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Trans-translation ensures timely initiation of DNA replication and DnaA synthesis in *Escherichia coli*

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ABSTRACT. The trans-translation pathway, mediated by the transfer messenger RNA (tmRNA; encoded by the *ssrA* gene) and the SmpB protein (tmRNA-binding protein expressed in *Salmonella enterica*), which is conserved in bacteria, is required for various cellular processes. A previous study has shown that trans-translation is required to ensure timely (non-delayed) *dnaA* transcription and consequent initiation of DNA replication in *Caulobacter crescentus*. In this study, we observed that initiation of chromosome replication was delayed in *Escherichia coli* lacking the *smpB* and/or *ssrA* genes (Δ*ssrA*, Δ*smpB*, or Δ*smpB*Δ*ssrA* mutants). We observed that the growth rate of the mutant cells was much slower than that of its wild-type counterpart. However, the delayed initiation of replication and slower growth in the Δ*ssrA* or Δ*smpB* mutants were reversed by ectopic expression of tmRNA or SmpB. A synchronized Δ*smpB*Δ*ssrA* cell culture containing the *dnaC*2 mutant allele showed delayed protein (total and DnaA) accumulation per cell; DnaA accumulation was also delayed in the Δ*smpB*. These

results indicated that absence of trans-translation leads to a delay in initiation of DNA replication, synthesis of total protein (including DnaA), and a decrease in *E. coli* growth rate. In summary, we propose that the trans-translation pathway is required to ensure timely initiation of replication, protein synthesis, and subsequent cell cycle progression.

Key words: Trans-translation; Replication initiation; *dnaA* expression; Cell cycle-dependent

INTRODUCTION

Trans-translation is a pathway employed by many bacteria to control the quality of translation (Keiler, 2008). One of the key components of trans-translation is the transfer-messenger RNA (tmRNA) encoded by the *ssrA* gene, also named 10Sa RNA in *Escherichai coli* (Lee et al., 1978). tmRNA is a small RNA with tRNA and mRNA moieties, and is therefore functionally analogous to both tRNA and mRNA (Tyagi and Kinger, 1992; Komine et al., 1994). Another important component of this process is the SmpB protein, a tmRNA-binding protein found in *Salmonella enterica* (Karzai et al., 1999). A moving ribosome can be stalled unexpectedly or even programmatically during translation (Tenson and Ehrenberg, 2002). When a ribosome is stalled at the end of an mRNA that lacks a stop codon, tmRNA bound to SmpB is charged with alanine via the alanyl tRNA-synthetase (AlaRS), EF-Tu, and the ribosomal protein S1. The tmRNA/SmpB/EF-Tu complex then enters the A site of stalled ribosome with assistance from PrsA, SAF, RNase R, and $tRNA^{ala}$, in order to transfer alanine from tmRNA to the nascent peptide chain. As a result, the nascent peptide chain is transferred from the original mRNA template to tmRNA. The mRNA moiety in the tmRNA molecule encodes the tag sequence of AANDENYALAA (Keiler et al., 1996), and the tagged peptide is then dissociated upon reaching the stop codon of tmRNA and subsequently degraded by ClpXP, RNase R, or other proteases (Gottesman et al., 1998; Levchenko et al., 2000; Shpanchenko et al., 2010).

The *ssrA* and *smpB* genes are conserved in bacteria; trans-translation is required for different processes, including sporulation in *Bacillus subtilis* (Abe et al., 2008), symbiosis in *Bradyrhizobium japonicum* (Ebeling et al., 1991), pathogenesis in *S. enterica* (Julio et al., 2000) and *Yersinia pseudotuberculosis* (Okan et al., 2006), and cell cycle control in *Caulobacter crescentus* (Keiler and Shapiro, 2003). A previous study has shown that trans-translation is required to ensure that *dnaA* transcription and consequent initiation of DNA replication in *C. crescentus* is not delayed (Cheng and Keiler, 2009). Mutations in the *dnaA* promoter partially suppress defects in the initiation of replication in the absence of trans-translation activity.

In this study, we investigated the possible involvement of trans-translation activity in ensuring the correct timing of protein synthesis and initiation of DNA replication; the latter appeared to be modulated by *dnaA* expression in a cell cycle-dependent manner.

