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Research Article

BlaB, a protein involved in the regulation of *Streptomyces cacaoi* β-lactamases, is a penicillin-binding protein

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Abstract. *Streptomyces cacaoi* β-lactamase genes are controlled by two regulators named *blaA* and *blaB*. Whereas BlaA has been identified as a LysR-type activator, the function of BlaB is still unknown. Its primary structure is similar to that of the serine penicillin-recognizing enzymes (PREs). Indeed, the SXXK and KTG motifs are perfectly conserved in BlaB, whereas the common SXN element found in PREs is replaced by a SDG motif. Site-directed mutations were introduced in these motifs and they all disturb β-lactamase regulation. A water-soluble form of BlaB was also overexpressed in the *Streptomyces lividans* TK24 cytoplasm and purified. To elucidate the activity of BlaB, several compounds recognized by PREs were tested. BlaB could be acylated by some of them, and it can therefore be considered as a penicillin-binding protein. BlaB is devoid of β-lactamase, D-aminopeptidase, DD-carboxypeptidase or thiolesterase activity.

Key words. *Streptomyces cacaoi*; induction; β-lactamase; penicillin-binding protein.

The ability of bacteria to produce β-lactamases is causing increasing problems in the clinical treatment of many infectious diseases [1]. In most cases, this production occurs in a constitutive way, being the same in the presence or absence of β-lactam compounds in the medium. However, some exceptions are known [2, 3]. Two different modes of control of β-lactamase genes have been studied for many years, one in enterobacteria and a few other Gram-negative bacteria [4], the second in *Bacillus* and *Staphylococcus* [5]. Regulation of β-lactamase synthesis has also been reported in *Aeromonas* spp. [6] and in *Streptomyces cacaoi* [7].

In *S. cacaoi*, β-lactamase production is induced by β-lactam compounds such as 7-deacetoxycephalosporanic acid (7-ADCA) or 6-aminocephalosporanic acid (6-APA) [7, 8]. *S. cacaoi* possesses two different unlinked β-lactamase structural genes called *blaL* and *blaU* [9]. The two genes encode two extracellular active-site serine class A β-lactamases sharing 50% strict identities [9–13] and they are under the control of the same regulatory system [14]. Sequence analysis and genetic study of the *blaL* regulatory region identified two genes, *blaA* and *blaB*, located just upstream of *blaL* but transcribed divergently and required for the induction and for the basal expression of BlaL and BlaU [8, 15, 16].

The primary structure of BlaA, as deduced from the nucleotide sequence of the gene, indicates that this protein is a member of the LysR family of regulator proteins [8, 16]. It binds to DNA through a helix-turn-helix motif and recognizes a T-N_{11}-A sequence including a double overlapping palindromic sequence [17]. This motif, characteristic of the LysR-type promoters, is present in

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the *blaA-blaL* intergenic region and upstream of *blaU* [14, 16].

To date, no function has been attributed to BlaB. Its amino acid sequence shows some similarities with that of the serine penicillin-recognizing enzymes (PREs). This superfamily of evolutionarily related proteins includes the DD-peptidases, enzymes involved in the biosynthesis and control of the cross-linking of the peptidoglycan and the serine β-lactamases [18]. All PREs react with penicillin and other β-lactam antibiotics according to the same general pathway which involves successive acylation and deacylation steps. However, there is a major difference between DD-peptidases and serine β-lactamases. The former are inactivated by β-lactam antibiotics as a result of the acylation of their active-site serine by the antibiotic. This covalent adduct is stable and, consequently, these enzymes are also referred to as penicillin-binding proteins (PBPs). On the other hand, serine β-lactamases inactivate penicillins and related antibiotics. In this case, the acyl enzyme is generally extremely unstable and is rapidly hydrolysed, regenerating the free enzyme and a product devoid of antibiotic activity. On the basis of their catalytic properties, serine β-lactamases are divided into three classes (A, C and D).

By sequence alignments and comparisons of three-dimensional (3D) structures, several conserved structural and functional elements have been identified in this family of proteins (table 1). The first conserved element (S*XXK) contains the active-site serine. After two variable residues whose side chains point away from the active site, a lysine is invariably found. The side chain of this Lys residue lies in the active site where it forms a hydrogen bond with the active-serine hydroxyl group. The second element [(S/Y)XN] forms one wall of the active site. The side chains of the first and third residues border the active site while that of the second is in the core of the protein. The first residue (Ser or Tyr) always bears a hydroxyl group, whereas the third is nearly always an asparagine. The third conserved element [(K/R)(T/S)G] forms the opposite wall of the catalytic cavity. The first residue is positively charged (Lys or Arg), the second is hydroxylated (Thr or Ser) and the third is always Gly. In fact, any side chain in this latter position would protrude into the active site and sterically hinder the interaction between most substrates and the active-site serine. In BlaB, the first and third conserved elements are also found (S^{47}LVK and K^{254}TG), whereas the second element is somewhat different, the asparagine being replaced by a glycine (S^{109}DG) (table 1).

