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Trans-Translation is Involved in the CcpA-Dependent Tagging and Degradation of TreP in *Bacillus subtilis*

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TreP [trehalose-permease (phosphotransferase system (PTS) trehalose-specific enzyme IIBC component)] is one of the target proteins of tmRNA-mediated *trans*-translation in *Bacillus subtilis* [Fujihara *et al.* (2002) Detection of tmRNA-mediated *trans*-translation products in *Bacillus subtilis*. *Genes Cells*, 7, 343–350]. The TreP synthesis is subject to CcpA-dependent carbon catabolite repression (CCR), and the *treP* gene contains catabolite-responsive element (*cre*) sequence, a binding site of repressor protein CcpA, in the coding region. Here, we demonstrated that the tmRNA-tagging of TreP occurs depending on the gene for CcpA. In the presence of CcpA, the transcription of *treP* mRNA terminates at 8–9 nucleotides upstream of the 5′-edge of the internal *cre* sequence, and translational switch to the tag-sequence occurs at the 101st amino-acid (asparagine) position from N-terminus of TreP. The results show that *trans*-translation reaction is involved in the tagging and degradation of the N-terminal TreP fragment produced by truncated mRNA, which is a product of transcriptional roadblock by CcpA binding to the *cre* sequence in the internal coding region.

Key words: *Bacillus subtilis*, CcpA, tmRNA, trans-translation, TreP.

Abbreviations: CCR, carbon catabolite repression; cDNA, complementary DNA; *cre*, catabolite-responsive element; RT–PCR, reverse transcription & polymerase chain reaction; RACE, rapid amplification of cDNA ends; tmRNA, transfer-messenger RNA; TreP, trehalose-permease; 2D gel, two-dimensional gel electrophoresis.

Bacterial transfer-messenger RNA (tmRNA, also known as 10Sa RNA or SsrA RNA) contains both a tRNA-like structure in the 5′- and 3′-end sequences and an internal reading frame encoding a 'tag' peptide (*1–3*). tmRNA is employed in a *trans*-translation reaction process to add a C-terminal peptide tag to incompletely translated nascent protein products from problematic mRNA, such as that lacking a stop codon (*4*, *5*). The tag is the target for specific proteases that hydrolyse incomplete peptides (*4*, *6*, *7*), and the degradation of problematic mRNA is enhanced by its release from the ribosome (*8*, *9*). Therefore, this process is regarded as a cellular system facilitating efficient translation by rescuing stalled ribosomes and establishing quality control by the degradation of incompletely synthesized peptides and damaged mRNA (see for reviews, refs. 10–14).

In a previous work (*15*), we have constructed an *in vivo* system for detecting the *trans*-translation products in *Bacillus subtilis*: a variant tmRNA gene encoding a tag sequence with six histidines (His-tag) and two aspartic acids (DD) at the C-terminus instead of two alanines was integrated in the genome; the cell containing the variant tmRNA(H6DD) accumulates *trans*-translation products without degradation, because a tag with a DD-terminus is not recognized by specific proteases for degradation (*4*, *7*, *16–18*); the His-tag-containing products, fractionated by Ni^{2+}-NTA column chromatography and gel electrophoresis, could be detected by western blot with antibody raised against His-tag (anti-His5). Using this system, we have identified 8 proteins which are specifically tagged by *trans*-translation reaction. Among them, we are especially interested in TreP protein, since it is known that the *treP*-operon is subject to the carbon catabolite repression (CCR) (see for reviews, refs 19–21).

The CCR in *B. subtilis* is mediated by a transcriptional regulator, CcpA, which belongs to the LacI repressor family. The repression occurs through the binding of CcpA on a *cis*-acting DNA sequence called catabolite-responsive element (*cre*) having a consensus sequence WTGNAANCGNWNNCW (N: A, T, G, or C; W: A or T) in the target genes (*22*, *23*). Several CCR genes contain *cre* sequences within the protein-coding regions, and binding of CcpA to *cre* leads to transcriptional roadblock of RNA polymerase before the authentic stop codon site (*24*, *25*). TreP is encoded by the first of the three genes in the trehalose operon, and a *cre* sequence of *treP* locates at 319–333 nucleotide (nt) position from the initiation codon,

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corresponding to the N-terminal 106th to 111th amino acids of TreP protein (*26*, *27*). Since the sizes of the tagged TrePs have been estimated from the mobility in SDS-gel electrophoresis as about 13 kDa (*15*), it is predicted that the arrest of transcription of *treP* by the binding of CcpA to the *cre* sequence would produce truncated mRNA lacking a stop codon, and that the translational stalling of ribosome at the 3′-terminus of the truncated mRNA leads to tmRNA-mediated *trans*-translation.

