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 α-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, 1993). 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...
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/phenotype</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
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<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR95</td>
<td>trpC2 pheA1 ilvC1</td>
<td>Our collection</td>
</tr>
<tr>
<td>LUX9595</td>
<td>trpC2 pheA1 ilvC1 ΔamyE::gldD leader-lacZ; KmR</td>
<td>This work</td>
</tr>
<tr>
<td>LUG0402</td>
<td>trpC2 pheA1 ilvC1 AglmP; PmR</td>
<td>Beijer et al. (1993)</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lacF[proAB+ lacZ]; KmR</td>
<td>Bullock et al. (1987)</td>
</tr>
<tr>
<td></td>
<td>Tn10 (ter')</td>
<td></td>
</tr>
<tr>
<td>MM294</td>
<td>pro thi endA hsr_ hsr_+</td>
<td>This work</td>
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<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHP13</td>
<td>CmR; EmR</td>
<td>Haima et al. (1987)</td>
</tr>
<tr>
<td>pMD433</td>
<td>KmR; ApR; 'lac2 (glpD leader from BR95 cloned in front of and in-frame with</td>
<td>Dahl &amp; Meinhof (1994)</td>
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<td></td>
<td>lacZ in pMD433)</td>
<td>Bron (1990)</td>
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<tr>
<td>pGDV1</td>
<td>CmR</td>
<td>This work</td>
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<tr>
<td>pLUM1041</td>
<td>KmR; ApR; 'lacZ (gldP leader from BR95 cloned in front of and in-frame with</td>
<td>This work</td>
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<td></td>
<td>lacZ in pMD433)</td>
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<tr>
<td>pLUM1047</td>
<td>T7 promoter gldP leader; CmR</td>
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<td>pGDV11</td>
<td>gldP leader from BR95; CmR</td>
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<td>pGDV14</td>
<td>gldP leader without -10 and -35 promoter regions; CmR</td>
<td>This work</td>
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<td>pPHis1</td>
<td>gldP-His; CmR</td>
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<tr>
<td>pPHis11</td>
<td>gldP-His; CmR</td>
<td>This work</td>
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* Ampicillin resistance expressed in E. coli.

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 LUX9595. 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 gldP leader from BR95 was amplified by PCR using primers GlpK3 and pDBam. The fragment obtained was cloned into pGDV1 between the EcoRI and BarnHI sites to give plasmid pGDV11. To get a promoterless gldP leader, a fragment was amplified from BR95 chromosomal DNA using primers UTLEco and pDBam. The EcoRI site of UTLEco is located at the -10 position. The fragment was cloned into pGDV1 between the EcoRI and BamHI sites, giving plasmid pGDV14.

Plasmid pLUM1041 was constructed as follows. Primers GfpDBamI and GfpDBamII were used to amplify a 400 bp fragment from BR95 chromosomal DNA. This fragment includes the gldP promoter and leader and the sequence encoding the first 33 aa of GlpD. The fragment was ligated to pMD433 which had been cleaved with BamHI 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.
To construct LUZ9595, B. subtilis BR95 was transformed with pLU1M041, which cannot replicate in B. subtilis but can integrate into amyE. The transformsants 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 BamHI and EcoRI sites in pHP13 (giving pPHisl) or pGDV1 (giving pPHisl1).

Plasmid pLU1M047 was constructed as follows. PCR was performed with the primers pT7Eco and pDBam. The resulting fragment was cloned into pHP13 between the EcoRI and BamHI sites. The 5' part of the primer pT7Eco contains AT followed by an EcoRI 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 BamHI 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 LU0402, the cells were induced with G3P instead of glycerol, as this strain lacks the glycerol uptake facilitator. β-Galactosidase activities of LUZ9595 were induced by glycerol.

Western blot analysis was performed using monoclonal 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 pLU1M047, which contains the glpD leader preceded by the T7 promoter, was linearized with BamHI, 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 μCi (1.85 x 10⁷ Bq) [α-³²P]UTP and 50 μM 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 μg 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 x TBE (0.045 M Tris/borate, 0.001 M EDTA), 2 mM DTT, 5 mM MgCl₂, 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 μl. 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 x TBE) was added and the mixture was loaded onto a 4% agarose gel (Metaphor XR; FMC) in 0.5 x TBE with 0.5 x 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 Sau3AI and a 112 bp internal 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, pGDV1, 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.

<table>
<thead>
<tr>
<th>Table 3. β-Galactosidase activities in <em>B. subtilis</em> LUZ9595 carrying pGDV1, pGDV11 or pGDV14</th>
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<tr>
<td>β-Galactosidase activity is expressed as nmol substrate (ONPG) converted min⁻¹ (mg protein)⁻¹. The extinction coefficient used was 7.5 x 10⁵ M⁻¹ cm⁻¹. The values represent the mean of at least two independent measurements. The deviation from the mean is less than 20%.</td>
</tr>
<tr>
<td><strong>Strain</strong></td>
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<tr>
<td>LUZ9595(pGDV1)</td>
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<td></td>
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<td>LUZ9595(pGDV11)</td>
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<td>LUZ9595(pGDV14)</td>
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**Purification of GlpP**

At the 3'-end of the *glpP* gene, six histidine codons [(CATCAC)₆] 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 Ni²⁺ 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.
Bacillus subtilis GlpP binds to glpD mRNA

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. 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 μM, 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.

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.
transcription of glpD affects the specificity of the GlpP-glpD leader mRNA in approximately 2-, 5-, 50- and 250-fold, respectively. Fig. 5 shows that increased concentrations of dsDNA from the glpD coding region at molar excesses of approximately 2-, 5-, 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 (Schnetz 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.
**Bacillus subtilis** GlpP binds to glpD mRNA

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**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.

The fact that the GlpP-His binds to _glpD_ leader mRNA is supported by the 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 $-22.8$ 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 free energy of the _bgZ/sac_ RATs has been reported to be $-17$ to $-21$ kJ mol$^{-1}$ (Aymerich & Steinmetz, 1996).

The sequence $5'\text{-}GGAAGGCAGCACGCGCA\text{-}3'$ has some similarity to the RAT sequences (Fig. 6). 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 $-22.8$ kJ mol$^{-1}$. The free energy of the _bgZ/sac_ RATs has been reported to be $-17$ to $-21$ kJ mol$^{-1}$ (Aymerich & Steinmetz, 1996).

**ACKNOWLEDGEMENTS**

We thank Ingrid Stål for expert technical assistance, Lars Rutberg for valuable discussions and Lars Hurberg 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.