MATERIAL AND METHODS

Bacterial strains

The *E. coli* K-12 bacterial strains used in this study are listed in Table 1. The cam^R cassette was amplified by PCR using the pKD3 plasmid as the template and the primers

5'-del-*ssrA*-P1 and 3'-del-*ssrA*-P2 (Table 2). The amplified *cam*^{R} cassette was used to replace chromosomal *ssrA* in BW25113, using a previously described one-step chromosomal gene inactivation method (Datsenko and Wanner, 2000), resulting in a Δ*ssrA*::*cam*^{R} mutant. The *kan*^{R} cassette was amplified by PCR, using the pKD4 plasmid as the template and the 5'-del-*smpB*-P1 and 3'-del-*ssrA*-P2 primers (Table 2); this was used to simultaneously replace the chromosomal genes *smpB* and *ssrA* in BW25113 by the method cited above, resulting in a Δ*smpB*Δ*ssrA*::*kan*^{R} double mutant. The *smpB* and *ssrA* genes can be removed simultaneously, because of their proximity on the *E. coli* chromosome. The *dnaC*2::*tet*^{R} allele was transduced using the P1 phage into BW25113 (wild-type); subsequently, the Δ*ssrA*::*cam*^{R}, Δ*smpB*::*kan*^{R}, or Δ*smpB*Δ*ssrA*::*kan*^{R} allele was P1 transduced into BW25113*dnaC*2::*tet*^{R}. Plasmid DNA was prepared using DH5α as the host.

Table 1. Strains and plasmids used in the study.

Strains	Genotype	Reference or source
BW25113	Wild-type *rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567*Δ(*rhaBAD*)*568 rph-1*	Baba et al., 2006
MOR568	BW25113Δ*smpB*::*kan*^{R}	Baba et al., 2006
MOR2363	BW25113Δ*ssrA*::*cam*^{R}	This study
MOR2461	BW25113Δ*smpB*Δ*ssrA*::*kan*^{R}	This study
MOR2471	BW25113*dnaC*2::*tet*^{R}(Ts)	This study
MOR2475	BW25113Δ*ssrA*:: *cam*^{R} *dnaC*2::*tet*^{R}(Ts)	This study
MOR2476	BW25113Δ*smpB*:: *kan*^{R} *dnaC*2::*tet*^{R}(Ts)	This study
MOR2477	BW25113Δ*smpB*Δ*ssrA*::*kan*^{R}*dnaC*2::*tet*^{R} (Ts)	This study
DH5α	F$^-$ *sup*E44Δ*lac*U169(Δ*lacZ*Δ*M15*) *hsdR*17 *recA*1 *endA*1 *gyrA*96	New England Biolabs
Plasmids		
pKD3	*rep*$_{R6K}$ *amp*^{R} FRT *cam*^{R} FRT	Datsenko and Wanner, 2000
pKD4	*rep*$_{R6K}$ *amp*^{R} FRT *kan*^{R} FRT	Datsenko and Wanner, 2000
pKD46	*rep*$_{pSC101^{ts}}$ *amp*^{R} P$_{araBAD}$ γβ exo	Datsenko and Wanner, 2000
pUHE21–2*lacI*^{q}	*rep*$_{pMB1}$ *amp*^{R} *lacI*^{q}	Soncini et al., 1995
pUHE21-*ssrA*	*ssrA* was inserted into pUHE21 at the *Bam*HI and *Hin*dIII sites	This study
pUHE21-*smpB*	*smpB* was inserted into pUHE21 at the *Bam*HI and *Hin*dIII sites	This study
pUHE21-*smpB*-*ssrA*	*smpB* and *ssrA* genes with their intergenic region were inserted into pUHE21 at the *Bam*HI and *Hin*dIII sites.	This study

Table 2. Primers used in the study.

