Volume 246, number 1,2, 83-88 FEB 06948 March 1989

Interaction of BlaI, the repressor for the β-lactamase gene of *Bacillus licheniformis*, with the *blaP* and *blaI* promoters

Matthew J. Grossman*, Ivan H.A. Curran+ and J. Oliver Lampen

Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, PO Box 759, Piscataway, NJ 08855, USA

Received 9 January 1989

BlaI repressor for the β-lactamase gene (*blaP*) of *Bacillus licheniformis* 749, was shown to repress expression of *blaP* and of the repressor gene (*blaI*), using the purified protein in a DNA-directed in vitro translation assay. Binding of BlaI to the promoter regions of *blaP* and *blaI* was examined by equilibrium and competitive binding assays. BlaI binds to the *blaP* promoter with an equal or only slightly higher affinity than to the *blaI* promoter. DNase I footprinting shows that BlaI binds directly to the *blaP* and *blaI* promoters, such that RNA polymerase binding and/or transcript elongation would be blocked.

Lactamase, β-; Repressor; Regulation; DNA-binding protein; (*Bacillus licheniformis*)

1. INTRODUCTION

Bacillus licheniformis 749 is inducible for the production of β-lactamase (BlaP) which is secreted into the medium after a series of processing steps (for reviews see [1,2]). The regulation of BlaP production occurs largely at the level of transcription [3]. Induction of BlaP is delayed and protracted; *blaP* mRNA and protein synthesis increase slowly to a maximum over a 1 h period and then decrease over the next hour, remaining above pre-induced levels for several more hours [3]. The half-life of the message is about 2 min and cannot account for the protracted induction period [3]. The time course of the *blaP* mRNA induction appears to be tied to cell growth, in that at a given temperature two to three cell divisions occur before maximum levels are attained [3].

The gene encoding the repressor (*blaI*) has been cloned from *Bacillus licheniformis* 749 and from a closely related strain 9945A and has been shown to lie 5′ to *blaP* [4,5]. Transcriptional analysis [6] demonstrated that *blaI* mRNA is transcribed divergently from *blaP* mRNA and that it is coinduced with *blaP* mRNA, suggesting that *blaI* expression is autoregulated.

We have recently purified the repressor protein (BlaI) and demonstrated that it binds specifically to DNA fragments containing the *blaP* and *blaI* promoters [7]. These regions contain highly homologous 23 bp regions of dyad symmetry believed to be the specific sites to which BlaI binds [5]. The *blaP* promoter contains two 23 bp regions of dyad symmetry whereas the *blaI* promoter contains only one.

Here we show that purified BlaI inhibits the production of BlaP and its own synthesis in vitro. In addition, we compare the relative binding affinity of BlaI for the isolated promoter regions and analyze BlaI-DNA binding by DNase I footprinting.

2. MATERIALS AND METHODS

2.1. *In vitro translation*

In vitro translation was performed using a prokaryotic DNA-directed in vitro translation kit (Amersham), according to the manufacturer's instructions.

Correspondence address: J.O. Lampen, Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, PO Box 759, Piscataway, NJ 08855, USA

* *Present address:* Exxon Research and Engineering, Clinton Township, Route 22 East, Annanandale, NJ 08801, USA

\+ *Present address:* National Research Council Canada, Sussex Drive, Ottawa, Ontario, Canada

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2.2. *DNA manipulation*

Plasmid DNA was isolated by the alkaline lysis procedure and purified by CsCl equilibrium density-gradient centrifugation [8]. DNA fragments were isolated from polyacrylamide gels by electroelution. DNA restriction enzyme digests and ligations were performed as previously described [7]. DNA fragments were ^{32}P-labeled by replacement synthesis using T_4 DNA polymerase (New England Biolabs) [9].

