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Polyphosphate induces the proteolysis of ADP-bound fraction of initiator to inhibit DNA replication initiation upon stress in *Escherichia coli*

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ABSTRACT

The decision whether to replicate DNA is crucial for cell survival, not only to proliferate in favorable conditions, but also to adopt to environmental changes. When a bacteria encounters stress, e.g. starvation, it launches the stringent response, to arrest cell proliferation and to promote survival. During the stringent response a vast amount of polymer composed of phosphate residues, i.e. inorganic polyphosphate (PolyP) is synthesized from ATP. Despite extensive research on PolyP, we still lack the full understanding of the PolyP role during stress. It is also elusive what is the mechanism of DNA replication initiation arrest in starved *Escherichia coli* cells. Here, we show that during stringent response PolyP activates Lon protease to degrade selectively the replication initiaton protein DnaA bound to ADP, but not ATP. In contrast to DnaA-ADP, the DnaA-ATP does not interact with PolyP, but binds to *dnaA* promoter to block *dnaA* transcription. The systems controlling the ratio of nucleotide states of DnaA continue to convert DnaA-ATP to DnaA-ADP, which is proteolysed by Lon, thereby resulting in the DNA replication initiation arrest. The uncovered regulatory mechanism interlocks the PolyP-dependent protease activation with the ATP/ADP cycle of dual-functioning protein essential for bacterial cell proliferation.

INTRODUCTION

Once a bacterial cell encounters stressful conditions, such as amino acid starvation, it switches from proliferating to survival mode, by launching the stringent response (1). The stringent response is a pleiotropic mechanism that alters the transcription profile and induces protein proteolysis to provide cell survival. The hallmark of stringent response is the accumulation of guanosine penta- or tetraphosphate [(p)ppGpp] molecule (2). Apart from rearranging gene transcription, (p)ppGpp inhibits the activity of exopolyphosphatase (PPX), thereby leading to the synthesis of long chains of inorganic polyphosphate (PolyP) by polyphosphate kinase (PPK) from ATP (3–5). The PolyP, which forms granules in cells (6), is abundant in all kingdoms of life and was proposed to participate in multiple processes (7). In *Escherichia coli*, PolyP directly interacts with highly conserved Lon protease, thus altering Lon substrate specificity to degrade ribosomal proteins, which affects protein translation (8). What is the influence of PolyP on other cellular processes, like DNA replication, during stress in *E. coli* is still elusive.

Although the control of DNA replication has been widely studied, how replication is arrested when a cell encounters adverse environmental changes is still not fully elucidated. Various mechanisms for DNA replication control were proposed to date. In *Caulobacter crescentus* it was shown that replication initiation protein (DnaA) is proteolyzed by ClpXP (9) and Lon during heat-shock and nutritional downshift (10,11). In *C. crescentus*, the accumulated (p)ppGpp leads to a change in DNA topology, which prevents replication initiation (12). In *E. coli*, the increased ppGpp level was also shown to be correlated with the inhibition of replication initiation (13). The binding of (p)ppGpp to primase decreases primase activity in *E. coli* and *Bacillus subtilis* (14,15). Yet, in *B. subtilis* DNA replication is arrested downstream from the *origin*, while in *E. coli* at the initiation stage (16). Notably, following the stringent response induction in *E. coli,* all the ongoing replication rounds are continued until completion (17). The immediate arrest of the initiation of DNA replication in *E. coli* was ascribed to be the result of the inhibition of *dnaA* transcription (18). However, neither the decreased activity of primase nor the inhibition of *de novo* DnaA synthesis, explains how DNA replication initiation is rapidly arrested in *E. coli*. We hypothesized thatPolyP-induced proteolysis by Lon contributes to the regulation of DNA replication during stress. Here, with the use of *in vivo* and *in vitro* exper-

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iments we uncovered regulatory system that interlocks inducible proteolysis and the regulation of DNA replication initiation during stress in *E. coli*.

MATERIALS AND METHODS

Oligonucleotides, plasmids and strains

Sequences of oligonucleotides used in this study are listed in Supplementary Table S1. Mutation in pBAD-*dnaA* (used for overproduction of DnaA R334A mutant) were introduced using QuickChange Site Directed Mutagenesis Protocol (Stratagene, USA) with the use of primers: R334A_F and R334A_R. Plasmid used for 6His-Hda overproduction (pET-*hda*) was created as follows: *hda* gene was PCR amplified by using primers Hda_F and Hda_R, which was cloned into pET15b (Novagen) by using NdeI and BamHI. The poriC plasmid contains *E. coli* 245bp-minimal *oriC* sequence cloned into pSP6 (19) by using KpnI and XbaI. Supercoiled form of poriC (also described as cccDNA) was obtained with the use of Plasmid Midi AX kit (A&A Biotechnology). The ϕx174 nicked circular DNA for *in vitro* RIDA Complex Assembly was purchased from New England Biolabs, UK.

Escherichia coli C600 wt strain and its *lon*-depleted derivative (described as *E. coli* C600 *Δlon*) (20), *E. coli* MG1655 wt strain and its derivatives (tj. MG1655 *Δppk* (21) and MG1655 *dnaC2* (22) and *E. coli* WM539 (23) were used for *in vivo* experiments. *Escherichia coli* BL21(DE3) (24) was used for overproduction of all protein, except for DnaA-6His and its mutant R334A, which were overproduced in *E. coli* JP313 (25).

Proteins, antibodies and reagents

Escherichia coli proteins were purified as described previously: subunits of DNA Polymerase III (i.e. α, ε, θ, τ, γ, δ, δ', χ and ψ) (26), β-clamp (26), DnaB-6His (27), DnaC (28), gyrase, HU, DnaG (26); Lon (20). *Escherichia coli* DnaA-6His and its R334A mutant were purified as described previously (27,28). Briefly, DnaA-6His or DnaA R334A were overproduced in *E. coli* JP313 strain with pBAD24 containing genes encoding either DnaA-6His or its R334A mutant. The cells were sonicated in sonication buffer [50 mM phosphate buffer (pH 8), 300 mM. NaCl, 2 mM imidazole, 0.1% (v/v) Triton X-100] Next, the supernatant after centrifugation was applied onto 1 ml of Nickel-nitrilotriacetic acid (Ni-NTA) resin. The resin was washed with sonication buffer containing 20 mM imidazole, followed by wash with storage buffer [500 mM potassium L-glutamate, 45 mM HEPES/KOH (pH 7.6), 10 mM Magnesium acetate, 20% sucrose, 0.1% (v/v) Triton X-100]. The DnaA-6His was eluted with the use of storage buffer containing 250 mM imidazole (0.5 ml fractions collected) and dialyzed against storage buffer without Triton X-100. *Escherichia coli* 6His-Hda protein was purified as follows: the *E. coli* BL21(DE3)/pET-*hda* was cultured in LB medium supplemented with ampicillin at 37°C. Once cells reached OD_{600} of 0.4, the overproduction of 6His-Hda protein was induced by the addition of IPTG (0.5 mM) and further cultured for 2 h. The next steps were as for DnaA-6His purification (26). Commercially available proteins were single-stranded DNA binding protein (SSB) (Promega), Bovine Serum Albumin (BSA) fraction V (Sigma-Aldrich) and creatine kinase (Roche Diagnostics). The subcomplexes of the holoenzyme of DNA Polymerase III (i.e. core and clamp loader complex) were reconstituted as described previously (26). The concentration and purity of proteins was estimated by resolving the protein of interest and BSA of known concentration by SDS-PAGE, followed by comparative densitometric analysis with the use of ImageJ or ImageLab softwares. The protein concentration was additionally verified by NanoDrop measurements at 280 nm. The proteins used in this study were highly pure (95% purity or higher). The molar concentration of proteins is shown for monomers. Immunodetection assays were performed with the use of polyclonal anti-DnaA antibodies (rabbit) and polyclonal anti-Lon antibodies (mouse), goat anti-mouse and anti-rabbit conjugated with Horseradish peroxide (HRP) secondary antibodies (BioRad). PolyP (biotinylated and non-biotinylated), synthetic long chain of ∼700 phosphate residues long (linear as stated by the producer), were purchased from Kerafast; Cardiolipin, Fluphenazine and creatine phosphate were from Sigma-Aldrich.

