Response of the bvg regulon of *Bordetella pertussis* to different temperatures and short-term temperature shifts

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*Bordetella pertussis* produces a number of virulence factors whose expression is coordinately regulated by the *bvgAS* locus. Transcription of virulence genes is repressed by environmental factors such as low temperature (25°C) and chemical stimuli. Temperature shift of bacterial cultures from 25°C to 37°C activates two classes of *bvg*-regulated virulence genes: the early genes, which are activated within 10 min, and late genes, which require 2–4 h for activation. During the interval between the activation of the early and late genes, the intracellular concentration of BvgA increases 50-fold. It has been proposed that this increased concentration may be required for the activation of the late genes. Here we have analysed the response of the *bvg* locus to intermediate temperatures and to repeated temperature shifts. Temperature shifts of *B. pertussis* cultures from 22°C to 28°C, 32°C or 35°C resulted in the synthesis of low, intermediate, and high amounts of BvgA. This implied that the intracellular concentration of BvgA is temperature-dependent. We have also observed that the amount of virulence factors produced correlates with the BvgA concentration. When bacteria grown at 37°C were shifted to 22°C, transcription from the adenylate cyclase toxin haemolysis promoter (PAC) was repressed after 30 min, while transcription from the *bvg* (Pvg) and filamentous haemagglutinin (Pfha) promoters was repressed after 2 h. During this time, the amount of BvgA did not decrease. A subsequent temperature shift from 22°C to 37°C induced transcription from the Pvg and Pfha promoters after 10 min and transcription from the PAC promoter after 20 min. This result shows that in the presence of a high concentration of BvgA, the time lag between temperature shift and late promoter transcription is reduced from 2–4 h to 20 min. The above data support the proposal that the concentration of BvgA plays a role in activating expression of the late genes.

**Keywords**: *bvg* locus, environmental regulation, thermoregulation, virulence genes, *Bordetella pertussis*

**INTRODUCTION**

The virulence genes of *Bordetella pertussis*, the human pathogen which causes whooping cough, are coordinately regulated by a sensory transduction system encoded by the *bvg* locus (Aricò et al., 1989; Stibitz & Yang, 1991; Weiss & Hewlett, 1986). This locus codes for two proteins, BvgA and BvgS, which are members of the two-component family of bacterial signal transduction proteins (Aricò et al., 1989; Coote, 1991; Miller et al., 1989a; Scarlato et al., 1993a; Stock et al., 1990). Transcription of the pertussis toxin operon (*ptx*), the adenylate cyclase toxin haemolysin gene (*cyaA*), the filamentous haemagglutinin gene (*phaB*), and others, requires the *bvgAS* gene products in trans (Domenighini et al., 1990; Gross et al., 1989; Laioide & Ullmann, 1990; Miller et al., 1989b; Roy et al., 1989, 1990; Scarlato et al., 1990). Transcription of the *bvg* locus is controlled by three autoregulated promoters (P₁, P₃, P₄) and one *bvg*-independent promoter (P₂) (Scarlato et al., 1990). The P₁, P₂ and P₃ promoters direct mRNA synthesis of the *bvg* operon and the P₄ promoter directs the synthesis of an anti-sense RNA strand of unknown function which has its
5'-end region complementary to the 5' untranslated region of the bvg mRNAs. The bvg-dependent transcription is induced at 35 °C and is repressed at a low temperature (25 °C), or in the presence of MgSO₄ or nicotinic acid in the culture medium (Domenighini et al., 1990; Gross & Rappuoli, 1988; Gross et al., 1989; Laode & Ullmann, 1990; Millet et al., 1989b).

bvgS encodes a 135 kDa sensory protein with a hydrophobic transmembrane sequence and an N-terminal region that is localized in the periplasm, as demonstrated by the analysis of alkaline phosphatase fusion proteins (Stibitz & Yang, 1991). The N-terminal periplasmic domain of BvgS is involved in signal recognition; via phosphorylation, it transduces the signals into the transcriptional regulation network (Uhl & Miller, 1994). Evidence that BvgS is involved in signalling events is provided by the finding of a class of mutations that make the system insensitive to environmental regulation (Miller et al., 1992; Manetti et al., 1994; Goyard et al., 1994).

