Trans-translation mediated by *Bacillus subtilis* tmRNA

Ken-ichi Ito[a], Toshimasa Tadaki[a], SungGa Lee[b], Kazuma Takada[a], Akira Muto[a,b], Hyouta Himeno[a,b,*]

[a] *Department of Biology, Faculty of Science, Hirosaki University, Hirosaki 036-8561, Japan*
[b] *Department of Biochemistry and Biotechnology, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561, Japan*

Received 24 January 2002; accepted 20 February 2002

First published online 14 March 2002

Edited by Horst Feldmann

Abstract ***Trans*-translation, in which a ribosome switches between translation of an mRNA and a tmRNA, produces a chimera polypeptide of an N-terminal truncated polypeptide and a C-terminal tag-peptide encoded by tmRNA. One of the tmRNA binding proteins, a ribosomal protein S1, has not been found in a group of Gram-positive bacteria. In this study, the *trans*-translation reaction with tmRNA from *Bacillus subtilis* belonging to this group was examined. When a truncated gene lacking a termination codon was expressed in *B. subtilis*, a 15-amino acid tag-peptide derived from tmRNA was identified in the C-termini of the *trans*-translation products. An identical tag-peptide was also found at the C-termini of the products from a truncated gene, when it was coexpressed with *B. subtilis* tmRNA in *Escherichia coli*. *B. subtilis* tmRNA was functional, although much less efficiently, in the in vitro poly(U)-dependent tag-peptide synthesis system of *E. coli*. A comparison of two bacterial tmRNAs suggests that the rule for determining the tag-initiation point on tmRNA may be the same in Gram-positive and Gram-negative bacteria.**

Key words: *Bacillus subtilis* tmRNA; *Trans*-translation; Tag-peptide; SsrA; S1

1. Introduction

tmRNA (also known as 10Sa RNA or SsrA RNA) is a novel molecule having both tRNA and mRNA properties.

Both terminal regions of this molecule can be folded into a partial tRNA-like structure comprising a T-arm and an acceptor stem leading to the 3′-terminal CCA [1,2] with two tRNA-specific modified nucleotides [3]. Chemical and enzymatic probing studies as well as comparative studies also support this tRNA-like structure [4,5], which can be recognized by AlaRS [1,2] and EF-Tu [6,7].

tmRNA has an additional domain, an mRNA domain, surrounded by four pseudoknot structures in the middle of the molecule. An 11-amino acid tag sequence, the last 10 of which are encoded in this domain, was first found on the C-terminus of a fraction of mouse IL-6 expressed in *Escherichia coli* [8] and was also found on other polypeptides when they are translated from mRNAs lacking a termination codon [9]. Together with several other findings, an unusual translation, *trans*-translation, has been established [10–16]. To relieve stalled translation, a ribosome switches from the translation of a problematic mRNA to the tag-encoded sequence of tmRNA with the addition of a specific tag-peptide to the truncated C-termini of polypeptides decoded.

tmRNA is widely distributed among eubacteria and has also been found in some chloroplasts [17]. In contrast to the high conservation of the tRNA-like structure, the central domain, in which the tag-encoded region starts at the position 12 nucleotides downstream of the first pseudoknot (PK1) and terminates in the loop of the fourth helix (H4), appears less conserved. Only the hydrophobic C-terminal sequence of the tag-peptide is highly conserved, probably because it serves as a specific target for cellular proteases [12,18,19], allowing deduction of the reading frame of the tag-peptide other than that from *E. coli*. However, the lower conservation of the remaining tag sequence as well as the variable length of the region between PK1 and H4 makes it difficult to identify the exact resuming codon on tmRNA from other species. The exact amino acid sequence of the tag-peptide has been identified in only two Gram-negative bacteria, *E. coli* and *Caulobacter crescentus*, which belongs to the α-proteobacterial group [20].

Although the mechanism underlying resumption of translation at a definite position during *trans*-translation remains elusive, several *trans*-acting factors involved in *trans*-translation have been identified in *E. coli*. One of them, the ribosomal protein S1, the largest protein component of the small subunit [21,22], has not been found in the ribosomes from the Clostridial group of Gram-positive bacteria [23]. In this study, we focused on the *trans*-translation reaction mediated by tmRNA from *Bacillus subtilis*, a Gram-positive bacterium belonging to this group. In this bacterium, tmRNA is thermoinducible, and its ability to recycle stalled ribosomes via *trans*-translation is involved in the stress tolerance of the cell [24].

2. Materials and methods

2.1. Overexpression of B. subtilis tmRNA in E. coli

The *B. subtilis* tmRNA gene was amplified by primer-directed PCR so as to add the 3′-terminal CCA sequence that is not encoded by the genome [1]. The resulting DNA fragment was ligated under the T7 promoter sequence of pALTER EX-2, which was then transformed into *E. coli* JM109 (DE3). Mutations were introduced by primer-directed PCR using the plasmid carrying the wild-type tmRNA gene as a template, and the amplified DNA fragment was ligated under the T7 promoter sequence of pALTER EX-2. tmRNA was purified from W3110 (Δ*ssrA*) strain harboring both pALTER EX-2 carrying the tmRNA gene and pACYC184 carrying the T7 RNA polymerase gene under the *lac*-promoter sequence, as described [25].

*Corresponding author. Fax: (81)-172-39 3593.
E-mail address: himeno@cc.hirosaki-u.ac.jp (H. Himeno).

0014-5793/02/$22.00
PII: S0014-5793(02)02561-9

2.2. Production and purification of tag-fusing chimera polypeptides in B. subtilis cells

The plasmid pWH1520, a shuttle vector for heterologous gene expression in *Bacillus megaterium* [26], was used for expression from a truncated mRNA lacking a termination codon in *B. subtilis*. The recognition sequence of factor Xa (IEGR), six successive histidine codons and a *Bacillus licheniformis* β-lactamase terminator sequence [27] with no termination codon in frame were designed to be aligned within the partial xylose isomerase gene under the *xyl*A promoter on pWH1520. This plasmid was then transformed into *B. subtilis* strain AMHG L1 (P*spac-ssrA*) or AMHG N1 (P*spac-ssrA*(*DD*)) [24].