Western blotting

Cell lysates were resolved by 9% SDS-PAGE and the proteins were transferred to the nitrocellulose blotting membrane (0.2 μm) with the use of transfer buffer [48 mM Tris, 39 mM glycine, 20% (v/v) methanol, 1.3 mM SDS]. The membrane was blocked for 1 h in a solution of 5% dry non-fat milk dissolved in PBST (pH 7.4) [137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% (v/v) Tween-20], followed by the incubation with diluted in PBST anti-DnaA antibodies (1:10 000 dilution) or anti-Lon antibodies (1:5000 dilution) for 1 h. After washing with PBST (3 × 15 min) the membrane was incubated for 1 h with appropriate HRP-conjugated secondary antibodies at a dilution of 1:30 000 in PBST buffer. The membrane was washed with PBST (5 × 30 min) and briefly incubated with chemiluminescent HRP antibody detection reagent WesternBright Quantum (Advansta) and developed by using the Chemidoc. Densitometric analysis was performed with the use of ImageLab or ImageJ softwares.

Determination of number of *E. coli* cells

Optical density. *Escherichia coli* cell culture (1 ml) was measured in spectrophotometer at OD_{600}. The number of *E. coli* cells was calculated by taking the estimate that 1 ml of cell culture at OD_{600} of 1 contains 8×10^8 cells.

Direct counting. *Escherichia coli* cell culture (5 μl) was put onto Petroff-Hauser chamber and the cells were observed under OLYMPUS BX51 microscope. The cells were counted from 25 random sectors (total number of sectors is 400, 50 μm square each). The calculated average number per sector was multiplied by 2×10^7, which gave the number of cells present in 1 ml of medium.

In vivo protein level analysis during stress

Overnight culture of *E. coli* was diluted 1:50 in 50 ml of LB medium and cultured at 30°C (150 RPM). Once cells reached OD_{600} of 0.6, either DL-serine hydroxamate (SHX) (at final concentration of 4 mM) was added; alternatively inductor was omitted to test DnaA level upon entry stationary phase. At particular time points, the cells (3.2×10^8 cells) were harvested and resuspended in 10 μl of 2× Laemmli buffer (29), followed by electrophoresis in 9% SDS-PAGE and western blotting. While testing DnaA stability in *E. coli* wt strains containing pBAD plasmid derivatives, 0.2% arabinose was added to induce plasmid-born DnaA expression. When testing DnaA stability in *E. coli* WM539 strains (containing hyper-unstable chromosome-derived DnaA I389N mutant) harbouring pBAD derivatives, the plasmid-born *dnaA* expression was induced at OD_{600} of 0.3 by the addition of 0.02% arabinose, followed by glucose addition at final concentration of 0.2% to inhibit *dnaA* expression and finally SHX addition (4 mM).

In vivo DNA replication assay

Overnight culture of *E. coli* cells was diluted 1:10 in LB medium and further incubated at 30°C (150 RPM). Once cells reached OD_{600} of 0.15, the DNA radiolabelling was initiated by the addition of 5 μCi/ ml tritium-labelled thymidine and continued until the end of the experiment. After 10 min, the stringent response was induced by adding SHX to the final concentration of 4 mM, when indicated. At particular time points the fractions were immediately collected and prepared as follows: 50 μl of cell culture was dropped on Whatmann paper, dried, incubated for 10 min with 10% TCA, followed by 10 min incubation with 5% TCA and wash with 96% ethanol. The samples (Whatmann papers) were dried, put into tubes, filled with 3 ml of scintillator liquid and the tritium-labelled thymidine incorporation was measured with the use of scintillator counter.

Chromatin immunoprecipitation (ChIP)

Escherichia coli wt strain was grown at 30°C (150 RPM) in 40 ml LB medium. At OD_{600} of 0.5, formaldehyde (at final concentration of 1%) was added to crosslink nucleoprotein complexes, followed by 20 min incubation at room temperature. The reaction was quenched by the addition of glycine to the final concentration of 500 mM. The cells were harvested by centrifugation (5 min; 4000 RCF; 4°C) and washed with 10 ml TBS buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl] and further with 1 ml of TBS buffer. The cells were suspended in 1 ml of ChIP-FA150 buffer [50 mM HEPES/KOH (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (v/v) sodium deoxycholate, 0.1% (w/v) SDS] supplemented with lysozyme (2 mg/ml) together with cOmplete Protease Inhibitor Cocktail (Roche) and incubated at 37°C for 30 min, followed by 10 min incubation on ice. Next, the lysate was sonicated on ice and centrifuged (5 min; 20 000 RCF; 4°C). The supernatant was diluted to the final volume of 2 ml with ChIP-FA150 buffer. The diluted supernatant was aliquoted to four portions and every portion was diluted to the final volume of 800 μl with ChIP-FA150 buffer. Protein A beads (12.5 μl, 50% slurry) (Protein A Sepharose 6MB, GE Healthcare) were equilibrated with TBS buffer and 0.8 μl of anti-DnaA antibodies were added, followed by 1.5 h incubation with rotation in a cold room. The immunoprecipitates were centrifuged for 1 min at 4,000 RCF, the supernatant was discarded and 750 μl of ChIP-FA150 buffer was added, followed by the incubation with rotation for 10 min in a cold room. The wash was further performed with the same volume of ChIP-FA150, ChIP-FA500 (ChIP-FA-150 containing 500 mM NaCl instead of 150 mM), ChIP-W [10 mM Tris/HCl (pH 8), 250 mM LiCl, 1 mM EDTA, 0.5% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate] and TE buffers [10 mM Tris/HCl (pH 8), 1 mM EDTA]. The beads were suspended in 100 μl of ChIP-E buffer [50 mM Tris/HCl (pH7.5), 10 mM EDTA, 1% (w/v) SDS], incubated at 65°C overnight, followed by the centrifugation (1 min; 4000 RCF; RT). The DNA was purified with the use of NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The eluted DNA sample was added to the PCR premix containing polymerase buffer (Marathon buffer, A&A Biotechnology), deoxynucleotides (dNTPs) and Polymerase Marathon (A&A Biotechnology). To the equal volumes of obtained PCR premix, primers were added (either dnaAp2_F and dnaAp2_R or terC_F and terC_R). The primers were used in excess. We tested the range of primers concentrations and ensured that they do not affect reaction efficiency. The PCR products were resolved in 1% agarose gel stained with ethidium bromide.

Synchronization of DNA replication in *E. coli* MG1655 *dnaC2* by temperature shift

The DNA replication in *E. coli* MG1655 *dnaC2* strain was synchronized similarly as described before (22). Briefly, the cell culture was grown at permissive temperature, i.e. 30°C (150 RPM) in LB medium until they reached OD_{600} of 0.05. After that, the cell culture was shifted to nonpermissive temperature (42°C) and cultured for 2 h. Next, the cultures were shifted back to permissive temperature. The synchronization of DNA replication was confirmed by flow cytometry.

Synchronization of DNA replication in *E. coli* C600 by SHX

Escherichia coli C600 wt cells were cultured at 30°C (150 RPM) in LB medium. Once cells reached OD_{600} of 0.2, SHX was added to the final concentration of 8 mM and the cells were further cultured for 1.5 h. Next, the cells were harvested by centrifugation (6000 RCF; 5 min) and resuspended in 50 ml of fresh LB. The synchronized cells were further used for the determination of the intracellular level of DnaA.

