Transcriptional regulation of the *Bacillus subtilis* *asnH* operon and role of the 5'-proximal long sequence triplication in RNA stabilization

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The *Bacillus subtilis* *asnH* operon, comprising *yxbB*, *yxbA*, *yxnB*, *asnH* and *yxam*, is induced dramatically in the transition between exponential growth and stationary phase in rich sporulation medium. The *asnH* operon is transcribed to produce an unstable long transcript covering the entire operon as well as a short one corresponding to the first three genes. Northern blot analysis revealed that the discrete band corresponding to the short transcript was detectable even 1 h after the addition of excess rifampicin, suggesting its unusual stability. The transcription start site of the operon was determined; its corresponding promoter was most likely sigma-A dependent and under tight control of AbrB and CodY. Within the 5'-proximal region of the transcript preceding *yxbB*, there is a mysterious long sequence triplication (LST) segment, consisting of a tandem repeat of two highly conserved 118 bp units and a less conserved 129 bp unit. This LST segment was not involved in regulation by AbrB and CodY. Transcriptional fusion of the 5'-region containing the LST segment to *lacZ* resulted in a significant increase in β -galactosidase synthesis in cells; the LST segment was thought to prevent degradation of the 5'-region-*lacZ* fusion transcript. These results suggest that the 5'-region containing the LST segment could function as an mRNA stabilizer that prolongs the lifetime of the transcript to which it is fused.

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INTRODUCTION

asnH is the fourth gene of the *Bacillus subtilis* *asnH* operon, which is composed of the genes *yxbB*, *yxbA*, *yxnB*, *asnH* and *yxam* (Yoshida *et al.*, 1999). *asnH* encodes an active asparagine synthetase, since its expression partially compensated asparagine auxotrophy of an *Escherichia coli* mutant (Yoshida *et al.*, 1999). However, it was observed that *asnH* contributed only partially to net asparagine synthesis because it was not required for growth in minimal medium lacking asparagine (Yoshida *et al.*, 1999). Two genes, *yxbB* and *yxam*, of the operon are predicted to encode a putative methyltransferase and an antibiotic-resistance-protein-like transporter, respectively, while *yxbA* and *yxnB* are functionally unknown and unique in databases (see the BSORF website: <http://bacillus.genome.ad.jp/>). The *asnH* operon was shown to produce

a 5.5 kb long transcript covering the entire operon as well as a mixture of various shorter fragments, presumably corresponding to its 5'-portions (Yoshida *et al.*, 2000).

During exponential growth, AbrB represses a number of transition-state genes. In the transition to stationary phase, the two-component system regulator Spo0A is activated to repress *abrB*, followed by expression of AbrB-repressed genes (Xu & Strauch, 2001). Since a previous transcriptome analysis indicated that *abrB* inactivation resulted in elevated expression of the *asnH* operon, transcription of the *asnH* operon was suggested to be under the repressive control of AbrB (Hamon *et al.*, 2004).

CodY is another global regulator that senses nutritional conditions of cells and functions as a GTP-binding repressor of many genes that are normally quiescent in cells growing in rich medium (Ratnayake-Lecamwasam *et al.*, 2001). In the transition to nitrogen-limited growth phase, when intracellular GTP levels decrease, CodY was shown to lose its repressor function, thereby inducing its targets. It is also known that branched-chain amino acids

Abbreviation: LST, long sequence triplication.

Two supplementary tables are available with the online version of this paper.

serve as co-effectors of CodY (Villapakkam *et al.*, 2009). Combined chromatin immunoprecipitation and transcriptome analyses previously demonstrated that CodY binds to a DNA segment containing *yxbB*, thereby repressing the *asnH* operon (Molle *et al.*, 2003). Therefore, it was suggested that the *asnH* operon could be under the control of CodY.

Upstream of *yxbB*, a mysterious long sequence triplication (LST) segment (Fig. 1) was found to consist of a tandem repeat of two highly conserved 118 bp units (LST1 and LST2, formerly known as TC and TB, respectively) and a less-conserved but related 129 bp unit (LST3, formerly known as TA) (Yoshida *et al.*, 1995). Since this segment is

unique within the genome sequence (Kunst *et al.*, 1997), it may have a significant biological function to be maintained during evolution. Previously at least a promoter-like sequence was predicted within each of the LST1 and LST2 stretches (Yoshida *et al.*, 1995), but its function was not examined.

In this study, we intensively examined transcriptional regulation of the *asnH* operon. We confirmed dual regulation of the *asnH* operon by both CodY and AbrB through *lacZ* fusion experiments. In addition, our data suggested that the LST segment most likely functions to stabilize the transcripts and to extend their intracellular lifetime.

Fig. 1. Promoter region of the *asnH* operon. Nucleotide sequences (upper and lower strands) of the *asnH* promoter region are represented. Predicted amino acid sequences of short peptides and the 5'-part of *yxbB* ORF are shown by three-letter symbols beneath nucleotide sequences, and possible ribosome-binding sites are in italics in the upper strand sequence. The transcription start base (+1) identified in this work and the presumed -35 and -10 regions of the promoter are indicated. Upper-strand sequences corresponding to each of the repeating units of LST (LST1, 2 and 3) are boxed. Positions of PCR primers (Supplementary Table S2) are indicated by arrows.