B. subtilis cells were cultured in 3 l of LB broth containing 3 mg erythromycin and 60 mg tetracycline with agitation at 37°C, and the culture was continued for 4 h after the addition of 0.5% D-xylose and 1 mM IPTG when OD_{600} had reached 0.3. After freezing and thawing, the collected cells were incubated at room temperature for 1 h in a mixture of 4 ml Y-PER yeast protein extraction reagent (Pierce), 4 ml of 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl (pH 8.0), 0.1 mg leupeptin, 0.01 mg pepstatin A and 0.01 mg antipain, and the cells were ruptured by sonication twice. The fraction containing a 6×His-tag sequence was purified from the cell lysate by affinity chromatography with a nickel-chelate spin column (Qiagen). This fraction was digested with factor Xa, and the peptide fragments containing a 6×His-tag sequence were further purified by nickel-chelate affinity chromatography.

2.3. Production and purification of tag-fusing chimera polypeptides in E. coli cells

The plasmid was constructed from pQE16 using synthetic DNA oligomers, so that the factor Xa recognition sequence, six consecutive histidine codons, followed by a trpA terminator, with no termination codon in frame, were fused to the 3′-terminal region of the DHFR gene. This plasmid was cotransformed into *E. coli* strain JM109(DE3) with another plasmid, pALTER EX-2, carrying the wild-type or mutant tmRNA gene under the T7 promoter sequence.

Cells were cultured in 150 ml of LB broth containing 7.5 mg ampicillin and 3 mg tetracycline with agitation at 37°C, and the culture was continued for 4 h after the addition of 1.0 mM IPTG when OD_{600} had reached 0.5. The collected cells were incubated at room temperature for 1 h in 1 ml of 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl (pH 8.0), 0.1 mg leupeptin, 0.01 mg pepstatin A and 0.01 mg antipain, and were ruptured by sonication.

2.4. Detection of polypeptides fusing the tag-peptide by mass spectroscopy

The chimera peptide fraction purified was digested with factor Xa, and the peptide fragments containing a 6×His-tag sequence were further purified by nickel-chelate affinity chromatography. The final peptide fragments containing a 6×His-tag sequence were desalted with ZipTipC18 (Millipore), and were then analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy (Voyager RP HU biospectrometry) using 3,5-dimethoxy-4-hydroxycinamic acid as a matrix.

2.5. In vitro aminoacylation with alanine

The aminoacylation reaction proceeded at 37°C in a 50-μl reaction mixture containing 80 mM Tris–HCl (pH 7.5), 150 mM ammonium chloride, 2.5 mM dithiothreitol, 2.5 mM ATP, 20 μM L-[U-^{14}C]alanine (6.5 GBq/mmol), 1.0 μM tmRNA variants and 9.1×10^{-2} U of AlaRS. At each specified time point, a 10-μl aliquot was withdrawn and spotted on Whatman 3MM filter paper, and radioactivity in the trichloroacetic acid-insoluble fraction was measured by a liquid scintillation counter.

2.6. Poly(U)-dependent tag-peptide synthesis in vitro

The preincubated S30 fraction was prepared from middle-log-phase cells of *E. coli* strain W3110 (Δ*ssrA*), as described previously [12]. The reaction mixture (100 μl) contained 80 mM Tris–HCl (pH 7.8), 5 mM magnesium acetate, 150 mM ammonium chloride, 2.5 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 20 μM L-[U-^{14}C]alanine and 0.05 mM each of the remaining unlabeled 19 amino acids, 5 μM or 35 μM tmRNA (when 1 A_{260} unit corresponds to 330 pmol), and 20 μl of the S30 fraction, in the presence of 250 μg of poly(U) (50–100-mer, Sigma). Each tmRNA was used in the reaction without any refolding procedure after purification from the gel. The reaction mixture was incubated at 37°C. At each specified time point, a 24-μl aliquot was withdrawn from the 100-μl reaction mixture and spotted on Whatman 3MM filter paper, and radioactivity in the hot trichloroacetic acid-insoluble fraction was measured by a liquid scintillation counter.

3. Results

B. subtilis and *E. coli* tmRNAs share several common structural features (Fig. 1), such as the presence of four pseudoknots (PK1–PK4) and a tandem repeat of a UAA triplet in the loop of H4, the first UAA of which functions as a termination codon for the tag-peptide in *E. coli*. The C-terminal four-amino acid sequence of the reading frame, which terminates at this first UAA triplet in *B. subtilis* tmRNA (ALAA), completely matches that of the tag-peptide of *E. coli*, suggesting that this reading frame is just the frame of the tag-peptide of *B. subtilis*. However, the first codon directing the second amino acid of the tag-peptide can only be predicted from the lower sequence conservation between Gram-positive and Gram-negative bacteria [17]. This prediction has been weakened by the fact that the span between PK1 and H4 in *B. subtilis* tmRNA is approximately 1.5-fold longer than that in *E. coli* tmRNA. We attempted to make a system to identify the first codon of the tag-encoded region on *B. subtilis* tmRNA.

It has been shown that a plasmid-encoding truncated protein gene lacking a stop codon produces chimera polypeptides fusing the tag-peptide at the C-termini as *trans*-translation products in *E. coli* [9]. In the present study, we developed a similar system to produce tag-fusing chimera polypeptides in *B. subtilis* using pWH1520, a shuttle vector for heterologous gene expression in *B. megaterium* [26]. The recognition sequence of factor Xa (IEGR), six successive histidine codons and a *B. licheniformis* β-lactamase terminator sequence [27] with no termination codon in frame were designed to be aligned within the xylose isomerase gene under the *xyl*A promoter on pWH1520 (Fig. 2a). This plasmid was transformed into *B. subtilis* strain AMHG L1 (P*spac-ssrA*) in which the expression of tmRNA encoded in the genome can be controlled by IPTG and into strain AMHG N1 (P*spac-ssrA*(*DD*)) in which mutations have been introduced into the tmRNA gene encoded by the genome of L1 so that the C-terminal AlaAla sequence of the tag-peptide is changed to AspAsp (DD mutant, Fig. 1b) [24]. This type of mutation in tmRNA has been shown to delay the decay of the *trans*-translation product due to the loss of a typical proteolytic signal [9,18,19,28]. The truncated xylose isomerase was expressed by the addition of D-xylose, and it was purified by using a nickel-chelate column. The factor Xa-digested polypeptide fragments having a 6×His-tag sequence were purified from another nickel-chelate column and were analyzed by MALDI-TOF mass spectroscopy.

