cells were harvested and fractionated as indicated in the Materials and methods section. The SDS/PAGE analysis revealed that BlaB was exclusively present in the insoluble fraction (inclusion bodies). By lowering the incubation temperature to 28 °C, it was possible to recover a small fraction (maximum 20%) of BlaB in the supernatant.

Maximal production of BlaB was obtained by adding the inducer IPTG at a concentration of 0.4 mM to a culture with an A_{600} of 0.8. Various periods of induction were tested. The yield of BlaB was 100 μ g/ml of culture after a 3 h induction and 300 μ g/ml after overnight induction. The insoluble fraction (80 %

Figure 2 Solubilization and purification of BlaB (SDS/PAGE and Coomassie Blue staining)

The samples contained approx. 10 μ g of BlaB. Lanes 1 and 6, size markers (kDa); lane 2, BlaB inclusion bodies solubilized in S buffer containing 4 M GdmCl; lane 3, the same preparation sequentially dialysed against decreasing concentrations of GdmCl, until plain S buffer, and the resulting precipitate resolubilized with 4 M urea in 20 mM Tris/HCl, (pH 8)/10% glycerol/1 mM DTT; lane 4, the material that remained insoluble from lane 3; lane 5, BlaB after purification on Mono P.

of BlaB) was used as starting material for purification because it contained very few contaminating proteins.

Solubilization and purification of BlaB

The Triton X-100-washed inclusion bodies were first solubilized in S buffer containing 4 M guanidinium chloride (GdmCl), the protein concentration being adjusted to 100 μ g of BlaB/ml. The solution was then dialysed sequentially against decreasing concentrations of GdmCl: 2, 1.5, 1, 0.5 and 0 M, in the same S buffer with twice-daily changes. Total precipitation of BlaB was observed when the GdmCl concentration was less than or equal to 0.5 M. This allowed the elimination of some remaining soluble contaminants (Figure 2). The material insoluble in the absence of GdmCl was washed in 20 mM Tris/HCl (pH 8)/10 mM EDTA/0.5% Triton X-100. After a 5 min incubation at room temperature, the material was pelleted (some more contaminant proteins were discarded with the washing supernatant) and used in the purification procedure described below.

The preparation obtained was resolubilized in a pH 8.3 buffer (25 mM Tris/acetate plus 2 M urea) and passed through a Mono P column equilibrated with the same buffer. Elution was carried out with Polybuffer 74/HCl (pH 4) containing 2 M urea, and BlaB was released at pH 6.2. The degree of purity reached after this step is shown in Figure 2, lane 5. The yield of the overall process was 78%.

BlaB solution devoid of urea was obtained by sequential dialysis in decreasing concentrations of urea (nine steps from 8 to 0 M), at various pHs (ranging from 4 to 9.5). BlaB was soluble in the absence of urea at pH 4 and pH 8 and above. At pH 5–7, precipitation occurred in 2 M urea or less.

In all these assays, a protein concentration of 100 μ g/ml was used. It was later shown that sequential dialysis in urea was efficient even at 500 μ g of protein/ml.

pI of BlaB

A theoretical pI of 6.08 was calculated from the primary structure of the protein, as deduced from the gene sequence. An experimental value of 6.5 was determined.

Figure 3 Membrane localization of BlaB (Western-blot)

(a) Anti-BlaB antibody diluted 1:1000 was mixed with BlaB overproduced in *E. coli* MJ4 (insoluble fraction corresponding to 1 μ l of MJ4 culture, induced with 0.4 mM IPTG) (lane 1), cytoplasmic fraction (20 μ l; 25 μ g of protein/ μ l) from *S. lividans* ML2 (lane 2) or membrane fraction (20 μ l; 25 μ g of protein/ μ l) from *S. lividans* MJ12 (lane 3). (b) Anti-BlaB antibody diluted 1:1000 was mixed with membrane fractions (20 μ l) from *S. coelicolor* (40 μ g of protein/ μ l) (lane 1), *S. lividans* TK24 (30 μ g of protein/ μ l) (lane 2), *S. cacaioi* (18 μ g of protein/ μ l) (lane 3), *S. lividans* ML2 (*blaB*⁺) (25 μ g of protein/ μ l) (lane 4) and *S. lividans* MJ12 (*blaB*⁻) (25 μ g of protein/ μ l) (lane 5).

