In vitro transcription of the cloned chromosomal crystal gene from *Bacillus thuringiensis*

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ABSTRACT. -

We have determined the conditions required for *in vitro* transcription of the cloned chromosomal crystal gene from *Bacillus thuringiensis* using either the homologous vegetative RNA polymerase or a sporulation specific form of this enzyme. The gene is actively transcribed by the latter enzyme (form II) but not by the vegetative one. Evidence for a specific recognition between the form II enzyme and the promoter site of the crystal gene was obtained by binding experiments. They showed that the binding is increased by the presence of some additional factors, which change the specificity of the vegetative core-enzyme. The sequence of the promoter has been determined and the startpoint of the transcription deduced. Two hexanucleotide sequences, TACAAT and CCTACG, centered at - 10 and - 35 bp are present, but are somewhat different from the consensus sequences previously described in other bacilli.

INTRODUCTION. -

The binding of RNA polymerase to the promoter site is the first step in gene expression. A large number of promoters present in *E. coli* have been sequenced and found to contain two highly conserved hexanucleotide sequences located at - 35 and - 10 bp, which are responsible for the specific binding of the enzyme (1).

In *B. subtilis* cells, and in bacteria infected with different phages, RNA polymerase holoenzyme exists in multiple forms which differ by a particular species of sigma factor (2). The promoters recognized by the vegetative *B. subtilis* holoenzyme are similar to those recognized by the RNA polymerase in *E. coli* (3). In the case of the promoters used by phage specific forms or sporulation forms of RNA polymerase, the - 35 and - 10 bp sequences differ significantly from each other and from the consensus sequence (4), but it appears that the mechanism by which the RNA polymerase interacts with its promoters is fundamentally the same (5).

The Gram positive sporulating bacterium *B. thuringiensis* is of special interest because it produces an insecticidal toxin (named δ

endotoxin) lethal to larvae of a wide range of lepidopterans as well as some dipterans (6). The δ endotoxin is synthesized as a crystalline inclusion only during sporulation. The mRNA coding for the toxin is relatively stable (7) and is produced by a particular form of RNA polymerase, as referred to as the form II enzyme (8). Depending on the strains, the crystal genes may be localized either on a large host plasmid, on the chromosomal DNA, or on both (10). The crystal DNA sequences from several strains have been recently cloned (9, 10, 11) and some of them have been expressed in *E. coli* or *B. subtilis* (10). It was shown that the chromosomal crystal gene from strain *berliner* was not transcribed by the *E. coli* RNA polymerase and the promoter sequence of this gene seems to be recognized only by the RNA polymerase from sporulating cells of *B. subtilis* (10).

In this paper, we report the conditions of the *in vitro* transcription of the chromosomal crystal gene from strain *berliner* and present the promoter sequence of this gene. We show that the RNA polymerase form II can bind specifically to this promoter for initiating the transcription, and that the hexanucleotide sequence centered approximately at - 10 bp upstream from the startpoint corresponds closely to the consensus sequence (1). The other hexanucleotide sequence centered at - 35 kb appears very different and has no equivalent so far, in *E. coli* or *B. subtilis* (1, 2, 3).

MATERIAL and METHODS.-

Recombinant plasmids.-

The construction of plasmid pBT 15-88 containing the chromosomal crystal gene and its derivatives pBT 88-3 and pBT 88-6 has been described (10). A schematic map of these plasmids is shown in fig. 1. The pBT 88-3 and pBT 88-6 was constructed by subcloning in both orientations, the 4 kb *EcoRI* fragment of pBT 15-88 harboring the crystal gene into the *PstI* site of the bifunctional vector pHV33 (12) by the poly(dG).poly(dC) tailing method (10).

Purification of RNA polymerase.-

The vegetative RNA polymerase and the two enzymes from t5 sporulating cells of *B. thuringiensis* strain *berliner* 1715 were purified as previously described (13). The last step of the purification procedure is a specific adsorption on a DNA-cellulose column.