Several signals of polypeptides derived from the partial xylose isomerase gene lacking a termination codon were identified in the mass spectrum (Fig. 2b). Their molecular weights correspond well with those of the factor Xa-digested fragments of chimera polypeptides comprising the truncated xylose isomerase fragments having heterologous C-termini around the transcription terminator region with and without an identical peptide with a 15-amino acid sequence (AGKTNSFNQNVALDD), the last 14 amino acid residues of which are encoded by the DD mutant of *B. subtilis*

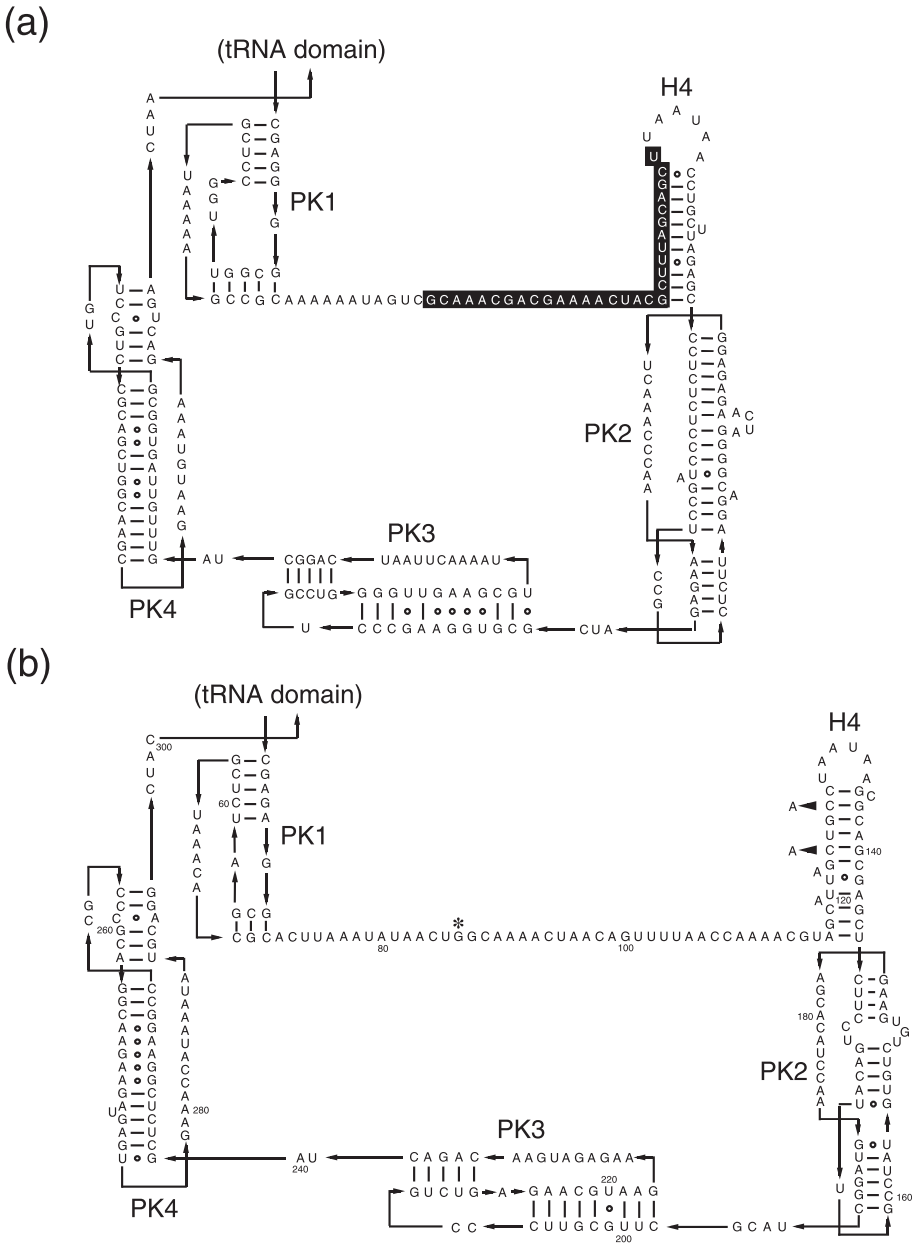


Fig. 1. The mRNA domains of tmRNAs of *E. coli* (a) and *B. subtilis* (b). The tag-encoding region in *E. coli* tmRNA is highlighted by white with a black background. Arrows indicate the base substitutions for the DD mutant. The tag-starting point in *B. subtilis* tmRNA identified in this study is designated by an asterisk.

tmRNA. The heterogeneity of the C-termini of the truncated xylose isomerase portion just prior to the tag-peptide sequence, which may be due to heterologous 3′ termini of the transcribed mRNA with no termination codon, is a typical feature of the *trans*-translation products in *E. coli* [8,9,29]. The signals of truncated xylose isomerase fragments without any tag-peptide were also identified. They may be produced by hydrolysis from peptidyl tRNA that had been abortively dissociated from the ribosome with the help of ribosome recycling factor and RF3 before completion of translation [30]. These results clearly show that the tag-peptide of *B. subtilis* has a 15-amino acid sequence of AGKTNSFNQNVALAA, in which the first alanine is from the alanine moiety aminoacylated to tmRNA and the C-terminal 14 amino acid residues are encoded by tmRNA. The tag-encoding region starts at G at position 87, 15 nucleotides downstream of PK1, and terminates at the first UAA triplet (129–131) in the loop of H4.