2.3. *Plasmids*

Fig.1 shows the structure of the promoter regions of *blaP* and *blaI*. pRWN101 contains the 4.2 kb *Eco*RI DNA fragment containing *blaP* and *blaI* from *Bacillus licheniformis* 749, the β-lactamase inducible strain [4]. pRWN121 contains the corresponding region from the β-lactamase constitutive strain 749/C which has an amber stop mutation at codon 32 of *blaI* [7]. The separate and contiguous *blaP* promoter (P_P) and *blaI* promoter (P_I) sequences were subcloned from pRWN101 into pUC119 [10] using the *Acc*I and *Sma*I sites within the multiple cloning site region. P_P was subcloned as a 117 bp *Rsa*I-*Hpa*II fragment, P_I was subcloned as a 244 bp *Hpa*II-*Rsa*I fragment, and the contiguous P_P and P_I sequence was subcloned as a 410 bp *Ssp*I-*Taq*I fragment. Promoter containing DNA used for binding studies was isolated from the resulting plasmids using flanking restriction enzyme sites.

2.4. *DNA-binding assay*

Binding of the repressor to DNA was examined by the gel electrophoresis mobility shift assay [11–13]. Binding reactions were performed in 20 μl, as previously described [7]. Analysis of bound DNA complexes was made by visual comparison. Repressor was maintained at −70°C until used.

The repressor concentrations indicated are based on a specific-binding activity of 20% calculated on the basis of one molecule of repressor per binding site. The specific-binding activity of the repressor stock solution was determined as that amount of repressor monomers required to completely bind the available binding sites present on a DNA fragment containing the *blaP* promoter (P_P). The DNA fragment was titrated at a concentration of 2×10^{-9} M, well above the expected equilibrium-binding constant, to allow for stoichiometric binding. Due to the presence of two regions of dyad symmetry within P_P, two binding sites were calculated per P_P containing DNA fragment.