In the present study, we first confirmed that the tmRNA-tagging of TreP occurs depending on the CcpA to verify the above prediction. Then, we attempted to determine the site of translational switching from TreP sequence to the tmRNA-tag sequence by mass-spectrometric analysis of the tagged products. We also detected truncated *treP* mRNA by northern hybridization, and determined the sites of their 3′-ends. The results showed that transcriptional roadblock by CcpA binding to *cre* resulted in the production of truncated mRNA, the 3′-end of which was 8 or 9 nt upstream from the 5′-edge of the *cre* sequence, and *trans*-translation acted on the truncated mRNA at the 101st amino acid (asparagine:N_{101}) position of TreP.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—*B. subtilis* strain AMHG (*pur, met, his*), a derivative of strain 168, was provided by Dr N. Ogasawara of Nara Institute of Science and Technology; plasmid pMUTin2 (*28*) was by Dr K. Nakamura of the University of Tsukuba. The deletion strain of tmRNA gene (*ΔssrA*: *ssrA::cat)* was described in Muto *et al*. (*16*); The mutant strain AMHG (*ssrA-H6DD*) encodes tmRNA having a tag-peptide with six histidine residues (His-tag) and two aspartic acids at the C-terminus (*15*).

B. subtilis strain AMHG with *ccpA* null mutant (*ΔccpA*: *ccpA::neo*) was obtained by transformation of the DNA from strain 168 with *ccpA::neo* (*29*), and selection on the LB-plate containing 15 μg/ml neomycin.

Protein Fractionation—Cells were grown in LB medium at 37°C, and harvested at mid-log phase by centrifugation. The total proteins were extracted from the cells and fractionated by Ni^{2+}-NTA-column chromatography as described in a previous paper (*15*). The fractions containing the tagged peptides were further purified by passing them through an Ni^{2+}-NTA spin column (Qiagen). The fraction was then fractionated by one-dimensional SDS or two-dimensional gel electrophoresis (*15*) and visualized by Coomassie Brilliant Blue staining or by western blotting.

The tagged TreP fragments fractionated by gel electrophoresis were subjected to in-gel digestion by lysine-specific endoproteinase Lys-C and to MALDI-TOF MS analysis, which was consigned to APRO Science Institute (Tokushima, Japan).

Western Blotting—After electrophoresis, the proteins in the gel were electroblotted onto a PVDF membrane (Millipore) and then probed with Penta-His antibody (anti-His5; Qiagen) or with anti-TreP antibody which was raised against the N-terminal 20 amino-acid sequence peptide of TreP. Antibody was visualized by chemiluminescence of ECL plus (GE Healthcare) after the binding of horseradish peroxidase-conjugated secondary antibody.

Northern Hybridization—Total RNA was prepared from exponentially growing cells by phenol extraction method as described in Fujihara *et al*. (*15*). RNA (5 μg) was separated on a 4% polyacrylamide-7M urea gel electrophoresis and blotted onto a nylon membrane (Millipore). The *treP* mRNA was detected by northern hybridization using a [^{32}P]UTP-labelled RNA probe (280 nt) complementary to the *treP* coding region upstream of the *cre* locus: the template DNA was prepared by polymerase chain reaction (PCR) with *treP* 5′-primer III (GGGA ACTGAACAAATCGGCACGTCAG), corresponding to the position 5–30 from the initiation codon (sense) and *treP* 3′-primer I (TAATACGACTCACTATAGGGGCCTTCTT CTTCACTTCATCC), corresponding to the position 284–265 (antisense) fused to the T7 promoter sequence (underlined). The product DNA was transcribed by T7 RNA polymerase, and the labelled RNA was isolated under the condition described in the manufacturer's manual (MAXIscript T7, Ambion). Hybridization was carried out at 55°C for 16 h in 1 ml ULTRAhyb (Ambion) per 10 cm^2 of membrane. The membrane was then washed twice in 2 × SSPE-0.1% SDS at 45°C for 15 min. The washed membrane was exposed to an Imaging Plate and analysed by BAS3000 (Fuji Film, Tokyo).