We then studied the difference between the *trans*-translation systems in *E. coli* and *B. subtilis*. Can *B. subtilis* tmRNA facilitate *trans*-translation in *E. coli*? If this is possible, the question arises as to whether the sequence of the tag-peptide produced in the heterologous system is identical to that produced in the homologous system. We attempted to coexpress a truncated polypeptide encoded by the *E. coli* DHFR gene lacking a termination codon with *B. subtilis* tmRNA in *E. coli* cells. The recognition sequence of factor Xa and six successive histidine codons, followed by a trpA terminator, a typical Rho-independent terminator, were inserted within the DHFR gene on pQE16 with no termination codon in frame (Fig. 3a). This plasmid was cotransformed with another plas-

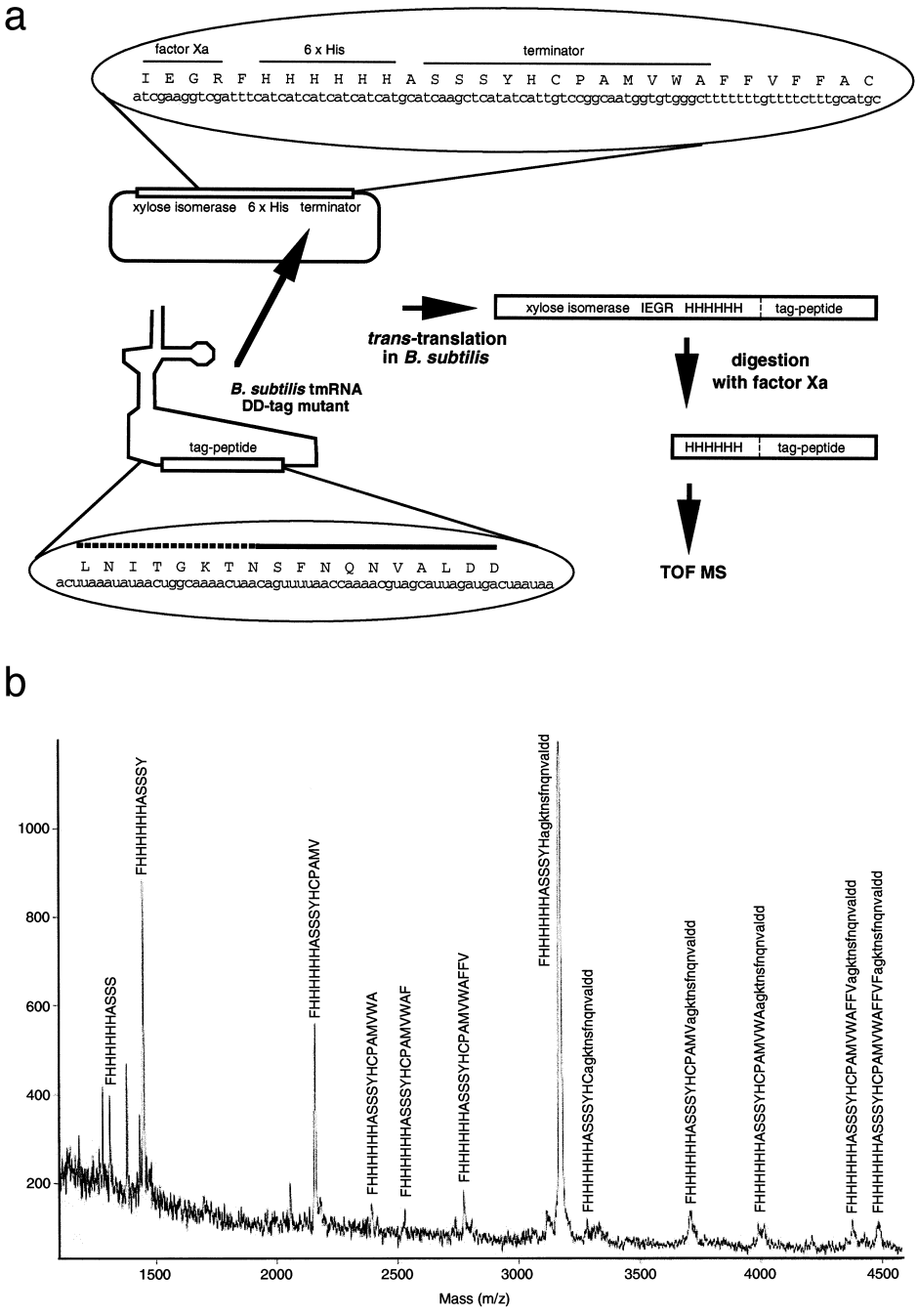
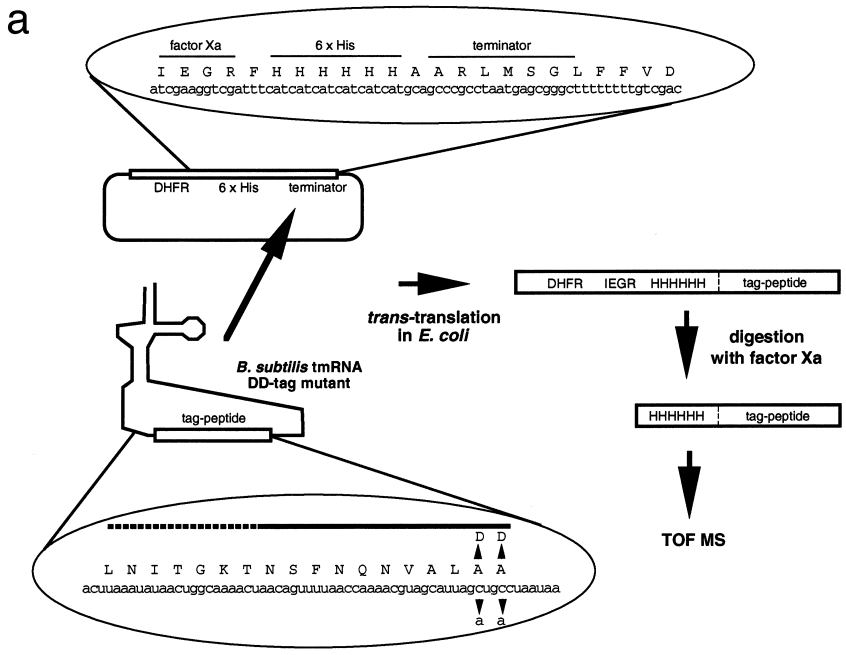
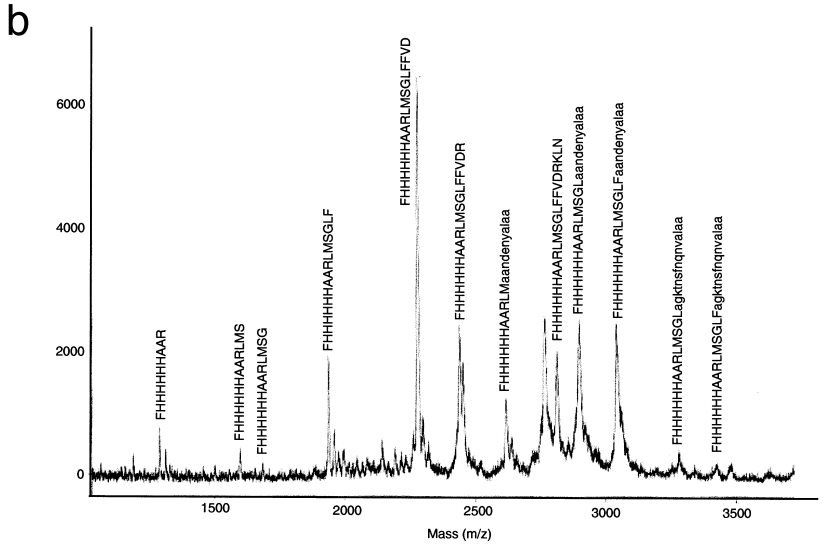


