Purification and DNA binding properties of the *blaI* gene product, repressor for the β -lactamase gene, *blaP*, of *Bacillus licheniformis*

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ABSTRACT

The location of the repressor gene, *blaI*, for the β -lactamase gene *blaP* of *Bacillus licheniformis* 749, on the 5' side of *blaP*, was confirmed by sequencing the *bla* region of the constitutive mutant 749/C. An amber stop codon, likely to result in a nonfunctional truncated repressor, was found at codon 32 of the 128 codon *blaI* open reading frame (ORF) located 5' to *blaP*. In order to study the DNA binding activity of the repressor, the structural gene for *blaI*, from strain 749, with its ribosome binding site was expressed using a two plasmid T7 RNA polymerase/promotor system (S. Tabor and C. C. Richardson. Proc. Natl. Acad. Sci. **82**, 1074-1078 (1985). Heat induction of this system in *Escherichia coli* K38 resulted in the production of Blal as 5-10% of the soluble cell protein. Repressor protein was then purified by ammonium sulfate fractionation and cation exchange chromatography. The sequence of the N-terminal 28 amino acid residues was determined and was as predicted from the DNA. Binding of Blal to DNA was detected by the slower migration of protein DNA complexes during polyacrylamide gel electrophoresis. Blal was shown to selectively bind DNA fragments carrying the promoter regions of *blaI* and *blaP*.

INTRODUCTION

Regulation of β -lactamase induction in *Bacillus licheniformis* 749 has been proposed to involve three regulatory loci (1). One of these loci, the repressor gene, *blaI*, was reported to be 90% linked to the β -lactamase structural gene, *blaP*. Recently it has been shown that *blaI* is located 5' to *blaP* and is transcribed in the opposite direction (N. J. Nicholls, Ph.D. Thesis, Rutgers University, 1986 and 2).

The function of the repressor appears to be more complex than simply shutting off transcription from the *blaP* promoter. This is demonstrated by the induction of both *blaP* and *blaI* mRNAs by β -lactam antibiotics and the presence of similar putative regulatory sites in the two promoters (A. J. Salerno and J. O. Lampen, submitted for publication, 2 and 3). In light of these findings it seems likely that *blaI* is autoregulated.

We present here supporting evidence for the location of *blaI*, 5' to *blaP*, by locating the changes in the sequence of the repressor from mutant 749/C, which is constitutive for β -lactamase production. We have gone on to clone *blaI* in a high level expression vector, purify the repressor, and demonstrate that Blal has DNA binding activity which is specific for

DNA fragments containing the *blaP* and *blaI* promoter sequences. In addition, the N-terminal amino acid sequence of BlaI has been determined and compared to that predicted from the DNA sequence.

MATERIALS AND METHODS

Plasmids and bacterial strains

The bacterial strains and plasmids used are listed in Table 1. pRWN9 is an Amp^S derivative of pBR322 (N. J. Nicholls, Ph.D. Thesis, Rutgers University, 1986), created by linearization at the unique *Pst*I site within the ampicillin resistance gene, position 3614 (4), followed by digestion with nuclease Bal-31 and religation of the shortened plasmid. pBR322 treated in the above manner was then used to transform *Escherichia coli* RR1 cells with selection on plates containing tetracycline (20 µg/ml), followed by screening for ampicillin sensitive isolates. pRWN9 was an Amp^S Tet^r transformant and the deletion was characterized by mapping with a variety of restriction enzymes. Approximately 500 bp including the unique *Pst*I site (position 3614) and the unique *Pvu*I site (position 3739) had been removed.