Name	Sequence (5'-3')	Usage
5'-del-*ssrA*-P1	CTGGTCATGGCGCTCATAAATCTGGTATACTTACCTTTACACATT GTGTAGGCTGGAGCTGCTTC	To delete *ssrA* gene on chromosome with 3'-del-*ssrA*-P2
3'-del-*ssrA*-P2	GGACTTCATCGGATGACTCTGGTAATCACCGATGGAGAATTTCA TATGAATATCCTCCTTAG	
5'-del-*smpB*-P1	GGGGTGTTTTCGATTTCAGATTACCGATGATTCACGACGCTTGTG TAGGCTGGAGCTGCTTC	To delete *smpB* and *ssrA* gene on chromosome with 3'-del-*ssrA*-P2
5'-pUHE21-*smpB*	CGGGATCCGATTACCGATGATTCACGAC(*Bam*HI)	To insert the *smpB* gene at the *Bam*HI and *Hin*dIII sites of pUHE21
3'-pUHE21-*smpB*	CCCAAGCTTCGCGGTGAGGAACTGGTC(*Hin*dIII)	
5'-pUHE21-*ssrA*	CGGGATCCAATCTGGTATACTTACC(*Bam*HI)	To insert the *ssrA* gene at the *Bam*HI and *Hin*dIII sites of pUHE21
3'-pUHE21-*ssrA*	CCCAAGCTTCTCTGGTAATCACCGATGG(*Hin*dIII)	

Growth conditions

Bacteria were cultured in ABTGcasa medium (Morigen et al., 2005; Yao et al., 2015) at 37°C; additionally, the cultures were incubated at 30° and 42°C for synchronization of *dnaC*2 cells. The culture medium was supplemented with antibiotics when required (ampicillin, chloramphenicol, tetracycline, and/or kanamycin at final concentrations of 100, 30, 15, and 50 μg/mL).

Plasmid construction

All plasmids used in this study are listed in Table 1. The *ssrA* gene was amplified by PCR using the wild-type chromosome as the template and the 5'-pUHE21-*ssrA* and 3'-pUHE21-*ssrA* primers; this was subsequently inserted into pUHE21-2*lacI*^{q} at the *Bam*HI and *Hin*dIII sites, resulting in the pUHE21-*ssrA* plasmid, which expresses the *ssrA* RNA. *smpB* was amplified by PCR at its native promoter region using the 5'-pUHE21-*smpB* and 3'-pUHE21-*smpB* primers, or at a region including the *smpB* and *ssrA* genes using the 5'-pUHE21-*smpB* and 3'-pUHE21-*ssrA* primers, using the wild-type chromosomal DNA as the template. The resultant fragment from each PCR cycle was inserted into the pUHE21-2*lacI*^{q} plasmid at the *Bam*HI and *Hin*dIII sites, respectively, resulting in the pUHE21-*smpB* and pUHE21-*smpB*-*ssrA* plasmids.

Flow cytometry

The cells were exponentially grown in ABTGcasa medium at 37°C up to an OD_{450} of 0.15; subsequently, the cells were treated with 300 μg/mL rifampicin and 10 μg/mL cephalexin for 4-5 generations in order to allow for the completion of the ongoing rounds of replication: rifampicin inhibits the initiation of replication by preventing transcription, while cephalexin inhibits cell division (Skarstad et al., 1986; Boye and Løbner-Olesen, 1991). In the presence of these drugs, the cells express an integral numbers of chromosomes, representing the number of origins per cell at the time of addition (of the drugs) (Skarstad et al., 1986). One milliliter aliquots of rifampicin- and cephalexin-treated cells were harvested, washed, and resuspended in 100 μL TE buffer, and fixed by adding 1 mL 77% ethanol. The fixed cells were stained with Hoechst 33258 (Invitrogen, Carlsbad, CA, USA) as described previously (Liu et al., 2014), and subsequently analyzed with a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The average DNA content/cell ratio was determined as the average Hoechst fluorescence per cell. The standard sample was prepared as described in a previous study (Morigen et al., 2003).

Synchronization of the *dnaC*2 cells

BW25113*dnaC*2 cells (and its derivatives) showing exponential growth in ABTGcasa medium at the permissive temperature (30°C) were cultured at a non-permissive temperature (42°C) for 2 h; after which the cells were synchronized at a stage prior to the initiation of chromosomal replication (Carl, 1970; Withers and Bernander, 1998).