bvgA encodes a 23 kDa transcriptional activator that binds upstream from the P₁ promoter of the bvg locus and upstream from the fhaB gene in vitro (Roy & Falkow, 1991) but does not bind upstream from the ptx and cyaA promoters, which are also regulated by BvgA. The same promoters are also activated differently in vivo. In fact, following a temperature shift of bacterial cultures from 25 °C to 35 °C, the P₁ and the fhaB promoters are activated within 10 min, while the ptx and cyaA promoters require 2-4 h for activation. Because of this behaviour, the bvg-activated genes have been divided into early and late, respectively.

During the interval between the activation of the early and late genes, the intracellular concentration of BvgA increases 50-fold. It has been proposed that this increased concentration may be required for the activation of the late genes. The difference between early and late genes can be observed also when the system is cloned in E. coli: the early promoters are readily activated by the bvg locus in trans, while the transcription of the late promoters depends upon the cloning configuration of the promoter (Scarlatò et al., 1993b). Further evidence of the differential recognition of the two classes of promoters by BvgA has been provided by single-amino-acid mutations in BvgA which affect the recognition of early and late promoters differently (Stibitz, 1994).

In this study we used growth at different temperatures and repeated temperature shifts to study the behaviour of early and late promoters and to ask whether a high concentration of BvgA is required for the expression of the late genes. The data obtained concord with the proposal that the expression of late genes requires a high concentration of BvgA.

**METHODS**

**Materials.** Restriction enzymes, calf intestinal alkaline phosphatase, T4 polynucleotide kinase and S1 nuclease were purchased from Boehringer Mannheim or New England Biolabs, and were used according to the manufacturer's specifications. Radiochemicals were from Amersham.

**Bacterial strains and growth conditions.** B. pertussis strain BPW28 (Bvg' SmR) (Aricò et al., 1993) was grown on BG agar plates containing 15% (v/v) defibrinated blood (Pittman, 1984) or in SS-modified liquid medium (Stainer & Scholte, 1970). E. coli DH5α was used for plasmid preparations and was grown on LB agar or in LB broth (Sambrook et al., 1989). Antibiotics were used at the following concentrations: ampicillin, 100 µg ml⁻¹; streptomycin, 400 µg ml⁻¹.

**Plasmids and probes.** The plasmids pFHA/EP, pVR/EP and pAD/BS are pGem3 (Promega) derivatives and have been described by Scarlatò et al. (1990, 1991). The probes used for S1 nuclease assays are described in the legend to Fig. 3. All DNA manipulations were carried out by standard techniques (Sambrook et al., 1989).

**RNA isolation.** B. pertussis was grown overnight with shaking in a 100 ml flask containing 10 ml SS-modified medium (Stainer & Scholte, 1970) at 35 °C, then diluted to an OD₅₀₀ of 0.1 and grown to mid-exponential phase. Bacteria (25 ml) were harvested at appropriate times and stored at −20 °C. Cells were lysed in 3.7 ml 100 mM Tris/HCl, pH 7.5, 1% (w/v) SDS, 2 mM Na₂EDTA for 5 min at 100 °C. After 5 min on ice in the presence of 75 mM KCl, cell debris was removed by spinning at 8000 r.p.m. for 10 min in a JA20 rotor. To 3.5 ml of sample, 4.56 g CsCl was added and the RNA was pelleted by sedimentation in an SW65 rotor at 35000 r.p.m. for about 20 h. The pellet was resuspended in 500 µl TE (10 mM Tris/HCl, pH 8.0, 1 mM EDTA), extracted with an equal volume of phenol/chloroform, ethanol precipitated, resuspended in 200 µl TE, reprecipitated with ethanol, and stored at −20 °C.

**S1 nuclease mapping.** S1 protection assays (Berk & Sharp, 1977) were performed by combining 6 µg RNA samples and about 20 fmol specific 5'-end labelled DNA probe (Fig. 3). The mixture was ethanol precipitated in the presence of 1 µg E. coli tRNA and 1 M LiCl and processed as previously described by Scarlatò et al. (1990, 1991). Hybridization was performed in a water bath for 16 h at appropriate temperatures (see legend to Fig. 4).

