RESEARCH LETTER

Region C of the *Escherichia coli* heat shock sigma factor RpoH (σ^{32}) contains a turnover element for proteolysis by the FtsH protease

Markus Obrist[1,2], Sina Langklotz[2], Sonja Milek[2], Frank Führer[2] & Franz Narberhaus[2]

[1]Institute of Microbiology, ETH Zürich, Switzerland; and [2]Microbial Biology, Ruhr-University Bochum, Germany

Correspondence: Franz Narberhaus, Lehrstuhl für Biologie der Mikroorganismen, Ruhr-Universität Bochum, Universitätsstraße 150, NDEF 06/783, D-44780 Bochum, Germany. Tel.: +49 234 322 3100; fax: +49 234 321 4620; e-mail: franz.narberhaus@rub.de

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Abstract

Transcription of most heat shock genes in *Escherichia coli* is initiated by the alternative sigma factor σ^{32} (RpoH). At physiological temperatures, RpoH is rapidly degraded by chaperone-mediated FtsH-dependent proteolysis. Several RpoH residues critical for degradation are located in the highly conserved region 2.1. However, additional residues were predicted to be involved in this process. We introduced mutations in region C of RpoH and found that a double mutation (A131E, K134V) significantly stabilized RpoH against degradation by the FtsH protease. Single-point mutations at these positions only showed a slight effect on RpoH stability. Both double and single amino acid substitutions did not impair sigma factor activity as demonstrated by a *groE–lacZ* reporter gene fusion, Western blot analysis of heat shock gene expression and increased heat tolerance in the presence of these proteins. Combined mutations in regions 2.1 and C further stabilized RpoH. We also demonstrate that an RpoH fragment composed of residues 37–147 (including regions 2.1 and C) is degraded in an FtsH-dependent manner. We conclude that in addition to the previously described turnover element in region 2.1, a previously postulated second region important for proteolysis of RpoH by FtsH lies in region C of the sigma factor.

Introduction

The adaptation to various stress conditions in *Escherichia coli* requires alternative sigma factors. Control of their cellular level via proteolysis is a common feature. Well-known examples are σ^{32}, σ^{S} and σ^{F} (Gottesman, 2003; Hengge & Bukau, 2003; Barembruch & Hengge, 2007). Although these sigma factors share the principle of regulated proteolysis, the mechanisms and proteases involved in these processes are different.

RpoH levels increase under heat shock conditions and the sigma factor initiates the transcription of >30 heat shock genes. These genes mainly code for chaperones and proteases counteracting the accumulation of unfolded proteins at high temperatures (Yura *et al.*, 2000). Regulation of the heat shock response at the post-translational level occurs via controlled proteolysis of RpoH involving different proteases (e.g. FtsH, Lon, HslVU) (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995; Kanemori *et al.*, 1997) and chaperones (DnaKJ, GroESL) (Gamer *et al.*, 1992; Blaszczak *et al.*, 1995; Guisbert *et al.*, 2004). At physiological temperatures, DnaK and DnaJ binding to RpoH makes it accessible to FtsH-dependent proteolysis. Unfolded or aggregated proteins titrate DnaK and DnaJ away from RpoH, leading to stabilization of the sigma factor, which in turn associates with the RNA polymerase core enzyme (RNAP) (Gamer *et al.*, 1992; Horikoshi *et al.*, 2004). Although point mutations in region 2.1 of RpoH led to stabilization of the protein (Horikoshi *et al.*, 2004; Obrist & Narberhaus, 2005; Yura *et al.*, 2007), we showed previously that this turnover element is not sufficient for efficient proteolysis by FtsH (Obrist *et al.*, 2007).

The closely related sigma factor RpoS (σ^{S}) is induced by diverse stresses such as starvation in the stationary phase, hyperosmolarity, oxidative stress or ultraviolet light (Hengge-Aronis, 2002). The amount of RpoS under nonstress conditions is tightly regulated through degradation by the ClpXP protease (Muffler *et al.*, 1996; Pratt & Silhavy, 1996; Schweder *et al.*, 1996; Zhou *et al.*, 2001). RpoS proteolysis depends on the phosphorylated adapter protein RssB, which specifically recognizes three amino acids in region 2.5 of the sigma factor, namely K173, E174 and V177 (Pratt & Silhavy, 1996; Becker *et al.*, 1999). Residue K173

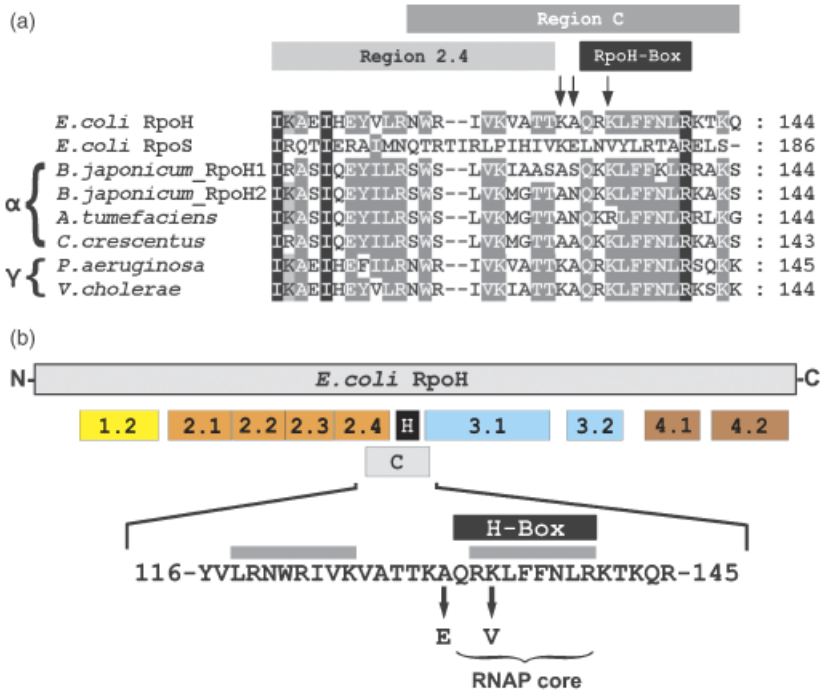

Fig. 1. Sequence alignment and conserved regions of *Escherichia coli* RpoH. (a) Sequences of selected RpoH homologs from *Alpha-* and *Gammaproteobacteria* are aligned with *E. coli* RpoH and RpoS. Arrows point out amino acids corresponding to the RssB-binding site of RpoS. (b) Organization of conserved regions in RpoH and detailed illustration of region C. Putative DnaK-binding sites are labeled in gray boxes (McCarty *et al.*, 1996).

