T-box-mediated control of the anabolic proline biosynthetic genes of *Bacillus subtilis*

Jeanette Brill, Tamara Hoffmann, Harald Putzer and Erhard Bremer

*Bacillus subtilis* possesses interlinked routes for the synthesis of proline. The ProJ–ProA–ProH route is responsible for the production of proline as an osmoprotectant, and the ProB–ProA–ProI route provides proline for protein synthesis. We show here that the transcription of the anabolic *proBA* and *proI* genes is controlled in response to proline limitation via a T-box-mediated termination/antitermination regulatory mechanism, a tRNA-responsive riboswitch. Primer extension analysis revealed mRNA leader transcripts of 270 and 269 nt for the *proBA* and *proI* genes, respectively, both of which are synthesized from SigA-type promoters. These leader transcripts are predicted to fold into two mutually exclusive secondary mRNA structures, forming either a terminator or an antiterminator configuration. Northern blot analysis allowed the detection of both the leader and the full-length *proBA* and *proI* transcripts. Assessment of the level of the *proBA* transcripts revealed that the amount of the full-length mRNA species strongly increased in proline-starved cultures. Genetic studies with a *proB–treA* operon fusion reporter strain demonstrated that *proBA* transcription is sensitively tied to proline availability and is derepressed as soon as cellular starvation for proline sets in. Both the *proBA* and the *proI* leader sequences contain a CCU proline-specific specifier codon prone to interact with the corresponding uncharged proline-specific tRNA. By replacing the CCU proline specifier codon in the *proBA* T-box leader with UUC, a codon recognized by a Phe-specific tRNA, we were able to synthetically re-engineer the proline-specific control of *proBA* transcription to a control that was responsive to starvation for phenylalanine.

**INTRODUCTION**

The amino acid proline serves several functions for the soil-dwelling Gram-positive bacterium *Bacillus subtilis*. Exogenously provided proline can be used as sole carbon and nitrogen source (Fisher & Debarbouille, 2002). The role of proline as a nutrient is underscored by the finding that it is an excellent chemoattractant for *B. subtilis* (Osmotically non-stressed conditions (Kempf & Bremer, 1998). The cellular pools of about 16 mM, and those subjected to a modest osmotic up-shock with 0.4 M NaCl amass proline to a concentration of about 0.5–0.7 M (Whatmore et al., 1990).

As in many other micro-organisms (Csonka & Leisinger, 2007), proline biosynthesis in *B. subtilis* proceeds from glutamate and involves three enzymes: the γ-glutamyl kinase (ProB), the γ-glutamyl-phosphate reductase (ProA) and the Δ1-pyrroline-5-carboxylase reductase (ProI) (Fig. 1) (Belitsky et al., 2001). The activity of the ProB enzyme from *B. subtilis* is subjected to feedback control by proline (Chen et al., 2006), thereby tying proline production to proline consumption by the protein biosynthetic activities of the cell (Csonka & Leisinger, 2007). Genetic studies have shown that in addition to the anabolic proline biosynthetic pathway formed by the ProB–ProA–ProI enzymes, a second route for proline biosynthesis is present in *B. subtilis* (Belitsky et al., 2001) that functions for the supply of the very large amounts of proline required for its use as an osmoprotectant (Bremer, 2002). This osmoadaptive proline biosynthetic route is formed by the ProJ–ProA–ProH enzymes, and relies on isoenzymes for the first
Protein also possesses In addition to the ProI and ProH enzymes, the ProG enzyme, the anabolic and the osmoregulatory proline biosynthesis (Fig. 1). Since no paralogous protein is present for the last steps of the anabolic proline biosynthetic route (Fig. 1). Since no paralogous protein is present for the ProA enzyme, the anabolic and the osmoregulatory proline biosynthetic routes in B. subtilis are interconnected by the y-glutamyl-phosphate reductase (ProA) (J. Brill and E. Bremer, unpublished results) (Fig. 1). Expression of the proBA operon is strongly upregulated in response to either suddenly imposed or sustained osmotic stress, whereas that of the proBA operon and that of the proA gene alone are not osmotically induced (Hahne et al., 2010; Steil et al., 2003). In addition to the ProI and ProH enzymes, the ProG protein also possesses Δ1-pyrroline-5-carboxylate reductase activity (Fig. 1), although the precise physiological function of the ProG protein is unresolved (Belitsky et al., 2001). Bioinformatic mining of the B. subtilis genome sequence had indicated that the expression of the proBA operon and that of the proI (ygiQ) gene are genetically controlled through a T-box regulatory system (Chopin et al., 1998). T-box systems are widespread in Gram-positive bacteria, and are sensory and regulatory devices that prevent wasteful overproduction and overaccumulation of amino acids (Green et al., 2010; Gutiérrez-Preciado et al., 2009; Henkin, 2008; Vitreschak et al., 2008; Wels et al., 2008; Winkler, 2007). The amino acid specificity of a given T-box system is conferred through a single codon, the specifier (Grundy & Henkin, 1993), which is present in the 5′-untranslated region of each T-box-controlled gene (Foy et al., 2010; Green et al., 2010; Gutiérrez-Preciado et al., 2009; Henkin, 2008; Vitreschak et al., 2008; Wels et al., 2008). The decision between premature termination of transcription and transcription of the full-length gene is made through the combined interactions of the anti-codon of an uncharged cognate tRNA with the specifier codon and the highly conserved 5′-NCCA-3′ sequence in the acceptor stem of the non-acylated tRNA with a conserved 5′-UGGN-3′ sequence in the T-box. These interactions cannot occur when a tRNA is aminoacylated (Putzer et al., 2002). Interactions of the non-acylated tRNA with elements present in the mRNA leader sequence lead to a stabilization of the antiterminator configuration of the riboswitch and thus permit transcription of the full-length gene to proceed (Green et al., 2010; Gutiérrez-Preciado et al., 2009). T-box regulatory systems monitor the ratio between the charged and uncharged forms of a specific tRNA (Grundy et al., 2005; Yousef et al., 2005), and thereby allow an assessment of the cellular pool of a particular amino acid. Gene regulation via T-box-dependent regulatory switches prevents wasteful overproduction via synthesis or overaccumulation via transport of a particular amino acid (Green et al., 2010; Gutiérrez-Preciado et al., 2009; Henkin, 2008; Winkler, 2007).

