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Identification of BlaR, the 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*

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The *blaR* gene of *Bacillus licheniformis* encodes the signal transducer for induction of the class A β-lactamase. The protein product, BlaR, has a hydrophilic carboxy region that binds β-lactams and shows high sequence homology to the class D β-lactamases, particularly the OXA-2 β-lactamase of *Salmonella typhimurium*. The BlaR–β-lactam complex is stable and may provide the continuing stimulus needed for the prolonged production of the enzyme.

The β-lactamase of *Bacillus licheniformis* 749 is an inducible enzyme. Upon addition of a β-lactam, synthesis of the protein increases gradually, peaks at 1 to 1.5 h, and decreases slowly during the next 1 to 2 h (15). The *bla* genes comprise a cluster: *blaP*, the structural gene; *blaI*, for the repressor; and *blaR*, which is required for induction and encodes a 68-kilodalton (kDa) protein (12). BlaR contains five putative membrane-spanning segments and a carboxy region with a typical penicillin-binding sequence (Fig. 1). Another regulatory locus, termed R2, is unlinked to the cluster, and its function is not known (5, 18).

We have proposed (12) that BlaR is the acceptor for the inducing β-lactam. Formation of this complex might activate a cytoplasmic domain(s) of the protein to produce a signal that interacts with the repressor, or with another regulatory protein, to initiate the induction.

Expression of *blaR* in the *Escherichia coli* T7 two-plasmid system of Tabor and Richardson (19). To obtain the presumed 68-kDa product for characterization, *blaR* was obtained from pRWT8 (12) and inserted into pT7-6 to yield pRWZ1 (Fig. 2). Induction of *E. coli* K38(pGP1-2, pRWZ1) at 42°C and incubation for 2 h at 37°C were followed by separation of the cells into membrane and soluble fractions which were exposed to [^{3}H]benzylpenicillin. The procedures for preparing the fractions and labeling the penicillin-binding proteins (PBPs) were essentially those of Georgopapadakou and Liu (6). A light band of about the expected size was detected in the membrane but not in the soluble fraction (Fig. 3, lanes 1 and 4). However, the presence of the pRWZ1 insert led to a severe delay in the growth of the culture and to considerable lysis, and the *blaR* product was obviously heterogeneous.

To minimize the apparent toxicity of BlaR, we removed the portion of *blaR* that corresponds to four of the five presumed membrane-spanning regions, i.e., the *Ban*I-*Ban*I fragment of pRWZ1 (Fig. 1 and 2). The resulting plasmid, pRWZ2, carries Δ*blaR1*, which codes for ΔBlaR37-354 (M_r, 32,281). Expression of Δ*blaR1* in the *E. coli* system yielded considerable amounts of a somewhat heterogeneous 32-kDa PBP (Fig. 3, lanes 3 and 5). The truncated protein was distributed about evenly between the membrane and soluble fractions, which seems reasonable for a protein with a single highly hydrophobic region. On the basis of these observations, we conclude that the 68-kDa membrane-bound PBP is the product of *blaR* and thus is the signal transducer for the induction of β-lactamase in *B. licheniformis*.

Identification of the mutation in *B. licheniformis* 749/110 *pen28*. Additional evidence that the 68-kDa PBP detected in the *E. coli* system is the product of *blaR* and the signal transducer was obtained by the identification of the mutation in *pen28*. Portions of the wild-type *bla* gene cluster on pRWT20 (12) were replaced with corresponding portions from the mutant. Tests of the resulting chimeric clusters for their phenotypes showed the defect to be in *blaR* rather than in the repressor gene (*blaI*) as previously suggested (18). Sequencing of the *blaR* region from *pen28* by the dideoxy-chain termination method with [α-^{35}S]dATP (16) identified the mutation as a transition from G to A in the second nucleotide of codon 538. This replaces Gly-538 with Asp-538, immediately upstream of the triad Lys-539–Thr–Gly (Fig. 1). A negatively charged chain at position 538 is likely to interact with Lys-539, which is thought to be an important feature of the binding site. On this basis one would not expect mutant *pen28* to produce a 68-kDa PBP, and none was observed, although the mutant protein could be detected (data not shown).