METHODS

Bacterial strains, plasmids, media and oligonucleotides. Strains and plasmids used in this work are listed in Supplementary Table S1 (available with the online version of this paper). *B. subtilis* cells were grown on tryptose blood agar base (Difco) plates supplemented with 0.18% glucose at 30 °C and in Difco sporulation medium (DSM) (Schaeffer *et al.*, 1965) at 37 °C with shaking. *E. coli* cells were grown in Luria–Bertani (LB) medium (Sambrook & Russell, 2001) at 37 °C with shaking. Strain JM109 was used as the host in plasmid constructions. When needed to select and grow mutants and transformants, chloramphenicol (10 mg l⁻¹), erythromycin (0.3 mg l⁻¹), tetracycline (10 mg l⁻¹) and ampicillin (50 mg l⁻¹) were added to the culture media. Oligonucleotides used in this study are listed in Supplementary Table S2.

Construction of *B. subtilis* strains. The *yxbB* gene of strain 168 was disrupted by integration of plasmid pMUTIN2 as described previously (Yoshida *et al.*, 2000). Part of *yxbB* was amplified using PCR with a specific primer pair and strain 168 DNA as a template; nucleotide sequences of primers used are available at the BSORF website. PCR products were trimmed with *Hind*III and *Bgl*II and were ligated to the *Hind*III–*Bam*HI arm of pMUTIN2 (Vagner *et al.*, 1998). The recombinant plasmid was used to transform strain 168 to erythromycin resistance to obtain YXBBd (Supplementary Table S1). The promoter of the *asnH* operon was inactivated using a strategy similar to that described previously (Serizawa *et al.*, 2004). This was performed by inserting a tetracycline-resistance gene (*tet*) cassette between the –35 and –10 regions by the following procedure. A DNA fragment carrying the *tet* cassette was amplified from plasmid pTC177 (Yoshida *et al.*, 1993) by PCR using the primer pair TET-F/TET-R (Supplementary Table S2). The other two DNA fragments corresponding to upstream and downstream parts of the promoter region were amplified from strain 168 DNA by PCR using primer pairs PyxbB-F1/PyxbB-R1 and PyxbB-F2/PyxbB-R2, respectively (Supplementary Table S2). The 5'-end sequences of PyxbB-R1 and PyxbB-F2 were complementary to those of TET-R and TET-F, respectively. The three PCR fragments were mixed and ligated using PCR performed with the outside primer pair PyxbB-F1/PyxbB-R2. The resulting PCR fragment was used to transform YXBBd to tetracycline resistance to obtain KY001 (*PasnH::tet*, Supplementary Table S1). Similarly, the *abrB* gene of strain 168 was replaced with a chloramphenicol acetyltransferase gene (*cat*) cassette by the following procedure. A DNA fragment carrying the *cat* cassette was amplified from pCBB31 (Serizawa *et al.*, 2004) by PCR using the primer pair PUC-F and PUC-R (Supplementary Table S2). The other two DNA fragments corresponding to upstream and downstream regions flanking *abrB* were amplified from strain 168 DNA by PCR primer pairs *abrB*-F1/*abrB*-R1 and *abrB*-F2/*abrB*-R2, respectively (Supplementary Table S2). The 5'-end sequences of *abrB*-R1 and *abrB*-F2 were complementary to those of PUC-R and PUC-F, respectively. The three PCR fragments were mixed and ligated via PCR performed with the outside primer pair *abrB*-F1/*abrB*-R2. The resulting PCR fragment was used to transform strain 168 to chloramphenicol resistance to obtain TF2 (*abrB::cat*, Supplementary Table S1). DNA of YXBBd was used to transform NIG1131 (*spo0A34*), TF2 and PS37 (*ΔcodY*) to erythromycin resistance to obtain KY002, KY003 and KY004, respectively (Supplementary Table S1). DNA of TF2 was used to further transform KY002 and KY004 to chloramphenicol resistance to obtain KY005 and KY006, respectively (Supplementary Table S1). In order to evaluate the possible function of the LST segment, a series of *lacZ* reporter fusions were integrated into the *amyE* locus of the chromosome using the following method. Four DNA fragments corresponding to parts of the upstream region of the *asnH* operon were amplified by PCR using strain 168 DNA as a template and primer pairs PyxbBE/PyxbBB1 for a fragment of the *asnH* operon

