Introduction

Treatment of Staphylococcus aureus infections with β-lactams is often ineffectual as the majority of clinical strains are resistant because of the production of one or both of β-lactamase and an alternative penicillin-binding protein (PBP2a or PBP2). Production of β-lactamase is inducible in most isolates, and the genes for regulation and biosynthesis of β-lactamase are clustered together, frequently located on transposon Tn552.

The purified repressor (BlaI) of β-lactamase production has been shown to bind specifically to two regions of dyad symmetry, known as operators, and the genes for regulation and biosynthesis of β-lactamase are clustered together, frequently located on transposon Tn552.

The repressor proteins BlaI and MecI bind similarly to the bla operator implicated in the regulation of β-lactamase synthesis in Staphylococcus aureus. BlaI binds to two separate dyads but neither copper–phenanthroline footprinting nor dimethyl sulphate (DMS) methylation protection assays produced any evidence of a change in the geometry of the DNA between the two dyads. It is concluded that BlaI molecules bound at the dyads probably do not cause bending or looping of the intervening DNA. DMS protection assays of BlaI binding to the bla operator in vitro and in vivo gave similar results so that it is tentatively concluded that the in vitro results are an accurate reflection of the in vivo situation. Deletion of the dyad nearest to the blaZ gene resulted in decreased synthesis of the chloramphenicol acetyltransferase reporter protein synthesized from the blaZ promoter/translation inititiator. Explanations for this are considered.
Materials and methods

General microbiology

The bacteria and plasmids used are listed in Table 1. S. aureus was grown in casein hydrolysate (Oxoid, Basingstoke, UK)/yeast extract (Oxoid) (CY) medium or on CY agar without the addition of glucose or β-glycerophosphate. Escherichia coli was grown in Luria–Bertani (LB) medium (Oxoid) or on LB agar. For S. aureus the media contained, as required: 20 mg/L erythromycin, 5 mg/L chloramphenicol, 5 mg/L tetracycline. For E. coli, the media contained, as required, 100 mg/L ampicillin or 50 mg/L chloramphenicol. S. aureus was grown at 30°C and E. coli at 37°C. S. aureus was transformed by the method described by Gotz et al. and E. coli was transformed by the method of Hanahan.

Preparation of BlaI and MecI

BlaI was prepared from the GST–BlaI fusion protein. MecI was also prepared from a GST–MecI fusion protein as described by Lewis.

Recombinant DNA technology

All materials and methods were as in Sambrook et al. Wizard Plus SV Miniprep kit (Promega, Madison, WI, USA) was used to prepare DNA for cloning. T4 DNA ligase and all restriction enzymes were obtained from New England Biolabs (Hitchin, UK) or Boehringer-Mannheim (USA) was used to prepare DNA for cloning. T4 DNA ligase and all restriction enzymes were obtained from New England Biolabs (Hitchin, UK) or Boehringer-Mannheim (USA). Oligonucleotides OT (5'-GTACTTATAAATCAATAAT) and OB (5'-GTACTTATAAATCAATAAT) were obtained from Genosys (Cambridge, UK). DNA for footprinting and gel retardation was radioactively labelled by the method of Gregory et al.

Repressor–DNA binding reactions

Binding reactions were set up by preparing a reaction mix containing an amount of DNA appropriate for the footprinting reaction, 1 mg of non-specific DNA competitor [poly(dI–dC)] in 10% (w/v) glycerol, 10 mM Tris–glycine, 1 mM EDTA and 10 mM NaCl. This mix was dispensed into Eppendorf tubes, BlaI added and the reaction mixture was incubated at 37°C for 10 min. Samples were stored on ice and then loaded on to a pre-run acrylamide Tris–glycine gel (30 mM Tris, 10 mM glycine) and electrophoresed at a constant 300 V at 4°C. The position of the complexes was determined using StrataMarkers (Stratagene, La Jolla, CA, USA) and autoradiography.

