A Second Regulatory Gene, *blaR1*, Encoding a Potential Penicillin-Binding Protein Required for Induction of β -Lactamase in *Bacillus licheniformis*

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A second regulatory locus (*blaR1*) required for the induction of β -lactamase synthesis in *Bacillus licheniformis* 749 was cloned and sequenced. The gene was located on a 5.2-kilobase-pair *SphI* DNA fragment which also contained the β -lactamase (*blaP*) and repressor (*blaI*) genes. *Bacillus subtilis* BD224 carrying these three genes synthesized β -lactamase on exposure to cephalosporin C, whereas *Escherichia coli* HB101 carrying the genes did not show any detectable induction of the enzyme. An open reading frame of 1,803 bases was identified as the *blaR1* gene by subcloning and DNA sequencing. The gene started 2 bases downstream of the termination codon of *blaI* and was preceded by a putative Shine-Dalgarno sequence (AAGGA) with a spacing of 5 bases. The deduced *blaR1* product (601 amino acids) had a molecular weight of 68,425. Five transmembrane regions were predicted from the hydrophobicity profile. The region around Phe-Ala-Pro-Ala-Ser-Thr-Tyr-Lys (amino acids 398 to 405), which appeared to be located outside the membrane, was homologous to the binding regions of penicillin-binding proteins, including the β -lactamases. The segment of 22 amino acids from 400 to 421 showed more than 70% homology to the penicillin-binding region of PBP 2 of *E. coli*. The *blaR1* gene encodes a potential penicillin receptor which is required for the induction of β -lactamase in *B. licheniformis* 749.

The β -lactamase of *Bacillus licheniformis* is a secreted protein whose synthesis is induced by β -lactam antibiotics. On induction, the enzyme is synthesized very slowly. The rate of synthesis reaches a maximum after 1 to 1.5 h of induction and then decreases slowly over the next hour. The rate remains severalfold higher than that of the uninduced culture for 2 or 3 h longer (11). This prolonged induction is not the result of unusual mRNA stability, because the half-life of the β -lactamase mRNA is only 2 to 3 min (32).

Sherratt and Collins reported three possible regulatory loci for the synthesis of β -lactamase (35). The *blaI* gene, which is 90% linked to the β -lactamase gene (*blaP*), encodes the repressor to *blaP*. The *blaR1* locus is 50% linked to *blaP*, and mutations (R14 or R27) thought to lie in this region cause low induction of the enzyme. Mutation *blaR31* in the unlinked *blaR2* locus results in a low basal level of the enzyme and a defect in induction. Penicillins irreversibly bind to the cell surface of *B. licheniformis* (13). Consequently, there must be a system of signal transduction through the membrane, and the stimulus may reach the repressor protein through a novel process. The *blaR1* and *blaR2* loci are possibly involved in this transduction of the stimulus.

The *blaP* gene has been cloned and sequenced (5, 18, 22, 24, 30). Complementation analysis for *B. licheniformis* with the cloned 4.2-kilobase-pair (kbp) *EcoRI* DNA fragment has shown that the *blaI* gene is also encoded on this fragment (22). The nucleotide sequence of the *blaI* region has indicated that the gene is transcribed divergently to *blaP*, which lies 5' to the *blaI* gene (20; N. J. Nicholls, Ph.D. thesis, Rutgers University, New Brunswick, N.J., 1986). The promoter-operator regions of *blaI* and *blaP* share homologous sequences of 23 bp which provide possible binding sites for the repressor. Recently, the repressor protein was purified. The N-terminal amino acid sequence was identical to that

expected from the DNA sequence (M. J. Grossman and J. O. Lampen, unpublished results).

The mechanism of induction is still obscure, because *Escherichia coli* and *Bacillus subtilis* carrying the 4.2-kbp *EcoRI* DNA fragment do not show detectable induction of the β -lactamase (22). The absence of the *blaR1* and *blaR2* genes may be responsible for this lack of inducibility.

