Temporal activation of β -glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool

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β -Glucanase synthesis was temporally activated in *Bacillus subtilis* at the onset of stationary phase. This regulation was dependent upon a drop in the GTP concentration in response to nutrient limitation. The Spo0A and AbrB proteins were involved in the GTP-dependent temporal activation.

Introduction

In *Bacillus subtilis* nutrient limitations trigger the production of numerous extracellular and intracellular enzymes, possibly in order to scavenge alternative nutrient sources. Many of the genes encoding these enzymes are subject to carbon-catabolite repression (for a review see Fisher & Sonenshein, 1991) and are temporally activated when the cells enter the stationary phase after exhaustion of readily metabolizable nutrients (reviewed by Priest, 1977).

We are interested in the signal chain that leads from a nutrient limitation to the temporal activation of the extracellular enzyme β -glucanase (endo- β -1,3-1,4-glucanase, EC 3.2.1.73), the product of the *bglS* gene. The *bglS* gene has been cloned (Cantwell & McConnell, 1983) and sequenced (Murphy *et al.*, 1984). Here we show that the temporal activation of β -glucanase synthesis is a result of a sharp decrease in the intracellular GTP concentration after a nutrient limitation.

Methods

Bacterial strains. All *B. subtilis* strains used in this study are listed in Table 1. The genetic markers of the strains were verified by uridine incorporation experiments for the *relA* allele and by a test of sporulation for the *spo0A* allele.

Media and growth conditions. Cells of *B. subtilis* were grown at 37 °C in basal limitation medium (BLM) containing nutrients according to the type of limitation required. BLM consists of 50 mM-Tris, 15 mM-(NH₄)₂SO₄, 8 mM-MgSO₄, 27 mM-KCl and 7 mM-sodium citrate

(pH 7.5). The following substances were added from separate stock solutions: CaCl₂ to 2 mM, FeSO₄ to 1 μ M, MnSO₄ to 10 μ M and potassium glutamate to 4.5 mM. Amino acid starvation medium (ASM) contained 0.1% glucose, 0.4 mM KH₂PO₄ and 12.8 μ g ml⁻¹ of each of the required amino acids according to the auxotrophies of the strains. Glucose limitation medium (GLM) was supplemented with 0.05% glucose, 0.4 mM-KH₂PO₄ and 160 μ g ml⁻¹ of each of the required amino acids. All media were balanced to support growth to an OD₅₅₀ of 1.2. The actual starvations were regularly verified by the re-addition of the exhausted nutrient and re-initiation of growth.

For the experiments, 10 ml of medium (BLM containing 0.1% glucose, 0.6 mM-KH₂PO₄, and 160 μ g ml⁻¹ of each of the required amino acids) was inoculated with one glycerol stock culture. A dilution series of this culture was incubated overnight, and an exponentially growing culture from this series was used as inoculum for the experiments. Experiments were performed in 50 ml batches in 500 ml flasks under vigorous agitation (200 r.p.m.).

Determination of enzyme activity. β -Glucanase activity was assayed by determination of reducing sugar liberated from lichenan by the dinitrosalicylic acid method (Borriess *et al.*, 1980). One enzyme unit was defined as 1 mol glucose equivalent produced (OD₅₅₀ unit)⁻¹ ml⁻¹ and s⁻¹ (= 1 kat). Each enzyme assay was repeated twice. Experiments were performed in duplicate or triplicate. The deviation of results of different experiments was up to 5%. Representative results are shown in the figures.

Determination of nucleotide pools. Cultures were labelled with ³²PO₄³⁻ (100 μ Ci ml⁻¹; 200 mCi mmol⁻¹, 7.4 GBq mmol⁻¹). Nucleotides were

Table 1. *B. subtilis* strains used in this study

Strain	Genotype or phenotype	Reference
IS56	<i>trpC2 lys-3 relA</i>	Smith <i>et al.</i> (1980)
IS58	<i>trpC2 lys-3</i>	Smith <i>et al.</i> (1980)
JH642	<i>trpC2 phe</i>	Hoch & Mathews (1973)
JH646	<i>trpC2 phe spo0A</i>	Hoch & Mathews (1973)
ZB449	<i>trpC2 phe abrB703</i>	Zuber & Losick (1987)

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Fig. 1. Growth, intracellular concentration of GTP and β -glucanase synthesis in *B. subtilis* IS58 (*relA*⁺, $\square$, $\blacksquare$) and IS56 (*relA*⁻, $\triangle$, $\blacktriangle$) after glucose (a, b) and amino acid limitations (c, d). Cells were grown in GLM or ASM, respectively, and samples were taken as described in the text. $\square$, $\triangle$, OD₅₅₀; $\blacksquare$, $\blacktriangle$, GTP concentration (a, c) or β -glucanase activity (b, d).

extracted using formic acid, analysed by thin-layer chromatography and quantified as described previously (Hecker *et al.*, 1983).

Results and Discussion

Correlation of growth phase, nucleotide pools and β -glucanase synthesis

Many authors have presented evidence suggesting that the processes which occur at the onset of the stationary phase are paralleled by an accumulation of the highly phosphorylated guanine nucleotides (p)ppGpp and a sharp decrease of the intracellular GTP concentration (Lopez *et al.*, 1981; Riedel *et al.*, 1987). There are some indications that this decrease is a prerequisite for many

of the processes after the cessation of growth (Freese *et al.*, 1979; Nicholson & Chambliss, 1987). In *B. subtilis*, the synthesis of (p)ppGpp causes a drastic drop in the GTP concentration (Lopez *et al.*, 1979).