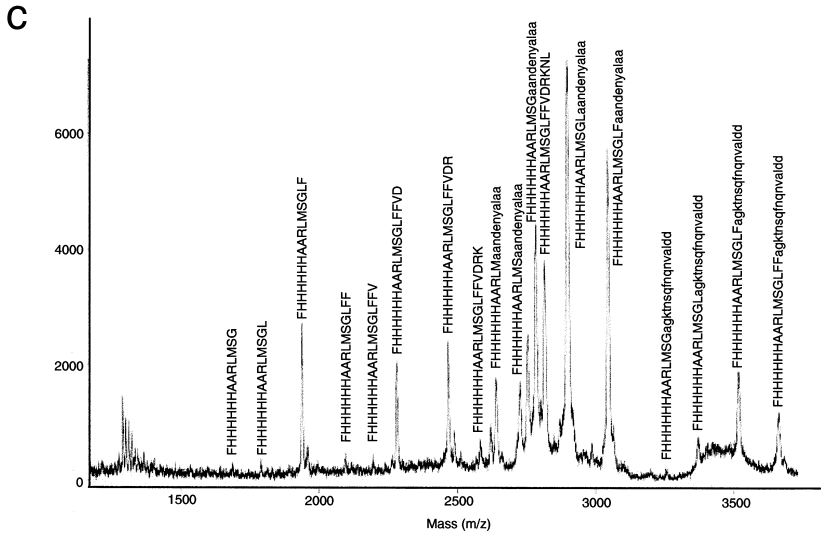
Fig. 2. *Trans*-translation in *B. subtilis* mediated by *B. subtilis* tmRNA. a: A schematic representation of the system for identification of the tmRNA-directed tag-peptide sequence in *B. subtilis*. The truncated C-terminal nucleotide and amino acid sequences of xylose isomerase are shown at the top. The putative tag-peptide sequence is designated at the bottom, in which the broken line above the sequence indicates that the exact start point had not been identified until this study. b: A mass spectrum of factor Xa-digested peptide fragments possessing a 6×His-tag upon induction of the truncated xylose isomerase gene in *B. subtilis*. Each signal is labeled with uppercase characters indicating the amino acid sequence of the factor Xa-digested fragment of truncated xylose isomerase with or without lowercase characters indicating the tag-peptide sequence.

→

Fig. 3. *Trans*-translation in *E. coli* mediated by *B. subtilis* tmRNA. a: A schematic representation of the system for identification of the *B. subtilis* tmRNA-directed tag-peptide sequence in *E. coli*. The truncated C-terminal nucleotide and amino acid sequences of DHFR are shown at the top. The putative tag-peptide sequence is designated at the bottom, in which the broken line above the sequence indicates that the exact start point had not been identified until this study. Arrowheads indicate the base substitutions for the DD mutant and the expected changes in its encoded tag-peptide. b,c: A mass spectrum of factor Xa-digested polypeptide fragments possessing a 6×His-tag upon induction of the truncated DHFR gene coexpressed with wild-type *B. subtilis* tmRNA (b) or the DD mutant tmRNA (c) in *E. coli*.


a
factor Xa
6 x His
terminator
I E G R F H H H H H H A A R L M S G L F F V D
atcgaaggtcgatttcatcatcatcatcatcatgcagccgcctaatgagcgggctttttttttgtcgac
DHFR
6 x His
terminator
trans-translation
in E. coli
B. subtilis tmRNA
DD-tag mutant
tag-peptide
DHFR IEGR HHHHHH
tag-peptide
digestion
with factor Xa
HHHHHH
tag-peptide
TOF MS
D D
L N I T G K T N S F N Q N V A L A A
acuuaaauauaacuggcaaaacuaacaguuuuaaccaaaacguagcauuagcugccuaauaa
a a


b
FHHHHHHAAR
FHHHHHHAARLMS
FHHHHHHAARLMSG
FHHHHHHAARLMSGLF
FHHHHHHAARLMSGLFFVD
FHHHHHHAARLMSGLFFVDR
FHHHHHHAARLMaandenyalaa
FHHHHHHAARLMSGLFFVDRKLN
FHHHHHHAARLMSGLaandenyalaa
FHHHHHHAARLMSGLFaandenyalaa
FHHHHHHAARLMSGLagktnsfnqnvalaa
FHHHHHHAARLMSGLFagktnsfnqnvalaa
6000
4000
2000
0
1500
2000
2500
3000
3500
Mass (m/z)


c
FHHHHHHAARLMSG
FHHHHHHAARLMSGL
FHHHHHHAARLMSGLF
FHHHHHHAARLMSGLFF
FHHHHHHAARLMSGLFFV
FHHHHHHAARLMSGLFFVD
FHHHHHHAARLMSGLFFVDR
FHHHHHHAARLMSGLFFVDRK
FHHHHHHAARLMaandenyalaa
FHHHHHHAARLMSaandenyalaa
FHHHHHHAARLMSGaandenyalaa
FHHHHHHAARLMSGLFFVDRKNL
FHHHHHHAARLMSGLaandenyalaa
FHHHHHHAARLMSGLFaandenyalaa
FHHHHHHAARLMSGagktnsqfnqnvaldd
FHHHHHHAARLMSGLagktnsqfnqnvaldd
FHHHHHHAARLMSGLFagktnsqfnqnvaldd
FHHHHHHAARLMSGLFFagktnsqfnqnvaldd
6000
4000
2000
0
1500
2000
2500
3000
3500
Mass (m/z)