The coregulated genes, *blaP* and *blaI*, from *Bacillus licheniformis* 749 (inducible for the synthesis of β-lactamase) were cloned, using ampicillin selection, as a 4.2 kb *Eco*RI fragment from *Eco*RI digested chromosomal DNA into the unique *Eco*RI site of pRWN9, producing pRWN101 (N. J. Nicholls, Ph.D. Thesis, Rutgers University, 1986). The corresponding 4.2 kb *Eco*RI region from the constitutive strain 749/C (*blaP*⁺, *blaI*⁻) was originally cloned from *Eco*RI digested chromosomal DNA into a lambda vector (5) and subsequently subcloned into the *Eco*RI site of pRWN9 producing pRWN121 (N. J. Nicholls, Ph.D. Thesis, Rutgers

TABLE 1
Bacterial strains and plasmids

<u>Strain</u>	<u>Genotype</u>	<u>Reference</u>
<i>E. coli</i> HB101	F ⁻ , <i>hsd</i> S20 (<i>r</i> _B ⁻ , <i>m</i> _B ⁻), <i>rec</i> A13, <i>ara</i> -14, <i>pro</i> A2, <i>lac</i> YI, <i>gal</i> K2, <i>rps</i> L20 (Str), <i>xyl</i> -5, <i>mtl</i> -1, <i>sup</i> E44, λ ⁻	2
<i>E. coli</i> K38	HfrC (λ)	25
<u>Plasmid</u>		
pRWN9	Tc ^r , Ap ^S	Nicholls, 1986
pRWN101	Tc ^r , Ap ^r , <i>bla</i> P ⁺ , <i>bla</i> I ⁺	Nicholls, 1986
pRWN121	Tc ^r , Ap ^r , <i>bla</i> P ⁺ , <i>bla</i> I ⁻	Nicholls, 1986
pRWΔOI	Tc ^r , Ap ^S , <i>bla</i> P ⁻ , <i>bla</i> IΔO	This work
pGP1-2	Kn ^r , T7 gene 1, λ <i>c</i> l857	13
pT7-6	Ap ^r , T7 Ø10	13
pRWT7I	Ap ^r , T7 Ø10 <i>bla</i> IΔO	This work

University, 1986). Both pRWN101 (749) and pRWN121 (749/C) have the 4.2 kb *EcoRI* *bla* fragment in the same orientation, with the *blaI* promoter transcribed in the direction of the *tet* promoter.

DNA isolation

Plasmid DNA was isolated from *E. coli* strains by the boiling method of Holmes and Quigley (6) for small scale preparations or by the alkaline lysis method for large scale preparations (7). DNA restriction fragments were isolated from low melting point agarose gels and purified on Bethesda Research Laboratories NACS ion exchange columns or extracted from polyacrylamide gels by electroelution.

DNA sequencing

The 1013 bp *PstI*-*SstI* fragment containing *blaI* from *B. licheniformis* 749/C was sequenced by the dideoxy chain terminating method (8). Restriction enzyme fragments for sequencing were subcloned for template production using the M13 mp vector system (9). Radio-labeling of DNA with dATP- α -³⁵S, was accomplished as described in the New England Nuclear protocol for ³⁵S Sanger sequencing (1983).

Construction of the *blaI* expression vector pRWT7I

Fig. 1 outlines the steps taken to produce pRWT7I. The primer used for primer repair (10) was synthesized by phosphoramidite chemistry using an Applied Biosystems DNA synthesizer (11, 12). pRWN101 DNA (55 μ g) was digested with *Sna*BI to produce a linearized plasmid opened between the promoters (for *blaI* and *blaP*) and the *blaP* translational start codon. Following linearization the plasmid was prepared for primer repair by treatment with 10 units of lambda exonuclease for 40 min at 37°C in a total reaction volume of 100 μ l. The single stranded regions produced by lambda exonuclease treatment were then available for hybridization with the phosphorylated oligonucleotide primer (TATAGGATGATTAAATGAAAAAATACCT) which covers the ribosome-binding site of *blaI* and the first 5 codons of the *blaI* structural sequence. Seventy picomoles of the primer was added to the plasmid DNA in T4 DNA polymerase buffer (70 mM Tris-HCl (pH 7.5), 10mM MgCl₂) at 70°C and allowed to cool slowly to 25°C. The hybridization mixture was then made 250 μ M in each dNTP, 5mM dithiothreitol, 100 μ g/ml bovine serum albumin. The annealed primer in the above mixture was extended and the unprotected single stranded regions were removed by treatment with 30 units of T4 DNA polymerase for 6 hr at 37°C. After primer repair the DNA was ligated using 6 units of T4 DNA ligase at 20°C for 12 hr. The ligated DNA was used to transform *E. coli* HB101 cells and transformants were selected on LB agar containing 20 μ g/ml tetracycline. In order to determine whether digestion had proceeded in the direction of the *blaP* and *blaI* promoters, plasmid DNA isolated from transformants was digested with *Nde*I which cuts within the *blaP* promoter (Fig. 1). The extent of the deletion within the *blaP* and *blaI*

