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

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The cellular level of the *Escherichia coli* heat-shock sigma factor RpoH (σ^{32}) is negatively controlled by chaperone-mediated proteolysis through the essential metalloprotease FtsH. Point mutations in the highly conserved region 2.1 stabilize RpoH *in vivo*. To assess the importance of this turnover element, hybrid proteins were constructed between *E. coli* RpoH and *Bradyrhizobium japonicum* RpoH₁, a stable RpoH protein that differs from region 2.1 of *E. coli* RpoH at several positions. Nine amino acids forming a putative α -helix were exchanged between the two proteins. Both hybrids were active sigma factors and showed intermediate protein stability. Introduction of RpoH region 2.1 into the general stress sigma factor RpoS, which is a substrate of the ClpXP protease, did not render RpoS susceptible to FtsH. Hence, region 2.1 alone is not sufficient to confer FtsH sensitivity to other proteins. Region 2.1 is not a major chaperone-binding site since DnaK and DnaJ bound efficiently to all RpoH variants. The *in vivo* stability of the mutated RpoH proteins correlated with their stability in a purified *in vitro* degradation system, suggesting that region 2.1 might be directly involved in the interaction with the FtsH protease.

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INTRODUCTION

All organisms react to heat stress with the expression of heat-shock genes in order to cope with accumulating unfolded proteins. In *Escherichia coli*, the heat-shock response is initiated at the transcriptional level by the alternative sigma factor σ^{32} (RpoH). During heat stress, RpoH associates with RNA polymerase (RNAP) and initiates transcription of the heat-shock regulon, coding mainly for molecular chaperones and proteases. The transient induction of the heat-shock response is tightly controlled by a regulatory feedback loop coupling the amount of heat-shock proteins to the cellular protein folding state (reviewed by El-Samad *et al.*, 2005; Gross, 1996; Yura *et al.*, 2000).

The activity and stability of RpoH is modulated by the chaperone systems DnaKJ and GroESL (Gamer *et al.*, 1996; Guisbert *et al.*, 2004; Straus *et al.*, 1990). At physiological temperature, e.g. 30 °C, DnaK and DnaJ associate with RpoH, rendering it inaccessible for RNAP. The sequestered sigma factor is made available for degradation by the

protease FtsH by a still unknown mechanism. Under heat-shock conditions, however, DnaK and DnaJ are titrated away by a high concentration of unfolded proteins. As a consequence, RpoH becomes available to associate with RNAP and to initiate transcription of heat-shock genes.

The major protease degrading RpoH is FtsH, a membrane-bound, hexameric, zinc- and ATP-dependent metalloprotease, which itself is a heat-shock protein (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995). Apart from general protein quality-control functions in degrading misfolded inner-membrane proteins, the FtsH protease has important regulatory functions (reviewed by Ito & Akiyama, 2005). Its active site is oriented towards the cytoplasm and it degrades specifically a number of soluble proteins such as LpxC, the key enzyme in LPS biosynthesis (Führer *et al.*, 2006; Ogura *et al.*, 1999), phage λ proteins cII, cIII and Xis (Herman *et al.*, 1997; Kihara *et al.*, 1997; Leffers & Gottesman, 1998; Shotland *et al.*, 1997), and the sigma factor RpoH. It is still largely unknown how this repertoire of entirely unrelated substrates is recognized and processed by the FtsH protease.

Multiple regions of RpoH have been implicated in its activity and stability (Arsène *et al.*, 1999; Bertani *et al.*,

Abbreviations: AC, adenylate cyclase; RNAP, RNA polymerase.

This work is dedicated to the memory of Amos Oppenheim, who was involved in the initial stages of this project.

2001; Blaszcak *et al.*, 1999; McCarty *et al.*, 1996; Nagai *et al.*, 1994; Narberhaus & Balsiger, 2003). Recently, an important turnover element was narrowed down to the highly conserved region 2.1 (Horikoshi *et al.*, 2004; Obrist & Narberhaus, 2005). Several point mutations in this region markedly stabilized RpoH against FtsH-mediated proteolysis. Horikoshi *et al.* (2004) provided evidence that the enhanced activity and stability of RpoH proteins mutated in region 2.1 is not the result of an increased interaction with RNAP core enzyme. Since the critical residues line up on the same face of a putative α -helix, we speculated that these amino acids might be exposed for interaction(s) with components of the proteolytic machinery, either chaperones or the FtsH protease itself (Obrist & Narberhaus, 2005).

Proteolysis of alternative sigma factors has emerged as a common principle in bacterial gene regulation (Gottesman, 1999; Hengge & Bukau, 2003; Jenal & Hengge-Aronis, 2003). Interestingly, even closely related sigma factors, like RpoH and RpoS (σ^{38}), are targeted to different proteases. Under optimal growth conditions, the starvation and general stress sigma factor RpoS is degraded by the ClpXP protease. Region 2.5 and the N-terminal region are critical for degradation and are recognized by the adaptor protein RssB and hexameric ClpX rings, respectively (Becker *et al.*, 1999; Stüdemann *et al.*, 2003). Region 2.1 of RpoS has not been implicated in ClpXP-mediated turnover of the sigma factor.