Flow cytometry

The flow cytometry experiments were performed similarly as previously described (12). Briefly, cells were fixed with the use of 95% ethanol, harvested and resuspended in sodium citrate containing RNase A, followed by incubation at 50°C for 5 h. The cells were stained with 0.5 μl/ml SYTOX green nucleic acid strain (Thermo Fisher) and analyzed by using Guava EasyCyte (Millipore) Flow Cytometer. 20 000 events were collected with the rate of ∼800 events per second.

Gel mobility shift assay (GMSA)

The PolyP (1.4 μM as polymer of 700 phosphate residues) was incubated with increasing concentration of DnaA, pre-heated for 20 min at 45°C DnaA, BSA proteins (0, 1.5, 3, 6 μM), or Lon (0, 0.75, 1.5, 3, 6 μM) in GMSA-P buffer [50 mM Tris/HCl (pH 7.6) and 10 mM Magnesium acetate] in 25 μl for 20 min at 37°C. Ficoll 4000 was added to the final concentration of 2.5%. The reaction mixtures were resolved in 1% agarose gel stained with PolyP-specific toluidine blue staining solution [0.5% (w/v) toluidine blue (Sigma-Aldrich) and 10% (v/v) glycerol], followed by incubation with destaining solution [25% (v/v) methanol and 10% (v/v) glycerol].

Bio-layer Interferometry (BLI) assays

BLI assays were performed with the use of BLItz instrument (ForteBIO, a division of PALL Life Sciences) at room temperature at 1500 RPM shaking. In all BLI assays the streptavidin-coated sensor tip was used (SA Dip and Read Biosensors), the buffer was PBS supplemented with 2% Tween-20 (PBST) and reaction mixture was 50 μl. For testing PolyP interactions, biotin-tagged PolyP (1.25 μM) was present in a test tube in PBST buffer during immobilization (300 s), then 60 s incubation with PBST alone was performed, followed by 1000 s association with DnaA (at concentrations as in figures) and 500 s of dissociation, where sensor tip was incubated with PBST buffer alone. For testing interaction with DnaA box R1, the 1 μM of biotinylated double-stranded DNA (hybridized R1_up with R1_bottom) in PBST was incubated with sensor tip for 100 s, then sensor tip was incubated with PBST alone, followed by association (50 s) and dissociation (60 s) as in figure. The results show only association and dissociations steps. The DnaA-ATP was obtained with the use of ATP-agarose resin similarly as previously described (30).

In vitro DNA replication assay reconstituted with purified proteins

The assay was performed essentially as described previously (26) with the use of poriC plasmid.

In vitro RIDA complex assembly

The RIDA complex was assembled similarly as described previously (31) with several modifications. The first reaction mixture containing β-clamp (25 pmol), the clamp loader (132 fmol) and ϕX174 open circular form (0.5 pmol) were incubated in buffer RIDA-β [20 mM Tris/HCl (pH 7.5), 10% (v/v) glycerol, 8 mM DTT, 0.01% (w/v) Brij-58, 8 mM Magnesium acetate, 20 mM potassium L-glutamate, 2 mM ATP] for 20 min at 32°C (final volume: 25 μl). The nucleoprotein complex was isolated by molecular filtration (0.63 ml MicroSpin S-400 HR column, GE Healthcare) in void volume. The second reaction mixture containing Hda (0.31 pmol) and 2 mM ADP was preincubated for 10 min at 32°C. The third reaction mixture contained DnaA (0.5 pmol), ATP (2 mM), cardiolipin (14 μg/ml), which was incubated for 10 min at 37°C; followed by the incubation with fluphenazine (0.15 mM) for 10 min at 37°C. The reaction mixtures were mixed together, incubated for 10 min and the nucleoprotein complex was isolated by molecular filtration as previously. The RIDA complex assembly was resolved in 12.5% SDS-PAGE, followed by silver staining.

In vitro reconstituted proteolysis assay

Post-RIDA DnaA isolated by *in vitro* RIDA complex assembly was incubated with Lon protease (350 nM) in the presence or absence of either supercoiled poriC (1 nM) or PolyP (1 μM as a 700 long polymer) in RIDA-A buffer [20 mM Tris/HCl (pH 7.5), 10% (v/v) glycerol, 8 mM DTT, 1 mM EDTA, 8 mM magnesium acetate, 80 mM potassium L-glutamate] for 2 h at 37°C (final reaction volume 12.5 μl). The reaction mixture was resolved in 9% SDS-PAGE, followed by DnaA detection by western blotting with the use of anti-DnaA antibodies.

RESULTS AND DISCUSSION

Since Lon protease is known to degrade ribosomal proteins in stringent response (8), we hypothesized that Lon contributes to the regulation of DNA replication during stress in *E. coli*. To determine if Lon is essential for the replication initiation arrest during stress, we performed *in vivo* DNA replication assay in *E. coli* C600 wt and *lon*-deficient *E. coli* strains (Figure 1A). We cultured *E. coli* and added the tritium-labelled thymidine to radiolabel newly synthesized DNA (Figure 1A, time point 0) and then induced stringent response by the addition of SHX (Figure 1A, time point 10). The analysis revealed that in *E. coli* C600 wt strain DNA replication was arrested during stress (Figure 1A, time points 20 and 30) as expected (13,32). However, in case of *lon*-depleted strain, DNA synthesis continued over time, which demonstrates that Lon is essential for replication initiation arrest during stress. As a control, DNA replication was tested in *E. coli* wt strain without SHX addition (Supplementary Figure S1). We observed lower (∼12%) increase in tritium-labelled thymidine incorporation after SHX addition in *E. coli* C600 wt, when compared to Tehranchi and others (∼25% increase) (32), which may be explained by prolonged incubation with tritium-labeled thymidine in our experiment that may be inhibitory to DNA replication (33).

Since in *C. crescentus* Lon degrades DnaA during stress, i.e. during heat shock and nutrient depletion (10,11), we asked whether the level of DnaA changes during stringent response in *E. coli*. To test this, we investigated DnaA level in *E. coli* in conditions during which stringent response is launched, i.e. amino acid starvation and stationary phase (34) (Figure 1B). We collected the samples of equal number of cells as determined by direct counting with the use of Petroff-Hauser chamber (Supplementary Table S2) at indicated time points and analyzed the level of DnaA by western blotting. We observed a stepwise decrease in DnaA level upon stress induction. The cell growth arrest in case of amino acid starvation after 0.5 h indicates that the starvation was successfully induced (Figure 1B, left bottom). We concluded that the level of DnaA decreases during stringent response regardless of the kind of stress induced. It was previously shown that under favorable growth conditions and when protein synthesis is inhibited DnaA level is stable (35). Under stringent response *dnaA* transcription was

