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Differential transcription of the *bla* regulatory region during induction of β-lactamase in *Bacillus licheniformis*

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Induction of β-lactamase (*blaP*) in *Bacillus licheniformis* involves the regulatory genes *blaI* (repressor), *blaR1* (coinducer) and R2 (function unknown). Transcription of the *bla* genes during induction was followed by Northern hybridization. In the first 30 min 2.3-kb transcripts encoding *blaI* and *blaR1* were present. Subsequently, *blaP* mRNA and short transcripts encoding only *blaI* accumulated and reached a peak at 1 h. All *bla* transcripts turn over rapidly. Active repressor is not required for the burst of *blaI-blaR1* mRNA. The production of *blaI-blaR1* mRNA, and thus of BlaR1, is probably controlled both at initiation of transcription and at a later step in its synthesis and degradation.

β-Lactamase; Regulation; Repressor gene; Transcription; (*Bacillus licheniformis*)

1. INTRODUCTION

The β-lactamase of *Bacillus licheniformis* has received considerable attention as a model of regulation in Gram-positive microorganisms [1,2]. Upon induction, the rate of synthesis of β-lactamase and of *blaP* mRNA increases slowly to a maximum at about 1 h, then decreases over the next hour, and remains several-fold that of uninduced cultures for 2 or 3 more hours [3].

Expression of *blaP* involves at least three regulatory loci: a repressor gene (*blaI*), 90% linked to *blaP* by transformation; a coinducer (R1), 50% linked; and R2, of unknown function, which is unlinked to *blaP* [4]. *blaI* is located 5′ to *blaP* [5,6] and is transcribed divergently from *blaP* and on the opposite DNA strand. The gene product is a 15 kDa hydrophilic protein. It binds specifically to the region between *blaI* and *blaP* which contains the two promoters [7]. Recently the positive effector R1 has been shown to lie immediately following *blaI* on a polycistronic message [8,9]. This gene (*blaR1*) codes for a 68 kDa hydrophobic protein which is essential for induction of β-lactamase in *B. licheniformis* and *B. subtilis*. The COOH-terminal region of BlaR1 probably lies on the outer surface of the cell membrane, and contains a sequence characteristic of penicillin-binding proteins [8]. We describe here the transcripts from the *blaI* promoter and the pattern of transcription of the three *bla* genes during the course of induction. A map of the *bla* region and the various transcripts is given in fig.1. The major *blaP* mRNA in vivo [3] and in vitro [10] is the 1.2-kb transcript. No role for the small amounts of long *blaP* mRNA has been identified.

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2. MATERIALS AND METHODS

2.1. *Strains and plasmids*

B. licheniformis 749 is inducible for synthesis of β-lactamase, and mutant 749/C is constitutive. pRSW17 (Ap^r) is a pUC9 derivative [11] carrying *blaI* in the multiple cloning site on a 1.4-kb *Eco*RI-*Nci*I fragment subcloned from pRWN1 [3]. The host was *Escherichia coli* GM215.

2.2. *Procedures*

The growth and induction of cultures, assay of β-lactamase, purification of plasmids and DNA fragments, radiolabeling of

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various probes, RNA isolation, S1 nuclease assay, and Northern analysis have been described in detail [3].

2.3. *Primer extension assay*

The oligonucleotide primer 5'-GGTATTTTTTTCATTTT-AATCATCC-3' was prepared using phosphoramidite chemistry [12,13]. To anneal this primer to *blaI* mRNA, 5 μg of RNA and 0.75 pmol of ^{32}P-end-labeled primer (about a 15-fold molar excess) were added to 3.2 μl of 6.25 × reverse transcriptase buffer (0.3 M Tris-HCl (pH 8.3), 0.375 M NaCl, 37.5 mM $MgCl_2$, 2.5 mM dithiothreitol), and H_2O to give a total volume of 18 μl. The reactions were brought to 100°C and allowed to cool to room temperature over 2.5 h. Then 0.8 μl of 25 mM dNTPs and 100 units (1 μl) of Molony murine leukemia virus reverse transcriptase were added, and the tubes were incubated at 42°C for 0.5 h. Next, EDTA was added to 24 mM, as well as yeast tRNA (20 μg), and the reaction mixtures were extracted with phenol/chloroform (50/50). Nucleic acids were precipitated, washed, dried and subjected to electrophoresis on sequencing gels containing 16% acrylamide in 8 M urea as described [14].