Fig. 1. Construction of the *blaI* expression vector, pRWT7I. P_P and P_I indicate the *blaP* and *blaI* promoters, respectively. The direction of transcription from the promoters is shown by arrows. Distance (in bp) from the ends of linearized DNA fragments is indicated below the line diagrams. The ssDNA removed from the 5' ends of *SnaBI* digested pRWN101 is indicated by dashed lines. The location of the oligonucleotide primer within this region is indicated by an arrow.

promoter region was then determined, for plasmids demonstrating the loss of this *NdeI* site, by digestion with *PstI* and *SstI* and sizing the liberated *blaI* containing fragment. The correctly tailored plasmid, pRWΔOI, was chosen in this manner and the exact extent of the deletion was determined (Fig. 2) by sequencing the 645 bp *PstI*-*SstI* *blaI* fragment liberated from this plasmid.

The 645 bp *PstI*-*SstI* fragment from pRWΔOI contains the *blaI* structural gene and ribosome binding site without either the *blaP* or *blaI* promoter sequences. This fragment was used to construct the *blaI* expression vector pRWT7I. The expression system employed was the dedicated T7 RNA polymerase/promoter dual plasmid system of Tabor and Richardson (13). The 645 bp *blaI* fragment from pRWΔOI was ligated into the *PstI*-*SstI* sites of pT7-6 in front of the T7 Ø10 promoter, creating pRWT7I. The size of pRWT7I was determined by digestion with *SstI* which should cut only once in this plasmid. A single band of approximately 2.85 kb (the expected size of pT7-6 with the 645 bp insert from pRWΔOI) was observed, indicating

Fig. 2. Nucleotide sequence of the *blaI* region of strain 749 and 749/C. The restriction enzyme sites used in this study are shown and the distance in bp from the *PstI* site is indicated. Numbering is from the first nucleotide of the *PstI* restriction site. P_p and P_i indicate *blaP* and *blaI* promoters, respectively. The location of transcriptional initiation sites are indicated by asterisks. The derived amino acid sequence is shown for the N-terminal regions of BlaI and BlaP. The Shine-Dalgarno (SD) and the promoter -10 and -35 sequences are indicated. Putative BlaI binding sites are boxed and direct, (—), and inverted, (→ ←), repeats are shown. The region deleted in the construction of pRWΔOI is located between triangles (Δ).

correct insertion of a single 645 bp *blaI* containing fragment. Digestion of pRWT7I with *Pst*I and *Sst*I released the 645 bp fragment as expected.

Isolation of Blal from SDS-polyacrylamide gels for amino acid sequencing

Cell lysate fractions containing Blal were electrophoresed through 15% polyacrylamide, 0.1% SDS gels according to Laemmli (14) and stained lightly with Coomassie Blue. The Blal band was excised from the stained gels and isolated by a crush and soak technique (Russell Durbin, personal communication). The soak solution used was 0.1 M Tris-HCl (pH 8.4), 0.2% SDS and 0.1% β -mercaptoethanol, at 37°C. The polyacrylamide was removed by centrifugation and the SDS bound protein was precipitated with 0.2 ml of a 0.1 M quinine-HCl solution per ml of supernatant. The precipitated protein was washed with 90% acetone prior to sequencing by Edman degradation using an Applied Biosystems Inc. model 470A protein sequenator (15).

