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Repressor gene, *blaI*, for *Bacillus licheniformis* 749 β-lactamase

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The repressor gene, *blaI*, for the β-lactamase of *Bacillus licheniformis* 749 was functional when cloned in *Escherichia coli*, but addition of a β-lactam did not lead to induction. One plasmid contained fragments from the inducible strain (source of repressor), the other carried fragments from the *blaI*⁻ mutant 749/C (target). *blaI* lies just 5′ to the promoter for the structural gene, *blaP*, and the target is the promoter region between the two genes. Interaction with both promoters seemed necessary for full repression. BlaI is a hydrophilic protein (M_r 15036) with the some structural similarities to repressors from Gram-negative bacteria.

β-Lactamase; Repressor; Regulation; (*Bacillus licheniformis, Escherichia coli*)

1. INTRODUCTION

The induction of β-lactamase synthesis in *Bacillus licheniformis* 749 is delayed and protracted even though the half-life of the specific mRNA is short [1–4]. These unusual features made it desirable to identify and examine the components of the regulatory system. The structural gene *blaP* (previously termed *penP*, see [3]) and its promoter/operator regions have been cloned and sequenced [5]. A negative regulatory element (*blaI*) is 90% linked to the structural gene and is encoded along with *blaP* on a 4.2-kbp *Eco*RI fragment [6,7]. The constitutive mutant 749/C does not form an active repressor. A second regulatory element is 50% linked to *blaP*, and a possible additional element is unlinked. *blaI* was tentatively positioned 3′ to *blaP* on a polycistronic message [8,9]. However, McLaughlin et al. [10] reported that the in vitro transcript of the *blaP* region was only 1.2 kb and terminated about 60 b beyond the 3′-end of the open reading frame. The major *blaP* transcript produced in vivo was also 1.2 kb, but 2 to 3% of the total message extended to about 3 kb [3,4]. The percentage of large *blaP* mRNA did not change significantly during induction, nor did it differ in the regulatory mutants.

Our observation that the repressor is functional when cloned in *Escherichia coli*, even though there is no induction following the addition of the β-lactam, enabled us to locate the gene and determine some of the properties of the repressor. One plasmid carried the 4.2-kbp *Eco*RI fragment from mutant 749/C as the target, while the other contained fragments from the inducible strain 749. The *blaI* locus of strain 749 has been identified and sequenced, and the nature of the deduced protein product has been examined.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E. coli HB101 was the host for the cotransformation experiments and strain RR1 for the other studies. *B. licheniformis* strains 749 and 9945A are inducible for high level β-lactamase production; mutant 749/C is constitutive. The 4.2-kbp *Eco*RI fragment (see fig.1) from 9945A containing *blaP* and *blaI* was obtained from pTTE21 [6]. The 749/C fragments used to construct pRWN132 and pRWN134 were from pRW83 [11].

Most of the vectors were pBR322 derivatives. PBR322 was made AP^S by removing 4 bp (pRW4) or about 500 bp (pRWN9) at the *Pst*I site within the Ap gene. The 4.2-kbp *Eco*RI fragments from 9945A and 749/C were ligated into the corresponding site of pRW4 to produce pRWN1 and pRW20, respectively. The 2.3-kbp *Pvu*II fragments from 749 and 9945A were ligated into pRWN9, producing pRWN21 and pRWN10, respectively.

Vector pRWN125 was designed to carry the 5′ 1.6-kbp *Eco*RI-*Pst*I or 3′ 2.3-kbp *Bgl*II-*Eco*RI fragment from 749. A translational stop codon had been introduced at the *Hin*dIII site within the *lacZ* gene of pUC9, resulting in pRWS17 [3]. The 0.424-kbp *Hae*II fragment from pRWS17 (containing the *lacZ* promoter and the stop codon) was blunt-ended and ligated into the (blunt-ended) *Hin*dIII site of pACYC184, resulting in pRWN125. This allows use of the *lacZ* promoter without the potential problem of a fusion protein.

All fragments were inserted in both orientations and, where appropriate, cultures were grown with and without a β-lactam or isopropylthiogalactoside as an inducer. In no instance was the production of β-lactamase affected.

2.2. *Growth media*

LB medium and agar plates contained 5 or 10 g NaCl/l. Antibiotics were added to 20 μg/ml [ampicillin (Ap), tetracycline (Tc), and chloramphenicol (CM)].

2.3. *DNA manipulations*

Plasmids were prepared as in [11], and the small scale isolation procedure of Holmes and Quigley [12] was used to identify colonies containing the appropriate plasmids. Restriction enzymes, *Bal*31 and T_4 DNA ligase were purchased from Bethesda Research Laboratories, Inc. and New England Biolabs, Inc. and used as recommended by the manufacturers.