Relation of BlaR to other penicillin-recognizing proteins. Proteins that bind or hydrolyze β-lactam antibiotics through an active-site serine, i.e., PBPs and β-lactamases, form a superfamily of evolutionarily related proteins or domains (11). From sequence comparisons and crystallographic evidence (4, 8), several loci in these proteins have been identified as conserved and important for the recognition of the ligands or for catalysis. Among the critical loci, the tetrad Ser-Xaa-Xaa-Lys contains the active-site serine, and downstream towards the carboxy terminus of the proteins, the triad His/Lys-Thr/Ser-Gly bears a positively charged side chain that orients itself towards the active-site serine (Fig. 1).

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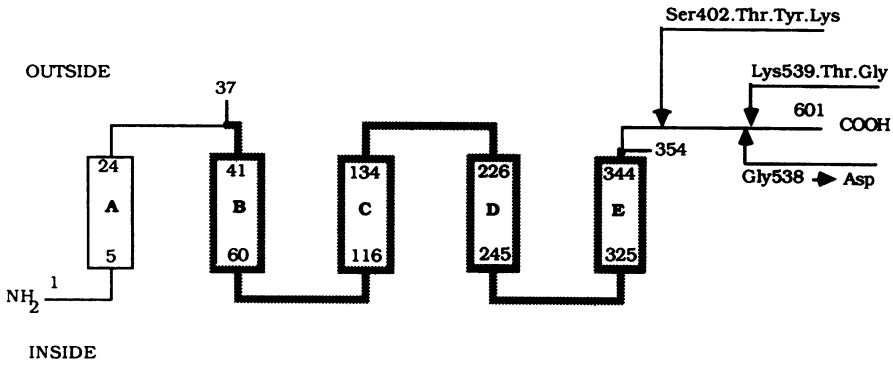

FIG. 1. Structure of mutant forms of BlaR. The numbers of the amino acids at the ends of the presumed transmembrane helices are shown, as are the typical penicillin-binding site (Ser-402 to Lys-405) and the essential triad Lys-539–Thr–Gly. The replacement of Gly-538 by Asp-538 in mutant *pen28* inserts a negatively charged residue immediately adjacent to the Lys-539. The hatched region was removed in constructing Δ*blaR1* (encodes ΔBlaR37-354). A through E are the five putative transmembrane regions. (Adapted from Fig. 5 in the work of Kobayashi et al. [12].)

The search for a possible relationship between Pro-354–Arg-601 (the putative external domain of BlaR) and the active-site serine penicillin-recognizing proteins (or domains of known primary structure) was made by comparing pairwise, without any editing, the corresponding amino acid sequences by the procedure of Goad and Kanehisa (7; for details, see reference 11). The carboxy domain of BlaR lacked homology (i.e., had very low score and standard