**Immunoblots.** Bacteria from 15 ml culture were harvested and resuspended in 25% (w/v) saccharose, 50 mM Tris/HCl, pH 8.0, at an expected OD₅₀₀ of 0.3. The suspension (20 µl) was loaded on 12.5% (w/v) SDS-PAGE gels, subjected to electrophoresis and blotted to nitrocellulose for immunoblot analysis. Western blots were processed by standard procedures and stained with the chemiluminescence detection system (Amersham). BvgA was detected with a 1:2500 dilution of a polyclonal mouse antiserum raised against the purified protein (Scarlatò et al., 1990).

**RESULTS**

**Accumulation of BvgA correlates with the growth temperature.** To investigate whether at intermediate temperatures B. pertussis expresses intermediate amounts of BvgA and virulence factors, cells were grown to exponential phase at 22 °C for 7–10 d, and then shifted to 25 °C, 28 °C, 32 °C or 35 °C for 8 h. Aliquots of bacteria were withdrawn after 8 h and tested by Western blotting to evaluate the amount of BvgA and the outer-membrane protein ptx.

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A large amount of BvgA shortens the time response of the late-induced genes

To further study the role of BvgA concentration in late-gene activation, we designed a time-course experiment of temperature variation by shifting bacterial cultures from 37 °C to 25 °C for 2 h and then again to 37 °C for an additional 2 h. Aliquots of bacterial samples were harvested at 37 °C, after 10, 30 and 120 min at 25 °C, and after 10, 20, 60 and 120 min at 37 °C. Total protein extracts and RNA samples were extracted to evaluate the amount of BvgA and the pattern of RNA accumulation of the bvg-regulated promoters, respectively.

First we used Western blotting to analyse whether the amount of BvgA varied during the course of the experiment. Fig. 2 shows that the amount of BvgA did not decrease in a detectable manner during the 2 h at 25 °C, and no increase was found when the cells were shifted again at 37 °C. Then we used S1 nuclease mapping to study the transcriptional regulation of the bvg-regulated promoters in the cells that had undergone a temperature shift while the concentration of BvgA was constant. DNA probes (Fig. 3) were 5'-end labelled, hybridized with total RNA, digested with S1 nuclease (Berk & Sharp, 1977) and the S1-resistant DNA–RNA duplexes were sized by polyacrylamide/urea gel electrophoresis.

Fig. 4 shows the electrophoretic pattern of signals generated by the P$_{AS}$ (a), P$_{FHA}$ (b) and P$_{AC}$-associated (c) transcripts. As expected, the P$_{1}$, P$_{3}$, P$_{FHA}$ and P$_{AC}$ promoters were transcriptionally active at 37 °C (lanes 4), while transcription from the P$_{2}$ promoter was repressed (Fig. 4a, lane 4). Following a temperature transition from 37 °C to 25 °C, the pattern of RNA accumulation associated with these promoters changed in the following 2 h at 25 °C. The amounts of the P$_{1}$ (Fig. 4a) and P$_{FHA}$ (Fig. 4b) RNAs decreased after 120 min at 25 °C (lanes 7), the amount of the P$_{AC}$ RNA decreased after 30 min (Fig. 4c, lane 6), while the amount of the P$_{2}$ RNA was already detectable after 10 min at 25 °C (Fig. 4a, lane 5).

