Antiterminator protein GlpP of *Bacillus subtilis* binds to *glpD* leader mRNA

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The *Bacillus subtilis* *glpD* gene encodes glycerol-3-phosphate (G3P) dehydrogenase. Expression of *glpD* is mainly controlled by termination/antitermination of transcription at an inverted repeat in the *glpD* leader. Antitermination is mediated by the antiterminator protein GlpP in the presence of G3P. In this paper, interaction between GlpP and *glpD* leader mRNA *in vivo* and *in vitro* is reported. *In vivo*, the antiterminating effect of GlpP can be titrated in a strain carrying the *glpD* leader on a plasmid. GlpP has been purified and gel shift experiments have shown that it binds to *glpD* leader mRNA *in vitro*. GlpP is not similar to other known antiterminator proteins, but database searches have revealed an *Escherichia coli* ORF which has a high degree of similarity to GlpP.

Keywords: antitermination, GlpP, mRNA binding, glycerol-3-phosphate dehydrogenase, *Bacillus subtilis*

INTRODUCTION

Control of gene activity by premature termination of transcription and various mechanisms of antitermination were originally discovered in phage λ (Roberts, 1988) but are also found increasingly among bacteria. Attenuation in amino acid biosynthetic operons was the first bacterial termination/antitermination system to be described (Yanofsky & Crawford, 1987). Here, the frequency of translation of a short peptide encoded by the mRNA leader determines whether a full-length or a truncated transcript is produced by affecting the formation of alternative secondary structures in the leader. A mechanistically similar mode of regulation has been found for aminoacyl-tRNA synthetases, for example, where binding of uncharged tRNA to the mRNA leader of the cognate aminoacyl synthetase gene prevents formation of a transcriptional stop signal in the leader (Henkin, 1994). Expression of a number of catabolic operons in *Bacillus subtilis* and other bacteria is controlled by termination/antitermination of transcription, where binding of an activated antiterminator protein permits readthrough of a transcriptional stop signal (Rutberg, 1997).

The *B. subtilis* *glp* regulon is involved in the catabolism of glycerol and glycerol 3-phosphate (G3P). The regulon

contains six genes which constitute four operons: *glpP*, *glpFK*, *glpD* and *glpTQ* (Lindgren, 1978; Holmberg & Rutberg, 1989; Holmberg *et al.*, 1990; Beijer *et al.*, 1993; Nilsson *et al.*, 1994). The operons all have constitutive σ^A -type promoters. The leaders of the *glpFK*, *glpD* and *glpTQ* operons contain inverted repeats followed by runs of ATs, i.e. sequences with the potential to form transcription terminators. Sequence comparisons between the *glpFK*, *glpD* and *glpTQ* leaders reveal several conserved regions (Nilsson, 1995). The inverted repeat of the *glpD* leader functions as a transcription terminator *in vitro* (Holmberg & Rutberg, 1991, 1992). *glpP* encodes an antiterminator protein which, together with G3P, induces readthrough of the transcription terminators of *glpFK*, *glpD* and *glpTQ*. Besides causing antitermination, GlpP is also involved in stabilization of *glpD* mRNA and in glucose repression of *glp* genes (Glatz *et al.*, 1996). We have proposed that binding of GlpP to *glp* mRNA is essential for antitermination and mRNA stabilization. In the present paper, we present evidence that GlpP binds specifically to the leader of *glpD* mRNA *in vivo* and *in vitro*.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Media, transformation and DNA techniques. These are described by Glatz *et al.* (1996). *amyE* activity was checked on

