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

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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* (Ordal & Gibson, 1977). Proline also serves as an important osmostress protectant (Bremer, 2002). For this task, proline can either be taken up by *B. subtilis* from the environment via the osmotically inducible OpuE transporter (Spiegelhalter & Bremer, 1998; von Blohn *et al.*, 1997) or be produced in very large quantities from the precursor glutamate (Belitsky *et al.*, 2001; Whatmore *et al.*, 1990). Proline belongs to a selected group of organic osmolytes, the compatible solutes, which are amassed by many micro-organisms to adapt to high-osmolarity growth conditions (Kempf & Bremer, 1998). The cellular pools required for proline as a building block for protein synthesis and as an osmostress protectant are very different. Osmotically non-stressed *B. subtilis* cells have a proline pool 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 Δ^1-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 osmostress protectant (Bremer, 2002). This osmoadaptive proline biosynthetic route is formed by the ProJ–ProA–ProH enzymes, and relies on isoenzymes for the first

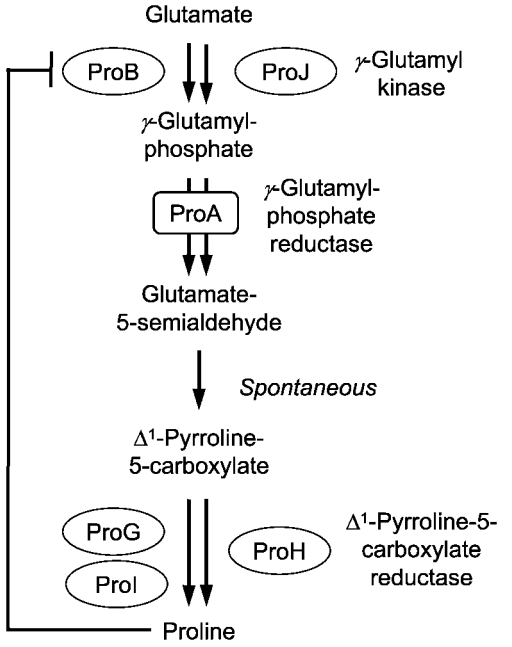

Fig. 1. Anabolic and osmoregulatory proline biosynthesis in *B. subtilis*. The proline biosynthetic enzymes and the intermediates of the enzymic conversion of glutamate into proline are indicated. Feedback inhibition of proline on the activity of the ProB enzymes is highlighted.

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 osmodaptive proline biosynthetic routes in *B. subtilis* are interconnected by the γ-glutamyl-phosphate reductase (ProA) (J. Brill and E. Bremer, unpublished results) (Fig. 1). Expression of the *proHJ* operon is strongly upregulated in response to either suddenly imposed or sustained osmotic stress, whereas that of the *proBA* operon or 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 Δ^1-pyrroline-5-carboxylase 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* (*yqjO*) 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; Vitreschak *et al.*, 2008; Wels *et al.*, 2008; Winkler, 2007). T-box systems are RNA-based regulatory switches (Henkin, 2008) that are used in many Gram-positive micro-organisms to regulate the expression of aminoacyl-tRNA synthetase genes and of genes involved in amino acid biosynthesis and uptake (Green *et al.*, 2010; Gutiérrez-Preciado *et al.*, 2009; Winkler, 2007).

T-box-controlled genes typically possess very long 5′-untranslated mRNA segments that function as tRNA-responsive riboswitch elements and which can fold into mutually exclusive terminator or antiterminator secondary structures (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 JH642 (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

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

Strain*	Relevant genotype*	Source or reference
JH642	*trpC2 pheA1*	BGSC† 1A96
FSB1	Δ(*treA*::*neo*)*1*	Spiegelhalter & Bremer (1998)
TRB0	Δ(*treA*::*neo*)*1 amyE*:: [Φ(*treA*) *cat*]	Spiegelhalter & Bremer (1998)
JSB5	*amyE*::[Φ(*proB′-treA*)1 *′ykkE cat*] Δ(*treA*::*neo*)*1*	This study
JSB8	Δ(*proHJ*::*tet*)*1*	This study
JSB9	Δ(*proI*::*spc*)*1*	Belitsky *et al.* (2001)
JSB11	(Δ*proBA*::*cat*)*1*	This study
JSB13	Δ(*proHJ*::*tet*)*1* Δ(*proI*::*spc*)*1*	This study
JSB17	*proA* × pEPV1T (*tet*)	This study
JSB31	*amyE*:: [Φ(*proB′-treA*)*1 ′ykk cat*] (Δ*treA*::*neo*)*1 proA* × pEPV1T (*tet*)	This study
JSB31-mut1	*amyE*:: [Φ(*proB′-treA*)*2 ′ykkE, cat*] (Δ*treA*::*neo*)*1 proA* × pEPV1T (*tet*)	This study
JSB34	*proA* × pEPV1T (*tet*) (Δ*treA*::*neo*)*1*	This study
JSB41	Δ(*proHJ*::*tet*)*1* Δ(*proI*::*spc*)*1* Δ(*proG*::*ble*)*1*	This study
BB1951	SMY Δ(*proG*::*ble*)*1*	Belitsky *et al.* (2001)