Here we demonstrate that the transcription of the anabolic proBA and proI proline biosynthetic genes of B. subtilis is sensitively regulated via a T-box system in response to proline starvation. Although proline-responsive T-box systems are predicted to occur widely in Gram-positive bacteria (Gutiérrez-Preciado et al., 2009; Vitreschak et al., 2008; Wels et al., 2008), the data reported here on the regulation of the proBA and proI genes of B. subtilis provide, to the best of our knowledge, the first experimental proof for the functionality of a proline-responsive T-box system in any micro-organism.

**METHODS**

**Bacterial strains.** For routine cloning experiments we used the Escherichia coli strain DH5α (Clontech). All B. subtilis mutant strains used in this study are derivatives of the wild-type strain H642 (BGSC 1A96; a kind gift of J. Hoch, Scripps Research Institute, CA, USA) and are listed in Table 1.

**Growth conditions.** The B. subtilis strains were cultivated in Spizizen’s minimal medium (SMM) with 0.5% (w/v) glucose as the biosynthesis and uptake (Green et al., 2010; Gutiérrez-Preciado et al., 2009; Winkler, 2007).

**Fig. 1.** Anabolic and osmoregulatory proline biosynthesis in B. subtilis. The proline biosynthetic enzymes and the intermediates of the enzymic conversion of glutamate into proline are indicated. Feedback inhibition of proline on the activity of the ProB enzymes is highlighted.

and last steps of the anabolic proline biosynthetic route (Fig. 1). Since no paralogous protein is present for the ProA enzyme, the anabolic and the osmoregulatory proline biosynthetic routes in B. subtilis are interconnected by the y-glutamyl-phosphate reductase (ProA) (J. Brill and E. Bremer, unpublished results) (Fig. 1). Expression of the proBA operon is strongly upregulated in response to either suddenly imposed or sustained osmotic stress, whereas that of the proBA operon and that of the proA gene alone are not osmotically induced (Hahne et al., 2010; Steil et al., 2003). In addition to the ProI and ProH enzymes, the ProG protein also possesses Δ1-pyrroline-5-carboxylate reductase activity (Fig. 1), although the precise physiological function of the ProG protein is unresolved (Belitsky et al., 2001). Bioinformatic mining of the B. subtilis genome sequence had indicated that the expression of the proBA operon and that of the proI (ygiQ) gene are genetically controlled through a T-box regulatory system (Chopin et al., 1998). T-box systems are widespread in Gram-positive bacteria, and are sensory and regulatory devices that prevent wasteful overproduction and overaccumulation of amino acids (Green et al., 2010; Gutiérrez-Preciado et al., 2009; Henkin, 2008; Vitreschak et al., 2008; Wels et al., 2008; Winkler, 2007). The amino acid specificity of a given T-box system is conferred through a single codon, the specifier (Grundy & Henkin, 1993), which is present in the 5′-untranslated region of each T-box-controlled gene (Foy et al., 2010; Green et al., 2010; Gutiérrez-Preciado et al., 2009; Henkin, 2008; Vitreschak et al., 2008; Wels et al., 2008). The decision between premature termination of transcription and transcription of the full-length gene is made through the combined interactions of the anti-codon of an uncharged cognate tRNA with the specifier codon and the highly conserved 5′-NCCA-3′ sequence in the acceptor stem of the non-acylated tRNA with a conserved 5′-UGGN-3′ sequence in the T-box. These interactions cannot occur when a tRNA is aminoacylated (Putzer et al., 2002). Interactions of the non-acylated tRNA with elements present in the mRNA leader sequence lead to a stabilization of the antiterminator configuration of the riboswitch and thus permit transcription of the full-length gene to proceed (Green et al., 2010; Gutiérrez-Preciado et al., 2009). T-box regulatory systems monitor the ratio between the charged and uncharged forms of a specific tRNA (Grundy et al., 2005; Yousef et al., 2005), and thereby allow an assessment of the cellular pool of a particular amino acid. Gene regulation via T-box-dependent regulatory switches prevents wasteful overproduction via synthesis or overaccumulation via transport of a particular amino acid (Green et al., 2010; Gutiérrez-Preciado et al., 2009; Henkin, 2008; Winkler, 2007).