(the first arrow in Fig. 1a) is crucial for binding of RssB to RpoS, whereas E174 and V177 have auxiliary functions. However, this motif is not sufficient for targeting the RssB–RpoS complex to the protease ClpXP (Stüdemann *et al.*, 2003). Another site in the very N-terminal region around position nine of RpoS is necessary for degradation. This motif, which is otherwise cryptic and inaccessible, is exposed to the surface upon binding of RssB to RpoS and serves as a binding site for ClpX promoting proteolysis by ClpXP (Stüdemann *et al.*, 2003).

Unlike RpoS, RpoH does not have a region 2.5 but contains the so-called RpoH box in region C instead (Fig. 1a and b). This highly conserved stretch of nine amino acids is unique to the RpoH family and is involved in binding of RNAP (Nakahigashi *et al.*, 1995; Joo *et al.*, 1998; Arsène *et al.*, 1999). We found that the introduction of residues equivalent to the RssB-binding site of RpoS into region C of RpoH affected the stability but not the activity of the heat shock sigma factor. Residues A131 and K134 of RpoH were identified as a second turnover element critical for degradation by the FtsH protease.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. All strains were grown in Luria–Bertani (LB) medium (Sambrook & Russell, 2001). Antibiotics and chemicals were added as follows: ampicillin (100–200 μg mL^{-1}), chloramphenicol (200 μg mL^{-1}), kanamycin (30–50 μg mL^{-1}) and isopropyl-thio-β-D-galactoside (IPTG) (1 mM final concentration).

Plasmids and recombinant DNA techniques

Plasmids used in this study are presented in Table 1, and oligonucleotide sequences are shown in Table 2. DNA manipulations were performed according to standard protocols (Sambrook & Russell, 2001). To introduce the RssB motif (A131E/K134V) of RpoS into RpoH, pBAD18-RpoHΔ11 (Regine Hengge, Berlin), carrying a truncated *rpoH* allele with the corresponding mutations, was digested with NcoI/PstI and the isolated 476-bp fragment carrying the mutations was introduced into the pEC5217 replacing the wild-type fragment. The new plasmid was named pEC5386. The mutations that code for the single amino acid exchanges in RpoH were introduced into pEC5217 carrying the wild-type allele by site-directed mutagenesis (Quik-Change™ Site-Directed Mutagenesis Kit, Stratagene). To construct pBO703 and pBO716, the template pEC5217 and the primer pairs MO31/MO32 and MO41/MO42 were used, respectively (Table 2). Histidine-tagged RpoH proteins were expressed from pEC5217 derivatives, in which internal 526 bp MluI/PstI or 476 bp NcoI/PstI fragments were replaced by the corresponding mutated fragments (Table 1). A combination of point mutations in regions 2.1 and C was achieved by introducing the 385 bp EcoNI/PstI fragment from pEC5386, carrying both mutations in region C, into pEC5356 and pEC5357, replacing the

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference or source	Oligonucleotides
E. coli strains			
DH5α	*supE44*, Δ*lacU169* (Ψ80*lacZ* ΔM15), *hsdR17, recA1, gyrA96, thi1, relA1*	Sambrook & Russell (2001)	
Δ*rssB*	MC4100 F⁻Δ(*arg-lac*)*U169 araD139 rpsL150 ptsF25 flbB5301 rbsR deoC relA1* Δ*rssAB*::*cat*	Muffler *et al*. (1996)	
Δ*rpoH*	MC4100 Δ*rpoH30*::*kan zhf-50*::Tn10 [λpF13-(*groE*$_p$-*lacZ*$^+$)]	Zhou *et al*. (1988)	
BL21 DE3	F$^-$, *ompT, gal, (dcm), (lon), hsdS*$_B$ (r$_B$- m$_B$-), λ(DE3)	Karata *et al*. (1999), Studier *et al*. (1990)	
AR5088	BL21[DE3] *sfhC21 zad220*::Tn10 Δ*ftsH3*::*kan*	Karata *et al*. (1999)	
Plasmids			
pEC5217	pUC18 carrying *E. coli rpoH*, Ap^R	Narberhaus & Balsiger (2003)	
pBAD18-RpoH Δ11	pBAD18 carrying *E. coli rpoH* with A131E-A134V lacking the last 11 residues, Ap	Regine Hengge, unpublished	
pEC5356	pEC5217 derivative coding for RpoH-A50, Ap^R	Obrist & Narberhaus (2005)	
pEC5357	pEC5217 derivative coding for RpoH-L47Q, Ap^R	Obrist & Narberhaus (2005)	
pEC5386	pEC5217 derivative coding for RpoH-A131E-K134V, Ap^R	This study	
pBO703	pEC5217 derivative coding for RpoH-A131E, Ap^R	This study	MO31/MO32
pBO716	pEC5217 derivative coding for RpoH-K134V, Ap^R	This study	MO41/MO42
pBO717	pEC5217 derivative coding for RpoH-A50V-A131E-K134V, Ap^R	This study	
pBO718	pEC5217 derivative coding for RpoH-L47Q-A131E-K134V, Ap^R	This study	
pEC5261	pET24b coding for RpoH-His$_6$, Km^R	Obrist *et al*. (2007)	
pBO704	pEC5261 derivative coding for RpoH-A131E-His$_6$, Km^R	This study	
pBO720	pEC5261 derivative coding for RpoH-K134V-His$_6$, Km^R	This study	
pEC5398	pEC5261 derivative coding for RpoH-A131E-K134V-His$_6$, Km^R	This study	
pBO974	pET24b(+) derivative coding for RpoH-37-147-His$_6$, Ap^R	This study	MO39/FF111
pBO998	pET24b(+) derivative coding for RpoH-His$_6$, Ap^R	This study	