Although region 2.1 is a rather conserved region in all σ^{70} -type sigma factors (Lonetto *et al.*, 1992), it must be sufficiently diverse to allow for its critical role in protease discrimination. We asked whether transfer of RpoH region 2.1 to FtsH-resistant sigma factors would render them susceptible to degradation by FtsH. The corresponding regions were exchanged between *E. coli* RpoH and *Bradyrhizobium japonicum* RpoH₁, which is known to be active and stable in *E. coli* (Urech *et al.*, 2000). Introduction of RpoH region 2.1 into RpoS and into a bacterial one-hybrid system, developed to screen for protease recognition sites (Dautin *et al.*, 2000), was used to examine whether region 2.1 would confer FtsH sensitivity to these hybrid proteins. Finally, the interaction between RpoH and its partner proteins was assessed. Our results suggest that region 2.1 is involved in direct interaction with FtsH. In addition we conclude that regions outside region 2.1 are required for efficient turnover of the sigma factor.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. All strains were grown in Luria-Bertani medium (Sambrook & Russell, 2001). Antibiotics and chemicals were added as follows: ampicillin (Ap) (200 μ g ml⁻¹), chloramphenicol (Cm) (200 μ g ml⁻¹), kanamycin (Km) (30 μ g ml⁻¹), nalidixic acid (Nal) (15 μ g ml⁻¹) and IPTG (1 mM final concentration), unless indicated otherwise. *E. coli* DH5 α , GB2 and GB28 were grown at 37 °C, *E. coli* DHM1 at 30 °C, *E. coli* Δ ftsH at 23 °C and *E. coli* Δ rpoH at 25 °C.

Plasmids and recombinant DNA techniques. Plasmids used in this study are presented in Table 1 and primer sequences are shown in Table 2. DNA manipulations were performed according to standard protocols (Sambrook & Russell, 2001). To introduce single amino acid exchanges and to swap nucleotides coding for region 2.1 between *E. coli* rpoH and *B. japonicum* rpoH₁ primer-based mutagenesis was used (Quik-Change Site-Directed Mutagenesis kit, Stratagene). The plasmids pEC5364, pEC5377 and pEC5388 resulted from site-directed mutagenesis with template plasmid pEC5217 using primer pairs MO11/12, MO19/20 and MO21/22, respectively (exchanged nucleotides in Table 2 are underlined twice). To construct pRJ5389, plasmid pRJ5000 served as template and primers MO23 and MO24 were used. By transfer of the *NcoI*–*PstI* fragment of pEC5385 into pEC5217, replacing the wild-type fragment, plasmid pEC5387 was obtained. Plasmids coding for C-terminally histidine-tagged RpoH proteins were based on a PCR fragment amplified from pEC5217 using primers MO3 and MO4. The 870 bp fragment was cloned via *NdeI*/*XhoI* into the expression vector pET24b(+) (Novagen), resulting in pEC5261. Point mutations were transferred from rpoH into rpoH-His by digesting pEC5217 derivatives with *MluI*/*PstI*. A 526 bp fragment was used to replace the equivalent wild-type fragment in pEC5261. To generate pRJ5399, plasmid pRJ5389 carrying *B. japonicum* rpoH₁ with the 2.1 region of *E. coli* rpoH was digested with *SacI*/*HindIII* and the 296 bp fragment was cloned into pRJ5086, replacing the *SacI*–*HindIII* wild-type fragment. To construct plasmids pBO711, pBO712, pBO713, pBO714 and pBO715, PCR was performed with primers MO39 and MO40 using pEC5217, pEC5357, pEC5356, pEC5360 and pEC5387 as templates. PCR fragments were digested with *NheI*/*KpnI* and introduced into pEC5352, replacing the rpoH fragment between the subdomains of the *Bordetella pertussis* adenylate cyclase gene.

The construction of plasmid pRpoS18 was described previously (Becker *et al.*, 1999). Codons for amino acids 47–55 of helix 12a of *E. coli* σ^{32} were introduced into pRpoS18 by a four-primer/two-step PCR mutagenesis protocol as described previously (Germer *et al.*, 2001). The ‘internal’ primer with the coding sequence for helix 12a of σ^{32} and the ‘external’ primers with an *EcoRI* restriction site at the start of the rpoS coding sequence and an *AatII* restriction site 672 bp downstream of the *EcoRI* site in the coding sequence of rpoS are listed in Table 2. For PCR reaction, primer Int1 was used with Ext1, and Int2 with Ext2. These two restriction sites (*EcoRI*, *AatII*) were used for cloning the PCR fragment into pRpoS18, resulting in pRpoS18(2.1_H).

In vivo degradation assay and immunoblot analysis. *In vivo* degradation and sample preparation of *E. coli* Δ rpoH cells carrying pEC5217 derivatives was performed as previously described (Obrist & Narberhaus, 2005). Equivalent amounts of protein (10 μ g) were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-C; Amersham). RpoH proteins were detected using an anti-RpoH antibody (polyclonal rabbit anti-RpoH antibody; 1:4000 dilution) and a secondary antibody [goat anti-rabbit immunoglobulin G(H+L)-horseradish peroxidase conjugate; Bio-Rad; 1:3000 dilution], followed by chemiluminescence detection (SuperSignal, Pierce). RpoH bands on X-ray films (ECL Hyperfilm, Amersham) were scanned and quantified using the AIDA program (Advanced Image Data Analyser, version 4.13, raytest).

