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Purification and Characterization of the *Proteus vulgaris* BlaA Protein, the Activator of the β-Lactamase Gene[1]

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Induction of the expression of the β-lactamase gene, *blaB*, is regulated by the *blaA* gene located just upstream of *blaB* in the opposite direction in *Proteus vulgaris*. The expression of the *blaA* gene is negatively autoregulated by its own product BlaA, the activator of the *blaB* gene. The *P. vulgaris* BlaA protein shares high amino acid homology with the LysR family members, which are prokaryotic transcriptional activators that possess a putative helix-turn-helix DNA binding motif. To characterize its function, we purified the BlaA protein to homogeneity from *Escherichia coli* carrying the expression plasmid of the *blaA* gene driven by the tac promoter. The gel shift assay and DNaseI footprinting showed that purified BlaA specifically bound to the *blaA* promoter region, which resides immediately upstream of that of *blaB*. The binding region contained an inverted repeat, including a T-N_{11}-T sequence which is similar to the LysR motif (T-N_{11}-A) that is conserved in some LysR family members [Goethals *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89, 1646-1650]. We also showed that the BlaA protein forms a dimer in solution, using glycerol gradient centrifugation and glutaraldehyde crosslinking.

Key words: β-lactamase, BlaA, DNA binding protein, LysR family.

β-Lactamases are produced constitutively or inducibly in many Gram-positive and Gram-negative bacteria (*1-4*). Some Gram-negative enterobacteria have class-C chromosomal β-lactamase genes (*ampC*) and their expression is induced by β-lactam antibiotics (*3-7*). In *Citrobacter freundii* and *Enterobacter cloacae*, the inducible expression of the *ampC* β-lactamase gene is controlled by the transcriptional regulator AmpR (*6, 8*), the transmembrane protein AmpG (*9, 10*), and the negative regulator AmpD (*11*). AmpR is thought to interact with an inducing ligand, which has not been identified, to express the *ampC* gene. The *Escherichia coli ampD* mutant accumulates a murein peptide, 1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid, in the cytoplasm (*12*) and AmpD hydrolyses the murein peptide to release a tripeptide, L-alanyl-D-glutamyl-meso-diaminopimelic acid (*13*). Since the *ampC* β-lactamase from *C. freundii* is expressed even in the absence of β-lactams in the *E. coli ampD* mutant, concomitantly with accumulation of the murein peptide, the murein peptide seems to be an activating ligand for AmpR (*12*).

The Gram-negative bacterium *Proteus vulgaris* has a chromosomal and inducible class-A β-lactamase gene (*14-17*). We studied the regulation of the class-A β-lactamase production of *P. vulgaris* 5E78-1. The *blaA* gene is located immediately upstream of the *blaB* β-lactamase gene in the opposite direction and is autoregulated by its own product, BlaA. Since the *blaA* gene is required for *blaB* gene expression, the *blaA* gene product is considered to be an activator for *blaB* transcription in the presence of β-lactams. The *blaB* β-lactamase is not expressed in *E. coli* carrying the cloned *blaB* and *blaA* region from *P. vulgaris* even in the presence of β-lactam antibiotics. However, in the *E. coli ampD* mutant strain, the *blaB* β-lactamase is produced dependently upon BlaA during the stationary phase even in the absence of β-lactam antibiotics (Sugimoto, K. *et al.*, unpublished data). Thus, the *E. coli ampD* gene product is likely to be a negative regulator for the induction of the *blaB* β-lactamase as well as *ampC* β-lactamases of *C. freundii* and *E. cloacae* in *E. coli*. The *E. coli ampD* mutant is considered to accumulate the BlaA ligand during the stationary phase and AmpD would be involved in the degradation pathway of the ligand. The *blaD* gene, which is highly homologous with *ampD*, has been isolated from *P. vulgaris* (Itoh, T., unpublished data). Datz *et al.* have cloned and sequenced a class-A β-lactamase gene, *cumA*, and the transcriptional activator protein, *cumR* from *P. vulgaris* B317 (*18*). They also showed that *cumD* and *cumG*, analogous to *ampD* and *ampG*, respectively, are present in *P. vulgaris* B317 (*18*).

