

Structure, Function, and Fate of the BlaR Signal Transducer Involved in Induction of β -Lactamase in *Bacillus licheniformis*

YINGFANG ZHU,^{1,2} SERGE ENGLEBERT,³ BERNARD JORIS,³ JEAN-MARIE GHUYSEN,³ TETSUO KOBAYASHI,^{1,4} AND J. OLIVER LAMPEN^{1*}

Waksman Institute, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08855-0759¹; Centre d'Ingénierie des Protéines, Université de Liège, B6 Sart-Tilman, Liège, Belgium³; Department of Radiation Oncology, University of California, San Francisco, San Francisco, California 94143-0806²; and RIKEN, The Institute of Physical and Chemical Research, Wako, Saitama, 351-01, Japan⁴

Received 16 March 1992/Accepted 26 July 1992

The membrane-spanning protein BlaR is essential for the induction of β -lactamase in *Bacillus licheniformis*. Its nature and location were confirmed by the use of an antiserum specific for its carboxy-terminal penicillin sensor, its function was studied by genetic dissection, and the structure of the penicillin sensor was derived from hydrophobic cluster analysis of the amino acid sequence by using, as a reference, the class A β -lactamases with known three-dimensional structures. During the first 2 h after the addition of the β -lactam inducer, full-size BlaR, bound to the plasma membrane, is produced, and then β -lactamase is produced. By 2 h after induction, BlaR is present in various (membrane-bound and cytosolic) forms, and there is a gradual decrease in β -lactamase production. The penicillin sensors of BlaR and the class D β -lactamases show strong similarities in primary structures. They appear to have the same basic spatial disposition of secondary structures as that of the class A β -lactamases, except that they lack several α helices and, therefore, have a partially uncovered five-stranded β sheet and a more readily accessible active site. Alterations of BlaR affecting conserved secondary structures of the penicillin sensor and specific sites of the transducer annihilate β -lactamase inducibility.

Bacillus licheniformis produces an inducible β -lactamase (penicillinase, BlaP) belonging to class A. Induction is regulated by a repressor (BlaI), a signal acceptor-transducer (the BlaR penicillin sensory transducer) (28), and a signal carrier, R2 (1, 22). BlaR has two major domains with an amino-terminal transducer that consists of several transmembrane segments and a carboxy-terminal penicillin sensor, BlaR-CTD, that protrudes from the outer face of the membrane (12). The sensor has the specific markers of the penicilloyl serine transferases (6) and shows high homology to that of the class D OXA-2 β -lactamase of *Salmonella typhimurium* (28). On this basis, the transducer (which extends from M-1 to A-345) is assumed to terminate and the sensor (which extends from M-346 to R-601) is assumed to start 57 residues upstream from the essential serine of the tetrad S402TYK. Accordingly, BlaR-CTD, which comprises essentially the penicillin sensor of BlaR fused to the secretion sequence of the OXA-2 β -lactamase of *S. typhimurium* (12), is a soluble penicillin-binding protein of M_r 26,000. It has been purified to protein homogeneity (15).

The membrane-bound location of BlaR in *B. licheniformis* has now been studied by cell fractionation and Western immunoblotting with antibody to BlaR-CTD and by labelling with radioactive penicillin. For functional analysis of the domains of BlaR during induction of β -lactamase, plasmids with mutant *blaR* genes have been constructed and their inducibilities in *Bacillus subtilis* have been tested. Finally, hydrophobic cluster analysis by using class A β -lactamases with known three-dimensional structures as a reference has allowed us to identify most of the secondary structures in the

penicillin sensor of BlaR as well as sites for signal generation.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains and plasmids used are listed in Table 1.

Materials. Enzymes for the manipulation of DNA were purchased from New England BioLabs, Inc. (Beverly, Mass.) or Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). [³⁵S]methionine was a product of Amersham Corp. (Arlington Heights, Ill.). [³H]benzylpenicillin and En³Hance were obtained from New England Nuclear Research Products (Boston, Mass.). Phenylmethylsulfonyl fluoride (PMSF) was obtained from United States Biochemical Corp. Cephalosporin C was a gift from E. Lilly and Co. (Indianapolis, Ind.). Goat anti-immunoglobulin G rabbit-alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals. The BCA* protein assay reagent was obtained from Pierce (Rockford, Ill.). The rProtein A purification kit was obtained from Beckman Instruments, Inc. (Fullerton, Calif.).

Antibody to BlaR-CTD. One milligram of purified BlaR-CTD (see above) was injected intracutaneously into two rabbits over a period of 42 days (Pocono Rabbit Farm and Laboratory, Pocono, Pa.). Sera from days 0, 21, and 42 were tested (17) against membrane proteins from *B. licheniformis* 749/110. With membrane fractions from induced cultures, large amounts of a 68-kDa protein were detected with both day-21 and day-42 sera but not with the preimmune serum. Moreover, *Escherichia coli* K38 cultures carrying pRWZ1 (Km^r Ap^r *blaR*) and labelled with [³⁵S]methionine produced a labelled 68-kDa cross-reacting protein, while those without

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Source or reference
Strains		
<i>B. licheniformis</i>		
749/110 (wild type)	<i>blaP blaI blaR</i>	4
749/110/C3 <i>pen-22</i>	<i>blaP22 blaIC3 blaR</i>	4
749/110 <i>pen-27</i>	<i>blaP blaI blaR27</i>	4
749/110 <i>pen-28</i>	<i>blaP blaI blaR28</i>	4
749/110 <i>pen-31</i>	<i>blaP blaI blaR R2</i>	4
<i>B. subtilis</i> BD224	<i>trpC2 thr-5 recE4</i>	Bacillus Genetic Stock Center, Ohio State University, Columbus
Plasmids		
pT7-6/pGP-2	Km ^r Ap ^r	24
pRWZ1	Km ^r Ap ^r <i>blaR</i>	28
pUB110	Km ^r	16
pRWC5	Km ^r <i>pen-28</i>	28
pRWT20	Km ^r <i>blaP-blaI-blaR</i>	13
pRWT24	Km ^r <i>pen-27</i>	This study
pRWT28	Km ^r <i>pen-31</i>	This study
pRWT203	Km ^r <i>blaR203</i>	This study
pRWT204	Km ^r <i>blaR204</i>	This study
pRWT205	Km ^r <i>blaR205</i>	This study
pRWT206	Km ^r <i>blaR206</i>	This study
pRWT207	Km ^r <i>blaR207</i>	This study
pRWT208	Km ^r <i>blaR208</i>	This study
pRWT209	Km ^r <i>blaR209</i>	This study
pRWT210	Km ^r <i>blaR210</i>	This study
pRWT211	Km ^r <i>blaR211</i>	This study
pRWT212	Km ^r <i>blaR212</i>	This study

