Heat-shock sigma factor RpoH from *Geobacter sulfurreducens*

Toshiyuki Ueki and Derek R. Lovley

Department of Microbiology, Morrill Science Center IV North, University of Massachusetts Amherst, 639 North Pleasant Street, Amherst, MA 01003-9298, USA

Recent studies with *Myxococcus xanthus* have suggested that homologues of the *Escherichia coli* heat-shock sigma factor, RpoH, may not be involved in the heat-shock response in this *δ*-proteobacterium. The genome of another *δ*-proteobacterium, *Geobacter sulfurreducens*, which is considered to be a representative of the Fe(III)-reducing *Geobacteraceae* that predominate in a diversity of subsurface environments, contains an *rpoH* homologue. Characterization of the *G. sulfurreducens rpoH* homologue revealed that it was induced by a temperature shift from 30 °C to 42 °C and that an *rpoH*-deficient mutant was unable to grow at 42 °C. The predicted heat-shock genes, *hrcA*, *grpE*, *dnaK*, *groES* and *htpG*, were heat-shock inducible in an *rpoH*-dependent manner, and comparison of promoter regions of these genes identified the consensus sequences for the −10 and −35 promoter elements. In addition, DNA elements identical to the CIRCE consensus sequence were found in promoters of *rpoH*, *hrcA* and *groES*, suggesting that these genes are regulated by a homologue of the repressor HrcA, which is known to bind the CIRCE element. These results suggest that the *G. sulfurreducens* RpoH homologue is the heat-shock sigma factor and that heat-shock response in *G. sulfurreducens* is regulated positively by RpoH as well as negatively by the HrcA/CIRCE system.

**INTRODUCTION**

The Gram-negative *δ*-proteobacterium *Geobacter sulfurreducens* is considered to be a representative of the Fe(III)-reducing *Geobacteraceae* that predominate in a diversity of subsurface environments where Fe(III) reduction is important (Lovley *et al.*, 2004). *Geobacter* species also play critical roles in bioremediation of groundwater contaminated with organic compounds or metals (Lloyd & Lovley, 2001; Lovley, 1997, 2003; Lovley & Coates, 1997, 2000) and in electricity production from waste organic matter (Bond *et al.*, 2002; Bond & Lovley, 2003; Lovley, 2006a, b). However, the mechanisms they use to deal with the multiple stresses they encounter in these environments are poorly understood.

*Geobacter* species face various environmental changes and growth conditions in the subsurface. Heat shock is a common stress to which all organisms adapt by inducing heat-shock proteins. Many heat-shock proteins are well conserved among organisms (Arrigo & Iandry, 1994; Lindquist & Craig, 1998). In bacteria, the expression of heat-shock genes is regulated in various fashions (Gross, 1996; Hecker *et al.*, 1996; Narberhaus, 1999; Rosen & Ron, 2002; Schumann, 2000, 2003; Servant & Mazodier, 2001; Yura *et al.*, 2000). The Gram-negative bacterium *Escherichia coli* uses two sigma factors, RpoH and RpoE, to activate transcription of heat-shock genes (Gross, 1996; Yura *et al.*, 2000). The sigma factor, which is a subunit of RNA polymerase (RNAP), recognizes specific promoter elements and is essential for initiation of transcription. RpoH-dependent transcription is also found in other Gram-negative bacteria (Gross, 1996; Yura *et al.*, 2000; Rosen & Ron, 2002). The regulation of heat-shock gene expression in the Gram-positive bacterium *Bacillus subtilis* is rather complex (Hecker *et al.*, 1996; Schumann, 2003). In the *B. subtilis* heat-shock response transcription of heat-shock genes is regulated by both activation via a sigma factor, SigB, and repression via two different transcription factors, HrcA and CtsR. Both HrcA and CtsR repressors are also found in other bacteria (Narberhaus, 1999; Rosen & Ron, 2002). Other repressor proteins, HspR and RheA, have been shown to be involved in heat-shock gene expression in some bacteria (Servant & Mazodier, 2001).