BlaA as well as AmpR shows amino acid similarity with the members of the LysR family (*19, 20*), which consists of over 50 DNA binding proteins having a putative helix-turn-helix motif positioned near their N-termini. These proteins bind to the regulatory regions of related genes and some of them bend the DNA. Many members of this family activate the transcription of linked target genes in response to inducing ligands (*20*), but a ligand for BlaA has not been

[1] The sequences of the *blaA* and the *blaB* genes from *P. vulgaris* 5E78-1 have been submitted to the GSDB/DDBJ/EMBL/NCBI nucleotide sequence data base with the accession number D37831.

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Abbreviations: dATP, deoxyadenosine-5′-triphosphate; dCTP, deoxycytidine-5′-triphosphate; IPTG, isopropyl-β-D-*thio*-galactopyranoside.

identified yet.

To understand the BlaA-mediated activation of the *blaB* transcription, we purified BlaA and studied its biochemical properties including DNA binding and oligomerization.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media—*P. vulgaris* 5E78-1 was obtained from Dr. Arisawa, M. (Nippon Roche Research Center, Kamakura). The ampicillin hypersensitive mutant, *E. coli* AS226 was derived from C600 r^-m^- {F^-, *thr-1, leuB6, thi-1, hsdS1, lacY1, tonA21, supE44*, λ^-} (*21*) by deleting the region from the *Xho*I to the *Hin*dIII restriction sites in the *ampC* gene (*22*). AS226-51 is an *ampD* spontaneous mutant derived from AS226. A plasmid pCR-1 has the *blaA-blaB* promoter region within the *Dra*I-*Hin*dIII fragment from *P. vulgaris* in the *Sma*I-*Hin*dIII site of pHC624 (*23*). A plasmid pHSG-ACR has the *P. vulgaris Cla*I-*Bgl*II fragment including the entire *blaA* gene in pHSG396 digested with *Cla*I and *Bam*HI. A plasmid (pbAtp2) which overproduces the BlaA protein under the tac promoter was constructed as follows. A plasmid pMC1871 (*24*) was digested with *Pst*I and the fragment containing the *lacZ* gene was introduced to the *Pst*I site in pUC12. The resulting plasmid pKI-1 was digested with *Hin*dIII and *Xba*I, then the *Hin*dIII-*Xba*I fragment was ligated to pCR-1 digested with *Hin*dIII and *Xba*I. The resulting plasmid pCR-lacZ had a *blaA'-'lacZ* fusion gene. The expression vector pTA-1 (obtained from Dr. Aoyama, T., Kyoto University, Kyoto) was digested with *Bam*HI and *Xba*I, then the protruding end of the *Bam*HI site was deleted by S1 nuclease digestion and repaired by the Klenow fragment. The *Ssp*I-*Xba*I fragment of pCR-lacZ was placed just downstream of the tac promoter of pTA-1. The plasmid plac-tacP2, which showed the highest β-galactosidase activity (*25*) among the plasmids that produced BlaA-β-galactosidase fusion protein, was cut by *Hin*dIII and *Xba*I, then the *Hin*dIII-*Xba*I fragment of plac-tacP2 was replaced with that of pHSG-ACR. The resulting plasmid pbAtp2 was used to overproduce the BlaA protein.

Sources of Enzymes and Reagents—[α-^{32}P]dCTP and [γ-^{32}P]dATP were purchased from ICN (USA). Molecular standard markers, poly(dI)-(dC) and pUC12 were from Pharmacia (Uppsala, Sweden). Polyethyleneimine (Polymin P) was from Bethesda Research Laboratories (Rockville, MD). Hydroxylapatite (BIO-GEL HT) was from Bio-Rad Laboratories. Enzymes, pBR322 and pHSG396 were from Takara Shuzo (Kyoto).