a *blaR*⁺ gene did not (17, 24, 27). Bands corresponding to smaller but not larger proteins were found under conditions in which proteolysis might be expected. On the basis of these results, it was concluded that the antibody to BlaR-CTD was specific for the penicillin sensor of BlaR. The antibody was purified on an rProtein A column.

Induction of β -lactamase. Cultures of *B. licheniformis* or *B. subtilis* grown in LB broth (containing, per liter, tryptone, 10 g; yeast extract, 5 g; and NaCl, 5 g) with shaking at 37°C to the early log phase were induced by adding cephalosporin C (final concentration, 5 μ M) and allowing growth to continue. At various times after induction, samples of the cultures were gently sonicated, and the β -lactamase activity was assayed by the colorimetric method (21). One unit of β -lactamase is the amount of enzyme hydrolyzing 1 μ mol of benzylpenicillin in 1 h at 30°C. The protein concentration was estimated by the BCA* method (23).

Cell fractionation. Cells of *B. licheniformis* and *B. subtilis* were harvested, and the supernatants were concentrated by acetone precipitation (culture fluid). The cell pellets were washed with 50 mM KPO₄ (pH 7.0) and suspended in 50 mM Tris-HCl (pH 7.5)–10 mM MgCl₂–1 mg of lysozyme per ml–40 μ g of PMSF per ml–10 μ g of DNase I per ml. The suspensions were incubated at room temperature for 30 min and then sonicated for 12 min in 50-s pulses. The sonicated suspensions were separated by centrifugation at 120,000 \times g and 4°C for 1 h. The supernatant was termed the cytoplasmic fraction. The membrane fractions were obtained by solubilization of the remaining pellets in 50 mM KPO₄ (pH 7.0)–1 mM NaCl–2% Triton X-100–1 mM β -mercaptoethanol and

centrifugation to remove cell wall remnants as described by Zhu et al. (28).

Detection of penicillin-binding proteins. *B. licheniformis* and *B. subtilis* were grown at 37°C to the early log phase in the absence of the β -lactam inducer. Membrane fractions (100 μ g of protein in a 50- μ l final volume) were incubated with [³H]benzylpenicillin (0.2 μ g; 10 μ Ci) for 20 min at room temperature. The proteins were precipitated with acetone, resuspended in cracking buffer, and separated on sodium dodecyl sulfate (SDS)–13% polyacrylamide gels. The gels were fixed in 30% methanol–10% acetic acid, treated with En³Hance, dried, and fluorographed at –70°C for 2 to 3 days.

Construction of *blaR* Mutants. (i) *blaR203*, *blaR204*, *blaR205*, and *blaR206*. For *blaR203*, *blaR204*, *blaR205*, and *blaR206*, plasmid pRWT20 (Km^r *blaP-blaI-blaR*); for a restriction map, see Kobayashi et al. [13] and Zhu [27]) was digested with *Bal*I, *Hpa*I, *Sst*I, and *Oxa*NI, respectively, which recognize a single site in pRWT20 (within *blaR*). The *Bal*I and *Hpa*I digests were ligated with an *Sma*I hexamer (dCCCCGGG) and recircularized with T4 DNA kinase and ligase. The *Sst*I digests were treated with T4 DNA polymerase to produce blunt ends, ligated with a *Bam*HI decamer (dCGGGATCCCCG), and recircularized. The *Oxa*NI digests were also treated with T4 DNA polymerase, ligated with the *Sma*I hexamer, and recircularized.

(ii) *blaR207*, *blaR208*, and *blaR209*. For *blaR207*, *blaR208*, and *blaR209*, pRWT20 was partially digested with *Eco*RI, *Eag*I, and *Ban*I, respectively. The ends were repaired with T4 DNA polymerase, and an *Sma*I octamer (dCCCCGGGG) was ligated with the ends. The singly cut fragments were purified from agarose gels and recircularized.

(iii) *blaR210*, *blaR211*, and *blaR212*. For *blaR210*, *blaR211*, and *blaR212*, an *Sst*I-*Bam*HI fragment of pRWT20 containing a portion of *blaR* was subcloned into pUC19, and the resultant plasmids were partially digested with *Taq*I, *Acc*I, and *Dra*I, respectively. The *Taq*I and *Acc*I digests were repaired with T4 DNA polymerase and ligated with an *Sma*I decamer (dCCCCGGGG). The singly cut fragments were purified from agarose gels, recircularized, and used to transform *E. coli*. Plasmids that contained a single *Sma*I site in this portion of *blaR* were digested with *Sst*I and *Bam*HI and ligated with pRWT20 digested with the same enzymes.

The constructs were introduced into *B. subtilis* by protoplast transformation, and the transformants were screened for the desired restriction enzyme patterns of the plasmids. To locate and identify the mutations, we replaced portions of the wild-type *bla* gene cluster in pRWT20 with corresponding portions from the mutants or vice versa using appropriate restriction enzymes and tested the resultant chimeric clusters for their phenotypes. The DNA fragments that contained a mutation were sequenced by the dideoxy chain termination method (20) with [α -³⁵S]dATP.

Hydrophobic cluster analysis. Hydrophobic cluster analysis (5, 8) is based on a duplicated representation of amino acid sequences on an α -helical two-dimensional pattern in which the hydrophobic residues tend to form clusters that usually correspond to secondary structures. Proteins whose hydrophobic clusters have similar shapes, sizes, and positions along the amino acid sequences show similarities in polypeptide folding. Compared with methods based on single amino acid properties or identities, hydrophobic cluster analysis allows distant information to become more visible and, for purposes of amino acid alignment, allows deletions or insertions to be introduced more easily between secondary structures.