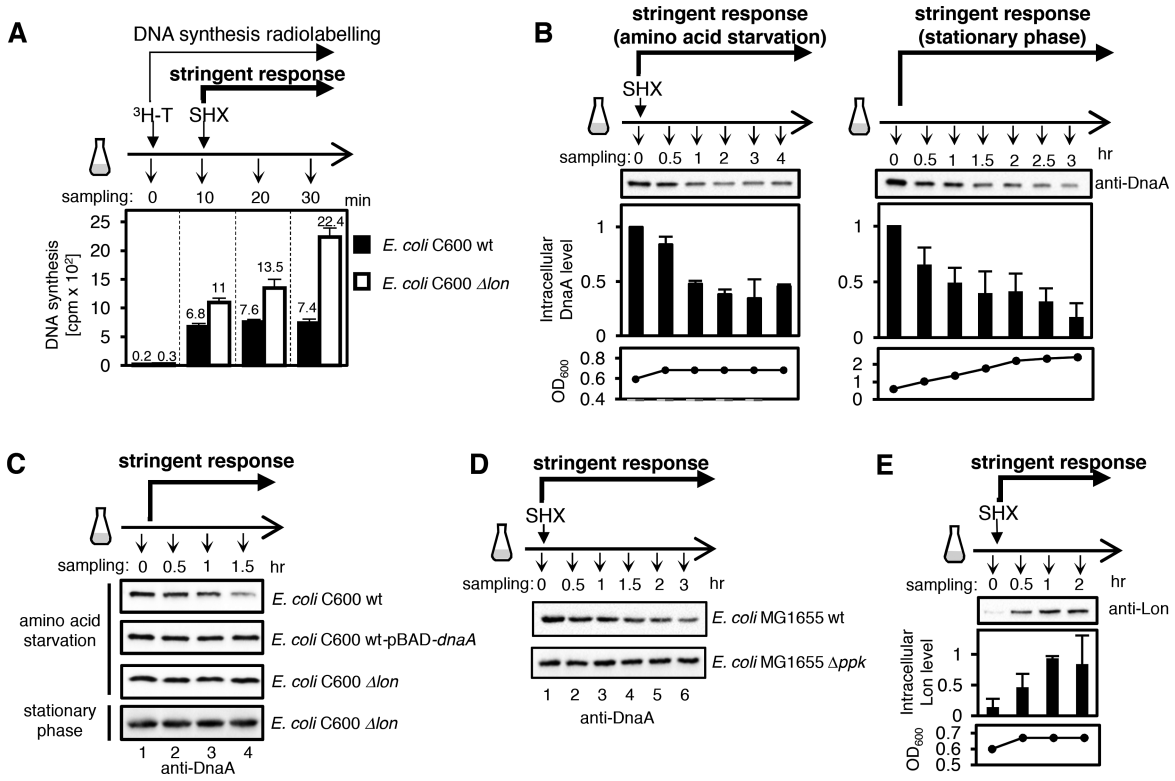

Figure 1. Stringent response induces Lon-dependent DnaA degradation and DNA replication initiation arrest. (**A**) The *in vivo* DNA replication was tested by measuring the incorporation of tritium-labelled thymidine (3H-T) before and after stress induction in *E. coli* C600 wt and *E. coli* C600 Δ*lon* (see Materials and Methods for details). Results are means ± SD ($n = 3$). The mean values are shown above the bars. The control for *E. coli* C600 wt strain when no stress was induced is presented in Supplementary Figure S1. (**B**) The DnaA level was analysed in *E. coli* during stringent response, i.e. amino acid starvation (left) and stationary phase (middle). Equal number of cells (see Supplementary Table S2) were collected at indicated time points and analysed by Western blotting (top). Bar charts show densitometric analysis of Western blots. Results are means ± SD ($n = 3$). The representative OD600 measurement are shown (bottom). (**C**) *In vivo* DnaA level was tested during stringent response (i.e. amino acid starvation and stationary phase) in *E. coli* strains as for panel A. The experiment was performed similarly as described for panel B. (**D**) DnaA level was analysed during SHX-induced stringent response in *E. coli* MG1655 wt and *E. coli* MG1655 Δ*ppk* as described in panel B. (**E**) The level of Lon was tested similarly as for panel B, but anti-Lon antibodies were used. The western blots (top) were analysed by densitometry (bar charts). The Lon level represents means ± SD ($n = 3$). The representative OD_{600} measurements are shown (bottom).

demonstrated to be inhibited (18). Therefore, we concluded that the decrease in DnaA level during stringent response, observed by us, is due to DnaA proteolysis. Since we do not observed DNA replication arrest in *lon*-depleted strain (Figure 1A) we assumed that Lon protease could be responsible for DnaA degradation during stringent response in *E. coli*. To determine whether it is the Lon protease that is responsible for DnaA degradation during stress, we measured DnaA level in the *E. coli lon*-depleted strain during stringent response (Figure 1C). We observed that, in contrast to *E. coli* wt strain, DnaA was stable during amino acid starvation and stationary phase when cells lacked Lon protease (Figure 1C). Moreover, the proteolysis of DnaA in *E. coli* wt strain carrying pBAD-*dnaA* plasmid was masked (Figure 1C). Together, we concluded that Lon protease degrades DnaA, thereby inhibiting replication initiation during stringent response. Because Lon degrades ribosomal proteins during stress only in the presence of PolyP (8), we asked whether the depletion of enzyme synthetizing PolyP (PPK) would result in DnaA stabilization during stress. Indeed, we observed that in the absence of PolyP the level of DnaA remained at constant level after stress induction (Figure 1D). Notably, we also observed the decrease in DnaA level during stringent response in another *E. coli* wild-type strain, i.e. *E. coli* MG1655 wt. Since the level of Lon protease increases during heat-shock (36), we examined whether it also changes during stringent response (Figure 1E). We observed an increase in Lon level under applied conditions, which implies that during stress the changing level of Lon and DnaA contributes to the control of replication initiation during stress.

Although in our experiments the decrease in DnaA level during stress is evident, a fraction of DnaA molecules is still remaining (Figure 1B). Hence, we examined DnaA level during prolonged stationary phase (Supplementary Figure S2) and we still did not observe the complete clearance of DnaA, which may be due to either continuous DnaA synthesis or because a pool of DnaA molecules is somehow resistant from degradation by Lon. Presumably, the proteolysis-resistant DnaA molecules perform an important role during stress survival. Apart from initiating DNA replication, DnaA, as a dual-functioning protein, regulates the transcription of various genes in bacteria (37). Knowing that DnaA autoregulates its own transcription in a nutrient-rich environment and that *dnaA* promoter (*dnaAp2*) is stringently controlled in *E. coli* (18,38), we speculated that DnaA

binds its promoter during stringent response to block *dnaA* expression. To test this hypothesis, we applied chromatin immunoprecipitation (ChIP) technique to pull down nucleoprotein complexes formed during stringent response with the use of anti-DnaA antibodies (Supplementary Figure S3). Indeed, during stress we observed DnaA interacting with *dnaAp2* promoter region (Supplementary Figure S3, i, lanes 2–4), thus implying that it is the DnaA that autoregulates its transcription during stringent response. Notably, DnaA occupied *dnaAp2* sequence throughout the stress duration, thereby suggesting that DnaA is protected from proteolysis when bound to its promoter region. We expect that also other promotors are occupied by DnaA in such conditions. Indeed, DnaA was shown to stimulate the expression of gene encoding DNA repair Polymerase I during stationary phase (39). The binding of initiator proteins to promoters was previously shown to regulate various gene transcription not only in *E. coli,* but also in other organisms like *C. crescentus* and *Saccharomyces cerevisiae* (37,40,41) and it remains to be tested which are inhibited or activated during stringent response. Furthermore, it was previously shown that it is the DnaA bound to ATP, not ADP, that preferentially represses *dnaA* promoter under favourable growth conditions (38).

