**Short Communication**

**Expression of Two RpoH Sigma Factors in Sinorhizobium meliloti upon Heat Shock**

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The plant symbiotic α-proteobacterium Sinorhizobium meliloti has two RpoH-type sigma factors, RpoH1 and RpoH2. The former induces the synthesis of heat shock proteins and optimizes interactions with the host. Using a Western blot analysis, we examined time course changes in the intracellular contents of these factors upon a temperature upshift. The RpoH1 level was relatively high and constant, suggesting that its regulatory role in the heat shock response is attained through the activation of the pre-existing RpoH1 protein. In contrast, the RpoH2 level was initially undetectable, and gradually increased. These differential patterns reflect the functional diversification of these factors.

**Key words:** α-proteobacteria, heat shock protein, root-nodule symbiont, sigma factor, Sinorhizobium meliloti

Upon exposure to stresses including heat, cells of virtually all living organisms transiently increase the synthesis of a set of heat shock proteins (hsps), which are typically molecular chaperones and proteases. This response, known as the heat shock response, minimizes damage from thermal denaturation and the aggregation of proteins. In the γ-proteobacterium Escherichia coli, an RNA polymerase holoenzyme containing the sigma factor σ32 (the rpoH gene product) transcribes many of the hsp genes (7). Following a sudden shift from 30°C to 42°C, the σ32 level increases approximately 17-fold by 6 min (the initial phase) and then drops to 5 times that at 30°C by 15 min (the adaptation phase); the transcription rate of the σ32 regulon member dnaKJ changes in parallel with the change in the σ32 level (29). The synthesis of σ32 is mainly modulated at the translational level (14). In addition, the σ32 protein is unstable during steady-state bacterial growth (half-life: approx. 1 min at 30°C) due to proteolytic degradation mediated by the chaperone systems DnaK/DnaJ/GrpE and GroEL/GroES and the ATP-dependent protease FtsH, all of which belong to the σ32 regulon. Upon heat shock, misfolded or aggregated proteins titrate these chaperones away, which results in transient σ32 stabilization (6, 28, 29, 32). The same chaperones also inactivate the σ32 protein in the adaptation phase of the heat shock response (6, 33).

Homologs of σ32, referred to here as RpoHs, are present in diverse proteobacteria (16). Similar to E. coli σ32, α-proteobacterial RpoHs control the expression of hsps (9, 17, 19, 24, 34). Notably, some α-proteobacteria have more than one RpoH species, which are sometimes not functionally equivalent (4, 11, 20, 22, 23). Two RpoHs, RpoH1 and RpoH2, are encoded in the genome of the α-proteobacterium Sinorhizobium meliloti, which lives either as a saprophyte in soil or as a root-nodule nitrogen-fixing symbiont of alfalfa (22, 23). In wild-type S. meliloti, the synthesis of several hsps peaks 5–10 min after a temperature upshift, whereas this induction is abolished in a mutant lacking rpoH1 (23). More than 300 genes have been identified as RpoH1 regulon members by transcription profiling during heat shock (1, 26). In contrast, the rpoH2 null mutation has no appreciable effects on the heat shock response in either the wild-type or rpoH1 mutant background (1, 13). Instead, the expression of at least 44 genes depends on RpoH2 in the late stationary phase (1). Notably, the rpoH1 mutant elicits the formation of nodules with no nitrogen-fixing activity (13, 22, 23). Although the rpoH2 single mutant has no symbiotic defects, the rpoH1 rpoH2 double mutant shows more severe defects, including the lack of nodule formation, than the rpoH1 single mutant (22, 23).

In several α-proteobacteria, RpoHs are transcriptionally up-regulated upon heat shock (19, 20, 24, 34, 35). In some bacteria with multiple RpoH species, the level of each rpoH mRNA responds differently to stress (19, 20). In the plant pathogenic α-proteobacterium Agrobacterium tumefaciens, the level of its single RpoH factor increases approximately 5-fold by 15 min of heat shock (17). Notably, activation of the pre-existing RpoH protein is sufficient for the normal induction of hsp synthesis, and the contribution of this increase in the RpoH1 level is negligible (18).