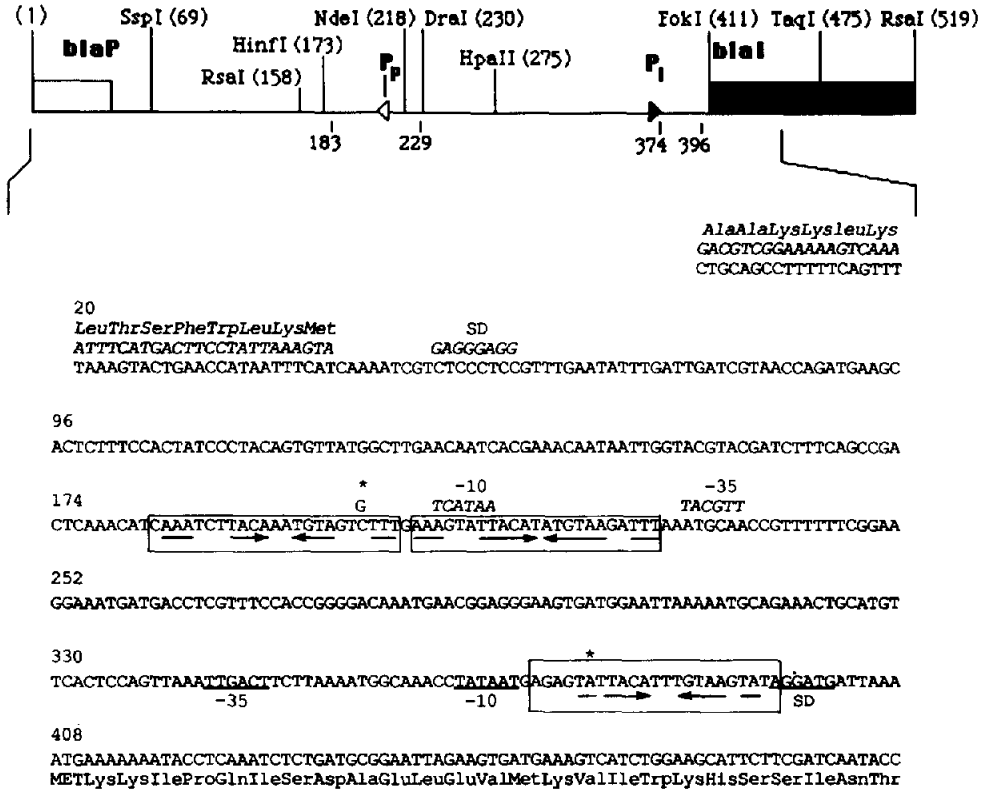

Fig.1. Physical arrangement of the *blaP* and *blaI* promoter region. The top line drawing shows the restriction enzyme site used in this work and the arrangement of promoter and coding sequences for *blaP* and *blaI*. The extent of the regions of dyad symmetry within the two promoters is shown beneath the line. The corresponding sequence is shown below. The regions of dyad symmetry associated with BlaI binding are boxed; direct (– –) and inverted repeats (→ ←) are indicated. Promoter consensus sequences, transcriptional start sites (*) and ribosome-binding sites (SD) are shown.

The relative affinity for nonspecific competitor (DNA not containing *bla* promoter sequences) versus specific competitor was determined by comparing the concentration of competitor-binding sites required to eliminate binding to a specific BlaI/DNA complex. The simple alternating copolymer poly(dI-dC)·(dI-dC) (Pharmacia) was used as the nonspecific DNA competitor. The concentration of nonspecific sites was calculated as the concentration in base pairs.

Equilibrium-binding analysis was performed using labeled specific DNA fragments at concentrations of 10^{-12} to 5×10^{-13} M, well below that of the repressor which was varied within a range spanning the equilibrium-binding constant. Under these conditions, the apparent binding constant for a given repressor/DNA complex is essentially equal to the concentration of active protein required to produce half-maximal formation of that complex [14].

2.5. *DNA footprinting*

Binding reactions were performed using a constant level of DNA fragment (2.5×10^{-10} M), labeled at one 3′-end with ^{32}P, and poly(dI-dC)·(dI-dC) (100 μg/ml) with various levels of repressor. After incubation of the binding mixture at 30°C for 70 min, 1 μl of a 10 μg/ml DNase I (Sigma) solution, freshly diluted in 25 mM $CaCl_2$, 50 mM $MgCl_2$, was added and allowed to incubate for 60 s. The DNase I reaction was stopped by addition of one half volume of 95% formamide, 0.1% bromophenol blue, 0.1% xylene cylanol and heated at 85°C for 5 min prior to loading on an 8% sequencing gel.

Size markers were generated by digestion of the labeled fragments with restriction enzymes that cleave at known positions.

3. RESULTS

3.1. *Demonstration of BlaI activity in vitro*

To confirm a repressor function for BlaI and to determine whether autoregulation occurs we tested the ability of purified repressor to inhibit expression from P_P and P_I in a DNA-directed in vitro translation assay. pRWN121, which contains an inactive truncated *blaI* [7], was chosen as template for the production of BlaP in order to eliminate repression of *blaI* from BlaI produced in the in vitro reaction. In vitro translation of pRWN121 in the presence of a 15-fold molar excess of BlaI monomers resulted in a dramatic reduction in the amount of a 34 kDa protein, the correct size for unprocessed BlaP (BlaP with intact signal sequence) (fig.2, cf. lanes 8 and 9). In addition the production of a protein of about 3 kDa was also selectively repressed. This is the correct size for the truncated BlaI protein expected as result of the amber stop mutation in *blaI*.