2.4. *Cloning of* blaP *from* B. licheniformis *749*

B. licheniformis 749 chromosomal DNA (*blaP*$^+$, *blaI*$^+$) was digested with *Eco*RI and fragments of 2.3 to 6.6 kbp were ligated into the *Eco*RI site of pRWN9. The resulting plasmids were transformed into *E. coli* RR1 cells made competent by the calcium chloride shock procedure [13]. A plasmid from one of the Ap^R colonies was isolated as pRWN101.

2.5. *Production of β-lactamase*

Overnight cultures were used to inoculate LB medium (containing the appropriate antibiotics) at 35–37°C. At indicated times the cells were collected, suspended in TDC buffer (0.1 M KH_2PO_4 and 0.1% taurodeoxycholic acid, pH 7) and sonicated for 20 s on ice. Liquid samples were assayed by the method of Sargent [14]. Colonies were tested for β-lactamase activity using the semi-quantitative iodine/polyvinyl alcohol plate assay [8]. One unit of β-lactamase hydrolyzes 1 μmol benzyl penicillin in 1 h at 30°C.

3. RESULTS

3.1. *Preliminary efforts to locate* blaI

We inserted 4.2-kbp *Eco*RI fragments from the inducible strains 749 and 9945A and from the constitutive mutant 749/C into the *Eco*RI site of pRWN9, and cloned the plasmids in *E. coli* RR1. With a clone containing fragments from the inducible strains (e.g. pRWN1), production of β-lactamase was repressed (20 to 80 U/ml) and there was no induction following addition of ampicillin. When the segment from 749/C was present (pRW20), yields of 10000–20000 U/ml were obtained. Cutting the 4.2-kbp *Eco*RI fragment from strain 9945A down to the 2.3-kbp *Pvu*II segment (plasmids pRWN21 and pRWN10) eliminated the repression (not shown).

3.2. *Location of the repressor gene*

For definitive location of *blaI* a plasmid containing the 749/C *blaP* gene (usually the 4.2-kbp *Eco*RI segment from pRW20) was cotransformed

into *E. coli* HB101 along with a plasmid carrying portions of the *Eco*RI fragment from an inducible strain. Initially, *blaP* from strain 749, on the 4.2-kbp *Eco*RI fragment, was cloned into the *Eco*RI site of pRWN9 to produce pRWN101, and the corresponding 749/C fragment was inserted into pRWN9 to yield pRWN121. Then the 1.6-kbp *Eco*RI-*Pst*I and 2.3-kbp *Bgl*II-*Eco*RI fragments from pRWN101 were blunt-ended and inserted into the (blunt-ended) *Pst*I site of pRWN125 to produce pRWN127 and pRWN129, respectively. Finally, each plasmid was cotransformed with pRWN121 into *E. coli* HB101, and Ap^R, Cm^R colonies were assayed for β-lactamase production. β-Lactamase production in cells carrying the 749/C *blaP* gene (pRWN121) and the 5'-fragment from strain 749 (pRWN127) was reduced more than 70-fold as compared to cells containing pRWN121 and the 3'-fragment from strain 749 (pRWN129) (fig.1A).

3.3. *Target site for the repressor*

Further cotransformation experiments refined the location of *blaI* and indicated the size of its target. There were large amounts of 749/C DNA on both sides of *blaP* in the target used for the experiments in fig.1A, and it was conceivable that the repressor was 3' to *blaP* with a corepressor located 5', or vice versa. To rule this out, the 1.2-kbp *Eco*RI*-*Bcl*I fragment was tested as the target (fig.1B). The 3' *Bcl*I site is close to the *blaP* translational termination codon. The fragment on pRWN132 was transformed along with pRWN127 which contains the 749 1.6-kbp *Eco*RI-*Pst*I fragment, the apparent source of repressor. β-Lactamase production (1320 U/ml) was reduced as

