

Characterization of HeID, an interacting partner of RNA polymerase from *Bacillus subtilis*

Jana Wiedermannová^{1,2}, Petra Sudzinová¹, Tomáš Kovač³, Alžbeta Rabatinová¹, Hana Šanderová¹, Olga Ramaniuk¹, Šimon Rittich¹, Jan Dohnálek^{3,4}, Zhihui Fu⁵, Petr Halada⁶, Peter Lewis⁵ and Libor Krásný^{1,*}

¹Laboratory of Molecular Genetics of Bacteria, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague 14220, Czech Republic, ²Department of Genetics and Microbiology, Faculty of Science, Charles University in Prague, Prague 12843, Czech Republic, ³Department of Structure Analysis of Biomacromolecules, Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague 16206, Czech Republic, ⁴Laboratory of Structure and Function of Biomolecules, Institute of Biotechnology, Academy of Sciences of the Czech Republic, Prague 14220, Czech Republic, ⁵School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia and ⁶Laboratory of Molecular Structure Characterization, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague 14220, Czech Republic

Received June 18, 2013; Revised January 14, 2014; Accepted January 16, 2014

ABSTRACT

Bacterial RNA polymerase (RNAP) is an essential multisubunit protein complex required for gene expression. Here, we characterize YvgS (HeID) from *Bacillus subtilis*, a novel binding partner of RNAP. We show that HeID interacts with RNAP-core between the secondary channel of RNAP and the alpha subunits. Importantly, we demonstrate that HeID stimulates transcription in an ATP-dependent manner by enhancing transcriptional cycling and elongation. We demonstrate that the stimulatory effect of HeID can be amplified by a small subunit of RNAP, delta. *In vivo*, HeID is not essential but it is required for timely adaptations of the cell to changing environment. In summary, this study establishes HeID as a valid component of the bacterial transcription machinery.

INTRODUCTION

RNA polymerase (RNAP) in bacteria is the key enzyme responsible for transcription of DNA into RNA. The bacterial RNAP core consists of $\alpha_2\beta\beta'\omega$ subunits and it is capable of transcription elongation but not initiation. Binding of an appropriate sigma factor to RNAP core enables the holoenzyme to recognize promoter DNA and initiate transcription (1). Unlike in gram-negative bacteria, RNAP from *Bacillus subtilis* and other gram-positive bacteria contains an additional subunit, δ . δ affects both transcription initiation and RNAP recycling,

the latter depending on the ability of RNAP to be efficiently released from nucleic acids after transcription termination (2,3).

Regulation of RNAP is a complex process involving other factors besides the bona fide subunits. These factors (e.g. Gre, Nus and Rho factors) interact with RNAP and affect its function under various conditions and in various ways (4–6). Understanding the function of these factors and identification of new factors interacting with RNAP is imperative for understanding transcription and gene expression regulation.

Recently, HeID (YvgS) was identified as a binding partner of *B. subtilis* RNAP (7) and is the main copurifying band in preparations of RNAP from this organism. It is a putative helicase, and based on sequence homology, belongs to the superfamily I of DNA and RNA helicases, most closely related to HeIV helicases from gram-positive bacteria. The best characterized helicases, belonging to the same superfamily but only distantly related to HeID or HeIV, are UvrD and Rep helicases from *Escherichia coli*, or PcrA helicase from *Geobacillus stearothermophilus*. These helicases unwind DNA duplexes in an ATP-dependent manner, inchworming along the nucleic acid (8). HeID is strongly expressed during the exponential phase of growth with a further increase in expression in stationary phase (9). However, the cellular role(s) of HeID are poorly understood; it has been implicated in DNA repair and homologous recombination (10) but it has neither been characterized biochemically nor has its role(s) in transcription been investigated.

*To whom correspondence should be addressed. Tel: +420 241 063 208; Fax: +420 241 722 257; Email: krasny@biomed.cas.cz

In this study, we set out to characterize the function of HelD in transcription. We confirmed that HelD interacts with RNAP, and we identified the form of RNAP with which it interacts, and the region of RNAP to which it binds. Importantly, we found a functional link between HelD and δ and showed that these two proteins act synergistically to stimulate transcription.

MATERIALS AND METHODS

Bacterial strains and plasmids

Strains and plasmids are listed in Table 1. Competent *E. coli* cells [DH5 α used for cloning or BL21 (DE3) used for overproduction of proteins] were prepared according to Hanahan (11). Competent *B. subtilis* cells were prepared as described (12).

All polymerase chain reactions (PCRs) were performed using the Expand High Fidelity System (Roche). All constructs were verified by sequencing. Wild-type *helD* was amplified by PCR from the genomic DNA of *B. subtilis* MH5636 (forward primer: 5'-caccatgaatcagcaggataagg-3', reverse primer: 5'-tcattcagcaatctgatataag-3'), and cloned into the expression vector pET151/D-TOPO (Invitrogen) allowing in-frame fusion of a His₆ tag at the N-terminus of HelD. The resulting plasmid was named pHelD-His6 (LK800, see Table 1).

Table 1. List of strains and plasmids

Strain/plasmid	Relevant characteristics ^a	Source
<i>B. subtilis</i>		
MH5636	<i>rpoC</i> -His10	(13)
MGNA-A456	<i>helD</i> ::MLS	(14)
LK637	<i>rpoC</i> -10His, <i>rpoE</i> :: <i>kan</i>	(15)
LK782	<i>rpoC</i> -10His, <i>helD</i> ::MLS	This work
LK1032	<i>rpoC</i> -10His, <i>rpoE</i> :: <i>kan</i> , <i>helD</i> ::MLS	This work
LK1401	<i>amyE</i> :: <i>helD</i> -His6, <i>spe</i>	This work
BSB1	wt BaSysBio	(9)
<i>E. coli</i>		
LK22	BL21 pCD2/Bsu_ sigA	(16)
RLG770	pRLG770	(17)
LK1	pRLG770 with Pveg (-38/+1,+1G)	(18)
LK1109	pRLG770 with PhelD	This work
LK888	pRLG770 with PglpD	This work
RLG7023	BL21/pFL31/Bsu_rpoE	(3)
LK800	BL21/pHelD-His6	This work
LK1413	pSG1721-HelD-His6	This work
pET151	pET151/D-TOPO	Invitrogen
pNG213	pETMCSIII/His6-rpoA	(6)
pNG490	pETMCSIII/His6-rpoB ₁₋₆₀₈	(6)
pNG479	pETMCSIII/His6-rpoB ₄₀₀₋₇₆₀	(6)
pNG480	pETMCSIII/His6-rpoB ₇₅₀₋₁₀₄₀	(6)
pNG481	pETMCSIII/His6-rpoB ₉₅₀₋₁₁₉₃	(6)
pNG482	pETMCSIII/His6-rpoC ₁₋₄₃₃	(6)
pNG483	pETMCSIII/His6-rpoC ₂₅₃₋₆₁₀	(6)
pNG484	pETMCSIII/His6-rpoC ₆₀₀₋₉₁₅	(6)
pNG485	pETMCSIII/His6-rpoC ₈₀₀₋₁₁₉₉	(6)
pNG492	pETMCSIII/His6-yloH (ω_2)	(6)
pNG579	pETMCSIII/His6-ykzG (ω_1)	(6)
pNG613	pETMCSIII/His6-rpoB _{750-846, 875-1040}	(6)

^aMLS, macrolide-lincosamide-streptogramin B resistance; kan, kanamycin; spe, spectinomycin.

The *B. subtilis* *helD*-null knockout strain (LK782) was prepared by transformation of *B. subtilis* strain MH5636 containing a His₁₀-tagged β' subunit (13) with chromosomal DNA from MGNA-A456, kindly provided by the National BioResource Project (Japan). A double knockout strain LK1032 (for *helD* and *rpoE* encoding the δ subunit of RNAP) was obtained by transformation of strain LK637 (15) with MGNA-A456 chromosomal DNA.

Supercoiled plasmids and linear DNA for *in vitro* transcription assays were obtained using the Wizard Midiprep Purification System (Promega) and subsequently phenol-chloroform extracted, precipitated with ethanol, and dissolved in water. The plasmids used in *in vitro* transcriptions contained promoter fragments cloned into p770 (17). Transcription terminated at a Rho-independent terminator. Linear DNA templates were prepared by PCR from the plasmid containing Pveg (LK1). All linear templates started at -118 relative to the transcription start site. The template containing the Rho-independent terminator (at +145) ended at +255. The template without the Rho-independent terminator ended at +111. The template with the short transcribed region (Figure 6D) ended at +20.

For *in vitro* transcription assays, pRLG770 with Pveg (-38/-1,+1G) was used (18) unless stated otherwise.

Media and growth conditions

For plasmid and protein purifications, appropriate strains were grown in Luria-Bertani (LB) medium at 37°C. For *in vivo* experiments, the cells were grown in defined 3-(N-morpholino)propanesulfonate (MOPS) - buffered medium (18) supplemented with 0.4% glucose and all 20 amino acids at 25 μ g/ml.

Purification of proteins

Bacillus subtilis RNAP with a His₁₀-tagged β' subunit or His₆-HelD was purified from the strain LK782 (strain without *helD*), LK1032 (strain without *helD* and *rpoE*) or LK1401 (strain with HelD-His6). The purifications were performed as described (13). Induction of HelD-His6 in strain LK1401 was carried out at OD₆₀₀ = 0.5 with 0.08% xylose for 2 h.

Plasmid pHelD-His6 was transformed into *E. coli* BL21 (DE3) and the production of HelD-His₆ induced following the addition of 1 mM IPTG for 2 h at room temperature. Cells were harvested and protein was purified by affinity chromatography as described for RNAP.