To investigate the involvement of the stringent response in the expression of the *bglS* gene, the synthesis of β -glucanase in the wild-type strain IS58 and in the *relA* mutant IS56 was compared. Cells were grown in ASM and GLM respectively, and enzyme activities were assayed. In both strains the β -glucanase activity increased only when the cells stopped growth after either glucose limitation or amino acid starvation (Fig. 1b, d). These results indicate that the presence of the *relA* allele does not cause a difference in the expression of β -glucanase.

Fig. 2. β -Glucanase synthesis in strain IS58 (*relA*⁺) grown in ASM ($\square$, $\blacksquare$). Mid-exponential cultures were divided and decoyinine (a) ($\diamond$, $\blacklozenge$), or either guanine ($\triangle$, $\blacktriangle$) or guanosine ($\circ$, $\bullet$) (b) was added to parts of the culture (indicated by arrows). $\square$, $\diamond$, $\triangle$, $\circ$, OD₅₅₀; $\blacksquare$, $\blacklozenge$, $\blacktriangle$, $\bullet$, β -glucanase activity.

To determine if there was concomitance of temporal activation and a decrease in the GTP concentration, the nucleotide pools of the stringent and the relaxed strains were analysed. IS58 (*Rel*⁺) and IS56 (*relA*) were grown in either ASM or GLM. Nutrient limitations resulted in a sharp decrease of intracellular GTP concentration. This applied to both strains, but the drop was slightly more pronounced in the wild-type strain (Fig. 1*a, c*). Simultaneously, both ATP and phosphoribosyl phosphate concentrations dropped in both strains (data not shown).

Manipulation of purine nucleotide concentration

In order to verify the significance of the GTP pool for temporal activation, the GTP concentration was manipulated by adding to exponential-phase cultures either decoyinine, an inhibitor of GMP synthetase (Mitani *et al.*, 1977), or purine nucleotides. The responses of β -glucanase synthesis to these manipulations were measured.

Decoyinine (final concentration 1 mg ml⁻¹) was added to cultures of IS58 grown in the mid-exponential phase of growth (OD₅₅₀ = 0.5) in a non-repressing medium (ASM). This resulted in an immediate cessation of growth and in the production of β -glucanase (Fig. 2*a*). This experiment could not prove whether growth inhibition or the drop of the GTP concentration was responsible for temporal activation of β -glucanase. Therefore either guanine or guanosine was added to mid-

exponential cultures of *B. subtilis* IS58 (OD₅₅₀ = 0.6) to 1 mM, to prevent a drop of the GTP concentration at the end of growth. In both cases, the addition resulted in a complete loss of temporal activation (Fig. 2*b*). To obtain further evidence for the involvement of guanine nucleotides the effect of adding adenosine to the culture was investigated. Adenosine addition did not influence the kinetics of β -glucanase synthesis (data not shown).

Involvement of the *spo0A* and *abrB* gene products

It is well known that the Spo0A protein is involved in signalling nutrient starvation (Grossman, 1991). As a first step in the elucidation of the regulatory pathway from the signal (drop of GTP concentration) to the expression of the β -glucanase gene, the involvement of the *spo0A* gene in this pathway was addressed.

Strain JH646 (*spo0A*) produced significantly less β -glucanase than its isogenic parent JH642 in both ASM and GLM. Furthermore, the synthesis of the enzyme after the onset of stationary phase was delayed in the *spo0A* strain by 2.5 h compared to JH642 (Fig. 3*a*).

To obtain further evidence for the involvement of the *spo0A* gene product in GTP-dependent regulation of β -glucanase, enzyme synthesis by JH646 after the addition of decoyinine was measured. No stimulation of enzyme synthesis was observed (Fig. 3*b*).

The Spo0A protein is a repressor of the *abrB* gene (Strauch *et al.*, 1990), whose product in turn represses

Fig. 3. (a) β -Glucanase synthesis during growth in strains JH642 (*spo0A*⁺ *abrB*⁺) (∇ , $\blacktriangledown$), JH646 (*spo0A*, $\triangle$, $\blacktriangle$) and ZB449 (*abrB*, $\square$, $\blacksquare$). Bacteria were cultivated in ASM. (b) Decoyinine was added to late-exponential cultures of JH646 ($\diamond$, $\blacklozenge$) as indicated by the arrow. ∇ , $\triangle$, $\square$, $\diamond$, OD₅₅₀; $\blacktriangledown$, $\blacktriangle$, $\blacksquare$, $\blacklozenge$, β -glucanase activity.

many genes during exponential growth (Robertson *et al.*, 1989). The *abrB* mutant strain ZB449 showed a fivefold greater activity of β -glucanase during exponential growth than did the isogenic wild-type JH642 (Fig. 3a).

Concluding remarks

We have shown that the expression of *B. subtilis* β -glucanase is regulated by GTP-concentration-dependent temporal activation. A role for GTP has also been implicated in the regulation of α -amylase synthesis (Nicholson & Chambliss, 1987).

Whereas an inhibition of guanine nucleotide synthesis led to the induction of β -glucanase synthesis, synthesis of the enzyme was abolished by addition of a guanine nucleotide. These studies allowed the possible effect of the cessation of growth to be distinguished from the effect of a drop of GTP concentration.

The data presented here clearly demonstrate that the decrease in the intracellular GTP concentration at the end of exponential growth is the necessary prerequisite for temporal activation of β -glucanase synthesis. The *spo0A* and *abrB* genes are probably involved in the signal transduction pathway leading from the decreased intracellular GTP concentration to temporal activation of β -glucanase synthesis.

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