The 3′-end Analysis of Truncated treP mRNA—The 3′-ends of truncated treP mRNAs were analysed by 3′-RACE (Rapid Amplification of cDNA Ends) according to the method described in Elbashir *et al*. (*30*) and Hokii *et al*. (*31*) with some modifications: total RNA (10 μg) was ligated with 5 μM 3′-adapter oligonucleotide, pUUUaa ccgcatccttctctx (upper case, RNA; lower case, DNA; p, phosphate; x, 4-hydroxymethylbenzyl), in 50 μl of reaction mixture containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 0.002% bovine serum albumin, 10% DMSO, 12.5% PEG6000, 40 units RNase inhibitor (Takara) and 80 units T4 RNA ligase (Takara), at 10°C for 14 h, and the products were recovered by phenol/chloroform extraction and ethanol precipitation. Reverse transcription of the ligation products with RT3′-primer I (AGAAGGATGCGGTT: complementary to the 3′-adapter), was followed by the first PCR with the *treP* 5′-primer I (CCAAGAGATGCTTGATCAAATTGATG TGG), corresponding to the position 126th nucleotides from the initiation codon) and the RT 3′-primer I. The second PCR was performed with treP 5′-primer II (GCT GAACTGGTTAAGGAAACGGGGATTGGC: forward) and 3′-primer (GACTAGCTGGAATTCAAGGATGCGGTTAAA: reverse). The products were directly ligated to pCR2.1 vector using TA cloning kit (Invitrogen), and transformed to *Escherichia coli* DH10B. The sequences of the inserts were determined using M13 primer by DNA sequencer (Hitachi SQ5500). The 3′-end of mRNA was obtaind from the boundary sequence between treP mRNA and the ligated 3′-adapter.

RESULTS

CcpA-dependent Tagging of TreP—In the *B. subtilis* strain AMHG having tmRNA (H6DD) with a tag sequence of AGKTNSHHHHHHLDD, His-tag containing

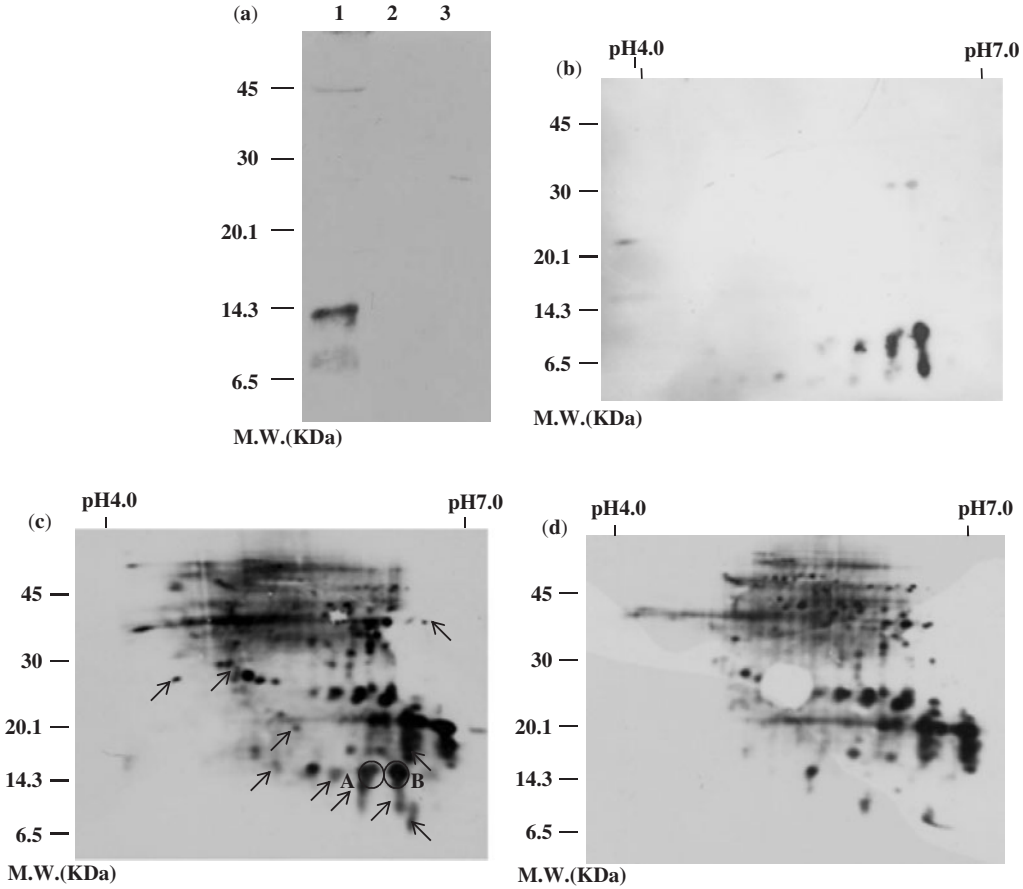
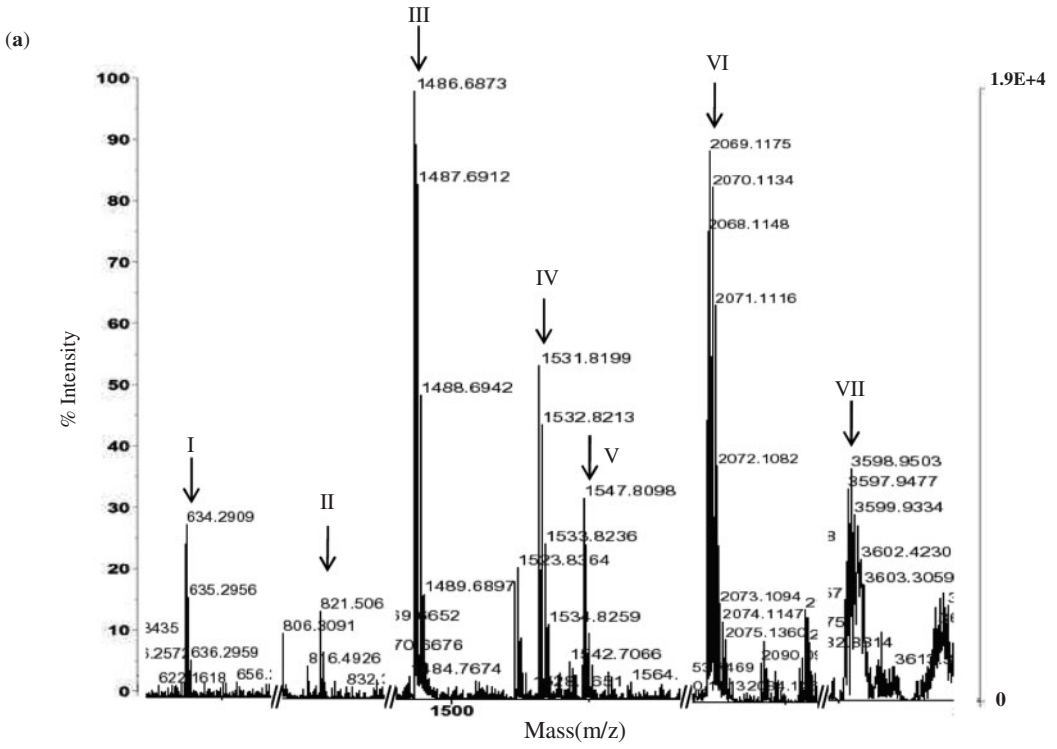