promoter region alone, PyxbBE/PyxbBB2 for a fragment of the promoter region plus the LST1 segment as well as another fragment of the promoter region plus both the LST1 and LST2 segments and PyxbBE/PyxbBB4 for a fragment of the promoter region plus the entire LST segment (Supplementary Table S2; see Figs 1 and 5). Another PCR fragment containing the promoter region plus the LST3 segment alone was obtained by recombinant PCR using outside primers PyxbBE and PyxbBB4 and inside overlapping primers PyxbBB14R and LST3F (Supplementary Table S2; see Figs 1 and 6). Each of the five fragments was trimmed with *Eco*RI and *Bam*HI and then ligated to the arm of pCRE-test (Miwa *et al.*, 2000) that had been cleaved with the same two enzymes, thereby eliminating an *Eco*RI–*Bam*HI segment containing the *spac* promoter. Correct construction of the five recombinant plasmids by placing *lacZ* under the control of the *asnH* operon promoter region was confirmed by DNA sequencing. Each plasmid was linearized by *Pst*I digestion and then used to transform strain 168 to chloramphenicol resistance, thereby integrating the *lacZ* reporter fusion into the *amyE* locus. Resulting transformants were further transformed with pCm::Tc (Steinmetz & Richter, 1994) to change their antibiotic resistance from chloramphenicol to tetracycline resistance, yielding strains KY007, KY008, KY009, KY010 and KY023 (Supplementary Table S1). Chromosomal DNAs prepared from KY007, KY008, KY009 and KY010 were used to transform TF2 (*abrB::cat*) to tetracycline resistance to obtain KY011, KY012, KY013 and KY014, respectively (Supplementary Table S1). Similarly, KY015, KY016, KY017 and KY018 were obtained by transformation of PS37 (*ΔcodY*) (Supplementary Table S1). KY015, KY016, KY017 and KY018 were further transformed to chloramphenicol resistance with TF2 DNA to obtain KY019, KY020, KY021 and KY022, respectively (Supplementary Table S1). Within each of the repeating units, a putative ORF was found, encoding the same short peptide of 22 amino acid residues, which is associated with a probable ribosome-binding site (Fig. 1). *B. subtilis* strains TM101 and TM102 (Supplementary Table S1) were designed to test the possible translation of the short ORF by *lacZ* translational fusions integrated into the *amyE* locus and constructed as follows. To construct TM101, a DNA fragment covering the promoter region and a proximal part of LST1 corresponding to the entire ORF (Fig. 1) was amplified using PCR with the primer pair PyxbBE/ORF1 (Fig. 1; Supplementary Table S2), which was designed to make an in-frame fusion of the ORF and *lacZ* reporter. For TM102, another fragment from the promoter to the stop codon of the ORF was amplified with the primer pair PyxbBE/ORF2 similarly (Fig. 1; Supplementary Table S2). Each of the fragments was trimmed with *Eco*RI and *Hind*III and ligated with the *Eco*RI and *Hind*III arm of ptpBGI (Shimotsu & Henner, 1986). The resulting recombinant plasmid, whose correct construction was confirmed by DNA sequencing, was linearized by *Pst*I digestion and then used to transform 168 to chloramphenicol resistance, thereby integrating the *lacZ* reporter fusion into the *amyE* locus.

RNA techniques. *B. subtilis* strains 168, KY007, KY008, KY009, KY010 and KY023 were grown in DSM. Cells were harvested at several time points after inoculation or addition of excess rifampicin (200 mg l⁻¹), and were disrupted to extract total RNA by vigorous shaking with glass beads in the presence of SDS and phenol (Yoshida *et al.*, 2001). Primer extension experiments were performed as described previously (Yoshida *et al.*, 2002). RNA samples prepared from strain 168 were used as templates for reverse transcription initiated by the specific primer PyxbBP (Supplementary Table S2, Fig. 1) that had been 5'-end labelled with [γ -³²P]ATP (Amersham) using a MEGALABEL kit (Takara Shuzo). Sequence ladders were produced from the same primer by dideoxy sequencing reactions using a template of PCR fragment amplified from strain 168 DNA with primers PyxbBup and PyxbB-R2 (Supplementary Table S2, Fig. 1). Northern blot analysis was done as described previously (Yoshida *et al.*, 2003). Probe DNA, parts of *lacZ*, *asnH*, *yxbB* and

ynb, were PCR products amplified from pMUTIN2 DNA using the primer pair LacZ1/LacZ2. Similarly, strain 168 DNA was amplified using primer pairs *asnH*H/*asnH*B, *yxbB*-F/*yxbB*-R and *ynb*-F/*ynb*-R (Supplementary Table S2), and was labelled with [α - 32 P]dCTP (ICN Biomedicals) using a BcaBest labelling kit (Takara Shuzo).

RESULTS

Two transcripts of the *asnH* operon with different length and stability

Strain 168 cells were grown in DSM, and further mRNA synthesis was arrested by addition of excess rifampicin when the *asnH* operon was induced early in the stationary phase (Yoshida *et al.*, 1999, 2000). Total RNA samples were prepared from these cells and subjected to Northern blot analysis using *yxbB*, *ynb* and *asnH* probes to reveal at least two bands with different lengths (Fig. 2). The longer band corresponding to the 5.5 kb transcript hybridized with all probes, indicating that this transcript could cover the entire operon as described previously (Yoshida *et al.*, 2000). The shorter band corresponding to a 2 kb transcript was not detected by the *asnH* probe but by both *yxbB* and *ynb* probes, which could cover only the proximal three genes *yxbB*, *yxbA* and *ynb*. It is likely that the 5.5 kb transcript was unstable and disappeared within 30 min after transcription arrest, while the short one was still detectable

even after 1 h. These results suggest specific and unusual stability of the 2 kb transcript.

Identification of the *asnH* operon promoter

Strain YXBBd had *yxbB* disrupted through integration of pMUTIN2, and the pMUTIN2-borne *lacZ* reporter gene (Vagner *et al.*, 1998) was used to monitor transcription of the *asnH* operon. Drastic increase in β -galactosidase synthesis in YXBBd cells indicated that the *asnH* operon was induced in the transition between exponential growth and stationary phase (Fig. 3). To determine the transcription start site of the *asnH* operon, a primer extension experiment was performed by localizing the 5'-end of the transcript at 409 bp upstream of the translation start site of *yxbB* (data not shown). We found a presumed promoter of the *asnH* operon with -35 (TTGACA) and -10 (TAATAT) sequences separated by 17 bp, which was likely to be recognized by sigma-A RNA polymerase (Fig. 1). When the predicted promoter was disrupted by insertion of the *tet* cassette between the presumed -35 and -10 regions (Fig. 1), β -galactosidase synthesis arrested completely (Fig. 3). These results indicate that the promoter actually functions to drive transcription of *yxbB* at least and presumably the entire *asnH* operon. The 5'-proximal region of the *asnH* transcript upstream of *yxbB* contains the LST segment, which consists of a tandem repeat of two

Fig. 2. Northern blot analysis of the *asnH* transcript. The analysis was performed using RNA samples from strain 168 and (a) *yxbB*-, (b) *ynb*- and (c) *asnH*- specific probes. RNA samples were prepared 5 h after inoculation, when induction was established and when excess rifampicin was added to the culture (lane 1) as well as 30 min (lane 2) and 1 h (lanes 3) after rifampicin addition. Each lane contained 15 μ g RNA. Long (L) and short (S) transcripts are indicated by arrows on the right. Positions of size marker RNA are indicated on the left. (d) Schematic summary of the Northern blot analysis.