*All strains, except BB1951, are derivatives of JH642 and therefore also carry the *trpC2 pheA1* mutations.
†Bacillus Genetic Stock Center.

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 pre-cultures in pre-warmed minimal media to OD_{578} 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 pre-grown 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 (0.2, 0.4, 0.8 or 1.2 mM) of proline. For experiments that involved starvation for phenylalanine, cultures were grown either with excess (0.1 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-α-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 *Bam*HI and *Eco*RI sites of the low-copy-number vector pHSG575 (B. Kempf and E. Bremer, unpublished data). A 2.75 kb *Sal*I–*Eco*RI DNA fragment (*′ykkE–proB–proA′*) from pBKB26 was cloned into the *Sal*I- and *Eco*RI-restricted pBluescriptSK$^-$ vector (Stratagene), thereby yielding plasmid pJS3. For the construction of the *proBA* gene disruption mutation, a *Hin*dIII and *Bcl*I fragment (224 bp) was deleted from plasmid pJS3 and replaced with a chloramphenicol-resistance cassette derived from plasmid pRMK59 (Kappes *et al.*, 1999), resulting in plasmid pJS8. A *proBA–treA* reporter gene fusion was constructed as follows. The 1.05 kb *Mun*I–*Sau3*A 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 *Eco*RI and *Bam*HI; this construction yielded plasmid pJS2. The 1.02 kb *Eco*RV–*Sau3*AI *proB* fragment derived from pJS2 was then inserted into the *Sma*I and *Bam*HI sites present in the polylinker of the low-copy-number *treA* reporter plasmid pJMB1 (M. Jebbar and E. Bremer, unpublished results), thereby yielding a *proB–treA* operon fusion (present on plasmid pJS5) with *treA* as the reporter gene (Gotsche & Dahl, 1995; Schöck *et al.*, 1996). To construct a *proHJ*::*tet* gene disruption mutation, we first amplified the *′rtp–proH–proJ–gltC′* region from the *B. subtilis* chromosome of strain JH642 via PCR, cleaved it with *Sca*I and *Mun*I, and then inserted the resulting restriction fragment (2578 bp) into plasmid pBluescriptSK$^-$ that had been cleaved with *Eco*RI and *Eco*RV; this yielded plasmid pJS13. A 1.9 kb *Pst*I–*Xba*I restriction fragment, carrying a tetracycline-resistance cassette, was isolated from plasmid pBEST307 (Itaya, 1992) and ligated into the plasmid backbone of pJS13 cleaved with *Nsi*I and *Avr*II. In the resulting plasmid, pJS19, an internal DNA segment of the *proHJ* operon (1.089 kb) was replaced by the *tet* resistance cassette.

Construction of *B. subtilis* strains. Strain JSB11 [Δ(*proBA*::*cat*)*1*] was isolated after transformation of strain JH642 with DNA of plasmid pJS8 (linearized with the restriction enzymes *Bam*HI and *Eco*RI) and subsequent selection for chloramphenicol-resistant transformants. Strain JSB17 [*proA* × pEPV1T (*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*)*1*] (Spiegelhalter & Bremer, 1998) by DNA transformation with chromosomal DNA from strain JSB17 and subsequent selection for tetracycline-resistant colonies. This yielded strain JSB34 [Δ(*treA*::*neo*) (*proA* × pEPV1T (*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 *Pst*I and *Xho*I) and selecting for chloramphenicol-resistant transformants. In this way, the *proB–treA* gene fusion was

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(*proHJ*::*tet*)*1*] was isolated by transforming linear DNA of plasmid pJS19 (cleaved with *Bam*HI) into strain JH642 and subsequently selecting for tetracycline-resistant transformants. The [Δ(*proHJ*::*tet*)*1*] 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(*proI*::*spc*)*1* Δ(*proHJ*::*tet*)*1*] (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(*proI*::*spc*)*1* Δ(*proHJ*::*tetI*) Δ(*proG*::*ble*)*1*].