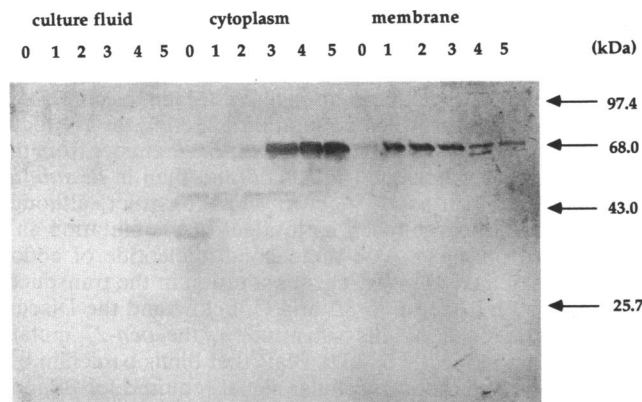


FIG. 1. Formation and distribution of BlaR during the induction of β -lactamase in *B. licheniformis* 749/110. Samples taken at 0 to 5 h after induction were separated into culture fluid, cytoplasm, and membrane fractions. The proteins were separated on an SDS-13% polyacrylamide gel, and 30 μ l of purified antibody to BlaR-CTD was used for Western blotting.

RESULTS

Nature and location of BlaR during the β -lactamase induction cycle of *B. licheniformis* 749/110. Direct evidence that BlaR is a membrane-bound protein was obtained by cell fractionation. Parallel cephalosporin C-induced and uninduced cultures were sampled hourly and separated into culture fluid, cytoplasm, and membrane fractions. Sixty micrograms of protein from each sample was loaded on a medium-sized SDS-13% polyacrylamide gel and separated at 60 mA for 8 h before Western blotting (25, 26) with purified antibody to BlaR-CTD (Fig. 1). BlaR was quantitated by computerized densitometry of the blots (Fig. 2).

Membranes from uninduced cultures contained trace amounts of 68-kDa BlaR material (Fig. 1). At 1 to 3 h after induction, the amount of BlaR was increased considerably; BlaR was almost exclusively in the 68-kDa form and occurred only in the membrane fraction (Fig. 1). At 4 and 5 h after induction, the total amount of membrane-bound BlaR was decreased, and about one-third of that material was 60 to 63 kDa in size (Fig. 2), suggesting proteolytic cleavage of the extracellular domain of the transducer (see Fig. 8B and C, below, and the Discussion). In parallel to these changes,

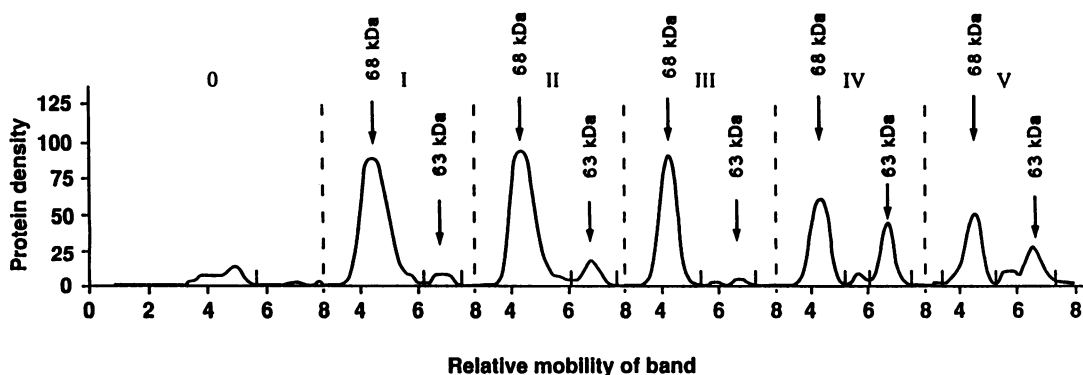


FIG. 2. Quantitation by a densitometric analysis of the level of membrane-bound BlaR during the induction of β -lactamase in *B. licheniformis* 749/110. Antibody to BlaR-CTD was used for detection. The vertical axis represents the density of the BlaR bands in the membrane fraction. The horizontal axis shows the mobility of the BlaR bands in samples obtained throughout induction. 0, I, II, III, IV, and V indicate, respectively, samples induced for 0 to 5 h. BlaR bands of 68 and 63 kDa are marked.

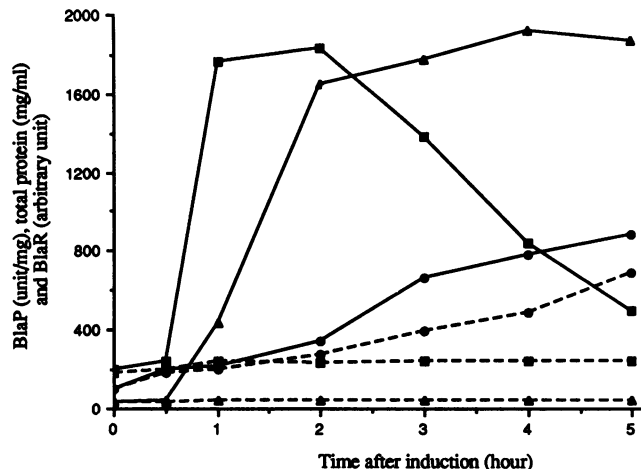


FIG. 3. Relative levels of membrane-bound 68-kDa BlaR (■), BlaP (β -lactamase) (\blacktriangle), and total cell protein (\bullet) during induction of β -lactamase in *B. licheniformis* 749/110. Results for induced cultures are shown in solid lines, and those for uninduced cultures are shown in broken lines. BlaP values (β -lactamase units per milligram of protein) were obtained by a Sargent (21) β -lactamase assay; BlaR arbitrary units were obtained by a densitometric analysis (Fig. 2); and total protein was estimated by use of a Bio-Rad assay.

the bulk of the synthesized 68-kDa BlaR failed to undergo membrane integration and accumulated in the cytoplasm (Fig. 1). At no time was BlaR detected in the culture fluid fraction (Fig. 1).