Pulse labelling of cells with L-[³⁵S]methionine and immunoprecipitation of σ^S was described previously (Lange & Hengge-Aronis, 1994). *E. coli* GB2 bearing plasmid pRpoS18(2.1_H), grown in M9/0.4% (v/v) glycerol supplemented with 100 μ g ampicillin ml⁻¹ was harvested at an optical density (578 nm) of 0.6 and pulse-labelled for 1 min. Chase times were between 1 and 10 min. For immunoprecipitation, a polyclonal antiserum against RpoS was used. RpoS bands were quantified on a FLAG2000G phosphoimager (Fuji Photo Film Co.). The intensity of the RpoS band was calculated relative to the intensity of bands representing stable proteins that cross-reacted with the RpoS antiserum.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference or source
<i>E. coli</i> strains		
BL21 DE3	F ⁻ <i>ompT gal (dcm) (lon) hsdS_B (r_B⁻ m_B⁻) λ(DE3)</i>	Studier & Moffatt (1986)
DH5α	<i>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 gyrA96 thi1 relA1</i>	Sambrook & Russell (2001)
DHM1	F ⁻ <i>cya-854recA1 gyrA96 (Nal^r) thi1 hsdR17 spoT1 rfbD1 glnV44(AS)</i>	D. Ladant
Δ <i>ftsH</i>	W3110 [F ⁻ IN(<i>rrnD-rrnE</i>)1] <i>zad220::Tn10 sfhC21 ΔftsH3::kan</i>	Tatsuta <i>et al.</i> (1998)
Δ <i>rpoH</i>	MC4100 Δ <i>rpoH30::kan zhf-50::Tn10</i> [λpF13-(<i>groE_p</i> - <i>lacZ</i> ⁺)]	Zhou <i>et al.</i> (1988)
GB2	MC4100 Δ(<i>ara-leu</i>)7697 <i>csi5(osmY)::lacZ rpoS::Tn10</i>	Becker <i>et al.</i> (1999)
GB28	GB2 <i>clpX::cat</i>	This study
Plasmids		
pKACp5	pKAC derivative, carrying p5 HIV protease cleavage site between codons 224 and 225 of the catalytic domain of the <i>Bordetella pertussis</i> AC, Km ^r	Dautin <i>et al.</i> (2000)
pCD951	pKAC carrying <i>rpoH</i> -N-terminal fragment coding for AC-RpoH-M1-M156, Km	Obrist & Narberhaus (2005)
pEC5352	pKACp5 derivative, p5 replaced by <i>E. coli rpoH</i> , coding for AC-RpoH-WT, Km ^r	Obrist & Narberhaus (2005)
pBO711	pKACp5 derivative, p5 replaced by <i>E. coli rpoH</i> fragment coding for AC-RpoH-2.1-WT, Km ^r	This study
pBO712	pKACp5 derivative, p5 replaced by <i>E. coli rpoH</i> fragment coding for AC-RpoH-2.1-L47Q, Km ^r	This study
pBO713	pKACp5 derivative, p5 replaced by <i>E. coli rpoH</i> fragment coding for AC-RpoH-2.1-A50V, Km ^r	This study
pBO714	pKACp5 derivative, p5 replaced by <i>E. coli rpoH</i> fragment coding for AC-RpoH-2.1-I54F, Km ^r	This study
pBO715	pKACp5 derivative, p5 replaced by <i>E. coli rpoH</i> fragment coding for AC-RpoH-2.1-I54T, Km ^r	This study
pEC5217	pUC18 carrying <i>E. coli rpoH</i> , Ap ^r	Narberhaus & Balsiger (2003)
pEC5356	pEC5217 derivative coding for RpoH-A50, Ap ^r	Obrist & Narberhaus (2005)
pEC5357	pEC5217 derivative coding for RpoH-L47Q, Ap ^r	Obrist & Narberhaus (2005)
pEC5360	pEC5217 derivative coding for RpoH-I54F, Ap ^r	Obrist & Narberhaus (2005)
pEC5364	pEC5217 derivative coding for RpoH-L47P, Ap ^r	This study
pEC5377	pEC5217 derivative coding for RpoH-L47C, Ap ^r	This study
pEC5387	pEC5217 derivative coding for RpoH-I54T, Ap ^r	This study
pEC5388	pEC5217 derivative coding for RpoH _{2.1} -H ₁ , Ap ^r	This study
pRJ5000	pUC18 carrying <i>Bradyrhizobium japonicum rpoH₁</i> , Ap ^r	Narberhaus <i>et al.</i> (1996)
pRJ5389	pRJ5000 derivative coding for RpoH ₁ -2.1-H, Ap ^r	This study
pET24b(+))	Km	Novagen
pEC5261	pET24b carrying <i>E. coli rpoH</i> with C-terminal His-tag, Km ^r	This study
pEC5367	pEC5261 derivative coding for RpoH-A50V-His, Km ^r	This study
pEC5368	pEC5261 derivative coding for RpoH-L47Q-His, Km ^r	This study
pEC5369	pEC5261 derivative coding for RpoH-I54F-His, Km ^r	This study
pEC5385	pEC5261 derivative coding for RpoH-I54T-His, Km ^r	This study
pEC5397	pEC5261 derivative coding for RpoH _{2.1} -H ₁ -His, Km ^r	This study
pRJ5086	pET24b carrying <i>Bradyrhizobium japonicum rpoH₁</i> with C-terminal His-tag, Km ^r	Narberhaus <i>et al.</i> (1998)
pRJ5399	pRJ5086 derivative coding for RpoH ₁ -2.1-H-His, Km ^r	This study
pGST-FtsH	pGEX-2T carrying <i>E. coli ftsH</i> with N-terminal GST-tag, lacking the first three amino acids of FtsH, Ap ^r	Shotland <i>et al.</i> (2000b)
pRpoS18	pBAD18 carrying <i>E. coli rpoS</i> under the control of the arabinose-inducible p _{BAD} promoter, Ap	Becker <i>et al.</i> (1999)
pRpoS18(2.1_H)	pRpoS18 derivative coding for RpoS with amino acids 47–55 of <i>E. coli</i> RpoH, helix 12a of region 2.1, Ap	This study