Since Kurokawa and Katayama observed the accumulation of DnaA–ADP as the replication forks progress (42), we asked if the time at which stress is induced after initiation of DNA replication affects the DnaA degradation. To answer this question, we synchronized and restarted the DNA replication initiation in *E. coli* MG1655 *dnaC2* (as confirmed by Flow Cytometry), followed by stringent response induction after 10, 20, 30, 40 and 50 min and measured DnaA level (Figure 2). The *E. coli* MG1655 *dnaC2* is a temperature-sensitive strain, which is commonly used for synchronization of DNA replication initiation (22). When stress was induced shortly after the DNA replication initiation synchronization DnaA proteolysis was inefficient (see timepoint T10). However, with the progression of DNA replication (as confirmed by Flow Cytometry) more and more of DnaA molecules were proteolyzed. We observed the most efficient DnaA degradation at timepoint T30, but after 10 min (timepoint T40) the degradation was less efficient (compare also bars below Western blots). This may be explained by new synthesis of DnaA-ATP between timepoints T30 and T40. In experiments published by Kurokawa *et al.* (42) temporal increase in the level of DnaA-ATP was observed. When we synchronized the *E. coli* C600 wt strain with the use of SHX (Supplementary Figure S4A) more efficient DnaA degradation was also observed with the progression of DNA replication. It was previously demonstrated that the DnaA-ATP/DnaA-ADP ratio varies before and after replication initiation (42). At the preinitiation stage ∼80% of DnaA molecules are bound to ATP. Following the initiation onset the ratio of DnaA-ATP to DnaA-ADP shifts gradually, eventually resulting in 20% of DnaA in ATP form, which is mainly caused by Regulatory Inactivation of DnaA (RIDA), which hydrolyses DnaA-ATP to DnaA-ADP during ongoing DNA synthesis (42). In our experiment, we observe that the later the stress was induced the more DnaA molecules were degraded, which suggest that it is the DnaA-ADP, but not DnaA-ATP, that is de-

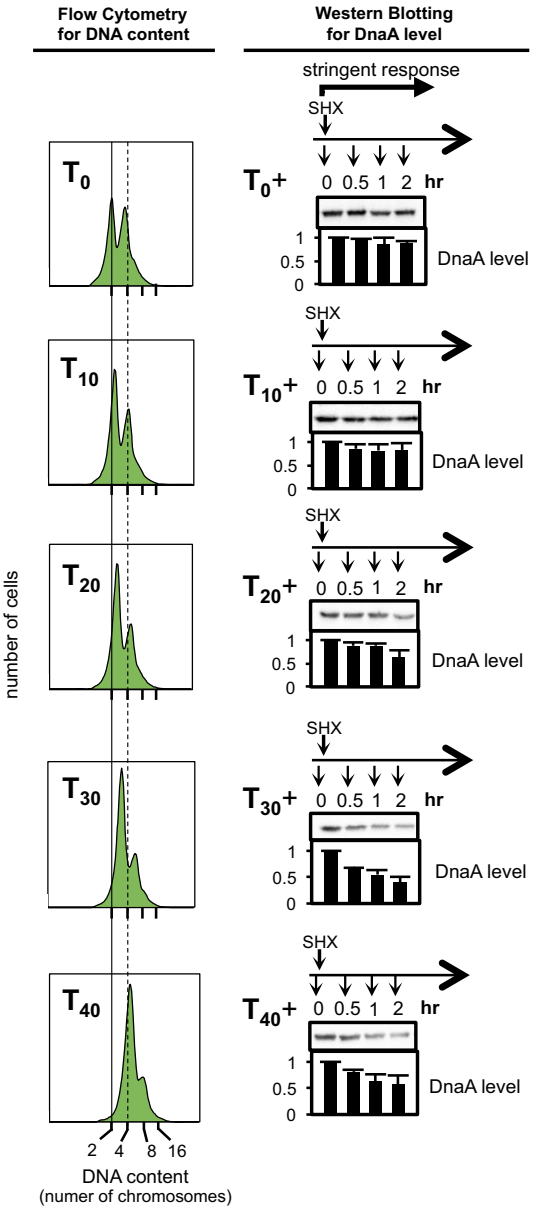

Figure 2. With the progression of DNA replication the stress-induced proteolysis of DnaA is more efficient. Analysis of DnaA intracellular level after stringent response induction in synchronized *E. coli* cells. DNA replication initiation was synchronized in *E. coli* MG1655 *dnaC2* cells by incubation at 42°C for 2 h, followed by immediate transfer to 30°C (T_0). After the synchronization of DNA replication (T_0), the stringent response was induced at indicated time points (T_0–T_{40}). The DnaA level was measured in stressed cells for 2 h. The DnaA level was determined by western blotting and analysed by densitometry (bar charts) (right). The results are means ± SD ($n = 3$). The synchronization of replication initiation was confirmed by flow cytometry, which measured DNA content at indicated time points (left). The flow cytometry profiles of DNA content in unsynchronized cells n are shown in Supplementary Figure S4C (top). To interfere the chromosome number equivalents, cells were grown in 'run out' conditions with the use of rifampicin and cefalexin (see Supplementary Figure S4C, bottom) (56). The solid and dashed lines indicate the DNA content that equal two and four chromosomes per a cell respectively. See Materials and Methods for details.