Purification of Blal

To locate Blal in the various cell lysate fractions, samples were subjected to SDS-polyacrylamide gel electrophoresis (14) and stained with Coomassie Blue. The amount of Blal in samples was determined by scanning densitometry of the stained gels using a Joyce-Loebl scanning densitometer. Protein concentrations were determined by the Lowry assay (16) or by the Bradford dye binding assay (17).

A single colony of *E. coli* K38 containing pGP1-2 and pRWT7I was used to inoculate 250 ml of LB broth containing 50 μ g/ml ampicillin and kanamycin. After overnight growth at 30°C the culture was added to 12 liters of LB broth containing 10 g/l Difco tryptone, 5 g/l Difco yeast extract, 10 g/l NaCl and 50 μ g/ml ampicillin and kanamycin. The culture was grown at 30°C to an OD₅₉₀ of 1.15, followed by heat induction of T7 RNA polymerase at 42°C for 30 min. Rifampicin was then added to 100 μ g/ml and the culture was shifted to 37°C for 3 hr to allow expression of *blaI* from the T7 ϕ 10 promoter. The cells were then harvested and washed twice with 10 mM Tris-HCl (pH 7.5), 1% NaCl. The cell pellet was suspended in 100 ml of 10 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgSO₄ containing 40 μ g/ml phenylmethyl sulfonyl fluoride (PMSF) and 1 μ g/ml pepstatin and was stored at -70°C.

Fifty ml of the cell suspension was thawed and mixed with 50 ml of 20 mM Tris-HCl (pH 8.0), 100 mM KCl and 10 mM MgSO₄ and the cells were broken in a French press. The resulting lysate was highly viscous. Two mg of DNaseI and 100 ml of 30 mM Tris-HCl (pH 7.5), 10 mM MgSO₄ containing 40 μ g/ml PMSF and 1 μ g/ml pepstatin were added and the solution was maintained at 25°C with intermittent swirling for 30 min, then centrifuged at 47,800 $\times$ g for 2 hr at 4°C. The soluble fraction, containing virtually all of the Blal protein, was concentrated by ultrafiltration using a Amicon Pellicon filter under N₂ pressure. The concentrated solution was brought up in 80 ml of dilution buffer (10 mM Tris-HCl (pH 7.5 at

22°C), 100 mM KCl, 10 mM MgSO₄, 10% glycerol). Thirty three ml of the above fraction was brought up to 50ml with dilution buffer. The diluted solution (12mg protein/ml) was made 45% saturated in ammonium sulfate by the addition of 14 grams of solid ammonium sulfate over a 60 min period while stirring on ice. The pH was maintained at 7.5 by addition of 1M NaOH as needed. Proteins were precipitated at 0°C overnight, then centrifuged at 14,500 x g for 40 min at 0°C. The resulting supernatant (53ml), containing the majority of the Blal protein, was dialyzed against 20mM Tris-HCl (pH 8.0), 1 mM Na₄EDTA and 5% glycerol. The dialyzed solution (88ml, 3.2 mg protein/ml) was made 30 µg/ml in PMSF and 0.5 µg /ml in leupeptin and stored at -20°C.

Final purification was carried out using a Pharmacia MonoS HR 5/5 cation exchange FPLC column with OD₂₈₀ detector and automatic gradient controller. The dialyzed fraction was used directly or was redialyzed against 50 mM HEPES (pH 7.6), 1 mM Na₄EDTA, 5% glycerol (buffer A). All reagents were Aldrich Gold Label except the Na₄EDTA which was Sigma Grade. 500 µl of sample (1.6 mg protein) was loaded onto the column equilibrated with buffer A. Blal was eluted by a linear NaCl gradient from buffer A to buffer B (buffer A with 1M NaCl) over a 25 minute period. Blal eluted as an isolated peak between 0.55 and 0.60 M NaCl yielding approximately 9.0 mg of pure Blal per liter of culture.