The heat-shock response in *δ*-Proteobacteria is poorly understood. *Myxococcus xanthus* has three homologues of RpoH (Ueki & Inouye, 2001). However, these homologues were shown to be dispensable for the production of heat-shock proteins and adaptation to heat shock, and instead to be involved in multicellular development in *M. xanthus*.
The G. sulfurreducens genome contains homologues of the sigma factor genes rpoD, rpoS, rpoH, rpoE, flA and rpoN (Methé et al., 2003). In this study, we characterized the role of the rpoH homologue in the heat-shock response of the model organism G. sulfurreducens in order to understand the stress responses utilized by Geobacter communities in the subsurface. Our results indicate that G. sulfurreducens RpoH is indeed the heat-shock sigma factor. In addition, it is likely that the HrcA/CIRCE system is also involved in the G. sulfurreducens heat-shock response.

**METHODS**

**Bacterial strains and growth conditions.** *G. sulfurreducens* DL1 (Caccavo et al., 1994) was used as the parent (wild-type) strain for the construction of the rpoH mutant. *G. sulfurreducens* strains were grown anaerobically in NBAF (acetate and fumarate as an electron donor and an acceptor, respectively) medium (Coppi et al., 2001), supplemented with appropriate antibiotics when necessary. *E. coli* DH5α (Hanahan, 1983) was used for plasmid preparation and grown in LB medium (Miller, 1972), supplemented with appropriate antibiotics when necessary.

**Construction of the rpoH mutant.** The gene encoding RpoH was replaced with a kanamycin-resistance gene, such that the coding region for amino acid residues from Ile-19 to Leu-208 was deleted. These flanking DNA fragments were amplified by PCR with primers 5′-TCTCTAGATGCCGCGATGAAAGATC-3′ and 5′-TCGAATTCGGCTACGGTAGAAC-3′ (XbaI and EcoRI sites are underlined), and 5′-TCAAGCTTCGAGGCGGAGTAGGC-3′ and 5′-TCGAATTCGGCTACGGTAGAAC-3′ (HindIII and BamHI sites are underlined), respectively. The DNA fragment of the kanamycin-resistance gene was amplified by PCR with primers 5′-GACTAAGATTCGGGAGAAGCGGAGAGTTCCAAGC-G-3′ and 5′-GCTTATGAAGCTTCTATAGAAGCGGGC-3′ (EcoRI and HindIII sites are underlined) and pBBR1MCS-2 (Kovach et al., 1994) as a template. The replacement was confirmed by PCR amplification.

**Construction of the expression vector for rpoH.** The DNA fragment containing the 286 bp upstream region of the initiation codon of RpoH, the coding region of RpoH, and the 102 bp downstream region of the termination codon of RpoH including the putative transcription termination signal was amplified by PCR with primers 5′-TCAAGCTTCGAGGCGGAGTAGGC-3′ and 5′-TCGAATTCATACCGTTCGTGTACC-3′ (HindIII and EcoRI sites are underlined). The spectinomycin-resistance gene was amplified with pS985Q (Sandler & Clark, 1994) as a template and primers 5′-TCGAATTCACGGATGCCTAAGC-3′ and 5′-TCGAATTCATACCGTTCGTGTACC-3′ (HindIII and EcoRI sites are underlined). The HindIII–EcoRI fragment of the rpoH gene and the EcoRI–XhoI fragment of the spectinomycin-resistance gene were cloned into the XhoI–HindIII fragment from pCM66 (Marx & Liddstrom, 2001). The resultant plasmid was introduced into the rpoH mutant by electroporation (Coppi et al., 2001).

**RESULTS AND DISCUSSION**

**G. sulfurreducens RpoH**

The *G. sulfurreducens* genome contains a single *rpoH* homologue (GSU0655) (Methé et al., 2003). *G. sulfurreducens* RpoH shows similarity to RpoH homologues from other bacteria including *G. metallireducens*, *Anaeromyxobacter dehalogenans* and *E. coli*. In addition, *G. sulfurreducens* RpoH contains the sequence QKLLFKLN, which is highly homologous to the RpoH box, a stretch of nine amino acid residues, Q(R/K)(R/L)LFNLR, that is only conserved in RpoH homologues (Nakahigashi et al., 1995).