Isolation and analysis of plasmid DNAs.-

E. coli recombinant plasmid DNAs were purified from 1 to 2 l

Fig. 1 : Schematic restriction map of plasmids pBT 15-88 containing the crystal gene from *B. thuringiensis* and its derivatives pBT 88-3 and pBT 88-6. Only the restriction sites used in this work were indicated: $\square$ *EcoRI*; $\blacktriangle$ *HindIII*; $\triangle$ *PstI*.

cultures according to the procedure of ISH-HOROWICZ and BURKE (14). DNA fractions were analyzed by electrophoresis on 0.6 % agarose slab gels. Restriction fragments were recovered after electrophoresis by electroelution in a dialysis bag.

Radioactive probes.-

DNA was labeled by nick-translation using *E. coli* DNA polymerase (BRL) in the presence of ($\alpha^{32}\text{P}$) dCTP and ($\alpha^{32}\text{P}$) dATP (NEN 400 Cie/mmole) as described by RIGBY et al. (15).

Blotting and hybridization procedures.-

The technique used was essentially that described by SOUTHERN (16) using Schleicher and Schull nitrocellulose filters BA85. Hybridization of the filters with RNA probes was performed as described (17).

In vitro transcription.-

RNA was synthesized *in vitro* by the following procedure. RNA polymerase (0.25 unit) was preincubated for 10 min at 37°C with about 4 μg of hybrid plasmid DNA or a similar amount of a purified restriction fragment in a total volume of 50 μl lacking ribonucleotide triphosphates but containing 15 % glycerol (8). These conditions represent an enzyme to template ratio from 0.4 to 1.2. Ribonucleotides were added (0.15 mM CTP, GTP, ATP and 0.4 μM ($\alpha^{32}\text{P}$) UTP (NEN, 20 μCie , 400 Cie/mmole)) and the incubation was continued. After 3 min, 0.15 mM unlabeled UTP was added and the reaction was stopped after 1 min by adding phenol and RNA was isolated as described (18). The RNAs resulting from *in vitro* transcription were used for hybridization reactions or subjected to polyacrylamide gel electrophoresis in the presence of 7M urea (19).

RNA polymerase binding.-

Conditions of RNA polymerase binding to the restriction fragments

were essentially as described by TALKINGTON et al. (20) and JONES et al. (21). RNA polymerase was incubated at 37°C for 30 min with 5 µg of restriction fragments in 0.5 ml of pBBX (0.01 M Tris-HCl pH 7.9, 0.01 M MgCl₂, 0.1 mM dithiothreitol, 0.1 mM EDTA, 50 µg/ml BSA) and 0.1 M KCl at a RNA polymerase to DNA molar ratio between 3 and 5. Heparin (400 µg/ml final concentration) and 50 µg/ml calf thymus DNA were added. After incubation for 10 min the mixture was passed through a Schleicher and Schu11 BA85 nitrocellulose filter. The filter was washed with 2.5 ml of PBBX (37°C) containing 0.05 M KCl. DNA fragments were eluted from the filter by soaking it for 20 min in 20 mM Tris-HCl pH 7.9 containing 0.2 % SDS. The eluted DNA fragments were then analyzed by electrophoresis.

DNA sequencing.-

Nucleotide sequences were determined by the method of MAXAM and GILBERT (19), with DNA strands radioactively labeled at their 5' termini or at their 3' ends. Labeling of the 5' ends of DNA fragments with (γ ³²P) ATP (3000 Cie/mmol, Amersham) and T4 polynucleotide kinase was performed by exchange reaction (19). Restriction fragments were labeled at their 3' ends with (α ³²P) dNTP in the presence of DNA polymerase I according to COSSART and GICQUEL-SANZEY (22).