Fig. 3. Identification of the *asnH* operon promoter. Growth curves and β -galactosidase activities in protein extracts of cells of strains YXBBd (carrying the intact promoter of *asnH* operon; ■ and ●, respectively) and KY001 (in which the *asnH* operon promoter is inactivated; □ and ○, respectively) grown in DSM. Enzyme activities were measured as described by Yoshida *et al.* (2000). Experiments were repeated at least three times, and similar results were obtained each time. Representative results are shown.

highly conserved 118 bp LST1 and LST2 units as well as a less conserved 129 bp LST3 unit (Fig. 1). This finding implies that our previous prediction that promoter-like sequences within LST1 and LST2 units might drive transcription of this operon was not correct (Yoshida *et al.*, 1995). Since the Northern analysis revealed that both the long and short transcripts contained the *yxkB* region (Fig. 2), it is likely that the identified promoter directs transcription of both transcripts. Several plausible terminator-like sequences were predicted just downstream of *yxnB* by a computer program (Ermolaeva *et al.*, 2000) (data not shown), and it is possible that the short transcript was produced through premature termination. However, we cannot exclude the possibility that it could be a by-product of the degraded long transcript.

Dual regulation of the *asnH* operon by AbrB and CodY

Hamon *et al.* (2004) suggested that the *asnH* operon was under the control of AbrB. In an *abrB* knockout strain, β -galactosidase synthesis, which reports the expression of the *asnH* operon, was more elevated in the transition to stationary phase of this strain than in the wild-type strain, suggesting that AbrB repressed *asnH* expression (KY003 in Fig. 4). In addition, a defective mutation of *spo0A* (*spo0A34*) completely shut off the synthesis (KY002 in Fig. 4). This is probably due to constitutive expression of *abrB*, as activated Spo0A represses *abrB* transcription (Perego *et al.*, 1988). As expected, simultaneous inactivation of *abrB* and *spo0A* resulted in induction of β -galactosidase activity similar to its induction due to *abrB* mutation (KY005 in Fig. 4). These results indicated that

Fig. 4. The *asnH* operon is under the dual control of AbrB and CodY. β -Galactosidase activities in protein extracts of cells of strains YXBBd (○), KY002 (*spo0A34*, ●), KY003 (*abrB::cat*, □), KY004 (Δ *codY*, ■), KY005 (*abrB::cat* and *spo0A34*, △) and KY006 (*abrB::cat* and Δ *codY*, ▲) grown in DSM medium are indicated. All strains exhibited growth comparable to that shown in Fig. 3. Experiments were performed similarly as in Fig. 3 and repeated at least three times, and similar results were obtained each time. Representative results are shown.

AbrB acts as a negative regulator of the *asnH* operon. However, the expression was still repressed even in the absence of AbrB during the exponential growth phase (KY003 and KY005 in Fig. 4), suggesting that there might be a second regulator besides AbrB.

A possible second regulator was CodY, since Molle *et al.* (2003) reported that CodY binds to a DNA segment containing *yxkB* and suggested that this regulator protein could be involved in repression of the *asnH* operon. To test this possibility, the *lacZ* reporter system was introduced into strain PS37, a *codY* mutant, revealing that the expression was slightly elevated even during exponential growth despite the presence of active AbrB (KY004 in Fig. 4). The observation that simultaneous inactivation of *codY* and *abrB* resulted in completely constitutive expression provided more conclusive evidence for the regulatory role of CodY (KY006 in Fig. 4). These results clearly indicated that *asnH* transcription was under the dual control of AbrB and CodY.

Neither AbrB nor CodY requires the LST segment

We suspected that the LST segment played a role in the dual regulation of the *asnH* operon by AbrB and CodY. To test this possibility, the promoter region with the 5'-proximal region containing a deletion series of the LST segment was fused to the *lacZ* reporter and then integrated into the *amyE* locus (Fig. 5a). Strain KY010 carrying the *asnH* promoter with the 5'-proximal region containing the entire LST segment fused to *lacZ* (LST1-2-3, Fig. 5a) exhibited significant induction of β -galactosidase synthesis in the transition to stationary phase (Fig. 5b). The highest activity was almost eightfold lower than that observed in strain YXBBd, which might be due to the difference in the