Abbreviations: G3P, glycerol 3-phosphate; RAT, ribonucleotide antiterminator.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/phenotype	Source/reference
Strains		
<i>B. subtilis</i>		
BR95	<i>trpC2 pheA1 ilvC1</i>	Our collection
LUZ9595	<i>trpC2 pheA1 ilvC1 ΔamyE::glpD leader-lacZ</i> ; Km ^R	This work
LUG0402	<i>trpC2 pheA1 ilvC1 ΔglpP</i> ; Pm ^R	Beijer <i>et al.</i> (1993)
<i>E. coli</i>		
XL-1 Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lacF'[proAB⁺ lacI^q lacZΔ M15 Tn10 (tet^r)]</i>	Bullock <i>et al.</i> (1987)
MM294	<i>pro thi endA hsr_k hsr_{m+}</i>	Amann <i>et al.</i> (1983)
Plasmids		
pHP13	Cm ^R Em ^R	Haima <i>et al.</i> (1987)
pMD433	Km ^R Ap ^{R*} ; <i>ΔamyE::lacZ</i>	Dahl & Meinhof (1994)
pGDV1	Cm ^R	Bron (1990)
pLUM1041	Km ^R Ap ^R ; <i>'lacZ (glpD leader from BR95 cloned in front of and in-frame with lacZ in pMD433)</i>	This work
pLUM1047	T7 promoter <i>glpD leader</i> ; Cm ^R Em ^R	This work
pGDV11	<i>glpD leader from BR95</i> ; Cm ^R	This work
pGDV14	<i>glpD leader without -10 and -35 promoter regions</i> ; Cm ^R	This work
pPHis1	<i>glpP-His</i> ; Cm ^R Em ^R	This work
pPHis11	<i>glpP-His</i> ; Cm ^R	This work

* Ampicillin resistance expressed in *E. coli*.

Fig. 1. (a) Schematic representation of the *glpK-glpD* region. Plasmid inserts are indicated by thick lines. The insert of pLUM1047 starts with the T7 promoter which is not shown in the figure. The line for pLUM1047 also denotes the size of the run-off product when linearized pLUM1047 is used as template for *in vitro* transcription with T7 RNA polymerase. (b) DNA sequence of the *glpD* promoter and leader. The thick arrows indicate the inverted repeat that gives rise to a transcription terminator. The thin arrows indicate a sequence that has some similarity to RAT sequences (Fig. 6). The resulting RNA secondary structure is proposed to be a target for GlpP. The arrowheads denote the 5'-ends of the inserts of pGDV14 and pLUM1047 (excluding the T7 promoter). The GGATCC at 121-126 is the BamHI site that was introduced into pLUM1047 to allow linearization of the plasmid before it was used in *in vitro* transcription.

Tryptose Blood Agar Base plates containing 1.5% starch. After incubation for 2 d, the plates were sprayed with an iodine solution (1 g iodine and 2 g potassium iodide in 300 ml distilled water). Electroporation of *Escherichia coli* was performed according to Dower *et al.* (1988).

Construction of plasmids and *B. subtilis* LUZ9595. Fig. 1 shows the inserts of plasmids pGDV11, pGDV14, pLUM1041 and pLUM1047. Primers used are listed in Table 2.

Plasmids pGDV11 and pGDV14 were constructed as follows. The *glpD* leader from BR95 was amplified by PCR using primers GlpK3 and pDBam. The fragment obtained was cloned into pGDV1 between the *Eco*RI and *Bam*HI sites to give plasmid pGDV11. To get a promoterless *glpD* leader, a fragment was amplified from BR95 chromosomal DNA using primers UTLEco and pDBam. The *Eco*RI site of UTLEco is located at the -10 position. The fragment was cloned into pGDV1 between the *Eco*RI and *Bam*HI sites, giving plasmid pGDV14.

Plasmid pLUM1041 was constructed as follows. Primers GlpDBamI and GlpDBamII were used to amplify a 400 bp fragment from BR95 chromosomal DNA. This fragment includes the *glpD* promoter and leader and the sequence encoding the first 33 aa of GlpD. The fragment was ligated to pMD433 which had been cleaved with *Bam*HI and treated with shrimp alkaline phosphatase. The ligate was introduced into *E. coli* XL-1 Blue. Transformants were selected on TBAB plates containing ampicillin (75 µg ml⁻¹) and X-Gal (40 µg ml⁻¹). Blue colonies were picked, plasmids were isolated, and a plasmid containing an insert of the expected size was selected and named pLUM1041. The insert was sequenced to verify that it was correctly oriented in relation to *lacZ*.