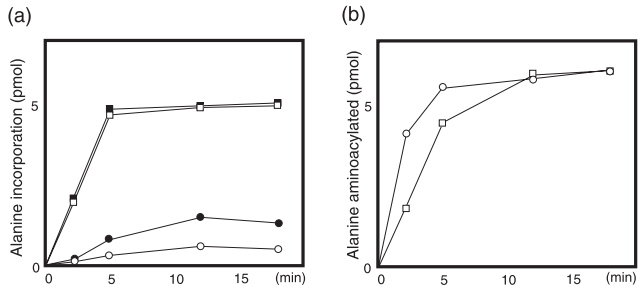


Fig. 4. a: In vitro poly(U)-dependent tag-peptide synthesis in the presence of *B. subtilis* tmRNA using the *E. coli* S30 fraction. Incorporation of alanine into the polypeptide fraction in the presence of 5 μM (open circles) or 35 μM (filled circles) *B. subtilis* tmRNA and 5 μM (open squares) or 35 μM (filled squares) *E. coli* tmRNA. The mean value obtained from at least two independent experiments is plotted. b: In vitro aminoacylations with alanine for *B. subtilis* tmRNA (open circles), as compared to *E. coli* tmRNA (open squares). The mean value obtained from at least two independent experiments is plotted.

mid encoding *B. subtilis* tmRNA into *E. coli* strain JM109 (DE3). *B. subtilis* tmRNA or the DD mutant was successfully expressed in *E. coli*, and the expression level upon induction was roughly 100-fold higher than that of endogenous *E. coli* tmRNA. The factor Xa-digested polypeptide fragments having a 6×His-tag sequence were purified from another nickel-chelate column and were analyzed by mass spectroscopy.

Many signals of polypeptides derived from the DHFR gene lacking a termination codon were able to be identified in the mass spectrum (Fig. 3b,c). The molecular weights of some signals correspond well with those of the factor Xa-digested fragments of chimera polypeptides comprising the truncated DHFR fragments having heterologous C-termini around the transcription terminator region with a typical tag-peptide sequence of *E. coli*, AANDENYALAA. These signals may be derived from endogenous *E. coli* tmRNA, since they appeared even in the absence of the second plasmid encoding *B. subtilis* tmRNA (data not shown). Several signals were identified as those of the factor Xa digests of the truncated DHFR fragments having heterologous C-termini without any tag sequence, as observed in the *trans*-translation in *B. subtilis* described above. In addition to these signals, we were able to identify several signals with molecular weights corresponding to the factor Xa digests of the truncated DHFR fragments fused with an identical peptide with a 15-amino acid sequence (AGKTNSFNQNVALAA for wild-type or AGKTNSFNQNVALDD for the DD mutant), the last 14 amino acid residues of which are encoded by *B. subtilis* tmRNA or its mutant. These results demonstrate that *B. subtilis* tmRNA has the ability to facilitate *trans*-translation in *E. coli* cells, and that the *B. subtilis* tmRNA-encoded tag-peptide produced in *E. coli* has a 15-amino acid sequence of AGKTNSFNQNVALAA. This sequence is completely the same as the sequence of the *B. subtilis* tmRNA-encoded tag-peptide expressed in *B. subtilis*. The ribosome stalled on the 3′ end of truncated mRNA can serve as a target of both *E. coli* and *B. subtilis* tmRNAs. The intensities of the signals derived from *B. subtilis* tmRNAs, especially from the wild-type, were apparently lower than those derived from endogenous *E. coli* tmRNA. Considering that the level of *B. subtilis* tmRNA in *E. coli* upon induction was roughly 100-fold higher than that of endogenous *E. coli* tmRNA, the *trans*-translation efficiency in *E. coli* cells by *B. subtilis* tmRNA is thought to be much lower than that by *E. coli* tmRNA. The low signal intensities of chimera polypeptides fusing the wild-type tag-peptide may reflect the fact that the C-terminal AA sequence of tag-peptide derived from *B. subtilis* tmRNA, like that derived from its *E. coli* counterpart, is a preferential target of cellular proteases in *E. coli*.

E. coli tmRNA-dependent tag-peptide synthesis in vitro can be evaluated by monitoring the incorporations of the tag-specific amino acids into the polypeptide in the presence of poly(U) [12]. We examined the in vitro incorporation of alanine, a major constituent of the *B. subtilis* tag-peptide, depending on *B. subtilis* tmRNA using the S30 fraction extracted from an *E. coli* Δ*ssrA* strain. As shown in Fig. 4a, alanine was substantially incorporated in the presence of wild-type tmRNA of *B. subtilis*. The level of alanine incorporation was about 10-fold lower than that in the presence of wild-type tmRNA of *E. coli*. When a seven-fold larger amount of *B. subtilis* tmRNA was added to the reaction mixture, the level of alanine incorporation was increased by about two-fold. In contrast, the addition of a seven-fold larger amount of *E. coli* tmRNA had almost no effect. *E. coli* tmRNA, but not *B. subtilis* tmRNA, was saturated in the reaction, suggesting an insufficient interaction between *B. subtilis* tmRNA and some *E. coli* machinery.

We also examined the aminoacylation ability with alanine using *E. coli* AlaRS (Fig. 4b). *E. coli* AlaRS could aminoacylate *B. subtilis* tmRNA with an efficiency comparable to that of *E. coli* tmRNA. The observed efficient heterologous aminoacylation may probably be due to the presence of major identity determinants for *E. coli* AlaRS in *B. subtilis* tmRNA [1].

4. Discussion

The present study reveals that *B. subtilis* tmRNA can facilitate *trans*-translation not only in *B. subtilis* but also in *E. coli*. It was also found that the *B. subtilis* tmRNA-encoded tag-peptide produced in *E. coli* is the same as that produced in *B. subtilis*, although the *trans*-translation in *E. coli* directed by *B. subtilis* tmRNA was much less efficient than that directed by *E. coli* tmRNA. The tag sequence starts at G, 15 nucleotides downstream of PK1, and terminates at the first UAA triplet in the loop of H4.