DNase I footprinting

A binding reaction mix containing at least 400 counts per second (cps) of the DNA probe per reaction was set up.

The DNase I enzyme stock solution was diluted to c. 75 mg/L with a 0.9% NaCl solution, and 2 μL of this was added to 33 μL of a freshly prepared solution of 5 mM CaCl2 and 5 mM MgCl2. Ten microlitres of the binding reaction mix was then added and incubated at room temperature for 20 s, before addition of 50 μL of equilibrated phenol, vigorous vortexing for 20 s and storage on ice until all reactions were completed. Ten microlitres of chloroform was added, the tube was then briefly vortexed and microfuged at 12 000g for 15 min at 4°C. The aqueous layer was recovered and a 1/10 volume of 3 M sodium acetate and 2.5 vols of fresh ethanol were added, and the tube kept at −70°C overnight. Precipitated DNA was pelleted by microcentrifugation and resuspended in sequencing gel loading buffer. The samples were resolved on 15% acrylamide gels.

Copper–phenanthroline footprinting

Reaction conditions were adapted from the method of Sigman et al. BlaI–DNA binding reactions were electrophoresed through 6% (w/v) polyacrylamide gels, and complexes visualized by autoradiography. Excised gel fragments were immersed in 100 μL of 50 mM Tris–HCl pH 8.0. Ten microlitres of OP-Cu mix (2 mM 1,10-phenanthroline, 0.45 M CuSO4) and 10 μL of 58 mM mercaptothiolic acid were added and the mixture incubated for 15 min at room temperature. The reaction was stopped by addition of 20 μL 2,9-dimethyl-1,10-phenanthroline and SDS to a final concentration of 0.2%. The DNA was eluted, ethanol precipitated and resolved on a denaturing acrylamide gel.

Dimethyl sulphate footprinting in vitro

Reactions were carried out essentially as described by Sasse-Dwight & Gralla. Probe DNA (c. 500 cps) was dissolved in 100 μL of Tris–glycine binding buffer containing c. 5 μg BlaI. Samples without added protein were made up to the final volume with 1:1 phosphate-buffered saline and glycerol. After incubation at 37°C for 20 min, 6.7 μL of 150 mM dimethyl sulphate (DMS) was added and the mixture incubated for 5 min at 37°C. The reaction was stopped by the addition of 200 μL of cold stop buffer (3 M ammonium acetate, 1 M 2-mercaptoethanol, 20 mM EDTA, 250 mg/L yeast tRNA). The DNA was precipitated by addition of 600 μL of cold absolute ethanol and incubation of samples at −70°C for 30 min, followed by centrifugation. The pellets were washed in 70% (v/v) icedcold ethanol and dried. The DNA was then elaved by an alkaline method adapted from Strauss & Orkin. Deionized water was mixed with c. 500 cps of labelled DNA to a final volume of 50 μL. An equal volume of 20 mM NaH2PO4 was added, the reaction incubated at 90°C for 15 min and then transferred to ice. Twenty microlitres of 1 M NaOH was added and the reaction was incubated at 90°C for a further 30 min. The solution was neutralized with 20 μL of 1 M HCl and...
Table I. Strains and plasmids

