Supplemental Material

RpoS proteolysis is controlled directly by ATP levels in *Escherichia coli*

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Supplemental Materials and Methods

Bacterial strains

Metabolic mutant alleles from the Blattner collection (Kang et al. 2004) were introduced by P1 transduction. For transduction of the nadA::Tn10 allele selection was on media supplemented with nicotinic acid. For construction of CNP245, zdi-925::Tn10 was moved into the ppsA::kan strain by P1 transduction with selection on tetracycline media, followed by screening on kanamycin media which showed ~50% linkage between the alleles. A P1 lysate was made from the double mutant strain and brought into MC4100 pckA::kan, selecting on tetracycline and screening for lack of growth on M63 pyruvate and succinate media.

Western blot analysis of stability assay samples

The 1-ml samples from the stability assays were added directly to 50 µl cold trichloroacetic acid and chilled on ice for over three hours. Next, the samples were pelleted at 4°C, washed with 500 µl cold acetone, and then resuspended in sodium dodecyl sulfate (SDS) loading buffer in a volume equal to OD600/10. After boiling for 10 minutes, equal volumes of the protein samples were loaded onto 12% polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes and probed with either a 1:6,000 dilution of anti-RpoS antibody (our laboratory stock) or a 1:10,000 dilution of GFP antibody (AbCam). For secondary antibody, donkey anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Amersham Pharmacia Biotech) was used at 1:6000. The bands were detected using the ECL antibody detection kit (Amersham Pharmacia Biotech) and XAR film (Kodak) or a Laser Scanner 9400 Typhoon scanner and ImageQuant software (GE Healthcare). The intensity of the bands was analyzed by gel image analysis software (Kodak 1D Image Analysis Software or GE Healthcare).
ImageQuant) and it was determined that the lanes were not overexposed. All figures are printed using a linear gray scale unless otherwise noted.

**RpoS in vitro degradation assay**

Proteins were purified according to previously described protocols for SprE (Zhou et al. 2001), RpoS (Zhou et al. 2001), ClpX (Kim et al. 2000) and ClpP (Kim et al. 2000). For the endpoint experiments, reaction mixtures were assembled in 50 μL of Buffer SD (20 mM Tris-HCl at pH 7.5, 10 mM MgCl₂, 140 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 5% glycerol [v/v]) (Zhou et al 2001) containing 25 mM acetyl phosphate, 0.1 μM SprE, 0.3 μM ClpX₆, 0.9 μM ClpP₁₄ and different concentrations of ATP from 0 μM, 50 μM, 100 μM, 200 μM and 400 μM. First, SprE was incubated with acetyl-phosphate in the SD buffer for 15 minutes and then it was added to the ClpXP/ATP mix, 5 μM RpoS and a creatine kinase (5 mM) /creatine phosphate (50 μg) ATP regeneration mix. The 30°C incubation was allowed to go 16 hours and then endpoint samples were taken. For the kinetic experiments, the ATP mix included different combinations of ATP and ATPγS in a final concentration of 5mM. After incubating SprE with acetyl-phosphate, the phosphorylated adaptor was added to 0.3 μM ClpX₆ and 0.9 μM ClpP₁₄ with the different ATP/ATPγS mixes for 2 minutes. Subsequently, RpoS was added at 5 μM concentration and time points were taken as noted. No ATP regeneration was used in the kinetic experiments and all incubations were done at 30°C. Degradation of RpoS was monitored by SDS-PAGE, followed by staining with SYPRO Orange (Sigma Aldrich) and use of a Typhoon Laser Scanner 9400 (GE Healthcare). Intensity of the bands was analyzed by ImageQuant5 and the average and standard deviation from the results of two replicates were calculated using Microsoft Office Excel. ATPase assays were measured under similar conditions as above using a NADH-coupled ATP-hydrolysis assay as previously described (Burton et al. 2001).