The σ^A subunit of RNAP was overproduced from the pCD2 plasmid (16) and purified as described (2).

The δ protein was purified from RLG7023 as described (3). Proteins were dialyzed against storage buffer containing 50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 50% glycerol, 3 mM 2-mercaptoethanol and stored at -20°C. Proteins were visualized on NuPAGE 4-12% Bis-Tris gels (Invitrogen) with Novex Sharp Pre-Stained Protein Standard as a marker.

Determination of experimental pI

The experimental pI of HelD was determined by isoelectric focusing (IEF) using precast 5% polyacrylamide

Vertical Novex® IEF Mini Gels pH 3–10 and XCell SureLock™ mini-cell electrophoresis system (Life Technologies Corp.). IEF was performed according to the manufacturer's instructions.

Western blotting

Proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (SimplyBlue, Invitrogen) and detected by Western blotting using mouse monoclonal antibodies to σ^{70} [2G10] or to the β subunit of RNAP [8RB13] (both from Santa Cruz) and secondary antibodies conjugated with horseradish peroxidase. Signal was created using SuperSignal West Femto Chemiluminiscent Substrate (Thermo Scientific) and exposing blots to photographic film.

Far western blotting

Purified proteins were biotinylated using the EZ-Link sulfo-NHS-biotinylation system (Thermo Scientific). Far western blots were performed as detailed by Yang *et al.* (6), except protein–protein interactions were detected using Horseradish peroxidase-conjugated streptavidin and the Opti4CN system (BioRad). Binding affinity to RNAP fragments was determined from digitized scans of blots using ImageJ (NIH) where maximal binding (100%) was set as the intensity of the signal of HelD bound to the $\beta'_{600-915}$ fragment. HelD binding sites on RNAP were mapped onto the *B. subtilis* RNAP homology model (19) and visualized using PyMol (Schrödinger).

Native PAGE assays

Five picomoles of RNAP and 25 pmol of HelD, T4 DNA ligase (TaKaRa) or MLV reverse transcriptase (Promega), respectively, were used. Proteins tested for mutual interactions were incubated for 15 min at 30°C in 10 μ l in the storage buffer. After incubation samples were mixed with 3 μ l of Native PAGE 4 \times Sample buffer (Invitrogen) and loaded onto the Native PAGE 4-16% Bis-Tris Gel (Invitrogen) and electrophoresed. The gels were subsequently Coomassie stained.

Enzymatic digestion for mass spectrometry

Coomassie blue-stained protein bands were excised from the gel, cut into small pieces and destained using 50 mM 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). The proteins were further reduced with 30 mM tris(2-carboxyethyl)phosphine (TCEP) in 100 mM Tris-HCl (pH 8.0) at 65°C for 30 min and alkylated with 30 mM iodacetamide in 100 mM Tris-HCl (pH 8.0) for 60 min in the dark. The gel was washed with water, shrunk by dehydration in MeCN and re-swelled again in water. The supernatant was removed and the gel was partly dried in a SpeedVac concentrator. The gel pieces were then incubated overnight at 37°C in cleavage buffer containing 25 mM 4-ethylmorpholine acetate, 5% MeCN and trypsin (100 ng; Promega). The resulting peptides were extracted into 40% MeCN/0.3% trifluoroacetic acid

(TFA). An aqueous 50% MeCN/0.1% TFA solution of α -cyano-4-hydroxycinnamic acid (5 mg/ml; Sigma) was used as a MALDI matrix. One microliter of the peptide mixture was deposited on the MALDI plate, allowed to air-dry at room temperature and overlaid with 0.4 μ l of the matrix.

MALDI mass spectrometry and protein identification

Mass spectra were measured on an Ultraflex III MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) in the mass range of 700–4000 Da and calibrated internally using the monoisotopic $[M+H]^+$ ions of trypsin autoproteolytic fragments (842.5 and 2211.1 Da). The peak lists created using flexAnalysis 3.3 were searched using an in-house MASCOT search engine against the SwissProt 2013_09 database subset of *B. subtilis* proteins with the following search settings: peptide tolerance of 30 ppm, missed cleavage site value set to one, variable carbamidomethylation of cysteine, oxidation of methionine and protein N-term acetylation. Proteins with MOWSE scores over the threshold value of 56 calculated for the used settings were considered as being positively identified. If the score was lower or only slightly higher than the threshold value, the identity of protein candidate was confirmed by tandem mass spectrometry analysis.

ATPase activity

ATPase activity was measured by the hydrolysis of inorganic phosphate from $[\gamma\text{-}^{32}\text{P}]$ ATP. The reaction mixture in 110 μ l of 50 mM Tris-Cl, pH 8; 5 mM MgCl_2 , 1 M KCl contained 550 pmol HelD or 550 pmol bovine serum albumin (BSA) or 275 pmol *Bacillus stearothermophilus* EF-Tu G-domain and 3450 pmol $[\gamma\text{-}^{32}\text{P}]$ ATP (specific activity 1800 cpm/pmol). The reaction was performed at 30°C and followed kinetically for 90 min at 30°C. Aliquots of 20 μ l were withdrawn at appropriate time intervals (0, 30, 60 and 90 min) and liberated Pi determined by the charcoal method (20). Five microliter of the product was spotted on Whatman 3MM filter paper, dried and scanned using a Molecular Imager_FX (Bio-Rad). The amounts were quantified with QuantityOne software (Bio-Rad). Blank samples were run simultaneously to determine background values of ATP hydrolysis. The experiments were repeated three times. The amounts of hydrolyzed ATP were calculated and plotted as the function of time.

In vitro transcription assays

Initiation competent enzyme was reconstituted using RNAP isolated from LK782 or LK1032 with a saturating concentration of σ^A in storage buffer (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 50% glycerol) for 15 min at 30°C.

Multiple round transcription assays were carried out essentially as described by (13,18) unless stated otherwise. Briefly, reactions were carried out in 10 μ l: 30 nM holoenzyme (RNAP σ^A), 2.5 nM supercoiled or 50 nM linear DNA template unless stated otherwise, transcription buffer (40 mM Tris-HCl, pH 8.0, 10 mM MgCl_2 , 0.1 mg/ml BSA and 1 mM dithiothreitol (DTT)), 150 mM KCl

and NTPs (ATP, CTP and GTP were 200 μ M; UTP was 10 μ M plus 2 μ M of radiolabeled [α - 32 P]-UTP).

RNAP was reconstituted with HelD at a 1:4 ratio unless stated otherwise. The RNAP: δ ratio used in experiments where δ was added was 1:4 (saturating concentration). Reconstitution experiments were carried out in storage buffer for 15 min at 30°C. When denatured HelD was used, native HelD protein was denatured at 90°C for 5 min.

All transcription reactions were allowed to proceed for 15 min at 30°C (unless stated otherwise) and stopped with equal volumes of formamide stop solution (95% formamide, 20 mM EDTA, pH 8.0). Transcription assays on 20 nt linear templates were allowed to proceed for 30 min.

In single round transcription assays, and in the multiple round assays (Figure 6), RNAP was preincubated with plasmid DNA and HelD and δ were subsequently added and incubated for 10 min at 30°C. Reactions were carried out in transcription buffer supplemented with 150 mM KCl and started by the addition of NTPs (concentration of NTPs was the same as in multiple round *in vitro* transcription assays) together with 600 nM double-stranded DNA (dsDNA) competitor. The competitor dsDNA with a full-consensus promoter sequence (21) was used to sequester free RNAP and allow only one round of *in vitro* transcription (22). Stock dsDNA competitor was prepared by annealing equimolar amounts of complementary primers (LK 923: 5'-ccggaattcaaatattgtgttgaactctgacaaaagtgttaaatgtgctatactgtattggttctcaagctccg-3' and LK 924: 5'-cggaagcttgagaaccaatacagatagcacaatttaacactttgtcaagagtaacaacaataatttgaattccgg-3') in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM NaCl, which were denatured at 95°C for 5 min and then cooled down to 30°C (1°C per min). In negative controls, whole reaction mix with plasmid DNA and competitor dsDNA was started with RNAP to ensure that all RNAPs were sequestered by the dsDNA competitor.

In vitro transcription restart assays (Figure 5E and F) were carried out in two steps. The first 15-min step was basically the same as described above for multiple round reactions; KCl was used at 100 mM concentration. At the beginning of step II, the main compounds (water, NTPs, template DNA, δ , HelD, both δ and HelD, RNAP) were added to respective reactions in the same amounts as they were at the beginning of step I. Reactions were then allowed to proceed for another 15 min and were stopped with 10 μ l of stop solution. In experiments with two templates (Figure 6A), 10 nM final concentrations of the plasmids were used.

Samples were loaded onto 7 M Urea 7% polyacrylamide gels and electrophoresed. The dried gels were scanned with a Molecular Imager_FX (BioRad). The amount of transcript (originating from the cloned promoters) was quantified with QuantityOne software (BioRad). All calculations and data fitting were carried out using SigmaPlot (Jandel Scientific).

In vivo experiments: outgrowth from the lag phase

Wild type *B. subtilis*, δ and HelD knockout, and the double knockout strains (MH5636, LK637, LK782 and LK1032, respectively) were cultivated in LB medium for

24 h at 37°C under continuous shaking to ensure entry into stationary phase and then diluted into fresh LB medium to OD₆₀₀ = 0.03. OD₆₀₀ was measured during outgrowth to compare the duration of the lag phase of the respective strains.

Protein sequence and domain analysis

Protein sequence searches were performed with the BLAST protein-protein service (23,24). Sequence alignments were carried out with Clustalx, using the Gonnet 250 weight matrix (25).