To elucidate the function of the rpoH homologue in *G. sulfurreducens*, the expression of the rpoH gene was examined by a primer extension assay (Fig. 1). *rpoH* mRNA was detected only in cells heat-shocked at 42°C for 10 min (Fig. 1a). A single 5′ end of the mRNA was detected and the putative −35/−10 promoter elements were assigned (Fig. 1b). In addition, a sequence identical to the CIRCE (controlling inverted repeat of chaperon expression) consensus sequence (TTAGACTC-N6-GAGTGCTAA), which is the operator sequence bound by HrcA in other bacteria (Narberhaus, 1999; Mogk et al., 1997; Schulz & Schumann, 1996; Zuber & Schumann, 1994), was found to be located in the rpoH promoter, indicating that *rpoH* expression is regulated by HrcA as discussed below. It should be noted that a putative transcription termination signal is located downstream of the stop codon (Fig. 1c), indicating that the *rpoH* gene is probably monocistronic.
In order to examine the role of rpoH in heat-shock response in G. sulfurreducens an rpoH mutant was constructed. The mutant grew normally in NBAF medium at 30°C (data not shown). However, the mutant was unable to adapt to an elevated growth temperature of 42°C (Fig. 2). The wild-type grew to an OD600 of approximately 0.8 after the temperature shift. In contrast, the mutant stopped growing at an OD600 of approximately 0.5 after the temperature shift and cell lysis was observed in the mutant culture. The plasmid containing the rpoH gene allowed the rpoH mutant to grow at 42°C similarly to the wild-type, indicating that the defect in the adaptation of the rpoH mutant was solely due to the absence of the rpoH gene. These results suggest that the rpoH gene is essential for adaptation to an elevated growth temperature in G. sulfurreducens.

Expression of heat-shock genes

To identify genes regulated by RpoH in G. sulfurreducens, the expression of genes known to be heat-shock inducible in other bacteria was investigated by primer extension assays (Fig. 3). G. sulfurreducens hrcA (Fig. 3a), grpE (Fig. 3b), dnaK (Fig. 3c), htpG (Fig. 3d), and groES (Fig. 3e) were induced by heat shock at 42°C for 10 min. One 5’ end of htpG mRNA was detected, two 5’ ends of mRNA were detected for hrcA, grpE and dnaK, and three 5’ ends of groES mRNA were detected. The expression of these genes was dependent on the rpoH gene, as it was undetectable or drastically decreased in the rpoH mutant. Interestingly, groES mRNA was observed even before heat shock, although the expression level was low. This suggests that groES and most likely groEL, which is located downstream of groES and appears to be co-transcribed with groES, are required at physiological temperatures. Molecular chaperons such as the GroE system and molecular chaperones such as the DnaK/DnaJ/GrpE and the HtpG systems are crucial to protein folding (Riggs et al., 2004; Young et al., 2004; Zhang et al., 2002). In addition, the GroE system is thought to activate the HrcA repressor (Mogk et al., 1997; Schumann, 2000).

The putative −35/−10 promoter elements were assigned for these genes from an analysis of the 5’ ends identified by the primer extension assays (Fig. 3). A sequence identical to the CIRCE consensus sequence (Narberhaus, 1999) was found to be located in the hrcA promoter, indicating that HrcA represses the transcription of its own gene. Such auto-regulation of hrcA is also found in other bacteria, such as the ε-proteobacterium Helicobacter pylori (Spohn et al., 2004). In addition, the groES promoter contains a sequence identical to the CIRCE consensus sequence, as other groES promoters do in bacteria that utilize HrcA. The −35/−10 elements in the hrcA P2, the grpE P2, the dnaK P2, the groES P3 and the htpG promoters show high similarity to one another and those in the rpoH promoter (Fig. 4a). The groES P1 promoter has −35/−10 elements similar to those in the RpoD-dependent promoters, while the other promoters exhibit no apparent similarity to known promoters. These results indicate that these heat-shock genes and rpoH are regulated by the rpoH gene. Moreover, it is likely that the absence and/or the reduced expression of heat-shock genes
caused by the lack of the rpoH gene results in inadequate adaptation to the higher temperature.

The consensus sequences for the −35/−10 promoter elements of heat-shock genes in G. sulfurreducens are proposed (Fig. 4a). The −35 element shows high similarity to that of the E. coli RpoH consensus recognition sequence (Gross, 1996; Yura et al., 2000), whereas the −10 element exhibits low similarity. This difference is supported by the extent of similarity in amino acid sequences of RpoH from G. sulfurreducens and E. coli (Fig. 4b). Region 4.2, which recognizes the −35 element, is highly similar between G. sulfurreducens and E. coli RpoH homologues, while region 2.4, which recognizes the −10 element, is less conserved between G. sulfurreducens and E. coli RpoH homologues.