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Resistances</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> JM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F [traΔ36 proAB+ LacIq lacZΔM15]</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> RN4220</td>
<td>NCTC8325 modified to accept <em>E. coli</em> DNA</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>E. coli</em> cloning vector</td>
<td>12</td>
</tr>
<tr>
<td>pHSG398</td>
<td>Cm</td>
<td><em>E. coli</em> cloning vector</td>
<td>14</td>
</tr>
<tr>
<td>pRB394</td>
<td>Ap, Cm, Kn</td>
<td>promoter probe vector</td>
<td>15</td>
</tr>
<tr>
<td>pE194</td>
<td>Em</td>
<td>small plasmid of <em>S. aureus</em></td>
<td>16</td>
</tr>
<tr>
<td>pT181MCS</td>
<td>Tc</td>
<td>small plasmid of <em>S. aureus</em></td>
<td>17</td>
</tr>
<tr>
<td>pOX490</td>
<td>Em, Ap, Cm</td>
<td>4.8 kb <em>HincII</em> fragment of Tn552 cloned into pE194/pHSG398 fusion plasmid</td>
<td>18</td>
</tr>
<tr>
<td>pOX491</td>
<td>Em, Ap, Cm</td>
<td>pOX490 digested with <em>Asp</em>718, blunted and religated</td>
<td>this paper</td>
</tr>
<tr>
<td>pOX617</td>
<td>Ap</td>
<td>minimum operator in pUC18</td>
<td>5</td>
</tr>
<tr>
<td>pOX618</td>
<td>Ap</td>
<td>operator in opposite orientation to pOX618</td>
<td>18</td>
</tr>
<tr>
<td>pOX637</td>
<td>Em, Cm</td>
<td>intact <em>blaRI</em> and <em>bla</em> operator in pE194/pHSG398</td>
<td>18</td>
</tr>
<tr>
<td>pSRC500</td>
<td>Ap</td>
<td>pUC18 with ΔZ operator</td>
<td>this paper</td>
</tr>
<tr>
<td>pSRC520</td>
<td>Ap, Cm, Kn</td>
<td>ΔZ operator in pRB394</td>
<td>this paper</td>
</tr>
<tr>
<td>pSRC522</td>
<td>Ap, Cm, Tc</td>
<td>pT181MCS in pSRC520</td>
<td>this paper</td>
</tr>
<tr>
<td>pSRC523</td>
<td>Ap, Cm, Kn</td>
<td>pSRC520 plus <em>bla</em> operon</td>
<td>this paper</td>
</tr>
<tr>
<td>pSRC521</td>
<td>Ap, Cm, Tc</td>
<td>pT181MCS in pSRC521</td>
<td>this paper</td>
</tr>
<tr>
<td>pSRC900</td>
<td>Em, Ap, Cm</td>
<td>derived from pOX491</td>
<td>this paper</td>
</tr>
<tr>
<td>pSRC920</td>
<td>Ap, Cm, Kn</td>
<td>operator cloned into pRB394</td>
<td>this paper</td>
</tr>
<tr>
<td>pSRC922</td>
<td>Ap, Cm, Tc</td>
<td>pT181MCS in pSRC920</td>
<td>this paper</td>
</tr>
<tr>
<td>pSRC921</td>
<td>Ap, Cm, Kn</td>
<td>pSRC920 plus <em>bla</em> operon</td>
<td>this paper</td>
</tr>
<tr>
<td>pSRC923</td>
<td>Ap, Cm, Tc</td>
<td>pT181MCS in pSRC921</td>
<td>this paper</td>
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</tbody>
</table>

<sup>a</sup>Ap, ampicillin; Cm, chloramphenicol; Kn, kanamycin; Em, erythromycin; Tc, tetracycline.
the DNA precipitated in 0.3 M sodium acetate and 2.5 vols of absolute ethanol. The DNA was collected after incubation at –70°C for 1 h by centrifugation at 12 000g for 30 min at 4°C, dried and resuspended in formamide loading buffer before electrophoresis in a denaturing polyacrylamide gel.

**DMS footprinting in vivo**

The method was based on that of Sasse-Dwight & Gralla.24 Cells to be footprinted were grown overnight in L-broth [16 g/L tryptone (Oxoid), 10 g/L yeast extract, 5 g/L NaCl] with appropriate antibiotic selection and the culture diluted 1:100 the next morning. At an A$_{675}$ of c. 0.6, 10 mL of culture were induced (where required) with 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid (CBAP) at 5 mg/L, and the induction was allowed to proceed for 10 min. Cultures were then treated with DMS at a final concentration of 4 mM for 5 min with shaking at 30°C. Following treatment, the cells were removed to –20°C,

![Diagram showing construction of plasmid pSRC501](image)