RESULTS

HelD copurifies with RNAP

In our preparations of *B. subtilis* RNAP by affinity chromatography, we regularly observed a major contaminating band of ~90 kDa (Figure 1A). This band did not appear in control experiments where the lysate made from *B. subtilis* cells containing no His-tagged protein was incubated with the affinity matrix, indicating that the protein does not have an intrinsic ability to bind the matrix (Figure 2A). This protein could not be removed by gel filtration and its level in the preparation decreased only after ion exchange chromatography [(26) and data

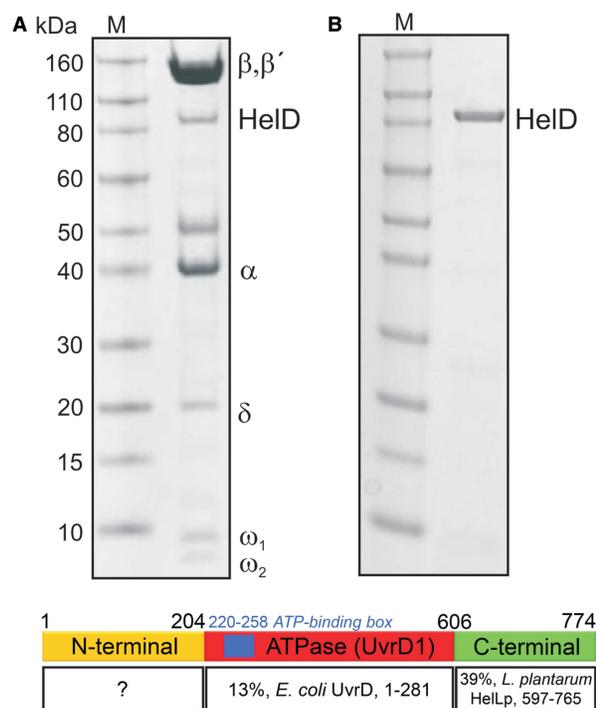


Figure 1. HelD copurifies with RNAP. (A) RNAP purified by Ni-affinity chromatography via the 10 \times N-terminal histidines on the β' subunit. Subunits of RNAP and the band corresponding to HelD are indicated. (B) HelD purified by Ni-affinity chromatography via the 6 \times N-terminal histidines. The apparent molecular weights are shown by Novex Sharp Pre-Stained Protein Standard, M. Panel C. Putative domain structure of *B. subtilis* HelD. Amino acid identity with respective protein fragments is indicated. The percent values show the level of sequence identity to the marked protein segment.

not shown]. This band was identified by MALDI mass spectrometry as the HelD protein, which was recently reported as a binding partner of RNAP (7).

The HelD protein is encoded by the *yvgS* (*helD*) gene and consists of 774 amino acids (aa). Based on its aa sequence, HelD is a putative UvrD-like helicase. Helicase from *Lactobacillus plantarum* HelLp, with a sequence identity of 39%, is the closest related protein for which any 3D structure (C-terminal domain, PDB ID 3DMN, unpublished) is available. The closest structurally related helicase of this class, UvrD from *E. coli*, for which the complete 3D structure has been determined (27), shows only 12% sequence identity with HelD. Based on domain arrangement of HelLp and UvrD and sequence comparisons of these two proteins with HelD, it is postulated that HelD comprises three domains (Figure 1C). The first, N-terminal domain (residues 1–204) may be involved in DNA binding. The second domain (205–606) contains the ATP-binding box and is related to the ATPase domain of UvrD helicase (residues 1–281 in UvrD, domain marked as UvrD1). The C-terminal domain (607–774) is related to the domain of UvrD helicase involved in DNA unwinding and shares significant similarity with the C-terminal domain of HelLp. However, in the case of UvrD this domain is formed by two distant parts of the protein chain, whereas the HelLp C-domain is formed by a single section of the protein chain. In summary, HelD is a unique protein only part of which resembles proteins characterized to date.

For further studies, we prepared recombinant HelD. The recombinant protein was purified by nickel affinity chromatography via the introduced N-terminal 6xHis

tag (Figure 1B) and the experimentally determined pI of the protein under native conditions was ~7.1 (theoretical pI = 6.1). HelD was able to bind DNA (Supplementary Figure S1), and as it was predicted to be a helicase, we attempted to detect this activity in strand displacement assays. Using an array of DNA templates [5' or 3' overhangs, forked DNA or blunt-ended DNA; (28)] we detected no strand-displacement activity for this protein (data not shown).

Interaction of HelD with RNAP

To confirm that HelD binds to RNAP and to establish to which form (core or holoenzyme), we performed *in vivo* and *in vitro* experiments. *In vivo*, we overproduced C-terminally His-tagged HelD in *B. subtilis* cells (strain LK1401) and subsequently purified it via the His-tag. In parallel, we purified RNAP that was His-tagged at β' (MH5636) and we also performed a control purification from a strain without any His-tagged protein (BSB1). Figure 2A shows that RNAP core subunits copurified with the His-tagged HelD, and that RNAP is the main interacting partner of this protein. Western blot analysis (Figure 2B) showed that HelD interacts predominantly with the core form of RNAP as virtually no σ^A was detected.

To verify that purified HelD binds to RNAP *in vitro* we performed gel-shift experiments under native conditions in nondenaturing PAGE gels. Figure 2C lane 1 shows that HelD forms oligomers in solution as also seen by size exclusion chromatography (Supplementary Figure S2). The addition of HelD to RNAP resulted in reduced migration of RNAP, suggesting binding of HelD to

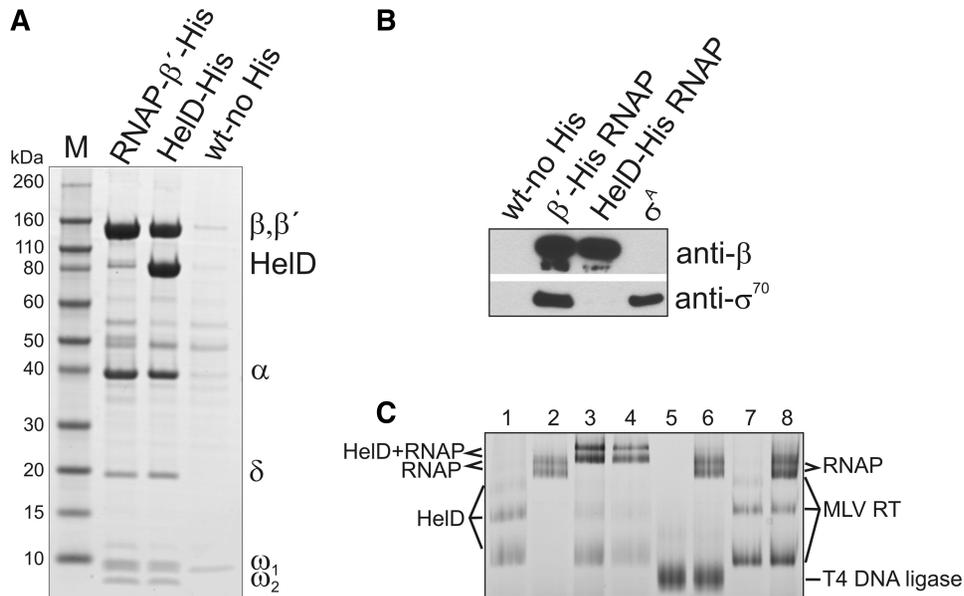


Figure 2. HelD interacts with RNAP core. (A) Coomassie stained gel of proteins purified by nickel affinity chromatography. Lane 1—proteins purified from strain MH5636 containing His-tagged β' subunit of RNAP; Lane 2—proteins purified from strain LK1401 containing His-tagged HelD; Lane 3—proteins purified from strain (BSB1) without any His-tagged protein. (B) Western blot of the gel from (A). The antibodies used are indicated. In the last lane purified σ^A was used as a marker. (C) Band shift assay on native PAGE gel. RNAP was incubated with potential interacting partners: HelD, T4 DNA ligase (68 kDa), MLV reverse transcriptase (71 kDa). Samples were separated on 4–16% gradient native PAGE Bis–Tris gels. Lane 1—HelD, lane 2—RNAP, lane 3—HelD with RNAP, lane 4—DNaseI-treated HelD+DNaseI-treated RNAP, lane 5—T4 DNA ligase, lane 6—RNAP with T4 DNA ligase, lane 7—MLV reverse transcriptase, 6—RNAP with MLV reverse transcriptase. In all experiments, RNAP was prepared from the $\Delta helD$ strain (LK782).

RNAP (Figure 2C, lane 3). To confirm results from the gel-shift experiment, we excised the two bands predicted to be RNAP from lane 2 and lane 3 and determined the protein identity by mass spectrometry. The analysis confirmed the presence of the β , β' and α subunits as well as HelD in the bands in lane 3. It is not clear why RNAP and RNAP-HelD complexes ran as two bands on the gel (Figure 2C, lanes 2 and 3), but they appeared identical by mass spectrometry analysis, and we speculate that they may represent two conformational states of RNAP, both capable of HelD binding. We also verified by mass spectrometry that the bands in lane 1 consist of HelD

(probably in different oligomeric states). As an additional control to exclude the possibility that HelD binds to RNAP via traces of DNA remaining in complex with the proteins, DNaseI was added to the RNAP and HelD preparations before the experiment and we obtained the same result (Figure 2C, lane 4). As specificity controls, we used T4 ligase and murine reverse transcriptase and neither of these proteins bound to RNAP (Figure 2C, lanes 5–8).