RESULTS.-

In vitro transcription of the cloned chromosomal crystal gene from B. thuringiensis strain berliner 1715.-

The efficiency of transcription of the recombinant plasmid pBT 15-88 harboring the chromosomal crystal gene or its two derivatives, pBT 88-3 and pBT 88-6, was measured using either the *B. thuringiensis* vegetative RNA polymerase or the sporulation form II of the enzyme isolated from t5 sporulating cells. The specific activities obtained in these conditions are reported in Table 1. The recombinant plasmids were efficiently transcribed by the sporulation form II RNA polymerase, while the standard plasmid vectors alone pBR322 or pHV33 were better templates for the vegetative enzyme. This result confirmed a previous observation concerning the presence of the mRNA coding for the parasporal protein among only the transcription products of the form II RNA polymerase using total *B. thuringiensis* DNA as a template (8).

To study the in vitro transcription of the crystal gene, which was located in a 4 kb Eco RI fragment of pBT 15-88, labeled RNA copied from the native hybrid plasmid DNA of pBT 15-88 was hybridized to restric-

Table 1 : Specific activities of the different form of RNA polymerase using the vector alone and the recombinant plasmids carrying the crystal gene as templates. See the methods section for experimental details.

	pBR322	pHV33	pBT 15-88	pBT 88-3	pBT 88-6
Vegetative enzyme (A)	120	130	110	120	110
Sporulation form II enzyme (B)	80	70	170	210	200
Ratio B/A	0.86	0.54	1.5	1.75	1.8

tion fragments of pBT 15-88 separated by gel electrophoresis. This experiment, which is described in fig. 2, shows hybridization of the labeled RNA synthesized by form II RNA polymerase with the 4 kb EcoRI fragment containing the crystal gene and with the 2.8 and 1.3 fragments generated by two HindIII sites located inside the gene. Hence, the form II RNA polymerase

Fig. 2 : In-vitro transcription of the plasmid pBT 15-88 by the vegetative or by the sporulation form II RNA polymerase. RNA was synthesized in vitro (using pBT 15-88 as template) and hybridized with restriction fragments of pBT 15-88 obtained by digestion with HindIII (1), EcoRI (2) and HindIII-EcoRI (3). Panels A and C show the results obtained with RNA produced by vegetative and form II enzyme respectively. Panel B shows the electrophoretic patterns of the digests.

Fig. 3 : Size of the in vitro RNA transcripts obtained by the vegetative or by the form II RNA polymerase. RNA was synthesized in vitro using the 4 kb EcoRI fragment of pBT 15-88 (panel C) this fragment digested by HindIII (panel B) or the purified 0.15 kb fragment HindIII-PstI fragment of pBT 88-3 (panel A) by the vegetative (lanes 1) or the sporulation form II enzymes (lanes 2). RNAs were subjected to a 8 % polyacrylamide gel electrophoresis in the presence of 7M urea.

is able to transcribe the crystal gene when it is located in the pBT 15-88 plasmid. No such hybridization was observed with RNA synthesized by the vegetative enzyme. Other sequences of the plasmids such as the two larger HindIII fragments (8 and 5 kb) were transcribed by both enzymes, but other fragments did not seem to be transcribed at all. Therefore, the purified vegetative RNA polymerase failed to recognize the sporulation controlled sequence located within the 4 kb EcoRI fragment and corresponding to the crystal gene.

If, in this experiment, a HindIII digest of pBT 15-88 was used instead of native DNA as a template for the form II RNA polymerase, no hybridization was found between the resulting labeled RNA transcripts and the 4 kb EcoRI or the two HindIII fragments (2.8 and 1.3 kb). Therefore the mRNA coded by the crystal gene has not been synthesized. The pattern of the autoradiogram is identical in this case to that obtained in fig. 2A.

Another experiment using an EcoRI digest of pBT 15-88 as a template gave the same results as those obtained with the native DNA, suggesting that the gene with its control region is located entirely in the 4 kb EcoRI fragment (data not shown).