In this paper, we describe the cloning and sequencing of the *blaR1* gene, which is required for inducibility of β -lactamase in *B. subtilis*. The role of the *blaR1* product, based on its homology to penicillin-binding proteins (PBPs), is discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. licheniformis* 749, which is inducible for β -lactamase production, was obtained from M. R. Pollock (31) and was the source of the *bla* genes. *E. coli* HB101 (F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 supE44* λ^{-}) (3) and *B. subtilis* BD224 (*trpC2 thr-5 recE4*) (15) were the recipient strains for recombinant plasmids. *E. coli* JM101 [*supE thi* Δ (*lac-proAB*) F' *traD36 proAB lacI^qZ* Δ M15] was used as the host of M13 clones for DNA sequencing. pRW33, which is a pBR325 derivative containing an inactive *bla* gene (28), and pUB110 (26) were used as vectors in *E. coli* and *B. subtilis*, respectively. pRWN101 (Nicholls, Ph.D. thesis), which contains the *blaP-blaI* region on the 4.2-kbp *EcoRI* DNA fragment from *B. licheniformis* 749, was the source of the fragment. The chloramphenicol resistance gene which is expressed in *B. subtilis* was obtained from pC194 (21).

DNA manipulations. Chromosomal DNA from *B. licheniformis* 749 and plasmid DNAs from *E. coli* and *B. subtilis* were prepared as described elsewhere (10, 14, 19). All reactions of DNA-related enzymes were done according to the instructions of the supplier. Recombinant plasmids were introduced into competent cells of *E. coli* (25) and proto-

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FIG. 1. Location of the *blaR1* gene. The top line shows the restriction map of the DNA region around the *blaP-blaI-blaR1* gene cluster. The positions of *blaP*, *blaI*, and *blaR1* are shown. pRWT13, pRWT15, pRWT17, and pRWT19 have *blaP* in the same orientation as the kanamycin resistance gene of pUB110, whereas in pRWT14, pRWT16, pRWT18, and pRWT20 the orientation is opposite. pRWT192 and pRWT202 carry the chloramphenicol acetyltransferase gene (CAT) in the *SstI* site of pRWT19 and pRWT20, respectively. Abbreviations and symbols: P_p and P₁, promoters of *blaP* and *blaI*, respectively; □, DNA, regions carried by the plasmids used to locate the *blaR1* gene; ■, *blaR1*.

plasts of *B. subtilis* (8). Transformants into *E. coli* were selected on L agar containing 20 µg of chloramphenicol and 10 µg of ampicillin per ml. *B. subtilis* transformants were selected on DM3 regeneration plates containing 500 µg of kanamycin per ml and then were screened for production of the β-lactamase on L agar containing 10 µg of kanamycin per ml and 0.75% polyvinyl alcohol by the method of Sherratt and Collins (35).

β-Lactamase assay. Cultures in early log phase in L broth containing appropriate antibiotics were induced by adding cephalosporin C (final concentration, 2 µg/ml) and grown at 37°C. After 3 h of induction, the culture was sonicated and the β-lactamase activity was assayed by the method of Sargent (34) with minor modifications. Uninduced cultures were included to determine the basal level of the enzyme. One unit of the β-lactamase was defined as the amount of enzyme hydrolyzing 1 µmol of benzylpenicillin in 1 h at 30°C. The protein concentration was estimated by the method of Bradford (4), with bovine plasma gamma globulin as the standard.

DNA sequencing and computer analysis. Specific DNA fragments were cloned into M13 mp18 and mp19 with *E. coli* JM101 as the host and then sequenced by the dideoxy chain termination method (27, 33) with α-³⁵S-dATP. The method of Dale et al. (12) was also used. The DNA sequence and the derived amino acid sequence were analyzed with the BIONET program. The hydrophobicity profile was determined with the GES program for membrane proteins (16).

Materials. Enzymes for DNA manipulation and primers for DNA sequencing were purchased from New England BioLabs, Inc. (Beverly, Mass.) and Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). α-³⁵S-dATP was a product of Amersham Corp. (Arlington Heights, Ill.).