The number of amino acids comprising the tag-peptide is not conserved between the two bacteria: 11 amino acids in *E. coli* versus 15 amino acids in *B. subtilis*. The number of amino acids between the start point and H4 is also different. According to recent alignments of the deduced tag-peptide, the second amino acid defined by the start codon is highly conserved as an alanine [17]. In *E. coli*, this second alanine is required for recognition by ClpA and a ribosome-associated protein, SspB, which binds specifically to the *trans*-translation product to enhance specificity for degradation by ClpXP protease [31]. A recent study has raised the possibility of the involvement of the first alanine codon in resumption of translation based on the finding that a portion of *E. coli* $tRNA^{Ala}$ binds tmRNA in vitro [32]. The first codon of *B. subtilis* tmRNA designates glycine. Nevertheless, the *E. coli* machineries can select the authentic initiation point of *B. subtilis* tmRNA.

The nucleotide number of the span between the resuming nucleotide and PK1 is not conserved: 11 for *E. coli* versus 14 for *B. subtilis*. This indicates that the number of nucleotides

between PK1 and the tag-initiation point is not necessarily invariant among species and that PK1 does not serve as a determinant for the tag-initiation point. This is consistent with earlier findings showing that neither deletion nor addition of a nucleotide just downstream of PK1 affects the frame of in vitro tag translation in *E. coli* [25,33]. The second and third nucleotides of the tag-encoding region are diverged, while the first nucleotide, G, is identical. The nucleotide number of the span between the resuming nucleotide and the H4 helix is also not conserved: 17 for *E. coli* versus 28 for *B. subtilis*. The redundancy of the length of this region has been shown in an *E. coli* in vitro system in which an addition of a nucleotide downstream of the tag-initiation point does not shift the site of tag initiation [33]. The GG sequence between the two stems of PK1 as well as the specific conformation of PK1 is important for *trans*-translation efficiency in *E. coli* [25,34]. The absence of this GG sequence between the two stems of *B. subtilis* PK1 (Fig. 1b) seems a likely reason for the inefficiency of cross-species *trans*-translation. It has recently been shown that some nucleotides in the tRNA domain are crucial for *trans*-translation, but not for aminoacylation [35]. These nucleotides are conserved in *B. subtilis* tmRNA.

Typically, translation in *B. subtilis* requires a specific interaction between the SD sequence on mRNA and 16S rRNA that is stronger than that in *E. coli* [36]. However, neither *E. coli* nor *B. subtilis* tmRNA has a homolog of the SD sequence within the span between PK1 and the tag-initiation point. Instead, the sequence from −6 to −3 ($A_{-6}U_{-5}A_{-4}Pu_{-3}$) is conserved between the two bacteria. A part of this sequence, $U_{-5}A_{-4}Pu_{-3}$, as well as G1 is included in a highly phylogenetically conserved UAPuNNG sequence predicted as a potential determinant of the tag-initiation point, which is supported by a functional selection from a pool of randomized sequences on the *E. coli* tmRNA framework [29]. Some mutations around this sequence inactivate the tag translation or shift the initiation point in vitro [33].

A comparison of the locations around the tag-encoding region in the two bacterial tmRNAs confirms the significance of the highly conserved sequence downstream of PK1 rather than its structural context. The rule for determining the tag-initiation point on tmRNA may be the same in Gram-positive and Gram-negative bacteria. This rule should be governed by some general translational and/or *trans*-translation-specific machinery/machineries. The tag sequences derived from *B. subtilis* tmRNA in *E. coli* and in *B. subtilis* were identical, suggesting that such a presumed machinery as well as its recognition mode of the conserved sequence is universal. On the other hand, the poor efficiency of *B. subtilis* tmRNA-dependent *trans*-translation in *E. coli* may reflect the lack of an optimal interaction between *B. subtilis* tmRNA and some of the *E. coli* machineries. The efficiency of aminoacylation by *E. coli* AlaRS was comparable between *E. coli* and *B. subtilis* tmRNAs. Thus, the observed poor efficiency of cross-species *trans*-translation may be due to the processes after the step of aminoacylation. *B. subtilis* tmRNA, like *E. coli* tmRNA [6,7], seems to be a good substrate for *E. coli* EF-Tu, considering the apparent fulfillment of the structural requirement for EF-Tu [37]. The determination of the tag-initiation point may be facilitated by interaction of the highly conserved sequence downstream of PK1 with ribosome or a *trans*-translation-specific factor(s) after the process of EF-Tu binding.

The ribosome protein S1, which is usually involved in the initiation step of translation [34,38], can cross-link with tmRNA on and off the ribosome [21], and form a complex with tmRNA in *E. coli* [22]. Cross-linking occurs mainly in H4, PK2 and PK3 on and off the ribosome, although neither of these structural units is essential for correct initiation of the tag translation in vitro [33,39]. U_{-5} is also cross-linked off but not on the ribosome, raising the possibility that S1 recognizes the *cis*-element on tmRNA to induce the tag-initiation point into the decoding region on the ribosome. This possibility, however, is not supported by the results of a recent cryo-electron microscopic study indicating that S1 can interact with mRNA upstream of the SD sequence at the platform region on the 30S subunit outside the 3′ end of the 16S rRNA rather than around the decoding region [40]. Note that S1 is missing in the ribosomes from *B. subtilis*. *B. subtilis* genome has an S1 homolog, although it is dissimilar to the *E. coli* counterpart in that it is not essential for cell viability and it has only two S1 motifs instead of four [41]. It is possible that this protein functions as an S1 substitute in *B. subtilis* for *trans*-translation but not for canonical translation. Otherwise, we should assume a universal rule of the initiation point determination governed by another *trans*-acting factor that recognizes the *cis*-element on tmRNA.

The small basic protein SmpB, the gene of which is located immediately upstream of the tmRNA gene in the genome, has been identified in *E. coli* as a protein factor essential for *trans*-translation [42], and it is included in the complex involving tmRNA and S1 [22]. The *B. subtilis* genome also encodes an SmpB homolog juxtaposed to the tmRNA gene. Future study of the function and the mode of interaction of SmpB as well as those of other factors will help to clarify the molecular mechanism of *trans*-translation.

Acknowledgements: We thank Gene Research Center and Center of Joint Research of Hirosaki University for allowing us to use their facilities. This work was supported by a grant-in-aid for scientific research and grants ('Research for the Future' Program, JSPS-RFTF96100305 and JSPS-RFTF97L00593) from the Japan Society for the Promotion of Science and a Human Frontier Science Program research grant (RG0291/2000-M 100).