Table 2. Oligonucleotides used in this study

Name	Sequence (5′ → 3′)	Restriction site
MO31	GTTGCGACCACCAAAGAGCAGCGCAAACTGTTC	
MO32	GAACAGTTTGCGCTGCTCTTTGGTGGTCGCAAC	
MO39	aaaagctagcCTGGCTGAAAAGCTGCATTACC	NheI
MO41	GCGACCACCAAAGCGCAACGCGTACTGTTCTTCAACCTG	MluI
MO42	CAGGTTGAAGAACAGTACGCGTTGCGCTTTGGTGGTCGC	MluI
FF111	TTCTCGAGGCCCAGACGCTGCTTGGT	XhoI

Introduced restriction sites are underlined once; exchanged nucleotides for site-directed mutagenesis are underlined twice.

wild-type fragment and resulting in plasmids pBO717 and pBO718, respectively. Truncated *rpoH* coding for RpoH-37-147 were amplified using primers MO39 and FF111 (Table 1) with pEC5217 as a template. The 339 bp fragment was cloned via NheI/XhoI into a pET24b(+) derivative confirming ampicillin resistance. The final construct codes for RpoH-37-147 with a C-terminal histidine tag.

Analysis of RpoH sigma factor activity

Escherichia coli Δ*rpoH* cells were transformed with plasmids coding for the RpoH variants, which should be tested for sigma factor activity. The Δ*rpoH* strain carries a chromosomally coded and RpoH-dependent *groE–lacZ* fusion. The cells were grown to the mid-logarithmic phase in LB medium containing 0.5 mM IPTG for protein expression. The activity of the sigma factor was then defined by measuring the β-galactosidase activity as described previously (Miller, 1972).

In vivo degradation assay and immunoblot analysis

In vivo degradation and sample preparation of *E. coli* Δ*rpoH*, Δ*rssB*, BL21 [DE3] and AR5088 cells expressing plasmid-encoded RpoH derivatives were performed as described previously (Obrist & Narberhaus, 2005). Protein samples were separated by 12–15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Hybond-C; Amersham) using standard protocols (Sambrook & Russell, 2001). RpoH proteins were detected using a polyclonal rabbit anti-RpoH

antibody and a goat anti-rabbit immunoglobulin G(H+L)–HRP conjugate (Bio-Rad) as the secondary antibody, followed by chemiluminescence detection (SuperSignal, Pierce). Autoradiography films (ECL Hyperfilm, Amersham) were scanned and RpoH bands were quantified using the AIDA program (Advanced Image Data Analyzer, version 4.13, raytest). Detection of RpoH and RpoH-L37-G147 in the BL21 [DE3] backround was realized using a Penta-His–HRP conjugate (Qiagen). Chemiluminescence signals were detected using the ECL Western blotting system (Amersham) and a ChemiImager^{TM} Ready (Alpha Innotech), and quantified with AlphaEase^{TM} FC software (Alpha Innotech).

Protein expression and copurification

Plasmids encoding C-terminally histidine-tagged RpoH proteins were freshly transformed into *E. coli* BL21 cells. Hundred milliliters of cell cultures were grown at 37 °C to an $OD_{600\,nm}$ of 0.6 before production of recombinant proteins was induced with 0.02 mM IPTG. Cells were harvested after incubation for 2 h at 30 °C. Pellets were dissolved in 4 mL binding buffer (0.5 M KCl, 20 mM Tris-HCl and 5 mM imidazole, pH 7.9), 1 mM phenylmethylsulfonylfluoride and 10 μg mL^{-1} DNAseI were added and cells were disrupted by sonication (6 × 15 s at 20% output level; Branson Sonifier 250). Cell extracts were centrifuged at 15 000 ***g*** for 30 min and the supernatant was loaded onto a 0.5 mL Ni-NTA column (Qiagen). The proteins were washed with 1.5 mL washing buffer and eluted with 1 mL elution buffer, consisting of binding buffer supplemented with increasing imidazole concentrations: W1 (5 mM), W2 (25 mM), W3 and W4 (50 mM), E1 (100 mM), E2 (125 mM), E3 (150 mM) and E4 (1 M). From each fraction, a 15-μL sample was analyzed by 12% SDS-PAGE and proteins were visualized by immunodetection with specific antisera. The polyclonal antisera from rabbit were diluted as follows: anti-DnaK, 1 : 3000; anti-DnaJ, 1 : 2000; and anti-GroEL, 1 : 5000.

Structural modeling of RpoH and RpoS

RpoH and RpoS were modeled using the SWISS-MODEL SERVER program (version 36.0003), which calculates structures on the basis of homology (Guex & Peitsch, 1997; Schwede *et al.*, 2003). Modeling was based on the solved structures from a σ^{70} fragment of *E. coli* RNA polymerase [protein data bank (PDB) entry 1sig], a fragment containing regions 1.2–3.1 from the *Thermus aquaticus* RNA polymerase sigma subunit (PDB entry 1ku2a), the *Thermus thermophilus* RNA polymerase holoenzyme in complex with an inhibitor tagetitoxin (PDB entry 2be5p) and from the *T. thermophilus* RNA polymerase holoenzyme without inhibitor (PDB entry 1iw7F). The entire protein sequences of RpoH and RpoS were submitted. The structure models were graphically prepared with the program SWISS PDB VIEWER (version 3.7) (Guex & Peitsch, 1997).

Results and discussion

Amino acid substitutions in region C stabilize RpoH

In search of mutations that might affect the stability of RpoH, we changed alanine 131 into glutamate and lysine 134 into valine by site-directed mutagenesis to engineer a region equivalent to the RssB-binding site of RpoS (Fig. 1a). The lysine residue corresponding to K173 of RpoS is already present in RpoH at position 130. Degradation experiments in *E. coli* Δ*rpoH* cells revealed that the RpoH-A131E-K134V protein was stabilized 10-fold against proteolysis. It reached a calculated half-life of 20 min (Fig. 2a and b). The stability of the singular substitutions A131E and K134V was only slightly enhanced, resulting in half-lives of 3.3 and 2.3 min, respectively. This might explain why region C of RpoH containing a second turnover element escaped identification in all of the three previously performed independent genetic screens (Horikoshi *et al.*, 2004; Obrist & Narberhaus, 2005; Yura *et al.*, 2007).