2.4. *DNA sequencing*

The 0.2-kb *Sau*3A-*Nci*I DNA fragment includes the promoter region of *blaI* (fig.2) and part of the structural gene. The sizing ladder for the primer extension analysis was generated from this fragment by annealing the ^{32}P-end-labeled primer to the denatured DNA fragment and extending the primer with the Klenow fragment of DNA polymerase I in the presence of dideoxynucleotide triphosphates. Primer (90 ng) and template (65 ng) were prepared for sequencing in the manner described by Zagursky et al. [15] for supercoiled plasmid DNA. Sequencing reactions were as described [16] except that no radiolabeled dNTPs were used in the reactions.

3. RESULTS

3.1. *Transcription of the* blaI *(repressor) region specifically induced by cephalosporin C*

To determine the pattern of mRNA transcribed from the *blaI* region, total RNA was prepared from cultures of wild-type *B. licheniformis* 749 which were serially sampled during induction. Total RNA samples were subjected to Northern analysis by hybridization to a 0.25-kb *Sau*3A-*Sau*3A DNA fragment which lies completely within the *blaI* gene and is separated from the *blaP* gene by 0.44 kb. Several *blaI* mRNAs were specifically induced by cephalosporin C (fig.3). The largest transcript was 2.3 kb, and on longer exposures a small amount of 1.9-kb transcript could be detected. The 2.3-kb mRNA was barely detectable before induction. By 0.5 h after induction it had attained its maximum level and over the next 0.5 h it fell by two thirds. At 1.5 h it was barely detectable. In addition, two small, apparently discrete transcripts were observed with sizes of 0.95 and 0.75 kb. These transcripts also were low before induction, rose only slowly during the first 30 min, and reached a maximum at 1.0 h. During the next 0.5 h, their levels dropped to roughly 10% of maximum and decreased slowly thereafter.

We have previously reported that the *blaP* transcripts induced by cephalosporin C turn over rapidly, with half-lives of about 2 min [3]. Similar experiments using probes homologous to the *blaI* region, showed that the mRNAs initiated at *blaIp* also undergo rapid breakdown with comparable half-lives (not shown).

3.2. *Start site of* blaI *mRNAs*

To determine whether all *blaI* mRNAs were transcribed from the same strand, the Northern blot shown in fig.3 was subjected to single-

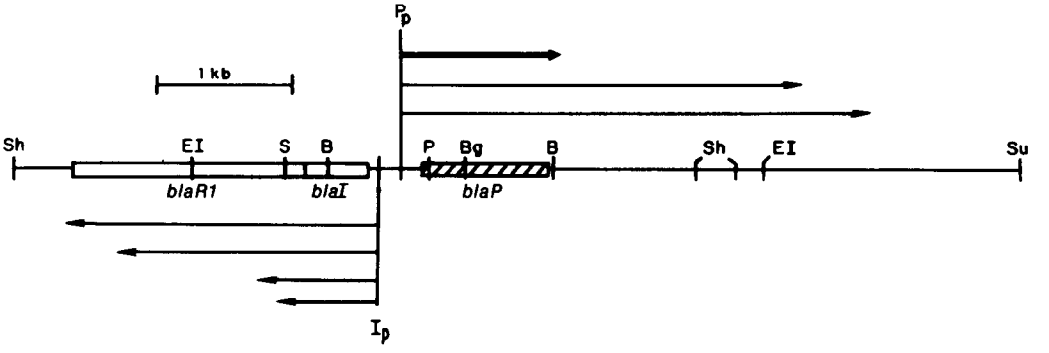

Fig.1. Physical organization of the cephalosporin C induced *bla* region in *B. licheniformis*. The thick crosshatched area is the *blaP* gene, the two open boxes are *blaR1* and *blaI*. The transcripts initiated from the *blaP* promoter (P_P) or the *blaI* promoter (I_p) are indicated by arrows showing the direction and length of transcription. The heavy arrow indicates the major *blaP* mRNA (1.2 kb). Restriction endonucleases are abbreviated as follows: B, *Bcl*I; Bg, *Bgl*II; EI, *Eco*RI; P, *Pst*I; S, *Sst*I; Sh, *Sph*I; and Su, *Stu*I.