These results have been confirmed by analyzing the size of the in vitro transcripts. After synthesis, the RNAs were purified and subjected to electrophoresis (fig. 3). Among the mRNA species synthesized by the form II RNA polymerase using either intact pBT 15-88 or an EcoRI digest of the plasmid as templates, an RNA of about 23S was detected. This size corresponds closely to the 26S species observed in vivo (10). In the case of a HindIII-EcoRI digest of pBT 15-88, a small RNA of 80 nucleotides was observed, which corresponds to the transcription of the DNA sequence between the promoter site and the first HindIII site (fig. 1). We concluded that the promoter site is located into the 0.15 kb EcoRI-HindIII fragment of the inserted crystal gene sequence. This conclusion was confirmed by the results of the transcription of the purified 0.15 kb PstI-HindIII fragment of pBT 88-3. An RNA of similar size (80 nucleotides) was found among the in vitro transcripts. No similar RNA could be detected when the vegetative enzyme was used in these experiments, indicating that the promoter site is only recognized by the sporulation form II RNA polymerase. Another experiment was performed using the same 0.15 kb PstI-HindIII fragment of pBT 88-3 and the form II RNA polymerase, but replacing ($\alpha^{32}\text{P}$) UTP with one of the ($\gamma^{32}\text{P}$) NTP's. The 80 nucleotide RNA was detected only when ($\gamma^{32}\text{P}$) ATP was used, indicating that the first nucleotide of the RNA is an adenine residue (data not shown).

Binding of RNA polymerase to the promoter sequence.-

The location of the promoter site of the crystal gene was determined by studying the binding of the different forms of RNA polymerase to different restriction fragments of pBT 88-3. The plasmid pBT 88-3 was cleaved by PstI and the fragments were examined for their binding capacity for RNA polymerase extracted from either B. thuringiensis vegetative cells or from t5 sporulating cells. As seen in fig. 4, the 4 kb fragment containing the crystal gene bound the sporulation form II enzyme but not the vegetative enzyme or the form I enzyme. The vegetative core-enzyme, which has a similar pattern to the form II enzyme on SDS-polyacrylamide gel electrophoresis, had no affinity for the DNA fragment. The vegetative σ factor had no effect on the specific binding of the form II enzyme and E. coli RNA polymerase did not recognize the promoter site of this gene. The 7 kb frag-

Fig. 4 : Binding of *B. thuringiensis* RNA polymerase to *Pst*I restriction fragments of pBT 88-3.

A : Binding of *Pst*I fragments of pBT 88-3 with : the sporulation form II enzyme (lane 1), the sporulation form I enzyme (lane 2), the vegetative core-enzyme (lane 3), the vegetative holoenzyme (lane 4), the sporulation form II enzyme + vegetative σ factor (lane 5) or the *E. coli* enzyme (lane 6). B : Binding of *Hind*III fragments from pHV33 with the sporulation form II enzyme (lane 8) or with the *E. coli* enzyme (lane 9). Mixture of DNA fragments obtained by digestion of pHV33 with *Hind*III and digestion of pBT 88-3 with *Pst*I was loaded in lane 7 to serve as a control.

ment corresponding to the pHV33 vector bound all of the different forms of RNA polymerase that were tested (fig. 4). The *E. coli* RNA polymerase had a high affinity for the pBR322 part of the pHV33, while the form II RNA polymerase bound the pC194 region of the vector plasmid.

Similar experiments were performed using the purified labeled 0.15 kb *Pst*I-*Hind*III fragment of pBT 88-3 (fig. 5 and 6). Only the sporulation form II enzyme can bind to this fragment, indicating that this fragment contains a major part of the promoter sequence of the crystal gene. The other forms of RNA polymerase did not recognize this sequence and the vegetative σ factor had no effect on the binding of the form II enzyme. However, the stability of the complex between the fragment and the form II RNA polymerase was increased by adding polypeptides from the flow-through of the DNA-cellulose column used to separate form I and form II enzymes (13). These polypeptides are able to promote a change in the specificity of the vegetative core-enzyme, but this effect was inhibited by the vegetative σ factor (fig. 5).