In the present study, we compared the responses of RpoH1 and RpoH2 levels to heat shock in S. meliloti in order to gain insights into the functional diversification of these factors. The bacterial strains and plasmids used are listed in Table 1. The rpoH1 and rpoH2 coding regions were amplified by PCR. The primers 5'-TGCCATATGCGCAGATACCTTG-3' and 5'-GGTAAGCCTTAGGCCCTTCAACCACCG-3' were used for rpoH1; the primers 5'-CAACATATGAGAACACCTCACAGCA-3' and 5'-TACAGCCTATGATCGACGCACCG-3' were used for rpoH2. PCR products were cloned into the expression plasmid pET-20b(+) (17); restriction sites used for cloning are underlined. The hexahistidine-tagged recombinant proteins RpoH1-His (calculated molecular mass, 36.1 kDa) and RpoH2-His (33.6 kDa) were produced in E. coli BL21(DE3). We prepared antisera against the recombinant proteins. In the Western blot analysis, these antisera recognized authentic RpoH1 (34.6 kDa) and RpoH2 proteins (32.1 kDa) in S. meliloti cell extracts; however, the anti-RpoH2 antiserum also reacted with other proteins including RpoH1 (Fig. S1).
RpoH2 was only detectable in heat-shocked cells. RpoH1 and RpoH2 were also detected in *S. meliloti* cells isolated from alfalfa nodules (Fig. S1).

Using a Western blot analysis, we examined time course changes in RpoH1 and RpoH2 levels in growing *S. meliloti* cells upon a temperature upshift (from 25°C to 37°C). Protein bands were immunodetected with an ECL Detection System (GE Healthcare, Little Chalfont, England), and images of blots were captured with a LAS-1000plus image analyzer (Fuji Film, Tokyo, Japan). Protein amounts were estimated using calibration curves generated using purified RpoH1-His (1.0–2.5 ng) and RpoH2-His proteins (0.1–2.5 ng). A set of calibration samples was always included along with cell lysate samples in the Western blot analysis. The RpoH1 level was relatively constant before and after the temperature upshift (Fig. 1A). This is consistent with the absence of a significant increase in *rpoH1* transcription upon heat shock (1, 22). The RpoH1 level ranged between 40 and 50 fmol microgram⁻¹ of total protein (Fig. 1B), which is markedly higher than that of σ²² under non-stress conditions (0.44 fmol microgram⁻¹ of total protein at 30°C [5]) and similar to the content of the primary sigma factor σ²⁰ (50–80 fmol microgram⁻¹ of total protein in cells grown at 37°C [8]) in *E. coli*. In contrast, the RpoH2 level was below the detection limit (<1 fmol microgram⁻¹ of total protein) at 25°C, increased gradually after the temperature upshift (up to approx. 5 fmol microgram⁻¹ of total protein by 40 min), and remained at this level until 60 min (Fig. 1). The RpoH2 level was significantly higher at 60 min in the *rpoH1* mutant than in the wild type, whereas the RpoH1 level was not significantly affected by the *rpoH2* mutation (Fig. 1B).

The constantly high level of RpoH1 indicates that this protein level is not a limiting factor in hsp synthesis under non-stress conditions, similar to *A. tumefaciens* RpoH (18). The most likely explanation is that RpoH1 is stable but inactive in non-stressed cells, and is activated by heat shock. In *A. tumefaciens*, the DnaK/DnaJ chaperone appears to inactivate RpoH (18). In *S. meliloti*, five genomic loci encode GroEL homologs, of which GroEL5 is induced upon stress in an RpoH1-dependent manner, whereas GroEL1 is present at the highest level and plays a housekeeping role (2, 3, 13). The mutational loss of GroEL1 increases the GroEL5 level in non-stressed cells (3), suggesting that GroEL1 inactivates RpoH1. Studies that assess the half-life of the cellular RpoH1 protein before and after heat shock and examine the effects of engineered reductions in the levels of the major chaperones on the expression of RpoH1-dependent genes will be useful for confirming the above explanation. On the other hand, the increase in the RpoH2 level following heat shock is attributable, at least in part, to an increase in its mRNA level, which is regulated by RpoE2, an extracytoplasmic function sigma factor involved in the general stress response in *S. meliloti* (27). It currently remains unclear whether the higher level of RpoH2 in the *rpoH1* mutant than in the wild type at the late stage of heat shock was caused by further enhanced transcription or a post-transcriptional mechanism such as stabilization of the RpoH2 protein. The sequence similarity between RpoH1 and RpoH2 is lower (41% identical at the amino acid level) than that between RpoH1 and *A. tumefaciens*.
RpoH2 contributes to the induction of a particular set of genes. It is of interest to examine the effects of extended heat shock on the expression of known RpoH2 regulon members, which were identified based on their expression in the stationary phase (1).