In vivo degradation of RpoS was also assayed by using spectinomycin to stop protein biosynthesis. After addition of spectinomycin (1.5 mg ml⁻¹) at an OD₅₇₈ of 0.6, samples were taken at the

indicated time points, precipitated with 10 % cold trichloroacetic acid, prepared for SDS-PAGE and transferred to a PVDF-membrane (Carl Roth) as previously described (Lange & Hengge-Aronis, 1994); 30 μg

Table 2. Oligonucleotides used in this study

Name	Sequence*
MO3	5'-ACTCCGCTAGCCATATGACTGACAAAATGCAAAGTTTAG-3'
MO4	5'-CACCAGGTACCCTCGAGCGCTTCAATGGCAGCACGC-3'
MO11	5'-CCATGGCGATCCGGAAGCAGCTAAAACGCTGATCC-3'
MO12	5'-CGTTTTAGCTGCTTCCGATCGCCATGGTAATGCAGC-3'
MO19	5'-CCATGGCGATTGCGAAGCAGCTAAACCGCTGATCC-3'
MO20	5'-CGTTTTAGCTGCTTCGCAATCGCCATGGTAATGCAG-3'
MO21	5'-GCTGCATTACCATGGCGATCGCGACGCAGCGTACCACCTGGTGACCTCTCACCTGCGGTTTG-3'
MO22	5'-CAAACCGCAGGTGAGAGGTACCAGGTGGTACGCTGCGTCGCGATGCCATGGTAATGCAGC-3'
MO23	5'-GGCGCGAGCACCGGGATCTAGAAGCAGCTAAAACGCTGATCCTGAGTCATCTCAGGCTCG-3'
MO24	5'-CGAGCCTGAGATGACTCAGGATCAGCGTTTTAGCTGCTTCTAGATCCCAGGCTCGCGCC-3'
MO39	5'-AAAAGCTAGCCTGGCTGAAAAGCTGCATTACC-3'
MO40	5'-AAAGGTACCGCCCGCATAATTACGAGCAATATG-3'
Int1	5'-CCAGACGCAAGTTACTCAGGATCAGCGTTTTAGCTGCCTCGAGATCTCCACGCAGTGC-3'
Int2	5'-GCACTGCGTGGAGATCTCGAGGCAGCTAAAACGCTGATCCTGAGTAACTTGCCTCTGG-3'
Ext1	TAGCGAATTCGGCGGAACCAG
Ext2	CGGCTGACGTCATCAACTGGCTTATCCAGTTGC

*Introduced restriction sites are underlined. Exchanged nucleotides for site-directed mutagenesis are underlined twice.

of total cellular protein was applied per lane. A polyclonal serum against σ^S , a goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) and a chromogenic substrate (BCIP/NBT, Carl Roth) were used for visualization of the σ^S bands.

Protein expression and purification. Plasmids coding for C-terminally histidine-tagged RpoH proteins were freshly transformed into *E. coli* BL21(DE3) cells. Cultures (500 ml) were grown at 37 °C to an OD₆₀₀ of 0.6, then production of recombinant proteins was induced by the addition of 0.5 mM IPTG. After incubation for 2 h at 30 °C the cells were harvested. Pellets were resuspended in 20 ml binding buffer (0.5 M KCl, 20 mM Tris/HCl, 5 mM imidazole, pH 7.9), 1 mM PMSF and 5 µg DNase I ml⁻¹ were added and cells were disrupted by sonication (6 × 15 s at 20 % output level; Branson Sonifier 250). Cell extracts were centrifuged at 15000 g for 30 min and the supernatant was loaded onto a 0.5 ml Ni-NTA-agarose column (Qiagen). Proteins were eluted with increasing imidazole concentration and stored in 20 % (v/v) glycerol at -80 °C. GST-FtsH was expressed, purified and stored as described previously (Shotland *et al.*, 2000b).

Cell growth, harvesting and preparation of protein extract for co-purification experiments were essentially performed as described above except that cells from a culture of 100 ml were resuspended in 4 ml binding buffer. Extracts were loaded on a column of 0.5 ml and bound 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 sample of 15 µl was analysed by 12 % SDS-PAGE and proteins were visualized by Coomassie Brilliant Blue staining or immunodetection with specific antisera. Anti-DnaK, -DnaJ and -GroEL sera (Stressgen Bioreagents) were diluted 1:3000, 1:2000 and 1:5000, respectively, and detected by secondary anti-rabbit sera as described above.

In vitro degradation assay. Degradation tests with purified GST-FtsH and RpoH-His derivatives were carried out essentially as described previously (Tomoyasu *et al.*, 1995; Urech *et al.*, 2000). The total volume was adjusted to 85 µl and contained 17 µg GST-FtsH and 4.25 µg RpoH-His. The reaction was started by addition of 5 mM

ATP and incubated at 42 °C. Samples of 10 µl were taken at different time points and analysed on 10 % SDS-polyacrylamide gels followed by staining with Coomassie Brilliant Blue. The relative amount of RpoH proteins on scanned gels was quantified with the AIDA program (Advanced Image Data Analyser, version 4.13, raytest).

Other methods. SDS-PAGE and β -galactosidase assays were performed as described by Sambrook & Russell (2001) and Miller (1972), respectively.