Fig. 1. **Western blot of tagged TreP.** (a) The strains AMHG having *ssrA-H6DD* (lane 1), *ssrA-H6DD;ΔccpA* (lane 2) and *ΔssrA* (lane 3), were cultured in LB medium at 37°C. The tmRNA-tagged proteins were fractionated by Ni^{+2}-NTA affinity column chromatography, and 40 μg proteins were separated by SDS-gel electrophoresis. The peptides containing the sequence of TreP were detected by western blot with anti-TreP antibody. (b) About 3 mg proteins from the cells with *ssrA-H6DD* were separated by 2D gel electrophoresis and subjected to western blot with anti-TreP antibody. About 3 mg proteins from the cells with *ssrA-H6DD* (c), and *ssrA-H6DD; ΔccpA:* (d) were separated by 2D gel electrophoresis and subjected to western blot with anti-His5 antibody. The arrows represent the spots disappeared in the cell with *ssrA-H6DD; ΔccpA* (d). The tagged TreP spots (A) and (B) are circled in (c).

trans-translation products accumulate in the cell. One of the products, tagged TrePs of about 13 kDa, was identified by the N-terminal sequence of the tagged peptides (*15*). In the present study, we used an antibody raised against synthetic polypeptides with the N-terminal 20 amino-acid sequence of TreP protein (anti-TreP antibody) to detect tagged TreP peptides. To see the effects of CcpA and of tmRNA on the TreP tagging, the strains AMHG-*ssrA(H6DD)*, its *ΔccpA(ccpA::neo)* derivative and AMHG-*ΔssrA(ssrA::cat)* were separately cultured at 37°C in LB medium. The His-tag containing peptides were fractionated by Ni^{2+}-NTA column chromatography and separated by SDS-gel electrophoresis, followed by western blot with anti-TreP antibody. As shown in Fig. 1a, a distinct band at about 13 kDa was observed in the cells with *ssrA(H6DD)* (lane 1). A broad and faint band was also observed around 10 kDa region. On the other hand, no positive band was observed in AMHG-*ssrA(H6DD)* cells with *ΔccpA* or with *ΔssrA* cells (lanes 2 and 3), showing that the tagging of TreP is CcpA- and tmRNA-dependent. Figure 1b shows the western blot of 2D gel with anti-TreP antibody of the fraction prepared from *ssrA(H6DD)* cells. Two major spots at about 13 kDa and some other spots with apparently smaller molecular weights could be detected. Figure 1c and d compares the western blot profiles of 2D gel with anti-His5 antibody of the fractions prepared from cells with *ssrA(H6DD)* and *ssrA(H6DD); ΔccpA*, respectively. Several spots, which were present in the *ssrA(H6DD)* cell, were absent in the *ΔccpA* derivative cell (arrows in Fig. 2c), showing the presence of other CcpA-dependent tagged proteins.