A quantitative analysis of the patterns of RNA accumulation associated with these promoters after a temperature shift from 37 °C to 25 °C is reported in Fig. 5. Surprisingly, the amount of the P$_{2}$, P$_{FHA}$, and P$_{AC}$-associated RNA increased after 10 min at 25 °C. The P$_{FHA}$-associated RNA also increased after 30 min at 25 °C, while at this time the amount of the P$_{2}$ and P$_{AC}$ RNAs decreased. From the slope of the curves shown in Fig. 5 it can be deduced that between 10 and 30 min the amount of the P$_{AC}$ RNA decreased with a half-life of 9 min. The amounts
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Fig. 4. Time course of transcription for promoters $P_1$, $P_{PHA}$ and $P_{AC}$ upon temperature shifts from 37 °C to 25 °C to 37 °C. (a) $P_1$. The Avall–PvuII DNA fragment (273 bp) from plasmid pVIR/EP was used for hybridization and S1 nuclease digestion analysis. The hybridization temperature was 48 °C. (b) $P_{PHA}$. The MluI–EcoRI fragment (356 bp) of plasmid pPHA/EP was used as probe. The hybridization temperature was 54 °C. (c) $P_{AC}$. The Sall–BamHI fragment (513 bp) of plasmid pAD/B5 was used as a probe. The hybridization temperature was 55 °C. RNAs were collected at the indicated times after temperature shifts. Control samples were processed identically but contained no RNA, and were (lanes C), or were not (lanes P), digested with S1 nuclease. A denatured 1 kb ladder (Gibco-BRL) served as size markers (lanes M).

DISCUSSION

Following the discovery that the virulence genes can be divided into early- and late-induced genes, a number of studies have tried to address the question of how one transcriptional activator may differentially activate two types of promoters. Since this behaviour has never been described for other transcriptional activators, the question is very interesting, not only for B. pertussis, but it may provide a better understanding of the basic mechanisms of gene transactivation. So far, two major hypotheses have been proposed. One claims that transcription of the early genes is mediated directly by BvgA, while transcription of the late genes may require a second molecule specific for the late genes. To demonstrate this hypothesis, extensive studies have been performed to identify, either by expression cloning or by classical genetics, the putative factor responsible for the activation of the late genes. So far these studies have been inconclusive. The only factors that have been found to have an effect on the transcription of the late genes are mutations in the $\sigma$ subunit of the RNA polymerase. These mutants play a general role in transcription, but are not specific for the late genes (Carbonetti et al., 1994).

The second hypothesis to explain the differential regulation of early and late genes proposed that both early and late genes were regulated directly by BvgA, without the need of additional specific factors. The hypothesis claimed that early genes could be activated by a very low concentration of BvgA, such as that present when the bacteria are grown for a long period of time at 22 °C, while activation of the late genes required large amounts of BvgA, which are present only in bacteria grown for a long period of time at 35 °C. This theory was initially supported only by the observation that bacteria grown at 22 °C have a basal level of BvgA which, following temperature shift to 37 °C, increases progressively with time, until it reaches a concentration that is 50-fold the
initial one. Transcription of the late genes initiates when the concentration of BvgA is 20-fold over the basal level and then increases proportionally with the concentration of BvgA. Today, this theory is supported by several other experimental observations. For instance, the presence of two copies of the bvgA locus in B. pertussis was sufficient to increase the basal level of BvgA and to induce the expression of pertussis toxin at 25 °C (Scarlato et al., 1991). Then it was shown that the pertussis toxin promoter can be activated in E. coli by the bvgA locus alone, without additional factors deriving from B. pertussis (Scarlato et al., 1993b). Finally, it was shown that mutations in the bvgA gene could affect only the transcription of the late genes (Stibitz, 1994). The above data support, but do not prove, the proposal that BvgA is sufficient to activate both early and late promoters. Moreover, they suggest that BvgA has a high affinity for the early promoters, so that BvgA can bind them at low concentration, while BvgA has a low affinity for late promoters and can bind them only when it is present in high amounts.

In this work, we have provided additional evidence that BvgA may be the sole regulator of early and late genes, and that a high concentration of BvgA may be necessary for the activation of the late genes. In fact, we have shown that the amount of BvgA protein present in the cells is temperature-dependent. By Western blot analysis, we also assayed the expression of the products coded by the late genes and found that it correlates with the concentration of BvgA (data not shown). In addition, when BvgA was kept constantly high, we have been able to show that the time required to activate the late genes can be shortened to 20 min. These data strongly support the theory that BvgA is sufficient for the activation of both early and late genes, and encourage studies to understand the molecular basis of this extremely interesting mechanism of gene transactivation. It is likely that a fine regulation of the two classes of promoters is at the level of BvgA phosphorylation.

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REFERENCES


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