Purification of the BlaA Protein—*E. coli* AS226 carrying the plasmid pbAtp2 was cultured at 30°C to an OD_{600} of about 0.5 in 1 liter of LB medium containing ampicillin (50 μg/ml). The culture was continued for 12 h after the addition of IPTG (0.5 mM) and the cells were harvested by centrifugation. Subsequent steps were performed at 4°C. Fractions containing the BlaA protein were monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The wet cell paste was suspended in 20 ml of extract buffer (20 mM Tris-HCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, pH 8.0), and the cells were disrupted by sonication. After centrifugation, NaCl was added to the supernatant to 100 mM, and Polymin P (pH 7.9) was added to 0.6%. The centrifuged pellet was suspended in 20 ml of T-buffer (10 mM Tris-HCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 10% glycerol, pH 8.0) containing 1.0 M NaCl and the suspension was centrifuged. Ammonium sulfate was added to the supernatant to a final concentration of 30% saturation, then the mixture was stirred and centrifuged. The pellet was suspended in 5 ml of P-buffer (10 mM sodium phosphate, 2 mM 2-mercaptoethanol, 10% glycerol, pH 6.8) containing 100 mM NaCl and dialyzed against the same buffer. The precipitate formed during the dialysis was collected by centrifugation and the pellet was dissolved in P-buffer containing 400 mM NaCl and dialyzed against the same buffer. The dialyzate was applied to a hydroxylapatite column equilibrated with P-buffer containing 400 mM NaCl, and eluted with 40 ml of a linear gradient of 10 to 200 mM sodium phosphate buffer. The fractions that eluted at about 100 mM phosphate contained the BlaA protein. These fractions were pooled and dialyzed against storage buffer (10 mM Tris-HCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 50% glycerol, pH 8.0) containing 0.4 M NaCl. The protein concentration was determined as described by Bradford (*26*).

Glycerol Gradient Analysis of the BlaA Protein—The BlaA protein (10 μg) and molecular weight marker proteins (chymotrypsinogen A, ovalbumin, bovine serum albumin, and aldolase) in 250 μl of T-buffer containing 400 mM NaCl were applied to a 10-30% linear glycerol gradient in T-buffer (4 ml). After centrifugation in a Hitachi RPS55T-2 rotor at 43,000 rpm at 4°C for 24 h, fractions of 150 μl were collected. Proteins were separated by 10% SDS-PAGE and visualized by silver staining.

Protein Cross-Linking—The BlaA protein was incubated at room temperature with various amounts of glutaraldehyde at a protein concentration of 0.27 mg/ml in 10 mM T-buffer containing 0.4 M NaCl. The reaction was stopped by the addition of the SDS polyacrylamide gel sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 0.001% Bromophenol Blue, 10% glycerol). Samples were resolved by electrophoresis on an 8% polyacrylamide gel containing SDS and stained with Coomassie Brilliant Blue R250.

Gel Shift Assay—The *Eco*RI-*Xba*I fragment (240 bp) from plasmid pCR-1 was labeled with [α-^{32}P]dCTP using the Klenow fragment. The gel shift reaction mixture (10 μl) contained 6.7 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM KCl, 3.3 mM sodium acetate, 1 mM DTT, 5% glycerol, 0.005% BSA, 50 ng poly(dI)-(dC), ^{32}P-labeled fragment (5 ng), and the indicated amount of protein. Reactions were carried out at room temperature for 20 min and the reaction mixtures were resolved by electrophoresis through a 6% polyacrylamide gel. The gel was dried and the labeled DNA fragment was visualized by autoradiography.