Analysis of Tagged TreP Fragment—About 5 mg protein fraction prepared from the cells with *ssrA(H6DD)* was separated by 2D gel and stained with Coomassie Brilliant Blue [see Fig. 4 of Fujihara *et al*. (*15*)]. The two major spots of tagged TreP with about 13 kDa (corresponding to the spots A and B in Fig. 1c) were excised from the gel and digested with lysine-specific endoproteinase (Lys-C). Figure 2a shows the MALDI-TOF MS spectrum of the digestion products of spot A, exhibiting at least seven peptides supposed to be derived from tagged TreP. The molecular mass of peak I, 634.2909, matches well with the molecular weight of NMNAGK (634.2983), the first three amino-acid residues of the tag-peptide (AGK) fused to the



(**b**)

MGELNK SARQ IVEAVGGAEN IAAATHCVTR LRFALIDESK VDQEMLDQID VVK GSFSTNG QFQVVIGQGT VNK
1 10 20 30 40 50 60 70
VII IV, V(Met-ox) VI

VYAELVK ETGIGESTKD EVKKASEK NM N (**AGK TNSHHHHHHHLDD**)
80 90 100 **tag**
II I III

Fig. 2. **MALDI-TOF MS of tagged TreP.** (a) Mass spectrum of MALDI-TOF of tagged TreP. About 5 mg of the proteins from the cells with *ssrA-H6DD* fractionated by Ni^{+2}-NTA affinity column chromatography were separated by 2D gel electrophoresis and stained with Coomassie Brilliant Blue. Tagged TreP spots, corresponding to spot A and B in Fig. 1 (c), were digested with lysine-specific proteinase Lys-C and subjected to MALDI-TOF MS analysis. Five parts of spectrum of spot A (Figure 1c) are combined. The peaks matching to the predicted molecular weights of the digestion products of tagged TreP are indicated by arrows with Roman numbers. (b) The sequence of tagged TreP fragment deduced from the MALDI-TOF MS analysis. The tmRNA-tag sequence is in parenthesis. The numbers (Roman) under the sequence correspond to the peaks in (a). The peak V is a derivative of peak IV, in which a methionine residue (45th amino acid) is oxidized. The amino acid numbers from the N-terminus of TreP are also shown below the sequence.

99th to 101st residues (NMN) of TreP. The other six peaks were those seemed as lysine-specific proteinase products derived from the N-terminal upstream region of the *cre* sequence of TreP (Fig. 2b). The peak V is the modified form, containing oxidized methionine, of the peak IV, corresponding to 41st to 53rd amino acids of TreP. No peptide including the sequence from downstream of the *cre* sequence of TreP was detected. We could not find any significant differences between the spectra of the spots, A and B. Probably, the difference reflects the different amino-acid modifications in the peptides not detected in this analysis. The results indicate that the tmRNA-tagging of both peptides predominantly occurred at the 101st amino acid, asparagine (N_{101}), site of TreP (Fig. 2b), corresponding to 301–303 nt position from the initiation codon of *treP* mRNA, and to 16–18 nt upstream of the 5′-edge of the internal *cre* sequence. The deduced tagged TreP is 116 amino acids long and the molecular weight is 12 640 Da.

Analysis of Truncated treP mRNA—The CcpA-roadblock of RNA polymerase may result in transcriptional termination somewhere upstream of the *cre* sequence. To detect the mRNA fragment including the sequence 5′-upstream *cre* within the coding region of *treP*, northern hybridization was performed towards total RNAs from the cells with or without *ccpA* using ^{32}P-labelled RNA probe complementary to the sequence of 5–279 nt position of *treP*. A distinct band of about 300 nt was detected only in the cells containing CcpA (Fig. 3a, lane 1), suggesting that the transcription termination occurred around the *cre* sequence depending on CcpA. Smear bands with higher molecular weights, which are probably full-size mRNA of