Competitive DNA binding gel electrophoresis assay

The binding of Blal to DNA was assayed using a gel electrophoresis technique in which protein-DNA complexes are detected as discrete bands with mobilities reduced as a function of the number of protein units bound to a given fragment (18, 19, 20). FPLC purified Blal (6 pM/µl) was the source of repressor for the binding assay. Blal in the FPLC fraction (0.58 M NaCl) retained binding activity and specificity without noticeable decline after 2 months at -20°C.

The 1013 bp *Pst*I-*Sst*I fragment from pRW101, containing P_P and P_I (the promoters for *bla*P and *bla*I) and the *bla*I structural gene from *B. licheniformis* 749, was digested with *Taq*I and ³²P-labeled by replacement synthesis using T4 DNA polymerase (21). The 470 bp *Taq*I fragment containing P_P and P_I was also isolated and then ³²P-labeled as above. Labeled DNA was incubated with FPLC purified Blal and either cold *Hae*III digested øX174 DNA (as nonspecific competitor) or cold 470 bp *Taq*I fragment DNA (as specific competitor), at the indicated concentrations, for 40-60 min at 30°C in 20 µl of binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 µg/ml bovine serum albumin). Two µl of a 50% glycerol, 0.1% bromophenol blue solution was then added and the samples were immediately applied to a vertical 5% acrylamide, 0.13% bisacrylamide gel and

Fig. 3. Expression of *blaI*. Prestained standards, 5 μ g each (lanes 1 and 8). *E. coli* K38/pGP1-2 containing pT7-6 (lanes 2 and 3), or pRWT71 (lanes 4 and 5), were grown in enriched medium, containing 50 μ g/ml ampicillin and kanamycin. Cells were grown at 30°C to OD₅₉₀ = 1.4, and harvested (lanes 2 and 4) or induced at 42°C, as described in methods (lanes 3 and 5). Soluble cell lysates were prepared, and samples containing 40-50 μ g of total protein were used for gel electrophoresis. Lane 6, 21 μ g of FPLC purified BlaI. Lane 7, 9 μ g of FPLC purified BlaI. Samples were boiled for 3 min after addition of 1 volume of sample buffer (2% SDS, 5% β -mercaptoethanol, 10% glycerol, 0.0005% bromophenol blue, and 0.0625 M Tris-HCl, pH 7.5), and loaded onto a 15% polyacrylamide, 0.1% SDS gel. Protein bands were stained with Coomassie Brilliant Blue.

electrophoresed for 2-3 hours at 30 mAmp with buffer circulation. The gel and running buffers were 10 mM Tris (pH 7.5 at 22°C), 1 mM Na₄EDTA. Following electrophoresis the gel was dried and autoradiographed.

RESULTS

Sequencing of *blaI* from the constitutive mutant 749/C: Confirmation of the location of *blaI* 5' to *blaP*

The mutation responsible for the constitutive phenotype of β -lactamase expression in *B. licheniformis* 749/C, was determined by sequencing the 1013 bp *Pst*I-*Sst*I fragment of pRW121. In the wild type inducible strain 749, the sequence of this DNA fragment contains a 128 codon ORF which encodes the repressor for *blaP*, designated *blaI*, as determined by cis-trans analysis (N. J. Nicholls, Ph.D. Thesis, Rutgers University, 1986). Three nucleotide differences were seen between the 128 codon ORF region from the constitutive strain

(pRWN121) and that from the inducible strain (pRWN101). A transversion (G to T) within codon 32 produces an amber stop codon. In addition, two adjacent transversions (GG to TC) change amino acids 97 and 98 from Met to Ile and Val to Leu, respectively. It is likely that the amber mutation accounts for the constitutive phenotype of strain 749/C due to the production of a nonfunctional truncated repressor. However, if read-through of this codon occurs, a nonfunctional repressor might still be formed as a result of the other amino acid changes. As there are no nucleotide changes in the promoter region of either *blal* or *blaP*, the constitutive phenotype is not the result of an operator mutation.