Table 2. Primers used in PCR

Restriction sites are underlined. The T7 promoter and the His-tag sequence are italicized.

Primer	Sequence	Restriction site and other features
pT7Eco	5'-ATGAATTCGCGTAATACGACTCACTATAGAGTTAATAAGAACGGTCCTGAGATGAGG-3'	<i>Eco</i> RI, T7 promoter
pDBam	5'-ATTGAGGATCCATCATTACGTTTCCTCC-3'	<i>Bam</i> HI
HisC3	5'-CCGGATCCAACAATGTCATCCACGTTGC-3'	<i>Bam</i> HI
HisC4	5'-AGAAATCTCAGTGATGGTGATGGTGATGATCACTTTCCGTCAAAAAG-3'	<i>Eco</i> RI, six His anticodons
GlpK3	5'-GTTCTGAATTCGCGAACCAGTG-3'	<i>Eco</i> RI
UTLEco	5'-CCGAATTCGGTTACAAGTTAATAAGAACGGTCC-3'	<i>Eco</i> RI
GlpDBamI	5'-CTGTGGGATCCTGGATCCTGGAAGGACC-3'	<i>Bam</i> HI
GlpDBamII	5'-GCTGTTCCGGATCCTGTAATTC-3'	<i>Bam</i> HI
GlpDI	5'-CGACCTGCAGTCCCCTTGATG-3'	<i>Pst</i> I

To construct LUZ9595, *B. subtilis* BR95 was transformed with pLUM1041, which cannot replicate in *B. subtilis* but can integrate into *amyE*. The transformants were tested for amylase activity; one amylase-negative transformant was chosen and the strain derived from it was named LUZ9595. β -Galactosidase activities of LUZ9595 were induced by glycerol.

Plasmids pPHis1 and pPHis11 were constructed as follows. *glpP* was His-tagged and amplified using primers HisC3 and HisC4. The resulting fragment was inserted between the *Bam*HI and *Eco*RI sites in pHP13 (giving pPHis1) or pGDV1 (giving pPHis11).

Plasmid pLUM1047 was constructed as follows. PCR was performed with the primers pT7Eco and pDBam. The resulting fragment was cloned into pHP13 between the *Eco*RI and *Bam*HI sites. The 5' part of the primer pT7Eco contains AT followed by an *Eco*RI site and 21 bases of the T7 promoter including the T7 polymerase startpoint (G). Then there is a sequence that is complementary to the +2 to +29 bp of the *glpD* leader. The second primer, pDBam, was positioned at the *glpD* start codon. A *Bam*HI site, just downstream of ATG, was introduced into the primer as shown in the sequence (Table 2). This site was used to linearize the plasmid for use in *in vitro* transcription.

Growth of cells, preparation of cell extracts and enzyme assays. *B. subtilis* was grown and cell extracts were prepared as described previously (Glatz *et al.*, 1996). When G3P dehydrogenase activity was measured in strain LUG0402, the cells were induced with G3P instead of glycerol, as this strain lacks the glycerol uptake facilitator. β -Galactosidase activity was measured as follows. Cell extract (80 μ l) was diluted in 720 μ l Z-buffer (Miller, 1972) containing 0.05 M β -mercaptoethanol in a 1 ml cuvette. Two hundred microlitres of ONPG (4 mg ml⁻¹ in Z-buffer) was added and the change in A_{420} was measured for 60 s. G3P dehydrogenase activity was measured as described by Lin *et al.* (1962). The protein concentration of cell extracts was determined with BCA Protein Assay reagent (Pierce), using BSA as the standard.