We conclude that HelD interacts with RNAP core *in vivo* and that purified HelD interacts directly with RNAP *in vitro*.

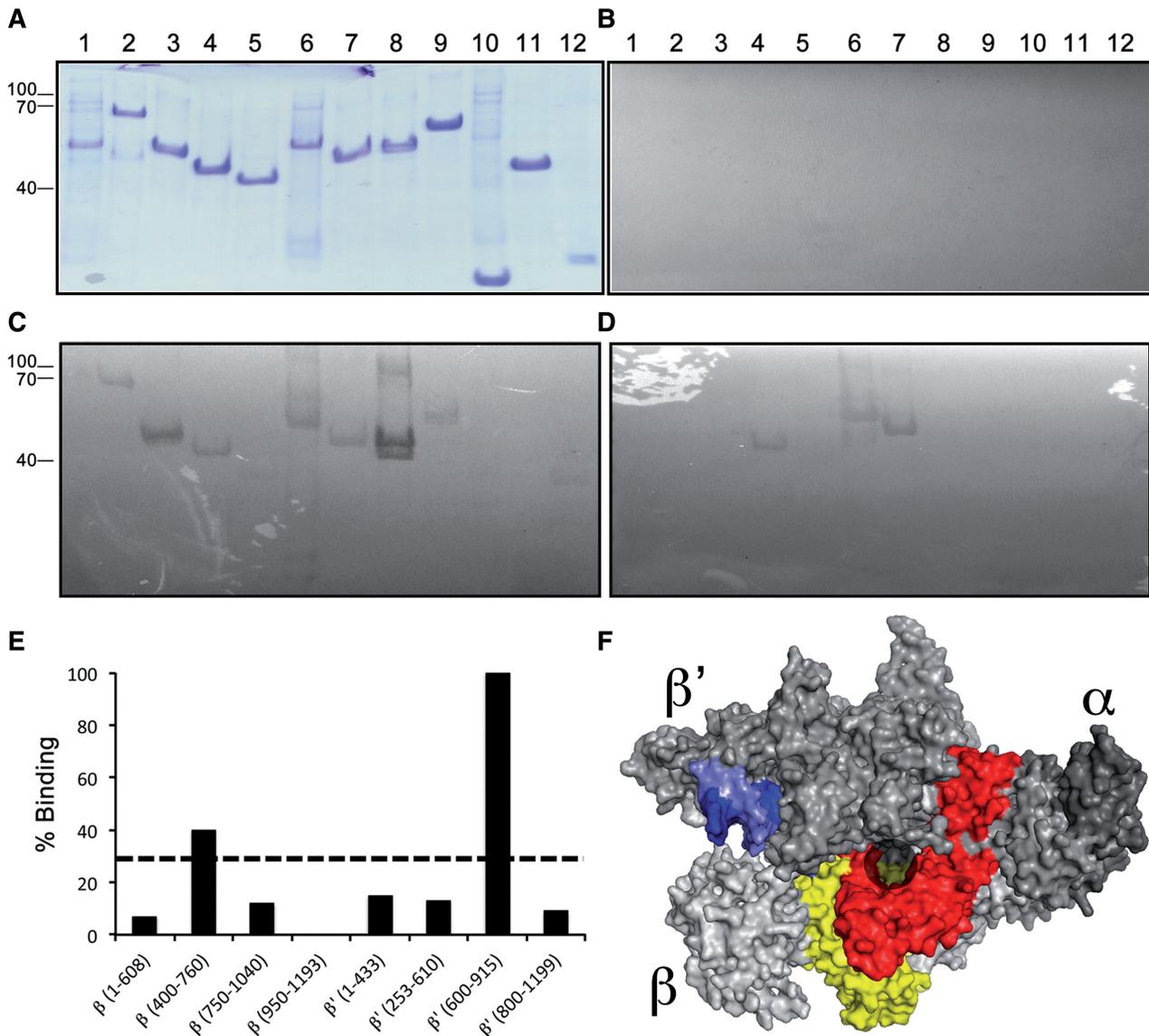


Figure 3. HelD binds on the downstream side of RNAP. (A) Coomassie blue stain of RNAP subunits and fragments showing approximate equal loading in all lanes. (B) Negative control blot containing all components except biotinylated ligand. (C) Blot performed using biotinylated HelD. (D) Control blot using biotinylated σ^A . Molecular weights (kDa) are shown down the left hand side. Lane 1— α ; lane 2— β_{1-606} ; lane 3— $\beta_{400-760}$; lane 4— $\beta_{750-1040}$; lane 5— $\beta_{950-1193}$; lane 6— β'_{1-433} ; lane 7— $\beta'_{253-610}$; lane 8— $\beta'_{600-915}$; lane 9— $\beta'_{800-1199}$; lane 10— ω_2 ; lane 11— $\beta_{750-1040}$ Δ flap; lane 12— ω_1 . (E) Quantification of binding signal of HelD to β and β' fragments normalized to the strongest binding fragment, $\beta'_{600-915}$ (set at 100%). Dotted line represents 30%. (F) Surface rendered model of *B. subtilis* RNAP with the strongest HelD binding fragments labeled in yellow ($\beta_{400-760}$) and red ($\beta'_{600-915}$). α Subunits are shown in dark gray, β subunit in light gray and β' subunit in medium gray. DNA is shown in blue; template strand in light blue and nontemplate strand in dark blue. The approximate location of the secondary channel is indicated by the transparent gray circle.

Localization of HelD on RNAP

Next, we addressed the topology of the binding of HelD to RNAP. We used far western blot analysis using fragments of subunits of *B. subtilis* RNAP probed with biotinylated HelD (Figure 3). Controls were performed with no protein (Figure 3B) and with biotinylated σ^A that bound to the N-terminus of the β' subunit as well as the fragment of the β subunit containing the β -flap tip (Figure 3D). These data are consistent with previous far western blot results using anti- σ^A antibodies (6) and structural data (29). When using HelD as a probe, signal was observed for most of the fragments of the β and β' subunits (Figure 3C), but as shown in Figure 3E and F, it bound most strongly to two fragments that form the rim of the secondary channel by which it is believed that NTPs enter the active site (gray circle, Figure 3F). Mapping of the fragments to which HelD bound weakly enabled us to identify portions that all triangulated to the surface of RNAP close to the secondary channel (not shown) consistent with HelD forming extensive contacts with RNAP on the downstream side in close proximity to DNA (light and dark blue, Figure 3F). Assuming HelD binds on the downstream side of RNAP it is unlikely there is significant steric hindrance preventing σ from being simultaneously bound.

HelD affects transcription *in vitro*

Because HelD directly interacts with RNAP, we wished to determine whether it was important for the DNA-dependent synthesis of RNA. Therefore, we performed a panel of *in vitro* transcription experiments with RNAP purified from a HelD knockout strain, and studied the effect of addition of HelD. As a model promoter, we used *Pveg*, a strong constitutive promoter that is well characterized (18). From this promoter RNAP transcribes a 145 nt transcript and transcription is terminated at a Rho-independent terminator (17,30). In *in vitro* multiple round transcription assays HelD displayed a stimulatory effect on transcription (Figure 4A, black columns; Supplementary Figure S3). However, a large stoichiometric excess of HelD over RNAP abolished transcription, possibly by nonspecific binding of HelD to DNA, resulting in the formation of transcription roadblocks (31). Negative control experiments using heat-denatured HelD failed to produce any stimulatory effect on transcription activity (Figure 4A; gray columns). We also tested the effect of HelD with two other templates containing two other promoters, *PhelD* and *PglpD* (32), and we obtained similar results to those obtained with *Pveg* (Supplementary Figure S3AB). Moreover the effect of HelD on transcription was salt-concentration dependent, as increasing the amount of salt in the reaction rendered the effect more pronounced (Supplementary Figure S4).

We conclude that HelD in low stoichiometric excess over RNAP stimulates transcription.

The effect of HelD is ATP-dependent

As HelD contains an ATP binding motif, we investigated whether the effect of HelD on transcription depends on ATP. First, we tested whether HelD possesses an ATPase

activity. While none of the two control proteins, the GTPase domain of *B. stearothermophilus* elongation factor Tu or BSA, was able to hydrolyze ATP, HelD displayed significant ATPase activity (Figure 4B and C). This effect was independent of the presence of RNAP (data not shown). Next, we performed transcription assays with increasing concentrations of ATP either in the presence or absence of HelD. As a control, we performed the same type of experiment with increasing concentrations of CTP. When ATP or CTP concentrations were increased (from 1 to 200 μ M), the other NTP concentrations were kept constant (100 μ M). Figure 4D and E shows that by increasing the concentration of ATP from 1 to 20 μ M (where the effect plateaued) the level of transcription increased \sim 6-fold in the presence of HelD. When HelD was not present, this stimulation was absent. Furthermore, increasing the CTP concentration had no significant effect on transcription either in the presence or absence of HelD. The overall higher level of transcription in the presence of HelD when CTP concentration was being varied was due to the 100 μ M ATP that was present in the reaction.

We conclude that HelD is an ATPase and the stimulatory effect of HelD on transcription is ATP-dependent.

HelD and δ have a synergistic stimulatory effect on transcription cycling

The next question was which part of the process of RNA synthesis is affected by HelD. Even though Figure 2B shows that HelD binds to core and not holoenzyme, we wanted to test whether it has any effects on steps important for transcription initiation—RNAP association with σ^A and promoter binding. Unsurprisingly, the presence or absence of HelD had no effect either on the affinity of σ^A for RNAP or the affinity of RNAP for promoter DNA (Supplementary Figure S5A and B).