Fig.2. DNA sequence of the intergenic region of the cephalosporin C induced *bla* region from *B. licheniformis*. The DNA sequence has been reported [5,17]. Direction of transcription of the *blaP* and *blaI* genes and the sites of initiation are indicated by bold arrows. The −10 and −35 regions are underlined. The I_p promoter should be read on the bottom strand from right to left. The direct repeat sequence is boxed. Inverted repeats are indicated by dashed or solid lines with arrowheads.

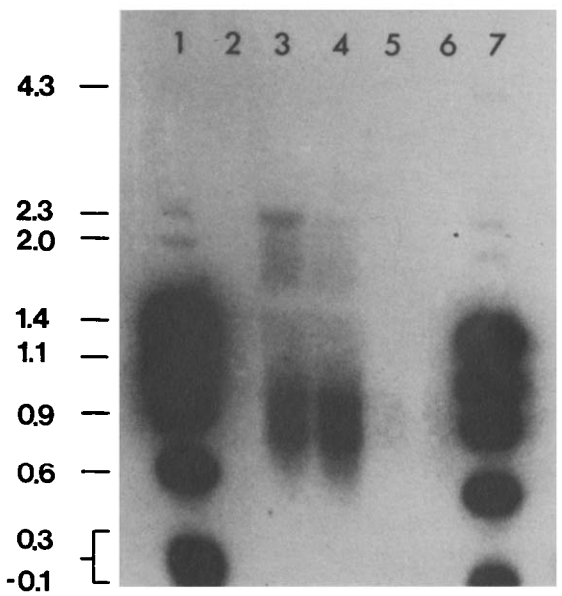

Fig.3. Induction by cephalosporin C of transcription in the *blaI* region of *B. licheniformis*. Northern analysis of total RNA (10 μg) prepared from cells grown at 37°C either before adding cephalosporin C (lane 2), or 0.5 h (lane 3), 1.0 h (lane 4), 1.5 h (lane 5) and 2.0 h (lane 6), respectively, after the addition. Samples were hybridized to the 0.25-kb *Sau*3A-*Sau*3A *blaI*-specific DNA probe. Markers (lanes 1 and 7) consisted of glyoxylated λ-*Hin*dIII- and φX174 *Hae*III-restricted DNA of the indicated lengths (kb).

stranded probing. All the RNAs were derived from the strand encoding *blaI* and *blaRI*. S1 mapping of the 5′-end of these transcripts showed a clustered start site in a hexanucleotide region (Salerno, A.J., PhD Thesis, Rutgers University, 1985). To determine the site more accurately, *blaI* mRNA was subjected to primer extension analysis using a synthetic oligonucleotide that anneals to a site separated by 16 bases from the aforementioned region. When the extended primer was sized, RNA from induced *B. licheniformis* strains 749 and 9945A gave rise to a DNA fragment 43 bases in length (fig.4, lanes 1 and 2). Reactions containing RNA from uninduced cultures of *B. licheniformis* (lane 3), or containing no *B. licheniformis* RNA (lanes 4 and 5), gave no such DNA fragment. Thus *blaI* mRNA is initiated at an A that is located as shown in fig.2. Appropriately positioned upstream of this A is a promoter for the σ^{43}-containing vegetative form of RNA polymerase holoenzyme.

3.3. *Level of* blaI *mRNA in the absence of a functional repressor*

B. licheniformis 749/C constitutively produces *blaP* mRNA and β-lactamase [3]. This mutant phenotype is presumably due to the formation of a truncated inactive repressor [7]. The presence of similar DNA sequences in *blaPp* and *blaIp* (fig.2) is indicative of coordinate regulation. To determine whether active repressor is required for the burst of *blaI-blaR1* mRNA formation following addition of the inducer, cultures of *B. licheniformis* 749/C were grown in the presence and absence of inducer, cephalosporin C. RNA samples from these cultures were subjected to Northern analysis using a *blaI* specific probe. *blaI* transcripts were at a maximal level prior to the addition of inducer to half of an exponential phase culture (fig.5A, lane 4). Densitometry of the autoradiograms revealed that by 1.5 h later the level of total *blaI* mRNA had fallen, in both induced and control cultures, to about 10% of the preinduction value. The addition of cephalosporin C had, however, caused a striking increase at 0.5 h in the amount of 2.3-kb *blaI-blaR1* mRNA (lane 5) as compared with the control culture (lane 3) or the preinduction sample (lane 4). The total amount of *blaI* transcripts had, however, declined slightly over this period. Thus, the increase in large *blaI-blaR1* mRNA was not the result of increased transcription from the *blaI* pro-