Purification of GlpP. To purify GlpP-His from *B. subtilis* BR95(pPHis11), cells were grown as described previously

(Glatz *et al.*, 1996) except that G3P was added to the culture at OD₆₀₀ 0.5 (Hitachi U-1100). After 2 h, the cells were spun down at 3600 g for 20 min and then resuspended in 10 ml of 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 0.1% Tween 20 (sonication buffer) per g of pellet. The cells were incubated with lysozyme (4 mg ml⁻¹) at 37 °C for 30 min before they were disrupted by two passages through an Aminco French press cell at 16000 p.s.i. (110400 kPa). The extract was sonicated for 2 min, centrifuged at 20000 g for 20 min and loaded onto a column (see below). To purify GlpP-His from *E. coli* MM294(pPHis1), the cells were grown in LB until late exponential phase. The cells were spun down and treated as described for *B. subtilis*, except that the lysozyme step was omitted. The supernatant from the final centrifugation was loaded onto a 4 ml Ni²⁺ NTA agarose (Qiagen) column equilibrated with 40 ml sonication buffer. The flow rate was approximately 0.5 ml min⁻¹. The column was washed with 60 ml sonication buffer followed by 80 ml wash buffer (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 10% glycerol, 0.1% Tween 20) and GlpP-His was eluted with an imidazole gradient of 0.05–0.30 M in wash buffer. Most GlpP-His eluted at 0.30 M imidazole. Fractions were analysed by denaturing SDS-PAGE. Purified GlpP-His was stored in the elution buffer at 4 °C. Regardless of source, purified GlpP-His had a strong tendency to precipitate. This happened during 3–6 weeks of storage at 4 °C or –20 °C. Attempts to dialyse or change buffer using Amicon centrprep resulted in precipitation of the protein. Therefore, all experiments with purified GlpP-His were performed in the presence of small amounts of imidazole and Tween 20 in the storage buffer. Different dilutions of the eluted GlpP-His were run on SDS-PAGE next to known concentrations of carbanhydrase, which has a similar size. The gels were stained with Coomassie brilliant blue and the concentration of GlpP-His was estimated by comparing the intensities of the bands. SDS-PAGE was run according to Neville (1971). Western blot analysis was performed using polyclonal anti-GlpP antibodies from rabbit and a Western Exposure Chemiluminescent Detection system (Clontech).

In vitro RNA synthesis. The template for *in vitro* RNA synthesis was prepared as follows. Plasmid pLUM1047, which contains the *glpD* leader preceded by the T7 promoter, was linearized with *Bam*HI, run on a 0.8% agarose gel and purified using a Jet Sorb Gel Extraction kit (Genomed). The template

was transcribed using the SP6/T7 Transcription kit (Boehringer Mannheim) with 50 μ Ci (1.85×10^6 Bq) [α - 32 P]UTP and 50 μ M UTP. The DNA was removed by incubating with 20 U RNase-free DNase at 37 °C for 15 min. The mRNA was separated from unincorporated nucleotides by ethanol precipitation and resuspended in diethylpyrocarbonate-treated double-distilled water containing 25 μ g yeast tRNA ml $^{-1}$. The mRNA was analysed on a denaturing 6% polyacrylamide sequencing gel.

RNA-binding assay. mRNA (at a concentration of approximately 5 nM) was incubated at 80 °C for 3 min in 0.5 $\times$ TBE (0.045 M Tris/borate, 0.001 M EDTA), 2 mM DTT, 5 mM MgCl $_2$, and then transferred to an ice bath. Purified GlpP-His diluted in the above buffer was mixed with the mRNA to a final volume of 9 μ l. The mixture was incubated at 16 °C for 10 min and then at 0 °C for 10 min. Three microlitres of sample buffer (0.3 g Ficoll 400 ml $^{-1}$, 0.04% bromophenol blue in 0.5 $\times$ TBE) was added and the mixture was loaded onto a 4% agarose gel (Metaphor XR; FMC) in 0.5 $\times$ TBE with 0.5 $\times$ TBE as running buffer. Electrophoresis was carried out for 120 min at 150 V and room temperature. The gel was dried and analysed by autoradiography. The DNA fragment from within the *glpD* coding region which was used in the *in vitro* binding experiments was made as follows. A 409 bp fragment was amplified by PCR using primers GlpK3 and GlpDI. The fragment was digested with *Sau*3AI and a 112 bp internal *glpD* coding fragment was isolated.