Based on the finding that two point mutations localized in region C stabilized RpoH against degradation by FtsH, it was tempting to speculate that the combination of these mutations with mutations in region 2.1 would further stabilize the heat shock sigma factor. Mutations in region 2.1 alone result in moderately stabilized proteins, for example L47Q has a half-life of 8.6 min and A50V of 6.2 min (Obrist & Narberhaus, 2005). As expected, the RpoH triple mutants with amino acid substitutions in regions 2.1 and C exhibited a much higher stability than all currently known RpoH derivatives (Fig. 2a and b). In particular, RpoH-L47Q-A131E-K134V was barely degraded with a half-life of > 40 min.

To exclude that RssB has an influence on the stability of RpoH mutated at positions A131E and K134V, degradation experiments were also performed in Δ*rssB* cells (Fig. 2c). The chromosomally encoded *rpoH* wild-type allele in this strain is not expected to interfere with detection of the plasmid-encoded RpoH proteins, as it is known to be degraded rapidly. The stability of the mutated RpoH variants in the Δ*rssB* strain was comparable to the stability observed in *rssb*$^{+}$ strains, indicating that RssB is not involved in stabilization of RpoH-A131E-K134V.

The stability of RpoH is affected by a cellular protein network comprised of at least the RNAP, the FtsH protease and the chaperones DnaK, DnaJ and GroEL (Gamer *et al.*, 1992; Blaszczak *et al.*, 1995; Nakahigashi *et al.*, 1995; Tomoyasu *et al.*, 1995; Joo *et al.*, 1998; Arsène *et al.*, 1999; Guisbert *et al.*, 2004). Altered RpoH stability might be

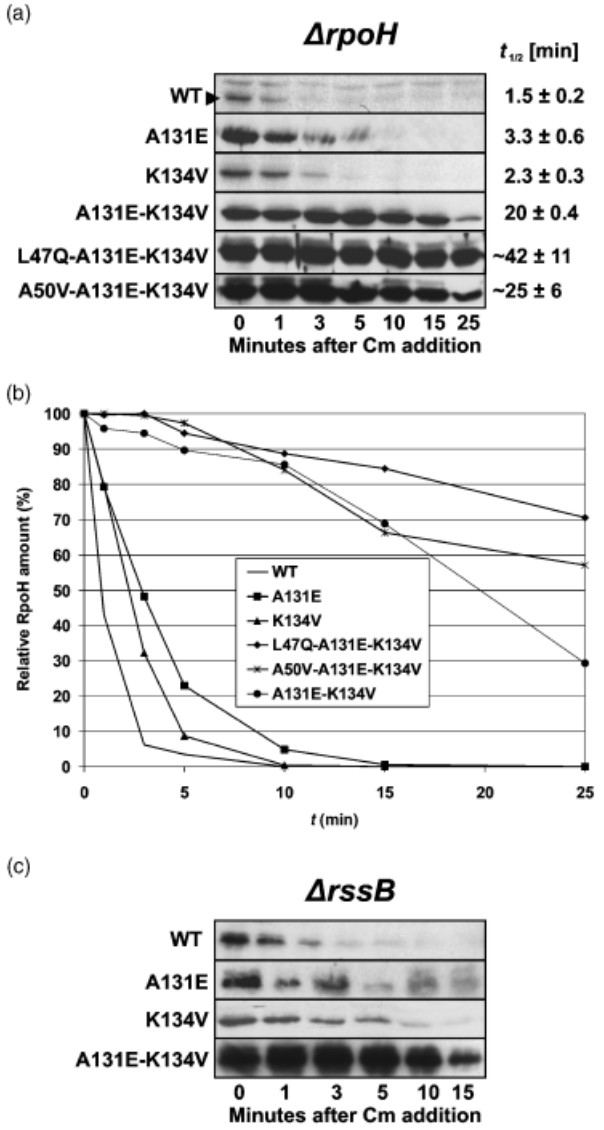

Fig. 2. Amino acid substitutions in region C stabilize RpoH. Degradation of RpoH derivatives was analyzed by immunodetection after protein synthesis was blocked by addition of chloramphenicol in *Escherichia coli* Δ*rpoH* (a) and Δ*rssB* (c). RpoH bands were quantified and half-lives were calculated in minutes from three independent experiments.

caused by enhanced or decreased interaction with any of these partners. A role of region C in DnaK binding is disputed (Nagai *et al.*, 1994; McCarty *et al.*, 1996; Joo *et al.*, 1998; Arsène *et al.*, 1999; Tatsuta *et al.*, 2000). Hence, we conducted pull-down experiments with histidine-tagged RpoH-A131E-K134V, RpoH-A131E and RpoH-K134V. DnaK, DnaJ and GroEL did not bind to the column resin in the absence of RpoH proteins and RpoH eluted mainly in fractions E2 to E4 (data not shown; Obrist *et al.*, 2007). Because all three mutated RpoH proteins bound DnaK, DnaJ and GroEL like RpoH-WT (Fig. 3), we conclude that (i) residues A131 and K134 are not involved in chaperone binding and (ii) stabilization of RpoH-A131-K134 cannot be attributed to altered interaction with the DnaK, DnaJ and GroEL chaperone network. The results fully agree with previous reports showing that region C is not involved in chaperone binding (Arsène *et al.*, 1999).

Amino acid substitutions in region C do not impair the sigma factor activity of RpoH

Although the correlation is not always very strict, resistance toward degradation by the FtsH protease is usually accompanied by elevated RpoH activity *in vivo* (Horikoshi *et al.*, 2004; Obrist & Narberhaus, 2005; Yura *et al.*, 2007). Because certain residues in region C play a role in the interaction with RNAP (Nakahigashi *et al.*, 1995; Joo *et al.*, 1998; Arsène *et al.*, 1999), it was important to test whether the sigma factor activity was affected by mutations in this region.