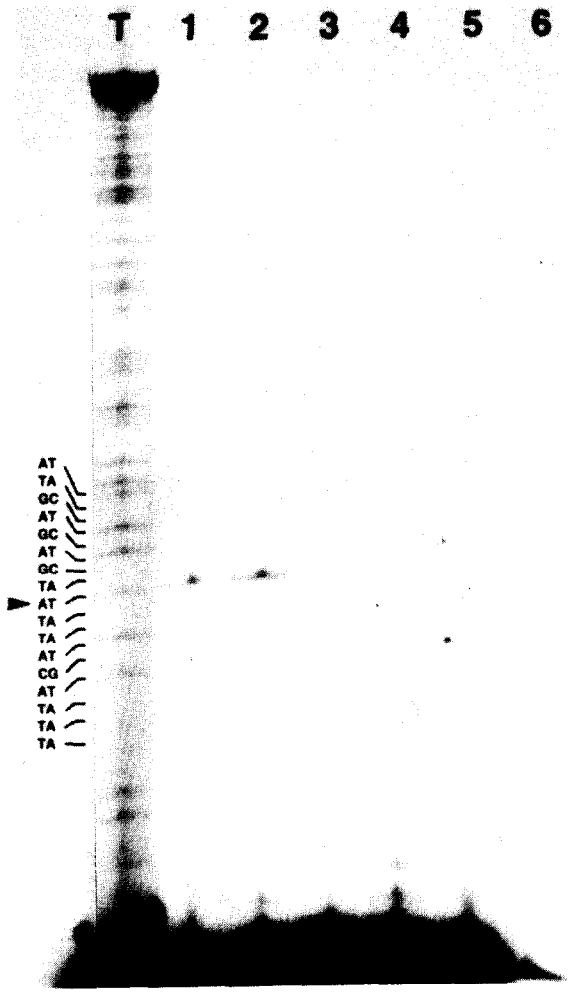

Fig.4. Primer extension analysis of the initiation site for *blaI* mRNA. Radiolabeled oligonucleotide primer was annealed to samples of total RNA from cultures of *B. licheniformis* strains 9945A (lane 1) or 749 (lane 2) grown at 37°C and induced with cephalosporin C, or from an uninduced culture of *B. licheniformis* strain 749 (lane 3). Primer was then extended with Molony murine leukemia virus reverse transcriptase and electrophoresed in parallel to a sizing ladder (lane T) which was generated from the same primer. Controls include a complete reaction containing yeast tRNA (lane 4) in lieu of *B. licheniformis* RNA, a reaction containing only primer and Molony murine leukemia virus reverse transcriptase (lane 5), and a reaction containing primer alone (lane 6). The transcription initiation site is indicated by an arrowhead.