Fig. 5 : Binding of *B. thuringiensis* RNA polymerase to the 0.15 kb *Pst*I-*Hind*III fragment of pBT 88-3. Binding of this fragment to the vegetative enzyme (lane 2), the sporulation form I enzyme (lane 3), the sporulation form II enzyme (lane 4), the *E. coli* enzyme (lane 5), the sporulation form II enzyme + 2 μ g (lane 6) or 4 μ g (lane 7) of vegetative σ factor, the sporulation form II enzyme + 10 μ g (lane 8) or 20 μ g (lane 9) polypeptides of the flow-through of the DNA-cellulose column, the vegetative-core enzyme + 10 μ g (lane 10) or 20 μ g (lane 11) polypeptides of the same flow-through of the DNA-cellulose column. The position of the 0.15 kb fragment is indicated in lane 1.

From the binding experiments, it is clear that the only sequence in the 4 kb *Pst*I fragment able to bind the form II RNA polymerase is located in the 0.15 kb *Pst*I-*Hind*III fragment, because the other parts of the fragment did not bind to the enzyme (fig. 6). The interaction between the form II RNA polymerase was inhibited at high ionic strength. Furthermore this enzyme lost some factors during chromatography on a phosphocellulose column, as the 0.15 kb fragment bound smaller amount of the enzyme after phosphocellulose chromatography. The material found in the flow-through converts the specificity of the vegetative core-enzyme to that of the form II enzyme (fig. 6).

The results described above confirmed that the 0.15 kb fragment contains a sequence able to promote the specific binding of sporulation RNA polymerase form II. The RNA polymerase pBT 88-3 complex that was formed during the polymerase binding experiment was digested with both *Hind*III and *Pst*I (fig. 7). At a low polymerase : plasmid ratio, both restriction enzymes efficiently cut the plasmid. At a high ratio, the *Hind*III digestion was not changed. However, under these conditions *Pst*I did not cut the plasmid and the amount of 0.15 kb fragment decreased whereas a

Fig. 6 : Binding of *B. thuringiensis* RNA polymerase to digests of pBT 88-3. Panel A : The form II RNA polymerase was bound to the 2.4 kb PstI-HindIII fragment (lane 1), the 1.3 kb HindIII fragment (lane 2), the 0.15 kb PstI-HindIII fragment (lane 3), the 0.15 kb PstI-HindIII fragment at 10^{-2} M KCT (lane 4), at 10^{-1} M KCl (lane 5) or at $2 \cdot 10^{-1}$ M KCl (lane 6). Panel B : Binding of the 0.15 kb fragment to the form II RNA polymerase that was passed either one time (lane 7) or two times (lane 8) on a phosphocellulose column, the vegetative core-enzyme (lane 9), the vegetative core-enzyme + 1 μ g (lane 10) or 3 μ g (lane 11) proteins non-adsorbed from the phosphocellulose column, the vegetative core-enzyme + 3 μ g proteins non adsorbed on the phosphocellulose column + 10 μ g of vegetative σ factor (lane 12). The position of 0.15 kb fragment is indicated in lane c.

larger PstI/HindIII fragment (about 2 kb) appeared and was detected in the wells of the gel (lanes 4 and 5). These results suggest that the binding site of the RNA polymerase overlaps with the PstI site but not with the HindIII site.

Sequence of the promoter site of the chromosomal crystal gene.-

The sequence of the promoter site was determined using the recombinant plasmid pBT 88-3. The plasmid was first digested by HindIII and then labeled either by phosphate exchange at both 5' termini or by filling in at both 3' ends using DNA polymerase I. The 0.8 kb DNA fragment was recovered and further digested by PstI giving a 0.15 kb PstI-HindIII fragment labeled at one side either the 5' or the 3' end. The fragment was sequenced, and the nucleotide sequence of the non coding strand is shown in fig. 8.