Here we demonstrate that the transcription of the anabolic proBA and proI proline biosynthetic genes of B. subtilis is sensitively regulated via a T-box system in response to proline starvation. Although proline-responsive T-box systems are predicted to occur widely in Gram-positive bacteria (Gutiérrez-Preciado et al., 2009; Vitreschak et al., 2008; Wels et al., 2008), the data reported here on the regulation of the proBA and proI genes of B. subtilis provide, to the best of our knowledge, the first experimental proof for the functionality of a proline-responsive T-box system in any micro-organism.
carbon source and l-tryptophan (20 mg l⁻¹) and l-phenylalanine (18 mg l⁻¹) to satisfy the auxotrophic growth requirements of strain JH642 (trpC2 pheA1) and its derivatives (Table 1). A solution of trace elements was added to SMM (Harwood & Archibald, 1990). All B. subtilis cultures were inoculated from exponentially growing precultures in pre-warmed minimal media to OD₆₀₀ 0.1 and the cultures were subsequently propagated at 37 °C in a shaking water bath set to 220 r.p.m. The B. subtilis cells were grown in either 20 or 75 ml culture volumes in 100 or 500 ml Erlenmeyer flasks, respectively. For amino acid limitation experiments, the B. subtilis cells were pregrown in SMM with an excess of the appropriate amino acid, and the cultures were washed twice with SMM without added amino acids and then used to inoculate the main culture in SMM containing either excess (10 mM) or limiting amounts (0.016 mM) of this amino acid. The antibiotics chloramphenicol (5 µg ml⁻¹), zeocin (35 µg ml⁻¹), tetracycline (15 µg ml⁻¹), erythromycin (1 µg ml⁻¹) and spectinomycin (100 µg ml⁻¹) were used for the selection of gene disruption mutations in B. subtilis (Table 1) after DNA transformation with chromosomal DNA. Ampicillin was used at a final concentration of 100 µg ml⁻¹ for E. coli cultures carrying plasmids.

**Chemicals.** Ampicillin, chloramphenicol, tetracycline, erythromycin and spectinomycin were purchased from Sigma-Aldrich. Zeocin was purchased from Invitrogen. The chromogenic substrate [para-nitrophenyl-alpha-D-glucopyranoside (PNPG)] for TreA enzyme assays was purchased from Sigma-Aldrich.

**Construction of plasmids.** Plasmid pBKB26 carries a 2.75 kb `ykkE–proB–proA` DNA fragment cloned into the BamHI and EcoRI sites of the low-copy-number vector pHSG575 (B. Kempf and E. Bremer, unpublished data). A 2.75 kb Sall–EcoRI DNA fragment (`ykkE–proB–proA`) from pBKB26 was cloned into the Sall- and EcoRI-restricted pBluescriptSK⁺ vector (Stratagene), thereby yielding plasmid pJS8. For the construction of the proBA gene disruption mutation, a HindIII and Bcl fragment (224 bp) was deleted from plasmid pJS3 and replaced with a chloramphenicol-resistance cassette derived from plasmid pRMK39 (Kappes et al., 1999), resulting in plasmid pJS8. A proBA–treA reporter gene fusion was constructed as follows. The 1.05 kb MunI–SacI3A DNA fragment derived from pBKB26, carrying the proB regulatory region and part of the proB coding sequence (B. Kempf and E. Bremer, unpublished data), was inserted into the vector pBluescriptSK⁺ that had been cleaved with EcoRI and BamHI; this construction yielded plasmid pJS2. The 1.02 kb EcoRV–SacI3A proB fragment derived from pJS2 was then inserted into the Smal and BamHI sites present in the polylinker of the low-copy-number treA reporter plasmid pMB1 (M. Jебbar and E. Bremer, unpublished results), thereby yielding a proB–treA operon fusion (present on plasmid pJS5) with treA as the reporter gene (Gotsch et al. & Dahl, 1995; Schöck et al., 1996). To construct a proH–tet gene disruption mutation, we first amplified the `rpp–proH–pro–glic` region from the B. subtilis chromosome of strain JH642 via PCR, cleaved it with Scal and MunI, and then inserted the resulting restriction fragment (2578 bp) into plasmid pBluescriptSK⁺ that had been cleaved with EcoRI and EcoRV; this yielded plasmid pJS13. A 1.9 kb Pur–XbaI restriction fragment, carrying a tetracycline-resistance cassette, was isolated from plasmid pBEST307 (Itaya, 1992) and ligated into the plasmid backbone of pJS13 cleaved with NsiI and AvrII. In the resulting plasmid, pS19, an internal DNA segment of the proH operon (1.089 kb) was replaced by the tet resistance cassette.

**Construction of B. subtilis strains.** Strain JSB11 [A(proBA::cat)] was isolated after transformation of strain JH642 with DNA of plasmid pJS8 (linearized with the restriction enzymes BamHI and EcoRI) and subsequent selection for chloramphenicol-resistant transformants. Strain JSB17 [proA × EPV1T (tet)] was isolated after transformation of strain JH642 with the circular plasmid pEPV1T (Ogura et al., 1994) and selection for tetracycline-resistant colonies; in this strain, plasmid pEPV1T is inserted into the proA gene via a Campbell-type single-crossover integration event, thereby disrupting the integrity of proA. The proA mutation present in strain JSB17 was introduced into strain FSB1 [treA::neo] (Spiegelhalter & Bremer, 1998) by DNA transformation with chromosomal DNA from strain JSB17 and subsequent selection for tetracycline-resistant colonies. This yielded strain JSB4 [Δ(proA::neo) (proA × EPV1T (tet))]. The proB–treA operon fusion present in plasmid pJS5 was introduced into various B. subtilis strains by transforming linearized plasmid DNA (pJS5 cleaved with PstI and XhoI) and selecting for chloramphenicol-resistant transformants. In this way, the proB–treA fusion was