RESULTS AND DISCUSSION

Cloning of the *blaP-blaI-blaR1* region. Sherratt and Collins reported that a second regulatory locus, *blaR1*, is 50% linked to *blaP* (35). This result suggests the existence of a *blaP-blaI-blaR1* gene cluster. To examine this possibility, *SphI*

digests of the chromosomal DNA of *B. licheniformis* 749 were shotgun cloned into *E. coli* HB101 with pRW33 as the vector. A single transformant was obtained by selection on L agar containing 10 µg of ampicillin and 20 µg of chloramphenicol per ml. The plasmid isolated from the transformant, pRWT8, consisted of the 5.2-kbp *SphI* fragment in pRW33. A restriction map of the fragment was constructed (Fig. 1). The 3.6-kbp *SphI-EcoRI* region of the fragment showed a map identical to that of the 4.2-kbp *EcoRI* fragment cloned previously, which indicated that the desired fragment was obtained. This conclusion was confirmed by DNA hybridization with the ³²P-labeled *HpaI-EcoRI* portion of the 4.2-kbp *EcoRI* fragment used as a probe (data not shown). The *blaI* gene of pRWT8 had the same orientation as the tetracycline resistance gene of pRW33, which was inactivated by the insertion. pRWT9 has the 5.2-kbp *SphI* fragment in the opposite direction from that in pRWT8.

β-Lactamase inducibility in *E. coli* and *B. subtilis*. Previous work (22) has shown that the 4.2-kbp *EcoRI* DNA fragment is not sufficient for inducibility of the β-lactamase in *E. coli* and *B. subtilis*. To examine the involvement of the region downstream of *blaI*, β-lactamase inducibility was examined for *E. coli* and *B. subtilis* carrying the 5.2-kbp *SphI* fragment. In *E. coli* strains carrying pRWT8 or pRWT9, no significant induction was detected (Table 1), although the induction ratios were slightly higher than those with pRWN101, which contains the shorter 4.2-kbp *EcoRI* DNA fragment. Inducibility in *E. coli* was also examined with various concentrations (0 to 80 µM) of a gratuitous inducer, 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid. No induction was detected (data not shown).

To examine induction in *B. subtilis*, the 5.2-kbp *SphI* DNA fragment was made blunt ended with T4 DNA polymerase and was then introduced into the *PvuII* site of pUB110 (pRWT15 and pRWT16). *B. subtilis* carrying these plasmids showed β-lactamase induction with ratios of 14 (pRWT15 and 24 (pRWT16), while pRWT13 and pRWT14, which contain the 4.2-kbp *EcoRI* fragment in the *EcoRI* site of pUB110, did not confer the inducible phenotype on the host

TABLE 1. Induction of β-lactamase in *E. coli*, *B. subtilis*, and *B. licheniformis*

Species and plasmids	Sp act of β-lactamase (U/mg of protein)		Induction ratio
	Uninduced	Induced	
<i>E. coli</i>			
pRW33	ND ^a	ND	
pRWN101	14	15	1.1
pRWT8	16	19	1.2
pRWT9	19	29	1.5
<i>B. subtilis</i>			
pUB110	ND	ND	
pRWT13	63	73	1.2
pRWT14	78	84	1.1
pRWT15	140	1,900	14
pRWT16	130	3,100	24
pRWT17	290	260	0.9
pRWT18	89	96	1.1
pRWT19	160	530	3.3
pRWT20	53	2,500	47
pRWT192	97	100	1.0
pRWT202	45	51	1.1
<i>B. licheniformis</i> 749	11	1,300	120

^a ND, Not detected.

FIG. 2. Sequencing strategy for the *blaR1* gene. Abbreviation: Del1, Del2, and Del3, regions sequenced with the clones which were obtained by the method of Dale et al. (12). Symbols: →, direction and length sequenced by the dideoxy-chain-terminating method; ████, *blaR1*.

(Table 1). This result indicates that another regulatory locus responsible for β -lactamase induction was present on the 5.2-kbp *SphI* DNA fragment as a gene cluster with *blaP* and *blaI*. The gene was designated *blaR1* because it is possibly identical to the 50%-linked *blaR1* locus reported by Sherratt and Collins (35). This fragment encodes all genes required for induction in *B. subtilis*. The inability of *E. coli* to be induced may result from a lack of the *blaR2* locus (*B. subtilis* may naturally have the locus) or a difference in its membrane

structure (gram negative) from that of *B. subtilis* (gram positive).