TreA enzyme assays. The expression of chromosomal *proB–treA* fusions was monitored by assaying the TreA [phospho-α-(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 pJMB1 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′ end 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 (OD_{578} 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 (A_{260}); an A_{260} 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 *proI*, 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 cm^2 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 *proI* 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 (OD_{578} 0.8) was isolated as described above. Reverse-transcriptase assays for the *proBA* and *proI* mRNAs were carried out with 15 μg total RNA and about 2 pmol of either a ^{32}P-5′-end-labelled *proBA*-specific oligonucleotide (5′-TCTCTGTCACAGAAAGGTCTGC-3′) or an IRD-800-fluorescence-end-labelled *proI*-specific oligonucleotide (5′-TTTTCCTAACCG-CGACTTGAGGGC-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 *proB* mRNA. The size of the *proI* reverse transcript was analysed on a LI-COR DNA sequencer (model 4000) (Eurofins). The DNA primer employed for the *proI* reverse transcription reaction was used with plasmid pJS18 (Belitsky *et al.*, 2001) to determine the exact position of the 5′ end of the *proI* 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 QuikChange 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-mut1 was linearized by digestion with *Xho*I and *Pst*I, 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 *proI* 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 *proI* transcription initiation sites

To map the transcription initiation sites of the *proBA* and *proI* 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

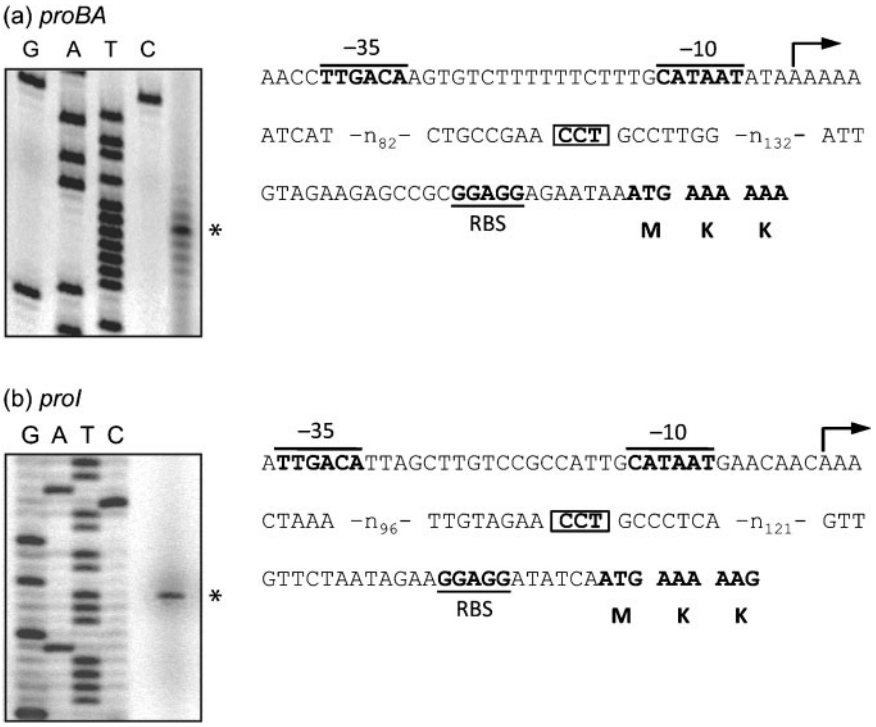

Fig. 2. Mapping of the *proBA* (a) and *proI* (b) transcription initiation sites by primer extension analysis. The 5′ ends of the *proBA* and *proI* transcripts were mapped using mRNA isolated from the proline-prototrophic strain JH642; the asterisk indicates the cDNA fragment generated via the primer 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.

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).

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.