However, another putative helicase, the RapA protein of *E. coli*, has been shown to associate with RNAP and stimulate transcription by promoting cycling of RNAP by enhancing the release of RNAP from nucleic acids after termination and also by stimulating the dissociation of RNA–DNA hybrids (30). HelD and RapA are not sequence homologs; RapA belongs to the Swi2 family of helicases, but we speculated that these two proteins may be functional analogs. As the δ subunit of RNAP was previously also shown to enhance cycling of RNAP, we included δ as a positive control. We used RNAP from a double knockout strain (δ -null HelD-null, LK1032) in *in vitro* transcription assays from a *Pveg*-based supercoiled template. First, we tested the effects of HelD and δ separately and observed moderate stimulatory effects for each (2-fold increase) (Figure 5A and B; multiple rounds). Surprisingly, when we combined HelD and δ together, we detected a strong increase in RNA synthesis. The \sim 10-fold stimulation by HelD and δ together was more than the sum of the stimulatory effects of HelD and δ alone, suggesting a synergistic effect of these proteins.

Next, we decided to directly test whether the synergistic effect of HelD and δ is due to a more efficient cycling of transcription. If this was true, then limiting transcription

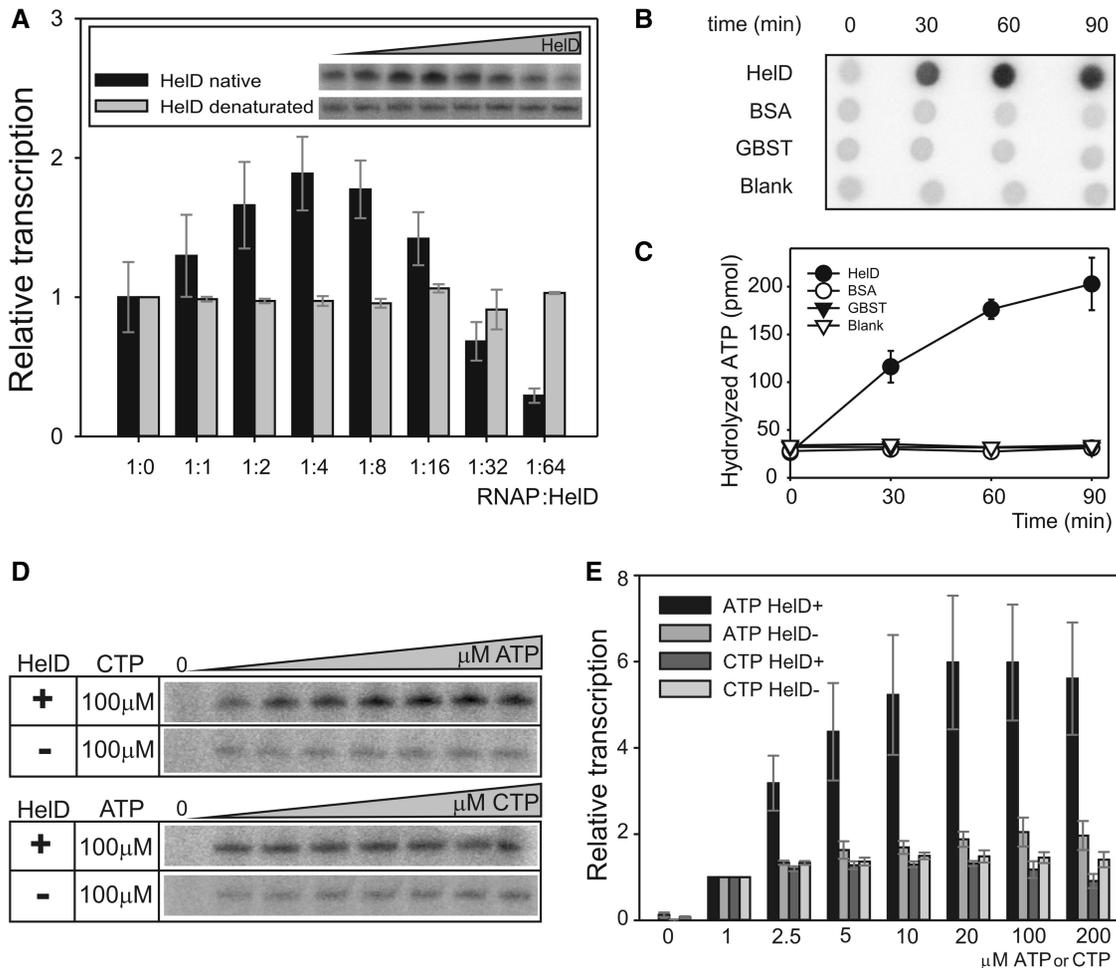


Figure 4. Effect of HelD on *in vitro* transcription. (A) RNAP (from HelD knockout strain LK782) was reconstituted with saturating concentrations of σ^A . Holoenzyme was incubated with increasing amounts (molar ratio from 1:0 to 1:64) of HelD (black bars) or heat denatured HelD (gray bars) and used to initiate transcription. Primary data are shown in the inserted box and show a representative experiment. The data were normalized to the 1:0 ratio set as 1. In this and the following experiments, the graphs represent data from three independent experiments \pm SD. (B) HelD hydrolyzes ATP. Primary data show spots with hydrolyzed γ - 32 P (for details see 'Materials and Methods' section). (C) Graph showing the quantification, with the symbol key presented in the graph. (D) The effect of HelD is ATP-dependent- representative primary data. RNAP from HelD knockout strain (LK 782) was tested in *in vitro* multiple round transcription assays in the presence/absence of HelD and increasing amounts of ATP (from 1 to 200 μ M total ATP). As a control, CTP was varied. The concentration of CTP was 100 μ M when [ATP] was varied and *vice versa*. (E) The effect of HelD is ATP-dependent quantification of the data normalized to transcription at 1 μ M ATP or CTP. The color coding of the bars is defined in the graph.

to a single round should abolish the effect. As shown in Figure 5A and B, the pronounced stimulatory effect of these factors was cancelled, consistent with the stimulatory effect of HelD and δ being due to cycling of transcription.

To examine the cycling in more detail, we performed multiple round transcription experiments where we followed transcription in the presence/absence of δ , HelD or δ and HelD as a function of time. In the absence of HelD or δ , transcription stopped at about the 5-min time point. The addition of either protein modestly prolonged this time. The addition of both proteins then markedly increased the time with transcription still continuing at the final 25-min point (Figure 5C and D).

This experiment raised two principal questions: (i) Why does the cycling of transcription stop after 5 min in the absence of δ and HelD? and (ii) Why is the cycling more

efficient with δ and HelD? We address these questions in the following two sections.

Transcription cycling stops because the template is not functional

There are three possibilities to explain why transcription stops after 5 min: first, depletion of NTPs prevents RNA synthesis; second, RNAP is in complex with RNA and/or DNA and inactive; third, the template DNA is not functional either because it is blocked by RNAP, or because it forms interactions with RNA [such as R-loops (33)].

To distinguish between these possibilities we allowed the *in vitro* transcription assays to proceed for 15 min without HelD and δ and then divided the mixture into several aliquots. Next, equal amounts of the main components (NTPs, template DNA, RNAP) were added as at the