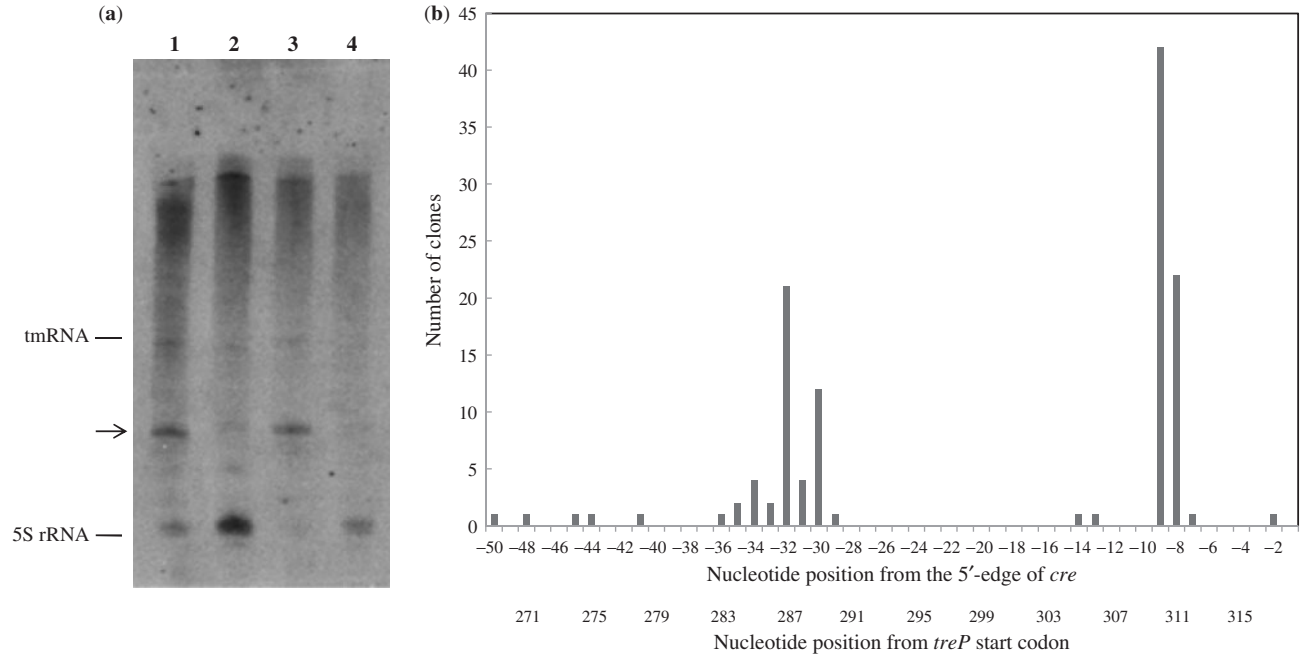


Fig. 3. **Analysis of truncated *treP* mRNA.** (a) The strains AMHG with *ssrA-H6DD* and its *ΔccpA* derivative were cultured in LB medium (lanes 1 and 2) or in LB medium with 0.5% glucose (lanes 3 and 4). The total RNA were prepared from mid-log phase cells and 5 μg RNA each was separated by 4% acrylamide gel electrophoresis containing 7 M urea. The *treP* mRNA was detected by northern hybridization with ^{32}P-labeled *treP* probe. The positions of tmRNA and 5S rRNA on the gel determined by northern hybridization are shown by bars. (b) The 3′-ends of *treP* mRNA from *ssrA-H6DD* cell cultured in LB medium were analysed by 3′-RACE method. The distribution of the 3′-ends from –1 to –50 position from the 5′-edge of the *cre* sequence is shown.

the *treP* operon and its degradation products (*27*) and a faint band of about 100 nt were also observed. In *ΔccpA* cells, the shorter 100 nt band was predominated instead of the 300 nt band (lane 2). The CcpA-dependent 300 nt band was also observed in the sample of the cell with wild type *ccpA* cultured in LB medium in the presence of 0.5% glucose (Fig. 3a, lanes 3 and 4). However, the intensity of the 300 nt band did not significantly change by addition of glucose, while that of the higher molecular weight bands apparently decreased.