Plasmids expressing various *rpoH* alleles were transformed into *E. coli* Δ*rpoH* cells, and the corresponding sigma factor activities were measured using the

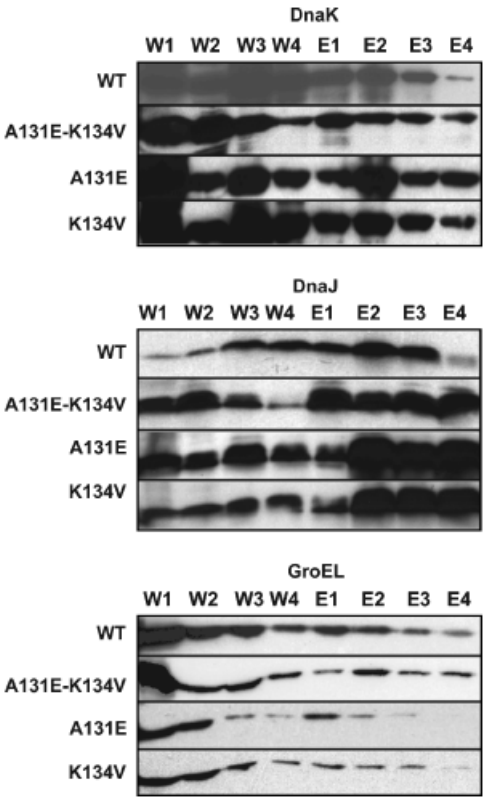

Fig. 3. Coelution of chaperones with RpoH derivatives. RpoH proteins were purified by Ni-NTA chromatography. Protein bands of DnaK, DnaJ and GroEL in the washing (W) and elution (E) fractions were visualized by immunodetection. Imidazole concentrations were as follows: W1 = 5 mM, W2 = 25 mM, W3 and W4 = 50 mM, E1 = 75 mM, E2 = 100 mM, E3 = 125 mM and E4 = 150 mM.

chromosomally integrated *groE–lacZ* fusion. RpoH-A131E-K134V showed a significantly elevated activity (Fig. 4a), indicating that the two-point mutations did not inactivate RpoH although they are close to the RNAP interaction site (Fig. 1b). The intermediate sigma factor activity of the single-point mutations ranging between wild-type RpoH and the double mutant suggests that the elevated *in vivo* activity of RpoH-A131E-K134V in the *groE-lacZ* assay results from an additive effect of both individual point mutations (Fig. 4a). The single-point mutations L47Q and A50V in region 2.1 of RpoH have previously been shown to enhance sigma factor activity in this assay (Obrist & Narberhaus, 2005). To investigate a possible cumulative effect between region 2.1 and C on the activity of RpoH, triple mutations were constructed. The already high sigma factor activity of RpoH-A131E-K134V was not further increased by addition of the point mutation L47Q or A50V (Fig. 4a). Although the stability and activity of RpoH are usually positively correlated, it has to be kept in mind that (i) the *groE–lacZ* activity test is semi-quantitative, (ii) not all RpoH variants (e.g. N83D) with higher activities are stabilized (Obrist & Narberhaus, 2005), (iii) the overall activity of all RpoH molecules in the cell might reach a maximum and (iv) that the correlation between activity and stability is not linear (c.f. K51N and I54N) (Yura *et al.*, 2007).

To further analyze the activity of the RpoH variants, we monitored the production of heat shock proteins in the Δ*rpoH* strain under the same conditions as in the β-galactosidase activity test. While the vector control (pUC18) shows no detectable production of heat shock proteins, induction of GroEL, DnaK, DnaJ, GrpE and the small heat shock proteins IbpA and B can be observed using Coomassie-stained polyacrylamide gels and Western blot analysis (Fig. 4b). It is evident that all RpoH variants are able to induce heat shock protein synthesis at least as efficiently as the wild-type RpoH.

Expression of the RpoH variants in the Δ*rpoH* strain also complemented the temperature-sensitive growth defect (Fig. 4c). The strain harboring the vector control did not survive at 30, 37 and 42 °C. All complemented Δ*rpoH* strains expressing either WT-RpoH or mutated RpoH proteins were able to grow at 30 °C or at even higher temperatures (Fig. 4c). Subtle differences in complementation efficiency, in particular poor complementation by the L47Q protein at 37 and 42 °C, remain unexplored. All three activity assays collectively show that the stabilized RpoH proteins are active