To determine the role of these three conserved motifs in BlaL regulation, a site-directed mutagenesis program was initiated and four plasmids bearing the *blaA* gene, the *blaL* gene and the mutated *blaB* gene were constructed. The serine of the S^{47}LVK motif was replaced by an alanine or a cysteine; the glycine of the S^{109}DG motif was changed to asparagine and the lysine of the K^{254}TG motif was mutated to alanine. BlaL production in *S. lividans* TK24 transformed by each of these four plasmids was measured in the presence or absence of 7-ADCA. All changes in these conserved motifs disturbed the basal production or the induction of the β-lactamase, suggesting that they play an important role in the β-lactamase regulatory process.

Table 1. The conserved elements found in the three classes of active-site serine β-lactamases, in PBPs and in BlaB.

Class	Element 1	Element 2	Element 3
Class A	^{70}S*-X-X-K	S^{130}-D-(N/S)	(K^{234}/R)-(T/S)-G
Class C	^{64}S*-X-X-K	Y^{150}-(A/S)-N	K^{315}-T-G
Class D	^{70}S*-X-X-K	S^{144}-X-V	K^{215}-T-G
PBPs	S*XXK	(S/Y)-X-(N/C)	K-(T/S)-G
BlaB	S^{47}-L-V-K	S^{109}-D-G	K^{254}-T-G

S* represents the active-site serine. X corresponds to a variable amino acid residue.

In this paper, we also describe the overexpression of BlaB as a soluble protein in the *S. lividans* TK24 cytoplasm and its purification. To determine the function of BlaB, some possible activities were tested. We demonstrate that BlaB can be referred to as a PBP and that it does not act as a β-lactamase, a D-aminopeptidase, a DD-carboxypeptidase or a thiolesterase.

Materials and methods

Plasmids, bacterial strains and growth conditions

Plasmids and strains are described in table 2. *Escherichia coli* strains were cultivated at 37 °C in Luria-Bertani (LB) medium or plated on LB agar (1.5% w/v) [21]. *Streptomyces* liquid cultures were grown with vigorous orbital shaking at 28 °C in YEME medium; *Streptomyces* plating medium was R2YE [22]. The medium used for the overexpression of BlaB was composed of 0.5 g l^{-1} L-asparagine, 0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4.7H_2O$, 0.01 g l^{-1} $FeSO_4.7H_2O$, 0.12% casamino acids Difco and 2% fructose. *S. lividans* TK24 protoplasts were prepared as described previously [22]. Selective pressure was maintained by ampicillin (100 µg ml^{-1}), chloramphenicol (50 µg ml^{-1}), thiostrepton (25 µg ml^{-1}), spectinomycin (20 µg ml^{-1} in *E. coli* or 250 µg ml^{-1} in *Streptomyces*) or by streptomycin (100 µg ml^{-1}).

Enzymes, antibiotics and chemicals

Benzonase and T4 DNA ligase were from Merck and Roche Diagnostics, respectively. Lysozyme was from Belovo. Restriction endonucleases and *Pfu* polymerase were purchased from Promega Benelux. Oligonucleotides and primers for DNA sequencing were obtained from Amersham Biosciences or Eurogentec. Fluorescent ampi-

Table 2. Plasmids and strains used or constructed in this study.

	Characteristics	Source or reference
Plasmid		
pBR322	*E. coli* cloning vector (Amp^R/Tc^R)	[19]
pACYC184	*E. coli* cloning vector (Tc^R/Cm^R)	[20]
pUC18	*E. coli* cloning vector (Amp^R)	Boehringer
pCR-Script Amp SK(+)	*E. coli* PCR cloning vector (Amp^R)	Stratagene
Vpro p145	*E. coli*/*Streptomyces* shuttle vector used to overexpress BlaB (Spm^R/Str^R). It contains the I19 phage strong promoter from *S. ghanaensis*.	Giannotta [unpublished data]
Vpro p145.10	Vpro p145 with the *blaB* gene cloned in the *Spe*I and *Pst*I sites	this paper
pIJ702	*Streptomyces* vector (Tsr^R)	John Innes Institute
pDML1164	pACYC184 derivative with the *S. cacaoi bla* divergon on a 4.4-kb *Bcl*I-*Sph*I fragment	Gérard [unpublished data]
pDML72	pBR322 derivative with the *S. cacaoi bla* divergon on a 6.6-kb *Sph*I fragment	[10]
pMCP38	pIJ702 derivative with the *S. cacaoi bla* divergon on a 4.4-kb *Bcl*I-*Sph*I fragment	Gérard [unpublished data]
pDML52	pIJ702 derivative with the *S. cacaoi bla* divergon on a 6.6-kb *Sph*I fragment	[10]
pDML1176	mutated pDML72 (S47A in BlaB)	this paper
pDML1177	mutated pDML52 (S47A in BlaB)	this paper
pDML1178	mutated pDML1164 (K254A in BlaB)	this paper
pDML1179	mutated pMCP38 (K254A in BlaB)	this paper
pDML1180	mutated pDML72 (G111N in BlaB)	this paper
pDML1181	mutated pDML52 (G111N in BlaB)	this paper
pDML1182	mutated pDML72 (S47C in BlaB)	this paper
pDML1183	mutated pDML52 (S47C in BlaB)	this paper
Strain		
E. coli JM110	non-methylante strain	Stratagene
S. lividans TK24	host strain for the cloning of the *S. cacaoi bla* divergon	John Innes Institute
S. lividans ML2	*S. lividans* TK24 transformed with pDML52	[10]
S. lividans CMA38	*S. lividans* TK24 transformed with pMCP38	[10]
S. lividans CG10	*S. lividans* TK24 transformed with pDML1177	this paper
S. lividans CG11	*S. lividans* TK24 transformed with pDML1179	this paper
S. lividans CR1	*S. lividans* TK24 transformed with pDML1181	this paper
S. lividans CR2	*S. lividans* TK24 transformed with pDML1183	this paper