Determination of the total protein content per cell

Cell cultures (4.5 mL) were harvested at the indicated time points after synchronization by centrifugation at 4°C, washed in 1 mL TE buffer, resuspended in 100 mL TE buffer containing 1% sodium dodecyl sulfate (SDS) and glycerol, and finally boiled for 5 min. Total protein content in the 4.5 mL cell extract was determined using a standard BCA kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer instructions. The number of cells in the cell cultures (fixed volume) harvested at the various time points were determined by plating a series of dilutions of the cultures on LB agar plates with the requisite antibiotics, and counting the colonies formed on the next day. The protein content and cell count in the 4.5-mL culture (at each time point) was used to calculate the protein concentration per cell.

Western blotting and determination of DnaA protein content per cell

The cell extracts were separated by 12% SDS-polyacrylamide gel electrophoresis (40 mA, 2-3 h) for western blot analysis. Subsequently, the DnaA concentration and the DnaA content per cell was determined using a DnaA-antibody and an ECF fluorescence kit (Amersham Biosciences, Amersham, UK) as described previously (Liu et al., 2014).

RESULTS

Absence of trans-translation delays initiation of chromosomal replication

The tmRNA and SmpB proteins are key components of the trans-translation process. The deletion of the *ssrA* or/and *smpB* genes has been previously shown to delay initiation of replication in *C. crescentus* (Keiler and Shapiro, 2003). In order to determine if trans-translation exerts a similar effect in *E. coli*, we developed Δ*ssrA*, Δ*smpB*, and Δ*smpB*Δ*ssrA* mutant cells and monitored the replication pattern of cells lacking tmRNA, SmpB, or both by flow cytometry. Wild-type, Δ*ssrA*, Δ*smpB*, or Δ*smpB*Δ*ssrA E. coli* cells in the exponential phase of growth were treated with rifampicin and cephalexin for 4-5 generations. Therefore, the number of chromosomes per cell indicates the number of origins per cell after drug treatment (Skarstad et al., 1986). The wild-type culture was chiefly composed of 4-chromosome cells (65%); the remaining cells were composed of 2 chromosomes (10%) or 8 chromosomes. The cell mass of this culture doubled every 33 min and the average number origins per cell was 4.4 (Figure 1) at the permissive culture conditions. In comparison, the Δ*ssrA*, Δ*smpB*, and Δ*smpB*Δ*ssrA* mutant cell cultures were composed of a slightly higher number of 2-chromosome cells (and a lower number of 4-chromosome cells) and a lower average number of origins per cell (3.8-4.1); moreover, the doubling time (40-55 min) of these cultures was also seen to be higher (Figure 1). These results indicate that deletion of the *ssrA* or *smpB* genes, or both, results in delayed initiation of chromosomal replication and slow growth. This in turn suggested that the absence of trans-translation delays the initiation of chromosomal replication, and subsequently, cell division, consistent with the mechanism previously reported in *C. crescentus* (Keiler and Shapiro, 2003).

Delay in initiation of replication in the Δ*ssrA* and Δ*smpB* mutants is reversed by ectopic expression of tmRNA and SmpB

The plasmids pUHE21-*ssrA* (p*ssrA*), pUHE21-*smpB* (p*smpB*), and pUHE21-*smpB-ssrA* (p*smpB-ssrA*), which ectopically express tmRNA, SmpB, or both tmRNA and SmpB, respectively, under IPTG induction, were used to investigate the possible rescue of delayed initiation of replication in the absence of trans-translation. The p*ssrA*, p*smpB*, and p*smpB-ssrA* plasmids were subsequently introduced to Δ*ssrA*, Δ*smpB*, or Δ*smpB*Δ*ssrA* mutant cells, respectively. The resulting cells were cultured in ABTGcasa medium up to the exponential phase of growth, and tmRNA and/or SmpB expression was subsequently induced with IPTG (final concentration 0.5 mM). The cells were then treated with rifampicin and cephalexin and analyzed by flow cytometry. We discovered that Δ*ssrA* or Δ*smpB* expressing tmRNA or SmpB showed a wild-type-like replication pattern and growth rate (Figure 2); however, tmRNA and SmpB co-expression in the p*smpB-ssrA* plasmid-containing mutant cells did not induce any reversal in the delay in initiation of replication, because of an unknown reason.