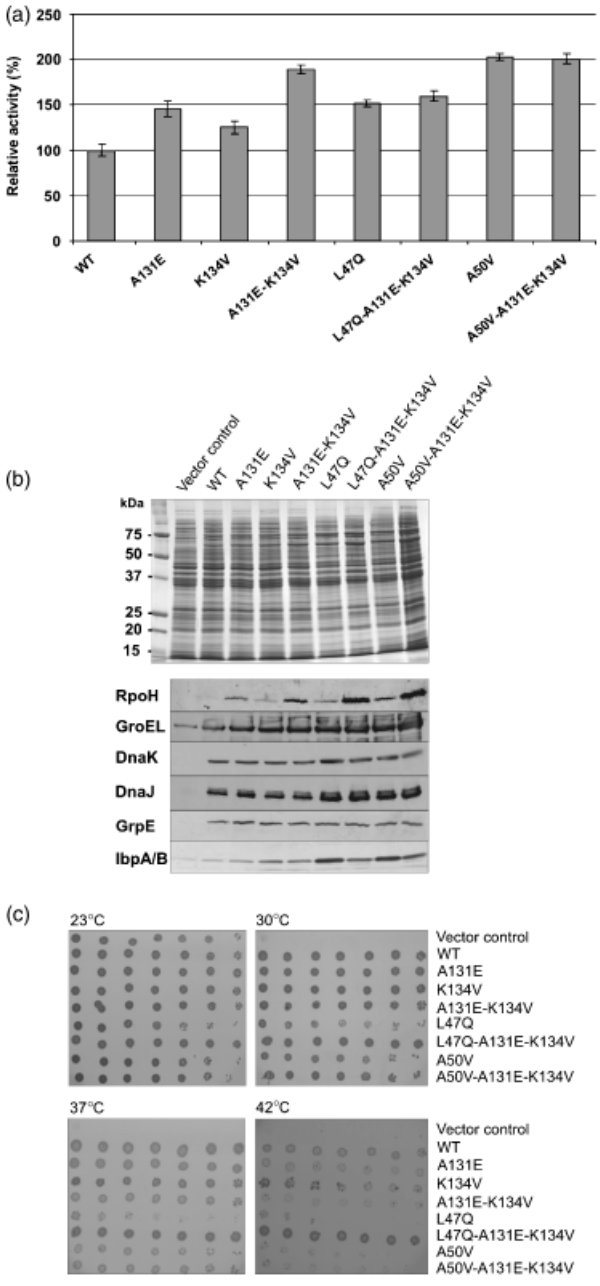

Fig. 4. RpoH variants carrying amino acid substitutions in region C are active as a sigma factor. (a) Activity was tested by β-galactosidase assays of different RpoH variants in *E. coli* Δ*rpoH* carrying a *groE–lacZ* fusion. The cells were grown to the mid-logarithmic phase in LB–ampicillin–kanamycin medium containing 0.5 mM IPTG for protein expression. Relative values from at least three independent assays including the SDs are shown. (b) Immunodetection of heat shock proteins in samples of cultures used for the activity test in (a). Crude cell extracts were diluted in protein sample buffer to equivalent optical densities and separated on 12–15% polyacrylamide gels. A Coomassie blue-stained gel is shown at the top. Identical gels were used for immunodetection of RpoH and the heat shock proteins GroEL, DnaK, DnaJ, GrpE and IbpA/B after Western transfer. (c) Expression of the RpoH variants in the Δ*rpoH* strain complements its temperature-sensitive growth. *Escherichia coli* Δ*rpoH* harboring pUC18 (vector control), pEC5217 (WT-RpoH) or plasmids coding for the indicated RpoH variants were grown overnight in liquid LB–ampicillin–kanamycin. Cultures (2 μL) adjusted to an $OD_{580\,nm} = 0.5$ and of consecutive 1 : 10 dilutions thereof were spotted from left to right on solid LB–ampicillin–kanamycin containing 0.2 mM IPTG and incubated for 2 days at the indicated temperatures.

sigma factors, indicating that the introduction of mutations in region C did not perturb the structural integrity of these proteins.

A minimal RpoH variant composed of residues L37–G147 (including region 2.1 and C) is a sufficient FtsH substrate

Having found that region 2.1 and region C are important turnover elements, we asked whether a minimal RpoH fragment (RpoH-37-147) containing these two regions would be sufficient to be degraded by FtsH. The RpoH fragment was expressed in *E. coli* BL21 [DE3] using the pET system and the stability of the protein was measured. As a control, the half-life of full-length RpoH was determined to be 2.7 min in this background (Fig. 5). RpoH-37-147 was degraded rapidly with a half-life of about 1 min. It is conceivable that the missing regions have a stabilizing effect on the wild-type protein due to intramolecular interactions. Such intramolecular interactions of the N-terminus were already shown to modulate DNA binding of sigma factors (Dombroski *et al.*, 1993). Degradation of RpoH-37-147 can be attributed to the FtsH protease, because the stability was increased 10-fold in an *ftsH* knockout strain in the BL21 [DE3] background. A degradation of RpoH-37-147 within the quality control pathway is unlikely because (i) the proteolysis is FtsH-dependent and (ii) the BL21 [DE3] strain is deficient in the expression of the major protein control protease Lon (Rosen *et al.*, 2002). A similar FtsH-dependent 10-fold stabilization is also typical for the full-length protein, indicating that the short fragment contains all major sites required for FtsH-mediated degradation of RpoH.

Region 2.1 and region C might serve as interaction surfaces for FtsH-mediated degradation

Region 2.1 of RpoH and other sigma factors of the Sigma70 family is predicted to be an α-helix (Malhotra *et al.*, 1996; Narberhaus & Balsiger, 2003; Obrist & Narberhaus, 2005). RpoH residues L47, A50 and I54, critical for degradation by FtsH are predicted to line up on one face of this helix (Fig. 6). The RpoH box is also predicted to be mostly α-helical. The RssB-binding motif of RpoS is also located within an α-helix and the critical residues K173, E174 and V177 are oriented in the same direction (Becker *et al.*, 1999) (Fig. 6). Structural modeling of RpoH, RpoS and RpoH-A131E-K134V predicts that introduction of the A131E and K134V mutations into RpoH causes an extension of the RpoH-box helix (Fig. 6 shown in yellow), positioning residues 131 and 134 slightly away from its original orientation. Hence, interaction with components of the proteolytic machinery might not be possible.

Region C is predicted to serve multiple functions in heat shock gene expression in *E. coli* mediating interaction with RNAP and with DnaK (Nagai *et al.*, 1994; Nakahigashi *et al.*, 1995; McCarty *et al.*, 1996; Joo *et al.*, 1998; Arsène *et al.*, 1999; Tatsuta *et al.*, 2000). According to structural models (based on crystal structures of σ^{70}), residues A131 and K134 of RpoH turn away from the RNAP-binding site (Fig. 6) which is also the case for the RssB-binding motif in RpoS (Fig. 6). As the introduction of point mutations did not deplete sigma factor activity (Fig. 4a–c), A131 and K134 do not seem to interact with the transcription machinery. The RpoH derivatives even showed an elevated activity in the *groE–lacZ* assay as it is common for stabilized RpoH proteins (Horikoshi *et al.*, 2004; Obrist & Narberhaus, 2005).

In addition to its important role in the proteolysis of RpoH, region 2.1 was recently described to be important for chaperone-mediated inactivation of the sigma factor (Yura *et al.*, 2007). RpoH mutants (i.e. I54N) did not show altered binding either to RNAP or to the members of the chaperone network DnaK, DnaJ and GroEL/S. Consequently, these mutants were proposed to be defective in an unknown regulatory step downstream of chaperone binding, which affects inactivation as well as degradation (Yura *et al.*, 2007). We cannot rule out that this is also the case for residues A131 and K134 of region C. However, residues A131 and K134 are roughly oriented in the same direction like L47, A50 and I54, which form the turnover element in region 2.1. Hence, we propose that both regions of RpoH interact with the same protein(s), probably FtsH itself.