The gene cluster containing hrcA, grpE, dnaK and dnaJ

In G. sulfurreducens, hrcA, grpE, dnaK and dnaJ are located in this order on the chromosome and appear to constitute an operon, because the lengths of intergenic regions between these genes are short (data not shown). A similar gene organization of these homologues is also found in other bacteria such as B. subtilis (Wetzstein et al., 1992). grpE is typically located downstream of hrcA on the chromosome in bacteria that contain hrcA. Despite the proximity between hrcA and grpE, the transcription of grpE is often driven by its own promoter (Wetzstein et al., 1992; Narberhaus et al., 1992; Roberts et al., 1996). In contrast, the location of dnaK and dnaJ, which is usually located downstream of dnaK, is diversified. When located downstream of grpE, dnaK and dnaJ are co-transcribed with grpE (Wetzstein et al., 1992; Narberhaus et al., 1992). However, in G. sulfurreducens, not only grpE but also dnaK were individually transcribed (Fig. 3). It appears likely that dnaJ is co-transcribed with dnaK in G. sulfurreducens, as a 5' end specific to dnaJ mRNA was not detected by a primer extension assay (data not shown). Separate transcription may facilitate more efficient expression of these genes than co-transcription, resulting in proper function of these genes during heat shock. However, it is possible that these genes can be both co-transcribed and individually transcribed under different conditions.

Transcriptional regulation of rpoH

To investigate the transcriptional regulation of the rpoH gene in G. sulfurreducens, in vitro transcription assays were conducted with the rpoH promoter as a template (Fig. 5). The in vitro transcripts were analyzed by primer extension assay to confirm that the 5' end of the in vitro transcripts was the same as that of the in vivo transcripts. Holo RNAP/RpoH recognized the rpoH promoter and initiated transcription in vivo from the same position as in vitro (Fig. 5b), indicating that RNAP/RpoH transcribes its own gene. Furthermore, it is likely that RNAP/RpoH initiates the transcription of the other heat-shock genes, as their expression was dependent on rpoH and their promoter elements show high similarity to rpoH promoter elements (Fig. 4a). Surprisingly, E. coli holo RNAP/RpoD also initiated transcription in vitro from the same position as in vivo, although it produced fewer transcripts than RNAP/RpoH. The regulation of rpoH expression in vivo was further examined in the rpoH mutant (Fig. 5c). It was found that rpoH expression was still induced by heat shock in the rpoH mutant, although its expression level decreased. It should be noted that the rpoH promoter region as well as the 5' end of the coding region, to which the primer used in the primer extension assays hybridized, were not deleted in the rpoH mutant, allowing one to measure the rpoH promoter activity in the rpoH mutant. These results suggest that the rpoH promoter is also recognized by RNAP/RpoD and that RNAP/RpoD is capable of transcribing the rpoH gene in the absence of RpoH. rpoD is known to be heat-shock inducible in some bacteria such as E. coli (Taylor et al., 1984). Thus, it is possible that G. sulfurreducens rpoD is also heat-shock inducible and that the induction of RpoD is sufficient to account for transcription of rpoH in the rpoH mutant. The rpoH promoter region contains the sequences TTGATT and TACATT (Fig. 1b), which show similarity to the E. coli RpoD consensus sequences TTGACA and TATAAT, respectively (Harley & Reynolds, 1987; Hawley & McClure, 1983). In addition, the groES P1 promoter also contains the RpoD recognition sequence-like −35/−10 elements, TTGATT and TATAGT, respectively, and groES expression dependent on the P1 promoter was still induced by heat shock in the rpoH mutant (Fig. 3e). These results further suggest that RpoD is involved in heat-shock response in G. sulfurreducens. However, it is also possible that instead of RpoD, RpoS is involved in their expression in G. sulfurreducens. RpoS is the stationary phase sigma factor involved in responses to various stresses including heat shock (Hengge-Aronis, 2002) and G. sulfurreducens possesses a homologue of RpoS (Núñez et al., 2004). Furthermore, RpoD and RpoS recognize similar −35/−10 promoter elements (Yan et al., 2006).
The expression of RpoH is mainly controlled at the level of translation in the Gram-negative γ-proteobacterium *E. coli* (Gross, 1996; Yura et al., 2000). Upon heat shock the cellular level of RpoH increases by both enhanced translation of *rpoH* mRNA and stabilization of RpoH in *E. coli*. The *rpoH* homologues from γ-Proteobacteria share common structural characteristics with *E. coli* *rpoH*, such as a downstream box, mRNA secondary structure and highly conserved amino acid sequence of region C, all of which are important for thermoregulation of *rpoH* translation and for stability and activity of RpoH in *E. coli* (Nakahigashi et al., 1995). In contrast, α- and β-Proteobacteria have diverged from γ-Proteobacteria in their mechanisms of regulation of *rpoH* expression. *rpoH* genes from α- and β-Proteobacteria do not contain characteristics found in those from γ-Proteobacteria. Instead, some *rpoH* genes from α-Proteobacteria, such as *Agrobacterium tumefaciens* (Nakahigashi et al., 1999), *Bradyrhizobium japonicum* (Narberhaus et al., 1997) and *Caulobacter crescentus* (Reisenauer et al., 1996; Wu & Newton, 1996), contain an RpoH-dependent promoter that can be induced by heat shock. In *G. sulfurreducens* the downstream box and mRNA secondary structure found in
Proteobacteria are absent (data not shown), while the rpoH gene has an RpoH-dependent promoter (Figs 1 and 4). Thus, the regulation of rpoH expression in G. sulfurreducens appears to be more closely related to that in γ-Proteobacteria. However, it is likely that rpoH expression in G. sulfurreducens is more tightly regulated, as G. sulfurreducens rpoH also contains a DNA element identical to the CIRCE consensus sequence (Fig. 1), indicating negative regulation of rpoH expression by HrcA.