Localization of the *blaR1* gene. Several pairs of plasmids containing a DNA fragment in opposite orientations were constructed to localize the *blaR1* gene (Fig. 1). pRWT17 and pRWT18 contain the 3.9-kbp *SphI*-*AccI* fragment, and pRWT19 and pRWT20 contain the 4.0-kbp *FspI* fragment in the *PvuII* site of pUB110. The *SphI*-*AccI* fragment was made blunt ended with T4 DNA polymerase. pRWT192 and pRWT202 contain the chloramphenicol resistance gene of pC194 in the *SstI* site of pRWT19 and pRWT20. In the construction of these plasmids, the 1.0-kb *HpaII*-*Sau3AI* DNA fragment was made blunt ended and ligated to *SstI*-digested pRWT19 or pRWT20 with an *SstI* linker.

B. subtilis carrying pRWT19 or pRWT20 could be induced for β -lactamase (Table 1), which shows that *blaR1* is located on the *FspI* fragment. pRWT192 and pRWT202 did not confer this inducibility, nor did pRWT13, pRWT14, pRWT17, or pRWT18. Therefore, the *SstI*, *EcoRI*, and *AccI* sites are located within the *blaR1* gene. From these results, the *blaR1* gene was located between the termination codon of *blaI* and the *FspI* site 3' to *blaI* (Fig. 1). Some differences in the induction ratio of the enzyme were observed (Table 1). This diversity may reflect the influence of the vector. The effect might be dependent on the distance between the *blaP*-*blaI*-*blaR1* cluster and the vector.

DNA sequencing of the *blaR1* region. The strategy for sequencing the *blaR1* gene is shown in Fig. 2. The DNA sequence revealed a long open reading frame (1,803 bases)

1
CAGCTGTCAGGTGAAGAAATTAATGAATGTATCAATATTAGAAGACATAAGAACAGAAAGAGCTTAA
PvuII
100
ATGAGCAGTCTCTTCTTATCCCTTTTGGTTAGTCAAATCTATGTCTCTGTTTTCTATCATCATA
METSerSerPhePheIleProPheLeuValSerGlnIleLeuLeuSerLeuPhePheSerIleIleIle
1 24
200
CTCATTAAAAAAGCTCAGGACTCAATCACTAGTACGCCACTATTATATTAGCGTGATTTCTCTTTG
LeuIleLysLysLeuLeuArgThrGlnIleValGlyThrHisTyrTyrIleSerValIleSerLeuLeu
48
300
GCATTGATCGCTCTTTTATCCCTTTTCTTCTAAAAAGCCATCATTTGACTGGATTCTGAATTTAGGA
AlaLeuIleAlaProPheIleProPheHisPheLeuLysSerHisHisPheAspTrpIleLeuAsnLeuGly
72
400
GGAGCTCAATCGGCCTTATCCCAACCCACTCCACAGACAGCAGAGCAATCGGCCAACATGTAAAC
GlyAlaGlnSerAlaLeuSerGlnThrHisSerThrAspLysThrThrGluAlaIleGlyGlnHisValAsn
96
500
TGGGTGCGAGCTTTTCTGTGCTGATTGAACAATCGTCTTCCAAATGATAGATTACGCTTTTTCGAGTT
TrpValGlnAspPheSerLeuSerIleGluGlnSerSerSerLysMETIleAspSerAlaPheAlaVal
120
600
TGGATATTGGCGGTTGCGGTTATGCTTTTGGCTACTCTTATAGCAATCAAAAATCGGCAAAATAAAAAG
TrpIleLeuGlyValAlaValMETLeuLeuAlaThrLeuTyrSerAsnLeuLysIleGlyLysIleLysLys
144
700
AACCTGCAATTTGTTAACAACAAAGAGCTATTATCCCTTTTTCACACATGTAAAGAGGAAATACGCTTTCAT
AsnLeuGlnIleValAsnAsnLysGluLeuLeuSerLeuPheHisThrCysLysGluGluIleArgPheHis
145 168
800
CAAAAAGTCATTTTGGCGGTTCTCCATTGATAAAATCCCCATTACATTTGGGGTCATTGCTCCATATTT
GlnLysValIleLeuSerArgSerProLeuIleLysSerProIleThrPheGlyValIleArgProTyrIle
192
900
ATCTTGCCAAAGATATTTCAATGTTTTCAGCCGACGAAATGAAATGTGTTTGGCTTCATGAACGTACCAT
IleLeuProLysAspIleSerMETPheSerAlaAspGluMETLysCysValLeuLeuHisGluLeuTyrHis
216
1000
TGCAACGAAAGATATGCTTATCACTATTTCCTTGGCTGTTGAAATCGTCTATTGGTTTAACTCTCTC
CysLysArgLysAspMETLeuIleAsnTyrPheLeuLysLeuLysIleValTyrTrpPheAsnProLeu
240
1100
GTATGCTATTATCAAGGAAGCGAAACCGAGATGGAGATTTCTGCGACTTTGCCGTTATTAAGACGCTT
ValTrpTyrLeuSerLysGluAlaLysThrGluMETGluIleSerCysAspPheAlaValLeuLysThr
264
1200
GATAAAAAATGCACTCAATATGCGAGGTGATTTTAAATCACTTCAATTAAGCAAGAACATCGTCT
AspLysLysLeuHisLeuLysTyrGlyGluValIleLeuLysPheThrSerIleLysGlnArgThrSerSer
288
1300
TTGTTAGCGCTTCTGAATTCAGCAGCTCATCAACATATCAAAAGCGATTGTTACAGTTGTCAACTTT
LeuLeuAlaAlaSerGluPheSerSerSerTyrLysHisIleLysArgArgIleValThrValValAsnPhe
312