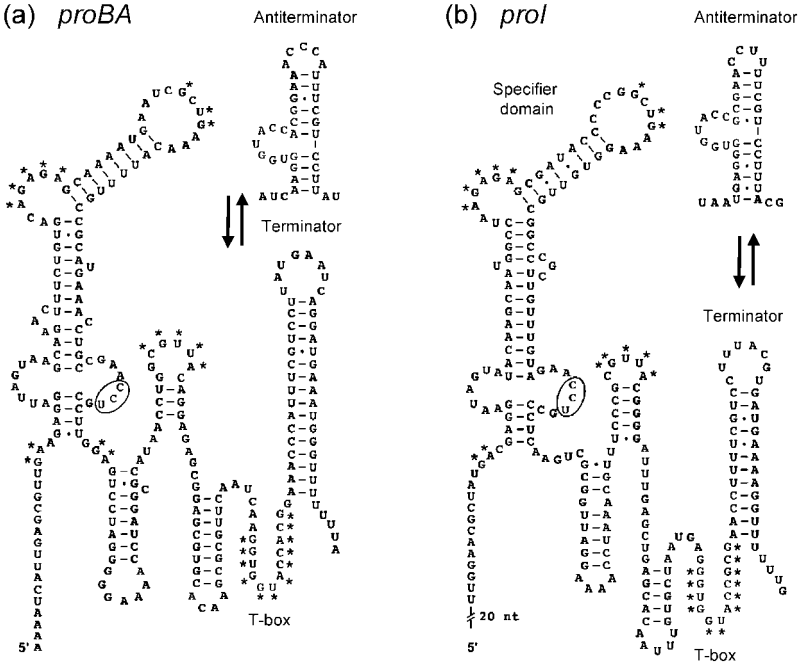

Fig. 3. Predicted secondary structures of the *proBA* (a) and *proI* (b) leader transcripts. The secondary structure of the 5′ regions of the *proBA* and *proI* transcripts were predicted with Mfold and edited manually for their termination and antitermination configurations. The suggested proline-specific specifier codons for the *proBA* and *proI* genes and the T-box sequences are indicated.

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; Gutiérrez-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 Δ^1-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

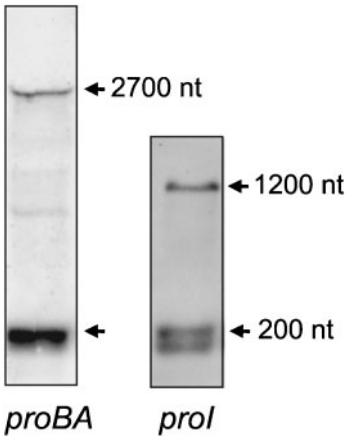

Fig. 4. Northern blot analysis of the *proBA* and *proI* transcripts in cells that are not starved for proline. Cells of the proline-prototrophic strain JH642 were grown to the mid-exponential growth phase, and total RNA was prepared and separated according to size under denaturing conditions on a 1.4 % agarose gel; then the *proBA*- and *proI*-specific transcripts were detected by hybridization with DIG-labelled single-stranded DNA probes. Arrows mark the *proBA* and *proI* leader and full-length transcripts.