DNaseI Footprinting—The *Eco*RI-*Xba*I fragment from plasmid pCR-1 was labeled with [α-^{32}P]dCTP using the Klenow fragment or with [γ-^{32}P]dATP using T4 polynucleotide kinase after dephosphorylation with the calf intestinal alkaline phosphatase, and cleaved with *Nla*III. The reaction mixture was the same as that used for the gel shift reaction except for the addition of 5 mM $CaCl_2$ and 3 mM $MgCl_2$. The reaction mixture was incubated at room temperature for 20 min. DNaseI digestion was initiated by the addition of 10 ng of DNaseI. The mixture was incubated at room temperature for 1 min, then the reaction was stopped

with 4 mM EDTA, 2 M ammonium acetate, and 20 μg/ml *E. coli* rRNA. DNA was precipitated with ethanol, washed with 70% ethanol, and resolved by electrophoresis through an 8% polyacrylamide sequencing gel containing 8 M urea.

RESULTS AND DISCUSSION

Purification of the P. vulgaris BlaA Protein—To purify the overexpressed BlaA protein from *E. coli*, we constructed the expression plasmid, pbAtp2, that contained the *blaA* gene from *P. vulgaris* under the control of the inducible tac promoter. In pbAtp2, the *blaA* open reading frame was connected 7 bp downstream of the Shine-Dalgarno sequence of the tac promoter. Since the *blaA* promoter was deleted, the expression induced with IPTG was not repressed by BlaA protein.

E. coli AS226 carrying pbAtp2 overexpressed a protein of 32 kDa (estimated from SDS-PAGE) after induction with 0.5 mM IPTG at 30°C; this was consistent with the molecular weight (33.4 kDa) of the BlaA protein predicted from the DNA sequence (Fig. 1, lane 2). The overexpressed BlaA protein accounted for about 12% of the total soluble protein as determined by densitometric analysis. The induced lysate (Fig. 1, lane 2) was fractionated by precipitation with Polymin P (Fig. 1, lane 3), and by subsequent precipitation with 30% ammonium sulfate (Fig. 1, lane 5). The suspended pellet was dialyzed against the low salt buffer; BlaA remained in the undissolved precipitate, whereas most other proteins dissolved (Fig. 1, lane 6). This property of BlaA was useful in the purification procedure. The precipitate was then dissolved in the high salt buffer (Fig. 1, lane 7), and eluted through hydroxylapatite. No other proteins were found in the pooled fraction by Coomassie Blue staining (Fig. 1, lane 8). The amino acid sequence from the amino terminus of the purified protein (Met-Arg-Thr-His-Leu-Pro-Leu-Asn-Ala-Leu) was exactly the same as that deduced from the nucleotide sequence of the *blaA* gene.

Dimerization of the BlaA Protein—The molecular mass of the native BlaA protein in solution was examined by glycerol gradient centrifugation (Fig. 2). BlaA sedimented in a peak which corresponds to a molecular weight of 75 kDa. Since the molecular weight of the monomer protein was 33.4 kDa and there was no BlaA protein in the fractions corresponding to other oligomers, we considered that BlaA formed a stable dimer in solution.

Glutaraldehyde crosslinking supported the notion that BlaA forms a dimer (Fig. 3). A protein of about 66 kDa, corresponding to the dimer, migrated on SDS-PAGE in addition to a 32 kDa monomer after crosslinking of the purified BlaA protein with glutaraldehyde. These results were consistent with the oligomerization of other LysR family proteins. For example, NodD (*28*), AmpR (*29*), and CatR (*30*) form dimers, and CysB (*31*), NahR (*32*), and TrpI (*33*) form tetramers.