**Figure 1.** Construction of plasmid pSRC501.
Staphylococcus aureus β-lactamase operator

while DNA was isolated using the Wizard Plus SV DNA purification system. DNA samples were processed as in the alkaline denaturation primer extension method.24

Piperidine cleavage of DNA

Modified DNA was suspended in 100 μL of 1 M piperidine and placed at 90°C for 30 min. Butan-1-ol (1 mL) was added, the mixture vortexed and centrifuged at 12 000g for 5 min. The supernatant was removed and the DNA resuspended in 100 μL of 0.5% SDS. One millilitre of butan-1-ol was added and the DNA precipitated as before. The DNA was resuspended in formamide loading buffer for electrophoresis on a denaturing gel.

Construction of a plasmid to investigate the effect of the blaZ dyad on expression from the blaZ promoter

Oligonucleotides OT and OB were annealed, mixed with pUC18 that had been digested with SspI and Asp718, ligated and used to transform E. coli JM109 with ampicillin selection. The resultant plasmid (designated pSRC500) was digested with SspI, mixed with SspI-digested pOX617, ligated and used to transform E. coli JM109 to ApR. The plasmid with the 0.6 kb SspI fragment from pOX617 inserted into pSRC500 in the required orientation was designated pSRC501 (Figure 1). Figure 2 shows the sequences of the operator regions of the wild type and the clone with the Z-dyad deleted.

To measure the strength of the promoter and the effect of repressor on the mutant operator, the mutant operator was cloned into pRB394 at a site downstream of the promoterless cat gene. The mutant operator from pSRC501, subsequently referred to as the ΔZ operator, was prepared by digesting the plasmid with EcoRI and BamHI gel purified and mixed with EcoRI–BamHI-digested pRB394, ligated and used to transform E. coli JM109 to CmR. The resultant plasmid was designated pSRC520 (Figure 3). A plasmid similar to pSRC520 but without the Z-dyad deletion was needed for comparison. pRB394 and pOX617 were both digested with EcoRI and PstI, then ligated together and used to transform E. coli JM109 to CmR. A transformant that had the intact bla operator upstream of the cat gene was designated pSRC920.

To enable pSRC520 and pSRC920 to replicate in S. aureus, a staphylococcal plasmid had to be inserted. BglII-digested pSRC520 and pSRC920 were separately mixed with BamHI-digested pT181MCS,17 ligated and used to transform E. coli JM109 to CmR. The required plasmids were designated pSRC522 and pRSC922 and were separately transformed into a strain that does not contain the bla operon (S. aureus RN4220) with selection for TcR.
Construction of plasmids to investigate the effect of BlaI on CAT production

To construct a plasmid from which the bla genes could be easily removed as a unit, pOX491 was digested with AvaII and AccI, the ends ‘filled’ using Klenow polymerase and dNTPs and ligated. The ligation mix was used to transform E. coli JM109 to ApR and the resulting plasmid designated pSRC900. The bla genes next had to be inserted into the plasmids carrying the bla-derived promoters downstream of the cat reporter gene. The 3.6 kb Psrl fragment of pSRC900 containing the bla genes was purified and cloned into Psrl-digested pSRC920 and pSRC520. The ligation mixtures were transformed into E. coli JM109 with selection for CmR. The new plasmids were designated pSRC921 and pSRC521, respectively (Figure 4). To provide staphylococcal replicons, pT181MCS was ligated into the BglII site of both plasmids, to make pSRC923 and pSRC523, which were separately transformed into S. aureus RN4220 with selection for TcR.