The level of membrane-bound BlaR reached its peak at 1 to 2 h after induction (7.5- and 9-fold higher than the uninduced level at 1 and 2 h, respectively) (Fig. 3). The ratio was down to fivefold by 3 h and twofold by 5 h. The increase in the specific activity of β -lactamase occurred between 1 and 2 h and reached 64-fold. This result is consistent with the finding of Salerno and Lampen (18, 19) that the peak level of *blaI-blaR* mRNA occurred 30 to 60 min before the peak level of *blaP* mRNA.

Forms of BlaR in mutants of *B. licheniformis* 749/110. Membranes from uninduced and 2-h-induced cells of wild-type strain 749/110 and the *pen-22*, *pen-27*, *pen-28*, and *pen-31* mutants (Table 1) were subjected to Western blotting with purified antibody to BlaR-CTD (Fig. 4). Membranes

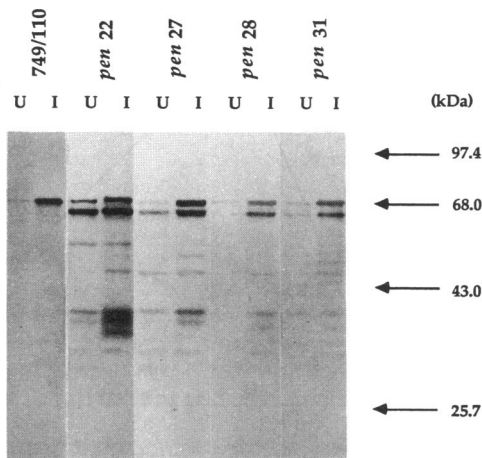


FIG. 4. BlaR-CTD antibody detection of forms of membrane-bound BlaR produced by *B. licheniformis* strains carrying mutations in *blaR*. Membrane proteins from induced (I) or uninduced (U) cultures were separated on an SDS-polyacrylamide gel. Purified antibody to BlaR-CTD was used for the Western blotting. Pre-stained protein molecular mass markers were used.

from uninduced cultures were also submitted to labelling with [3 H]benzylpenicillin (28) (Fig. 5). The following observations were made.

Immunoreactive 68-kDa BlaR material occurred in trace amounts in the membranes of uninduced mutants, except the *pen-22* mutant, in which the basal level was relatively high (Fig. 4), and was overproduced by induction in all of the mutants (Fig. 4). BlaR (at the basal level) behaved as a penicillin-binding protein, except that BlaR produced by the *pen-28* mutant did not react with penicillin (see below) and thereby served as a negative control (Fig. 5). Note that the 94- and 43-kDa radioactive bands seen in Fig. 5 are the main penicillin-binding proteins involved in cell wall peptidoglycan synthesis in *B. licheniformis*. The membranes of all the mutant strains but not those of the wild-type strain also possessed several immunoreactive membrane-bound proteins with molecular masses smaller than 68,000 Da (Fig. 4), suggesting that mutations in *blaR* result in the production of protein products that are unstable.

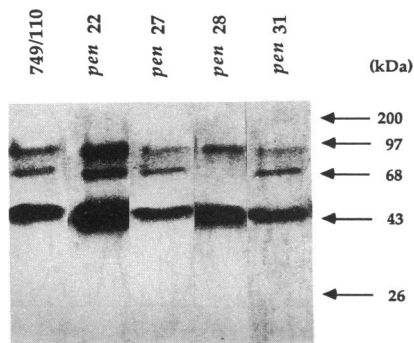


FIG. 5. [3 H]benzylpenicillin detection of forms of membrane-bound BlaR produced by *B. licheniformis* strains carrying mutations in *blaR*. Solubilized membrane proteins (100 μ g) from uninduced cultures were incubated with [3 H]benzylpenicillin in a 50- μ l total volume, separated on an SDS-polyacrylamide gel, and fluorographed. [14 C]-labeled protein molecular mass markers were used.

The properties of the mutants were consistent with their genotypes. The *pen-22* mutant has a normal *blaR* and a mutated *blaP*. Hence, it synthesizes an inducible penicillin-binding BlaR but produces an inactive β -lactamase (1).

The *pen-27* mutant has a mutation affecting the *R1* locus (4, 22), now called *blaR*. The *blaP-blaI-blaR* cluster from the *pen-27* mutant was cloned in *E. coli* and then in *B. subtilis* with pUB110 (16) as the vector. This construct, although weakly expressed, allowed us to identify the mutation as a transition from G \rightarrow A in the second nucleotide of codon 124, causing a Gly-124 \rightarrow Asp substitution in the transducer domain of BlaR (see Fig. 8B and C, below and the Discussion). As a result of this substitution, the *pen-27* mutant produces an inducible 68-kDa BlaR that binds penicillin but fails to generate the intracellular signal required for β -lactamase synthesis. Accordingly, the *pen-27* mutant shows poor induction of β -lactamase (60 U/mg of protein at 3 h, instead of 1,300 U/mg of protein for the wild type).

The *pen-28* mutant was suggested to carry a superrepressor (22). The *blaP-blaI-blaR* cluster of the *pen-28* mutant was cloned in *E. coli* and then in *B. subtilis* (28). β -Lactamase production was not inducible in this construct. The mutation proved to be a G \rightarrow A transition in the second nucleotide of codon 538, causing a Gly-538 \rightarrow Asp substitution in the sensor of BlaR, immediately upstream from the essential K539TG541 triad (see Fig. 8B and C, below, and the Discussion). As a result of this alteration, the *pen-28* mutant still produces an immunoreactive 68-kDa protein, but the protein fails to bind penicillin and thereby is nonfunctional for β -lactamase induction.

The *pen-31* mutant has a mutation within the locus that codes for the signal carrier R2 which is not linked to *blaP* (22) and thereby does not induce β -lactamase synthesis in *B. licheniformis*. In *B. subtilis* carrying the *bla* cluster from the *pen-31* mutant, β -lactamase production is inducible (showing that the mutation is outside the *bla* cluster), as is 68-kDa BlaR production.