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

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Relevant genotype*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH642</td>
<td>trpC2 pheA1</td>
<td>BGSC† IA96</td>
</tr>
<tr>
<td>FSB1</td>
<td>Δ(proA::neo)</td>
<td>Spiegelhalter &amp; Bremer (1998)</td>
</tr>
<tr>
<td>TRB0</td>
<td>Δ(proA::neo) amyE:: [Φ(proA) cat]</td>
<td>Spiegelhalter &amp; Bremer (1998)</td>
</tr>
<tr>
<td>JSB5</td>
<td>amyE:: [Φ(proB–treA)I] ‘ykkE cat’ Δ(proA::neo)</td>
<td>This study</td>
</tr>
<tr>
<td>JSB8</td>
<td>Δ(proH::tet)</td>
<td>This study</td>
</tr>
<tr>
<td>JSB9</td>
<td>Δ(pro::spc)</td>
<td>Bellitsky et al. (2001)</td>
</tr>
<tr>
<td>JSB11</td>
<td>(ΔproBA::cat)</td>
<td>This study</td>
</tr>
<tr>
<td>JSB13</td>
<td>Δ(proH::tet) Δ(pro::spc)</td>
<td>This study</td>
</tr>
<tr>
<td>JSB17</td>
<td>proA × EPV1T (tet)</td>
<td>This study</td>
</tr>
<tr>
<td>JSB31</td>
<td>amyE:: [Φ(proB–treA)I] ‘ykk cat’ Δ(proA::neo) proA × EPV1T (tet)</td>
<td>This study</td>
</tr>
<tr>
<td>JSB31-mut1</td>
<td>amyE:: [Φ(proB–treA)I] ‘ykk cat’ Δ(proA::neo) proA × EPV1T (tet)</td>
<td>This study</td>
</tr>
<tr>
<td>JSB34</td>
<td>proA × EPV1T (tet)</td>
<td>This study</td>
</tr>
<tr>
<td>JSB41</td>
<td>Δ(proH::tet) Δ(pro::spc) Δ(proG::ble)</td>
<td>This study</td>
</tr>
<tr>
<td>BB1951</td>
<td>SMY Δ(proG::ble)</td>
<td>Bellitsky et al. (2001)</td>
</tr>
</tbody>
</table>

*All strains, except BB1951, are derivatives of JH642 and therefore also carry the trpC2 pheA1 mutations.
†Bacillus Genetic Stock Center.
recombined via its flanking 5’ and 3’ amyE DNA sequences into the B. subtilis chromosome, yielding a genetically stable, single-copy amyE::proB–treA–cat::amyE insertion. Strain JSB8 [Δ(proH::tet)] was isolated by transforming linear DNA of plasmid pJS19 (cleaved with BamHI) into strain JH642 and subsequently selecting for tetracycline-resistant transformants. The [Δ(proH::tet)] mutation was then introduced into strain JSB9 (Belitsky et al., 2001) by transforming it with chromosomal DNA of strain JSB8 and selecting for tetracycline-resistant colonies. This yielded strain JSB13 [Δ(pro::spa)1 Δ(proH::tet)] (Table 1). For the construction of a strain defective in all three genes encoding the enzyme for the last step in proline biosynthesis (Fig. 1), chromosomal DNA of the B. subtilis strain BB1951 (Belitsky et al., 2001) carrying the Δ(proG::ble) allele was transformed into strain JSB13, and zeocin-resistant transformants were selected; this yielded strain JSB41 [Δ(pro::spa)1 Δ(proH::tet) Δ(proG::ble)].

TreA enzyme assays. The expression of chromosomal proB–treA fusions was monitored by assaying the TreA [phospho-x-(1,1)-glucosidase] enzyme activity using the chromogenic substrate PNPG (Gotsche & Dahl, 1995). TreA enzyme activity is expressed as U (mg protein)^-1 according to the definition used for the quantification of β-galactosidase (Miller, 1992). Protein concentrations of the samples were estimated from the optical density of the cell culture. In each of the proB–treA fusion strains, the natural copy of treA in the B. subtilis chromosome is disrupted (Table 1), so that the measured TreA enzyme activity in the reporter strain reflects solely that encoded by the reporter fusion. Strain TRB0 (Table 1) carries a promoterless treA gene derived from plasmid pMB1 inserted into the chromosomal amyE locus, and this strain was always used as a control for the proB–treA fusion experiments. Background TreA activity exhibited by strain TRB0 was subtracted from the TreA activities exhibited by proB–treA fusion strains.

Methods used with nucleic acids. Routine manipulations of plasmid DNA, PCR, the construction of recombinant plasmids and the isolation of chromosomal DNA from B. subtilis were carried out according to standard procedures (Sambrook et al., 1989). The nucleotide sequences of cloned PCR fragments and of mutations generated via site-directed mutagenesis were verified by the chain-termination method using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech). The DNA-sequencing reactions were primed with synthetic oligonucleotides labelled at their 5’ ends with the infrared dye IRD-800 (Eurofins), and the products were analysed on a LI-COR DNA sequencer (model 4000) (Eurofins).