BlaB is a membrane-bound protein

Total protein extracts were prepared from the strains of *S. lividans* ML2 (*blaB*⁺) and MJ12 (*blaB*⁻) and analysed by Western blot with anti-BlaB antibody. Immunoblots revealed that BlaB was present in *S. lividans* ML2 but not in MJ12. ML2 and MJ12 cells were fractionated as described in the Materials and methods section. Membrane and cytoplasmic fractions were immunologically tested for the presence of BlaB. From Figure 3(a), it can be concluded that BlaB is located in the membrane. Induction by β -lactam of the *S. lividans* ML2 culture did not modify the cellular localization of BlaB. Cytoplasmic and membrane fractions from other *Streptomyces* strains were also examined. *S. coelicolor* A3(2) membrane contained a component that cross-reacted with the anti-BlaB antibody. This was also the case for *S. cacaioi* and *S. lividans* ML2 (see above), but not for *S. lividans* TK24 (Figure 3b). None of the cytoplasmic fractions gave any cross-reaction.

Analysis of the membrane attachment of BlaB

Effect of salt concentration

Salt treatments were applied to *S. lividans* ML2 protoplasts: 50 μ l of protoplast suspension were diluted to 500 μ l with 0.25, 0.5, 0.75 or 1 M NaCl or NaI. Incubation was for 30 min on ice. The pellet (37000 g; 30 min) was analysed by Western blot. BlaB remained attached to membranes under all the conditions tested (not shown).

Effect of NaOH and EDTA

The effects of NaOH (1, 10 and 100 mM; 15 min on ice) and EDTA (0.01, 0.1, 1 and 10 mM; 15 min at 37 °C) on protoplasts were also tested; neither had any effect on the attachment of BlaB (results not shown).

Effect of trypsin and proteinase K

S. lividans ML2 protoplasts (40 μ l of sample) were diluted with 100 μ l of P buffer, 10 μ g of trypsin was added and the mixture

Figure 4 Effect of trypsin treatment on the attachment of BlaB to protoplasts (Western blot)

Anti-BlaB antibody diluted 1:1000 was mixed with protein extracts from membranes treated with trypsin (10 μ g; 3 h; 37 °C; lanes 3 and 6), trypsin plus 5% glycerol (lanes 4 and 7) or trypsin plus 1% Triton X-100 (lanes 5 and 8). The membranes were prepared from 40 μ l of intact ML2 protoplasts (lanes 3–5) or 40 μ l of lysed ML2 protoplasts (lanes 6–8). Protein extracts of membranes from untreated protoplasts are in lane 2, and BlaB overproduced in *E. coli* MJ4 is in lane 9 (see caption of Figure 3). Size markers (kDa) are indicated in the margin (lane 1). BlaB is indicated by the arrow.

incubated at 37 °C for 3 h. In parallel treatments, 5% glycerol or 1% Triton X-100 was added with the trypsin. The same amount of protoplasts was lysed by dilution with 100 μ l of water and then treated with trypsin as described for the intact cells. Digestion was stopped by the addition of trypsin inhibitor (50 μ g) and a 20 min incubation at 4 °C. All eight samples (no trypsin, trypsin, trypsin plus glycerol, trypsin plus detergent with intact and lysed protoplasts) were centrifuged at 37000 g for 30 min. The pellets were analysed by Western blot. The BlaB band was still visible in intact protoplasts treated with trypsin or trypsin plus glycerol but had disappeared from the protoplasts treated with Triton X-100, as well as in all the samples of previously lysed protoplasts (Figure 4).

The same preparations of intact or lysed protoplasts were also treated with proteinase K, giving similar results.

Possible activities of the overproduced protein

β -Lactamase and D-aminopeptidase activities

Purified BlaB overproduced in *E. coli* was tested for β -lactamase and D-aminopeptidase activity using nitrocefin and D-Ala-p-nitroanilide as substrates respectively. No hydrolysis was observed in either case.

Penicillin fixation

The capacity of overproduced BlaB to bind penicillin was investigated. No BlaB–penicillin complex was found.

Phosphorylation

Three different preparations of BlaB were tested for susceptibility to phosphorylation: (i) cellular supernatant fractions from *E. coli* MJ4, cultivated overnight under four different conditions (in minimal M9 medium or in Luria–Bertani broth in the presence or absence of IPTG); (ii) pellet from a sonicated IPTG-induced MJ4 culture solubilized in 8 M urea and cleared of urea by sequential dialysis at pH 8 (see above); (iii) membrane suspension obtained from protoplasts of *S. lividans* ML2. Membranes from *S. lividans* TK24 were also tested. All the assays gave negative results.

We thank Dr. Moreno Galleni, Christine Jacobs, Dr. Martina Datz and Dr. Karin Goraj for many helpful suggestions and discussions. This work was supported in part by

the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy programming (PAI no. 19), the Fund for Medical Research of Belgium (contract no. 3.4531.92) and the Fund for Joint Basic Research of Belgium (contract no. 2.4525.92). J. D. is Research Associate of the National Fund for Scientific Research of Belgium.

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