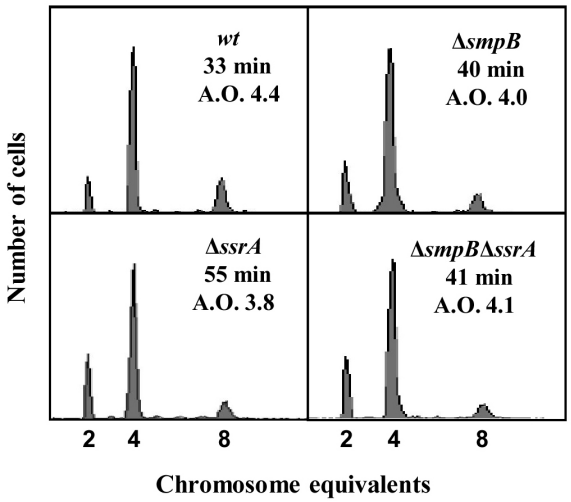

Figure 1. Absence of trans-translation delays the initiation of chromosome replication. Wild-type, Δ*ssrA*, Δ*smpB*, and Δ*ssrA*Δ*smpB* mutant cells at the exponential phase of growth in the ABTGcasa medium at 37°C were treated for 4-5 generations with rifampicin and cephalexin to inhibit replication initiation and cell division, while allowing the completion of the ongoing replication cycles. The cells were then fixed in 70% ethanol, stained with Hoechst 33258 for 30 min, and analyzed by flow cytometry. The x- and y-axes indicate chromosome equivalents per cell and the number of measured cells, respectively. Each measurement includes 10,000 cells. A.O. = average number of origins per cell, as described in a previous study (Liu et al., 2014). The doubling time and genotype of cells are also shown.

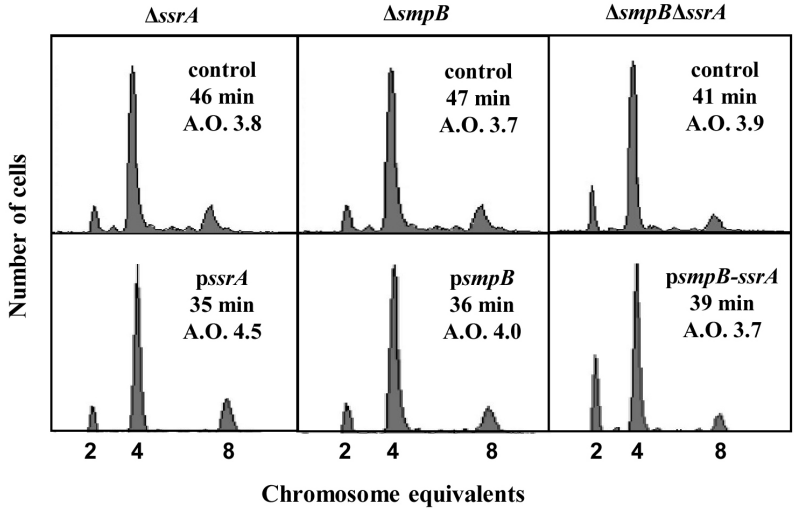

Figure 2. Ectopic expression of tmRNA or SmpB reverses the delayed initiation and slow growth of Δ*ssrA* and Δ*smpB* mutant. Δ*ssrA*, Δ*smpB*, and Δ*ssrA*Δ*smpB* mutant cells, carrying the pUHE21-*ssrA*, pUHE21-*smpB*, and pUHE21-*smpB*-*ssrA* plasmids, in the exponential phase of growth in ABTGcasa medium at 37°C were treated for 4-5 generations with rifampicin and cephalexin, collected, fixed in 70% ethanol, stained with Hoechst 33258 for 30 min, and analyzed by flow cytometry. The x- and y-axes indicate the chromosome equivalents per cell and the number of measured cells, respectively. Each measurement includes 10,000 cells. A.O. = average number of origins per cell. The doubling time of the cells is also shown.