RESULTS

Additional point mutations in region 2.1 that render *E. coli* RpoH more active *in vivo*

Point mutations in RpoH region 2.1 at positions 47, 50, 51, 54 or 55 were shown to turn the sigma factor into a stabilized protein, resulting in enhanced transcription of an RpoH-dependent promoter *in vivo* (Horikoshi *et al.*, 2004; Obrist & Narberhaus, 2005). The predicted three-dimensional structure suggested that amino acids at positions 47, 50 and 54 are positioned on the same side of the putative α -helix 12a in region 2.1 of RpoH (Obrist & Narberhaus, 2005). The sequence of region 2.1 from 21 bacterial species shows that several RpoH proteins contain a proline residue at position 47 (Fig. 1). It is known that proline can act as a helix breaker introducing a kink into the α -helix. To assess whether a proline residue would affect the activity and stability of the *E. coli* protein, we introduced the corresponding codon at this position. In another mutant, leucine 47 was changed to a cysteine residue. An RpoH protein with an isoleucine to threonine exchange at position 54 that derived from our previous mutagenesis screen but had not been analysed yet was included in this study. The different *rpoH* alleles coding for RpoH-L47P,

Fig. 1. Sequence alignment, generated with CLUSTAL W (Thompson *et al.*, 1994), of RpoH region 2.1 from different proteobacteria. Subdivisions of the listed proteobacteria are indicated on the left. The predicted secondary structure derives from our previous study (Obrist & Narberhaus, 2005). Residues 47, 50 and 54, which are critical for turnover of the protein, are indicated. The RpoH sequences belong to: E.co, *Escherichia coli*; B.ja_1, *Bradyrhizobium japonicum* RpoH₁; B.ja_2, *Bradyrhizobium japonicum* RpoH₂; S.me, *Sinorhizobium meliloti*; A.tu, *Agrobacterium tumefaciens*; C.cr, *Caulobacter crescentus*; R.sp, *Rhodobacter sphaeroides*; R.ca, *Rhodobacter capsulatus*; Z.mo, *Zymomonas mobilis*; H.th, *Hydrogenophilus thermoluteolus*; R.me, *Ralstonia metallidurans*; C.fr, *Citrobacter freundii*; C.ma, *Colwellia maris*; E.cl, *Enterobacter cloacae*; P.ae, *Pseudomonas aeruginosa*; P.pu, *Pseudomonas putida*; P.mi, *Proteus mirabilis*; S.ma, *Serratia marescens*; V.ch, *Vibrio cholerae*; X.ca, *Xanthomonas campestris*; M.xa, *Myxococcus xanthus*; S.au, *Stigmatella aurantiaca*. For comparison, the *E. coli* RpoS sequence is given at the bottom of the alignment. Amino acids identical to conserved RpoH residues are underlined.

RpoH-L47C and RpoH-I54T were transformed into an *E. coli* Δ rpoH strain carrying a chromosomally integrated RpoH-dependent *groE-lacZ* fusion. Functionality of the mutated sigma factors was assessed by β -galactosidase assays. Mean values of three independent experiments showed that all RpoH derivatives tested were functionally intact. They induced higher β -galactosidase activities than the wild-type (WT) sigma factor (set to 100 %) (Fig. 2). Sigma factor activities differed substantially among RpoH derivatives mutated at the same position. Reproducibly,

Fig. 2. Sigma factor activity of RpoH variants derived from *E. coli* RpoH and *B. japonicum* RpoH₁. β -Galactosidase activity was measured in *E. coli* Δ rpoH cells carrying a chromosomally encoded transcriptional *PgroE-lacZ* fusion. An activity of 100 % corresponds to $\approx$ 1000 Miller units. Error bars indicate standard deviations from at least three independent assays. RpoH-WT is wild-type *E. coli* RpoH and RpoH1-WT is the *B. japonicum* RpoH₁ protein. RpoH-L47Q, -L47C, -I54F and -I54T contain the indicated amino acid exchanges in *E. coli* RpoH. In RpoH-2.1_H1, amino acids 47–55 (helix 12a) of *E. coli* RpoH are exchanged for the equivalent *B. japonicum* RpoH₁ region. Conversely, in RpoH1-2.1_H *B. japonicum* helix12a was replaced by the *E. coli* RpoH region.

RpoH-L47C (274 %) and RpoH-L47P (246 %) were more active than RpoH-L47Q (142 %), which originates from the original genetic screen (Obrist & Narberhaus, 2005). Variants at position 54 showed 213 % (RpoH-I54T) and 135 % (RpoH-I54F) activity in comparison to RpoH-WT.

Exchange of the turnover element in region 2.1 between *E. coli* RpoH and *B. japonicum* RpoH₁ affects sigma factor activity and stability

The amino acid sequence of the comparatively stable RpoH₁ protein of the α -proteobacterium *B. japonicum* deviates from its enterobacterial homologue at several positions in region 2.1 (Fig. 1). Only two alanine residues (A49 and A50) and L53 are identical in the predicted α -helix 12a, which is flanked by a conserved aspartic acid residue (D46) and an SHLR-motif. Nevertheless, the corresponding region of RpoH₁ is also predicted to form an α -helix (data not shown).

To test whether *E. coli* RpoH could be stabilized by the corresponding RpoH₁ helix and vice versa, the nine amino acids located between the conserved residues D46 and S56 were swapped between the two proteins. Both hybrid proteins were active sigma factors (Fig. 2). *E. coli* RpoH containing RpoH₁ region 2.1 (designated RpoH-2.1_H1)

exhibited strongly enhanced activity (256 %) as compared to RpoH-WT (100 %), whereas RpoH₁-2.1_H was almost as active (356 %) as RpoH₁-WT (395 %).