Amp^R, Tc^R, Cm^R, Spm^R, Str^R and Tsr^R refer to ampicillin, tetracycline, chloramphenicol, kanamycin, spectinomycin, streptomycin and thiostrepton resistance, respectively.

cillin (AmpFlu) was prepared as described elsewhere [23]. Ampicillin, cefoxitin, cephalexin, cephaloridine, cephalothin, chloramphenicol, cloxacillin, spectinomycin and streptomycin were from Sigma-Aldrich. 7-ADCA, benzylpenicillin, cephacetrile, nitrocefin and thiostrepton were from Gist-Brocades, Rhône-Poulenc, Novartis, Unipath and Squibb and Sons, respectively. Aztreonam and imipenem were from Bristol-Myers and Merck Sharp Dohme, respectively. D-Ala-p-nitroanilide was purchased from Bachem. Monoacetyl-L-Lys-D-Ala-D-Ala (AcKAA) and bisacetyl-L-Lys-D-Ala-D-Ala (Ac_2KAA) were from UCB BioProducts. The dipeptide glycyl-glycine (Gly-Gly) was from Sigma-Aldrich. Benzoyl-glycyl-thioglycolate (S2a) and benzoyl-alanyl-thioglycolate (S2d) were prepared as described elsewhere [24, 25].

DNA and protein methods

Routine DNA manipulations were carried out as previously described [21, 22]. DNA fragments and PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The pCR-Script Amp SK(+) Cloning Kit (Stratagene) was used for cloning PCR fragments. Polymerase chain reactions (PCRs) were carried out following Saiki et al. [26] in a Biometra apparatus (Eurogentec). DNA sequencing was performed by the dideoxy chain termination method using an ALFexpress DNA sequencer (Amersham Biosciences). Protein concentration was determined by the 2-bicinchoninic acid assay (BCA Protein Assay Kit, Pierce). The N-terminal sequence of purified BlaB (120 pmol) was determined by the Edman degradation re-

action with the help of a 477A pulsed liquid sequenator using a 120A Applied Biosystems analyser.

Overexpression and purification of BlaB

blaB was amplified by PCR with pDML72 as a template and with the primers O10 and O11 which introduce *Spe*I and *Pst*I sites just upstream of the *blaB* start codon and downstream of its stop codon, respectively (table 3). The purified PCR fragment was cloned in pCR-Script Amp SK(+) and sequenced (UP and RP). The *Spe*I-*Pst*I fragment corresponding to *blaB* was introduced just downstream of the GTG start codon of the Vpro p145 vector, giving rise to Vpro p145.10. This results in the addition of two amino acids at the NH_2 end of BlaB (Thr and Ser which correspond to the ACT and AGT codons of the *Spe*I site, respectively). Protoplasts of *S. lividans* TK24 were transformed by Vpro p145.10. Mycelium from a 3-day culture (250 ml) was pelleted, washed, resuspended in 5 ml of 20 mM Tris-HCl pH 8.5 (buffer A) and disrupted by sonication in a Branson ultrasonic disintegrator at 12-µm amplitude for nine 1-min bursts. The soluble cell fraction was obtained by centrifugation (16,000 g, 30 min) of the lysate and was treated with benzonase (10,000 U l^{-1}) before addition of 0.1% Triton X-100. It was then submitted to ion-exchange chromatography on a QAE-HP column (XK 16/20, 20 ml, Amersham Biosciences) equilibrated with buffer A supplemented with 0.1% Triton X-100 (buffer B). The adsorbed BlaB was eluted with a linear NaCl gradient (200 ml) from 0.25 to 0.55 M. The fractions were analysed by 12% polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) (0.1% w/v). Fractions containing BlaB were pooled, concentrated eightfold by centrifugation on an Amicon membrane (cutoff 10000) and loaded onto a Superdex 200 (HR 10/30, 24 ml, Amersham Biosciences) equilibrated with buffer B. The fractions were analysed by 12% SDS-PAGE and those containing BlaB were pooled. To obtain a purity >95%, an additional chromatography step was performed on a MonoQ (HR/R 5/5, 1 ml, Amersham Biosciences) equilibrated with buffer A. BlaB was eluted with a linear NaCl gradient (50 ml) ranging from 0.25 to 0.5 M and a 6 µM solution of purified BlaB was obtained (3.1 mg l^{-1}).

AmpFlu-binding assays

Purified BlaB (2 µM final) and AmpFlu (2 µM final) were incubated for different times at 37 °C. The reaction was stopped by addition of 5× SDS-PAGE loading buffer (1× final) and the sample was submitted to 12% SDS-PAGE. The fluorescent complexes were visualized using the FX-Imager with the Quantity One program (BioRad).