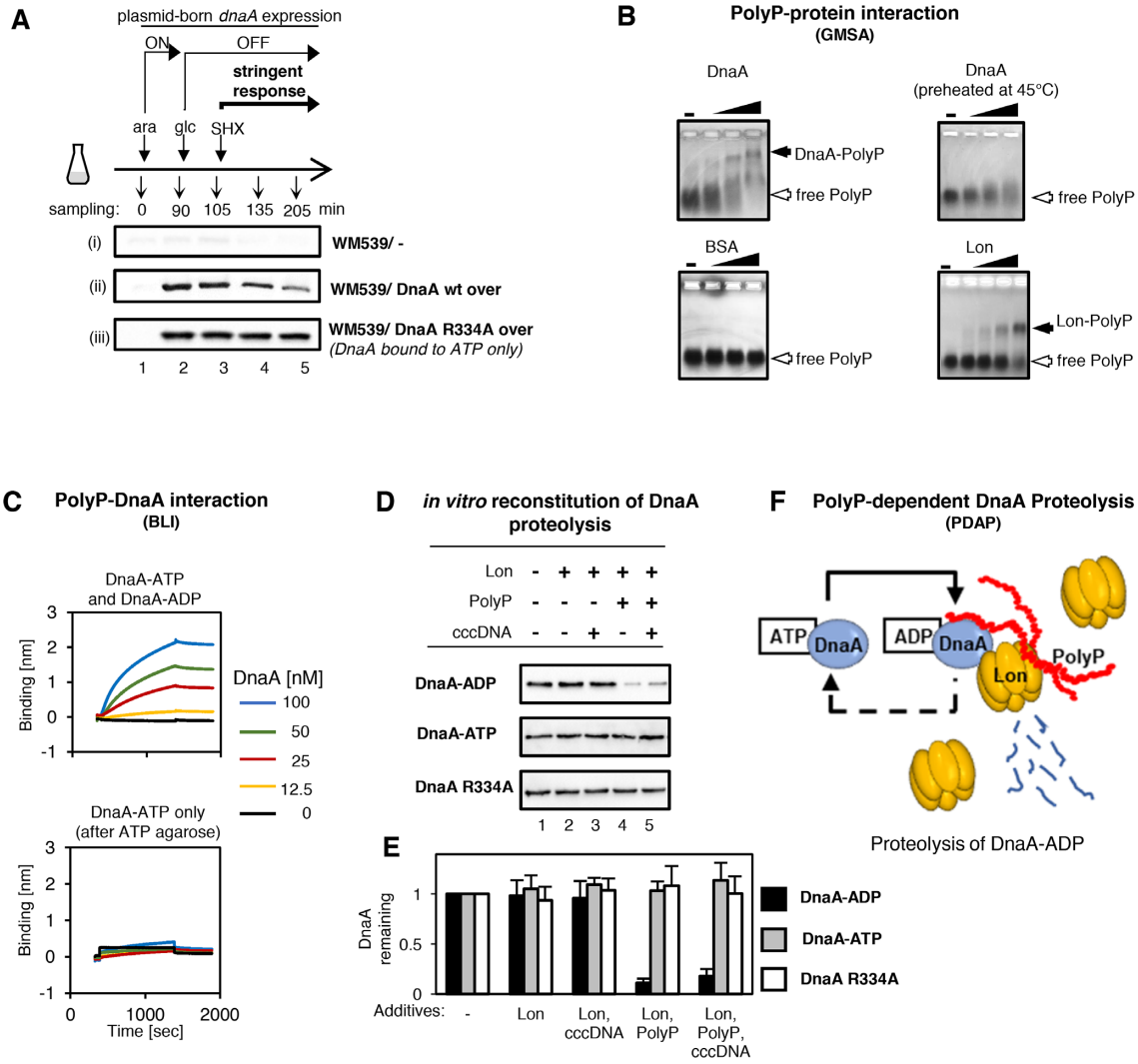

Figure 3. DnaA–ADP is selectively degraded by Lon in the PolyP-dependent manner. (**A**) DnaA mutant predominantly bound to ATP (DnaA R334A) is resistant to proteolysis during stringent response. As a genetic background *E. coli* WM539 strain containing hyper-unstable DnaA I389N mutant was used (i). The stability of plasmid borne DnaA wt (ii) and DnaA R334A mutant (iii) were tested during stringent response. Expression of genes encoding DnaA variants was induced by arabinose (ara), followed by glucose (glc) addition to inhibit *dnaA* expression. The DnaA level was determined before and after SHX addition as for Figure 1B (left). (**B**) DnaA and Lon interact with PolyP as examined by GMSA. DnaA (top left), DnaA preheated at 45°C (top right), BSA (bottom left) and Lon (bottom right) were added at increasing concentrations to PolyP, followed by 20 min incubation, agarose electrophoresis and toluidine blue staining (see Materials and Methods). White arrows indicate unbound PolyP, black arrows indicate protein–PolyP complex. For better visualization the images were modified by changing brightness and contrast, applied to whole images. (**C**) DnaA-ATP does not interact with PolyP, as opposed to a mix of DnaA–ATP and DnaA-ADP. Biotinilated PolyP was immobilized on a streptavidin coated sensor BLI tip and increasing concentrations of DnaA was added. The PolyP interaction was tested with DnaA before and after applying to ATP-agarose (top and bottom respectively). (**D**), DnaA-ADP, but not DnaA-ATP, is efficiently proteolysed by Lon in the presence of PolyP. Western blots of remaining DnaA-ADP, DnaA-ATP and DnaA R334A after *in vitro* reconstituted proteolysis in the presence or absence of supercoiled DNA (cccDNA) and PolyP. Results are means ± SD ($n = 3$). DnaA-ADP was obtained by using RIDA system; scheme of the RIDA assay and the control of RIDA assembly are presented in Supplementary Figure S6B and C. DnaA-ATP was obtained with the use of ATP-agarose, as for panel C, bottom. DnaA R334A mutant was found to predominantly bind ATP and was also tested in panel A (iii). (**E**) The densitometric quantification of results presented in panel D. (**F**) Our model of PolyP-induced DnaA proteolysis (PDAP). We propose that during stringent response in *E. coli* PolyP as well as Lon protease accumulate, Lon stimulated by PolyP proteolyses DnaA-ADP, but not DnaA-ATP to arrest replication initiation.

graded during stringent response. Hence, we anticipated that when DnaA is bound to ATP it is less prone to proteolysis. To test this hypothesis we established experiments in *E. coli* WM539 strain, encoding hyper-unstable DnaA mutant (DnaA I389N) (Figure 3A, i). In this strain, we examined the level of plasmid-born DnaA variants during stringent response. The level of DnaA wt protein decreased after stress induction (Figure 3A, ii). In contrast, the level of DnaA protein variant (DnaA R334A) which is permanently bound to ATP (43) did not change (Figure 3A, iii). The results indicate that when DnaA is bound to ATP, it is resistant to proteolysis during stringent response and implies that it may be the DnaA–ADP that is degraded by Lon. Consistently, corresponding DnaA R357A mutant (inactive in ATP-hydrolysis) was resistant to proteolysis in *C. crescentus* (44,45).

Since previously identified Lon substrates degraded during stringent response were shown to interact with PolyP (46), we aimed to determine whether DnaA interacts with PolyP. Therefore, we tested whether DnaA forms a complex with PolyP *in vitro* by using Gel Mobility Shift Assay (GMSA) (Figure 3B). We observed the retardation in bands migration when DnaA or Lon, but not BSA, was present in the reaction mixtures, which indicates DnaA-PolyP and Lon-PolyP complex formation. Almost no free PolyP was observed after incubation with high concentration of DnaA. Recently, it was proposed that PolyP acts as a molecular chaperone that binds partially unfolded proteins, thus preventing proteins from aggregation during stress (47,48). To exclude that PolyP recognizes unfolded DnaA, we preheated DnaA at 45°C and tested the interaction with PolyP (Figure 3B). We did not observed retarded band as it was observed when DnaA was not preheated (Figure 3B). We also confirmed that the DnaA preparation was active in helicase loading (Supplementary FigureS6A). Hence, it is the properly folded DnaA that specifically interacts with PolyP.

We hypothesized that the reason why DnaA–ATP is resistant to proteolysis is that it does not interact with PolyP. To test this we used Bio Layer Interferometry (BLI) technique in which we immobilized PolyP and investigated its interaction with DnaA. The increasing concentration of DnaA containing both ATP and ADP form, and DnaA after ATP–agarose, which contained ATP form only, were used in the experiment (Figure 3C). We observed that DnaA-ATP does not interact with PolyP. Notably, the DnaA–ATP preparation (obtained with the use of ATP-agarose) was active in DNA interaction (Supplementary Figure S5), helicase loading and DNA replication (Supplementary Figure S7).

We set out to reconstitute the reaction of DnaA proteolysis *in vitro.* Since Lon requires ATP for its activity and we expected that DnaA has to be in the ADP form, setting up such a reaction was challenging. In addition, the nucleotide exchange within DnaA is very slow and inefficient (49). Therefore, we obtained DnaA-ADP by using the most efficient physiological system for nucleotide exchange within DnaA, i.e. RIDA (50). We reconstituted the RIDA reaction *in vitro* to obtain post-RIDA DnaA bound to ADP exclusively (Supplementary Figure S6). Next, we added preincubated Lon-ATP and either supercoiled DNA (known to stimulate the proteolysis of plasmid initiator (20,51)) or PolyP and further incubated the reaction mixtures (Supplementary Figure S6B). The results revealed that post-RIDA DnaA is efficiently degraded by Lon in the presence of PolyP, but not in the presence of DNA. Also, DNA does not inhibit PolyP-induced DnaA proteolysis (Figure 3D and E). We also tested the proteolysis of DnaA-ATP (obtained by using ATP-agarose) and DnaA R334A mutant (predominantly bound to ATP). Neither the DnaA-ATP nor DnaA R334A mutant were proteolyzed by Lon in the presence of PolyP or DNA. Notably, obtained DnaA-ATP, but not DnaA-ADP, was active in its functional assays (Supplementary Figure S7). Collectively, the results of *in vivo* and *in vitro* experiments demonstrate that PolyP is essential for ADP-bound DnaA degradation by Lon. The uncovered regulatory mechanism we termed PolyP-induced DnaA Proteolysis (PDAP) (Figure 3F). It controls DNA replication initiation in *E. coli* in stressful environment when stringent response is induced. During favorable growth conditions the ratio of DnaA–ATP/DnaA–ADP changes (42). Once bacteria encounter stressful conditions PPX is inhibited, thereby resulting in the accumulation of PolyP (5). Lon protease level increases (Figure 1E) and PDAP is launched. PolyP binds Lon protease and shifts its substrate specificity, which results in DnaA proteolysis. Lon degrades DnaA, but only ADP form that interacts with PolyP. A fraction of DnaA, which does not bind PolyP, is resistant to proteolysis most likely binds *dnaA* promoter sequence and, as previously demonstrated (52), may cause the inhibition of *de novo* DnaA synthesis. Another fraction of remaining DnaA–ATP, is continuously converted to DnaA–ADP by RIDA and DDAH (*datA*-dependent DnaA–ATP hydrolysis) (53). In consequence, the level of DnaA–ATP decreases as well below the required threshold, leading to the arrest of DNA replication initiation.