Lack of trans-translation delays DnaA expression

The initiator protein DnaA, encoded by the *dnaA* gene, is a determinant for replication initiation (Braun et al., 1985; Løbner-Olesen et al., 1989); *dnaA* transcription is delayed in the *C. crescentus* Δ*ssrA* mutant. Therefore, it is reasonable to question whether the expression of DnaA is delayed in the absence of trans-translation during the *E. coli* cell cycle progression. In order to address this question, *dnaC*2 mutant cells, which can be synchronized during the B-period (before initiation of chromosome replication) via a temperature shift (Carl, 1970; Withers and Bernander, 1998), were utilized. BW25113*dnaC*2 cells, or its derivative cells expressing the Δ*ssrA*, Δ*smpB*, or Δ*smpB*Δ*ssrA* mutations, in the exponential phase of growth were shifted from a permissive temperature (30°C) to a non-permissive temperature (42°C) condition (Figure 3A). This stops a new round of initiation; however, the ongoing rounds of DNA synthesis and cell division continue, resulting in a single replicated chromosome being expressed in all cells after 120 min at the non-permissive temperature. Shifting the cell culture back to the permissive temperature leads to all cells initiating replication in a synchronous manner. Cell samples were taken at various time points (Figure 3) after shifting back to the permissive temperature; subsequently, the total protein content, cell count, and DnaA concentration was determined in a fixed culture volume. Protein production (quantified by amount per cell) in the wild-type cells peaked after culturing for 90 min in the permissive temperature, and decreased immediately afterward. Δ*ssrA* mutant cells showed a similar fluctuation in protein production (Figure 3B). Protein production (total amount per cell) in the Δ*smpB* cells followed a similar pattern as the wild-type cells (peak at the 90-min time point); however, these cells produced proteins at the peak level for approximately 60 min before downregulating the protein production (Figure 3B). On the other hand, Δ*smpB*Δ*ssrA* mutant cells showed a further delay in the total protein synthesis per cell (Figure 3B). These results suggested that the time-frame for general protein synthesis requires trans-translation.

DnaA protein content per cell was monitored by western blotting; in this process, we detected the DnaA concentration in the total protein samples taken at different time points during cell cycle progression in order to calculate the relative DnaA content per cell. The DnaA content per cell in the wild-type and Δ*ssrA* cells peaked after 90 min at the permissive temperature, decreasing immediately afterward, in agreement with the fluctuation of total protein content during the cell cycle (Figure 3C). However, the peak DnaA content per cell in the Δ*smpB* and Δ*smpB*Δ*ssrA* mutant cells was observed after 120 and 150 min in the permissive temperature, respectively (Figure 3C). These results indicated that DnaA accumulation could be delayed in the absence of trans-translation.

DISCUSSION

The initiation of chromosome replication in the Δ*ssrA*, Δ*smpB* and Δ*smpB*Δ*ssrA* mutant cells was delayed relative to the wild-type cells (Figure 1); moreover, this delay was reversed by the ectopic expression of the *ssrA* or *smpB* gene (Figure 2). Surprisingly, tmRNA and SmpB co-expression (due to the insertion of the p*smpB-ssrA* plasmid) did not reverse the delay in replication initiation in the Δ*smpB*Δ*ssrA* cells. This could be attributed to the lack of functionality or incorrect expression of the co-expressed tmRNA or/and SmpB.