Competition binding assays with β-lactam antibiotics, AcKAA and Ac_2KAA

Purified BlaB (4 µM final) was incubated at 37 °C for 1 h with an excess (3 mM final) of some penicillins (ampicillin, benzylpenicillin and cloxacillin), some cephalosporins (7-ADCA, cephacetrile, cephalexin, cephaloridine and cephalothin), a cephamycin (cefoxitin), a carbapenem (imipenem) and a monobactam (aztreonam). AmpFlu (16 µM final) was then added and the mixture was incubated at 37 °C for 2 min.

In the competition binding assays with the DD-carboxypeptidase substrates, BlaB (3 µM final) was mixed with different concentrations of AcKAA and Ac_2KAA (1.75, 3.5, 8.75 and 17.5 mM final) and the mixture was

Table 3. Sequence of the oligonucleotides used for site-directed mutagenesis of the BlaB conserved motifs (O1–O9), for BlaB overexpression (O10 and O11) and for DNA sequencing (UP and RP).

Oligonucleotide name	Sequence
O1	5′-GCGACC**TGTACA**CCGGCGAGGAACTCGGCATCGACCCGGACACCGAACTGCCCACCGCC<u>GCC</u>CTGGTCAAGC-3′
O2	5′-TGCCGGTCTTCGACG**ACCAGGT**CGCGG-3′
O3	5′-GCGACCTGGTCGTCG<u>GCG</u>ACCGGCACCCTGCTC-3′
O4	5′-CGCTGGACCAGCAGC<u>CGC</u>TGGCCGTGGGACGAG-3′
O5	5′-C**TGTACA**CCGGCGAGGAACTC-3′
O6	5′-GCGCGTCGGAGGCGGT<u>ATT</u>GTCGCTCACCGAGGTGCTCAGGTACAGCAGGTCGTCG-3′
O7	5′-TCGGTGAGCGAC<u>AAT</u>ACCGCCTCCGACGCGCTCTTCGAGATCACACCGCCCGCCCAG-3′
O8	5′-TGCCGGTCTTCGACG**ACCAGGT**CGCGG-3′
O9	5′-GCGACC**TGTACA**CCGGCGAGGAACTCGGCATCGACCCGGACACCGAACTGCCCACCGCC<u>TGC</u>CTGGTCAAGC-5′
O10	5′-CC**ACTAGT***GTG*CTGAACTCCGAGAGTCTGCTGCGCG-3′
O11	5′-GGGA**CTGCAG***TCA*CCACTCCCGGAGGCGGTCGCG-3′
UP	5′-GTAAAACGACGGCCAGT-3′
RP	5′-GGAAACAGCTATGACCATG-3′

The *Bsr*GI site is in bold in oligonucleotides O1, O5 and O9. The *Sex*AI site is in bold in oligonucleotides O2 and O8. The *Spe*I and *Pst*I sites are in bold in oligonucleotides O10 and O11, respectively. Mutated codons are underlined. The *blaB* GTG start codon and TCA stop codon are in italics in O10 and O11, respectively.

incubated for 30 min at 37 °C. AmpFlu (2 µM final) was then added and a 2-min incubation was performed at 37 °C. To determine if BlaB can form a covalent complex with AcKAA, it was incubated for 1 h at 37 °C with an excess of AcKAA (35 mM) which was then eliminated by the Ultrafree-MC Amicon system (cutoff 10000). AmpFlu (2 µM final) was added and the mixture was incubated for 2 min at 37 °C.

In all cases, fluorescent adducts were quantified as described above.

β-lactamase, D-aminopeptidase, DD-carboxypeptidase and thiolesterase activities of purified BlaB

Purified BlaB (0.6 and 3 µM, respectively) was tested for β-lactamase or D-aminopeptidase activities using the chromogenic substrates nitrocefin (90 µM final) [27] or D-Ala-p-nitroanilide (10 mM final) [28]. To probe for a DD-carboxypeptidase activity, purified BlaB (5.1 µM final) was incubated with AcKAA or Ac_2KAA (5 mM final) in the presence or absence of the acceptor Gly-Gly (10 mM final). The release of D-Ala was estimated by the D-amino acid oxidase method (DAAO) [29]. Thiolesterase activity of BlaB (0.12 or 0.23 µM final) was tested with S2a or S2d (200 µM final) and monitored at 250 nm in 50 mM phosphate buffer pH 7. Spectrophotometric measurements were performed with the help of an UVIKON 860 spectrophotometer interfaced to a Copam PC 88C microcomputer.

Site-directed mutagenesis of the three conserved motifs of BlaB

S47A mutation. This mutation was introduced by PCR with the oligonucleotides O1 and O2 (table 3) and pDML72 was used as template. O1 contains a *Bsr*GI site and the mutated codon (TCC→GCC), whereas O2 contains a *Sex*AI site. The purified PCR product (690 bp) was cloned in pCR-Script Amp SK(+) and sequenced with UP and RP (table 3). The non-methylated plasmid was digested by *Bsr*GI and *Sex*AI and the 662-bp fragment was cloned in pDML72, giving rise to pDML1176. The latter was cut by *Sph*I and the 6.6-kb fragment was cloned in pIJ702 to give pDML1177. *S. lividans* TK24 transformed by pDML1177 was called *S. lividans* CG10.