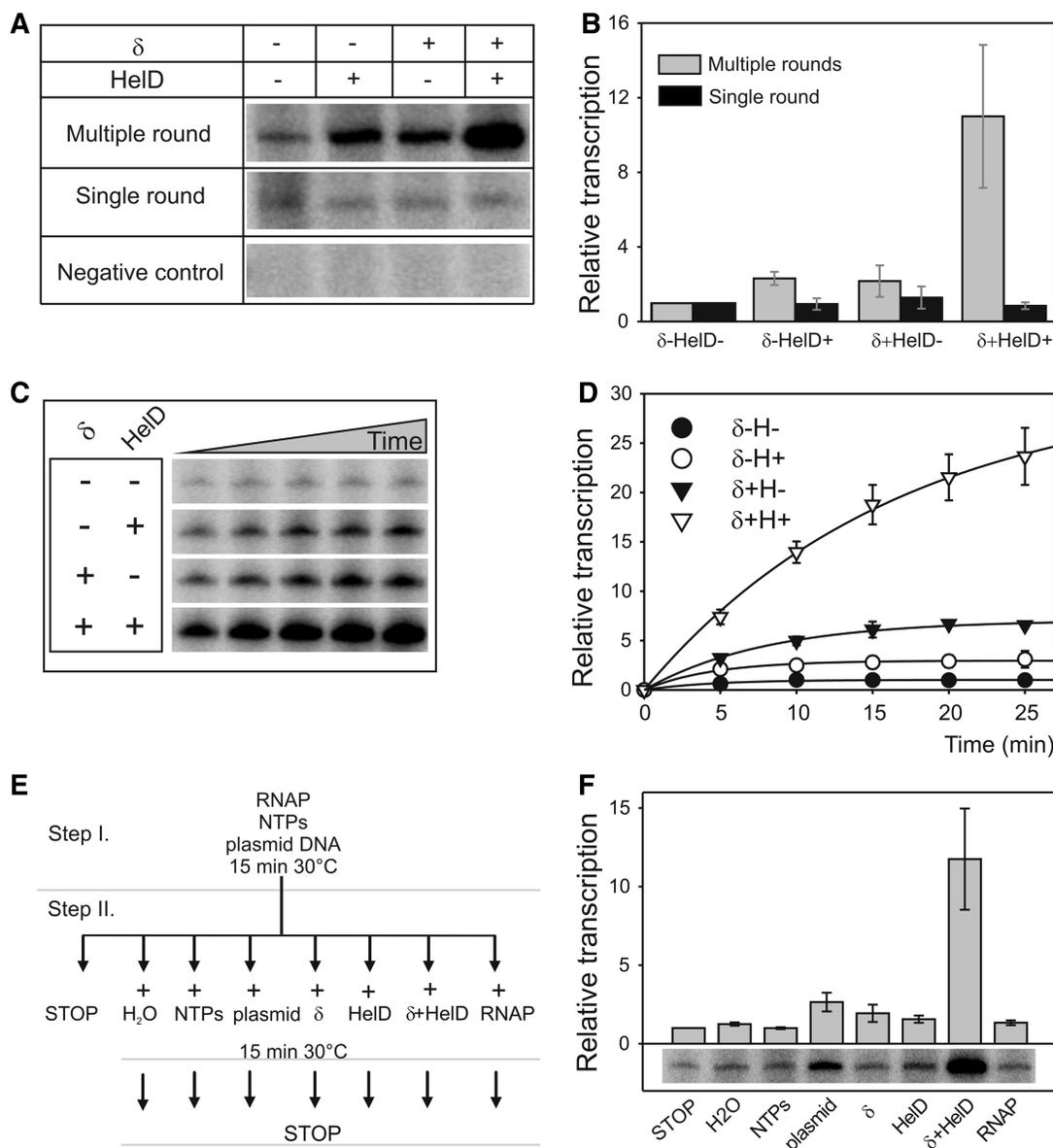


Figure 5. HelD and δ stimulate cycling of transcription. (A) Multiple round transcription assays were conducted in the absence of a competitor (dsDNA), whereas single round assays were conducted in its presence. In single round assays, the competitor was added to preform open complexes together with NTPs that were used to initiate transcription. The negative control shows the efficiency of the dsDNA competitor at preventing transcription of the 145-nt product when added to assay mixtures before formation of the open complex. (B) Quantification of data from multiple round (gray bars) and single round (black bars) transcription assays. Data from three independent experiments were quantified; the graph shows averages \pm SD. (C) Multiple round *in vitro* transcription is limited in time—representative data. RNAP was preincubated with/without δ and/or HelD. Reactions were started at time zero by NTPs. Aliquots were withdrawn at 5, 10, 15, 20 and 25 min, respectively, and stopped by formamide stop solution. (D) Multiple round *in vitro* transcription is limited in time—quantification. Data were quantified from two independent experiments normalized to the value of transcription without δ and HelD after 15 min (set as 1). The bars indicate the range. (E) HelD and δ restart halted *in vitro* transcription—a schematic drawing of the experiment. In step I, all the indicated components of the transcription reaction were combined together and transcription reactions were allowed to proceed for 15 min. At this time point, the mixture was distributed to eight tubes containing the indicated components, and the reactions (Step II) were allowed to proceed for further 15 min. (F) HelD and δ restart halted *in vitro* transcription—a representative result and quantitation of the data. The quantified data were normalized to lane 1, set as 1. The graph represents data from three independent experiments \pm SD.

beginning of the reaction, or HelD, δ or HelD and δ were added, and the reaction allowed to proceed for another 15 min. From the results (Figure 5E and F) we can exclude the first hypothesis: the addition of NTPs did not restart transcription (Figure 5F, lane 3). We can also exclude inactivation of all RNAP present in the experiment (second hypothesis) as addition of RNAP did not restart

transcription (Figure 5F, lane 8). This is in agreement with the fact that there was 12-fold molar excess of RNAP over the template DNA already at the beginning of the reaction. Finally, the addition of plasmid DNA resulted in \sim 2-fold increase in transcription, suggesting it is the lack of an available template DNA that prevents further transcription (third hypothesis).

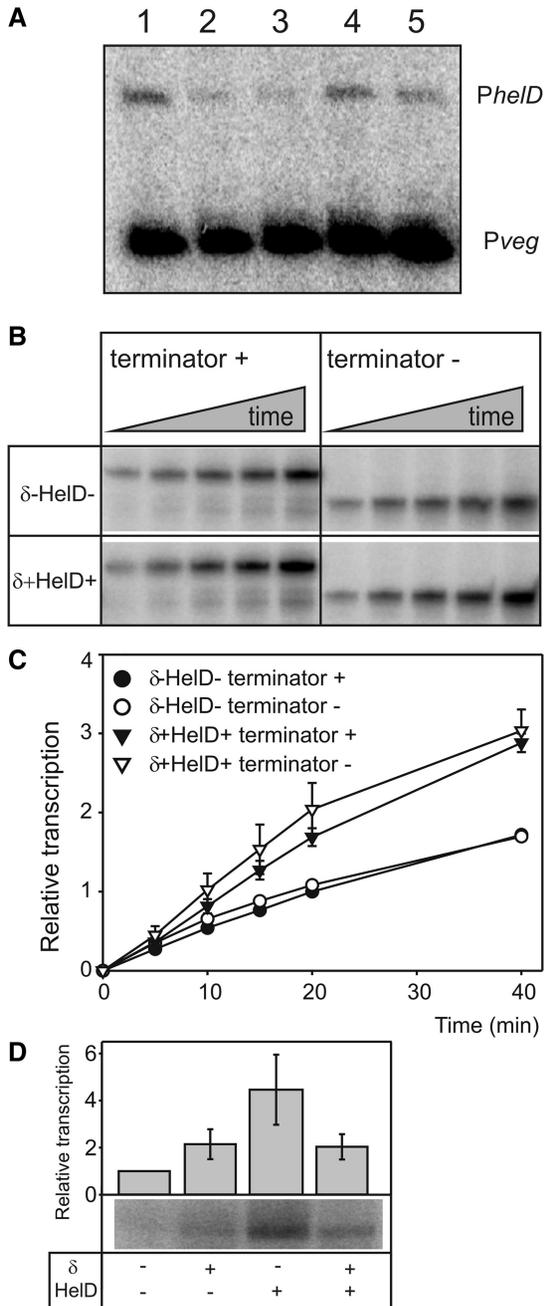


Figure 6. HelD liberates RNAP and affects elongation. (A) HelD liberates sequestered RNAPs—primary data. Transcription assays were performed with two supercoiled templates, either *Pveg* (145-nt transcript) or *PheID* (245 nt). *Pveg* was in all reactions from the beginning. *PheID* was added at the beginning (lane 1) or after 15 min (lanes 2–5) and reactions were allowed to proceed for another 15 min. RNAP contained no HelD or δ . These proteins were added after 15 min (lane 2—no protein; lane 3— δ ; lane 4—HelD; lane 5—HelD+ δ). (B) HelD and δ increase transcriptional rate on linear templates regardless of the presence/absence of a Rho-independent terminator—representative primary data. Two templates both containing the *Pveg* promoter were used (see ‘Materials and Methods’ section for details). One template contained a Rho-independent terminator, while the other lacked this terminator. With both templates, the experiment was conducted \pm HelD+ δ . (C) Quantification of the data (plus two more repeats) from the experiment shown in (B). Panel (D), HelD stimulates transcription of 20 nt RNA. Multiple round *in vitro* transcription reactions on a linear template with a 20 bp template were performed in the

The addition of HelD or δ alone had again a moderate stimulatory effect on transcription. However, a pronounced stimulatory effect was observed on the addition of HelD and δ together (Figure 5F, lane 7). This demonstrated that the lack of transcription-competent template was not caused by, for example, degradation or irreversible alteration of DNA.

HelD and δ release RNAP from DNA and affect elongation

The reason for the transcriptional inactivity of the plasmid in the previous experiment could be due to the presence of stalled (nonproductive) RNAP complexes either on the *Pveg* template or elsewhere on the plasmid. Another possibility is an alteration of the plasmid by transcription (e.g. a change of supercoiling), which would suggest an effect of HelD on elongation. Finally, the plasmid could be in complex with RNA (e.g. R-loops) and thus not functional in transcription. Previous experiments have already ruled out such possibilities as affecting promoter affinity (Supplementary Figure S5B) or affinity of σ^A for RNAP (Supplementary Figure S5A).

To test whether the presence of δ and HelD releases nonproductive RNAP from DNA we performed *in vitro* transcription assays with two templates producing RNAs of different length (from *Pveg* and *PheID* promoters). A subsaturating concentration of RNAP (with respect to the plasmid template) was used. When both templates were added at time zero, both were transcribed by RNAP in the absence of δ and HelD (Figure 6A, lane 1). When transcription started only from *Pveg*, and *PheID* was added after 15 min, free RNAP appeared to be unavailable, as only a small amount of transcript from *PheID* was generated (Figure 6A, lane 2), suggesting that RNAP may be sequestered on the *Pveg* template DNA. This effect was alleviated when HelD or HelD with δ were added together with the second template (Figure 6A, lanes 4 and 5). The presence of δ and HelD enabled RNAP to be reused for transcription from both templates. We note that δ alone did not display a stimulatory effect with the *PheID* template (Figure 6A, lane 3). This could be due to an effect of δ on transcription initiation at *PheID*, as δ is known to decrease transcription from promoters that form relatively unstable open complexes (important intermediates during transcription initiation) (15).

Next, we tested whether HelD and δ were able to release RNAP from posttermination complexes as suggested for RapA (30,34). We performed *in vitro* transcription reactions with linear templates containing or lacking Rho-independent terminators. Figure 6B and C shows that the presence or absence of the terminator had no effect on the stimulatory effect of HelD and δ . Interestingly,

Figure 6. Continued presence/absence of HelD and/or δ . The radioactively labeled transcripts were resolved on 20% polyacrylamide gels. The graph shows a representative result and quantification of the data. The quantified data were normalized to lane 1, set as 1. The graph represents data from three independent experiments \pm SD.