Figure 3. Cloning of the minimal operator from pSRC501 into pRB394 to give pSRC520.
**Staphylococcus aureus β-lactamase operator**

**CAT assays**

Reaction conditions used were adapted from those of Tomizawa.\(^{26}\) Cells (c. 500 μL from a mid-log phase culture) were harvested and resuspended in 500 μL of 0.1 M Tris–HCl pH 8.0. Twenty microlitres of lysis buffer (0.1 M EDTA, 100 mM dithiothreitol, 50 mM Tris–HCl pH 8.0) and a drop of toluene were added and the mixture vortexed. The sample was incubated at 30°C for 30 min and 10 μL of the mixture were incubated with 100 μL of 100 μM chloramphenicol and 2 μL of [1-14C]acytlyl-coenzyme A (60 mCi/mmol) at 30°C for 10 min. The reaction was terminated by addition of 200 μL of toluene and the amount of radiolabelled acetylchloramphenicol partitioned into the toluene was measured in a Beckman LS 5000TD scintillation counter (Palo Alto, CA, USA).

**Results**

**DNase I protection assays for MecI and BlaI**

The precise sequences within the *bla* operator to which BlaI binds have been determined\(^5\) by DNase I footprinting.\(^{27}\) To investigate by the same technique whether or not there are differences between the sites on the *bla* operators that are bound by MecI and BlaI, the purified proteins were bound to the 135 bp DNA *bla* operator probe, which contains the inter *blaZ-blaR1* sequence. This probe was prepared from plasmid pOX617.\(^5\) The results show that the two footprints are indistinguishable (Figure 5). The sequence protected by the repressors is a little longer than the dyads but this could be due to steric hindrance between the repressor and DNase I. The assays for the other strand with

![Figure 4](image-url)  
*Figure 4.* Cloning of the *bla* operon from pSRC900 into pSRC520 or pSRC920 to give pSRC521 and pSRC921, respectively.
the probe derived from pOX618\textsuperscript{18} gave similar results (data not shown).

**Copper–phenanthroline footprinting**

The 135 bp DNA \textit{bla} operator probes used in the DNase I footprinting experiments were also employed in copper–phenanthroline footprinting. The nuclease activity of the copper–phenanthroline complex efficiently induces single-strand breaks in the DNA through the minor groove of the double helix,\textsuperscript{23} but is prevented from doing so when protein is bound. If the DNA is bent in the region between the sites bound to BlaI then the copper–phenanthroline is likely to react differently and may produce a different pattern of breaks. The results (Figure 6) show the prevention of breaks where BlaI binds but no alteration in the pattern of breaks in the region between the sites of BlaI binding. It is concluded that, since no change in the geometry of the inter-operator DNA was detected, there is probably no interaction between the repressor molecules bound at the two operator sites.

**Methylation protection assays in vivo**

The technique of \textit{in vivo} methylation protection was also used to discover changes in the geometry of inter-operator DNA on induction of the \textit{\beta}-lactamase by whole bacteria. DMS methylates the N-7 of guanine and the N-3 of adenine in DNA provided that the sites are not blocked by protein. The assay was set up with \textit{S. aureus} RN4220 (pOX491) which has an intact \textit{bla} operon, and \textit{S. aureus} RN4220 (pOX637) in which \textit{blaI} has been deleted. The cultures were either induced with the gratuitous inducer, CBAP or not induced. The results (Figure 7) show that induction exposes guanines at positions 57 and 88 on the bottom strand and 52 and 83 on the top strand. In the culture in which \textit{blaI} is deleted these bases are exposed to methylation whether or not the culture has been induced. It is noted that the guanine at 54 is not protected in the absence of inducer although it is at the middle of the Z-dyad. Since all four guanines that are affected are within the two operator regions, it is concluded that, \textit{in vivo}, the repressor BlaI binds to the same sites as it does in \textit{in vitro} DNase I footprinting experiments. Since there are no changes in band intensity in the inter-operator DNA region, it is also concluded that there is no apparent alteration of the geometry of the DNA in this region on repressor binding.

**Methylation protection assays in vitro**

To establish whether methylation protection was the same \textit{in vitro} as \textit{in vivo}, purified BlaI was mixed with purified pOX491 DNA and then treated with DMS, followed by piperidine cleavage and primer extension. The results (Figure 8) indicate that there is no significant difference between the two, confirming that the \textit{in vivo} result can be...
Figure 6. Copper–orthophenanthroline footprint of the bla operator. Lanes 1–3, top strand probe: lane 1, no protein; lane 2, BlaI present; lane 3, Maxam–Gilbert sequencing ladder for A and G. Lanes 4–6, bottom strand probe: lanes as for top strand. The sequences are shown at the sides with the dyads in bold.