K254A mutation. This mutation was introduced by the Quick Change Site-Directed Mutagenesis Kit (Stratagene) using the pUC18 containing the 880-bp *Aat*II fragment of *blaB* as template and the oligonucleotides O3 and O4 which both contain the mutation (AAG→GCC) (table 3). The purified PCR fragment was sequenced (UP-RP) and cloned in pDML1164 to obtain pDML1178. This non-methylated plasmid was digested by *Bcl*I and *Sph*I and the 4.4-kb fragment was recovered. pIJ702 was cut with *Bgl*II and *Sph*I and the 5.5-kb fragment purified. pDML1179 was constructed by ligation of both fragments. Protoplasts of *S. lividans* TK24 were transformed by this plasmid and *S. lividans* CG11 was obtained.

G111N mutation. This mutation was introduced by PCR using the pDML72 as template and the oligonucleotides O5 to O8 (table 3). Three PCRs were performed. A 266-bp fragment containing a *Bsr*GI site and the mutation G111N (GGC→AAT) was obtained in a first PCR with O5 and O6. A 450-bp fragment which also included the mutation and a *Sex*AI site was amplified in a second PCR with O7 and O8. Both fragments were used in an overlapping PCR and a 685-bp fragment bearing the mutation and the *Sex*AI and *Bsr*GI sites was finally obtained. It was cloned in pCR-Script Amp SK(+) and sequenced (UP-RP). The mutated plasmid pDML1181 was obtained in the same way as pDML1177. *S. lividans* CR1 results from the transformation of *S. lividans* TK24 protoplasts by pDML1181.

S47C mutation. This mutation (TCC→TGC) was introduced as the S47A mutation and pDML1183 was obtained. Oligonucleotides O2 and O9 were used in the PCR (table 3). Transformation of *S. lividans* TK24 protoplasts by pDML1183 gave rise to *S. lividans* CR2.

Measurement of β-lactamase activity in *S. lividans* ML2, CG10, CG11, CR1 and CR2 in non-induced and induced conditions

YEME medium (25 ml) supplemented with thiostrepton (25 µg ml^{-1}) was inoculated with 1 ml of a 2-day culture of the reference *S. lividans* ML2 strain or of the *S. lividans* CG10, *S. lividans* CG11, *S. lividans* CR1 or *S. lividans* CR2 strains. Each culture was duplicated and inducer (7-ADCA) was added in only one flask (25 µg ml^{-1}). β-Lactamase activity was measured using nitrocefin as substrate [27].

Results and discussion

Overexpression and purification of BlaB

The *blaB* gene was amplified by PCR and cloned in the Vpro p145 vector as described in Materials and methods. BlaB was overexpressed in a soluble form in the cytoplasm of *S. lividans* TK24. Production was maximal after 72 h at 28 °C. The mycelium from a 250-ml culture was lysed by sonication and the soluble proteins recovered. BlaB was purified in a three-step procedure as described in Materials and methods. The sequence of the ten *N*-terminal amino acids of the purified protein corresponded exactly to that deduced from the nucleotide sequence of the *blaB* gene.

Identification of BlaB as a PBP

As BlaB possesses the three conserved motifs of the PREs, the penicillin-binding capacity of the purified protein was investigated. These experiments were based on

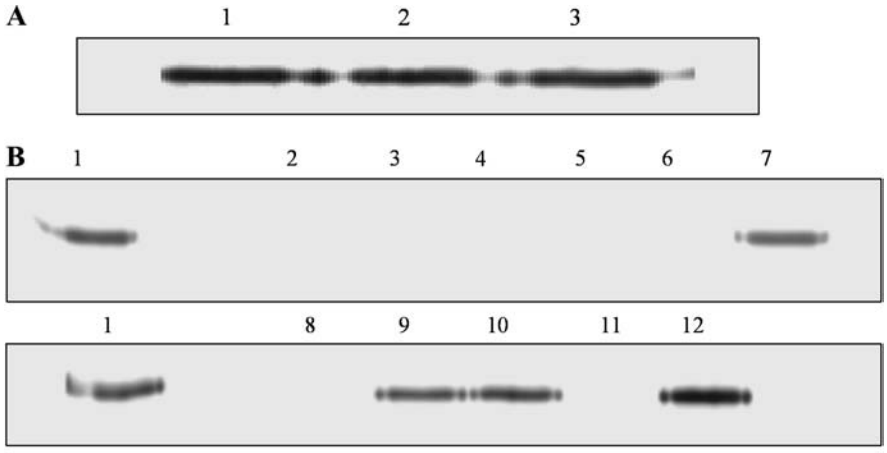

Figure 1. (*A*) AmpFlu-binding assay. Purified BlaB (2 µM final) was incubated with AmpFlu (2 µM final) for 2 (lane 1), 6 (lane 2) or 14 min (lane 3) at 37 °C. (*B*) Competition binding assays with some β-lactam antibiotics. Purified BlaB (4 µM final) was incubated at 37 °C for 1 h with 20 mM Tris-HCl pH 8.5 (lane 1; positive control) or with benzylpenicillin (lane 2), cloxacillin (lane 3), cephaloridine (lane 4), cefoxitin (lane 5), ampicillin (lane 6), cephacetrile (lane 7), imipenem (lane 8), 7-ADCA (lane 9), aztreonam (lane 10), cephalothin (lane 11) and cephalexin (lane 12) (each 3 mM final). AmpFlu (16 µM final) was then added and the mixture incubated for 2 min at 37 °C. In all cases, the fluorescent complexes were visualized as described in Materials and methods.