Interestingly in *C. crescentus* PolyP was shown as modulator of cell cycle (52). Also, PolyP was shown to be able to change the localization of proteins (54) as well as it was demonstrated that PolyP can interact with positively charged amino acids which results in a liquid–liquid phase separation (54). Arthur Kornberg work (8) demonstrated that Lon affinity to PolyP is higher than to DNA. We hypothesize that upon stress induction in *E. coli* DnaA–ADP binds to the accumulated PolyP, together with Lon and other proteins leading to the formation of PolyP granules, where DnaA proteolysis takes place.

To ensure the arrest of DNA replication PDAP must not be the only system which controls DNA replication during stress. The redundancy of regulatory systems controlling vital cellular processes was previously reported (55). Indeed, it was proposed that the (p)ppGpp, accumulated during stringent response, affects the activity of primase in *Bacillus subtilis* and *E. coli* (14,15). Recently, it was also shown that ppGpp has an influence on RNA polymerase activity and therefore superhelicity of replication *origin* leading to DNA replication initiation inhibition (12). Because Gray demonstrated that ppGpp is not required for PolyP synthesis in *E. coli* (21) we propose that the regulation by ppGpp-induced decrease in the supercoiling of *oriC* (previously described (33)) and PDAP (described here in our work) are independent mechanisms responsible for controlling DNA replication initiation during stress in *E. coli.* Since Lon protease, PolyP and replication initiators whose activity is modulated by nucleotides are present in all domains of life, it is intriguing if PDAP-like regulations occur in other organisms.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Boutte,C.C. and Crosson,S. (2013) Bacterial lifestyle shapes stringent response activation. *Trends Microbiol.*, **21**, 174–180.
2. Magnusson,L.U., Farewell,A. and Nystrom,T. (2005) ppGpp: a global regulator in Escherichia coli. *Trends Microbiol.*, **13**, 236–242.
3. Kuroda,A., Murphy,H., Cashel,M. and Kornberg,A. (1997) Guanosine tetra- and pentaphosphate promote accumulation of inorganic polyphosphate in Escherichia coli. *J. Biol. Chem.*, **272**, 21240–21243.
4. Rao,N.N., Gomez-Garcia,M.R. and Kornberg,A. (2009) Inorganic polyphosphate: essential for growth and survival. *Annu. Rev. Biochem.*, **78**, 605–647.
5. Traxler,M.F., Summers,S.M., Nguyen,H.T., Zacharia,V.M., Hightower,G.A., Smith,J.T. and Conway,T. (2008) The global, ppGpp-mediated stringent response to amino acid starvation in Escherichia coli. *Mol. Microbiol.*, **68**, 1128–1148.
6. Racki,L.R., Tocheva,E.I., Dieterle,M.G., Sullivan,M.C., Jensen,G.J. and Newman,D.K. (2017) Polyphosphate granule biogenesis is temporally and functionally tied to cell cycle exit during starvation in Pseudomonas aeruginosa. *Proc. Natl Acad. Sci. U.S.A.*, **114**, E2440–E2449.
7. Kornberg,A. (1999) Inorganic polyphosphate: a molecule of many functions. *Prog. Mol. Subcell. Biol.*, **23**, 1–18.
8. Kuroda,A., Nomura,K., Ohtomo,R., Kato,J., Ikeda,T., Takiguchi,N., Ohtake,H. and Kornberg,A. (2001) Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in E. coli. *Science*, **293**, 705–708.
9. Gorbatyuk,B. and Marczynski,G.T. (2005) Regulated degradation of chromosome replication proteins DnaA and CtrA in Caulobacter crescentus. *Mol. Microbiol.*, **55**, 1233–1245.
10. Jonas,K., Liu,J., Chien,P. and Laub,M.T. (2013) Proteotoxic stress induces a Cell-Cycle arrest by stimulating lon to degrade the replication initiator DnaA. *Cell*, **154**, 623–636.
11. Leslie,D.J., Heinen,C., Schramm,F.D., Thuring,M., Aakre,C.D., Murray,S.M., Laub,M.T. and Jonas,K. (2015) Nutritional control of DNA replication initiation through the proteolysis and regulated translation of DnaA. *PLoS Genet.*, **11**, e1005342.
12. Kraemer,J.A., Sanderlin,A.G. and Laub,M.T. (2019) The stringent response inhibits DNA replication initiation in E. coli by modulating supercoiling of oriC. *MBio*, **10**, e01330-19.
13. Schreiber,G., Ron,E.Z. and Glaser,G. (1995) Ppgpp-mediated regulation of DNA-replication and cell-division in Escherichia-coli. *Curr. Microbiol.*, **30**, 27–32.
14. Maciag,M., Kochanowska,M., Lyzen,R., Wegrzyn,G. and Szalewska-Palasz,A. (2010) ppGpp inhibits the activity of Escherichia coli DnaG primase. *Plasmid*, **63**, 61–67.
15. Wang,J.D., Sanders,G.M. and Grossman,A.D. (2007) Nutritional control of elongation of DNA replication by (p)ppGpp. *Cell*, **128**, 865–875.
16. Levine,A., Vannier,F., Dehbi,M., Henckes,G. and Seror,S.J. (1991) The stringent response blocks DNA replication outside the ori region in Bacillus subtilis and at the origin in Escherichia coli. *J. Mol. Biol.*, **219**, 605–613.
17. Ferullo,D.J. and Lovett,S.T. (2008) The stringent response and cell cycle arrest in Escherichia coli. *PLos Genet.*, **4**, e1000300.
18. Chiaramello,A.E. and Zyskind,J.W. (1990) Coupling of DNA replication to growth rate in Escherichia coli: a possible role for guanosine tetraphosphate. *J. Bacteriol.*, **172**, 2013–2019.
19. Perri,S., Helinski,D.R. and Toukdarian,A. (1991) Interactions of Plasmid-Encoded replication initiation proteins with the origin of DNA-Replication in the broad host range plasmid Rk2. *J. Biol. Chem.*, **266**, 12536–12543.
20. Kubik,S., Wegrzyn,K., Pierechod,M. and Konieczny,I. (2012) Opposing effects of DNA on proteolysis of a replication initiator. *Nucleic Acids Res.*, **40**, 1148–1159.
21. Gray,M.J. (2019) Inorganic polyphosphate accumulation in Escherichia coli is regulated by DksA but not by (p)ppGpp. *J. Bacteriol.*, **201**, e00664-18.
22. Withers,H.L. and Bernander,R. (1998) Characterization of dnaC2 and dnaC28 mutants by flow cytometry. *J. Bacteriol.*, **180**, 1624–1631.
23. Zahn,G., Tippe-Schindler,R. and Messer,W. (1977) Trans-dominance of dnaA mutants in Escherichia coli. *Mol. Gen. Genet.*, **153**, 45–49.
24. Studier,F.W. and Moffatt,B.A. (1986) Use of Bacteriophage-T7 Rna-Polymerase to direct selective High-Level expression of cloned genes. *J. Mol. Biol.*, **189**, 113–130.
25. Economou,A., Pogliano,J.A., Beckwith,J., Oliver,D.B. and Wickner,W. (1995) SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. *Cell*, **83**, 1171–1181.
26. Wawrzycka,A., Gross,M., Wasaznik,A. and Konieczny,I. (2015) Plasmid replication initiator interactions with origin 13-mers and polymerase subunits contribute to strand-specific replisome assembly. *Proc. Natl. Acad. Sci. U.S.A.*, **112**, E4188–E4196.
27. Caspi,R., Pacek,M., Consiglieri,G., Helinski,D.R., Toukdarian,A. and Konieczny,I. (2001) A broad host range replicon with different requirements for replication initiation in three bacterial species. *EMBO J.*, **20**, 3262–3271.
28. Caspi,R., Helinski,D.R., Pacek,M. and Konieczny,I. (2000) Interactions of DnaA proteins from distantly related bacteria with the replication origin of the broad host range plasmid RK2. *J. Biol. Chem.*, **275**, 18454–18461.
29. Laemmli,U.K. (1970) Cleavage of structural proteins during assembly of head of Bacteriophage-T4. *Nature*, **227**, 680–685.
30. Lipinska,N., Zietkiewicz,S., Sobczak,A., Jurczyk,A., Potocki,W., Morawiec,E., Wawrzycka,A., Gumowski,K., Slusarz,M., Rodziewicz-Motowidlo,S. *et al.* (2013) Disruption of ionic interactions between the nucleotide binding domain 1 (NBD1) and middle (M) domain in Hsp100 disaggregase unleashes toxic hyperactivity and partial independence from Hsp70. *J. Biol. Chem.*, **288**, 2857–2869.
31. Su'etsugu,M., Nakamura,K., Keyamura,K., Kudo,Y. and Katayama,T. (2008) Hda monomerization by ADP binding promotes replicase clamp-mediated DnaA-ATP hydrolysis. *J. Biol. Chem.*, **283**, 36118–36131.
32. Tehranchi,A.K., Blankschien,M.D., Zhang,Y., Halliday,J.A., Srivatsan,A., Peng,J., Herman,C. and Wang,J.D. (2010) The transcription factor DksA prevents conflicts between DNA replication and transcription machinery. *Cell*, **141**, 595–605.
33. Hu,V.W., Black,G.E., Torres-Duarte,A. and Abramson,F.P. (2002) 3H-thymidine is a defective tool with which to measure rates of DNA synthesis. *FASEB J.*, **16**, 1456–1457.
34. Rao,N.N. and Kornberg,A. (1999) Inorganic polyphosphate regulates responses of Escherichia coli to nutritional stringencies, environmental stresses and survival in the stationary phase. *Prog. Mol. Subcell. Biol.*, **23**, 183–195.
35. Slominska,M., Wahl,A., Wegrzyn,G. and Skarstad,K. (2003) Degradation of mutant initiator protein DnaA204 by proteases ClpP, ClpQ and Lon is prevented when DNA is SeqA-free. *Biochem. J.*, **370**, 867–871.
36. Goff,S.A. and Goldberg,A.L. (1987) An increased content of protease La, the lon gene product, increases protein degradation and blocks growth in Escherichia coli. *J. Biol. Chem.*, **262**, 4508–4515.
37. Messer,W. and Weigel,C. (1997) DnaA initiator–also a transcription factor. *Mol. Microbiol.*, **24**, 1–6.
38. Speck,C., Weigel,C. and Messer,W. (1999) ATP- and ADP-dnaA protein, a molecular switch in gene regulation. *EMBO J.*, **18**, 6169–6176.