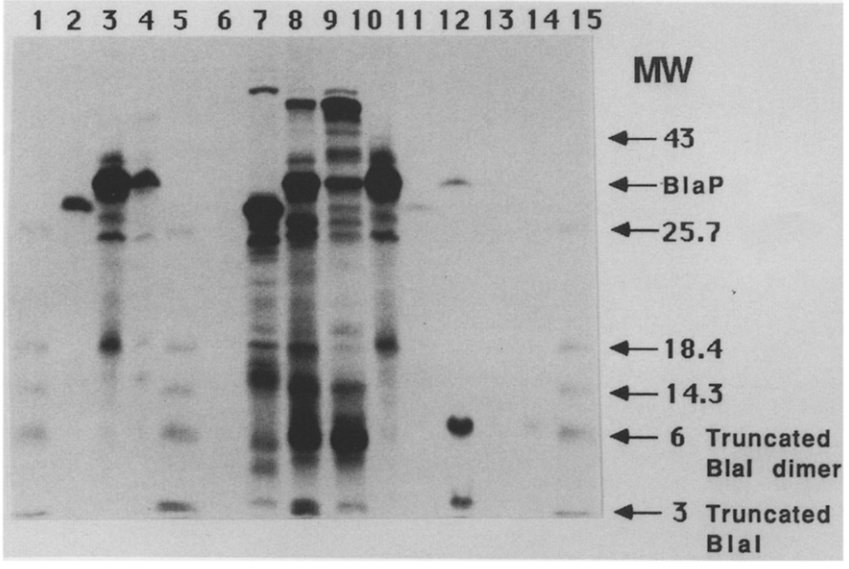

Fig.2. Immunoprecipitation of pRWN121 in vitro translated in the presence of BlaI. pRWN121 lanes 3, 8, 10, 12, and 14; and pAT153 (control plasmid without *blaP* or *blaI*) lanes 2, 7, and 11; in vitro translated in the absence of BlaI. pRWN121 in vitro translated in the presence of BlaI, lanes 4, 9, and 13. Lanes 7–9 in vitro translation samples not immunoprecipitated. Immunoprecipitations with BlaP or BlaI antiserum: pRWN121-BlaP antiserum, lanes 3, 4, and 10; pRWN121-BlaI antiserum, lanes 12 and 13; pRWN121-no antiserum lane 14; pAT153-BlaP antiserum, lane 2; pAT153-BlaI antiserum, lane 11. Lanes 1, 5 and 15, protein molecular mass standards, size is shown in kDa. Lane 6, blank.

The in vitro translation samples were subjected to immunoprecipitation with IgG purified from BlaP antiserum and with BlaI antiserum (fig.2). Anti-BlaP IgG specifically precipitated the 34 kDa protein and a number of smaller proteins apparently representing unfinished BlaP products (lane 3). The levels of BlaP precipitated from the samples translated in the presence and absence of BlaI demonstrated that BlaI repressed the production of BlaP (cf. lanes 3 and 4). Parallel immunoprecipitations using BlaI antiserum specifically brought down the 3 kDa BlaI protein (lane 12). A 6 kDa protein, the correct size of a possible dimeric form of the truncated BlaI protein, was also precipitated by the BlaI antiserum. The production of both proteins was repressed by the presence of BlaI in the in vitro translation reaction (cf. lanes 12 and 13).