RESULTS

In vivo titration of the antiterminating effect of GlpP

A major assumption in our model of how GlpP acts in antitermination and mRNA stabilization is that it can bind to the leader sequences of *glp* transcripts. As judged from Western blots (Nilsson, 1995), *B. subtilis* cells contain a relatively small amount of GlpP protein which does not increase upon induction of the *glp* regulon. We therefore did the following experiment to see if GlpP could be titrated by an excess of *glpD* leader mRNA. The *glpD* leader and promoter region (Fig. 1) was cloned into the high-copy-number vector pGDV1. The

resulting plasmid, pGDV11, was introduced into *B. subtilis* LUZ9595. This strain has one chromosomal copy of *glpP* and one chromosomal copy of *lacZ* under the control of the *glpD* promoter and leader. Expression of *lacZ* in this strain requires GlpP (and G3P). Another plasmid, pGDV14, was constructed which carries the *glpD* leader without its promoter region.

β -Galactosidase activities of LUZ9595(pGDV1), LUZ9595(pGDV11) and LUZ9595(pGDV14) were measured under non-inducing and inducing conditions. The results, presented in Table 3, show that the presence of the *glpD* leader on a high-copy-number vector in LUZ9595 decreases the inducibility of β -galactosidase when compared to the same strain carrying either only the vector or the *glpD* leader without a promoter. The decrease in β -galactosidase activity in a strain carrying many copies of the *glpD* promoter and leader indicates that GlpP is titrated due to interaction with *glpD* leader mRNA. That this is not due to an interaction with *glpD* leader DNA is shown by the fact that no titration was obtained with the non-transcribed *glpD* leader.

Purification of GlpP

At the 3'-end of the *glpP* gene, six histidine codons [(CATCAC) $_3$] were introduced by PCR. *glpP*-His was cloned into pHP13 to give pPHis1 and into pGDV1 to give pPHis11. pHP13 is an *E. coli*-*B. subtilis* shuttle vector with a copy number of approximately 200 in *E. coli* and five in *B. subtilis*. pGDV1 has a copy number of 150–200 in *B. subtilis*. To test if pPHis1 and pPHis11 give rise to functional GlpP, they were introduced into *B. subtilis* LUG0402, which has a deleted *glpP* and therefore shows no G3P dehydrogenase activity. The presence of either plasmid resulted in inducible G3P dehydrogenase activities, showing that the His-tagged GlpP is active *in vivo* (data not shown).

GlpP-His was purified by affinity chromatography on an Ni $^{2+}$ NTA agarose column from *E. coli* carrying pPHis1 and *B. subtilis* carrying pPHis11 as described in Methods. GlpP-His from *E. coli* was pure as judged by SDS-PAGE, while GlpP-His from *B. subtilis* co-purified with some other proteins which were present in small amounts (results not shown). The molecular mass of GlpP-His isolated from either bacterial species appeared to be around 25000 Da, which is higher than the 22300 Da calculated from the deduced amino acid sequence. Amino-terminal sequence analysis verified that the protein purified from *E. coli* was GlpP and also confirmed the proposed translational start.

When SDS-PAGE was performed with samples of GlpP-His which had not been boiled and without reducing agent in the sample buffer, GlpP-His from *E. coli* migrated mostly as a monomer but also to some extent as a dimer (54000 Da). The amount of dimers decreased as the concentration of DTT was increased. Since preparations of GlpP-His from *B. subtilis* contain more than one protein, in this case GlpP-His was detected by Western blotting. Most GlpP-His from *B. subtilis*

Table 3. β -Galactosidase activities in *B. subtilis* LUZ9595 carrying pGDV1, pGDV11 or pGDV14

β -Galactosidase activity is expressed as nmol substrate (ONPG) converted min $^{-1}$ (mg protein) $^{-1}$. The extinction coefficient used was 7.5×10^{-3} M $^{-1}$ cm $^{-1}$. The values represent the mean of at least two independent measurements. The deviation from the mean is less than 20%.