Construction of an expression vector for *blal*

The expression of *blal* transcripts in *B. licheniformis* is negatively regulated along with those of *blaP* (A. J. Salerno and J. O. Lampen, submitted for publication). In addition, the two promoter regions contain highly homologous 23 bp sequences of dyad symmetry, which strongly resemble the binding sites of regulatory proteins in *E. coli*, suggesting that *blal* is autoregulated (2). In addition, *blal* expression from the wild type promoter in maxicells was undetectable (data not shown). To avoid reduction of *blal* expression by autoregulation, we constructed the expression plasmid pRWT7I, as described in Materials and Methods. *Blal* was expressed in *E. coli* K38 from pRWT7I using the two plasmid T7 RNA polymerase/promoter system of Tabor and Richardson (13). Induction of *E. coli* K38/pGP1-2/pRWT7I resulted in the production of a novel protein of 15 kd, which is the expected MW of *Blal*. This protein represented 5-10% of the total protein in the soluble cell lysate (Fig. 3).

In order to prove that this protein was indeed *Blal*, the protein was extracted and purified from SDS-polyacrylamide gels and sequenced, as described in Materials and Methods. The N-terminal twenty eight amino acid residues were resolved, all of which matched the predicted sequence from the DNA.

Purification of the β -lactamase repressor

Cells from a heat induced culture of *E. coli* pGP1-2/pRWT7I were lysed and the insoluble material was removed by centrifugation. The soluble fraction was then made 45% saturated in ammonium sulfate (pH 7.5) and centrifuged. The supernatant fraction, representing 50% of the original soluble protein, contained the bulk of the *Blal* protein (10-20% of the protein in this fraction). Precipitation of *Blal*, by raising the ammonium sulfate concentration to 65% saturation, resulted in a precipitate which could not be solubilized without the addition of 1% SDS or 6 M guanidine. Final purification of the repressor was accomplished by cation exchange chromatography using a Pharmacia Mono S column and a linear (0.0-1.0 M) NaCl elution gradient. *Blal* in the 0.55 to 0.60 M NaCl fraction was judged to be pure based on SDS-polyacrylamide gel electrophoresis (Fig. 3).

DNA binding analysis of Blal

The promoter regions of *blal* and *blaP* possess highly homologous sequences with dyad symmetry. The consensus sequence is AAAGTATTACATATGTAAGNTTT (2). Similar structures have been reported for the binding sites of regulatory proteins found in *E. coli* and some eukaryotic systems (2, 22, 23). The DNA binding activity of Blal was analyzed using a sensitive polyacrylamide gel electrophoresis assay which detects the slower migration of DNA-protein complexes with respect to free DNA (18, 19, 20). To determine whether Blal would bind to DNA in a specific manner, we performed a competition binding assay using *TaqI* digests of the 1013 bp *PstI*-*SstI* fragment from pRWN101 as labeled binding substrate as described in Materials and Methods. *TaqI* digestion of the 1013 bp *PstI*-*SstI* fragment from pRWN101 (contains DNA from the inducible strain, 749) produced three fragments of 219 bp, 324 bp, and 470 bp (Fig. 2). A fourth fragment (689 bp) was generated as a result of incomplete *TaqI* digestion. The 470 bp and 689 bp fragments each contain three putative Blal binding sites (Fig. 2) (2), while the 219 and 324 bp fragments do not contain regions with homology to the complete consensus sequence.