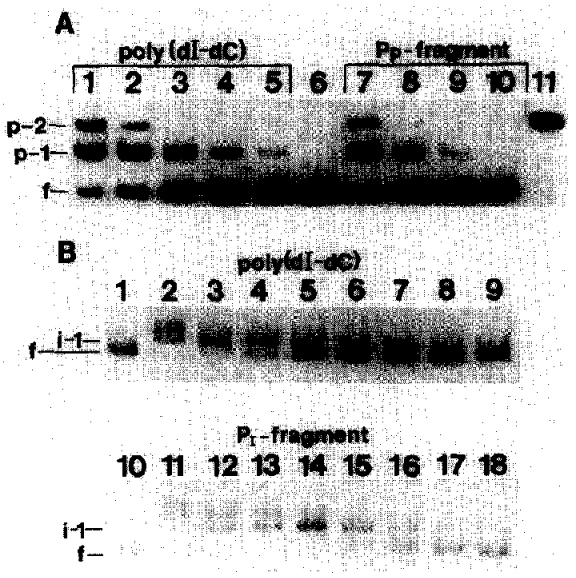

Fig.3. Relative affinity of BlaI for the separated promoters. Labeled DNA fragments were incubated with repressor and the indicated concentrations (given as the molar ratio in bp) of unlabeled nonspecific (poly(dI-dC)·(dI-dC)) or specific (promoter containing fragment), competitor DNA. (A) Labeled DNA fragment containing the *blaP* promoter (P_P). Lanes 1–5: competitor poly(dI-dC)·(dI-dC), 500:1, 1000:1, 2500:1, 5000:1 and 10 000:1, respectively. Lanes 7–10: competitor P_P containing fragment, 50:1, 100:1, 250:1 and 500:1, respectively. Lanes 6 and 11: no repressor and no competitor DNA, respectively. (B) Labeled DNA fragment containing the *blaI* promoter (P_I). Lanes 3–9: competitor poly(dI-dC)·(dI-dC), 78:1, 156:1, 313:1, 625:1, 1250:1, 2500:1 and 5000:1, respectively. Lanes 12–18: competitor P_I containing fragment, 8:1, 16:1, 31:1, 63:1, 125:1, 250:1 and 500:1, respectively. Lanes: 1 and 10, no repressor; 2 and 11, no competitor DNA. f = free DNA, p-1 and p-2, and i-1; specific protein/DNA complexes corresponding to BlaI binding to the promoter regions of *blaP* and *blaI*, respectively.

3.2. *Relative affinity of BlaI for the separated promoters*

Sequential binding of BlaI to a DNA fragment containing P_P, as the concentration of BlaI is increased, produces first the p-1 complex and then the p-2 complex (fig.3), which demonstrate high affinity in comparison to complexes formed at higher BlaI levels. This is shown by a greater stability in the presence of nonspecific DNA competitor, and is consistent with high-affinity specific binding of repressor to the two regions of dyad symmetry within P_P followed by low-affinity nonspecific binding. Similarly, BlaI binding to a DNA fragment containing P_I produces only one complex of high affinity (i-1) (fig.3), consistent with BlaI binding to the single region of dyad symmetry within P_I; and binding to a DNA fragment containing both promoters produces three high-affinity complexes c-1, c-2, and c-3 (table 1).

Fig.3 shows competitive-binding assays with DNA fragments containing separate P_P and P_I sequences. Results with the labeled P_P fragment (fig.3A) indicate that the specific competitor (P_P fragment) has approx. a 25-fold greater affinity (on a bp to bp basis) over nonspecific competitor for BlaI in the p-2 complex (lanes 3 and 8) and approx. a 40-fold greater affinity for BlaI in the p-1 complex (lanes 5 and 9). Calculated from the concentration of nonspecific sites versus the concentration of P_P promoter the P_P fragment competes approx. 4×10^3-fold more efficiently for BlaI when

Table 1

Relative equilibrium-binding constants for BlaI binding to P_P and P_I present on the same and separate DNA fragments

BlaI/DNA complex	Binding constant[a]	Variance
c-1	2.0×10^{-11} M	0.5×10^{-11} M
c-2	3.0×10^{-11} M	0.8×10^{-11} M
c-3	4.1×10^{-11} M	0.9×10^{-11} M
p-1	3.0×10^{-11} M	0.8×10^{-11} M
p-2	4.4×10^{-11} M	0.9×10^{-11} M
i-1	4.4×10^{-11} M	0.6×10^{-11} M

[a] The values for the c and p complexes are averages of four independent assays. The value for the i-1 complex is an average of two independent assays

measured with respect to the formation of the p-2 complex, and 6×10^3-fold more efficiently for BlaI when measured with respect to the formation of the p-1 complex.

A similar test with the labeled P_I fragment (fig.3B) showed a 5-fold greater affinity for the specific competitor (P_I fragment) over nonspecific competitor for the repressor in the i-1 complex (lanes 8 and 18). This corresponds to an approx. 1.5×10^3-fold more efficient binding to P_I than to nonspecific DNA.

If we compare BlaI affinities for P_P and P_I based on the number of specific sites (assuming two for P_P and one for P_I), we see a 2-fold greater affinity when measured with respect to the formation of the P_P-p-1 complex (initial binding to P_P), and an approximately equal affinity for formation of the P_P-p-2 complex and formation of the P_I-i-1 complex.