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References

1. Barnett, M.J., A.N. Bittner, C.J. Toman, V. Oke, and S.R. Long. 2012. Dual RpoH sigma factors and transcriptional plasticity in a symbiotic bacterium. J. Bacteriol. 194:4983–4994.

2. Bittner, A.N., and V. Oke. 2006. Multiple groESL operons are not key targets of RpoH1 and RpoH2 in Sinorhizobium meliloti. J. Bacteriol. 188:3507–3515.

3. Bittner, A.N., A. Foltz, and V. Oke. 2007. Only one of five groEL genes is required for viability and successful symbiosis in Sinorhizobium meliloti. J. Bacteriol. 189:1884–1889.

4. Green, H.A., and T.J. Donohue. 2006. Activity of Rhodobacter sphaeroides RpoH, a second member of the heat shock sigma factor family. J. Bacteriol. 188:5712–5721.

5. Grigorova, I., L.J. Phleger, V.K. Mutalik, and C.A. Gross. 2006. Insights into transcriptional regulation and σ competition from an equilibrium model of RNA polymerase binding to DNA. Proc. Natl. Acad. Sci. U.S.A. 103:5332–5337.

6. Guisbert, E., C. Herman, C.Z. Lu, and C.A. Gross. 2004. A chaperone network controls the heat shock response in E. coli. Genes Dev. 18:2812–2821.

7. Guisbert, E., T. Yura, V.A. Rhodius, and C.A. Gross. 2008. Convergence of molecular, modeling, and systems approaches for an understanding of the Escherichia coli heat shock response. Microbiol. Mol. Biol. Rev. 72:545–554.

8. Jishage, M., and A. Ishihama. 1995. Regulation of RNA polymerase subunit synthesis in Escherichia coli: intracellular levels of σ32 and σ32. J. Bacteriol. 177:6832–6835.

9. Karls, R.K., J. Brooks, P. Rossmeissl, J. Luedke, and T.J. Donohue. 1998. Metabolic roles of a σ32 family. J. Bacteriol. 180:10–19.

10. Lonetto, M., M. Gribskov, and C.A. Gross. 1992. The σ32 polypeptide is involved in DnaK-mediated negative regulation of the E. coli σ32-70 operon. J. Bacteriol. 174:3843–3849.

11. Martinez-Salazar, J.M., M. Sandoval-Calderón, X. Guo, et al. 2009. The Rhizobium etli RpoH1 and RpoH2 sigma factors are involved in different stress responses. Microbiology 155:386–397.

12. Meade, H.M., S.R. Long, G.B. Ruvkun, S.E. Brown, and F.M. Ausubel. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of Rhizobium meliloti induced by transposon Tn5 mutagenesis. J. Bacteriol. 149:114–122.

13. Mitsui, H., T. Sato, Y. Sato, N. Ito, and K. Minamisawa. 2004. Dual RpoH sigma factors and transcriptional plasticity in a symbiotic bacterium. J. Bacteriol. 188:3507–3515.

14. Nagai, H., H. Yuzawa, and T. Yura. 1991. Interplay of two cis-acting segments of the σ32 promoter of the heat shock response of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 88:10515–10519.

15. Nagai, H., H. Yuzawa, M. Kanemori, and T. Yura. 1994. A distinct promoter of σ32 synthesis during the heat shock response of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 91:10280–10284.

16. Nakahigashi, K., M. Kanemori, M. Morita, H. Yanagi, and T. Yura. 1998. Conservation of function and regulation of σ32 homologues in Gram-negative bacteria. J. Bacteriol. 175:407–407.

17. Nakahigashi, K., E.Z. Ron, H. Yanagi, and T. Yura. 1999. Differential and independent roles of a σ32 homolog (RpoH) and an HrcA repressor in the heat shock response of Agrobacterium tumefaciens. J. Bacteriol. 181:7509–7515.
18. Nakahigashi, K., H. Yanagi, and T. Yura. 2001. DnaK chaperone-mediated control of activity of a σ32 homolog (RpoH) plays a major role in the heat shock response of Agrobacterium tumefaciens. J. Bacteriol. 183:5302–5310.