Table II. Amount and specific activity of CAT produced by S. aureus RN4220 containing plasmids with wild-type or Z-dyad-deleted plasmids

<table>
<thead>
<tr>
<th>Test culture</th>
<th>Operator</th>
<th>cpm a</th>
<th>Protein mg/mL</th>
<th>Cpm/mg protein/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus RN4220 (pSRC922)</td>
<td>wild type</td>
<td>42012 ± 1392</td>
<td>23 ± 3</td>
<td>1826 ± 101</td>
</tr>
<tr>
<td>S. aureus RN4220 (pSRC522)</td>
<td>ΔZ</td>
<td>16727 ± 312</td>
<td>23 ± 3</td>
<td>727 ± 49</td>
</tr>
<tr>
<td>S. aureus RN4220</td>
<td>ΔZ</td>
<td>103 ± 10</td>
<td>24 ± 3</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td>66 ± 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Background (scintillation fluid only)</td>
<td></td>
<td>51 ± 15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are the mean of three independent experiments and standard errors are shown. 
a cpm, counts per minute.
attributed to the binding of BlaI. Enhancements of intensity of certain bases occurred, particularly at positions 55, 56, 62, 63 and 92 on the top strand. It would seem likely that this is due to hydrophobic regions of BlaI causing a ‘funnelling’ effect for DMS resulting in increased methylation of bases at these positions. A similar phenomenon caused by LacI has been reported.28

Deletion of the Z-dyad

The reason for the presence of two dyads: the Z-dyad nearest to blaZ and the RI-dyad nearest to blaRI (Figure 2), is not obvious. The RI-dyad includes elements of the promoters for both blaZ and blaRI and is presumably essential for expression of this operon. It was therefore decided to investigate the role of the Z-dyad by deleting it.

Assay for CAT in the absence of BlaI

To measure the strength of the wild-type promoter compared with that with the Z-dyad deleted, S. aureus RN4220 (pSRC922) and (pSRC522) were grown to mid-log phase (A600 of c. 0.5) at 30°C, harvested, lysed with toluene and the amount of CAT assayed. The results (Table II) indicate that the removal of the Z-dyad results in a significant decrease in the amount of CAT produced.

Figure 7. In vivo methylation protection assay of the bla operator region. Lanes 1–4 are bottom strand and lanes 5–8 are top strand. Lanes 1 and 5, intact bla operon induced with CBAP before treatment with DMS; lanes 2 and 6, intact bla operator uninduced; lanes 3, 4, 7 and 8 are with blaI deleted from the operon so that there is no repressor. Lanes 3 and 7, DMS added after induction with CBAP. The positions of the Gs that are protected from methylation by the repressor are marked by arrows on the sequence.
Staphylococcus aureus β-lactamase operator

Assay for CAT in the presence of BlaI

*S. aureus* RN4220 (pSRC923) and (pSRC523) were grown to mid-log phase (A_{680} c. 0.5) at 30°C. CBAP (5 mg/L) was added to half the cultures and samples taken every minute for 10 min. The samples were lysed with toluene and the amount of CAT present assayed. The production of CAT is induced by CBAP (Figure 9). There was a similar lag of about 4 min in both induced cultures before CAT was detected and more CAT was produced when the Z-dyad was present (i.e. with pSRC923).