RNA isolation and Northern blot analysis. Total RNA from B. subtilis was isolated by phenol extraction as described previously (Holtmann & Bremer, 2004). Cells were grown in SMM under the indicated conditions and samples were harvested either in mid-exponential phase (OD578 0.5–0.8) or at specified points during the growth cycle. RNA was routinely isolated from 20 ml cultures. The total amount of RNA isolated was determined spectrophotometrically (A260); an A260 of 1 corresponds to approximately 40 μg RNA ml^-1 (Sambrook et al., 1989). Samples (15 μg) of total RNA were electrophoretically separated on a 1.4% agarose gel, transferred to a Schleicher & Schuell NY13N membrane, and hybridized with a DIG-labelled single-strand DNA probe specific for either proB or proS, respectively, using conditions specified by the manufacturer of the Northern hybridization and digoxigenin detection kit (Roche Diagnostics). The hybridization products were visualized with the chemiluminescent reagent ECF-Vistra (12 μl per cm² of blotting membrane) (Amersham Pharmacia Biotech) and a Storm 860 Phosphorimager (Amersham Pharmacia Biotech). The 1056 nt DNA probe specific for proB (probe 1, Fig. 5b) was prepared from plasmid pJS2 using the Strip-EZ RNA kit (Ambion) in combination with DIG-UTP (Roche Diagnostics). The DIG-labelled single-strand 86 nt DNA probe specific for the proB leader (probe 2, Fig. 5b) was prepared from a PCR fragment, generated with oligonucleotides carrying an artificial T7-promoter sequence at the 3’ end. This probe begins 103 nt downstream of the proBA transcription initiation site and does not cover the specifier codon or the intrinsic terminator sequence; its 3’ end is localized at the –UGGU– sequence within the T-box.

**Primer extension analysis.** To map the 5’ ends of the proBA and proS transcripts, we used primer extension analysis. Total RNA from a 75 ml culture of the B. subtilis wild-type strain JH642 that had been grown in SMM to mid-exponential phase (OD578 0.8) was isolated as described above. Reverse-transcriptase assays for the proBA and proS mRNAs were carried out with 15 μg total RNA and about 2 pmol of either a 32P-5’-end-labelled proBA-specific oligonucleotide (5’-TCTCTGTCAACGAAAGGGTCTG-3’) or an IRD-800-fluorescence-end-labelled proS-specific oligonucleotide (5’-TTTTCTAAAGCCGGACTTTGAGGCC-3’). Reverse-transcriptase reactions were carried out with a Primer Extension System–AMV Reverse Transcriptase kit purchased from Promega. The size of the proBA reverse transcript was analysed on a denaturing 5% polyacrylamide DNA sequencing gel. The same DNA primer employed for the proBA reverse transcription reaction was used in a DNA sequencing reaction with plasmid pJS2 to determine the exact position of the 5’ ends of the proBA mRNA. The size of the proS reverse transcript was analysed on a LI-COR DNA sequencer (model 4000) (Eurofins). The DNA primer employed for the proS reverse transcription reaction was used with plasmid pJS18 (Belitsky et al., 2001) to determine the exact position of the 5’ end of the proS mRNA.

**Site-directed mutagenesis.** Site-directed mutagenesis was used to change the proline-specific specifier codon (CCT) present in the untranslated leader region of the proBA operon (Fig. 2a) to a phenylalanine-specific TTC codon. Site-directed mutagenesis was performed on the proB–treA wild-type fusion present in plasmid pJS5 using the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies) and a custom-synthesized oligonucleotide (Eurofins). The presence of the desired change in the proBA leader region was verified by DNA sequence analysis. The resulting mutant proB–treA reporter fusion plasmid pJS5-nptI1 was linearized by digestion with Xhol and PstI, and the fusion was recombined as a single copy into the amyE site of the B. subtilis Δ(treA::neo) strain FSB1 (Table 1) by selecting for chloramphenicol-resistant colonies. The resulting strain was then used for further genetic manipulations to introduce a proA mutation into this background (Table 1).

**Computer analysis of DNA, RNA and protein sequences.** Alignments of the amino acid and DNA sequences were performed with the Vector NTI software package (Invitrogen). The proBA and proS leader mRNA structures were predicted using the Mfold algorithm (Zuker, 2003). The mRNA secondary structures suggested by the program (http://mfold.rna.albany.edu) were then further manually adjusted based on phylogenetic considerations.

**RESULTS**

**Mapping of the proBA and proS transcription initiation sites**

To map the transcription initiation sites of the proBA and proS loci from B. subtilis, we carried out a primer extension analysis. Total RNA was isolated from exponentially growing cultures of the wild-type strain JH642 and...
hybridized with labelled single-stranded oligonucleotide DNA probes specific for either the proBA or proI mRNA transcripts. A single primer extension reaction product was found for both the proBA and the proI transcripts (Fig. 2a, b). The transcription initiation sites for the proBA operon and the proI gene are positioned 270 and 269 bp upstream of the predicted ATG start codons of proB and proI, respectively (Fig. 2a, b). Inspection of the DNA sequence upstream of the mapped 5′ ends of both transcripts revealed appropriately spaced −10 and −35 elements for both the proBA and the proI gene that closely resemble those of typical SigA-type housekeeping promoters of B. subtilis (Fig. 2a, b) (Helmann, 1995).

Fig. 2. Mapping of the proBA (a) and proI (b) transcription initiation sites by primer extension analysis. The 5′ ends of the proBA and proI transcripts were mapped using mRNA isolated from the proline-prototrophic strain JH642; the asterisk indicates the cDNA fragment generated via the primer extension procedure. The determined 5′ ends of the mRNAs (marked by arrows) are projected onto the DNA sequences of the proBA and proI regulatory regions. Putative SigA-dependent promoter sequences (−10 and −35 regions) are indicated. The proline-specific CCT specifier codons of the proBA and proI transcripts are boxed. The putative ribosome-binding sites (RBS) are underlined, and the translational initiation codons of the proB and proI genes are indicated.