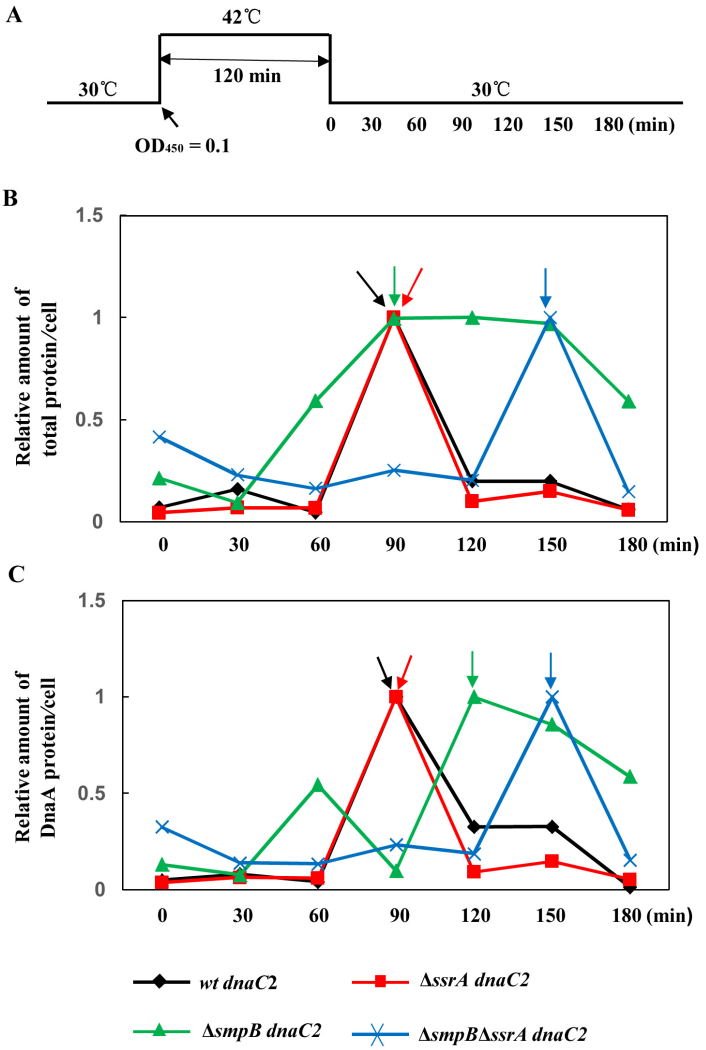

Figure 3. Protein synthesis and accumulation of DnaA per cell are delayed in the absence of trans-translation. **A.** Cells carrying the *dnaC*2 allele in the exponential phase of growth in the ABTGcasa medium were shifted from the permissive temperature (30°C) to the non-permissive temperature (42°C); at this temperature, the DnaC2 mutant was inactivated, thereby inhibiting the initiation of replication at *oriC*. The cells were synchronized after 120 min at 42°C. The synchronized cells were shifted to the permissive temperature and sampled at the indicated time intervals. **B.** Total protein content per cell was determined as described in Material and Methods. The x- and y-axes indicate time points at which the cell samples were taken and the total protein content at the indicated time points relative to the highest protein content per cell, respectively. The arrows indicate the peaks in accumulation of total protein per cell. The values are the average of three experiments. **C.** DnaA protein content per cell was determined as described in Material and Methods. The DnaA concentration was determined by western blotting, and the amount of protein cell was calculated. The relative DnaA content per cell was obtained by comparing the DnaA content per cell at each time point to the highest amount of DnaA per cell. The values are the average of three experiments. The x- and y-axes indicate the sampling times and the relative amount of DnaA per cell. The arrows indicate the peaks in DnaA accumulation. Black, red, green, and blue are representative of *wtdnaC*2, Δ*ssrAdnaC*2, Δ*smpBdnaC*2, and Δ*smpB*Δ*ssrAdnaC*2, respectively.

However, the growth rate of the Δ*ssrA*, Δ*smpB* and Δ*smpB*Δ*ssrA* mutant cells was slower than that of the wild-type cells. Moreover, total and DnaA protein accumulation in individual Δ*smpB*Δ*ssrA* mutant cells was delayed with respect to that in the wild-type cells (Figure 3). Therefore, we concluded that the trans-translation pathway ensures timely DnaA protein synthesis, which assists in the timely progression of the *E. coli* cell cycle. These conclusions are in agreement with those of a previous report, wherein trans-translation was shown to ensure timely *dnaA* transcription, and consequent initiation of DNA replication in *C. crescentus* (Cheng and Keiler, 2009).

The trans-translation pathway is conserved in all bacteria, with the reactions occurring over 13,000 times per cell division in *E. coli* cells in the exponential phase of growth (Moore and Sauer, 2005). Clearly, the trans-translation pathway plays a major role in various aspects of the cell cycle. Interestingly, eukaryotic cells express a similar pathway in the form of the ribosome quality control complex, whose Rqc2p and 60S ribosomal subunits mediate the mRNA-independent elongation of nascent chains (Shen et al., 2015).

Conflicts of interest

The authors declare no conflict of interest.

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