CAGACGGCATCACCTTTTGCTAAAGGCGAAAGCGCTTAGTGTCTCACTCTTGTCTCTGGGGCCATATTAGCC
GlnThrAlaSerProLeuLeuLysAlaLysSerAlaLeuValPheThrLeuValLeuGlyAlaIleLeuAla
336
1100
GGTACTCCTCTGTGTCTATATTGGCCATGCAAAAGAACACGCTTTTACCGCACCACATGAGAATAC
GlyThrProSerValSerIleLeuAlaMETGlnLysGluThrArgPheLeuProGlyThrAsnValGluTyr
360
1200
GAAATTACAGCAGCTTTTGTGATAAATTTTCAGCCTCAGGGGCTTTGTCTGTTTAACTTAATAGGAAA
GluAspTyrSerThrPhePheAspLysPheSerAlaSerGlyGlyPheValLeuPheAsnSerAsnArgLys
384
1300
AAGTATACAATATACATAGGAAAGAACACCTCCAGATTGCGACCTGCTCCACCTACAAGGTGTTTACG
LysTyrThrIleTyrAsnArgLysGluSerThrSerArgPheAlaProAlaSerThrTyrLysValPheSer
408
1400
GCATTGCTCGCACTGGAATCCGGATCATCAGCAAGAACACTCTACATGACTTGGGATGGGACTCAATAT
AlaLeuLeuAlaLeuGluSerGlyIleIleThrLysAsnAspSerHisMETThrTrpAspGlyThrGlnTyr
432
1500
CGGTATAAAGAATGGAATCAAGACGAGTATTATCTCTGCGATGAGCAGCTCCACACATGGTATTTTCAA
ProTyrLysGluTrpAsnGlnAspGlnAspLeuPheSerAlaMETSerSerSerThrTrpTyrPheGln
456
1600
AAATGGACCGGCAATTTGGGAGGATCATTACGTCAATTTCTCAATCTATACATTATGGAATGAGGAT
LysLeuAspArgGlnIleGlyGluAspHisLeuArgHisTyrLeuLysSerIleHisTyrGlyAsnGluAsp
480
1700
TTTTAGTCCCGCGGATTTTGGCTGGATGGCTCTCTCAAAATTTCTCCACTTGAACAGGTCAATATATTA
PheSerValProAlaAspTyrTrpLeuAspGlySerLeuGlnIleSerProLeuGluGlnValAsnIleLeu
504
1800
AAAAAGTTTATGATAACGAATTTGATTTTAAACAGTCTAATATTGAACTGTGAAGATTGCGATCGTTTA
LysLysPheTyrAspAsnGluPheAspPheLysGlnSerAsnIleGluThrValLysAspSerIleArgLeu
528
1900
GAAGAATCAAAATGCGAGGTTTATCCGGTAAACCGGAACCTCGGTAATCAACGGGAACCTCATGCGGT
GluGluSerAsnGlyArgValLeuSerGlyLysThrGlyThrSerValIleAsnGlyGluLeuHisAlaGly
552
2000
TGGTTATCGGATGTAGAACTGCGGATAACTTTTTCTTGTCTGTTTCATATTCAAGGTGAAACCGG
TrpPheIleGlyTyrValGluThrAlaAspAsnThrPhePhePheAlaValHisIleGlnGlyGluLysArg
576
2100
GCTGCGGGAAGCTCCGCTGCGGAAATGCACTTTTCCATTTTAGATAAAAAAGGATTTATCCCTCGGTTTC
AlaAlaGlySerSerAlaAlaGluLeuSerIleLeuAspLysLysGlyIleTyrProSerValSer
600
2200
CGATAACAGTTTACTTTTTATCTATGAACATGAATGATGCGCAATCGAT
Arg
624
ClaI

