The expression of the *bstVIM* gene from *Bacillus stearothermophilus* V is restricted to vegetative cell growth

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The activity of *BstVI* DNA methyltransferase was monitored during the sporulative cycle of *Bacillus stearothermophilus* V. Significant methylase activity was found only in bacteria growing vegetatively. This was confirmed by Northern hybridization, which indicated that the *bstVIM* gene was not transcribed in cells undergoing sporulation. Supporting evidence came from experiments which demonstrated that the RNA polymerase holoenzyme from these cells did not recognize the promoter elements upstream of the *bstVIM* gene.

Keywords: *Bacillus stearothermophilus*, sporulation, thermophilic promoters, differential gene expression

INTRODUCTION

Bacterial restriction and modification (R-M) systems have become the subject of great interest in the last decade. They are currently being used as model systems to study sequence-specific protein-DNA recognition.

We have been interested in studying R-M systems from thermophilic bacteria. In this context, we have previously characterized the *BstVI* R-M system, which is present in the thermotolerant, spore-forming rod *Bacillus stearothermophilus* V. Both the endonuclease, R·*BstVI*, and the DNA methyltransferase, M·*BstVI*, have been purified and characterized (Vásquez, 1985; Barra *et al.*, 1988) and their structural genes were cloned and expressed in *Escherichia coli* (Vásquez *et al.*, 1991). In addition, the complete nucleotide sequence of the system has been determined (González & Vásquez, 1993).

Earlier results showed that the synthesis of M·*BstVI* was restricted to early exponential growth, thus suggesting controlled expression of its structural gene (Barra *et al.*, 1988). The modulation of gene expression through the bacterial life cycle is a common feature of the genus *Bacillus*. In *Bacillus subtilis* for instance, a well-defined pattern of differential gene expression linked to the sporulation process has been established: several groups of genes are turned on or off depending on the sporulation stage (Piggot & Coote, 1976; Losick *et al.*, 1986). The mechanisms underlying such phenomena involve the

existence of different promoter structures, which are recognized by specific RNA polymerase σ factors (Losick & Pero, 1981; Losick *et al.*, 1986; Helmann & Chamberlin, 1988; Errington, 1993).

Two kinds of promoter structures have been identified upstream of *bstVIR* and *bstVIM* genes. One of them exhibits a degree of similarity with those from bacterial 'housekeeping' genes (González & Vásquez, 1993). The other is highly homologous to control elements of genes which are expressed at intermediate sporulation stages of *B. subtilis* (Helmann & Chamberlin, 1988). Since our interest was to determine if the above promoter-like elements were operative in their natural host, *B. stearothermophilus* V (Vásquez, 1985), we have analysed the expression of the *bstVIRM* system at defined stages during sporulation.

METHODS

Cell growth and induction of sporulation. *B. stearothermophilus* V was cultured in Müller–Hinton broth (Difco) with vigorous shaking at 65 °C. At an OD₆₅₀ of 1.2, cells were collected by centrifugation at 5000 g and transferred to a sporulation medium, which contained K₂HPO₄ (6 g l⁻¹), (NH₄)₂SO₄ (1 g l⁻¹), MnSO₄ (1 g l⁻¹), KNO₃ (1 g l⁻¹) and NaCl (5 g l⁻¹). An aliquot of the cell suspension was removed, sedimented as above and frozen at -20 °C (vegetative cells). The remainder of the culture was further incubated at 37 °C with shaking. Samples were taken at defined time intervals, and sporulation was monitored by endospore staining.

Enzyme assays. R·*BstVI* was assayed as described previously (Vásquez, 1985). The activity of the *BstVI* methyltransferase

Abbreviation: R-M, restriction and modification.

was determined by the protection assay described by Barra *et al.* (1988). The substrate was in both cases *Hind*III-digested lambda DNA (New England BioLabs).

RNA purification and Northern hybridization. Total RNA from *B. stearothermophilus* V was isolated according to the guanidinium/phenol method described by Maniatis *et al.* (1982), with minor modifications. Briefly, cells from 50 ml cultures were sedimented and frozen immediately in liquid nitrogen. Unless otherwise stated, all subsequent steps were carried out at 0–4 °C to minimize RNA degradation by endogenous thermophilic nucleases.