Expression of *blaR* mutations cloned in *B. subtilis* BD224. The mutations studied were those that occurred spontaneously in *B. licheniformis* (*pen-27*, *pen-28*, and *pen-31*; see above) and those that were made by inserting linkers in the *blaR* coding region (see Materials and Methods). Figure 6 shows the production of induced immunoreactive forms of BlaR in *B. subtilis* from a plasmid bearing wild-type *blaR* and from plasmids carrying mutated forms of *blaR* (27). Table 2 shows the amino acid changes at the altered sites (listed sequentially with reference to the amino acid sequence of BlaR, from the N terminus to the C terminus of the protein) and the observed effects that these changes had on β -lactamase production and inducibility. Alterations at sites 1 to 6 and at sites 7 to 12 affected the transducer and the sensor of BlaR, respectively. Alterations at sites 2, 4, and 6 (transducer) and at sites 7 and 10 (sensor) were neutral; an alteration at site 9 (sensor) decreased significantly β -lactamase inducibility; alterations at sites 1, 3, and 5 (transducer) and at sites 8, 11, and 12 (sensor) were highly detrimental. Attempts were made to translate these effects into structural terms.

Molecular organization of the BlaR penicillin sensor. The class A β -lactamases of *Staphylococcus aureus* (9) and *Streptomyces albus* G (3, 14) have known three-dimensional structures. They are two-domain proteins with an all- α domain and an α/β domain whose five stranded β sheet (β 1 to β 5) is protected on both faces by additional helices. The active site, at the junction between the all- α and α/β domains, is defined by four conserved motifs: the essential serine-containing tetrad S70TSK (or S70VFK) occurs in

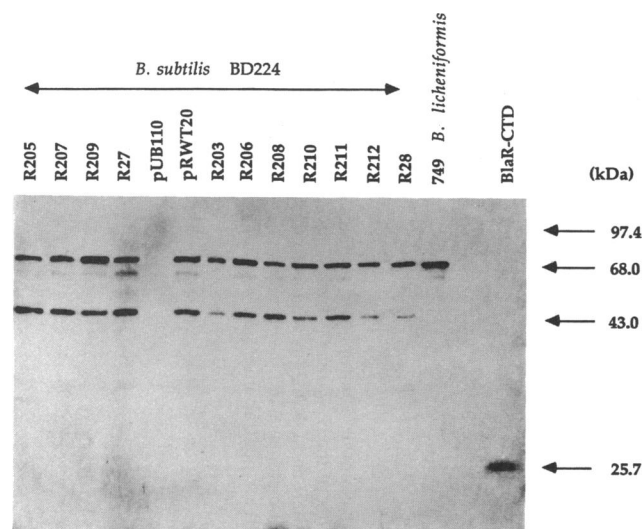


FIG. 6. Production of BlaR in *B. subtilis* BD224 from plasmids carrying mutated forms of *blaR*. Membrane fractions (60 μ g of protein) from induced cultures were separated on an SDS-polyacrylamide gel. Purified BlaR-CTD (0.5 μ g) was included as a positive control, and *B. subtilis* pUB110 (vector only) was included as a negative control. Thirty microliters of purified antibody to BlaR-CTD was used for the Western blotting. Prestained protein molecular mass markers were used.

helix α 2, the triad S130DN occurs on a loop between α 4 and α 5, the motif E166IELN (or E166PELN) occurs on a small helix between α 6 and α 8, and the triad K234SG (or K234TG) occurs on the innermost β 3 strand of the β sheet (6, 10, 11). The role played by the active site-defining motif in the catalyzed hydrolysis of β -lactam antibiotics has been studied (14).

With *S. aureus* β -lactamase as a reference (Fig. 7A), hydrophobic cluster analysis (see Materials and Methods)

allowed us to identify the secondary structures and active site-defining motifs along the amino acid sequence of the penicillin sensor of BlaR (from M-346 to R-601) (Fig. 7B) and OXA-2 β -lactamase (Fig. 7C). With *S. albus* G β -lactamase as a reference (Fig. 8A), these data led to the following conclusions regarding the three-dimensional structure of the BlaR penicillin sensor (Fig. 8B) and, by extension, that of the class D β -lactamases. (i) At variance with the scaffolding of the class A β -lactamases, helices α 4 and α 9 of the sensor are connected by a short \approx 30-amino-acid stretch, resulting in a more exposed active site and in a five-stranded β sheet that lacks α helices on one face. (ii) The tetrad S402TYK on α 2, the triad Y476GN downstream from α 4, and the triad K539TG on β 3 of the sensor are homologs of the corresponding active site-defining motifs of the class A β -lactamases. That the D490GSLQ motif between α 4 and α 9 of the sensor is equivalent to the E166IELN (or E166PELN) motif of the class A β -lactamases remains hypothetical.

On the basis of this model, it follows that alterations at sites affecting the conserved secondary structures β 2 (site 8; Table 2), α 10 (site 11), and β 3 (site 12) of the sensor yield altered BlaR proteins that are nonfunctional for β -lactamase induction. Alterations occurring outside the secondary structures are much less detrimental. Increasing the stretch that connects α 4 and α 9 (site 9) with four amino acid residues results in decreased β -lactamase inducibility. Increasing the loop that connects α 1 to β 1 with three amino acid residues (site 7) and increasing the loop that connects α 9 to α 10 with two amino acid residues (site 10) results in no change.