39. Quinones,A., Wandt,G., Kleinstauber,S. and Messer,W. (1997) DnaA protein stimulates polA gene expression in Escherichia coli. *Mol. Microbiol.*, **23**, 1193–1202.
40. Hottes,A.K., Shapiro,L. and McAdams,H.H. (2005) DnaA coordinates replication initiation and cell cycle transcription in Caulobacter crescentus. *Mol. Microbiol.*, **58**, 1340–1353.
41. Micklem,G., Rowley,A., Harwood,J., Nasmyth,K. and Diffley,J.F. (1993) Yeast origin recognition complex is involved in DNA replication and transcriptional silencing. *Nature*, **366**, 87–89.
42. Kurokawa,K., Nishida,S., Emoto,A., Sekimizu,K. and Katayama,T. (1999) Replication cycle-coordinated change of the adenine nucleotide-bound forms of DnaA protein in Escherichia coli. *EMBO J.*, **18**, 6642–6652.
43. Nishida,S., Fujimitsu,K., Sekimizu,K., Ohmura,T., Ueda,T. and Katayama,T. (2002) A nucleotide switch in the Escherichia coli DnaA protein initiates chromosomal replication: evidnece from a mutant DnaA protein defective in regulatory ATP hydrolysis in vitro and in vivo. *J. Biol. Chem.*, **277**, 14986–14995.
44. Wargachuk,R. and Marczynski,G.T. (2015) The caulobacter crescentus homolog of DnaA (HdaA) also regulates the proteolysis of the replication initiator protein DnaA. *J. Bacteriol.*, **197**, 3521–3532.
45. Liu,J., Francis,L.I., Jonas,K., Laub,M.T. and Chien,P. (2016) ClpAP is an auxiliary protease for DnaA degradation in Caulobacter crescentus. *Mol. Microbiol.*, **102**, 1075–1085.
46. Nomura,K., Kato,J., Takiguchi,N., Ohtake,H. and Kuroda,A. (2004) Effects of inorganic polyphosphate on the proteolytic and DNA-binding activities of Lon in Escherichia coli. *J. Biol. Chem.*, **279**, 34406–34410.
47. Gray,M.J., Wholey,W.Y., Wagner,N.O., Cremers,C.M., Mueller-Schickert,A., Hock,N.T., Krieger,A.G., Smith,E.M., Bender,R.A., Bardwell,J.C. *et al.* (2014) Polyphosphate is a primordial chaperone. *Mol. Cell*, **53**, 689–699.
48. Yoo,N.G., Dogra,S., Meinen,B.A., Tse,E., Haefliger,J., Southworth,D.R., Gray,M.J., Dahl,J.U. and Jakob,U. (2018) Polyphosphate stabilizes protein unfolding intermediates as soluble Amyloid-like oligomers. *J. Mol. Biol.*, **430**, 4195–4208.
49. Sekimizu,K., Bramhill,D. and Kornberg,A. (1987) ATP activates dnaA protein in initiating replication of plasmids bearing the origin of the E. coli chromosome. *Cell*, **50**, 259–265.
50. Katayama,T., Kubota,T., Kurokawa,K., Crooke,E. and Sekimizu,K. (1998) The initiator function of DnaA protein is negatively regulated by the sliding clamp of the E. coli chromosomal replicase. *Cell*, **94**, 61–71.
51. Karlowicz,A., Wegrzyn,K., Gross,M., Kaczynska,D., Ropelewska,M., Siemiatkowska,M., Bujnicki,J.M. and Konieczny,I. (2017) Defining the crucial domain and amino acid residues in bacterial Lon protease for DNA binding and processing of DNA-interacting substrates. *J. Biol. Chem.*, **292**, 7507–7518.
52. Chiaramello,A.E. and Zyskind,J.W. (1990) Coupling of DNA-Replication to Growth-Rate in Escherichia-Coli - a possible role for guanosine tetraphosphate. *J. Bacteriol.*, **172**, 2013–2019.
53. Kasho,K. and Katayama,T. (2013) DnaA binding locus datA promotes DnaA-ATP hydrolysis to enable cell cycle-coordinated replication initiation. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 936–941.
54. Zhao,J., Niu,W., Yao,J., Mohr,S., Marcotte,E.M. and Lambowitz,A.M. (2008) Group II intron protein localization and insertion sites are affected by polyphosphate. *PLoS Biol.*, **6**, e150.
55. Diffley,J.F. (2010) The many faces of redundancy in DNA replication control. *Cold Spring Harb. Symp. Quant. Biol.*, **75**, 135–142.
56. Ferullo,D.J., Cooper,D.L., Moore,H.R. and Lovett,S.T. (2009) Cell cycle synchronization of Escherichia coli using the stringent response, with fluorescence labeling assays for DNA content and replication. *Methods*, **48**, 8–13.