FIG. 3. Nucleotide sequence of the *blaR1* gene and derived amino acid sequence. Symbols: , possible Shine-Dalgarno (SD) sequence for *blaR1*; →, regions of dyad symmetry. The UGA stop codon for *blaI* is underlined.

TABLE 2. Homology of Bla1 to β -lactamases and PBPs^a

Proteins	Residues	Sequences	Reference
Bla1	(388-423)	I Y N R K E S T S R F A P A S T Y K V F S A L L A L E S G I I T K N D S	This paper
β-Lactamases			
<i>S. aureus</i>	(25-60)	K E V R F M S D K R F A Y A S T S K A I N S A I L L E Q V P Y N K L D G	(1)
<i>B. licheniformis</i>	(30-65)	R T V A Y R P D E R F A P A S T I K A L T V G V L L Q Q K S I E D L N Q	(2)
<i>B. cereus</i>	(33-68)	Q T I S Y R P N E R F A P A S T Y K A L A A G V L L Q Q N S I D S L N E	(36)
<i>E. coli</i> (R6K)	(31-66)	I L E S F R P E E R F P H N S T F K V L L C G A V L S R V D A G Q E Q L	(37)
<i>E. coli</i> (ampC)	(47-82)	K K Q P V T Q Q L L K L G S V S K T T G V L G G D A I A R G E I K L	(23)
PBPs			
PBP1A	(452-486)	K F N R A - T Q A L R Q V G S N I K F P L Y T A A M D K G L T L A S M L	(7)
PBP1B	(497-531)	G Y N R A - M Q A R S I G S L A K F A T Y L T A L S Q P K I Y R L M T	(7)
PBP2	(317-351)	L V N R A - T Q Q V Y P F A S T V K F Y V A V S A L S A G V I T R N T T	(2)
PBP3	(294-328)	M R N R T - I T D V F E P G S T V K F H V V M T A L Q R G V V R E N S V	(29)
PBP5	(30-65)	V L A E Q W A D V R R D P A S L T K M H T S Y V I G Q A N K A G K F K E	(6)
PBP6	(29-50)	G W A D E K L D P A S L T K I M T S Y V V G	
<i>B. subtilis</i>	(22-57)	I L Y S K N A D K L P I A S M T K M H T E Y L L L E A I D Q G K V K W	(38)
<i>B. stearothermophilus</i>	(22-40)	I L Y E K N I D T V L G I A S M T K M	(39)

^a Identical and structurally related amino acids are boxed. Equivalences assumed are Ile to Leu to Val, Ser to Thr, Phe to Tyr, Arg to Lys, and Asp to Glu. Symbol: *, position of the active-site serine residues. *S. aureus*, *Staphylococcus aureus*; *B. cereus*, *Bacillus cereus*; *B. stearothermophilus*, *Bacillus stearothermophilus*.

which started 2 bases downstream of the *blaI* termination codon (Fig. 3). Since this open reading frame was the only one which contained *SstI*, *EcoRI*, and *AccI* sites and terminated before the *FspI* site, this frame was identified as the *blaR1* gene. In this open frame, six possible translation initiation sites (ATG, positions 74 to 76; TTG, 101 to 103; TTG, 119 to 121; GTG, 203 to 205; TTG, 215 to 217; and TTG, 221 to 223) occur before the *SstI* site. According to published data, the average spacing between an initiation codon and a Shine-Dalgarno sequence is 7 bases, and spacings of less than 5 bases and longer than 9 bases are rare (17). Among the six possible initiation codons, the first ATG had a Shine-Dalgarno sequence (AAGGA) with a spacing of 5 bases, while the others had spacings of more than 30 bases. This observation makes it likely that the *blaR1* gene starts from the first ATG codon. Because no well-conserved -10 and -35 sequences were found 5' to *blaR1*, the gene probably is transcribed along with *blaI* as a polycistronic mRNA.