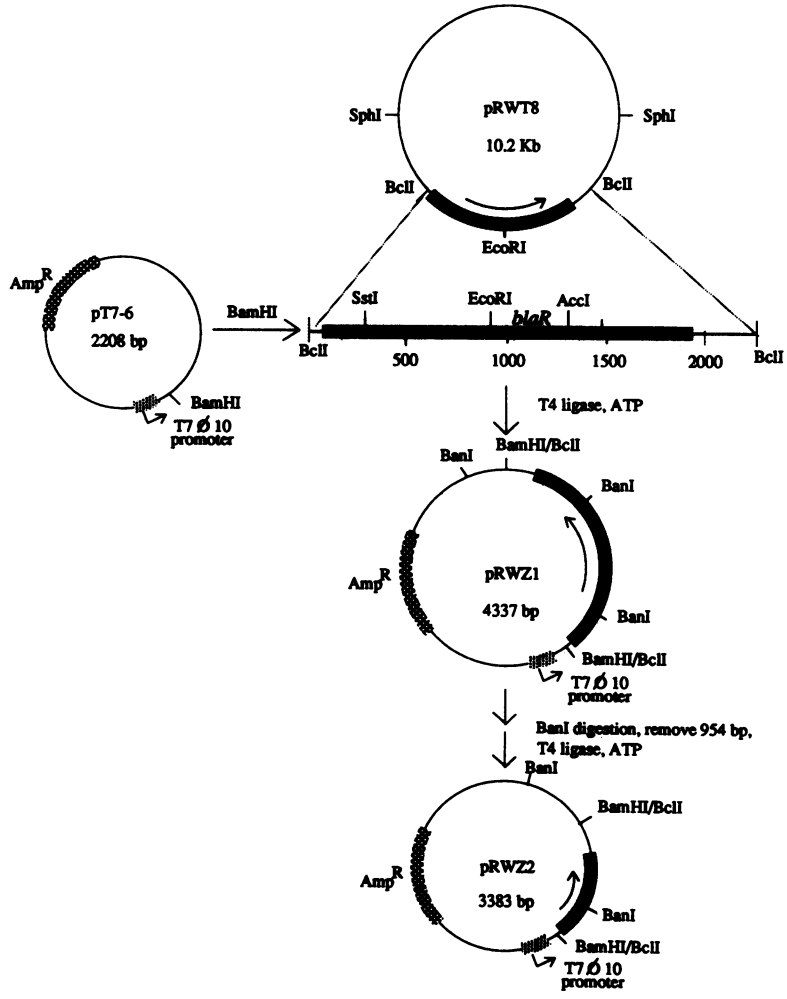

FIG. 2. Construction of *blaR* expression vectors pRWZ1 and pRWZ2. pRWT8 carries *blaP*, *blaI*, and *blaR* on a 5.2-kilobase-pair *Sph*I fragment (12). The wide solid regions represent *blaR* or Δ*blaR1*. bp, Base pairs.

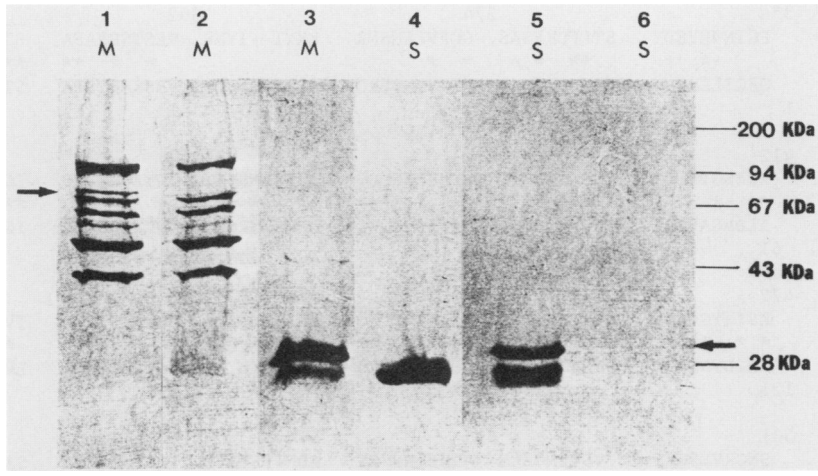

FIG. 3. Penicillin-binding activity of BlaR and ΔBlaR37-354 as expressed in an *E. coli* T7 system. Cultures of *E. coli* K38(pGP1-2) with pT7-6 (no insert), pRWZ1 (contains *blaR*), or pRWZ2 (contains Δ*blaR1*) were heat induced and processed into membrane (M) and soluble (S) fractions. Lanes 1 and 4, products of pRWZ1; lanes 2 and 6, from pT7-6; lanes 3 and 5, products of PRWZ2. Arrows indicate the expected products of *blaR* (68 kDa) and Δ*blaR1* (32 kDa). ^{14}C-labeled M_r standards (in kilodaltons): myosin (H-chain), 200; phosphorylase B, 94; bovine serum albumin, 67; ovalbumin, 43; and a-chymotrypsinogen, 28.