To determine the CcpA-dependent truncated sites of *treP* mRNA, the 3′-ends were analysed by 3′-RACE method: the 3′-ends of the total RNA from *ssrA(H6DD)* cells were ligated to synthetic RNA tag, and RT–PCR (reverse transcription & polymerase chain reaction) was carried out with the DNA primers having complementary sequence of the tag and the 5′-side sequence (226–250 nt position) of *treP*. The resulting products were cloned and sequenced. The 3′-end of mRNA was estimated from the ligation site of the tag. Among 194 cDNA clones sequenced, 150 encoded *treP* sequences, all of which located within 1–65 nt upstream from the 5′-edge of the *cre* sequence. The 3′-ends of 120 clones located 1–50 nt upstream from the *cre* sequence and those of 30 clones randomly distributed along 65–51 nt upstream from the *cre*. Figure 3b shows the distribution of the 3′-end of the clones between 1 and 50 nt upstream region of the *cre*. The 3′-ends of more than half (64 clones) in this region occurred at 8 or 9 nt upstream from the 5′-edge of *cre* sequence. There was also a broad peak around 30–33 nt upstream region, although we could not detect any band corresponding to this region by northern hybridization (Fig. 3a); probably these are degradation products during the preparation of cDNAs.

The same analysis with *ΔccpA* strain revealed that there is no specific cutting in this region (data not shown). Since the major *trans*-translation products of TreP occurred at 310–311 nt position (13–15 nt upstream from the *cre* sequence; Fig. 2), the tagging of them must occur at or downstream of this position. Thus, the results show that the major truncation of *treP* mRNA occurs at 8 or 9 nt upstream of the 5′-edge of *cre* sequence in a CcpA-dependent manner. The size of truncated mRNA estimated from the above data is about 330 nt long including 5′-leader sequence, and the 3′-end corresponds to the 104th amino acid, glutamine (Q_{104}), from the N-terminus (Fig. 4).

DISCUSSION

In this study, we have analysed one of the tmRNA-mediated *trans*-translation products, the tagged TreP, of *B. subtilis*. The expression of TreP, trehalose permease, is known to be subject to CcpA-dependent catabolite repression. CcpA is a major transcription factor mediating CCR in *B. subtilis*, and is widely conserved among Gram-positive bacteria of low GC content. The CcpA-dependent repression occurs at the transcription level by binding to a common DNA stretch, *cre* sequence. A computer search for *cre*s has picked up at least 126 putative and known *cre* sequences in the *B. subtilis* genome (*24*). Among them, 22 out of 32 genes tested for catabolite repression were found

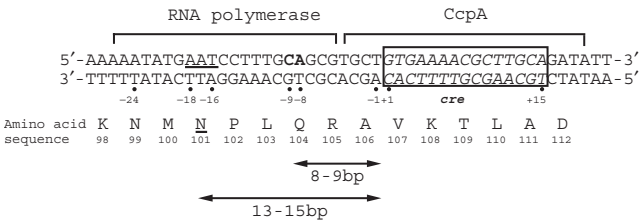


Fig. 4. **The sites of TreP-tagging and *treP* mRNA truncation.** The results of Fig. 2 and Fig. 3b are schematically shown. DNA sequence of *treP* is numbered from the 5′-edge of *cre*. The *cre* sequence (italic) is enclosed by box. Possible regions covered by CcpA binding and arrested RNA polymerase are shown by brackets. The translation switching site, 16–18 nt upstream of the *cre* sequence that encoded N_{101} and its codon, are underlined. Bold letters represent the 3′-end of truncated mRNA, 8 and 9 nt upstream of *cre* sequence. Two arrows, long and short, show the distances (base pairs) from the 5′-edge of the *cre* to the TreP tagging site and to the mRNA truncation site, respectively.

to function *in vivo*, and the *cre* sequences of 15 genes of them, including *treP*, locate in the protein-coding region of the targets. A whole-transcriptome analysis of the CcpA-dependent gene expression by Moleno *et al*. (*25*) has shown that about 330 genes exhibited more than a 3-fold glucose effect by a CcpA-dependent process; they listed *cre* containing 52 genes which are repressed by CcpA. Among them, 16 genes including *treP* have *cre* sequences in the protein-coding regions. The above two analyses revealed that at least 28 genes containing *cre*s in the coding regions are functioning in *B. subtilis* cell.

The *treP* gene is a major target for *trans*-translation in LB medium (*15*). The tmRNA-dependent tagging of TreP occurs in a CcpA-dependent manner (Fig. 1a), suggesting that the CcpA binding to the *cre* sequence results in *trans*-translation. The TreP gene contains an internal *cre*, GTGAAAACGCTTGCA, at 319–333 nt position in the reading frame, corresponding to the N-terminal 106th to 111th amino acids of TreP protein (*27*). The binding of CcpA to the *cre* sequence would cause transcriptional roadblock of RNA polymerase at upstream of the binding site (*24*, *32*, *33*), and the transcription elongation complex with stalled RNA polymerase may dissociate from DNA. Mfd protein, a transcription-repair coupling factor, dissociates stalled RNA polymerase transcription elongation complex from DNA (*34*,*35*). Zalieckas *et al*. (*36*) reported that Mfd participates in *cre*-dependent CCR at *hut* and *gnt* operon of *B. subtilis*. Thus, the roadblock of transcription elongation complex on *treP* gene would produce truncated mRNA whose 3′-end is upstream of the *cre* sequence. Northern hybridization of *treP* mRNA revealed a distinct band of about 300 nt long depending on the presence of CcpA (Fig. 3a), and the 3′-end sequence analysis showed that the mRNA is predominantly truncated at 8–9 nt upstream of the 5′-edge of *cre* sequence (Fig. 3b).