The specificity of Blal binding was demonstrated by the selective shifting of the 470 bp and 689 bp *TaqI* bands in the presence of a large molar excess of nonspecific competitor DNA, while little or no binding was observed with the 219 bp and 324 bp fragments (Fig. 4A). The relative affinity of a specific DNA site for the repressor protein was measured by the efficiency with which non-specific DNA competes for binding to the specific site. At a molar ratio of non-specific sites (taken as the concentration in bp) to putative binding sites (assuming 3 binding sites per 1013 bp *PstI*-*SstI* fragment) of 5.4×10^6 (lane 2, Fig. 4A), almost no band shifting is observed. As this ratio is decreased to 5.4×10^4 (lane 5), binding to the 470 bp and 689 bp fragments is clearly evident. The percentage of these fragments bound and the number of repressor units bound per fragment (as shown by the formation of new bands with slower migration rates) increase with decreasing competitor DNA as expected (lanes 2-7). At a 5.4×10^3 ratio of non-specific to specific sites (lane 7) virtually all of the specific DNA is complexed while none of the 219 bp or 324 bp DNA fragments are bound.

As the non-specific competitor is reduced to a ratio of 5.4×10^2 or is omitted (lanes 10 and 11), there is still no binding with the 324 bp fragment while a low level of binding to the 219 bp fragment is observed. This difference in affinity was more evident at higher Blal concentrations (data not shown), indicating that the 219 bp fragment may contain a specific binding site for Blal. Computer assisted analysis of the 1013 bp *PstI*-*SstI* fragment for sequences with homology to the putative Blal binding sites in the *blaP* and *blal* promoter regions has identified a sequence (AAAGTGATTATATA) which has strong homology to one half (AAAGTATTACATA) of the consensus sequence (2). This half-site lies within the *blal* coding sequence (628 bp from the *PstI* site) and is contained on the 219 bp *TaqI* fragment.

Fig. 4. Competitive Blal:DNA binding assay. **A.** The specificity of Blal binding is demonstrated by selective band shifting during polyacrylamide gel electrophoresis in the presence of a large molar excess of non-specific competitor DNA. ^{32}P -labeled *TaqI* digested 1013 bp *PstI*-*SstI* DNA (2.5×10^{-9} M bp (7.2×10^{-12} M putative Blal binding sites based on three sites per 1013 bp fragment)) was incubated with Blal (6×10^{-10} M), in the presence of the following concentrations (in bp) of *HaeIII* digested øX174 DNA as nonspecific competitor, as described in Materials and Methods. For lanes 2-7: 3.9×10^{-5} M, 3.9×10^{-6} M, 2.0×10^{-6} M, 3.9×10^{-7} M, 2.0×10^{-7} M, and 3.9×10^{-8} M, respectively. For lanes 9-11: 7.8×10^{-8} M, 3.9×10^{-9} M, and 0.0 M, respectively. Lane 8, no Blal and no competitor DNA. MW standards are shown in lane 1. **B.** Binding preference of Blal determined by the ability of competitor DNA to eliminate noticeable binding to the labeled 470 bp *TaqI* fragment. ^{32}P -labeled 470 bp *TaqI* fragment DNA (7.5×10^{-10} M bp (4.8×10^{-12} M putative Blal binding sites)) was incubated with Blal (1.2×10^{-9} M) and the following concentrations (in bp) of competitor DNA, as described in Materials and Methods. Lanes 3-6 *HaeIII* digested øX174 DNA (as nonspecific competitor): 7.5×10^{-6} M, 3.75×10^{-6} M, 3.75×10^{-7} M, and 7.5×10^{-8} M, respectively. Lanes 7-10 cold 470 bp *TaqI* fragment DNA (as specific competitor): 3.75×10^{-7} M, 7.5×10^{-8} M, 3.75×10^{-8} M, and 7.5×10^{-9} M, respectively. Lane 2, no competitor DNA. Lane 11, no Blal and no competitor DNA. MW standards are shown in lane 1 and 12. Samples were electrophoresed on a 5% polyacrylamide gel, prepared with 10mM Tris-HCl, pH 7.5, 1mM EDTA as running and casting buffers.