3.3. *Equilibrium-binding constants*

The relative affinity of BlaI for each of the two isolated *bla* promoters was further characterized by comparison of the apparent equilibrium-binding constants. We also wanted to compare the affinity of BlaI for the contiguous promoters to that for the separated promoters.

The constants averaged from a number of similar experiments are listed in table 1. These data support the competition data, indicating only slightly greater affinity for P_P than for P_I.

3.4. *Footprinting analysis*

DNase I footprinting analysis of BlaI binding to a DNA fragment containing both promoters confirmed that site-specific DNA binding is localized to the regions of dyad symmetry contained within the promoter regions of *blaI* and *blaP* (fig.4), as previously predicted [5]. Footprinting of the complementary strand and DNA containing the isolated promoters revealed similar protected regions (not shown).

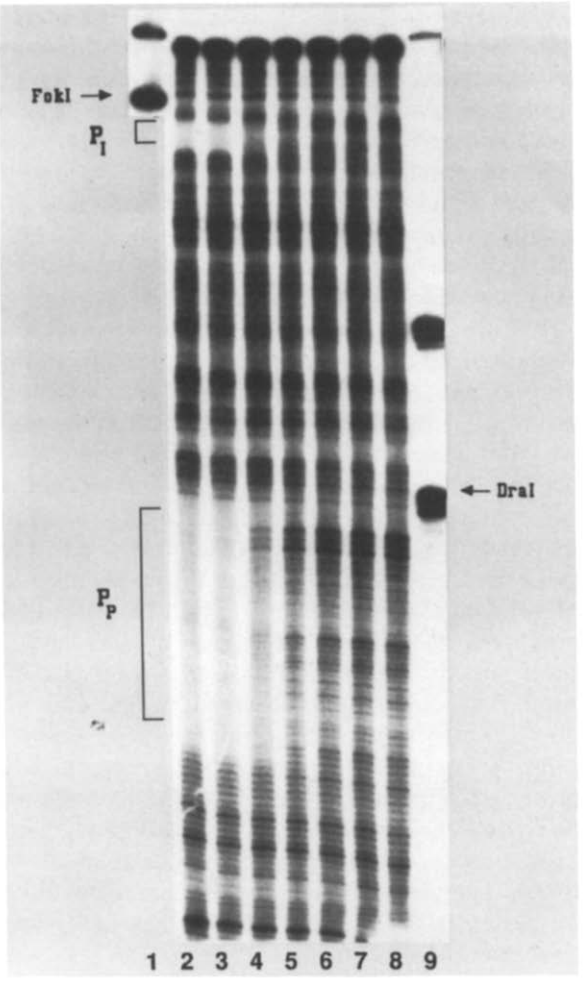

Fig.4. BlaI footprinting on the *blaI* antisense strand of a DNA fragment containing the contiguous *blaP* (P_P) and *blaI* (P_I) promoter sequences. Lanes 2–8: 1×10^{-7} M, 5×10^{-8} M, 2.5×10^{-8} M, 1.3×10^{-8} M, 6.3×10^{-9} M, 3.1×10^{-9} M, and no BlaI. Lanes 1 and 9: restriction enzyme generated fragments used for size markers. The regions of dyad symmetry associated with P_P and P_I are indicated by brackets.

4. DISCUSSION

The ability of purified BlaI to inhibit the synthesis of BlaP expressed from P_P in the in vitro translation system clearly demonstrates the repressor activity of this protein. Further, a similar effect on the production of truncated BlaI (3 kDa) expressed from P_I demonstrates that autoregulation of *blaI* does occur. The persistence of a dimer during SDS-PAGE is surprising, and may be a result of a frame shift allowing extension of truncated BlaI beyond the amber stop mutation. Analysis of the sequence of *blaI* reveals that a 6 kDa protein could be formed if an alternate reading frame was used 3′ to the amber stop. Alternatively the amino-terminal end of BlaI may be involved with dimerization, which is enhanced in the absence of a full-length protein.