the fact that the acyl enzyme formed between PBPs and β-lactam antibiotics can be analysed by SDS-PAGE as a stable adduct. As shown in figure 1 A, a fluorescent band corresponding to the acyl enzyme formed between BlaB and AmpFlu can be detected. Different incubation times for the reaction of BlaB with AmpFlu were tested and a maximal signal was already reached after 2 min (fig. 1 A). Prior heat denaturation of BlaB completely abolished the binding of AmpFlu, suggesting that BlaB must be correctly folded to interact with AmpFlu (data not shown). From these results, we concluded that the purified BlaB behaves as a PBP since it can form a stable covalent adduct with AmpFlu. This result is in contrast with that obtained by Magdalena et al. [30] who concluded that BlaB was not a PBP. This discrepancy is probably due to the fact that these authors used a BlaB preparation obtained by solubilization of inclusion bodies.

To test if BlaB can bind other β-lactam antibiotics, competition binding assays were carried out as described in Materials and methods. As shown in figure 1 B, all the tested penicillins, cefoxitin, cephaloridine, cephalothin and imipenem interact with BlaB and prevent binding of AmpFlu. Incubation with 7-ADCA, cephacetrile, cephalexin or aztreonam does not hinder the AmpFlu-BlaB interaction. Important to note is that 7-ADCA is a good inducer for the BlaL β-lactamase despite the fact that it does not acylate BlaB. This shows that the acylation of BlaB by a β-lactam compound is not the triggering factor for β-lactamase induction. Presumably, the presence of 7-ADCA must be detected by a membrane sensor not yet identified in the *Streptomyces* regulatory system or that, as in enterobacteria, a peptidoglycan degradation product might reenter the cell and trigger derepression of transcription of the β-lactamase gene. The fact that BlaL is inducible in *S. lividans* TK24 when cloned only with the *blaA* and *blaB* genes suggests that this system must be present in *S. lividans* TK24 as in *S. cacaoi*.

Possible activities of purified BlaB

To detect β-lactamase, D-aminopeptidase, DD-carboxypeptidase or thiolesterase activities, purified BlaB was incubated with the appropriate substrates as described in Materials and methods. Under the assay conditions used, BlaB did not act as a β-lactamase, a D-aminopeptidase, a DD-carboxypeptidase or a thiolesterase. DD-carboxypeptidase activity was also tested in

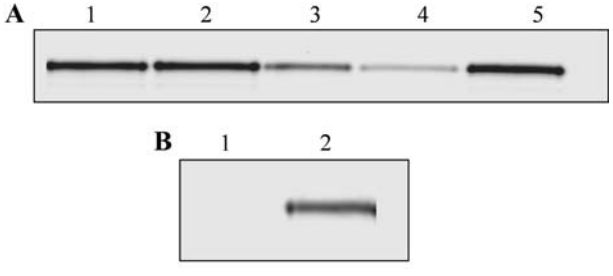

Figure 2. (*A*) Competition binding assays with AcKAA. Purified BlaB (3 µM final) was incubated with AcKAA [1.75 mM, lane 1; 3.5 mM, lane 2; 8.75 mM, lane 3; 17.5 mM, lane 4 and 0 mM final (positive control), lane 5] for 30 min at 37 °C. AmpFlu (2 µM final) was then added and the mixture incubated for 2 min at 37 °C. (*B*) Demonstration that the interaction between BlaB and AcKAA is covalent. Purified BlaB (3 µM final) was incubated with AcKAA [35 mM, lane 1; 0 mM (positive control) lane 2] for 1 h at 37 °C. Excess AcKAA was eliminated before AmpFlu (2 µM final) was added. The mixture was incubated for 2 min at 37 °C and the fluorescent complexes analysed as described in Materials and methods.