Fig. 5. β -Galactosidase synthesis in *B. subtilis* strains expressing *lacZ* fused to the LST-deletion series. (a) *lacZ* reporter constructs to evaluate the LST-deletion series. Each of the DNA fragments corresponding to the *asnH* operon promoter region alone (without LST), with LST1 (LST1), with both LST1 and 2 (LST1-2), with the entire LST (LST1-2-3) and with LST3 alone (LST3) was fused to a promoterless *lacZ* gene and integrated into the *amyE* locus as shown schematically. (b–e) β -Galactosidase activities in protein extracts of cells of strains grown in DSM. (b) Strains KY010 (○), KY014 (*abrB*::*cat*, ●), KY018 ($\Delta codY$, □) and KY022 (*abrB*::*cat* $\Delta codY$, ■) expressing the LST1-2-3 fusion. (c) Strains KY009 (○), KY013 (●), KY017 (□) and KY021 (■) expressing the LST1-2 fusion. (d) Strains KY008 (○), KY012 (●), KY016 (□) and KY020 (■) expressing the LST1 fusion. (e) Strains KY007 (○), KY011 (●), KY015 (□) and KY019 (■) expressing *lacZ* without the LST segment. All strains grew almost similarly as shown in Fig. 3. Experiments were repeated at least three times and similar results were obtained each time. Representative results are shown for each panel.

junction between the promoter and the *lacZ* reporter. In the KY010 background, *abrB* inactivation led to slightly higher β -galactosidase synthesis even during exponential growth and much stronger activity in the transition to stationary phase, whereas *codY* inactivation gave almost no effect. However, simultaneous inactivation of *abrB* and *codY* resulted in high levels of β -galactosidase synthesis and its nearly constitutive expression (Fig. 5b). These results indicated that the *lacZ* reporter system with the entire LST region was under the dual control of AbrB and CodY, as observed in the pMUTIN2-integrand (Fig. 4), although regulation by AbrB was more prominent than that in the former construct for unknown reasons. When the three repeating units of the LST segment were deleted in a stepwise fashion from the 3'-end (LST1-2 of KY009, LST1 of KY008, and without LST of KY007 in Fig. 5a), β -galactosidase synthesis became lower than that observed in strain KY010 (LST1-2-3) (Fig. 5c–e). In each mutant possessing one of the deletion series of the LST segment, *abrB* inactivation elevated β -galactosidase synthesis after the transition phase, and furthermore, simultaneous inactivation of *abrB* and *codY* made it much higher and constitutive. As shown in Fig. 5(e), the dual regulation by AbrB and CodY was seen even in the absence of the whole LST segment. Thus, we concluded that neither AbrB nor CodY required the LST segment for regulation of *asnH* expression.

The 5'-proximal region containing the entire LST segment as an mRNA stabilizer

We observed that *lacZ* fusion with the entire LST (LST1-2-3) segment had a tendency for enhanced β -galactosidase

synthesis as compared with other fusions (Fig. 5b–e), as described above. On the other hand, one of the authentic *asnH* operon transcripts, the short one covering *yxbB*, *yxbA* and *yxnB*, exhibited an extended lifetime (Fig. 2). These observations prompted us to examine the stability of the artificial LST–*lacZ* fusion transcripts. Cells of strains KY007 (without LST), KY008 (LST1), KY009 (LST1-2), KY010 (LST1-2-3) and KY023 (LST3), an additional construct carrying the promoter and LST3 alone, fused to *lacZ* (Fig. 5a), were subjected to a transcript-stability test, performed similarly to that described above (Fig. 2), and RNA samples from cells were subjected to Northern blot analysis to probe *lacZ* transcripts (Fig. 6). At the time of rifampicin addition, *lacZ* transcripts synthesized in cells of the five strains were detected as respective distinct bands of different sizes owing to various lengths of deleted regions. The signal density of the bands suggested that all fusion transcripts were initially synthesized in comparable amounts. However, transcripts in strains KY007 (without LST), KY008 (LST1), KY009 (LST1-2) and KY023 (LST3) rapidly decreased within 30 min after rifampicin addition and almost disappeared in the subsequent 30 min, while only the transcript in strain KY010 (LST1-2-3) was retained even after 1 h (at least more than 50% of the initial amount remained). Results indicated that the LST1-2-3–*lacZ* fusion transcript synthesized in strain KY010 was less degraded than those synthesized in the other strains, KY007, KY008, KY009 and KY023, suggesting that the transcript could be stabilized. Consequently, the 5'-proximal region containing the complete LST segment was able to extend the lifetime of artificial mRNA to which it was fused, resulting in higher β -galactosidase activity in this case.

Fig. 6. Northern blot analysis of *lacZ* mRNAs from strains of *B. subtilis* expressing *lacZ* fused with the 5'-proximal region containing the LST-deletion series. Northern blot analysis with the *lacZ*-specific probe was performed similarly to the method described in Fig. 2, except for using total RNA samples obtained from cells of strains KY007 (without LST, lanes 1–3), KY008 (LST1, lanes 4–6), KY009 (LST1-2, lanes 7–9), KY010 (LST1-2-3, lanes 10–12) and KY023 (LST3, lanes 13–15) at time points of rifampicin addition (5 h after inoculation when induction was established, lanes 1, 4, 7, 10 and 13) as well as 30 min (lanes 2, 5, 8, 11 and 14) and 1 h (lanes 3, 6, 9, 12 and 15) after rifampicin addition. Each lane contained 15 μ g of the RNA sample. The initial *lacZ* fusion transcripts detected when transcription was arrested are indicated by arrows. Positions of size marker RNAs are indicated on the left.