Molecular organization of the BlaR transducer. Figure 8C presents a tentative three-transmembrane-segment model of the transducer of BlaR (which extends from M1 to A435) (7). This model is consistent with the following observations. (i) Insertion of the tetrapeptide PGGT between T-38 and H-39 (site 1; Table 2), i.e., at the amino side of the membrane anchor, annihilates β -lactamase inducibility. (ii) The transmembrane segment from M-112 to L-137 has sequence similarity with signal transduction sequences in chemotactic proteins from *E. coli* (12). Altering this segment by changing

TABLE 2. Properties of *blaR* mutants of *B. subtilis* BD224

Plasmid in <i>B. subtilis</i> strain	Mutation	Amino acid changes and inserts (underlined)	Domain affected ^a	Site altered	β -Lactamase activity (U/mg of protein) in cultures that were ^b	
					Uninduced	Induced
pRWT20	None (wild type)				64	2,900
pRWT28	<i>pen-31</i>	Outside <i>bla</i> cluster			50	2,700
pRWT209	<i>bla-209</i>	T-38 <u>PGGT</u> H-39	Transducer	1	34	46
pRWT205	<i>bla-205</i>	A-74 \rightarrow GIP	Transducer	2	92	3,500
pRWT24	<i>pen-27</i>	G-124 \rightarrow D	Transducer	3	56	71
pRWT204	<i>bla-204</i>	V-149 <u>PG</u> N-150	Transducer	4	56	2,400
pRWT207	<i>bla-207</i>	F-295 <u>PGEF</u> S-296	Transducer	5	38	56
pRWT203	<i>bla-203</i>	A-355 <u>RA</u> M-356	Transducer	6	46	1,600
pRWT206	<i>bla-206</i>	S-373 <u>PGS</u> G-374	Sensor	7	54	2,200
pRWT210	<i>bla-210</i>	Y-386 <u>PPGD</u> T-387	Sensor	8	26	34
pRWT208	<i>bla-208</i>	A-485 <u>PRGA</u> D-486	Sensor	9	56	550
pRWT211	<i>bla-211</i>	F-514 <u>PG</u> K-515	Sensor	10	38	1,900
pRWT212	<i>bla-212</i>	S-525 <u>PPGA</u> I-526	Sensor	11	100	110
pRWC5	<i>pen-28</i>	G-538 \rightarrow D	Sensor	12	100	120

^a The transducer extends from M-1 to A-345. The sensor extends from M-346 to R-601.

^b Activity in induced cultures was measured after induction with 5 μ M cephalosporin C. With *B. licheniformis* 749/110 (wild-type strain), β -lactamase activities were 11 U (uninduced) and 1,300 U (induced).

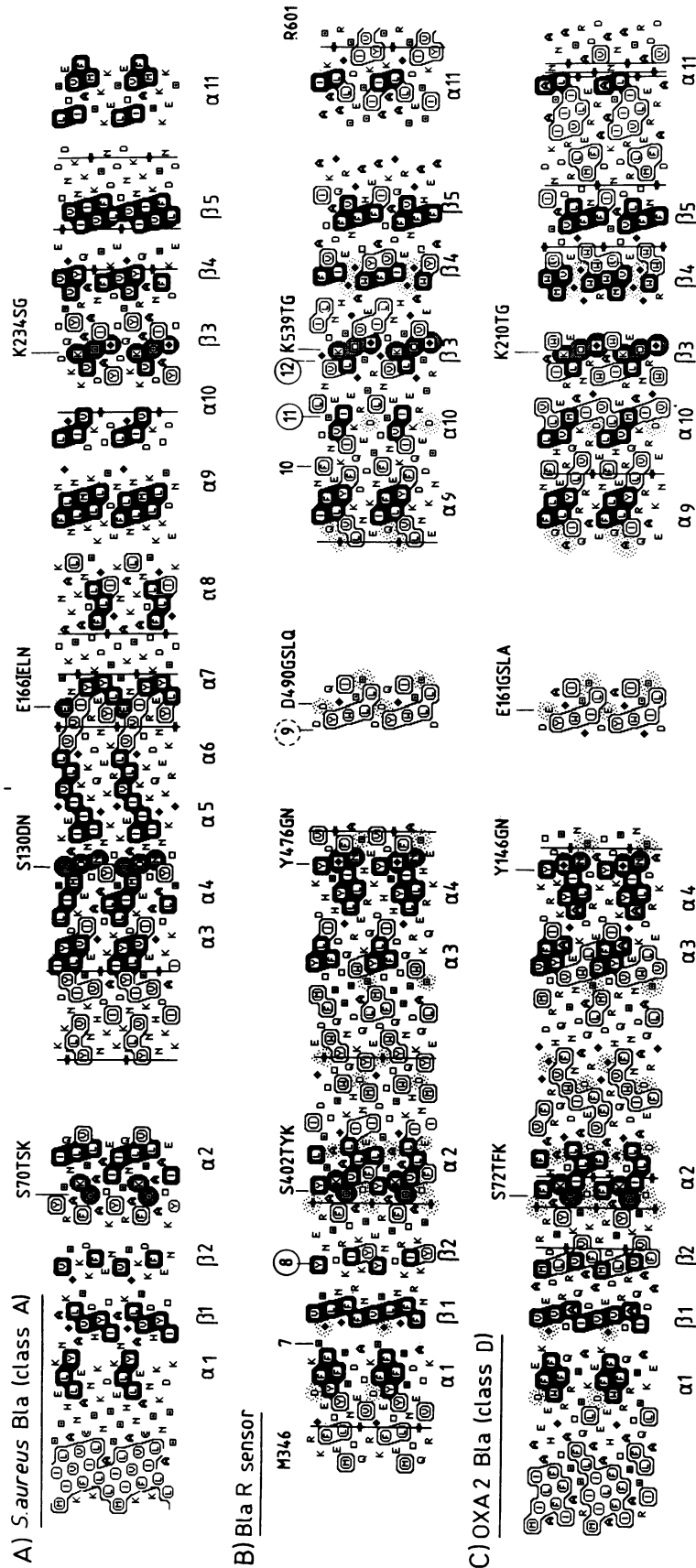


FIG. 7. Hydrophobic cluster analysis. (A) *S. aureus* class A β -lactamase. (B) BlaR penicillin sensor. (C) Class D OXA-2 β -lactamase. Hydrophobic residues are encircled, and hydrophilic clusters are also delineated. The hydrophobic residues and clusters known to be involved in the secondary structures of the *S. aureus* β -lactamase and those occurring at equivalent places along the amino acid sequences of BlaR and OXA-2 are in boldface type. Hydrophilic residues common to BlaR and OXA-2 (as well as to the OXA-1 and SSE-2 β -lactamases; data not shown) are indicated by scattered dots. The four active site-defining amino acid groupings of the *S. aureus* β -lactamase and the corresponding groupings of BlaR and OXA-2 are aligned. The locations of the mutations in the penicillin sensor of BlaR (Table 2) are indicated: mutations 7 and 10 are neutral; mutation 9 is damaging; and mutations 8, 11, and 12 are highly detrimental. Symbols: \blacklozenge , Gly; \square , Thr; \ast , Ser; \ast , Pro.