The half-life of the plasmid-encoded RpoH derivatives was determined in the *E. coli* $\Delta rpoH$ strain in order to avoid interference with chromosomally encoded sigma factor. The RpoH proteins mutated in region 2.1 were stabilized in comparison to the wild-type protein (Fig. 3a). RpoH-L47P was the most stable protein (about 10 times as stable as RpoH-WT), and RpoH-L47C and RpoH-I54T were stabilized approximately fivefold (Fig. 3b). Both RpoH-RpoH₁ hybrids showed intermediate stability (Fig. 3c). *E. coli* RpoH was stabilized by the RpoH₁ helix 12a (RpoH-2.1_H1) but did not reach the stability of RpoH₁. *B. japonicum* RpoH₁ was slightly destabilized by the corresponding *E. coli* segment (RpoH1-2.1_H). However, it remained much more stable than *E. coli* RpoH. The finding that both helix replacements caused intermediate protein stability suggests that amino acids outside region 2.1 contribute to efficient degradation of RpoH and high stability of RpoH₁.

Region 2.1 alone is not sufficient for FtsH-dependent proteolysis

All stabilized RpoH derivatives were still degraded by FtsH with slow kinetics, suggesting that other regions in RpoH also contribute to degradation. To test whether region 2.1 alone was sufficient for degradation by FtsH, residues 37–70 of RpoH, which include the C-terminal six amino acids of region 1.2 and the entire region 2.1, were introduced between two subdomains of the adenylate cyclase (AC) of

the bacterial one-hybrid system used previously (Obrist & Narberhaus, 2005). The system is based on two domains (T25 and T18) of the *Bordetella pertussis* AC, which produce cAMP if they are connected by a stable linker (Dautin *et al.*, 2000) (Fig. 4a). cAMP production can be scored by LacZ activity in the AC-negative *E. coli* reporter strain DHM1. If the linker between T25 and T18 is cleaved by a cellular protease, cAMP production is lost.

We introduced RpoH region 2.1 together with the final six residues of region 1.2 to ensure that the putative α -helix 12a was formed properly. The constructs were transformed into the AC-deficient reporter strain *E. coli* DHM1 and cAMP production was assessed by β -galactosidase assays. AC-RpoH-WT (containing full-length wild-type RpoH) and AC-RpoH-NT (containing the N-terminal half of RpoH) were degraded as described previously (Dartigalongue *et al.*, 2001; Obrist & Narberhaus, 2005). All region 2.1 fusions induced significantly higher β -galactosidase activities than the RpoH-WT fusion protein (set to 100 %) (Fig. 4b). Most constructs produced β -galactosidase activities as high as the positive control AC-p5, which is known to be stable in *E. coli* (Dautin *et al.*, 2000). The high activities suggest that introduction of region 2.1 between the AC subdomains is not sufficient to produce an FtsH substrate.

To further substantiate this finding, amino acids 47–55 of RpoH region 2.1 were used to replace the equivalent region in RpoS. The helix 12a region deviates substantially between RpoH and RpoS (Fig. 1). The product (RpoS-2.1_H) retained susceptibility towards the ClpXP protease. Turnover was slightly delayed (Fig. 5a) but remained strictly ClpXP-dependent. Neither RpoS nor RpoS-2.1_H

Fig. 3. *In vivo* stability of *E. coli* RpoH and *B. japonicum* RpoH₁ proteins. (a) RpoH decay was determined by immunoblot analysis after stopping translation by chloramphenicol (Cm) addition. (b) Quantification of the stability of RpoH variants carrying single amino acid exchanges. (c) Quantification of the stability of RpoH/RpoH₁ hybrids. All experiments were repeated three times with independent extracts and the corresponding mean values are shown.

Fig. 4. (a) Principle of the bacterial one-hybrid system. (b) β -Galactosidase activity resulting from different AC fusion proteins in *E. coli* DHM1. Relative activity is shown and AC-RpoH-WT is set to 100%. Error bars indicate standard deviations from three independent assays. AC-p5 is a positive control. AC-RpoH-WT contains full-length RpoH, AC-RpoH-NT the N-terminal half of RpoH, AC-2.1-WT region 2.1 of RpoH, AC-2.1-LQ region 2.1 of RpoH-L47Q, AC-2.1-AV region 2.1 of RpoH-A50V, AC-2.1-IF region 2.1 of RpoH-I54F and AC-2.1-IT region 2.1 of RpoH-I54T. For details of the constructs, see Table 1.

was degraded in a *clpX* mutant over a period of 80 min (Fig. 5b). Hence, introduction of region 2.1 from RpoH was not sufficient to commit the starvation sigma factor to degradation by FtsH. Despite being present at similar levels, RpoS-2.1_H was inactive in initiating transcription of the RpoS-dependent *osmY* promoter (Fig. 5c), suggesting that the contribution of region 2.1 to RNAP core binding is reduced in this mutated version of RpoS.