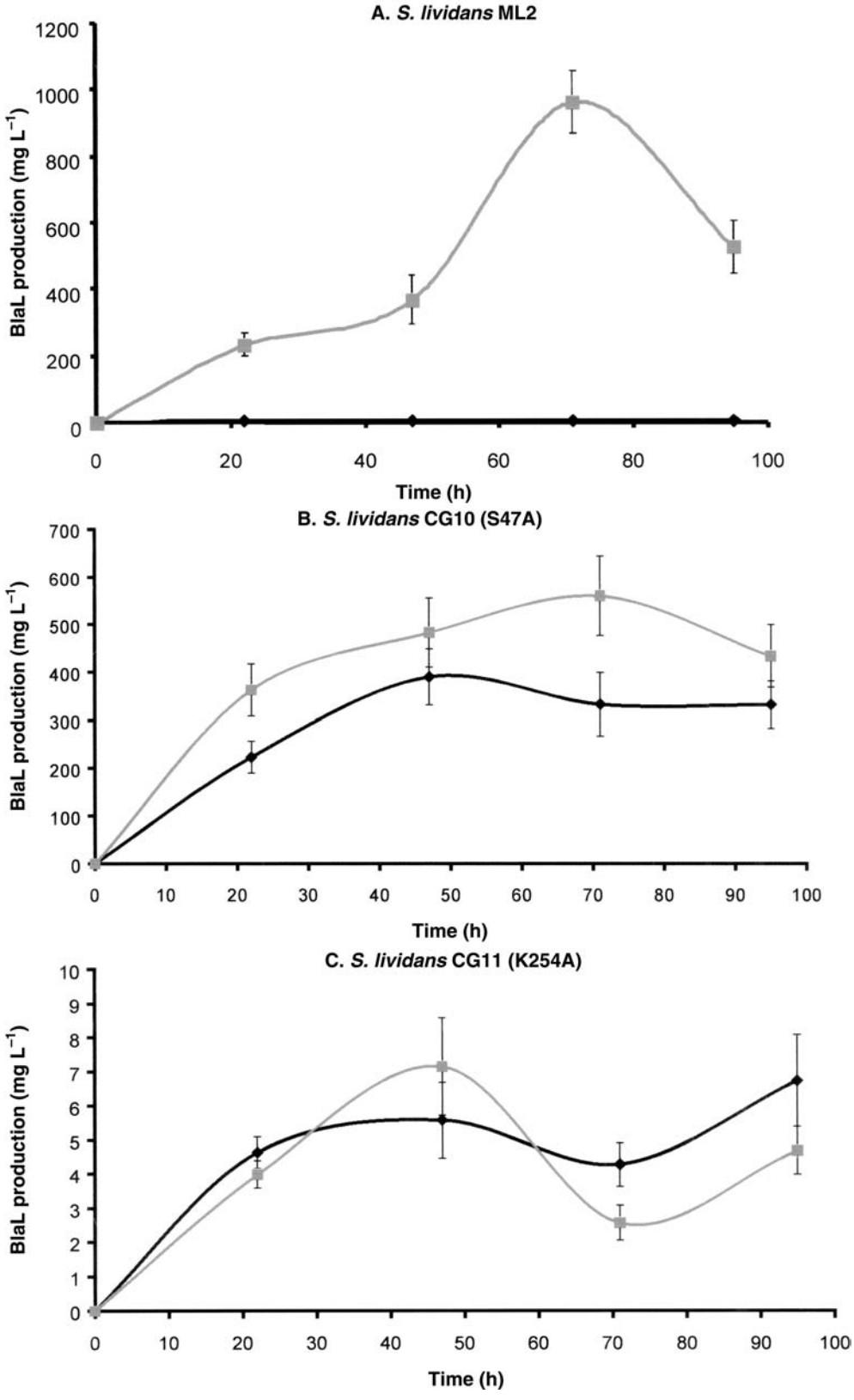

Figure 3. β-Lactamase production in *S. lividans* ML2 (*A*), in *S. lividans* CG10 (*B*) and in *S. lividans* CG11 (*C*) in non-induced (◆) and induced (■) conditions. 7-ADCA (25 µg ml^{-1}) was added as inducer at time zero.

the presence of Gly-Gly because, in some cases, the released D-alanine acts as an aminolytic acceptor in the enzyme deacylation step, the peptide donor is thus continuously regenerated and the enzyme is seemingly silent. Even in these conditions, BlaB was devoid of DD-carboxypeptidase activity. However, we showed that BlaB can covalently interact with AcKAA but not with Ac_2KAA (fig. 2).

The binding of β-lactam antibiotics and AcKAA to BlaB is important because most if not all the known PBPs are proteins involved in peptidoglycan metabolism and can bind some peptidoglycan moiety. This observation supports the hypothesis that the intracellular inducer of the *S. cacaoi* β-lactamases could be a peptidoglycan degradation product.

β-lactamase activity in *S. lividans* ML2 and in *S. lividans* CG10, CG11, CR1 and CR2 in the presence or absence of a β-lactam inducer

β-lactamase production by *S. lividans* CG10, CG11, CR1 and CR2 was measured in non-induced and induced conditions. It was compared to that of *S. lividans* ML2 used as reference. *S. lividans* ML2 is a *S. lividans* strain containing a plasmid which bears the *blaB*, *blaA* and *blaL* genes (table 2). 7-ADCA which is a poor substrate [31] of the BlaL β-lactamase was used as inducer.

In *S. lividans* ML2, the BlaL β-lactamase was produced at a basal level in the absence of inducer. When 7-ADCA was added, BlaL production increased, with a maximal induction factor of 200 after 71 h (fig. 3A).

In *S. lividans* CG10 (S47A), BlaL production was approximately the same with or without 7-ADCA (fig. 3B). In the absence of inducer, production of β-lactamase by this strain was higher than that obtained with *S. lividans* ML2 and it barely increased when 7-ADCA was added (mean induction factor: 1.5). So, replacement of the serine of the BlaB $S^{47}LVK$ motif by an alanine results in a high-constitutive phenotype.

In *S. lividans* CG11 (K254A), addition of 7-ADCA had no effect on β-lactamase production which remains at a basal level similar to that observed in *S. lividans* ML2 (fig. 3C). The K254A mutation thus abolishes the capacity of BlaB to promote induction of BlaL.