Strain	Addition	β -Galactosidase activity
LUZ9595(pGDV1)	–	< 0.5
	Glycerol	169.0
LUZ9595(pGDV11)	–	< 0.5
	Glycerol	26.0
LUZ9595(pGDV14)	–	< 0.5
	Glycerol	150.0

Fig. 2. Western blot showing monomers and dimers of GlpP-His purified from *B. subtilis*. SDS-PAGE was performed using a sample buffer lacking reducing agent (lane 1) and with increasing concentrations of DTT (lane 2, 1 mM; lane 3, 10 mM; lane 4, 200 mM).

Fig. 3. *In vitro* transcription of pLUM1047 with T7 RNA polymerase. The band at 128 bases represents the run-off transcript and the bands at 85 and 87 bases are the result of premature termination at the AT-rich region downstream of the inverted repeat in the *glpD* leader.

migrated as a dimer in the absence of DTT (Fig. 2). More slowly migrating bands, probably representing trimers and even larger complexes, could also be seen. When DTT was added, only monomers were seen.

Fig. 4. Gel shift showing binding of GlpP-His to *glpD* leader mRNA. All lanes contain approximately 5 nM *glpD* leader mRNA. Lanes: 1, mRNA only; 2–6, increasing amounts of GlpP-His (0.25, 0.50, 1.25, 2.50 and 5.00 μ M, respectively).

These results raise the question of whether GlpP exists as dimers *in vivo*.

GlpP binds to *glpD* leader mRNA *in vitro*

The *in vivo* titration experiments suggest that there is a specific interaction between GlpP and *glpD* leader mRNA. Gel shift experiments were thus performed to investigate if binding of GlpP to *glpD* leader mRNA could be detected *in vitro*.

glpD leader mRNA was synthesized in a T7 promoter/RNA polymerase system with pLUM1047 (Fig. 1) as a template as described in Methods. When the mRNA was analysed in a sequencing gel, three bands were seen (Fig. 3). The slowly migrating band represents the run-off transcript and the faster migrating bands are the result of premature termination at the AT-rich region immediately downstream of the inverted repeat in the *glpD* leader. The presence of these short transcripts shows that the T7 RNA polymerase recognizes a *B. subtilis* transcription terminator.

GlpP-His (from *B. subtilis* or *E. coli*) was incubated with radioactively labelled *glpD* leader mRNA (terminated and run-off transcripts) in the presence of at least a 40-fold excess of yeast tRNA. The relative mobility of the mRNA was studied in a native agarose gel. The results are shown in Fig. 4. When GlpP-His was added at increasing concentrations, a more slowly migrating RNA band appeared. If the concentration of GlpP-His was further increased, an even more slowly migrating band could be seen. Our interpretation is that these bands represent complexes of mRNA and different amounts of GlpP-His.

Fig. 5. Gel shift showing binding of GlpP-His to labelled *glpD* leader mRNA in the presence of increasing amounts of unlabelled *glpD* leader mRNA. All lanes contain approximately 5 nM labelled *glpD* leader mRNA. Lanes: 1, mRNA only; 2, GlpP-His (approximately 0.5 μ M); 3–6, GlpP-His and unlabelled *glpD* leader mRNA at a molar excess of approximately 5-, 10-, 50- and 250-fold, respectively.