Binding of the BlaA Protein to the Regulatory Region of

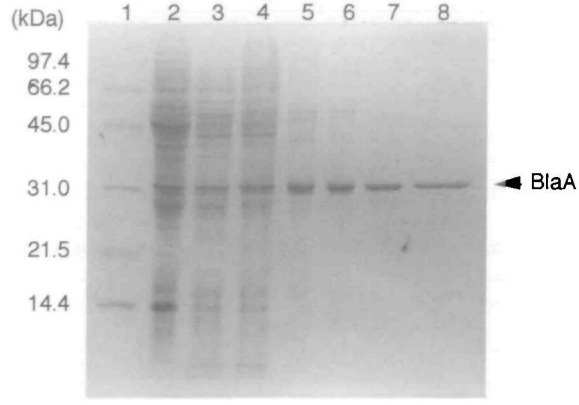

Fig. 1. **Purification of BlaA protein.** Samples were analyzed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. The position of BlaA is indicated by an arrowhead. Lane 1, Molecular weight markers; lane 2, soluble fraction after sonication; lane 3, Polymin P precipitate; lane 4, fraction eluted with T-buffer containing 1.0 M NaCl from Polymin P precipitate; lane 5, precipitate with 30% saturated ammonium sulfate; lane 6, precipitate formed during dialysis with P-buffer containing 0.1 M NaCl; lane 7, fraction before hydroxylapatite chromatography; lane 8, pooled peak fraction of hydroxylapatite column.

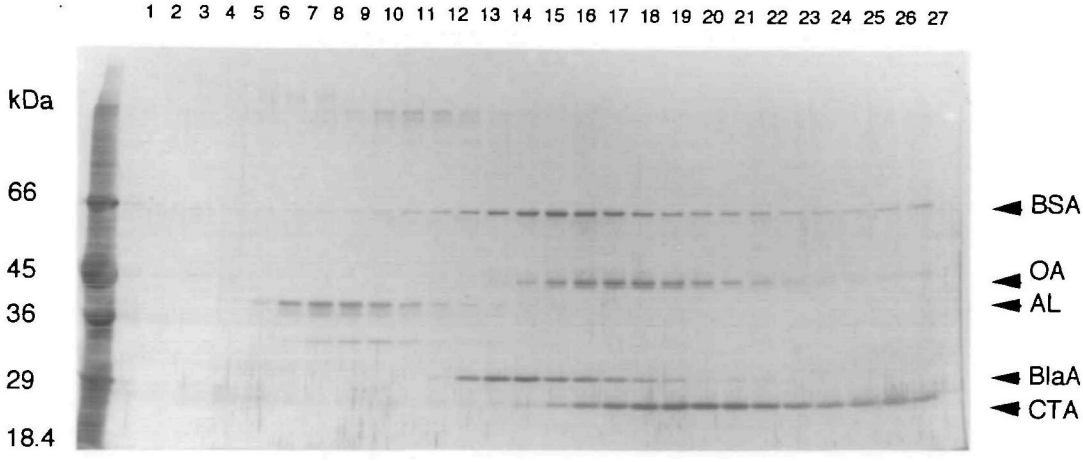

Fig. 2. **Glycerol gradient analysis of BlaA.** Fractions were collected from the bottom of the gradients. The proteins were separated by 10% SDS-PAGE and visualized by silver staining. The position of BlaA is marked, together with those of chymotrypsinogen A (CTA, $S_{20,w}$ 2.50), ovalbumin (OA, $S_{20,w}$ 3.66), bovine serum albumin (BSA, $S_{20,w}$ 4.30), and aldolase (AL, $S_{20,w}$ 7.60), which were included in the gradients as internal reference markers (27).