In *S. lividans* CR1 (G111N) and in *S. lividans* CR2 (S47C), the induction profile was similar to that observed in *S. lividans* ML2 (fig. 4). In non-induced conditions,

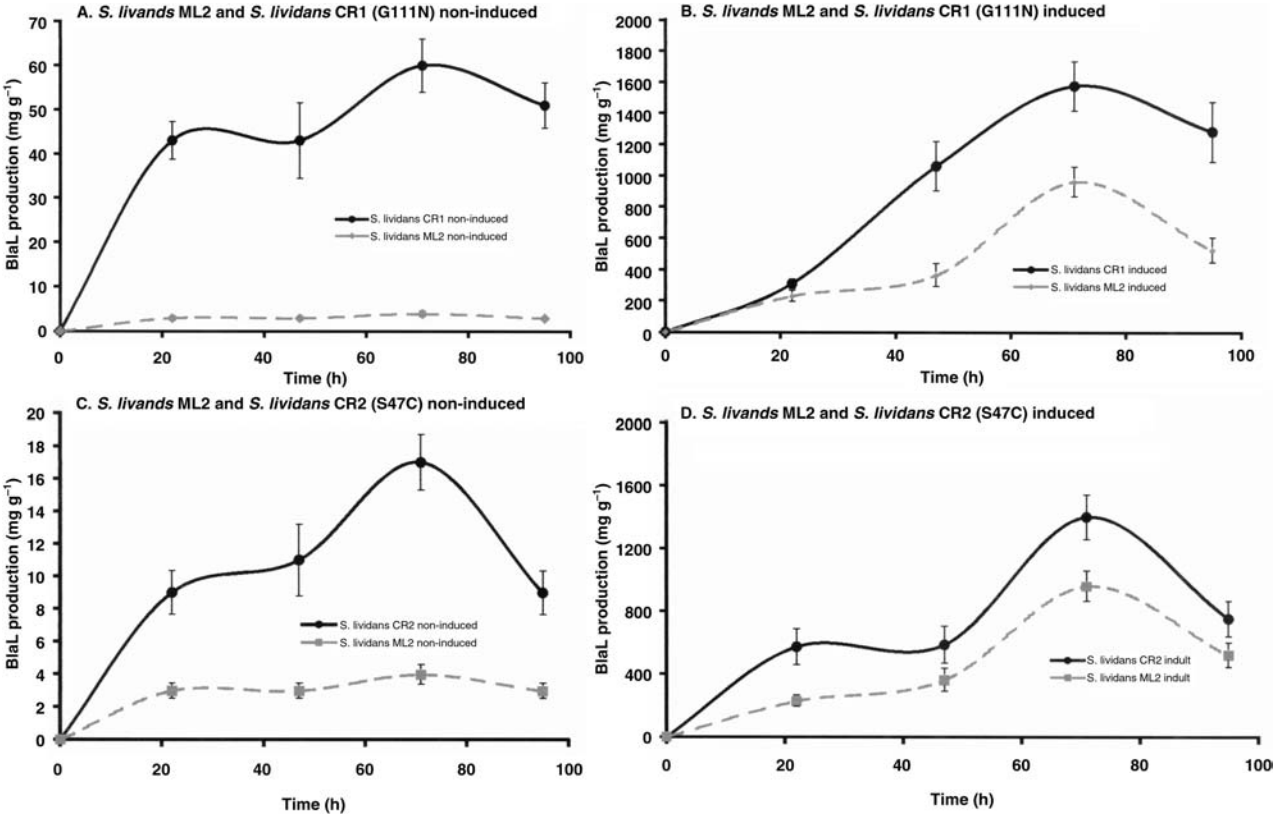

Figure 4. β-Lactamase production by *S. lividans* CR1 and *S. lividans* CR2 compared to that of *S. lividans* ML2 in non-induced and induced conditions. Dashed lines refer to *S. lividans* ML2, full lines are for *S. lividans* CR1 and CR2. (*A*, *B*) BlaL production in *S. lividans* CR1 and in *S. lividans* ML2 in the presence or absence of 7-ADCA, respectively. (*C*, *D*) β-Lactamase production by *S. lividans* CR2 and by *S. lividans* ML2 in non-induced and induced conditions. 7-ADCA was added (25 µg ml^{-1}) at time 0.

BlaL was produced at a basal level and the production increased when 7-ADCA was added. However, the β-lactamase production in these strains was comparatively higher than in *S. lividans* ML2.

These results suggest that these three conserved motifs of BlaB are important for the BlaL regulatory process.

When BlaB is inactivated, BlaL is no longer inducible and the basal production is reduced 30- to 60-fold [8]. This fact suggests an activator role for this protein. The high-constitutive production of BlaL observed in *S. lividans* CG10 (S47A) could be due to an altered conformation of BlaB that mimics the binding of the activator ligand. The constitutive basal phenotype of *S. lividans* CG11 (K254A) could be due to the fact that this mutation makes BlaB fold into a conformation that cannot be activated upon ligand binding.

Possible mechanism of *S. cacaoi* β-lactamases induction

The results presented in this paper suggest that the presence of a β-lactam antibiotic outside the cell could generate a stress which could be transmitted into the cell and to BlaA via BlaB. The nature of the intracellular signal remains unknown. However, as shown in this study, BlaB can be acylated by the AcKAA peptide and, thus, BlaB may also be able to interact with compounds derived from peptidoglycan and resulting from the penicillin stress. Although no peptidoglycan recycling has been highlighted in Gram-positive bacteria, small amounts of peptidoglycan degradation fragments could reenter the cell and acylate BlaB and, consequently, transcription of the β-lactamase genes might be directly or indirectly activated. To confirm this hypothesis, peptidoglycan degradation products such as tri-, tetra- and pentapeptides linked or not to a saccharidic moiety should be prepared and tested for their capacity to interact with BlaB.

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