Considering the previously determined orientation of transcription and the size of the mRNA synthesized from the startpoint of the transcription to the HindIII site (fig. 3), we can locate the startpoint, which must be an adenine residue on the non coding strand. About ten nucleotides before the

Fig. 7 : Overlapping of the promoter site of the crystal gene and the PstI site. The labeled plasmid pBT 88-3 was bound with the form II RNA polymerase at different plasmid enzyme molar ratio and then digested with HindIII and PstI. The bound fragments were recovered and subjected to electrophoresis. Molar ratio : 0.1 (lane 1), 0.2 (lane 2), 0.4 (lane 3), 0.8 (lane 4), 2 (lane 5). The position of the 0.15 kb fragment is indicated in lane c by an arrow.

startpoint, a hexanucleotide sequence TACAAT is found, which is closely related to the consensus sequence already described by MORAN *et al.* (3) for *B. subtilis* genes. Sixteen nucleotides upstream from this sequence, we can detect another hexanucleotide sequence CCTACG centered at - 35, which has no equivalent among the classical sequences already described (14). According to the result of fig. 7, this sequence CCTACG overlaps the restriction site

Fig. 8 : Nucleotide sequence of the 0.15 kb PstI-HindIII fragment of pBT 88-3. The nucleotide and amino-acid sequences of the putative N-terminal of the chromosomal crystal gene are presented. The ribosome binding site is indicated SD. The startpoint of transcription is indicated by 0. The - 35 and - 10 sequences are underlined.

of PstI. The sixteen nucleotide region between the two hexanucleotide sequences is relatively rich in AT residues.

Only one open reading frame from the startpoint of the transcription has been detected beginning with an ATG codon 45 bp downstream from the initial adenine residue. We can predict the first eleven aminoacids of the protein and this sequence contains five serine residues. About 17 bp upstream the ATG codon, there is a 4 bp sequence which might be related to a ribosome binding site, but this sequence seems to be slightly different from the sites already described in B. subtilis (3, 4).

DISCUSSION.-

We have previously demonstrated the existence of two new forms of RNA polymerase during sporulation of B. thuringiensis, which differ from one another and from the vegetative enzyme in their subunit composition and in some catalytical properties (13). In addition, two new low molecular weight polypeptides, Y and Z seem to be integrated into the subunit structure of the two sporulation enzymes. The mRNA coding for the δ endotoxin protein was found among the transcriptional products when the form II RNA polymerase was used to transcribe total B. thuringiensis DNA (8). In a previous paper (10) we have shown that the cloned crystal gene from chromosomal origin was not transcribed in E. coli cells or in vegetative B. subtilis cells. In sporulating B. subtilis cells, the crystal gene was transcribed, but not translated. Moreover, the crystal gene from host plasmid origin seemed to be submitted to the same transcriptional control in B. subtilis as in B. thuringiensis, but was also expressed at a high level in E. coli.

In the present work, the transcription of the cloned chromosomal crystal gene has been studied using purified B. thuringiensis RNA polymerase from vegetative and sporulating cells. The crystal gene is included in a 4 kb EcoRI fragment, which contains two HindIII sites. Digestion of this fragment with HindIII generates three fragments of approximately 2.4, 1.3 and 0.15 kb. Complementary RNAs of these fragments were detected only when transcribed by the form II RNA polymerase. This enzyme is similar to the vegetative core-enzyme according to SDS-gel electrophoresis but often contained non-stoichiometric amounts of two polypeptides of 35 and 27 kd. These additional subunits might be related to the σ 37 and σ 29 factors discovered in B. subtilis sporulating cells (2). The B. thuringiensis vegetative enzyme did not synthesize RNA which was able to hybridize with

the crystal coding sequence.

The inability of the vegetative enzyme to transcribe this gene was confirmed by in vitro binding studies. The absence of σ 55 factor was not sufficient to allow transcription of the gene by the vegetative core-enzyme. Functional differences between the vegetative core-enzyme and sporulation form II RNA polymerase were previously demonstrated, and limited or undetectable alteration of the core-enzyme has been assumed to occur during the sporulation phase (18). Template recognition is a function of the core-enzyme and these alterations could be responsible for change in template specificity of the enzyme (23).