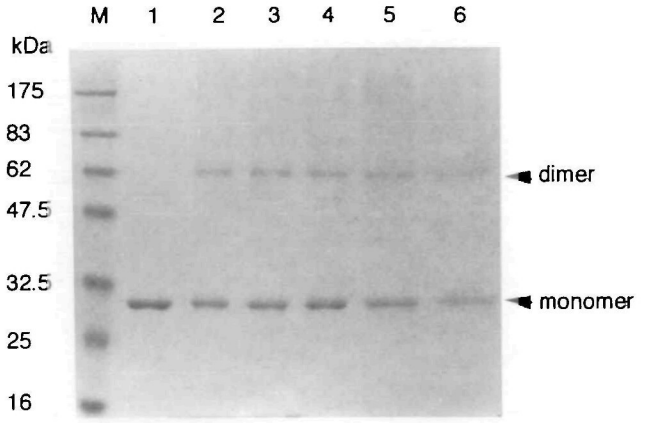

Fig. 3. **Glutaraldehyde cross-linking of BlaA.** The BlaA protein (0.27 mg/ml) was incubated with glutaraldehyde to 0% (lane 1), 0.0042% (lane 2), 0.0071% (lane 3), 0.011% (lane 4), 0.015% (lane 5), and 0.019% (lane 6). Reaction mixtures were resolved by 8% SDS-PAGE. Lane M shows the molecular weight marker proteins. Monomeric and dimeric BlaA are indicated.

the blaA and blaB Genes—We examined the DNA-binding activity of purified BlaA to the regulatory region of *P. vulgaris blaA* and *blaB* genes by gel shift analysis. The electrophoretic mobility of the end-labeled *Eco*RI-*Xba*I fragment, that contained the regulatory region of *blaA* and *blaB* genes, was retarded by binding of the BlaA protein (Fig. 4A). The shifted bands were decreased in the presence of unlabeled *Eco*RI-*Xba*I fragment as a competitor, whereas linearized pBR322 DNA had little effect (Fig. 4B). These results indicated that BlaA specifically bound to this fragment. As the quantity of the protein was increased, a second, slower-migrating complex appeared (Fig. 4A). Although the amount of protein bound to the DNA fragment in each band was not determined, BlaA may bind to DNA as a dimer in the faster band and as a tetramer or two dimers in the slower band. TrpI (*34*) and CatR (*30*) show increased binding affinity and the slower band predominates in the presence of their inducing ligands. It was suggested that the complex in the slower band is necessary for the transcriptional activation of the target gene. When a ligand for BlaA is identified, it will be interesting to test

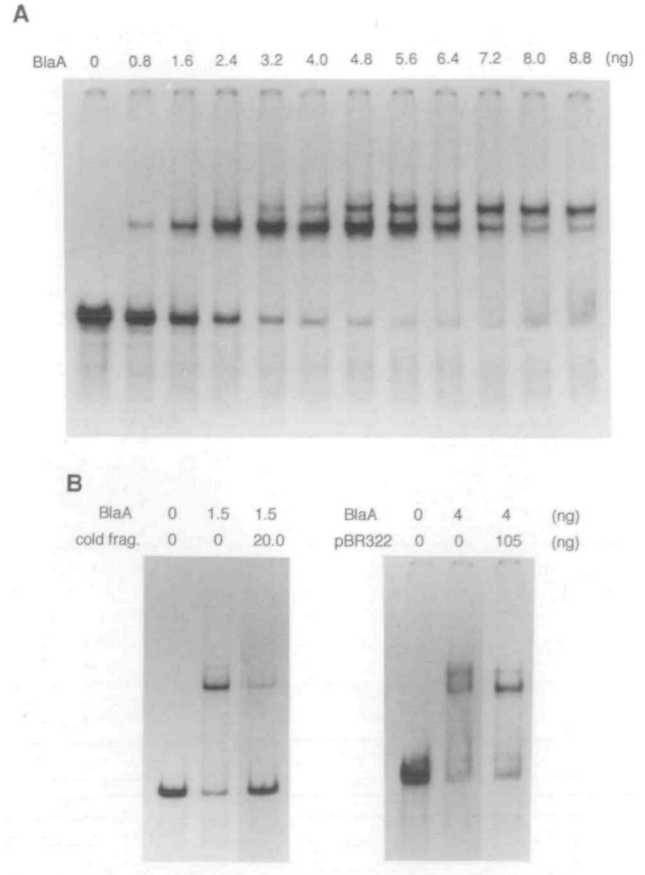

Fig. 4. **Gel shift assay.** (A) The end-labeled *Eco*RI-*Xba*I fragment (5 ng) carrying the *blaA* and *blaB* promoters was incubated with purified BlaA at various concentrations, then separated by 6% acrylamide gel electrophoresis. (B) Non-labeled *Eco*RI-*Xba*I fragment (cold fragment) or pBR322 was added as a competitor to the reaction mixture. Quantities of BlaA, unlabeled fragment and pBR322 are indicated.