The cell pellet was thawed and suspended in 1 ml of a solution which contained 100 mM Tris/HCl (pH 7.6), 20 mM EDTA, 100 mM 2-mercaptoethanol and 4 M guanidinium isothiocyanate. One volume of ice-cold saturated phenol was added and the phases mixed by vortexing. To shear the DNA, the mixture was passed through a 27-gauge needle and centrifuged at 10000 *g* for 10 min. The aqueous phase was transferred to a fresh sterile tube and extracted twice with 1 vol. phenol/CHCl₃ (1:1, v/v). Nucleic acids were precipitated by adding 3 vols ethanol. After 2 h at –20 °C, the mixture was centrifuged at 12000 *g* for 15 min. The pellet was air-dried and resuspended in 0.5 ml sterile water. The RNA was further purified by several selective precipitations with 3 M sodium acetate (pH 5.2). The RNA was finally resuspended in 100 µl sterile water.

RNA samples were electrophoresed in 1.8% agarose-formaldehyde gels and processed for Northern hybridization as described by Maniatis *et al.* (1982).

RNA polymerases. RNA polymerase holoenzymes were purified from vegetative cells of *B. stearothermophilus* V and from *B. stearothermophilus* V cells which were collected at defined sporulative stages. The procedure of Johnson *et al.* (1983) was followed in both cases. *E. coli* RNA polymerase was kindly provided by Dr. Luis Meza-Basso (Laboratory of Plant Biochemistry, Universidad de Talca).

Gel-retardation assay. Binding of RNA polymerase to DNA was analysed by the gel-retardation assay of Garner & Revzin (1988). A defined ³²P-labelled DNA fragment (see below) was incubated with the indicated RNA polymerase in 50 µl of a solution which contained 20 mM Tris/HCl (pH 8.0), 2 mM DTT, 10 mM MgCl₂ and 100 mM KCl. A protein/DNA molar ratio of 5 was used. After 15 min at 37 °C, heparin (100 µg ml^{–1} final concentration) was added to minimize non-specific binding. The protein/DNA complexes were electrophoresed in 5% (w/v) polyacrylamide gels and visualized by autoradiography.

RESULTS AND DISCUSSION

Induction of sporulation in *B. stearothermophilus* V

Since the sporulative process has not been characterized genetically or biochemically in this bacterium, it was monitored by light microscopy after endospore staining. The induction of sporulation was highly synchronous, yielding homogeneous populations in which over 90% of the cells were at the desired state. Four well-defined states were established: vegetative (V) cells and early (E), intermediate (I) and late (L) sporulative cells. At the E-stage, the bacterial cell was stained evenly and a random grouping was observed. On the other hand, I-stage cells were characterized by an accumulation of the dye at the bacterial pole and cell grouping was changed to a chain

Fig. 1. Activity of the *Bst*VI enzymes during the life cycle of *B. stearothermophilus* V. Extracts from cells collected at stages V (lanes A and G), E (lanes B and H), I (lanes C and I) and L (lanes D and J) were assayed for R-*Bst*VI (lanes A–D) and M-*Bst*VI (lanes G–J). Lane E, control DNA (*Hind*III-digested lambda DNA); lane F, control DNA digested with purified R-*Bst*VI. Reaction products were electrophoresed in 1% (w/v) agarose gels.

arrangement. Finally, at the L-stage the mature endospore was clearly visible and cells were now arranged as a palisade. By these criteria, states E, I and L were found to be equivalent to *B. subtilis* sporulation stages I, III–IV and VI–VII, respectively (Piggot & Coote, 1976; Losick *et al.*, 1986; Errington, 1993), and they occurred at 12, 36 and 60 h after the induction of sporulation.