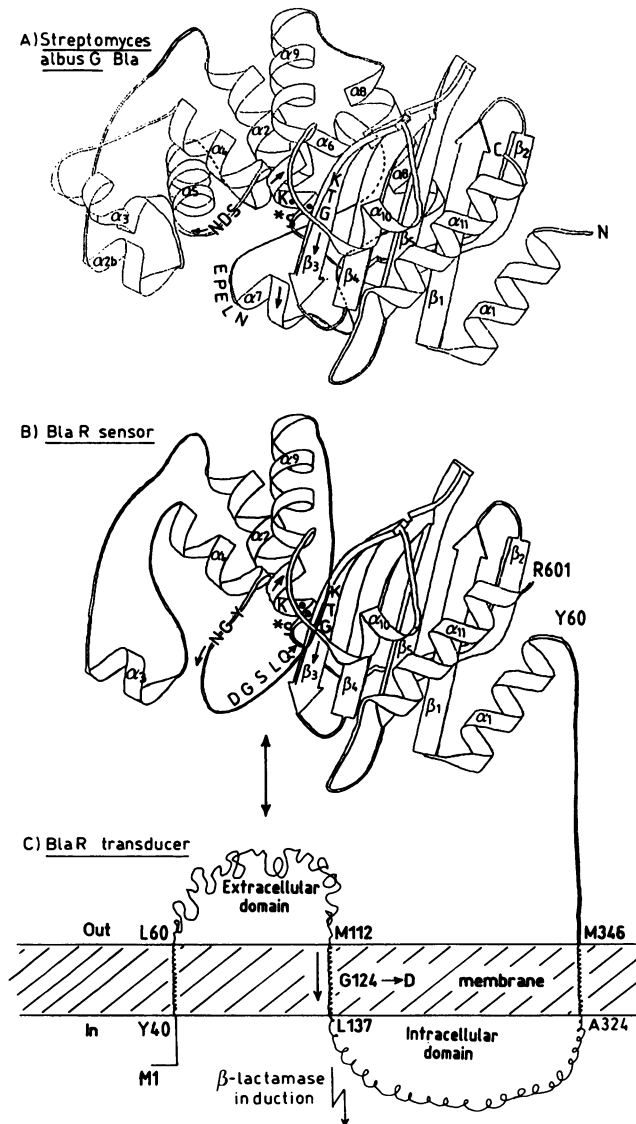


FIG. 8. (A) Three-dimensional structure of the *S. albus* G β -lactamase (14). (B) Three-dimensional model of the amino-terminal transducer of BlaR. The structure proposed for the sensor differs from that of the β -lactamases of class A in that $\alpha 4$ is linked directly to $\alpha 9$. *S, active site serine. For more details, see the text.

G-124 to D (site 3) also annihilates β -lactamase inducibility. (iii) Another detrimental alteration is made by inserting the tetrapeptide PGEF between F-295 and S-296 (site 5) in the intracellular domain.

DISCUSSION

Induction of β -lactamase synthesis in *B. licheniformis* is initiated by the binding of a β -lactam compound to the BlaR sensory transducer. The C-terminal penicillin sensor is extracellular and is fused at its amino side to a transducer whose three transmembrane segments define an extracellular domain and an intracellular domain.

The sensor possesses the active site-defining amino acid groupings (or homologs) that are common to the penicilloyl serine transferases (6) and appears to have the same basic

three-dimensional structure as that of the class A β -lactamases. However, the sensor binds the β -lactam inducer permanently, lacks several α helices, and therefore has a more readily accessible active site and a partially uncovered five-stranded β sheet. The class D β -lactamases probably have the same type of molecular architecture. This peculiarity is consistent with the dimeric nature proposed for the class D β -lactamases (2) and may provide BlaR with a site through which the sensor interacts with the extracellular domain of the transducer.

As a result of this interaction, the extracellular domain of the transducer would be responsible for the reception of the signal generated by permanent acylation of the essential serine of the sensor by the β -lactam inducer. After transmission of the signal via the transmembrane segment from M-112 to L-137, the intracellular domain of the transducer would be responsible for the generation of an intracellular signal that, via a carrier (presumably R2), would launch β -lactamase synthesis. The R2 locus has not been cloned. Whether R2 carries the signal generated by the transducer domain of BlaR and interacts with the repressor, BlaI, throughout the process of β -lactamase induction remains unknown.

Experimental evidence supports the structure proposed for BlaR. Altering sites that affect secondary structures of the sensor or the transmembrane segment of the transducer involved in signal transmission results in the production of BlaR forms that are nonfunctional for β -lactamase induction. Proteolytic cleavage at sites within the extracellular and/or intracellular domains of the transducer may give rise to truncated, nonfunctional BlaR forms that have decreased molecular masses (down to ≈ 35 kDa) but remain membrane bound and able to react with the antisensor antiserum. Under conditions in which the export machinery is saturated or impaired, BlaR and/or degraded forms of BlaR may accumulate in the cytoplasm in the form of nonfunctional water-soluble proteins.

ACKNOWLEDGMENTS

The work at Rutgers was supported in part by Public Health Service grant AI-23096 from the National Institute of Allergy and Infectious Diseases. T. K. held a Busch postdoctoral fellowship. The work at Université de Liège was supported in part by the Belgian Government through the Pôles Interuniversitaires (PAI 19), an action concertée with the Belgian Government (convention 86/91-90), the Fonds de Recherche Médicale (contract 3.4537.88), and a convention tripartite among the Région Wallonne, SmithKline Beecham, United Kingdom, and the Université de Liège. B.J. is chercheur qualifié of the Fonds National de la Recherche Scientifique, Brussels, Belgium.