The specificity of the GlpP-*glpD* leader mRNA interaction was investigated by studying the effect of adding unlabelled *glpD* leader mRNA or dsDNA from the *glpD* coding region at molar excesses of approximately 2-, 5-, 10-, 50- and 250-fold to the reaction mixture. A 40-fold molar excess of tRNA was always present. Fig. 5 shows that increased concentrations of unlabelled *glpD* leader mRNA caused the labelled mRNA to migrate as in the absence of GlpP-His, i.e. the unlabelled *glpD* leader competes with the labelled *glpD* leader for binding to the GlpP-His protein. Addition of dsDNA from the *glpD* coding region did not affect migration of the labelled mRNA; neither did replacement of GlpP-His with another His-tagged protein (adenovirus type 2 recombinant fibre protein; E. Rodriguez, unpublished) (data not shown). Similar results were obtained whether GlpP-His used in the experiments was purified from *E. coli* or *B. subtilis*.

DISCUSSION

The GlpP protein of *B. subtilis* causes antitermination of transcription of *glpD* and other *glp* genes and it also affects *glpD* mRNA half-life. We have proposed that the

effects of GlpP are due to specific binding of the protein to *glp* leader mRNA. The aim of this work was to find experimental evidence for a GlpP-mRNA interaction.

Several other catabolic operons are known where an antiterminator protein effects readthrough at a transcription terminator located in the leader (Rutberg, 1997). Examples are the *bgl* (β -glucoside) operon of *E. coli* and the *sac* (sucrose) regulons of *B. subtilis*. Expression of the *bgl* operon is controlled by the BglG protein which can bind to a sequence in the *bgl* leader mRNA and prevent formation of a terminator (Houman *et al.*, 1990). To be active in antitermination, BglG must be in an unphosphorylated, dimeric form. BglF, which is a transmembrane EII complex of the phosphotransferase system (PTS) that transports and phosphorylates β -glucosides, phosphorylates and inactivates the BglG protein in the absence of β -glucosides (Amster-Choder & Wright, 1993). The *B. subtilis* *sac* regulons are regulated similarly to the *E. coli* *bgl* operon (Crutz *et al.*, 1990; Débarbouillé *et al.*, 1990). The two antiterminator proteins SacT and SacY are homologous to BglG of *E. coli*. SacY activity is controlled by SacX, which is homologous to BglF, whereas the activity of SacT is controlled via the general PTS components (Arnaud *et al.*, 1992). SacT and SacY have been reported to bind to the SacT target in the *sacPA* leader mRNA *in vitro* and cause antitermination (Arnaud *et al.*, 1996). Of other antiterminator proteins discovered so far in *B. subtilis*, LicT (Schnetzer *et al.*, 1996) and GlcT (Stülke *et al.*, 1997) also belong to the BglG family. However, GlpP is not a member of this family.

The presence of a high-copy-number plasmid carrying the *glpD* promoter and leader in *B. subtilis* LUZ9595, which has the reporter gene *lacZ* under the control of the *glpD* promoter and leader, reduced the inducibility (by glycerol) of β -galactosidase activity by about 85% (Table 3). Removal of the *glpD* promoter from the plasmid resulted in normal β -galactosidase inducibility. Thus, overproduction of *glpD* leader mRNA interferes with expression of a single-copy gene under *glpD* control. The simplest explanation for this result is that GlpP binds to *glpD* leader mRNA.

A GlpP protein with a His sequence at the carboxy-terminus was shown to be biologically active *in vivo*. When SDS-PAGE was run under non-reducing conditions, isolated GlpP-His synthesized in *B. subtilis* migrated mostly as a dimer. Also, isolated GlpP-His synthesized in *E. coli* contained dimers, but most of the protein migrated as a monomer. The active form of the BglG antiterminator protein of *E. coli* is a dimer (Amster-Choder & Wright, 1992).