Predicted secondary structures of the T-box leaders in the proBA and proI mRNA sequences

Earlier bioinformatic inspection of the B. subtilis genome sequence had suggested the presence of elements characteristic of T-box regulatory systems in the 5′ mRNA regions of both the proBA and the proI gene (Chopin et al., 1998; Gutiérrez-Preciado et al., 2009; Vitreschak et al., 2008; Wels et al., 2008; Winkler, 2007). We analysed the proBA and proI leader sequences using Mfold (Zuker, 2003) and some manual adjustments to draw the secondary structures of the leader mRNAs (Fig. 3a, b). They revealed the presence of all major elements characteristically required for genetic control via tRNA-mediated antitermination. These include a factor-independent transcription terminator and the T-box sequence. The T-box sequence is part of a mutually exclusive terminator and antitermination mRNA structure present in both of the predicted mRNA leader elements of proBA and proI (Fig. 3a, b). Furthermore, the predicted mRNA secondary structures for both loci contain a CCU triplet (a proline-specific codon) in a bulged segment of the folded proBA and proI specifier domain, a region that typically contains the specifier codon for a given T-box system (Green et al., 2010; Gutiérrez-Preciado et al., 2009; Vitreschak et al., 2008). The mRNA secondary structures predicted for the proBA and proI leader regions closely resemble one another (Fig. 3a, b), and this is reflected in the considerable nucleotide sequence identity (59%) of the proBA and proI leader sequences. Neither the proG gene nor the proHJ operon is predicted to possess a T-box element.

The presented in silico data suggest that a T-box regulatory circuit controls the expression of the proBA and proI genes.
Consequently, short leader proBA and proI transcripts should be constitutively synthesized in B. subtilis cells that are not starved for proline. These transcripts should comprise the T-box leader mRNA element up to the intrinsic terminator sequence ending with a run of U nucleotides (Fig. 3a, b); both the prematurely terminated proBA and proI leader transcripts have a calculated length of 241 nt. To test for the presence of these short mRNA species, we carried out Northern blot analysis of total RNA isolated from B. subtilis cells of strain JH642 that were not starved for proline. As documented in Fig. 4, the predicted proBA and proI mRNA leader sequences were readily detected; their estimated lengths of about 200 nt are in good agreement with those predicted from the position of the intrinsic terminators in the proBA and proI mRNA leader sequences (Fig. 3a, b). In these Northern blot experiments, we also detected an approximately 2700 nt proBA and an approximately 1200 nt proI mRNA species. The sizes of these mRNA species correspond to those calculated for full-length mRNA transcripts of the proBA operon (about 2680 nt) and of the proI gene (about 1150 nt) initiating from the mapped promoters (Fig. 2a, b) and extending to putative factor-independent transcription terminators positioned immediately downstream of the proA and proI coding regions.

**Northern blot analysis of the proBA transcripts during proline starvation**

For further analysis of the T-box-mediated transcriptional control of the anabolic proline biosynthetic genes in B. subtilis, we focused on the proBA operon. In T-box-controlled genes, read-through transcription past the intrinsic terminator sequence present in the mRNA leader sequence will increase in cells that are starved for the particular amino acid determined by the specifier codon (Green et al., 2010; Gutierrez-Preciado et al., 2009; Henkin, 2008; Winkler, 2007). Hence, the level of the full-length transcript will increase and thereby provide the starved cell with proteins that will offset starvation for a particular amino acid. To visualize both the proBA mRNA leader and the full-length transcripts in cells starved of proline, we performed a Northern blot analysis of total RNA isolated from the B. subtilis mutant strain JSB41. This strain is a proline auxotroph in which the genes encoding three paralogous D1-pyrroline-5-carboxylate-reductases (ProG, ProH and ProI) (Fig. 1) are simultaneously disrupted (Table 1) (Belitsky et al., 2001). For this experiment, we grew strain JSB41 in SMM in the presence of either excess (10 mM) or limiting amounts (0.6 mM) of proline, and total RNA was isolated from these two cultures as soon as the growth of the proline-limited culture began to slow down (Fig. 5a). In cells that were not starved for proline, both the 200 nt leader transcript and the 2700 nt full-length transcript were detected with a single-stranded DNA probe (probe 1) that covered both the proBA leader region and 575 bp of the proB coding region (Fig. 5b). These two proBA transcripts were also present in proline-starved cells of strain JSB41, but the amount of the full-length proBA transcript was strongly increased in comparison with cells grown with excess proline (Fig. 5c). Hence, in agreement with the anticipated T-box-mediated control of proBA expression in B. subtilis, synthesis of the full-length proBA transcript is substantially enhanced in proline-starved cells.

In addition to the 200 nt proBA mRNA leader and the full-length mRNA species (2700 nt), we also detected an additional mRNA species (~2500 nt), whose amount was
also increased in proline-starved cells of strain JSB41 (Fig. 5c). This mRNA species represents in all likelihood a processed product of the full-length 2700 nt proBA mRNA, since it could not be detected with an 86 nt single-stranded DNA probe (probe 2) that covered only the region of the proBA mRNA leader region upstream of the intrinsic terminator sequence (Fig. 5b, c). Processing of mRNAs upstream of the intrinsic terminator present in the leader region has already been observed for the T-box-regulated thrS gene of B. subtilis, and contributes to the induced expression level in threonine-starved cells by stabilizing the downstream mRNA coding sequence (Condon et al., 1996; Even et al., 2005). We did not analyse the possible function of the processing step of the primary proBA transcript on mRNA stability.