References

[1] Ushida, C., Himeno, H., Watanabe, T. and Muto, A. (1994) Nucleic Acids Res. 22, 3392–3396.
[2] Komine, Y., Kitabatake, M., Yokogawa, T., Nishikawa, K. and Inokuchi, H. (1994) Proc. Natl. Acad. Sci. USA 91, 9223–9227.
[3] Felden, B., Hanawa, K., Atkins, J.F., Himeno, H., Muto, A., Gesteland, R.F., McCloskey, J.A. and Crain, P.F. (1998) EMBO J. 17, 3188–3196.
[4] Felden, B., Himeno, H., Muto, A., McCutcheon, J.P., Atkins, J.F. and Gesteland, R.F. (1997) RNA 3, 89–104.
[5] Williams, K.P. and Bartel, D.P. (1996) RNA 2, 1306–1310.
[6] Rudinger-Thirion, J., Giegé, R. and Felden, B. (1999) RNA 5, 989–992.
[7] Barends, S., Wower, J. and Kraal, B. (2000) Biochemistry 39, 2652–2658.
[8] Tu, G.-F., Reid, G.E., Zhang, J.-G., Moritz, R.L. and Simpson, R.J. (1995) J. Biol. Chem. 270, 9322–9326.
[9] Keiler, K.C., Waller, P.R.H. and Sauer, R.T. (1996) Science 271, 990–993.
[10] Muto, A., Sato, M., Tadaki, T., Fukushima, M., Ushida, C. and Himeno, H. (1996) Biochimie 78, 985–991.
[11] Muto, A., Ushida, C. and Himeno, H. (1998) Trends Biochem. Sci. 23, 25–29.

[12] Himeno, H., Sato, M., Tadaki, T., Fukushima, M., Ushida, C. and Muto, A. (1997) J. Mol. Biol. 268, 803–808.
[13] Nameki, N., Tadaki, T., Muto, A. and Himeno, H. (1999) J. Mol. Biol. 289, 1–7.
[14] Tadaki, T., Fukushima, M., Ushida, C., Himeno, H. and Muto, A. (1996) FEBS Lett. 399, 223–226.
[15] Roche, E.D. and Sauer, R.T. (1999) EMBO J. 18, 4579–4589.
[16] Roche, E.D. and Sauer, R.T. (2001) J. Biol. Chem. 276, 28509–28515.
[17] Williams, K.P. (2000) Nucleic Acids Res. 28, 168.
[18] Gottesman, S., Roche, E., Zhou, Y.N. and Sauer, R.T. (1998) Genes Dev. 12, 1338–1347.
[19] Herman, C., Thevenet, D., Bouloc, P., Walker, G.C. and D'Ari, R. (1998) Genes Dev. 12, 1348–1355.
[20] Keiler, K.C., Shapiro, L. and Williams, K.P. (2000) Proc. Natl. Acad. Sci. USA 97, 7778–7783.
[21] Wower, J., Zwieb, C., Guven, S.A. and Wower, I. (2000) EMBO J. 19, 6612–6621.
[22] Karzai, A.W. and Sauer, R.T. (2001) Proc. Natl. Acad. Sci. USA 98, 3040–3044.
[23] Robert, M.W. and Rabinowitz, J.C. (1989) J. Biol. Chem. 264, 2228–2235.
[24] Muto, A., Fujihara, A., Ito, K., Matsuno, J., Ushida, C. and Himeno, H. (2000) Genes Cells 5, 627–636.
[25] Nameki, N., Felden, B., Atkins, J.F., Gesteland, R.F., Himeno, H. and Muto, A. (1999) J. Mol. Biol. 286, 733–744.
[26] Rygus, T. and Hillen, W. (1991) Appl. Microbiol. Biotechnol. 35, 594–599.
[27] Hess, G.F. and Graham, R.S. (1990) Gene 95, 137–141.
[28] Wiegert, T. and Schumann, W. (2001) J. Bacteriol. 183, 3885–3889.
[29] Williams, K.P., Martindale, K.A. and Bartel, D.P. (1999) EMBO J. 18, 5423–5433.
[30] Heurgué-Hamard, V., Karimi, R., Mora, L., MacDougall, J., Leboeuf, C., Grentzmann, G., Ehrenberg, M. and Buckingham, R.H. (1998) EMBO J. 17, 808–816.
[31] Flynn, J.M., Levchenko, I., Seidel, M., Wickner, S.H., Sauer, R.T. and Baker, T.A. (2001) Proc. Natl. Acad. Sci. USA 98, 10584–10589.
[32] Gillet, R. and Felden, B. (2001) EMBO J. 20, 2966–2976.
[33] Lee, S., Ishii, M., Tadaki, T., Muto, A. and Himeno, H. (2001) RNA 7, 999–1012.
[34] Nameki, N., Chattopadhyay, P., Himeno, H., Muto, A. and Kawai, G. (1999) Nucleic Acids Res. 27, 3667–3675.
[35] Hanawa-Suetsugu, K., Bordeau, V., Himeno, H., Muto, A. and Felden, B. (2001) Nucleic Acids Res. 29, 4663–4673.
[36] Farwell, M.A., Roberts, M.W. and Rabinowitz, J.C. (1992) Mol. Microbiol. 6, 3375–3383.
[37] Nissen, P., Kjeldgaard, M., Thirup, S., Polekhina, G., Reshetnikova, L., Clark, B.F.C. and Nyborg, J. (1995) Science 270, 1464–1472.
[38] Tedin, K., Resch, A. and Bläsi, U. (1997) Mol. Microbiol. 25, 189–199.
[39] Nameki, N., Tadaki, T., Himeno, H. and Muto, A. (2000) FEBS Lett. 470, 345–349.
[40] Sengupta, J., Agrawal, R.K. and Frank, J. (2001) Proc. Natl. Acad. Sci. USA 88, 11991–11996.
[41] Sorokin, A., Serror, P., Pujic, P., Azevedo, V. and Ehrlich, S.D. (1995) Microbiology 141, 311–319.
[42] Karzai, A.W., Susskind, M.M. and Sauer, R.T. (1999) EMBO J. 18, 3793–3799.