The 3′-end of the major truncated *treP* mRNA is 310–311 nt position from initiation codon, corresponding to 103rd amino acid, leucine (L_{103}), from the N-terminus of TreP. If *trans*-translation reaction occurs at the 3′-end of truncated mRNA, the peptide tagging is most likely to occur at L_{103}. However, MALDI-TOF MS analysis of the tagged TreP indicated that tag-peptide was added at N_{101} (Fig. 2), which is 5–8 nt upstream of the 3′-end of truncated mRNA (Fig. 4). The result shows that translational pausing of the mRNA occurs at the N_{101} codon position leaving peptidyl-$tRNA^{Asn}$ on the P-site of ribosome, and alanyl-tmRNA enters the A-site, which is occupied by a next CCU (proline) codon followed by downstream 4–5 nt extension. Thus, the cleavage of mRNA at A-site on the stalled ribosome, observed in *E. coli* (*9*,*37–39*), did not occur in this case.

The reason for the discrepancy between the 3′-end of mRNA and the tagging position is not known. In *E.coli*, *trans*-translation occurs on stalled ribosomes not only at the 3′-end of mRNA lacking stop codon, but also at a tandem arginine rare codon (*40*), at an inefficient stop codon (*9*, *37*, *41*) or other translational pausing events (*39*, *41*). It is also shown *in vitro* that *trans*-translation can occur on stalled ribosome at either the nonsense codon (*38*, *42*) or the sense codon (*42*) without cleavage of mRNA at the A-site. In both cases, a shorter 3′-extension of mRNA from the A-site is preferable for *trans*-translation, suggesting the competition between the mRNA and alanyl-tmRNA on the A-site. Perhaps, the tight interaction between 3′-extension of mRNA and mRNA path encompassing the decoding region to the downstream tunnel on ribosome would be required for efficient codon–anticodon interaction on the A-site, and a short 3′-extension might destabilize the interaction, permitting entry of alanyl-tmRNA at upstream of the 3′-end of mRNA. In the case of truncated *treP* mRNA of *B. subtilis*, the length of 3′-extension after the N_{101} codon is 5–8 nt, the 3′-end of which is already out of the ribosome downstream tunnel. Probably, the poor occupancy of the A-site caused by the short 3′-extension results in the pausing of translation of treP mRNA at the N_{101} codon, facilitating *trans*-translation at upstream of the 3′-end of mRNA. Abo *et al*. (*43*) reported that tmRNA-mediated tagging of LacI occurs by transcriptional roadblock with LacI binding to its own DNA (*lacI*) in *E. coli*.

TreP senses trehalose outside the cell and transport the sugar into the cell, and the expression of the gene is repressed by glucose in the media. It has been reported that the *treP* transcription is activated by CcpA in LB medium without glucose, but is repressed to more than 30-fold by the addition of glucose (*25*). It is also known that the expression of *treP* gene is autogenously regulated by TreR encoded in the downstream region of *treP* in the same operon, and that the promoter region of the operon contains another *cre*-like sequence (*26*, *27*). We could not observe the increase of truncated *treP* mRNA by northern blot analysis in the cells cultured in the presence of glucose (Fig. 3(a)). It is thus unclear how the internal *treP* *cre* sequence is involved in the regulation of the gene expression in the cell cultured in LB medium. However, the present study clearly demonstrates that tmRNA-mediated *trans*-translation is involved in the tagging and degradation of truncated TreP peptide produced by CcpA binding to the *cre* sequence. In the previous work (*15*), we found two other target genes, *ytoQ* and *folA*, having internal *cre* sequences for *trans*-translation. A comparison of 2D gel patterns of the tagged peptides between the cells with and without CcpA showed that several spots were absent in the cells lacking *ccpA*

(Fig. 1c and d). These results suggest that *trans*-translation is widely involved in the tagging and degradation of truncated peptides subject to CcpA-dependent catabolite repression of the genes containing internal *cre* sequences.

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CONFLICT OF INTEREST

None declared.

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