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

E. González, C. Padilla, C. Saavedra and C. Vásquez

Author for correspondence: E. González. Tel: +56 71 227224 ext. 247. Fax: +56 71 224675.

The activity of BstVl 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 $\sigma$ 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 OD$_{660}$ of 1.2, cells were collected by centrifugation at 5000 $g$ and transferred to a sporulation medium, which contained K$_2$HPO$_4$ (6 g l$^{-1}$), (NH$_4$)$_2$SO$_4$ (1 g l$^{-1}$), MnSO$_4$ (1 g l$^{-1}$), KNO$_3$ (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
was determined by the protection assay described by Barra et al. (1988). The substrate was in both cases HindIII-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 arrangement. Finally, at the L-stage the mature endospore was 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·BstVI and M·BstVI 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 BstVI R-M enzymes was determined in extracts prepared from cells which were harvested at the sporulation states defined above. Both R·BstVI and M·BstVI 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·BstVI and 600 ± 64 U (mg protein)−1 for M·BstVI. About 10% of these values were found in E-stage 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 bstVIM genes at this stage.

Neither R·BstVI nor M·BstVI 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·BstVI and M·BstVI were detectable in extracts from V cells only.
Expression in vivo of the bstVIM gene

In addition to the putative control regions of the bstVIRAI genes described previously (González & Vásquez, 1993), sequence blocks showing high similarity to RNA polymerase $\sigma^k$-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 $\sigma$ 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 bst1 TM 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 $^{32}$P-labelled SalI–PstI DNA fragment (843 bp) which contains the coding region of the bst1 TM 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 C–H). 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 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 $^{32}$P-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. 2. Promoter structures upstream of bstVIR and bstVIM structural genes. P$^A$ and P$^E$ are promoters recognized by B. subtilis RNA polymerase $\sigma^A$ and $\sigma^E$ factors, respectively. See text for details. DNA sequences found upstream of the bstVIRM genes, which are analogous to P$^A$ and P$^E$ blocks, are indicated by one and two asterisks, respectively.

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.

Expression in vivo of the bstVIM gene (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·BstLV1 and M·BstLV1 from B. stearothermophilus LV (Lobos & Vásquez, 1993) was analysed (not shown).
E. GONZÁLEZ and OTHERS

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

ACKNOWLEDGEMENTS

This work was supported in part by Fondecyt grants 127/91 and 151/92.

REFERENCES


Received 13 September 1993; revised 4 January 1994; accepted 13 January 1994.