Discussion

The DNase I footprints for MecI and BlaI bound to the *bla* operator are indistinguishable (Figure 5). This provides the explanation for the ability of MecI to repress β-lactamase

Figure 8. Comparison of the methylation protection assay of the operator region of the *bla* operon top strand *in vivo* and *in vitro*. Lanes 1–4, *in vivo* assay; lanes 1 and 2, intact *bla* operon; lanes 3 and 4, *blaI* deleted therefore no repressor; lanes 1 and 3, induced with CBAP before treatment with DMS; lanes 2 and 4, not induced. Lanes 5 and 6, *in vitro* assay with intact operator; lane 5, no repressor; lane 6, repressor added. The bases 55, 56, 62, 63 and 92, which show enhancement, are labelled.
A fied form of BlaI, that it may bind differently the very high apparent dissociation constant for the puri-
was only detected operator regions (O1 and O3) in the Lac operon of actions; indeed the simultaneous binding of LacI to two proved entirely reliable for studying protein–DNA inter-
binding. The use of this experimental technique has not mobility shift assays of repressor–DNA complexes to study proved entirely reliable for studying protein–DNA inter-
conclusion that there is an absence of bending or looping DMS experiments produced negative results so that the interaction between repressor molecules bound to the two formed protein multimers when bound to a large mec operator molecule opening the possibility of protein–protein interactions between repressor molecules bound to the two separate dyads. Both the copper–phenanthroline and the DMS experiments produced negative results so that the conclusion that there is an absence of bending or looping must be tentative.

Previous studies have relied upon electrophoretic mobility shift assays of repressor–DNA complexes to study binding. The use of this experimental technique has not proved entirely reliable for studying protein–DNA interactions; indeed the simultaneous binding of LacI to two operator regions (O1 and O3) in the Lac operon of E. coli, was only detected in vivo on negatively supercoiled DNA molecules. Hence it seemed possible, especially given the very high apparent dissociation constant for the purified form of BlaI, that it may bind differently in vivo with a different ionic environment and a supercoiled DNA molecule. The results obtained with the in vivo system (Figure 7, lanes 5 and 6) are not significantly different from those obtained with the in vitro system (Figure 8, lanes 5 and 6). Thus it is concluded that the in vitro system reflects the situation found within the bacteria. On the bottom strand the guanine at position 54 (Figure 7, lanes 1 and 2) is not protected by the bound repressor although it is in the middle of the Z-dyad. This is also seen with the equivalent in vitro experiment (data not shown) so is not a specific feature of the in vivo position. One possibility is that a monomer binds to each half of the Z-dyad in such a way as to allow space for the methylating agent to attack the guanine.

The precise site for the initiation of blaZ mRNA has been determined by primer extension analysis (S. R. Clarke & K. G. H. Dyke, unpublished) and is indicated in Figure 2. This allowed the removal of the Z-dyad from operator DNA without loss of the wild-type transcription start site. The Z-dyad plus a further 27 bp was deleted and the resulting DNA was fused to the cat reporter gene precisely as for the construct including the wild-type operator/p promoter region. The deletion resulted in decreased synthesis of CAT (Table II). This could be explained by lower mRNA synthesis, greater instability of the mRNA or by poorer translation efficiency. One possibility is that the stem–loop structure that may be present when the Z-dyad is transcribed stabilizes the mRNA. In the absence of the stem–loop, RNase E or some similar RNase would hydrolise the mRNA. It is known that the half-life of mRNAs can be decreased by the removal of 5′ stem–loop structures at the 5′ untranslated region of transcripts. It is also possible that the presence of a stem–loop structure in the mRNA near to the ribosome binding site could increase the efficiency of initiation of translation. The presence of the genes specifying BlaI and BlaRI on the same plasmid resulted in repression of the synthesis of CAT. This repression was relieved about 4 min after addition of an inducer (Figure 9) and the proportion of the cell protein that was CAT was about three times higher in the presence of the Z-dyad plus an additional 27 bp untranslated section. This agrees with the results obtained in the absence of BlaI (Table II) and suggests that the system is fully induced. Further experiments are necessary to determine whether or not it is the Z-dyad that has this effect rather than the downstream 27 bp and to discover, for example, whether or not BlaI can bind to the upstream section of the blaZ mRNA.

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Staphylococcus aureus \(\beta\)-lactamase operator

References


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