Chaperone binding to RpoH

The rapid turnover of RpoH in *E. coli* depends on molecular chaperones. DnaK, DnaJ, GrpE and GroESL have been shown to interact with RpoH (Gamer *et al.*, 1996; Guisbert *et al.*, 2004; Liberek *et al.*, 1992). One possible explanation for the stabilization of RpoH mutated in region 2.1 might be an impaired interaction with the chaperones. To test this assumption, different *rpoH* alleles were transferred into a pET24b expression vector. Upon moderate expression, the His-tagged RpoH proteins were

purified on Ni-affinity columns and the elution fractions were analysed (Fig. 6). In each case, DnaK and DnaJ co-eluted with RpoH regardless of whether wild-type or mutated RpoH was used (shown as immunodetected DnaK and DnaJ in purified fractions of RpoH-WT and the hyperstable variants RpoH-L47Q and RpoH-I54F in Fig. 6). The amounts of DnaK were also visible in Coomassie-stained SDS-PAGE gels (data not shown). Quantification of these bands from three independent purification experiments for each RpoH protein demonstrated that the relative amount of DnaK/RpoH in the elution fractions ranged from 2 to 6%, with 4% for RpoH-WT (data not shown). All other RpoH derivatives, RpoH₁ as well as the hybrid proteins between *E. coli* RpoH and *B. japonicum* RpoH₁, showed similar results (data not shown). In control experiments with extracts from cells without histidine-tagged RpoH proteins, the chaperones did not bind to the column resin (data not shown). The interaction between RpoH and DnaK was also examined by glutaraldehyde cross-linking using Strep-tagged DnaK and His-tagged RpoH. RpoH-L47Q, L47C, L47P, A50V and I54F were able to interact with DnaK like RpoH-WT (data not shown).

Since Guisbert *et al.* (2004) recently reported an interaction between RpoH and GroESL, we subjected our RpoH elution fractions to immunodetection with anti-GroEL sera. GroEL indeed co-eluted with RpoH but did not discriminate between wild-type RpoH and the mutated RpoH proteins (data not shown). Thus, it appears that region 2.1 of RpoH is not critical for an interaction with molecular chaperones.

In vitro degradation of RpoH by FtsH

To analyse whether stabilization of the RpoH variants was due to an altered interaction with the protease FtsH, we performed *in vitro* degradation experiments using purified histidine-tagged RpoH proteins and GST-FtsH. Degradation of all proteins was ATP-dependent (data not shown). β -Casein, which is known to be a loosely folded protein, was readily degraded (Fig. 7) (Holt & Sawyer, 1988; Shotland *et al.*, 2000a). As observed previously, the kinetics of RpoH degradation *in vitro* was much slower than *in vivo* (Urech *et al.*, 2000). RpoH-WT was degraded with a half-life of approximately 20 min, whereas RpoH-L47Q, -A50V and -I54T were clearly stabilized (Fig. 7a, b). Consistent with previous *in vivo* results (Obrist & Narberhaus, 2005), RpoH-I54F was the most stable RpoH protein.

Degradation of the RpoH/RpoH₁ hybrids in the purified system was also in line with their stability *in vivo* (Fig. 8). The poor FtsH substrate RpoH₁ was destabilized by the presence of helix 12a from *E. coli* RpoH. On the other hand, RpoH was moderately stabilized by the equivalent RpoH₁ region. The sum of our *in vivo* and *in vitro* results suggests that proteolysis of RpoH might involve a direct interaction between RpoH and FtsH in region 2.1.

DISCUSSION

Initiation of transcription in bacteria is a multistep process involving binding of the sigma factor to other subunits of RNAP core enzyme and to the -10 and -35 regions of the

Fig. 5. *In vivo* degradation and sigma factor activity of *E. coli* σ^S with helix 12a from σ^{32} , RpoS(2.1_H). (a) *E. coli* GB2 harbouring the plasmids pRpoS18 or pRpoS18(2.1_H) was grown to an OD_{578} of 0.6 and pulse-labelled for 1 min with L-[35 S]methionine. Chase times (in min) are indicated. A polyclonal serum against σ^S was used for immunoprecipitation. Quantification: ●, RpoS-WT; ○, RpoS(2.1_H). (b) GB28 (GB2 *clpX::cat*) harbouring plasmid pRpoS18 or pRpoS18(2.1_H) was grown to an OD_{578} of 0.6. After addition of spectinomycin (1.5 mg ml $^{-1}$) samples were taken at the indicated time points and prepared for SDS-PAGE. The gel was blotted and developed with a polyclonal serum against σ^S and an anti-rabbit alkaline phosphatase conjugate. Quantification: ●, RpoS-WT; ○, RpoS(2.1_H). *In vivo* degradation experiments were performed at least three times with similar results. (c) Cells were grown in M9 minimal medium with 0.4 % glycerol as a carbon source at 37 °C. At the indicated time points arabinose was added to a final concentration of 0.02 % to induce pRpoS18 and pRpoS18(2.1_H) in *E. coli* strain GB2. ●, ▲, Growth curves of GB2 bearing pRpoS18 or pRpoS18(2.1_H), respectively; ○, △, β -galactosidase activity derived from the σ^S -dependent *osmY::lacZ* fusion of strain GB2 bearing pRpoS18 (○) or pRpoS18(2.1_H) (△).

promoter. Accordingly, sigma factors of the σ^{70} family (to which RpoH and RpoS belong) are characterized by several conserved regions. These functional domains are separated by flexible linkers (Gruber & Gross, 2003). The remarkable structural flexibility of free RpoH was demonstrated by amide hydrogen exchange studies (Rist *et al.*, 2003). Only in contact with interaction partners do sigma factors adopt a well-defined conformation amenable to structure determination (Campbell *et al.*, 2003; Murakami *et al.*, 2002; Vassilyev *et al.*, 2002).