19. Narberhaus, F., W. Weighhofer, H.-M. Fischer, and H. Hennecke. 1996. The Bradyrhizobium japonicum rpoH gene encoding a σ32-like protein is part of a unique heat shock gene cluster together with groESL, and three small heat shock genes. J. Bacteriol. 178:5337–5346.

20. Narberhaus, F., P. Krummenacher, H.-M. Fischer, and H. Hennecke. 1997. Three disparately regulated genes for σ32-like transcription factors in Bradyrhizobium japonicum. Mol. Microbiol. 24:93–104.

21. Obrist, M., and F. Narberhaus. 2005. Identification of a turnover element in region 2.1 of Escherichia coli σ32 by a bacterial one-hybrid approach. J. Bacteriol. 187:3807–3813.

22. Oke, V., B.G. Rushing, E.J. Fisher, M. Moghadam-Tabrizi, and S.R. Long. 2001. Identification of the heat-shock sigma factor RpoH and a second RpoH-like protein in Sinorhizobium meliloti. Microbiology 147:2399–2408.

23. Ono, Y., H. Mitsui, T. Sato, and K. Minamisawa. 2001. Two RpoH homologs responsible for the expression of heat shock protein genes in Sinorhizobium meliloti. Mol. Gen. Genet. 264:902–912.

24. Reisenauer, A., C.D. Mohr, and L. Shapiro. 1996. Regulation of a heat shock σ32 homolog in Caulobacter crescentus. J. Bacteriol. 178:1919–1927.

25. Rodriguez, F., F. Arsène-Ploetze, W. Rist, S. Rüdiger, J. Schneider-Mergener, M.P. Mayer, and B. Bukau. 2008. Molecular basis for regulation of the heat shock transcription factor σ32 by the DnaK and DnaJ chaperones. Mol. Cell 32:347–358.

26. Sasaki, S., K. Minamisawa, and H. Mitsui. 2016. A Sinorhizobium meliloti RpoH-regulated gene is involved in iron-sulfur protein metabolism and effective symbiosis under intrinsic iron limitation. J. Bacteriol. 198:2297–2306.

27. Sauviac, L., H. Philippe, K. Phok, and C. Bruand. 2007. An extracytoplasmic function sigma factor acts as a general stress response regulator in Sinorhizobium meliloti. J. Bacteriol. 189:4204–4216.

28. Straus, D., W. Walter, and C.A. Gross. 1990. DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of σ32. Genes Dev. 4:2202–2209.

29. Straus, D.B., W.A. Walter, and C.A. Gross. 1987. The heat shock response of E. coli is regulated by changes in the concentration of σ32. Nature 329:348–351.

30. Studier, F.W., and B.A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189:113–130.

31. Suzuki, H., A. Ikeda, S. Tsuchimoto, K. Adachi, A. Noguchi, Y. Fukumori, and M. Kanemori. 2012. Synergistic binding of DnaJ and DnaK chaperones to heat shock transcription factor σ32 ensures its characteristic high metabolic instability: implications for heat shock protein 70 (Hsp70)-Hsp40 mode of function. J. Biol. Chem. 287:19275–19283.

32. Tomoyasu, T., J. Gamer, B. Bukau, et al. 1995. Escherichia coli FtsH is a membrane-bound, ATP-dependent protease which degrades the heat-shock transcription factor σ32. EMBO J. 14:2551–2560.

33. Tomoyasu, T., T. Ogura, T. Tatsuta, and B. Bukau. 1998. Levels of DnaK and DnaJ provide tight control of heat shock gene expression and protein repair in Escherichia coli. Mol. Microbiol. 30:567–581.

34. Wu, J., and A. Newton. 1996. Isolation, identification, and transcriptional specificity of the heat shock sigma factor σ32 from Caulobacter crescentus. J. Bacteriol. 178:2094–2101.

35. Wu, J., and A. Newton. 1997. The Caulobacter heat shock sigma factor gene rpoH is positively autoregulated from a σ32-dependent promoter. J. Bacteriol. 179:514–521.