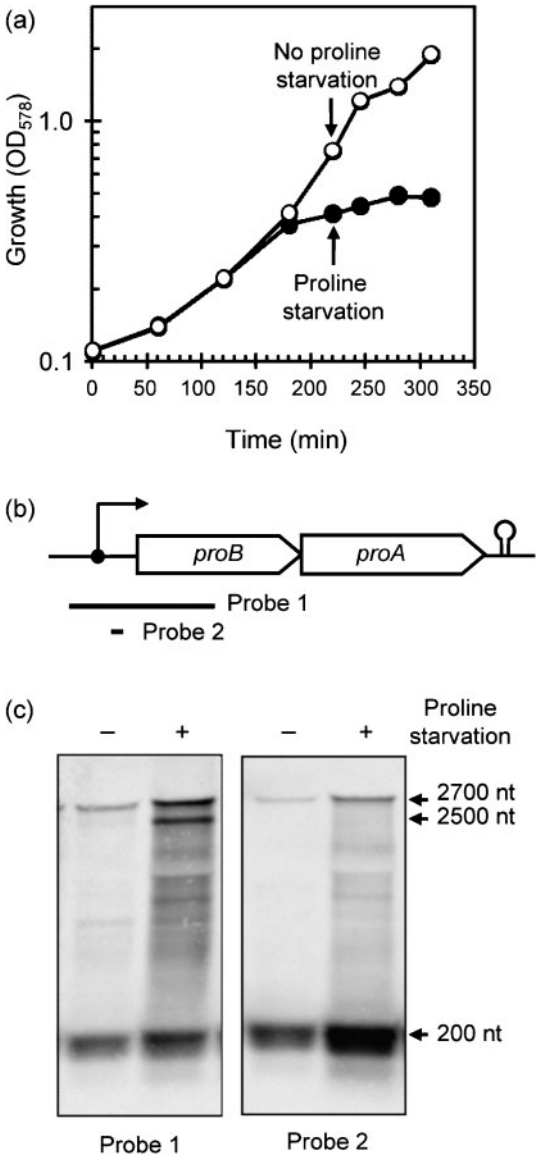

Fig. 5. Derepression of *proB* transcription in response to proline starvation. (a) The proline-auxotrophic strain JSB41 (*proG*, *proH*, *proI*) was grown in SMM with either 10 mM proline (no starvation for proline) or 0.6 mM proline (starvation for proline). As soon as proline starvation set in for the proline-limited culture, cells were harvested from both cultures and total RNA was isolated. The RNA was separated according to size under denaturing conditions on a 1.4 % agarose gel, and the *proBA*- and *proI*-specific transcripts were detected by hybridization with DIG-labelled single-stranded DNA probes (b) covering either the entire 5′ segment of the *proBA* transcript and part of the *proB* coding region (probe 1) or only part of the *proB* mRNA leader region (probe 2). The detected *proBA* mRNA leader, the full-length transcript and the processed mRNA species are displayed in (c).

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

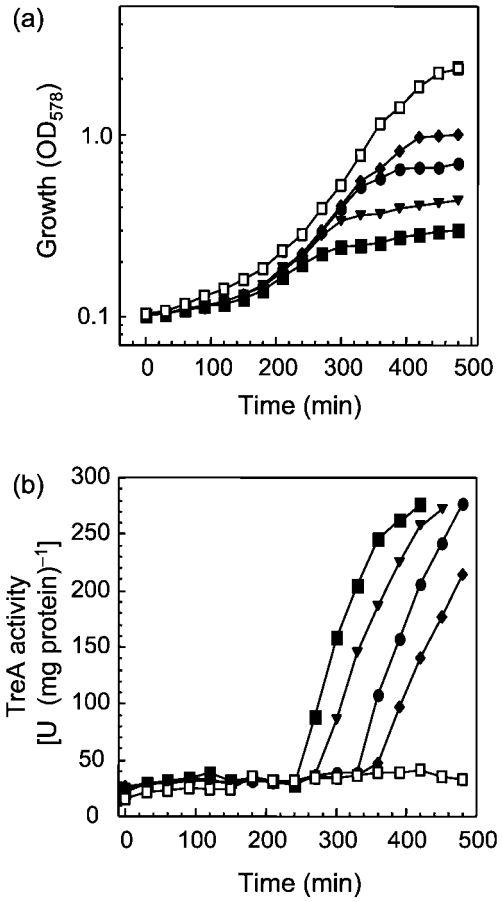

Fig. 6. Induction of *proB–treA* expression in response to proline starvation. The expression of a *proB–treA* reporter gene fusion was monitored in the proline prototrophic strain JSB5 (open symbols) and its proline-auxotrophic derivative strain JSB31 (filled symbols). Strain JSB5 was cultivated in SMM in the absence of proline (□) and strain JSB31 was propagated in SMM supplemented with 0.2 mM (■), 0.4 mM (▲), 0.8 mM (●) or 1.2 mM (◆) proline. Growth of the cultures was monitored (a), and by measuring the activity of the TreA reporter enzyme the expression of the *proB–treA* transcriptional gene fusion present in strains JSB5 and JSB31 was assessed (b).

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

Reporter strain*	Specifier codon	TreA activity [U (mg protein)$^{-1}$]					
		Starvation for proline		Fold-induction	Starvation for phenylalanine		Fold-induction
		−	+		−	+	
JSB31	CGAA **CCU** GCCU	21 ± 4	270 ± 21	12.9	12 ± 2	10 ± 1	0.8
JSB31-mut1	CGAA **UUC** GCCU	11 ± 2	13 ± 1	1.2	6 ± 1	40 ± 3	6.7

*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 JB31 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 0.1 mM phenylalanine to satisfy the auxotrophic needs of the strains. For the phenylalanine starvation experiment, the strains were cultivated either in the presence of excess phenyalanine (0.1 mM) or with limiting amounts of phenylalanine (0.016 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 that of the *proI* gene is regulated through a proline-responsive T-box regulatory system. This tRNA-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 *proI* 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 *proI* 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 *proI* transcript in proline-starved *B. subtilis* cells by Northern blot analysis did not reveal any evidence for a processing event of the full-length *proI* 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 *proI*-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 *proI* (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 $tRNA^{Pro}$ (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 osmostress protection (Bremer, 2002; Whatmore *et al.*, 1990).

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