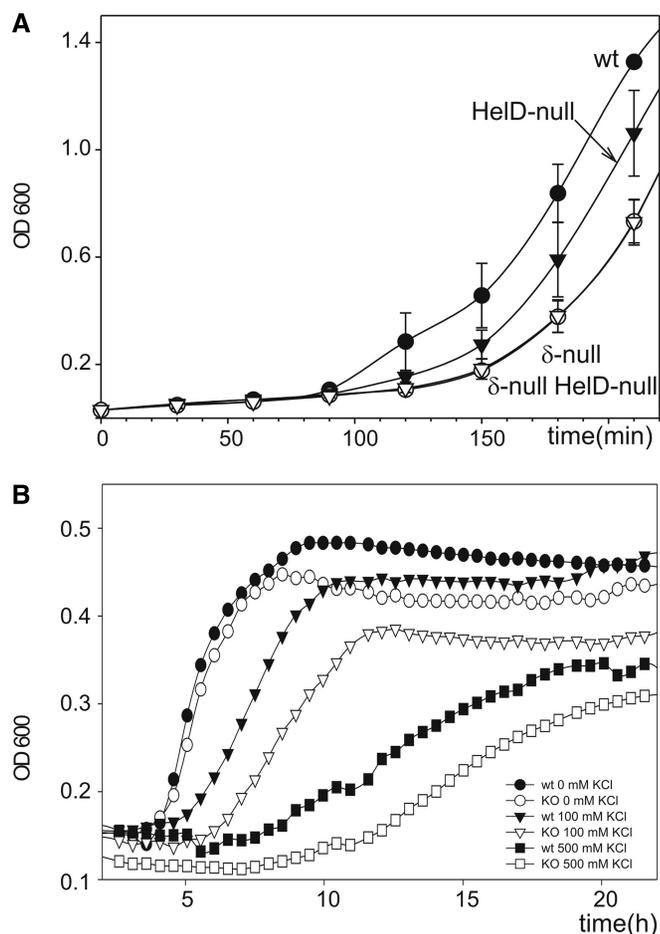


Figure 7. HelD and δ affect the growth phenotype. (A) wt, wild type *B. subtilis* strain (MH5636): closed circles; HelD-null (LK782): closed triangles; δ -null (LK637): open circles; δ -null HelD-null (LK1032): open triangles. The strains were grown for 24 h at 37°C. After 24 h, the cells were diluted into fresh LB medium at $OD_{600} = 0.03$ and the growth curves after the dilution are shown. (B) Growth in defined MOPS medium supplemented with all 20 amino acids in the presence 0, 0.5 or 1.0 M KCl as stated in the figure legend. Cells were cultivated in 1 ml of volumes at 37°C in 24-well EMSA plates in a TECAN Infinite® 200 Pro and OD 600 was measured every 10 min.

transcription did not stop on the linear templates after 5 min in the absence of these two proteins but continued throughout the duration of the experiment. However, the overall level of transcription was less in the absence of HelD and δ . We tested the effect of HelD and δ on releasing RNAP from the linear template and detected only a modest (20%) stimulation of this process (Supplementary Figure S6A and B). Hence, the main stimulatory effect of HelD on linear templates is most consistent with an effect on elongation.

To test whether the elongation effect could be due to the inability of RNAP to read through complexes of DNA with RNA, as also suggested for RapA (34), we performed experiments with a short linear DNA template where the transcribed region was only 20 nt long. This short length of transcribed template does not permit significant interactions of the nascent RNA with DNA (35). As shown in Figure 6D, HelD and δ were still capable of stimulating

transcription. Interestingly, the effect of δ with HelD was decreased relative to the effect of HelD alone. A possible explanation could be a parallel negative effect of δ on, for example, the affinity of RNAP for the promoter located on this short linear DNA fragment.

We conclude that the effect of HelD in conjunction with δ on transcriptional cycling on supercoiled templates appears to be caused, at least in part, by liberating nonproductive RNAPs from complexes with DNA. A parallel stimulatory effect of HelD on the rate of elongation that is more apparent with linear templates seems to also play a role.

HelD and δ display similar phenotypes *in vivo*

To assess the importance of HelD for the cell, we performed phenotypic experiments, using a HelD-null strain. In comparison with wild type, no difference in growth was observed in rich (LB) or nutritionally defined media (MOPS). Similarly, no difference was observed in response to various stresses, such as heat and ethanol (data not shown). However, the mutant strain displayed a prolonged lag phase for stationary cells diluted into fresh medium. This phenotype was reminiscent of the phenotype displayed by the δ -null strain (10,15). Therefore, we directly compared wild type, δ -null, HelD-null and the double knockout strain, HelD-null and δ -null (MH5636, LK637, LK782 and LK1032, respectively). The cells were grown for 24 h and then diluted into fresh LB medium. The outgrowth from lag phase of the δ -null strain was delayed compared with wild-type cells for ~ 30 min, as reported previously (Figure 7A). Growth of the HelD-null strain was delayed for ~ 15 min and the double knockout strain displayed an identical delay to the δ -null strain. The same phenotype was observed for wild-type versus HelD-null strain in defined rich medium (MOPS supplemented with all amino acids) and in poor M9 medium (Supplementary Figure S7A). Further, we also observed a minor effect on growth in the presence of increased amounts of salt (Figure 7B and Supplementary Figure S7B). Albeit relatively small, these phenotypes were highly reproducible. We concluded that the absence of HelD decreased the ability of the cell to rapidly adapt to nutritional changes in the environment.

DISCUSSION

In this study we confirmed HelD as a new interacting partner of RNAP from *B. subtilis*, we identified that it binds to the core form of the enzyme, localized its approximate binding region on RNAP and provided insights into the role of HelD in transcription. We have shown that HelD hydrolyzes ATP independently of RNAP and stimulates transcription in an ATP-dependent manner. This differs from RapA from *E. coli* where the ATPase activity of RapA without RNAP was minimal (36). On supercoiled templates, this effect can be enhanced by an additional subunit called δ and the two proteins promote more efficient cycling of RNAP. Furthermore, HelD appears to increase the transcription rate by stimulating

elongation. Finally, we demonstrated that the absence of HelD prolongs the lag phase of growth in a similar manner as the absence of δ does. This phenotype negatively affects the ability of the cell to rapidly react to nutritional changes in the environment.

Far western blotting experiments indicate that HelD binds to RNAP core on the downstream side, in close proximity to the secondary channel (Figure 3F). Interestingly, RapA, a putative helicase that stimulates transcription in an ATP-dependent manner in *E. coli* was found to cross-link to β' and α subunits on *E. coli* RNAP (37). These results are at low resolution but they are consistent with the possibility that HelD and RapA may bind to the same region of their respective RNAPs. Future studies will have to address the exact mode of binding of these two proteins.

RapA is a putative helicase, and, similarly to HelD, no helicase activity was detected for this protein (38). Further, both HelD from *B. subtilis* and RapA of *Pseudomonas putida* (39) bind to core RNAP although RapA of *E. coli* was shown to bind to the holoenzyme containing the main sigma factor (37,39). HelD and RapA, however, belong to different protein families with no significant amino acid sequence homology. Closer sequence analysis suggests that even if both proteins use ATP-binding domains, the structures of these domains and also of the whole proteins are likely entirely unrelated. The two proteins further differ in their effects during UV-induced DNA damage when in *B. subtilis* the lack of HelD was reported to have a negative effect (10), whereas the lack of RapA had no effect in *E. coli* (37). Nevertheless, both proteins stimulate transcription in an ATP-dependent manner, and HelD can be further assisted in this function by a small subunit of RNAP, δ , which is absent from gram-negative bacteria. Interestingly, both δ and HelD are conserved in the industrially and medically important Firmicutes. The stimulatory effect of RapA was shown to be caused by enhancing of the transcriptional cycling by releasing RNAP from the DNA of posttermination complexes (30,34), and possibly also by assisting with reading through RNA–DNA hybrids (34). HelD and δ enhance the release of stalled RNAP from DNA and this, at least partially, contributes to the more efficient cycling of transcription. The character of the RNAP–DNA complexes is not apparent but it seems that it is not a posttermination complex, as the presence or absence of the terminator had no effect (Figure 6B and C). Finally, it appears that HelD may also contribute to more efficient elongation. Interestingly, the level of expression of HelD inversely correlates with the level of expression of topoisomerases TopA and TopB (9) that are known to affect transcription elongation (9,40). The elongation effect is unlikely to be due to a more efficient reading through of RNA–DNA hybrids, such as R-loops, as experiments with short transcribed regions seem to argue against this possibility. Further studies will be required to address the mechanistic details of the interplay between HelD, δ , RNAP and nucleic acids.

The phenotype of the HelD-null strain is not dramatic but it is likely that the prolonged lag phase would adversely affect the competitive fitness of the cell in nature.

Further, it is possible that some other proteins, such as the PcrA helicase (7,41), may have overlapping functions with HelD and future studies will be required to decipher the role of these RNAP-associated helicases or helicase-like proteins in transcription. In conclusion, HelD is a novel type of RNAP-associated protein with an important role in transcription.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENT

The authors thank the National BioResource Project (NIG, Japan) for the *B. subtilis* HelD knockout strain MGNA-A456.