We have analyzed the binding of BlaI to the promoter sequences of *blaP* and *blaI* in order to gain perspective on the differences and possible interactions between them. The studies of competitive BlaI binding to the separate P_P and P_I sequences demonstrate that BlaI has an equal or slightly higher affinity for P_P in comparison to P_I. Equilibrium-binding analysis of the separated promoters and the two promoters on the same DNA fragment corroborates these results.

Wittman and Wong [15] recently reported a slightly stronger affinity for BlaI for binding to the P_P-binding domain, on the basis of DNase I footprinting. However, the binding conditions used were not indicated. Therefore, it is not possible to determine if differences in binding affinities for the two promoters could be attributed to differences in the binding system used by these researchers and those used here. They show that with a molar ratio of repressor to operator of 7.5 there was slight binding at P_P while no binding was observed at P_I. At a slightly higher ratio of 10, strong but incomplete binding was observed at P_P and no binding at P_I. The next increment was to a molar ratio of 50 and both promoters were completely protected. This is consistent with the data shown here and indicates that the difference in binding affinity between the two promoters is quite small. In fact, a likely explanation for the binding-affinity difference is the presence of two sites in P_P and only one in P_I.

Footprinting analysis confirms the prediction that the repressor binds to the regions of dyad symmetry present in both P_P and P_I (fig.3). The location of the binding domains suggests that bound repressor obstructs binding of RNA polymerase to P_P, and interferes either with binding to P_I or with elongation from this promoter. The difference in the positioning of the binding sites, with respect to the two promoters and the presence of two binding sites within P_P, result in differential regulation of P_P and P_I. Our footprinting experiments do not reveal binding to P_P without concomitant binding to P_I. In addition, the two regions of dyad symmetry within the P_P domain do not show differential binding, indicating that they bind BlaI with equal affinity.

Acknowledgements: This work was supported, in part, by Public Health Service Grant AI-23096 from the National Institute for Allergy and Infectious Diseases and by the Charles and Johanna Busch Memorial Fund. I.H.A. Curran held a Busch Postdoctoral Fellowship and M.J. Grossman held a Busch Predoctoral Fellowship. We thank Julia Sohm for excellent technical assistance.

REFERENCES

[1] Collins, J.F. (1979) in: Beta-lactamases (Hamilton-Miller, J.M.T. and Smith, J.T. eds) pp. 351–368, Academic Press, London.
[2] Lampen, J.O. and Nielsen, J.B.K. (1982) in: The Molecular Genetics of Bacilli (Ganesan, A.T. et al. eds) Academic Press, New York.
[3] Salerno, A.J. and Lampen, J.O. (1986) J. Bacteriol. 166, 769–778.
[4] Nicholls, N.J. and Lampen, J.O. (1987) FEBS Lett. 221, 179–183.
[5] Himeno, T., Imanaka, T. and Aiba, S. (1986) J. Bacteriol. 168, 1128–1132.
[6] Salerno, A.J. and Lampen, J.O. (1988) FEBS Lett. 227, 61–65.
[7] Grossman, M.J. and Lampen, J.O. (1987) Nucleic Acids Res. 15, 6049–6062.
[8] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[9] O'Farrell, P. (1981) Focus 3, 1.
[10] Vieira, J. and Messing, J. (1988) Methods Enzymol. 153, 3–11.
[11] Fried, M. and Crothers, D.M. (1981) Nucleic Acids Res. 9, 6505–6525.
[12] Garner, M.M. and Revzin, A. (1981) Nucleic Acids Res. 9, 3047–3061.
[13] Fried, M. and Crothers, D.M. (1984) J. Biol. Chem. 172, 241–262.
[14] Hendrickson, W. and Schleif, R.F. (1984) J. Mol. Biol. 174, 611–628.
[15] Wittman, V. and Wong, H.C. (1988) J. Bacteriol. 170, 3206–3212.