Several FtsH substrates have been described so far, among them, RpoH, LpxC, SsrA, λCII and λCIII. It was repeatedly found that nonpolar residues are enriched in regions

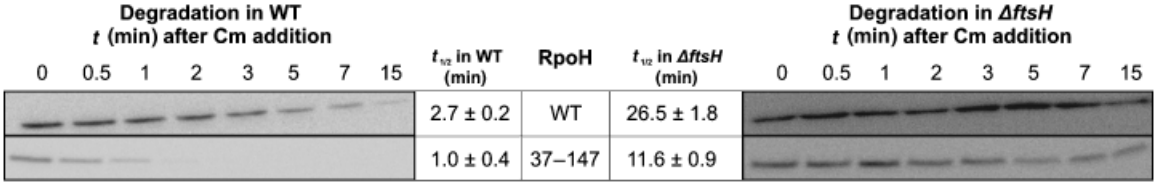

Fig. 5. Truncated RpoH composed of region 2.1 to C (L37–G147) serves as an FtsH substrate. Proteolysis of truncated RpoH derivatives was measured in *E. coli* BL21 [DE3] and the Δ*ftsH* strain AR5088 as described in Fig. 4.

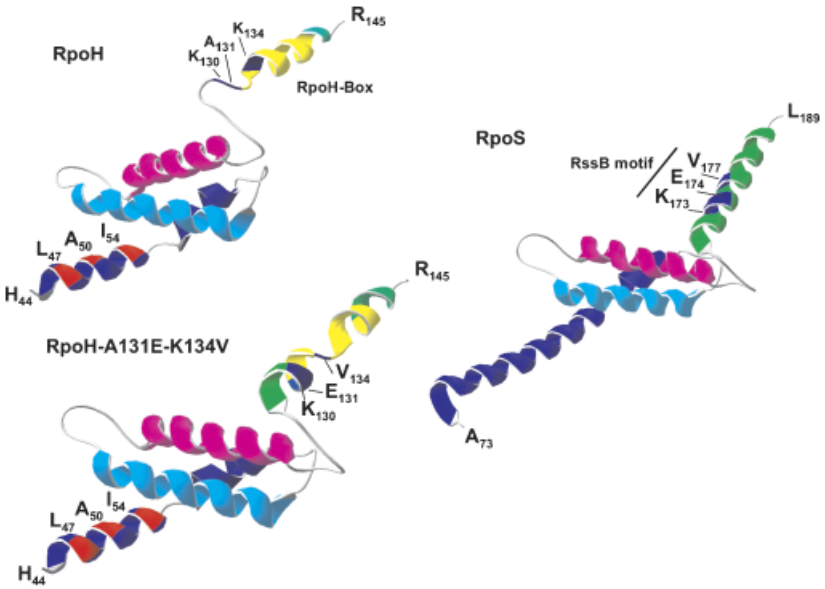

Fig. 6. Structure model of region 2 of *Escherichia coli* RpoH-WT, RpoH-A131E-K134V and RpoS. Modeling was performed as described in Materials and methods. Conserved α-helices are shown in an identical color in all three models.

important for turnover by FtsH. Exchange of nonpolar residues against polar amino acids often resulted in stabilization of substrate proteins (Herman *et al.*, 1998; Arsène *et al.*, 1999; Shotland *et al.*, 2000; Kobiler *et al.*, 2002; Horikoshi *et al.*, 2004; Führer *et al.*, 2006, 2007). In contrast to this common feature of many FtsH substrates, the situation in RpoH seems to be more complicated: (i) two distinct regions within RpoH are required for degradation; (ii) nonpolar as well as charged amino acids compose these turnover elements; (iii) exchange of nonpolar residues against a polar (i.e. L47Q; A131E) and *vice versa* in case of K134V renders RpoH stable. It is evident that substrate recognition by the FtsH protease and the regulation of the heat shock response is complex and far from being understood. Apparently, exact positioning of at least five residues in regions 2.1 and C of RpoH is critical for proteolysis by FtsH.

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References

Arsène F, Tomoyasu T, Mogk A, Schirra C, Schulze-Specking A & Bukau B (1999) Role of region C in regulation of the heat shock gene-specific sigma factor of *Escherichia coli*, σ^{32}. *J Bacteriol* **181**: 3552–3561.

Barembruch C & Hengge R (2007) Cellular levels and activity of the flagellar sigma factor FliA of *Escherichia coli* are controlled by FlgM-modulated proteolysis. *Mol Microbiol* **65**: 76–89.

Becker G, Klauck E & Hengge-Aronis R (1999) Regulation of RpoS proteolysis in *Escherichia coli*: the response regulator RssB is a recognition factor that interacts with the turnover element in RpoS. *P Natl Acad Sci USA* **96**: 6439–6444.

Blaszczak A, Zylicz M, Georgopoulos C & Liberek K (1995) Both ambient temperature and the DnaK chaperone machine modulate the heat shock response in *Escherichia coli* by regulating the switch between σ^{70} and σ^{32} factors assembled with RNA polymerase. *EMBO J* **14**: 5085–5093.

Dombroski AJ, Walter WA & Gross CA (1993) Amino-terminal amino acids modulate sigma-factor DNA-binding activity. *Genes Dev* **7**: 2446–2455.

Führer F, Langklotz S & Narberhaus F (2006) The C-terminal end of LpxC is required for degradation by the FtsH protease. *Mol Microbiol* **59**: 1025–1036.

Führer F, Müller A, Baumann H, Langklotz S, Kutscher B & Narberhaus F (2007) Sequence and length recognition of the C-terminal turnover element of LpxC, a soluble substrate of the membrane-bound FtsH protease. *J Mol Biol* **372**: 485–496.

Gamer J, Bujard H & Bukau B (1992) Physical interaction between heat shock proteins DnaK, DnaJ, and GrpE and the bacterial heat shock transcription factor σ^{32}. *Cell* **69**: 833–842.

Gottesman S (2003) Proteolysis in bacterial regulatory circuits. *Annu Rev Cell Dev Bi* **19**: 565–587.

Guex N & Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**: 2714–2723.

Guisbert E, Herman C, Lu CZ & Gross CA (2004) A chaperone network controls the heat shock response in *E. coli*. *Gene Dev* **18**: 2812–2821.

Hengge R & Bukau B (2003) Proteolysis in prokaryotes: protein quality control and regulatory principles. *Mol Microbiol* **49**: 1451–1462.

Hengge-Aronis R (2002) Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol R* **66**: 373–395.

Herman C, Thévenet D, D'Ari R & Bouloc P (1995) Degradation of σ^{32}, the heat shock regulator in *Escherichia coli* is governed by HflB. *P Natl Acad Sci USA* **92**: 3516–3520.