deviation unit values) with the β-lactamases of class A or class C, the low-M_r PBPs, and the penicillin-binding domains of the high-M_r PBPs (as defined by Joris et al. [11]). However, the carboxy domain of BlaR had very significant homology with the β-lactamases of class D, in particular the OXA-2 β-lactamase of *Salmonella typhimurium* (Fig. 4). There is no similarity between the *S. typhimurium* enzyme and the 350-residue amino-terminal domain of BlaR that contains the presumed transmembrane helices (results not shown). The search for the best possible match between the OXA-2 β-lactamase and the penicillin-recognizing domain of BlaR gave rise to the alignment shown in Fig. 5.

	E. coli AmpC	*S. albus*	*B. lich.*	*S. aur.* PC1	*E. coli* Oxa-1	*S. typh.* Oxa-2	*P. aeru.* PSE-2	BlaR (346-601)	S. D. U.
E. coli AmpC		-0.2	-0.2	1.7	-0.4	1.3	0.1	-0.1	
S. albus	-27		39.6	42.8	0.3	0.1	-0.7	-0.7	
B. lich.	-30	-513		52	-0.3	1.2	-0.3	4.5	
S. aur. PC1	-39	-332	-591		1.1	-0.6	0.1	1.3	
E. coli Oxa-1	-29	-25	-32	-48		25.1	23.3	20.1	
S. typh. Oxa-2	-23	-33	-44	-23	-234		43.2	28.8	
P. aeru. PSE-2	-35	-19	-28	-34	-279	-449		30.7	
BlaR (346-601)	-39	-18	-76	-60	-306	-439	-406		

SCORE

FIG. 4. Search for homology between the carboxy-terminal sequence of BlaR (residues 346 to 601) and selected active-site serine β-lactamases of class C (AmpC), class A (*S. albus*, *B. lich.* and *S. aur.*) and class D (Oxa-1, Oxa-2, and PSE-2) by the pairwise method of Goad and Kanehisa (7). The SEQHP and SEQDP programs yield a score and a standard deviation unit (S.D.U.). The lower the score (above the diagonal), the closer the relationship between the two sequences under comparison. A standard deviation unit value higher than 5 indicates a statistically significant homology ($P = 0.02$). Sequences used were *E. coli* AmpC (10) and OXA-1 (14), *Streptomyces albus* G (3), *B. licheniformis* (*B. lich.*) (13), *Staphylococcus aureus* (*S. aur.*) PC1 (1), *S. typhimurium* (*S. typh.*) OXA-2 (2), and *Pseudomonas aeruginosa* (*P. aeru.*) PSE-2 (9).