Upon re-examination of the LST segment, we found that each of the repeating units could contain an ORF encoding the same short peptide of 22 amino acid residues, which is associated with a probable ribosome-binding site (Fig. 1). No protein in the databases exhibited a significant sequence similarity to this short peptide (data not shown). Two *B. subtilis* strains, TM101 and TM102, were constructed to examine the possible translation of the short ORF. TM101 was designed to have an in-frame translational fusion of the short ORF to the *lacZ* reporter, whose transcription was driven by the identified promoter. On the other hand, TM102 had another *lacZ* translational fusion similar to the one in TM101 but a stop codon was inserted at the fusion point, thus no fusion protein is produced. The two strains and KY007 were cultivated under the conditions as described to detect the induction of the *asnH* operon early in the stationary phase; however, neither TM101 nor TM102 gave any elevation in β -galactosidase activity (Fig. 7) compared to KY007, which exhibited the lowest activities among the LST deletion series (Fig. 5). These results indicated that the short ORF was not translated, and it is not likely that the possible translation of the short ORF is involved in the mRNA stabilization.

DISCUSSION

A model for the mechanism of regulation of the *asnH* operon is summarized schematically in Fig. 8. *asnH* transcription is drastically induced in the transition between exponential growth and stationary phase in a rich sporulation medium (Fig. 3), and both AbrB and CodY, which are global regulators responsible for induction of numerous genes in the transition state,

Fig. 7. β -Galactosidase activities in protein extracts of cells of strains grown in DSM. Strains TM101 (*P_{asnH}*-driven in-frame translational fusion of the short ORF to *lacZ*; ●), TM102 (*P_{asnH}*-driven in-frame translational fusion similar to TM101 but with a stop codon in between; ○), and KY007 (*P_{asnH}*-driven transcriptional *lacZ* fusion without LST; □) grew almost similarly as shown in Fig. 3. One of the representative results is shown for each panel. Experiments were repeated at least three times and similar results were obtained on repetition.

Fig. 8. A model mechanism for regulation of the *asnH* operon.

negatively control the induction. AbrB and CodY work independently of each other (Fig. 4). During exponential growth, AbrB represses its target genes because it is present at higher concentrations in vegetatively growing cells than in cells entering the transition phase (O'Reilly & Devine, 1997). In the transition phase, the concentration of AbrB is reduced because transcription of *abrB* is repressed by activation of Spo0A in cells sensing a nutrient-depleted environment to trigger expression of sporulation genes (Phillips & Strauch, 2002). CodY is a GTP-binding regulator that senses intracellular GTP concentration as an indicator of nutritional conditions (Ratnayake-Lecamwasam *et al.*, 2001). During vegetative growth, a sufficient supply of nutrients elevates GTP concentration, thereby activating CodY for repression of early-stationary-phase genes as well as sporulation ones. Recently, it was demonstrated that branched-chain amino acids also enhance binding of CodY to its target sites (Shivers & Sonenshein, 2004). Based on these facts, it is conceivable that the *asnH* operon is induced by nutrient depletion in the transition phase.

Previous studies failed to reveal a clear DNA sequence consensus recognized by AbrB, and DNA targeting of AbrB has been thought to depend on recognition of some tertiary DNA structure, a novel DNA-binding and genetic-regulation model of AbrB proposed recently (Vaughn *et al.*, 2000). So far, no strictly conserved sequence has been identified for CodY binding (Fisher, 1999; Belitsky & Sonenshein, 2008). Moreover, it has been reported that AbrB- and CodY-binding sites overlap in some cases (Hamoen *et al.*, 2003). DNA binding of AbrB for *asnH* regulation has not been examined yet; however, Molle *et al.*

(2003) reported that CodY could bind to a DNA segment containing *yxbB*. Our results indicated that both these regulators required the promoter region and not the LST segment for controlling *asnH* transcription (Fig. 5), implying that binding sites for both may be located within or very close to the identified *asnH* promoter (Figs 1 and 3). Additional experiments such as DNase I footprinting are required to define AbrB- and CodY-binding sites for the dual regulation of the *asnH* operon.

Not only the authentic short transcript of the *asnH* operon but also artificial *lacZ* fusion with the 5'-proximal region containing the entire LST segment exhibited an extended lifetime in cells (Fig. 6). It is likely that RNase resistance and/or thermodynamic features of the 5'-proximal region conferred by the LST segment may contribute to stabilization of not only the authentic transcript but also the artificial fusion. However, in the case of authentic transcripts, both long and short transcripts could have possessed the same 5'-proximal region with the LST segment. Nevertheless, the long transcript was unstable from the beginning and degraded quickly, while the short transcript, corresponding to the 5'-half of the long one, was retained stably. The short transcript could be made through either possible premature termination or specific degradation of the 3'-half of the long transcript. Nevertheless, we could not explain the reason for stabilization of the 5'-half of the long transcript exclusively by the 5'-proximal region. It is possible that the 3'-half carries some destabilizing function; however, further study is required to investigate this possibility.