REFERENCES

- Collins, J. F. 1979. The *Bacillus licheniformis* β -lactamase system, p. 351-368. In M. T. Hamilton-Miller and J. T. Smith (ed.), *Beta-lactamases*. Academic Press, London.
- Dale, J. W., and J. T. Smith. 1976. Dimeric nature of an R-factor mediated beta-lactamase. *Biochem. Biophys. Res. Commun.* **68**:1000-1005.
- Dideberg, O., P. Charlier, J. P. Wery, P. Dehottay, J. Dusart, T. Erpicum, J. M. Frère, and J. M. Ghuyssen. 1987. The crystal structure of the β -lactamase of *Streptomyces albus* G at 0.3 nm resolution. *Biochem. J.* **245**:911-913.
- Dubnau, D. A., and M. R. Pollock. 1965. The genetics of *Bacillus licheniformis* penicillinase: a preliminary analysis from studies on mutation and inter-strain and intra-strain transformations. *J. Gen. Microbiol.* **41**:7-21.
- Gaboriaud, C., V. Bissery, T. Benchetrit, and J. P. Mornon. 1987. Hydrophobic cluster analysis: an efficient new way to

- compare and analyze amino acid sequences. *FEBS Lett.* **224**: 149–155.
6. **Ghuysen, J.-M.** 1991. Serine β -lactamase and penicillin-binding proteins. *Annu. Rev. Microbiol.* **45**:37–67.
 7. **Hardt, K., B. Joris, and J.-M. Ghuysen.** Unpublished data.
 8. **Herissat, B., M. Saloheimo, S. Lavaitte, and J. K. Knowles.** 1990. Structural homology among the peroxidase enzyme family revealed by hydrophobic cluster analysis. *Proteins* **8**:251–257.
 9. **Herzberg, O.** 1991. Refined crystal structure of beta-lactamase from *Staphylococcus aureus* pcl at 2.0 Å resolution. *J. Mol. Biol.* **217**:701–719.
 10. **Joris, B., J. M. Ghuysen, G. Dive, A. Renard, O. Dideberg, P. Charlier, J. M. Frere, J. A. Kelly, J. C. Boyington, P. C. Moews, and J. R. Knox.** 1988. The active-site serine penicillin-recognizing enzymes as members of the *Streptomyces* R61 DD-peptide family. *Biochem. J.* **88**:313–324.
 11. **Joris, B., P. Ledent, O. Dideberg, E. Fronzé, J. Lamotte-Brasseur, J. A. Kelly, J.-M. Ghuysen, and J. M. Frère.** 1991. Comparison of the sequences of class A β -lactamases and the secondary structure elements of penicillin-recognizing proteins. *Antimicrob. Agents Chemother.* **35**:2294–2301.
 12. **Joris, B., P. Ledent, T. Kobayashi, J. O. Lampen, and J.-M. Ghuysen.** 1990. Expression in *Escherichia coli* of the 346-carboxy terminal domain of the BlaR sensory transducer protein of *Bacillus licheniformis* as a water-soluble 26,000 Mr penicillin-binding protein. *FEMS Lett.* **70**:107–114.
 13. **Kobayashi, T., Y. F. Zhu, N. J. Nicholls, and J. O. Lampen.** 1987. A second regulatory gene, *blaR*, encoding a potential penicillin-binding protein required for induction of β -lactamase in *Bacillus licheniformis*. *J. Bacteriol.* **169**:3873–3878.
 14. **Lamotte-Brasseur, J., G. Dive, O. Dideberg, P. Charlier, J. M. Frère, and J.-M. Ghuysen.** 1991. Mechanism of acyl transfer by the class A β -lactamase of *Streptomyces albus* G. *Biochem. J.* **279**:213–221.
 15. **Ledent, P., and B. Joris.** Unpublished data.
 16. **McKenzie, T., T. Hoshino, T. Tansks, and N. Sueoka.** 1986. The nucleotide sequence of pUB110: some salient features in relation to replication and its regulation. *Plasmid* **15**:93–103.
 17. **Oliver, D. B., and J. Beckwith.** 1982. Regulation of a membrane component required for protein secretion in *Escherichia coli*. *Cell* **30**:311–319.
 18. **Salerno, A. J., and J. O. Lampen.** 1986. Transcriptional analysis of β -lactamase regulation in *Bacillus licheniformis*. *J. Bacteriol.* **166**:769–778.
 19. **Salerno, A. J., and J. O. Lampen.** 1988. Differential transcription of the *bla* regulatory region during induction of β -lactamase in *Bacillus licheniformis*. *FEBS Lett.* **227**:61–65.
 20. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 21. **Sargent, M. G.** 1968. Rapid fixed-time assay for penicillinase. *J. Bacteriol.* **95**:1493–1494.
 22. **Sherratt, D. J., and J. F. Collins.** 1973. Analysis by transformation of the penicillinase system in *Bacillus licheniformis*. *J. Gen. Microbiol.* **76**:217–230.
 23. **Smith, P. K., R. I. Hrohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85.
 24. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* **82**:1074–1078.
 25. **Winston, S. E., S. A. Fuller, and J. G. R. Hurrel.** 1988. Western blotting, p. 10.8.1–10.8.6. *In* F. M. Ausubel (ed.), *Current protocols in molecular biology*, John Wiley & Sons, Inc., New York.
 26. **Young, R. A., and R. W. Davis.** 1983. Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA* **80**:1194–1198.
 27. **Zhu, Y. F.** 1990. Ph.D. thesis. Rutgers, The State University of New Jersey, Piscataway.
 28. **Zhu, Y. F., I. J. H. Curran, B. Joris, J.-M. Ghuysen, and J. O. Lampen.** 1990. Identification of BlaR, signal transducer for β -lactamase production in *Bacillus licheniformis*, as a penicillin-binding protein with strong homology to the Oxa-2 β -lactamase (class D) of *Salmonella typhimurium*. *J. Bacteriol.* **172**:1137–1141.