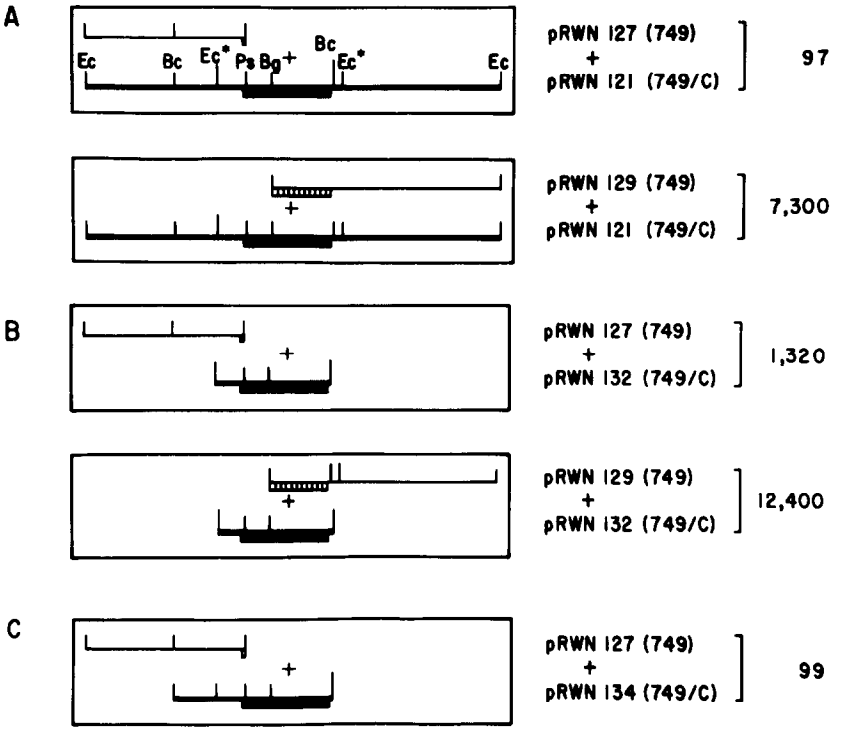

Fig.1. Location of the repressor gene *blaI* by complementation experiments in *E. coli* HB101. (A) Cells were cotransformed with pRWN121 containing the 749/C *blaP* region on the 4.2-kbp *Eco*RI fragment and with either the 5'1.6-kbp *Eco*RI-*Pst*I fragment on pRWN127 or the 3'2.3-kbp *Bgl*II-*Eco*RI fragment from 749 on pRWN129. (B) The 1.2-kbp *Eco*RI*-*Bcl*I fragment on pRWN132 along with pRWN129. (C) The 1.6-kbp *Bcl*I-*Bcl*I fragment on pRWN134 along with pRWN127. Solid bars and thick lanes, DNA from mutant 749/C); thin lines, DNA from strains 749 or 9945A. Values on the right, units of β-lactamase/ml at stationary phase. Abbreviations for restriction nuclease sites: Av, *Ava*I; Bc, *Bcl*I; Bg, *Bgl*II; Ec, *Eco*RI; Ec*, *Eco*RI*; Ps, *Pst*I; Pv, *Pvu*II; Ss, *Sst*I.

compared to cultures lacking the repressor plasmid but only by a factor of 6–10. As expected, the 3′-fragment from the inducible strain 749 (pRWN129) had no effect on expression.

Finally, the 1.6-kbp *BclI-BclI* fragment was tested as the target (fig.1C). This construction (pRWN134) includes 680 bp upstream from the start codon for *blaP*, which should be adequate to retain the entire target site for the repressor as well as any essential secondary DNA structure. A culture carrying only pRWN134 produced 55-fold more β-lactamase than a parallel culture which also contained a source of repressor (pRWN127). Thus a degree of repression characteristic of the wild-type strain 749 was obtained.

3.4. *Limits of* blaI

Our preliminary tests had indicated that *blaI* overlaps a *PvuII* site, presumably the one 5′ to *blaP*. To define the 5′-limit for this gene, pRWN101 (749 DNA) was linearized at the single *SstI* site (fig.1) which is 68 bp downstream of *Sau*3A and digested for varying periods with *Bal*31 nuclease. The digested samples were self-ligated and transformed into *E. coli* RR1. Plasmids containing deletions extending toward *blaI* up to or beyond the *PvuII* site produced maximum enzyme levels. Plasmids with deletions extending past the *Sau*3A site, but not as far as *PvuII* were low producers and must still contain an active repressor (not shown).

3.5. *Sequence of* B. licheniformis *749* blaI

The DNA sequence for both strands of the 639-bp *Sau*3A-*Eco*RI* fragment was determined by the dideoxy nucleotide termination procedure of Sanger et al. [15] using [α-^{35}S]dATP. Nucleotides 103 and 486 constitute an open reading frame (ORF) of 384 bp that encodes a protein of 128 amino acid residues (fig.2). A potential Shine-Dalgarno sequence (GTATAGGATG) is located 7 bp upstream from the initiation codon, and is preceded by a good promoter region (−35: TTGACT; −10: TATAAT). This protein terminates between the *PvuII* and *Sau*3A restriction sites, as predicted for BlaI. In addition, the *blaI* from the constitutive mutant 749/C contains an amber mutation in codon 32 (Grossman, M.J. and Lampen, J.O., unpublished).