Apart from binding to components of the transcription machinery, sigma factors interact with many other cellular proteins, which mediate post-translational control by influencing the activity and stability of the bacterial transcription initiation factors. Altogether at least seven cellular macromolecules (promoter DNA, the β and β' subunits of the RNAP core enzyme, DnaK, DnaJ, GroEL and FtsH) compete for binding to the heat-shock sigma factor RpoH. Some proteins might share overlapping binding sites because interaction of some partners is mutually exclusive. Incubation of RpoH with DnaK or GroEL recruits the sigma factor away from the RNAP and inhibits transcription initiation *in vitro* (Gamer *et al.*, 1996; Guisbert *et al.*, 2004; Liberek *et al.*, 1992). Conversely, in complex with RNAP the sigma factor is protected from attack by the FtsH protease *in vitro* (Blaszczak *et al.*, 1999; Urech *et al.*, 2000).

Region 2.1 of *E. coli* RpoH carries a critical turnover element promoting rapid degradation by FtsH *in vivo* (Horikoshi *et al.*, 2004; Obrist & Narberhaus, 2005). In this study, we provide further evidence for the importance of this region. Rationally designed point mutations stabilized the sigma factor and resulted in elevated RpoH-dependent gene expression. Swapping of the turnover element

Fig. 6. Co-elution of chaperones with RpoH derivatives. RpoH proteins were purified by Ni-NTA chromatography. In each top panel, RpoH (H) is indicated in Coomassie-stained SDS-PAGE gels. The corresponding lower panels show immunodetected DnaK (anti-K) and DnaJ (anti-J) bands. Imidazole concentrations were as follows: W3 and W4, 50 mM; E1, 75 mM; E2, 100 mM; E3, 125 mM; E4, 150 mM; E5, 1M.

between RpoH and the intrinsically stable RpoH₁ protein from *B. japonicum* produced proteins with intermediate stability. Differences in region 2.1, which contains highly conserved and some variable residues (Fig. 1), and yet undefined residues outside of region 2.1 seem to account for differences in stability of RpoH proteins from different bacteria (Narberhaus *et al.*, 1997; Urech *et al.*, 2000).

It is important to note that the mutated RpoH proteins were not entirely protected from proteolysis. Some residual degradation occurred both *in vivo* and *in vitro*, indicating that RpoH regions outside region 2.1 also contribute to efficient turnover. The fact that transfer of RpoH region 2.1 into the AC of *Bordetella pertussis* or the *E. coli* RpoS sigma factor did not suffice to convert these proteins into FtsH substrates supports this assumption.

As the key residues for protein turnover in RpoH region 2.1 are predicted to be exposed on one face of a α -helix, they might provide an interaction surface for proteins of the degradation machinery (Obrist & Narberhaus, 2005). There is a striking analogy in the turnover element of RpoS that is located in region 2.5 and interacts with RssB. Only amino acid exchanges on one side of an α -helix, in particular at position K173, have an effect on RpoS proteolysis (Becker *et al.*, 1999).

Fig. 7. FtsH-dependent *in vitro* degradation of RpoH proteins carrying single point mutations. (a) Coomassie-stained RpoH bands after SDS-PAGE. (b) Quantification of RpoH degradation. Mean values of three independent experiments are shown.

Little is known about the precise contact sites between DnaK, DnaJ, GroEL or FtsH with RpoH. Multiple DnaK interactions sites have been proposed by a peptide-scanning approach using cellulose-bound peptides (McCarty *et al.*, 1996). Region 2.1 of RpoH was among those potential DnaK sites, which led us to examine chaperone binding to our stabilized RpoH proteins. Binding of neither DnaK, DnaJ or GroEL was abolished by point mutations in amino acids 47, 50 or 54, suggesting that region 2.1 is not a major specific chaperone-binding site. Apparently, several of the membrane-displayed short peptides (13-mers) that served as good DnaK-binding sites in the peptide library do not bind the chaperone in the context of the native RpoH protein. This was also shown for region C (Arsène *et al.*, 1999). A remaining promising candidate as DnaK-binding site in RpoH lies around amino acid 200 in region 3.2 (McCarty *et al.*, 1996).

Since helix 12a did not seem to be a chaperone-binding site, the FtsH protease itself was left as potential interaction partner. The direct effect of mutations in region 2.1 on proteolysis was examined by using purified sigma factors

Fig. 8. FtsH-dependent *in vitro* degradation of RpoH/RpoH₁ hybrids with exchanged α -helix 12a of region 2.1. (a) Coomassie-stained RpoH bands after SDS-PAGE. (b) Quantification of RpoH/RpoH₁ degradation. Means of three independent experiments are shown.

and FtsH. Despite its low unfoldase activity, FtsH is able to degrade RpoH without the aid of chaperones (Herman *et al.*, 2003). As described previously, degradation *in vitro* was slower than *in vivo* (Urech *et al.*, 2000). Interestingly, the stability determined *in vitro* largely reflected the *in vivo* half-lives, i.e. proteins that were stabilized by mutations in region 2.1 were also stabilized *in vitro*. The RpoH₁ protein carrying the destabilizing *E. coli* RpoH helix 12a was rapidly degraded *in vitro*. These results strongly suggest that chaperones do not play a role in stability control by region 2.1. Based on our results we propose that residues 47, 50 and 54 of region 2.1 are important for establishing a contact to the FtsH protease.

Again, it should be noted that some residual degradation was observed with all constructs. Hence, it appears that region 2.1 is not the only RpoH region required for *in vitro* degradation by the FtsH protease. It will be interesting to track down additional amino acids involved in this process. It is remarkable that neither genetic screen came up with good candidates outside region 2.1 (Horikoshi *et al.*, 2004; Obrist & Narberhaus, 2005). Since the N-terminal half of RpoH mediates efficient turnover in the one-hybrid screen (Dartigalongue *et al.*, 2001; Obrist & Narberhaus, 2005), critical residues might be located in the first 156 residues of RpoH.

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