Activity of R-*Bst*VI and M-*Bst*VI at V, E, I and L states

As stated above, previous results suggested a modulated expression of the *bstVIM* gene during the *B. stearothermophilus* V life cycle (Barra *et al.*, 1988). It was therefore very interesting to analyse the expression of the whole R-M system in relation to the sporulation process of this bacterium. As a first approach, the activity of the *Bst*VI R-M enzymes was determined in extracts prepared from cells which were harvested at the sporulation states defined above. Both R-*Bst*VI and M-*Bst*VI were detected in significant amounts in V-stage cells (Fig. 1, lanes A and G). Enzyme titration gave a specific activity of 15000 ± 1500 U (mg protein)^{–1} for R-*Bst*VI and 600 ± 64 U (mg protein)^{–1} for M-*Bst*VI. About 10% of these values were found in E-state cells (Fig. 1, lanes B and H). These results could reflect either the unusual resistance of these enzymes to denaturation (Vásquez, 1985; Barra *et al.*, 1988) or the presence of a small amount of cells still in the V-state. It may also be explained by low-level expression of *bstVIRM* genes at this stage.

Neither R-*Bst*VI nor M-*Bst*VI activities were detected in extracts from I or L cells (Fig. 1, lanes C–D and I–J, respectively). In addition, and as determined by SDS-PAGE, polypeptide bands corresponding to R-*Bst*VI and M-*Bst*VI were detectable in extracts from V cells only

Fig. 2. Promoter structures upstream of *bstVIR* and *bstVIM* structural genes. P^A and P^E are promoters recognized by *B. subtilis* RNA polymerase σ^A and σ^E factors, respectively. See text for details. DNA sequences found upstream of the *bstVIR* genes, which are analogous to P^A and P^E blocks, are indicated by one and two asterisks, respectively.

(not shown). These results strongly suggest that the *BstVI* R and M genes are expressed only in cells which are undergoing vegetative growth. Identical results were obtained when the activity of R-*BstLVI* and M-*BstLVI* from *B. stearothermophilus* LV (Lobos & Vásquez, 1993) was analysed (not shown).

Expression *in vivo* of the *bstVIM* gene

In addition to the putative control regions of the *bstVIR* genes described previously (González & Vásquez, 1993), sequence blocks showing high similarity to RNA polymerase σ^E -factor-recognized *B. subtilis* promoters (Helmann & Chamberlin, 1988) were also identified at appropriate positions in this R-M system (Fig. 2). The presence of P^A and P^E-like promoters suggests that *BstVI* R and M genes could be expressed at different stages of the *B. stearothermophilus* V life cycle. Thus, a regulatory mechanism involving the replacement of RNA polymerase σ factors could operate in this bacterium as it occurs in *B. subtilis* (Losick & Pero, 1981; Losick *et al.*, 1986).

To analyse the above assumption, the expression *in vivo* of the *bstVIM* gene was investigated by Northern hybridization. RNA samples were obtained from bacteria arrested at the indicated sporulation states, as described in Methods. To rule out artifacts due to RNA degradation during purification, aliquots of two independent preparations of each stage were run in parallel in agarose-formaldehyde gels and transferred to nitrocellulose membranes. As a probe, a ³²P-labelled *SalI*-*PstI* DNA fragment (843 bp) which contains the coding region of the *bstVIM* gene was used (Vásquez *et al.*, 1991; González & Vásquez, 1993). Positive hybridization signals were obtained with RNA from V-state bacteria only (Fig. 3, lanes A and B). An RNA transcript of about 1900 nucleotides was detected. This was the expected size for a mRNA sequence starting near to the -10 block of the putative P^A promoter and ending at the rho-independent terminator of this gene (González & Vásquez, 1993). No RNA complementary to the probe was detected in samples from bacteria at E, I or L states (Fig. 3, lanes C-H). These

Fig. 3. Expression *in vivo* of the *bstVIM* gene. Total RNA samples from *B. stearothermophilus* V cells at stages V (lanes A and B), E (lanes C and D), I (lanes E and F) and L (lanes G and H) were electrophoresed in 1.8% agarose-formaldehyde gels, processed for Northern hybridization as described and autoradiographed. Molecular size markers are indicated on the left.

results suggest that transcription of the *bstVIM* gene *in vivo* is restricted to vegetative growth. Thus, the putative P^E promoter identified for this gene would be not functional in *B. stearothermophilus* V.

Binding of RNA polymerase to *bstVIM* gene-control elements

The ability of *B. stearothermophilus* V RNA polymerase holoenzyme to recognize and bind specific DNA sequences upstream of the *bstVIM* gene was investigated by the gel-retardation assay described in Methods. As substrate, a ³²P-labelled *HindIII*-*SalI* DNA fragment (485 bp, containing the upstream region of *bstVIM* gene) was used (González & Vásquez, 1993).