Triplification of such long nucleotide sequences found in the 5'-proximal region of the *asnH* operon is unique within the *B. subtilis* genome sequence (Kunst *et al.*, 1997). In addition, homology searches failed to identify any sequence similar to each of the LST repeating units in nucleotide sequence databases (data not shown). It is known that the 5'-proximal region of the long-lived *E. coli ompA* transcript functions as an mRNA stabilizer (Arnold *et al.*, 1998). In that case, there are at least two domains of this 5'-proximal region that work together for stabilization of mRNA. One is a 5'-terminal stem-loop, and the other is a single-stranded RNA segment that contains a ribosome-binding site highly complementary to 16S rRNA. The 5' stem-loop requires no sequence features or thermodynamic stability beyond the minimum necessary for stem-loop formation, and the single-stranded segment allows for a high degree of occupancy of this 5'-proximal region for ribosome binding, which enables ribosomes to periodically pass through the protein-coding region. Because a large number of possible stem-loop structures were predicted in the LST segment (data not shown), we are not able to deduce which is the most plausible one at present. Each of the repeating units could contain the ORF which encodes the same short peptide of 22 amino acid residues and is also associated with a probable ribosome-binding site (Fig. 1), but the test using *lacZ* translational fusion indicated that the ORF was not translated. Finally, translation might not be involved in

the mRNA stabilization, as it was previously reported that translation of a *B. subtilis* mRNA is generally not important for its stability; the situation seems different in *E. coli* (Hambraeus *et al.*, 2002). However, since it has been shown not only in *B. subtilis* but also in *Bacillus thuringiensis* that mRNA stabilization occurs by ribosome binding near the 5'-end without translation (Agaisse & Lereclus, 1996; Hambraeus *et al.*, 2002; Yao & Bechhofer, 2009), the putative ribosome-binding site is not for translation but might be for mRNA stabilization. Another possibility is that *trans*-acting factors, such as RNA-binding proteins, could recognize and bind to the LST segment of mRNA to stabilize it, as studied in *Saccharomyces cerevisiae* (Chen *et al.*, 1999; Ruiz-Echevarria & Peltz, 2000).

The biological and physiological significance of this mRNA stabilization is unknown at present. Moreover, the function of the *asnH* operon itself remains to be characterized. AsnH likely functions as a glutamine-dependent asparagine synthetase because it partially compensated asparagine auxotrophy of an *E. coli* mutant lacking asparagine synthetases (Yoshida *et al.*, 1999), and its enzymic activity to produce asparagine was also detectable *in vitro* (K. Yoshida, unpublished). Although drastic induction of the *asnH* operon could be required for asparagine production for cell survival in a growth-restricting environment, our previous results suggested that *asnH* contributed relatively little to net asparagine synthesis in cells and was also dispensable for normal sporulation (Yoshida *et al.*, 1999). The *asnH* operon was also suggested to be involved in biofilm formation (Hamon *et al.*, 2004). However, no obvious change in biofilm formation was seen upon inactivation of *asnH* (K. Yoshida, unpublished). So far, we have not been able to detect an obvious effect of *asnH* inactivation on cell survival in stationary phase or upon exposure to various stresses, such as high and low osmotic pressure, pH, temperature and various chemicals, including lysozyme and ethanol (T. Morinaga, unpublished). Recently, *Lactococcus lactis* *asnH* was shown to encode an asparagine synthase responsible for D-Asp amidation in the peptidoglycan interpeptide crossbridge (Veiga *et al.*, 2009), suggesting that *B. subtilis* *asnH* might encode an enzyme with a similar function. Further studies are currently ongoing to identify the physiological role of the *asnH* operon.