Regions of dyad symmetry are shown in Fig. 3 (arrows). The first region (A) is located at the 3' end of *blaI* and contains the putative Shine-Dalgarno sequence for *blaR1*. Although the predicted free energy was only -7.7 kcal/mol (1 kcal = 4.184 kJ), this region might be involved in translational control of the *blaR1* gene. The stem-loop structure might prevent direct ribosome binding to this region and

may only allow those ribosomes which translate *blaI* to translate *BlaR1*. The last region of dyad symmetry (F) is located near the end of *blaR1* and has a calculated free energy of -27.7 kcal/mol. Four additional regions of dyad symmetry are present in the *blaR1* gene: B, -11.9 kcal/mol; C, -12.0 kcal/mol; D, -16.8 kcal/mol; and E, -15.7 kcal/mol. The expression of the *blaR1* gene may be regulated by the potential stem-loops, either transcriptionally (by attenuation) or posttranscriptionally (by protecting a portion of the mRNAs from RNase digestion).

Putative product of the *blaR1* gene. The deduced *blaR1* product (Bla1) contains 601 amino acids and has a molecular weight of 68,425. The hydrophobicity profile (Fig. 4) was obtained with the GES algorithm designed for membrane proteins (16). Engelman et al. (16) reported that hydrophobic peaks corresponding to 20 kcal/mol correlate with stable insertion and anchoring of the membrane protein. Therefore, Bla1 was predicted to be a membrane protein with four definite transmembrane regions (Fig. 4A, B, C, and E) and one probable transmembrane region (Fig. 4D).

Homology between Bla1 and various PBPs was examined. The region around the active serine of the β -lactamases

FIG. 4. Hydrophobicity profile along the derived amino acid sequence of Bla1. The profile was obtained by the GES algorithm (15) with a modification of program FOAM84. A to E, Regions which have a hydrophobic peak (Gibbs free energy; ΔG) of more than 20 kcal/mol.

FIG. 5. Predicted transmembrane helices of Bla1. Transmembrane helices ($\square$) each represent a span of 20 amino acids which showed the highest ΔG value in regions A to E. The numbers of the amino acids at the ends of each helix are shown.

showed a high degree of homology to the region around Ser-402 of BlaR1 (Table 2). The Phe-X₃-Ser-X₂-Lys sequence conserved in these β -lactamases was also found in BlaR1 (Phe-398 to Lys-405). Among the PBPs other than β -lactamases, only the penicillin-binding region of PBP 2 in *E. coli* (2) showed significant homology to the region around Ser-402 of BlaR1. This homology was statistically very important because a long segment (22 amino acids) in the binding region showed more than 70% homology. However, the hydrophobicity profile of PBP 2 was not similar to that of BlaR1. PBP 2 has only a single transmembrane region at its NH₂ terminus (2). The function of BlaR1 may be different from that of PBP 2 except for penicillin binding, although this idea remains to be proved.

B. licheniformis has been reported to contain a variety of PBPs ranging in size from 20 to 128 kilodaltons on the basis of sodium dodecyl sulfate-acrylamide gel analysis (9, 11). It remains to be determined whether BlaR1 corresponds to one of these. Figure 5 shows the predicted model of the transmembrane structure of BlaR1. The possible penicillin-binding domain of BlaR1 is contained in the long COOH-terminal region (257 amino acids) outside the membrane. The NH₂-terminal sequence of BlaR1 has no charged amino acids, which suggests that the product does not have a removable signal peptide.

BlaR1 may play a role in the first stage of the induction process by serving as a receptor of penicillin. However, the following stages in which the stimulus reaches the repressor gene are not known. The unlinked *blaR2* locus may be involved in this process.

The *blaR1* locus was originally identified with the regulatory mutant R27 (35). We recently cloned the *blaR1* region from R27. It did not support induction of β -lactamase in *B. subtilis*, and there was at least one defect in the DNA (T. Kobayashi and J. O. Lampen, unpublished results).

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