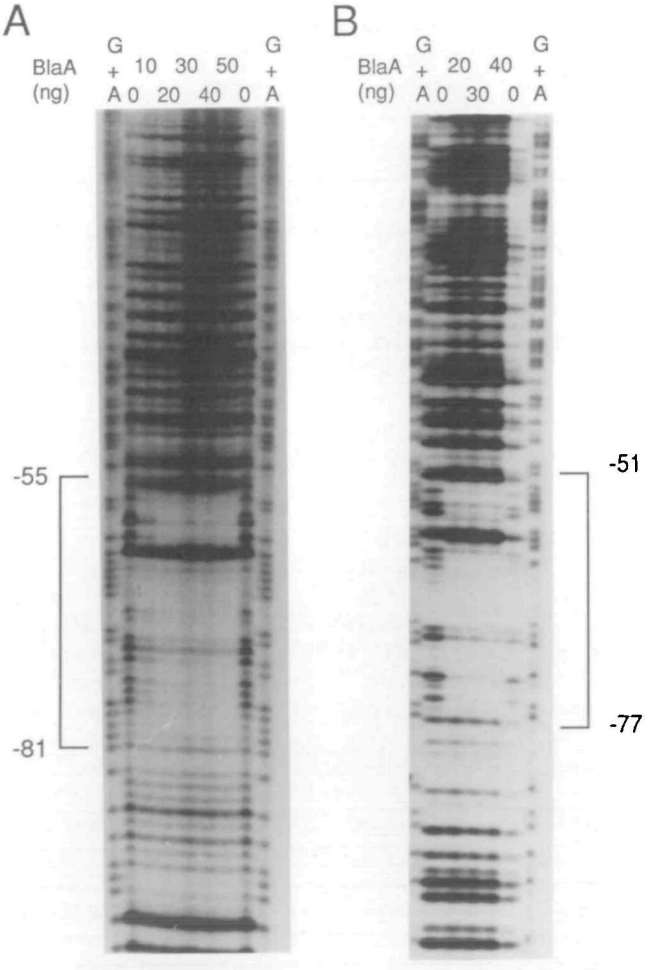

Fig. 5. **DNaseI footprinting of the regulatory region of *blaA* and *blaB*.** The *Eco*RI-*Xba*I fragment (24 ng) from plasmid pCR-1 was end-labeled on the antisense (A) or sense strands (B) of the *blaB* gene. The labeled fragment was incubated with BlaA, then digested with DNaseI. Quantities of BlaA are indicated. G+A lanes show the Maxam-Gilbert G+A reaction.