In order to confirm that *Blal* binds with a greater affinity to DNA bearing the *blaP* and *blaI* promoters, we performed a competition binding assay in which we compared the ability of the cold 470 bp *TaqI* fragment (specific competitor) to compete for repressor versus that of cold *HaeIII* digested ϕ X174 DNA (nonspecific competitor). In this experiment, shown in figure 4B, we determined the amount of competitor DNA (measured in bp) required to completely eliminate noticeable binding to the 470 bp *TaqI* fragment labeled with ^{32}P . At a molar ratio of cold ϕ X174 competitor DNA to labeled 470 bp *TaqI* fragment DNA of 10^4 (bp:bp) very little binding of the labeled specific fragment was observed (lane 3). At a molar ratio of cold 470 bp *TaqI* fragment (specific competitor) to labeled 470 bp *TaqI* fragment DNA of only 5×10^2 complete loss of noticeable binding of the labeled fragment occurred (lane 7). At a molar ratio of ϕ X174 competitor to labeled 470 bp fragment of 5×10^3 some binding was evident (lane 4), while at a molar ratio of 470 bp fragment competitor to labeled 470 bp fragment of 1×10^2 slightly more binding occurred (lane 8). This demonstrates a binding preference for the 470 bp (P_P and P_I containing) fragment over ϕ X174 nonspecific DNA, of 20 to 50 fold, compared on a bp to bp level. If compared as the concentration of putative operator sites (for the 470 bp *TaqI* fragment competitor) to nonspecific sites (for ϕ X174 DNA competitor) required to eliminate noticeable binding, a 3×10^3 to 8×10^3 fold greater binding preference is obtained.

DISCUSSION

The production of β -lactamase is negatively regulated at the level of transcription (3). It has been recently shown that the *blaI* gene, which encodes the repressor for the β -lactamase gene (*blaP*) of *B. licheniformis*, is located immediately 5' to *blaP* and is transcribed in the opposite direction (N. J. Nicholls, Ph.D. Thesis, Rutgers University, 1986, 2). The presence of three codon changes, one producing an amber stop signal, within the *blaI* ORF region of the constitutive mutant 749/C, further supports this location and function of *blaI*.

In addition to the increase in *blaP* mRNA upon induction with β -lactam antibiotics, *blaI* mRNA is also induced, so the two genes are to some extent coregulated (A. J. Salerno and J. O. Lampen, submitted for publication). The presence of highly homologous sequences with dyad symmetry within the promoter regions of *blaP* and *blaI*, which may be the sites to which repressor binds, suggests that *blaI* is autoregulated (2).

We have cloned the *blaI* gene, with the promoter region removed, into pT7-6 under the control of the T7 ϕ 10 promoter and have expressed *blaI* to levels enabling us to purify the repressor protein. The protein product produced has a MW of approximately 15 kd (the predicted size of the repressor) and was associated with the soluble fraction of the DNase I treated cell lysate. The amino acid sequence of the N-terminal 28 residues of the purified protein corresponded exactly to the sequence predicted from the DNA.

The selective binding of Blal to fragments containing the *blaP* and *blal* promoters indicates the presence of specific recognition sequences. In addition it is worth noting that at least three selectively shifted bands are formed with DNA fragments carrying both promoters. The significance of these bands is not as yet known. Selective binding to the 219 bp *TaqI* fragment is also observed, although with an apparent markedly lower affinity than for the fragments containing the promoters. The presence of a sequence with strong homology to a putative Blal binding half-site may explain this observation. It is possible that binding to this half-site plays some role in the regulation of β -lactamase expression. Regulation may be occurring at the level of synthesis or degradation of *blal* mRNA as well as at initiation. The location of a third ORF immediately following *blal*, which encodes a protein with a role in induction (24), indicates that regulation of the *blal* mRNA may be important in governing the induction response.

Further characterization of the interactions of repressor with DNA and its location and function within the *B. licheniformis* cell is required for an understanding of its role in the regulation of β -lactamase synthesis.

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