In gel shift experiments, GlpP-His bound to radioactively labelled *glpD* leader mRNA (Fig. 4). The terminated and run-off transcripts that were produced in *in vitro* transcription by T7 RNA polymerase both interacted with GlpP-His. This indicates that GlpP-His binds to *glpD* leader mRNA at the stem-loop or upstream of it. This fits well with a model where GlpP binds to and stabilizes an alternative secondary structure

Fig. 6. Comparison between the RAT sequence of the *sacPA* leader and the proposed GlpP target in the *glpD* leader. Bases in bold are conserved. The arrows show inverted repeats and the thick lines indicate the beginning of the proposed terminator structures. The *sacPA* transcription start has not been mapped.

thereby hindering formation of the terminator and allowing readthrough. The target for BglG of *E. coli* has been defined as a 32-nucleotide sequence upstream of and partially overlapping the inverted repeat of the *bgl* leader. It has the potential to form a stem-loop structure which would compete with formation of the terminator. Similar target sequences are found in, for example, the *B. subtilis* *sac* regulons and they have been named RATs (ribonucleic antiterminators) (Aymerich & Steinmetz, 1992). We have found a sequence in the *glpD* leader which has some similarity to the RAT sequences (Fig. 6). When RNA modelling was applied to the first 40 bases of *glpD* mRNA, the secondary structure indicated in Fig. 6 was predicted. This structure would overlap the terminator as shown in the figure and it has a predicted free energy of $-22.8 \text{ kJ mol}^{-1}$. The free energy of the *bgl/sac* RATs has been reported to be -17 to -21 kJ mol^{-1} (Aymerich & Steinmetz, 1992).

The fact that the GlpP-His binds to *glpD* leader mRNA *in vitro* in the absence of the effector molecule G3P might reflect an *in vivo* situation with two forms of GlpP (one inactive and one active in antitermination) that both bind to the *glpD* leader. Alternatively, it might reflect non-physiological conditions in the binding assay. The SacT and SacY proteins bind to the proposed *sacPA* target and cause antitermination *in vitro* even though they are in the inactive unphosphorylated form (Arnaud *et al.*, 1996).

Unlabelled *glpD* mRNA competed with radioactively labelled *glpD* leader mRNA in binding to GlpP-His while DNA corresponding to the *glpD* coding sequence did not compete. An unrelated His-tagged protein did not bind. From this, we conclude that GlpP binds specifically to *glpD* leader mRNA. However, a large excess (250-fold) of unlabelled RNA was necessary to fully compete with the labelled RNA. In a filter binding assay with BglG and its target mRNA, Houman *et al.* (1990) had to add unlabelled RNA at a 500-fold molar excess to see competition with the labelled RNA, whereas in a gel shift experiment with SacT and *sacPA* leader mRNA, only a 10-fold excess of unlabelled specific mRNA was needed for competition (Arnaud *et al.*, 1996).

We have transcribed the *glpD* leader *in vitro* using *B. subtilis* σ^A RNA polymerase (generous gift from G. Spiegelman, Dept of Microbiology, University of British

Columbia, Vancouver, Canada) and *E. coli* σ^{70} RNA polymerase and adding G3P and GlpP-His at different concentrations. These experiments did not demonstrate the antiterminating activity of GlpP-His, i.e. addition of GlpP-His and G3P did not increase the amount of run-off transcript (unpublished).

The GlpP protein is not similar to the antiterminator proteins of the BglG family or to any other known antiterminator proteins. A conserved phosphate-binding domain has been found in the carboxy-terminal part of GlpP (Bork *et al.*, 1995). Recently, an ORF of *E. coli* (orfo191; GenBank accession number U29579) was reported which has 28% identity and 56% similarity to GlpP. It is of the same size as GlpP (191 aa) and it contains a motif which is similar to the proposed phosphate-binding domain of GlpP. The mutations in the GlpP-negative mutants LUG2506 and LUG2512 (Glatz *et al.*, 1996) both lead to exchange of a conserved amino acid. It will be of interest to elucidate the function of the predicted *E. coli* protein.

ACKNOWLEDGEMENTS

We thank Ingrid Stål for expert technical assistance, Lars Rutberg for valuable discussions and Lars Rutberg and Lars Hederstedt for critically reading the manuscript. The authors thank the Institute for Genomic Research for availability of sequence data prior to publication. This project was supported by grants from the Swedish Medical Research Council, the Emil and Wera Cornell Foundation and the Crafoord Foundation.

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Received 1 September 1997; revised 28 October 1997;
accepted 3 November 1997.