Herman C, Thévenet D, Bouloc P, Walker GC & D'Ari R (1998) Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). *Gene Dev* **12**: 1348–1355.

Horikoshi M, Yura T, Tsuchimoto S, Fukumori Y & Kanemori M (2004) Conserved region 2.1 of *Escherichia coli* heat shock transcription factor σ^{32} is required for modulating both metabolic stability and transcriptional activity. *J Bacteriol* **186**: 7474–7480.

Joo DM, Nolte A, Calendar R, Zhou YN & Jin DJ (1998) Multiple regions on the *Escherichia coli* heat shock transcription factor σ^{32} determine core RNA polymerase binding specificity. *J Bacteriol* **180**: 1095–1102.

Kanemori M, Nishihara K, Yanagi H & Yura T (1997) Synergistic roles of HslVU and other ATP-dependent proteases in controlling *in vivo* turnover of σ^{32} and abnormal proteins in *Escherichia coli*. *J Bacteriol* **179**: 7219–7225.

Karata K, Inagawa T, Wilkinson AJ, Tatsuta T & Ogura T (1999) Dissecting the role of a conserved motif (the second region of homology) in the AAA family of ATPases. Site-directed mutagenesis of the ATP-dependent protease FtsH. *J Biol Chem* **274**: 26225–26232.

Kobiler O, Koby S, Teff D, Court D & Oppenheim AB (2002) The phage lambda CII transcriptional activator carries a C-terminal domain signaling for rapid proteolysis. *P Natl Acad Sci USA* **99**: 14964–14969.

Malhotra A, Severinova E & Darst SA (1996) Crystal structure of a sigma 70 subunit fragment from *E. coli* RNA polymerase. *Cell* **87**: 127–136.

McCarty JS, Rudiger S, Schonfeld HJ, Schneider-Mergener J, Nakahigashi K, Yura T & Bukau B (1996) Regulatory region C of the *E. coli* heat shock transcription factor, σ^{32}, constitutes a DnaK binding site and is conserved among eubacteria. *J Mol Biol* **256**: 829–837.

Miller JH (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Muffler A, Fischer D, Altuvia S, Storz G & Hengge-Aronis R (1996) The response regulator RssB controls stability of the σ^S subunit of RNA polymerase in *Escherichia coli*. *EMBO J* **15**: 1333–1339.

Nagai H, Yuzawa H, Kanemori M & Yura T (1994) A distinct segment of the σ^{32} polypeptide is involved in DnaK-mediated negative control of the heat shock response in *Escherichia coli*. *P Natl Acad Sci USA* **91**: 10280–10284.

Nakahigashi K, Yanagi H & Yura T (1995) Isolation and sequence analysis of *rpoH* genes encoding σ^{32} homologs from gram negative bacteria: conserved mRNA and protein segments for heat shock regulation. *Nucleic Acids Res* **23**: 4383–4390.

Narberhaus F & Balsiger S (2003) Structure-function studies of *Escherichia coli* RpoH (σ^{32}) by *in vitro* linker insertion mutagenesis. *J Bacteriol* **185**: 2731–2738.

Obrist M & Narberhaus F (2005) Identification of a turnover element in region 2.1 of *Escherichia coli* σ^{32} by a bacterial one-hybrid approach. *J Bacteriol* **187**: 3807–3813.

Obrist M, Milek S, Klauck E, Hengge R & Narberhaus F (2007) Region 2.1 of the *Escherichia coli* heat-shock sigma factor RpoH (σ^{32}) is necessary but not sufficient for degradation by the FtsH protease. *Microbiology* **153**: 2560–2571.

Pratt LA & Silhavy TJ (1996) The response regulator SprE controls the stability of RpoS. *P Natl Acad Sci USA* **93**: 2488–2492.

Rosen R, Biran D, Gur E, Becher D, Hecker M & Ron EZ (2002) Protein aggregation in *Escherichia coli*: role of proteases. *FEMS Microbiol Lett* **207**: 9–12.

Sambrook J & Russell DW (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schwede T, Kopp J, Guex N & Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res* **31**: 3381–3385.

Schweder T, Lee KH, Lomovskaya O & Matin A (1996) Regulation of *Escherichia coli* starvation sigma factor σ^s by ClpXP protease. *J Bacteriol* **178**: 470–476.

Shotland Y, Shifrin A, Ziv T, Teff D, Koby S, Kobiler O & Oppenheim AB (2000) Proteolysis of bacteriophage lambda CII by *Escherichia coli* FtsH (HflB). *J Bacteriol* **182**: 3111–3116.

Stüdemann A, Noirclerc-Savoye M, Klauck E, Becker G, Schneider D & Hengge R (2003) Sequential recognition of two distinct sites in σ^S by the proteolytic targeting factor RssB and ClpX. *EMBO J* **22**: 4111–4120.

Studier FW, Rosenberg AH, Dunn JJ & Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**: 60–89.

Tatsuta T, Joob DM, Calendar R, Akiyama Y & Ogura T (2000) Evidence for an active role of the DnaK chaperone system in the degradation of σ^{32}. *FEBS Lett* **478**: 271–275.

Tomoyasu T, Gamer J, Bukau B, Kanemori M, Mori H, Rutman AJ, Oppenheim AB, Yura T, Yamanaka K & Niki H (1995) *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat-shock transcription factor σ^{32}. *EMBO J* **14**: 2551–2560.

Yura T, Kanemori M & Morita MT (2000) The heat shock response: regulation and function. *Bacterial Stress Responses*

(Storz G & Hengge-Aronis R, eds), pp. 3–18. ASM Press, Washington, D.C.

Yura T, Guisbert E, Poritz M, Lu CZ, Campbell E & Gross CA (2007) Analysis of σ^{32} mutants defective in chaperone-mediated feedback control reveals unexpected complexity of the heat shock response. *P Natl Acad Sci USA* **104**: 17638–17643.

Zhou Y, Gottesman S, Hoskins JR, Maurizi MR & Wickner S (2001) The RssB response regulator directly targets σ^{S} for degradation by ClpXP. *Gene Dev* **15**: 627–637.

Zhou YN, Kusukawa N, Erickson JW, Gross CA & Yura T (1988) Isolation and characterization of *Escherichia coli* mutants that lack the heat shock sigma factor σ^{32}. *J Bacteriol* **170**: 3640–3649.