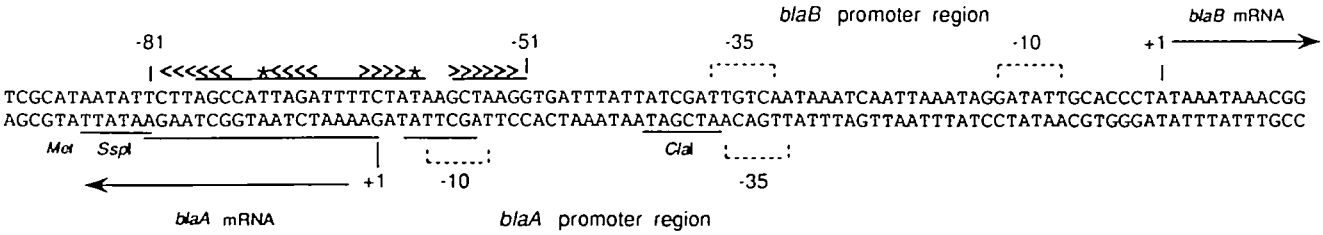

Fig. 6. **The *blaA-blaB* promoter region.** The promoter regions for *blaB* and *blaA* with their respective −10 and −35 regions are shown. The translational start position (Met) for BlaA and the *Cla*I and *Ssp*I restriction sites are indicated. Solid lines indicate the regions protected by BlaA from DNaseI digestion, as observed in Fig. 5. Arrows represent the direction of transcription from each promoter. Arrowheads (<) show inverted repeat sequences. T-N_{11}-T sequence is marked on T by an asterisk (*). The transcriptional start site (+1) of each gene was determined by primer extension (Itoh, T. and Ishiguro, K., unpublished data).

whether the second band formation is stimulated by adding the ligand in the gel shift analysis.

Binding Sequence of the BlaA Protein—The BlaA-binding site within the *Eco*RI-*Xba*I fragment was analyzed by DNaseI footprinting of both strands (Fig. 5). The BlaA protein protected the DNA region from −55 to −81 on the antisense strand and from −51 to −77 on the sense strand with respect to the *blaB* mRNA start site (Fig. 6). No significant difference was observed in the BlaA binding region by varying the concentration of BlaA (Fig. 5).

The protected region overlapped the *blaA* mRNA start site and the −10 region of *blaA* promoter (Fig. 6). This is consistent with the dual roles of the BlaA protein in the activation of *blaB* gene expression and in the repression (autoregulation) of *blaA*. This autoregulation will be responsible for the binding of BlaA to the promoter of its own gene, presumably preventing RNA polymerase from accessing the *blaA* promoter.

The binding region stretched over 30 bp and contained the motif characteristic of the LysR family protein recognition sequences. It contained an inverted repeat with the T-N_{11}-T sequence, which is similar to T-N_{11}-A (LysR) motif (*35*) of the binding site of some LysR proteins including TrpI, NodD, and MetR (*35*), and also contained A tract (T tract), 3 to 5 bp AT sequences in the middle of the LysR motif, conserved in the AmpR binding sequences of *C. freundii, E. cloacae, Yersinia enterocolitica*, and *Pseudomonas aeruginosa* (*29*).

Some members of the LysR family, including TrpI (*34*), NahR (*36*), and CatR (*30*), extend DNaseI protection to the −35 region of the target promoters in the presence of their ligands. Conversely, OccR with the ligand protects a promoter region shorter than that without the ligand (*37*). The conformational change of these proteins by their ligands would be necessary for interaction with the RNA polymerase. Tao *et al.* reported that OxyR, another LysR family protein, interacts with the RNA polymerase α subunit C-terminal region (*38*). The *blaB* β-lactamase from *P. vulgaris* is not induced in the presence of β-lactam antibiotics in *E. coli* wild-type strain harboring the *blaB* and *blaA* genes but is expressed dependently on the growth phase even in the absence of β-lactam antibiotics in *ampD* mutants (Sugimoto, K. *et al.*, unpublished data). We assumed that AmpD degrades the BlaA ligand and that the *ampD* mutant accumulates the ligand during the stationary phase. BlaA must be active for *blaB* expression in the stationary phase of the *ampD* mutant. Although we performed DNaseI protection assay using the extract of the stationary phase from the *ampD* strain (AS226-51) harboring a plasmid pbAtp2, this extract did not extend or shorten the protection region, as compared with purified BlaA (data not shown).

A peptidoglycan component seems to be a ligand of the activator in *ampC* β-lactamase expression (*12*). Studies to identify a ligand for BlaA and to investigate the DNA binding and conformational change of BlaA in the presence of the ligand should improve our understanding of the inducing mechanism of the *blaB* β-lactamase in *P. vulgaris*.

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