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REFERENCES

- Agaisse, H. & Lereclus, D. (1996). STAB-SD: a Shine-Dalgarno sequence in the 5' untranslated region is a determinant of mRNA stability. *Mol Microbiol* **20**, 633–643.
- Arnold, T. E., Yu, J. & Belasco, J. G. (1998). mRNA stabilization by the *ompA* 5' untranslated region: two protective elements hinder distinct pathways for mRNA degradation. *RNA* **4**, 319–330.
- Belitsky, B. R. & Sonenshein, A. L. (2008). Genetic and biochemical analysis of CodY-binding sites in *Bacillus subtilis*. *J Bacteriol* **190**, 1224–1236.
- Chen, W., Islas-Osuna, M. A. & Dieckmann, C. L. (1999). Suppressor analysis of mutations in the 5'-untranslated region of COB mRNA identifies components of general pathways for mitochondrial mRNA processing and decay in *Saccharomyces cerevisiae*. *Genetics* **151**, 1315–1325.
- Ermolaeva, M. D., Khalak, H. G., White, O., Smith, H. O. & Salzberg, S. L. (2000). Prediction of transcription terminators in bacterial genomes. *J Mol Biol* **301**, 27–33.
- Fisher, S. H. (1999). Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la différence. *Mol Microbiol* **32**, 223–232.
- Hambraeus, G., Karhumaa, K. & Rutberg, B. (2002). A 5' stem-loop and ribosome binding but not translation are important for the stability of *Bacillus subtilis* *aprE* leader mRNA. *Microbiology* **148**, 1795–1803.
- Hamoen, L. W., Kausche, D., Marahiel, M. A., van Sinderen, D., Venema, G. & Serrano, P. (2003). The *Bacillus subtilis* transition state regulator AbrB binds to the –35 promoter region of *comK*. *FEMS Microbiol Lett* **218**, 299–304.
- Hamon, M. A., Stanley, N. R., Britton, R. A., Grossman, A. D. & Lazazzera, B. A. (2004). Identification of AbrB-regulated genes involved in biofilm formation by *Bacillus subtilis*. *Mol Microbiol* **52**, 847–860.
- Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A. & other authors (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249–256.
- Miwa, Y., Nakata, A., Ogiwara, A., Yamamoto, M. & Fujita, Y. (2000). Evaluation and characterization of catabolite-responsive elements (*cre*) of *Bacillus subtilis*. *Nucleic Acids Res* **28**, 1206–1210.
- Molle, V., Nakaura, Y., Shivers, R. P., Yamaguchi, H., Losick, R., Fujita, Y. & Sonenshein, A. L. (2003). Additional targets of the *Bacillus subtilis* global regulator CodY identified by chromatin immunoprecipitation and genome-wide transcript analysis. *J Bacteriol* **185**, 1911–1922.
- O'Reilly, M. & Devine, K. M. (1997). Expression of AbrB, a transition state regulator from *Bacillus subtilis*, is growth phase dependent in a manner resembling that of Fis, the nucleoid binding protein from *Escherichia coli*. *J Bacteriol* **179**, 522–529.
- Perego, M., Spiegelman, G. B. & Hoch, J. A. (1988). Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. *Mol Microbiol* **2**, 689–699.
- Phillips, Z. E. & Strauch, M. A. (2002). *Bacillus subtilis* sporulation and stationary phase gene expression. *Cell Mol Life Sci* **59**, 392–402.
- Ratnayake-Lecamwasam, M., Serrano, P., Wong, K. W. & Sonenshein, A. L. (2001). *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. *Genes Dev* **15**, 1093–1103.
- Ruiz-Echevarria, M. J. & Peltz, S. W. (2000). The RNA binding protein Pub1 modulates the stability of transcripts containing upstream open reading frames. *Cell* **101**, 741–751.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schaeffer, P., Millet, J. & Aubert, J. P. (1965). Catabolic repression of bacterial sporulation. *Proc Natl Acad Sci U S A* **54**, 704–711.
- Serizawa, M., Yamamoto, H., Yamaguchi, H., Fujita, Y., Kobayashi, K., Ogasawara, N. & Sekiguchi, J. (2004). Systematic analysis of SigD-regulated genes in *Bacillus subtilis* by DNA microarray and Northern blotting analyses. *Gene* **329**, 125–136.
- Shimotsu, H. & Henner, D. J. (1986). Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. *Gene* **43**, 85–94.
- Shivers, R. P. & Sonenshein, A. L. (2004). Activation of the *Bacillus subtilis* global regulator CodY by direct interaction with branched-chain amino acids. *Mol Microbiol* **53**, 599–611.
- Steinmetz, M. & Richter, R. (1994). Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through in vivo recombination. *Gene* **142**, 79–83.
- Vagner, V., Dervyn, E. & Ehrlich, S. D. (1998). A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144**, 3097–3104.
- Vaughn, J. L., Feher, V., Naylor, S., Strauch, M. A. & Cavanagh, J. (2000). Novel DNA binding domain and genetic regulation model of *Bacillus subtilis* transition state regulator AbrB. *Nat Struct Biol* **7**, 1139–1146.
- Veiga, P., Erkelenz, M., Bernard, E., Courtin, P., Kulakauskas, S. & Chapot-Chartier, M. P. (2009). Identification of the asparagine synthase responsible for D-Asp amidation in the *Lactococcus lactis* peptidoglycan interpeptide crossbridge. *J Bacteriol* **191**, 3752–3757.
- Villapakkam, A. C., Handke, L. D., Belitsky, B. R., Levnikov, V. M., Wilkinson, A. J. & Sonenshein, A. L. (2009). Genetic and biochemical analysis of the interaction of *Bacillus subtilis* CodY with branched-chain amino acids. *J Bacteriol* **191**, 6865–6876.
- Xu, K. & Strauch, M. A. (2001). DNA-binding activity of amino-terminal domains of the *Bacillus subtilis* AbrB protein. *J Bacteriol* **183**, 4094–4098.
- Yao, S. & Bechhofer, D. H. (2009). Processing and stability of inducibly expressed *rpsO* mRNA derivatives in *Bacillus subtilis*. *J Bacteriol* **191**, 5680–5689.
- Yoshida, K., Fujita, Y. & Sarai, A. (1993). Missense mutations in the *Bacillus subtilis* *gnt* repressor that diminish operator binding ability. *J Mol Biol* **231**, 167–174.
- Yoshida, K., Seki, S., Fujimura, M., Miwa, Y. & Fujita, Y. (1995). Cloning and sequencing of a 36-kb region of the *Bacillus subtilis* genome between the *gnt* and *iol* operons. *DNA Res* **2**, 61–69.
- Yoshida, K., Fujita, Y. & Ehrlich, S. D. (1999). Three asparagine synthetase genes of *Bacillus subtilis*. *J Bacteriol* **181**, 6081–6091.
- Yoshida, K., Ishio, I., Nagakawa, E., Yamamoto, Y., Yamamoto, M. & Fujita, Y. (2000). Systematic study of gene expression and transcription organization in the *gntZ-ywaA* region of the *Bacillus subtilis* genome. *Microbiology* **146**, 573–579.
- Yoshida, K., Kobayashi, K., Miwa, Y., Kang, C. M., Matsunaga, M., Yamaguchi, H., Tojo, S., Yamamoto, M., Nishi, R. & other authors (2001). Combined transcriptome and proteome analysis as a powerful approach to study genes under glucose repression in *Bacillus subtilis*. *Nucleic Acids Res* **29**, 683–692.
- Yoshida, K., Yamamoto, Y., Omae, K., Yamamoto, M. & Fujita, Y. (2002). Identification of two myo-inositol transporter genes of *Bacillus subtilis*. *J Bacteriol* **184**, 983–991.
- Yoshida, K., Yamaguchi, H., Kinehara, M., Ohki, Y., Nakaura, Y. & Fujita, Y. (2003). Identification of additional TnrA-regulated genes of *Bacillus subtilis* associated with a TnrA box. *Mol Microbiol* **49**, 157–165.

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