Binding experiments performed between the form II RNA polymerase and the EcoRI digest of the pBT 15-88 shown that the 4 kb EcoRI fragment was recognized with a high affinity by the form II enzyme (data not shown). This result indicated that the specific binding observed between the 4 kb PstI fragment of pBT 88-3 and the form II enzyme was not due to the presence of the dG/dC tails used for the construction of the recombinant plasmid pBT 88-3 but to a specific recognition sequence present in the 4 kb DNA fragment.

The stability of the complex between form II enzyme and the 4 kb DNA fragment was enhanced by adding polypeptides excluded from the DNA-cellulose column used for the purification of the enzyme suggesting the role of factors partially or completely lost during this last purification step. These factors also seem to be removed from the form II enzyme by chromatography on a phosphocellulose column. We assume that these factors could be the polypeptides Y and Z (13) and that they can change the specificity of the vegetative core-enzyme to that of the form II enzyme. It is interesting to note that the σ -55 factor has no effect on the specific binding of the form II RNA polymerase to the promoter site of the gene, which further supports the hypothesis that an alteration of the properties of the core-enzyme occurs during the sporulation phase.

The in vitro transcription experiments have allowed us to determine the size of the transcripts from the 150 bp DNA fragment, which contains the promoter site of the crystal gene and the startpoint of the transcription. The startpoint is located 80 nucleotides upstream from the HindIII end, and the first nucleotide of the mRNA must be an adenine residue. A hexanucleotide sequence TACAAT was found ten nucleotides upstream from the transcription startpoint. This sequence is very similar to the consensus sequence found at - 10 for most of the promoter sites of E. coli (1), or for the vegetative promoters of B. subtilis (2). It is different from those reported by MORAN

et al. (3) for the promoters recognized by sporulation RNA polymerase containing the σ -37 and σ -29 factors. The promoter site of the crystal gene is not recognized by the *E. coli* or by the vegetative *B. subtilis* RNA polymerase, and we assume that the difference between a vegetative and a sporulation promoter sites resides in the hexanucleotide sequence located at - 35. This sequence CCTACG, located 16 nucleotides upstream the Pribnow box, has no equivalent in *E. coli* or in *B. subtilis*. These differences might provide a sufficient explanation for the failure of *E. coli* or *B. subtilis* RNA polymerase to transcribe the crystal gene, but leave unanswered the question as to which nucleotide sequences signal recognition by form II RNA polymerase.

An open reading frame from the transcription startpoint had been detected with an ATG initiation codon 45 bp downstream from the initial adenine residue. We assume that the ATG codon is the initial translation codon of the crystal gene, but there is no definitive proof because the N terminal sequence of the protein has not been determined in the strain used. If our assumption is correct, it is possible to deduce the beginning of the aminoacid sequence, which contains five Ser residues out of eleven aminoacids determined.

MAC LAUGHLIN et al. (24) have proposed that the ribosome site of Gram positive mRNAs are able to form highly stable complexes with the 3' terminal region of 16S rRNA. About 16 nucleotides upstream the ATG codon, we have found a putative ribosome binding site AAtcAGGtgA, similar to that found in *B. subtilis*. But the sequence of the 3' end of the 16S rRNA from *B. thuringiensis* has not been determined and the complementarity may be better. The calculated free-energy of interaction (ΔG) for this proposed ribosome binding site is about - 11 kcal as compared to - 9.4 kcal for the prototype *E. coli* ribosome binding sequence and from - 14 to - 23 kcal for different *B. subtilis* sites. This may explained the absence of translation of the chromosomal crystal gene in sporulating cells of *B. subtilis*. Another explanation for this fact may be the greater distance between the ribosome binding site and the initiation codon, which ranges from 7 to 14 bp in *B. subtilis*. The cloned crystal gene from host plasmid origin is expressed in *E. coli* or in sporulating cells of *B. subtilis* and we are currently trying to localize and sequence the promoter of this gene copy in order to explain the differences found at the transcriptional and translational levels between chromosomal and plasmid crystal genes.

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