RNA polymerases either from *E. coli* or V-state *B. stearothermophilus* V cells specifically bound the substrate DNA (Fig. 4, lanes A and B). Competitor DNAs lacking promoter structures did not affect the formation of protein/DNA complexes (Fig. 4, lane G). No binding was observed when the competitor DNA was the same unlabelled fragment (Fig. 4, lane E), or a *PstI*-*HindIII*

Fig. 4. Binding of different RNA polymerases to sequences upstream of the *bstVIM* gene. RNA polymerase holoenzymes from *E. coli* (lane A) and from *B. stearothermophilus* V at states V (lane B), E (lane C) and I (lane D) were incubated with the labelled probe DNA, electrophoresed in 5% (w/v) polyacrylamide gels and autoradiographed. In lanes E–G, polymerase/probe complexes were allowed to form as in B, but in the presence of an excess of the following competitor DNAs: unlabelled probe (lane E), a 646 bp *Pst*–*Hind*III fragment comprising the upstream region of the *bstVIR* gene (lane F) and a 843 bp *Sal*I–*Pst*I fragment containing part of the coding region of the *bstVIM* gene.

segment which contains the upstream region of the *bstVIR* gene (Fig. 4, lane F) (González & Vásquez, 1993). These results indicated that both holoenzymes interacted with the substrate DNA through the recognition of promoter structures. On the other hand, RNA polymerases isolated from E or I sporulation states failed to recognize either the P^A or P^E sequences found upstream of the *bstVIM* gene (Fig. 4, lanes C and D). These findings suggest that the core RNA polymerase from *B. stearothermophilus* V could bind different σ factors through the bacterial life cycle, thus allowing a differential gene expression.

Although the above results suggest that the putative P^E promoters were not functional during sporulation in *B. stearothermophilus* V, their presence upstream of *bstVIR* and *bstVIM* genes does not seem to be fortuitous. Further work is necessary, however, to unveil their role, if any, in the expression of these R and M genes. In this context, experiments to mobilize these genes into *B. subtilis*, and

thus analyse their expression in this bacterium, are now in progress.

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REFERENCES

- Barra, R., Chiong, M., González, E. & Vásquez, C. (1988). A DNA-modification methylase from *Bacillus stearothermophilus* V. *Biochem J* **255**, 699–703.
- Errington, J. (1993). *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. *Microbiol Rev* **57**, 1–33.
- Garner, M. & Revzin, A. (1988). Studying DNA-protein interactions by gel electrophoresis. *Focus* **10**, 26–28.
- González, E. & Vásquez, C. (1993). Characterization of the *bstVIRM* genes encoding the *Bacillus stearothermophilus* V restriction-modification system. *Gene* **101**, 103–106.
- Helmann, J. & Chamberlin, M. (1988). Structure and function of bacterial sigma factors. *Annu Rev Biochem* **57**, 839–872.
- Johnson, W., Moran, C. & Losick, R. (1983). Two RNA polymerase sigma factors from *Bacillus subtilis* discriminate between overlapping promoters for a developmentally regulated gene. *Nature* **302**, 800–804.
- Lobos, C. & Vásquez, C. (1993). Purification and characterisation of *Bst*LV1 restriction endonuclease, a thermostable isoschizomer of *Cla*I from *Bacillus stearothermophilus* LV. *Biochim Biophys Acta* **1171**, 295–298.
- Losick, R. & Pero, J. (1981). Cascades of sigma factors. *Cell* **25**, 582–584.
- Losick, R., Youngman, P. & Piggot, P. (1986). Genetics of endospore formation in *Bacillus subtilis*. *Annu Rev Genet* **20**, 625–669.
- Maniatis, T., Fritsch, E. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Piggot, P. & Coote, J. (1976). Genetic aspects of bacterial endospore formation. *Bacteriol Rev* **40**, 908–962.
- Vásquez, C. (1985). Isolation and partial characterization of *Bst*VI, a thermostable isoschizomer of *Xba*I. *Biochem Int* **10**, 655–662.
- Vásquez, C., Saavedra, C. & González, E. (1991). Cloning the *Bst*VI restriction-modification system in *Escherichia coli*. *Gene* **102**, 83–85.

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