A second ORF of undetermined length begins (nucleotide 491) after a gap of only one nucleotide and extends beyond the *Sau*3A site. Within the 3′-region of *blaI* there is a good Shine-Dalgarno sequence (AGAAAGAAG, nucleotides 475–484) that is 7 bp upstream from the ATG initiation codon. As there is no typical promoter sequence in

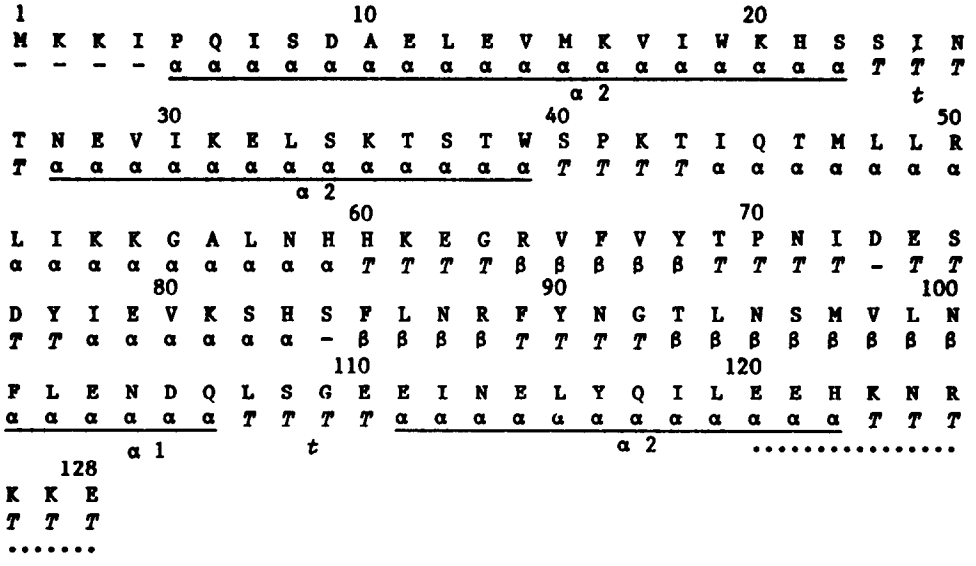

Fig.2. Probable secondary structure of the repressor for β-lactamase, predicted according to the principles of Chou and Fasman [1]. α, alpha-helical; T, β-turn; β, beta sheet; ....., charged COOH-terminal sequence. Two helix-turn-helix structures are indicated.

this region of *blaI*, the second ORF is probably transcribed along with *blaI* on a polycistronic message.

4. DISCUSSION

We have located the repressor gene, *blaI*, for the β-lactamase of *B. licheniformis* 749 within the 639-bp *Sau*3A-*Eco*RI* fragment that is directly 5′ to the structural gene, *blaP*. The sequence is identical to the gene from strain 9945A [16]. BlaI, the repressor, is functional in *trans* when cloned in *E. coli*. One plasmid carried by the target *bla* region from mutant 749/C, which does not produce an active repressor. The second plasmid contained DNA 5′ to the *blaP* gene from the inducible strain 749. The target was shown to lie in the region between *blaI* and *blaP* where their diverging promoters are located. No DNA 3′ to *blaP* was required for repression. With target fragments that contained only the *blaP* promoter, repression was partial, but when both promoters were included (1.6 kbP *Bcl*I-*Bcl*I sequence) full repression was achieved. We propose that the repressor can interact with both promoters, and probably must do so in order to produce full repression.

Sequencing the DNA 5′ to *blaP* showed that *blaI* is transcribed divergently from *blaP* and on the opposite strand. Closely following *blaI* there is another open reading frame which is probably translated along with *blaI* from a polycistronic message (fig.2).

The *blaI* product is a hydrophilic protein of M_r 15036, with a highly charged COOH-terminal region (nine residues). Prediction of the secondary structure of the protein (fig.2) by the principles of Chou and Fasman [17] indicated the existence of two helix-turn-helix segments of the type reported for most repressors [18,19]. The charged COOH-terminal region should form a large β-turn. Thus the *blaI* repressor exhibits the general characteristics of other repressors; however, the sequences of the helix-turn-helix regions do not show convincing homology to the consensus sequences reported by others [18,20]. Direct examination of mutant or modified forms of the protein will be needed to determine which structures interact with the promoter region. The two plasmid system described here should be valuable for assessing the effectiveness of various modified forms.

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