**Induction of proBA transcription is sensitively controlled by proline starvation**

The Northern blot experiment documented in Fig. 5(c) shows that the expression of the proBA operon is induced when the B. subtilis cells are starved for proline. To investigate this in greater detail, we constructed a transcriptional fusion of the proBA regulatory region to a promoterless treA reporter gene (Schöck et al., 1996). We recombined the resulting proB–treA reporter gene fusion as a single copy into the amyE locus in the genome of B. subtilis strains that were either prototrophic [strain JSB5 (proA+ proB–treA)] or auxotrophic [strain JSB31 (proA proB–treA)] for proline (Table 1). When the prototrophic strain JSB5 was grown in SMM minimal medium in the absence of proline (Fig. 6a), expression of the proB–treA reporter gene fusion remained at a low level during the entire growth cycle of the culture (Fig. 6b). This indicates that this Pro+ B. subtilis strain did not experience any
physiologically significant proline limitation during growth. In parallel, we grew the proline auxotrophic strain JSB31 in SMM containing 0.2, 0.4, 0.8 or 1.2 mM proline. Each of these concentrations of proline was limiting for cell growth and did not allow the cultures to reach the same cell densities as the wild-type strain (Fig. 6a). Strikingly, as soon as the growth of the proline-limited cultures of the Pro\(^-\) strain JSB31 started to slow down (Fig. 6a), transcription of the proB–treA reporter gene fusion was rapidly derepressed in each of these cultures, and proB–treA expression persisted at a strongly elevated level in the proline-starved B. subtilis cells (Fig. 6b). The amount of proline (more than 1.2 mM) required for the full growth of the Pro\(^-\) mutant strain JSB31 is substantially higher than that needed to satisfy the auxotrophy of this JH642 (pheA1 trpC2)-derived strain for either L-tryptophan (0.1 mM) or L-phenylalanine (0.1 mM). We attribute this observation to the fact that B. subtilis can efficiently use proline as a sole carbon or nitrogen source (Fisher & Debarbouille, 2002).

### Synthetic redesign of the proline specificity of the T-box control element in proBA

The predicted secondary structure of the proBA mRNA leader sequence suggests that a CCU codon (Fig. 3a) functions as the specifier element and confers proline specificity upon the regulation of proBA via the T-box system. Studies with several T-box-regulated genes have revealed that the amino acid specificity of a given T-box system can be altered through mutational changes of the specifier codon (Grundy & Henkin, 1993; Luo et al., 1997; Marta et al., 1996; Putzer et al., 1995). Hence, the redesign of the specificity of a given T-box system by manipulating the putative specifier codon is a genetic approach that can identify a particular codon in the 5’-leader region of the transcript as the specifier. To provide evidence that the CCU codon highlighted in Fig. 3(a) indeed confers proline specificity upon the proBA T-box element, we changed it via site-directed mutagenesis to UUC, a codon that interacts with an anti-codon of a Phe-tRNA. Consequently, the mutated proBA T-box element should cause derepression of proBA transcription in cells starved for phenylalanine and simultaneously eliminate their response to proline starvation. We inserted an appropriately modified proB–treA fusion (CCT changed to TTC) into a B. subtilis strain that is auxotrophic for both Pro and Phe (Table 1). In the proB–treA fusion strain (JSB31) carrying the wild-type CCU specifier codon, proline starvation induced the expression of the reporter gene fusion about 13-fold, but there was no derepression of proB–treA expression when the cells were starved for phenylalanine (Table 2). Conversely, the expression of the proB–treA fusion with the Phe-specific UUC codon instead of the natural CCU proline-specific specifier element no longer changed in response to proline starvation. Instead, transcription of the mutant proB–treA reporter fusion was induced about sevenfold in response to phenylalanine starvation (Table 2). This experiment thus provides solid evidence that the CCU codon highlighted in Fig. 3(a) is indeed the proline-specifier codon for the T-box control system of the proBA operon from B. subtilis.

### DISCUSSION

Two interconnected proline biosynthetic routes are present in B. subtilis, which serve for the supply of proline either as a building block for protein biosynthesis or as an osmostress protectant (Fig. 1) (Belitsky et al., 2001). Since the biosynthesis of amino acids is energetically expensive (Akashi & Gojobori, 2002), B. subtilis has to carefully adjust its proline biosynthetic capacities to produce the amounts needed for these very different physiological tasks (Bremer,
Table 2. Synthetic redesign of the proline specificity of the proBA T-box element

<table>
<thead>
<tr>
<th>Reporter strain*</th>
<th>Specifier codon</th>
<th>TreA activity [U (mg protein)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stav for proline</td>
<td>Fold-induction</td>
</tr>
<tr>
<td>JSB31</td>
<td>CGAA CCU GCCU</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>JSB31-mut1</td>
<td>CGAA UUC GCCU</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

*These strains are phenylalanine (pheA1) and proline auxotrophs (proA × pEPV1T), and both strains carry the same Φ(proB'–treA) reporter gene fusion. The two strains differ in that strain JSB31 carries the proline-specific CCT specifier codon, while its mutant derivative JSB31-mut1 carries instead a phenylalanine-specific TTC specifier codon in the Φ(proB'–treA) fusion construct. The strains were cultivated for the proline starvation experiment either in the presence of excess proline (10 mM) or with limiting amounts of proline (0.2 mM); these cultures contained 10 mM proline to satisfy the auxotrophic needs of the strains. Growth of the strains was monitored and the expression of the Φ(proB'–treA) reporter gene fusion was measured after starvation for proline or phenylalanine had set in (after about 7 h).