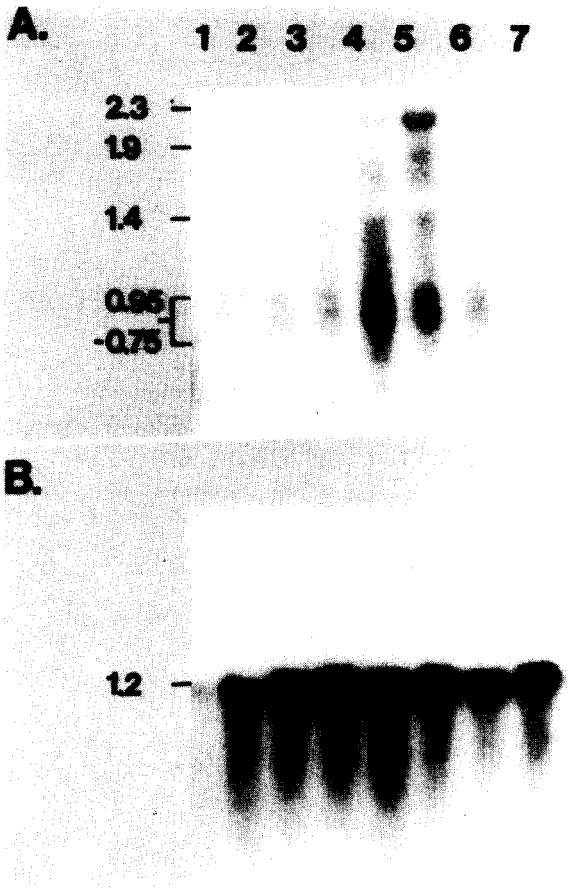

Fig.5. Comparison of transcripts produced by *B. licheniformis* 749/C in presence and absence of inducer. Total RNA (10 μg) was prepared from cells grown at 37°C. Following sampling (lane 4), the culture was divided in half, and one aliquot was induced with cephalosporin C (lanes 5–7). The other aliquot served as an uninduced control (lanes 1–3). Both cultures were sampled at 0.5 h (lanes 3 and 5), 1.0 h (lanes 2 and 6), and 1.5 h (lanes 1 and 7) after the addition. (A) Hybridized with a 0.45-kb *Bcl*I-*Nci*I *blaI* specific probe. (B) Hybridized with a 0.6-kb *Pst*I-*Cla*I probe specific for *blaP* DNA.

moter, but rather of a change in the proportion of large *blaI-blaR1* transcripts following the addition of the inducer. In contrast, the level of *blaP* mRNA, as detected with the 0.6-kb *Pst*I-*Cla*I *blaP* specific probe [3], showed only a gradual decrease of less than 2-fold over the 1.5 h of growth (fig.5B). By 2 to 2.5 h after induction, the level of *blaP* mRNA had dropped about 10-fold with or without inducer (not shown). Essentially equal levels of β-lactamase were produced by the uninduced (2.2×10^3 U/ml, 2.0×10^3 U/mg dry cell wt) and induced (2.5×10^3 U/ml, 2.2×10^3 U/mg dry cell wt) cultures of 749/C during the 1.5 h of growth.

4. DISCUSSION

The transcription map for the *bla* region of *B. licheniformis* as determined from this work shows that large and small leftward *blaI* transcripts are generated from one promoter, while three rightward *blaP* transcripts are produced from a second promoter (fig.1). The promoters are coordinately controlled since transcription from each is induced by cephalosporin C, constitutive transcription from each occurs in the *blaI*$^-$ mutant 749/C, and similar putative regulatory sites are present [5,7]. Furthermore, purified repressor specifically binds DNA fragments containing these sites [7]. The *blaI* gene is autoregulatory since *blaI* mRNA is synthesized at a high rate in the absence of an active repressor.

Two proteins involved in the induction of β-lactamase have recently been sequenced and characterized. *blaI* encodes a soluble repressor of 128 amino acid residues. Following *blaI* after a single nucleotide gap is *blaR1* which encodes a 601 residue protein [8,9]. The corresponding polycistronic mRNA must include 416 bases for *blaI* and its promoter region plus *blaR1* which would terminate at 2219–2221. Our experiments have shown that two distinct sets of leftward transcripts are produced. The small set (0.95 and 0.75 kb) can support only synthesis of BlaI, the repressor. The 2.3-kb mRNA should be large enough to yield both proteins. The 1.9-kb form could not produce the complete BlaR1. It was present only in low and variable amounts and may be a degradation product of the 2.3-kb transcript.

A major characteristic of transcription from the *blaI* promoter is that the large mRNAs are primarily produced early in induction. The level of the 2.3-kb transcript was at a maximum after 30 min and fell off rapidly thereafter. The small mRNAs did not peak until about 1 h and had returned to a low level by 2 h, a pattern which approximates that of the *blaP* transcripts during induction [3]. Assuming that the various mRNAs are actually translated, both BlaI and BlaR1 are produced during the first 30 min. After that the formation of BlaR1 should decrease rapidly while the repressor and β-lactamase are formed in substantial amounts. From these observations, we suggest that the 68-kDa protein BlaR1 has an essential function early in induction, possibly as the specific acceptor for the β-lactam through its potential penicillin-binding site [8]. The detection of large *blaI-blaR1* mRNA only during the initial stages of induction, despite continuing transcription from *blaIp*, suggests that the level of large *blaI-blaR1* mRNA is determined by control of transcript elongation, or by processing or selective degradation. Furthermore, cephalosporin C induces large *blaI-blaR1* transcripts in the constitutive mutant 749/C even though the level of transcription from *blaIp* is already maximal. Thus the functional repressor is not required in this regulatory process.

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