FUNDING

Czech Science Foundation [P302/11/0855, P305/12/G034, 13-16842S]; Science Foundation of Charles University [GAUK 425311]; Ministry of Education, Youth and Sports of the Czech Republic [EE2.3.30.0029]; European Regional Development Fund [BIOCEV CZ.1.05/1.1.00/02.0109]; National Health and Medical Research Council, Australia [APP1021479 to P.L.]; Operational Program Prague—Competitiveness [Project No.: CZ.2.16/3.1.00/24023 toward ‘Prague Infrastructure for Structure Biology and Metabolomics’]. Funding for open access charges: Czech Science Foundation [P302/11/0855, P305/12/G034, 13-16842S].

Conflict of interest statement. None declared.

REFERENCES

- Osterberg,S., del Peso-Santos,T. and Shingler,V. (2011) Regulation of alternative sigma factor use. *Annu. Rev. Microbiol.*, **65**, 37–55.
- Juang,Y.L. and Helmann,J.D. (1994) The delta subunit of *Bacillus subtilis* RNA polymerase. An allosteric effector of the initiation and core-recycling phases of transcription. *J. Mol. Biol.*, **239**, 1–14.
- Lopez de Saro,F.J., Woody,A.Y. and Helmann,J.D. (1995) Structural analysis of the *Bacillus subtilis* delta factor: a protein polyanion which displaces RNA from RNA polymerase. *J. Mol. Biol.*, **252**, 189–202.
- Epshtein,V., Dutta,D., Wade,J. and Nudler,E. (2010) An allosteric mechanism of Rho-dependent transcription termination. *Nature*, **463**, 245–249.
- Toulme,F., Mosrin-Huaman,C., Sparkowski,J., Das,A., Leng,M. and Rahmouni,A.R. (2000) GreA and GreB proteins revive backtracked RNA polymerase *in vivo* by promoting transcript trimming. *EMBO J.*, **19**, 6853–6859.
- Yang,X., Molimau,S., Doherty,G.P., Johnston,E.B., Marles-Wright,J., Rothnagel,R., Hankamer,B., Lewis,R.J. and Lewis,P.J. (2009) The structure of bacterial RNA polymerase in complex with the essential transcription elongation factor NusA. *EMBO Rep.*, **10**, 997–1002.
- Delumeau,O., Lecointe,F., Muntel,J., Guillot,A., Guedon,E., Monnet,V., Hecker,M., Becher,D., Polard,P. and Noirot,P. (2011) The dynamic protein partnership of RNA polymerase in *Bacillus subtilis*. *Proteomics*, **11**, 2992–3001.

8. Yang, W. (2010) Lessons learned from UvrD helicase: mechanism for directional movement. *Annu. Rev. Biophys.*, **39**, 367–385.
9. Nicolas, P., Mader, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., Bidnenko, E., Marchadier, E., Hoebeke, M., Aymerich, S. *et al.* (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science*, **335**, 1103–1106.
10. Carrasco, B., Fernandez, S., Petit, M.A. and Alonso, J.C. (2001) Genetic recombination in *Bacillus subtilis* 168: effect of DeltahelD on DNA repair and homologous recombination. *J. Bacteriol.*, **183**, 5772–5777.
11. Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.*, **166**, 557–580.
12. Dubnau, D. and Davidoff-Abelson, R. (1971) Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. Formation and properties of the donor-recipient complex. *J. Mol. Biol.*, **56**, 209–221.
13. Qi, Y. and Hulett, F.M. (1998) PhoP-P and RNA polymerase sigmaA holoenzyme are sufficient for transcription of Pho regulon promoters in *Bacillus subtilis*: PhoP-P activator sites within the coding region stimulate transcription *in vitro*. *Mol. Microbiol.*, **28**, 1187–1197.
14. Kobayashi, K., Ehrlich, S.D., Albertini, A., Amati, G., Andersen, K.K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P. *et al.* (2003) Essential *Bacillus subtilis* genes. *Proc. Natl Acad. Sci. USA*, **100**, 4678–4683.
15. Rabatinova, A., Sanderova, H., Matejkova, J.J., Korelusova, J., Sojka, L., Barvik, I., Papouskova, V., Sklenar, V., Zidek, L. and Krasny, L. (2013) The delta subunit of RNA polymerase is required for rapid changes in gene expression and competitive fitness of the cell. *J. Bacteriol.*, **195**, 2603–2611.
16. Chang, B.Y. and Doi, R.H. (1990) Overproduction, purification, and characterization of *Bacillus subtilis* RNA polymerase sigma A factor. *J. Bacteriol.*, **172**, 3257–3263.
17. Ross, W., Thompson, J.F., Newlands, J.T. and Gourse, R.L. (1990) *E. coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. *EMBO J.*, **9**, 3733–3742.
18. Krasny, L. and Gourse, R.L. (2004) An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. *EMBO J.*, **23**, 4473–4483.
19. Johnston, E.B., Lewis, P.J. and Griffith, R. (2009) The interaction of *Bacillus subtilis* sigmaA with RNA polymerase. *Protein Sci.*, **18**, 2287–2297.
20. Anborgh, P.H., Parmeggiani, A. and Jonak, J. (1992) Site-directed mutagenesis of elongation factor Tu. The functional and structural role of residue Cys81. *Eur. J. Biochem.*, **208**, 251–257.
21. Gaal, T., Ross, W., Estrem, S.T., Nguyen, L.H., Burgess, R.R. and Gourse, R.L. (2001) Promoter recognition and discrimination by E sigmaS RNA polymerase. *Mol. Microbiol.*, **42**, 939–954.
22. Barker, M.M., Gaal, T. and Gourse, R.L. (2001) Mechanism of regulation of transcription initiation by ppGpp. II. Models for positive control based on properties of RNAP mutants and competition for RNAP. *J. Mol. Biol.*, **305**, 689–702.
23. Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. and Madden, T.L. (2008) BLAST+: architecture and applications. *BMC Bioinformatics*, **10**, 421.
24. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403–410.
25. Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R. *et al.* (2007) Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**, 2947–2948.
26. Anthony, L.C., Artsimovitch, I., Svetlov, V., Landick, R. and Burgess, R.R. (2000) Rapid purification of His(6)-tagged *Bacillus subtilis* core RNA polymerase. *Protein Expr. Purif.*, **19**, 350–354.
27. Lee, J.Y. and Yang, W. (2006) UvrD helicase unwinds DNA one base pair at a time by a two-part power stroke. *Cell*, **127**, 1349–1360.
28. Cao, Z. and Julin, D.A. (2009) Characterization *in vitro* and *in vivo* of the DNA helicase encoded by *Deinococcus radiodurans* locus DR1572. *DNA Repair (Amst)*, **8**, 612–619.
29. Vassilyev, D.G., Sekine, S., Laptenko, O., Lee, J., Vassilyeva, M.N., Borukhov, S. and Yokoyama, S. (2002) Crystal structure of a bacterial RNA polymerase holoenzyme at 2.6 Å resolution. *Nature*, **417**, 712–719.
30. Sukhodolets, M.V., Cabrera, J.E., Zhi, H. and Jin, D.J. (2001) RapA, a bacterial homolog of SWI2/SNF2, stimulates RNA polymerase recycling in transcription. *Genes Dev.*, **15**, 3330–3341.
31. Epshtein, V., Toulme, F., Rahmouni, A.R., Borukhov, S. and Nudler, E. (2003) Transcription through the roadblocks: the role of RNA polymerase cooperation. *EMBO J.*, **22**, 4719–4727.
32. Holmberg, C. and Rutberg, B. (1991) Expression of the gene encoding glycerol-3-phosphate dehydrogenase (glpD) in *Bacillus subtilis* is controlled by antitermination. *Mol. Microbiol.*, **5**, 2891–2900.
33. Gowrishankar, J., Leela, J.K. and Anupama, K. (2013) R-loops in bacterial transcription: their causes and consequences. *Transcription*, **4**, 153–157.
34. McKinley, B.A. and Sukhodolets, M.V. (2007) *Escherichia coli* RNA polymerase-associated SWI/SNF protein RapA: evidence for RNA-directed binding and remodeling activity. *Nucleic Acids Res.*, **35**, 7044–7060.
35. Vassilyev, D.G., Vassilyeva, M.N., Zhang, J., Palangat, M., Artsimovitch, I. and Landick, R. (2007) Structural basis for substrate loading in bacterial RNA polymerase. *Nature*, **448**, 163–168.
36. Yawn, B., Zhang, L., Mura, C. and Sukhodolets, M.V. (2009) RapA, the SWI/SNF subunit of *Escherichia coli* RNA polymerase, promotes the release of nascent RNA from transcription complexes. *Biochemistry*, **48**, 7794–7806.
37. Sukhodolets, M.V. and Jin, D.J. (2000) Interaction between RNA polymerase and RapA, a bacterial homolog of the SWI/SNF protein family. *J. Biol. Chem.*, **275**, 22090–22097.
38. Sukhodolets, M.V. and Jin, D.J. (1998) RapA, a novel RNA polymerase-associated protein, is a bacterial homolog of SWI2/SNF2. *J. Biol. Chem.*, **273**, 7018–7023.
39. Stec-Dziedzic, E., Lyzen, R., Skarfstad, E., Shingler, V. and Szalewska-Palasz, A. (2013) Characterization of the transcriptional stimulatory properties of the *Pseudomonas putida* RapA protein. *Biochim. Biophys. Acta*, **1829**, 219–230.
40. Baranello, L., Kouzine, F. and Levens, D. (2013) DNA Topoisomerases: beyond the standard role. *Transcription*, **4** doi: 10.4161/trns.26598.
41. Gwynn, E.J., Smith, A.J., Guy, C.P., Savery, N.J., McGlynn, P. and Dillingham, M.S. (2013) The conserved C-terminus of the PcrA/UvrD helicase interacts directly with RNA polymerase. *PLoS One*, **8**, e78141.