2002; Whatmore et al., 1990). Our data show that the transcription of the anabolic proBA operon and show that of the prol gene is regulated through a proline-responsive T-box regulatory system. This RNA-responsive riboswitch (Green et al., 2010; Gutiérrez-Preciado et al., 2009; Henkin, 2008; Winkler, 2007) links the production of the anabolic ProB–ProA–ProI proline biosynthetic enzymes (Fig. 1) to the prevalent cellular proline pool so that no shortage of this amino acid as a building block for protein biosynthesis occurs (Fig. 6).

Comprehensive in silico surveys suggest that proline-responsive T-box systems are present in a large number of Gram-positive bacteria (Gutiérrez-Preciado et al., 2009; Vitreschak et al., 2008; Wels et al., 2008). Genetic control of the transcription of the proBA and prol genes via a T-box system has been predicted by the in silico mining of the B. subtilis genome sequence for the presence of T-box signature sequences (Chopin et al., 1998; Gutiérrez-Preciado et al., 2009; Vitreschak et al., 2008; Wels et al., 2008; Winkler, 2007). Our data show that the predicted T-box systems for the anabolic proBA and prol genes are indeed functional. Furthermore, they represent the first experimental proof, to our knowledge, of the functioning of a proline-responsive T-box regulatory system in any microbial species.

Northern blot analysis of the proBA transcripts in cells that are not starved of proline revealed the constitutive synthesis of the mRNA leader region and a limited amount of the full-length transcript (Fig. 5c). In agreement with the transcription pattern generally established for T-box-regulated genes (Green et al., 2010; Gutiérrez-Preciado et al., 2009), proline starvation did not appreciably reduce the amount of the proBA leader transcript but boosted the synthesis of the full-length proBA mRNA (Fig. 5c). In addition, in proline-starved cells, a proBA mRNA species was produced that lacked about 200 nt at its 5’ end. We have not studied how this processed proBA transcript is generated and what the physiological consequences of its production might be with respect to the cellular levels of the ProB and ProA proteins. However, there is a precedent for such a processing event in the context of a T-box regulatory system. The thrS gene encodes a threonyl-tRNA synthetase, and its transcript is processed at a site just upstream of the intrinsic transcription terminator in the leader mRNA sequence. This processing event increases the stability of the downstream mRNA segment and thereby enhances production of the threonyl-tRNA synthetase in threonine-starved cells (Condon et al., 1996). Given what has been reported in the context of the thrS T-box system, we are tempted to speculate that the processing of the primary proBA transcript prolongs the lifespan of the cleaved mRNA and thereby boosts ProB and ProA synthesis in B. subtilis cells starved for proline. A preliminary investigation of the prol transcript in proline-starved B. subtilis cells by Northern blot analysis did not reveal any evidence for a processing event of the full-length prol mRNA (J. Brill and E. Bremer, unpublished results).

The specificity of a given T-box system is conferred by a specifier codon strategically positioned in a bulged region of the first predicted stem structure of the mRNA leader region (the specifier domain), and by the interaction of this codon with the anti-codon of the uncharged cognate tRNA. As expected from the function of the B. subtilis proBA- and prol-encoded proteins in proline synthesis, the specifier codon in both loci is a proline-specific codon, CCU (Fig. 3a, b). Proline is encoded by four codons (CCA, CCG, CCU and CCC), and the CCU codon employed as the specifier in proBA and prol (Fig. 3) is one of the two proline-specific codons (CCG and CCU) that are preferentially used in protein-encoding genes of B. subtilis (Kanaya et al., 1999). It should be noted in this context that B. subtilis possesses only one tRNAPro (anticodon: 5-methoxyUGG) and that this single tRNA recognizes all four proline-encoding codons (Yamada et al., 2005).
Previous studies have shown that the amino acid specificity of a given T-box system can be synthetically redesigned by mutational changes in the specifier codon (Grundy & Henkin, 1993; Grundy et al., 1997; Luo et al., 1997; Marta et al., 1996; Putzer et al., 1995). Following these leads, we altered the CCU proline-specifier codon in the proBA mRNA leader sequence to a phenylalanine-specific UUC codon, and this change supplanted the original proline-specific response with a phenylalanine-specific regulatory response (Table 2). The degree of derepression of proBA transcription of the mutant T-box system in response to phenylalanine starvation (sevenfold) is similar to that of the original T-box system in response to proline starvation (13-fold) (Table 2). This experiment also illustrates the fact that simply starving B. subtilis cells for an amino acid other than proline does not trigger an indiscriminate derepression of the expression of the proBA and proI genes.

In addition to the proline-responsive transcriptional control of the proBA and proI genes through a T-box regulatory mechanism, the enzyme activity of the B. subtilis ProB protein is subject to feedback control by very low concentrations of proline (Fig. 1); 7.5 × 10−6 M proline is sufficient to inhibit 50 % of the activity of the purified ProB enzyme (Chen et al., 2007). Hence, combined transcriptional and post-transcriptional regulatory mechanisms allow the B. subtilis cell to sensitively control its proline pool produced via the anabolic ProB-ProA-ProI route. As a consequence, a wasteful overproduction of proline is prevented when it is made as a precursor for protein synthesis. It is immediately apparent that the above-discussed control mechanisms make the anabolic ProB-ProA-ProI proline biosynthetic route unsuitable for providing the B. subtilis cell with the very large amounts of proline it needs for osmotic stress protection (Bremer, 2002; Whatmore et al., 1990).

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