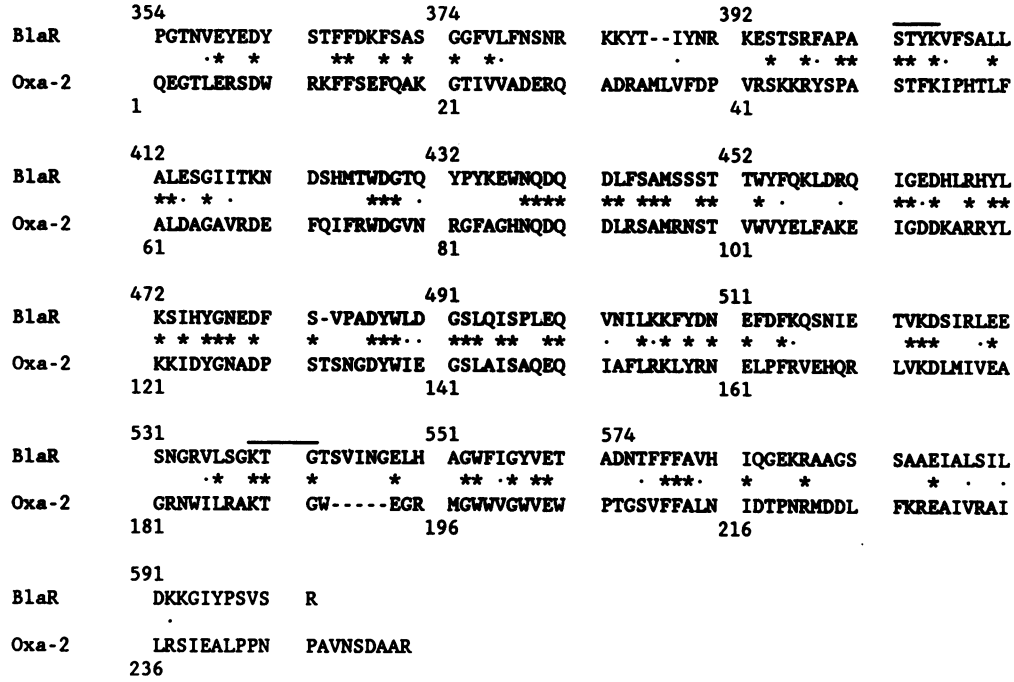

FIG. 5. Optimal alignment of the penicillin-binding domain of BlaR and the class D OXA-2 β-lactamase of *S. typhimurium*. Symbols: *, the 84 identical residues; ·, conservative replacements. The active-site serine-containing tetrad S-T-Y/F-K and the essential triad KTG are marked by overbars.

Is BlaR an oxacillinase? The close similarity between the penicillin-recognizing domain of BlaR and the class D β-lactamases raised the possibility that BlaR might have catalytic activity. To test this, we first compared the effectiveness of oxacillin and cephalosporin C in the standard *B. licheniformis* 749 induction system (12). At 5 μM, the two β-lactams induced similar levels of the enzyme (360 to 390 U/mg of protein, by the colorimetric method of Sargent with benzylpenicillin as the substrate [17]). At 23 μM oxacillin, growth was reduced by 20% and induction was reduced by 80%. Thus, oxacillin appears to be a typical inducer with both inducing and antibiotic activities. Nevertheless, no oxacillin-cleaving activity could be detected in either uninduced or induced cultures (<5 U of oxacillinase per mg of protein, as determined by the hydroxylamine procedure of Dale et al. [2]). We have also shown that mutant *B. licheniformis* 749/110 *pen* C3/22 (18), which has an inactive β-lactamase (*blaP* mutant) but an active receptor (*blaR*$^+$), did not hydrolyze oxacillin or benzylpenicillin.

Finally, as a test of the stability of the BlaR-oxacillin complex, we pretreated the membrane fraction from mutant *pen* C3/22 with unlabeled oxacillin at the β-lactam/protein ratio used in our test for the binding of [^{3}H]benzylpenicillin. The membranes were then washed and precipitated with acetone to remove free β-lactam and treated, as usual, with [^{3}H]benzylpenicillin. The fractions pretreated with oxacillin bound very little [^{3}H]benzylpenicillin, while control fractions treated in parallel but without the oxacillin bound large amounts of [^{3}H]benzylpenicillin (results not shown).

Gram-negative bacteria manufacture active-site serine β-lactamases of classes A, C, and D, while the known β-lactamases of gram-positive bacteria are exclusively of class A. Thus, it was unexpected that the PBP of *B. licheniformis*, BlaR, showed strong homology to the class D OXA-2 β-lactamase of *S. typhimurium*. There may be an advantage for the bacillus in using an unusual type of PBP for this specific regulatory function, or the critical characteristic may simply be that this receptor domain provides an efficient binding site without detectable hydrolytic activity.

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