Heat-shock promoters in G. sulfurreducens

The heat-shock promoters in G. sulfurreducens can be classified into four groups: promoters containing −35/−10 elements similar to the RpoH consensus recognition sequences (group 1; grpE P2, dnaK P2 and htpG), ones containing both −35/−10 elements similar to the RpoH consensus recognition sequences and the sequence identical to the CIRCE consensus sequence (group 2; rpoH, hrcA P2 and groES P3), ones containing −35/−10 elements similar to the RpoD consensus recognition sequences (group 1; groES P1), and ones containing −35/−10 elements different from the consensus recognition sequences for RpoH, RpoD or RpoS (group 4; hrcA P1, grpE P1, dnaK P1 and groES P2). The expression of group 1 during heat-shock response was undetectable in the rpoH mutant, while the expression dependent on the promoters containing the CIRCE

![Figure 3 (continued from facing page). Expression of heat-shock genes. (a) hrcA. (b) grpE. (c) dnaK. (d) htpG. (e) groES. Primer extension assays and their promoter regions are shown. Total RNA was prepared from the wild-type (WT) and the rpoH mutant (ΔrpoH) strains before (−) and after (+) heat shock. The 5' ends of mRNA identified by the primer extension assays are indicated by +1. The putative −35 and −10 elements and putative RBS are underlined. The arrows indicate the putative CIRCE element. The initiation codon is indicated by Met.](http://mic.sgmjournals.org)
sequence was still observed (Figs 3 and 5), suggesting that the expression of \( \text{rpoH} \), \( \text{hrcA} \) and \( \text{groES} \) is repressed by the HrcA/CIRCE system. The presence of the \(-35/-10\) elements similar to those in RpoD- or RpoS-dependent promoters suggests the involvement of RpoD and/or RpoS in heat shock. Because there is no apparent sequence similarity among \(-35/-10\) promoter elements in group 4, it is possible that another transcription factor is involved in heat-shock response transcription.

**Phylogenetic perspectives**

The division of \( \delta \)-Proteobacteria consists of a variety of Gram-negative bacteria including anaerobic metal-reducing...
bacteria such as *Geobacter* and *Desulfovibrio* species, bacteriolytic *Bdellovibrio* species, syntrophic bacteria and aerobic developmental myxobacteria. RpoH homologues were identified in other δ-Proteobacteria including *G. metallireducens* (CP000148), *D. vulgaris* (AE017285), *Bdellovibrio bacteriovorus* (BX842601), the syntrophic benzoate-oxidizing bacterium *Syntrophus aciditrophicus* (CP000252), *M. xanthus* (CP000113), and an anaerobic myxobacterium, *A. dehalogenans* (CP000251) (WWW.ncbi.nlm.nih.gov/Genomes). Most of the δ-Proteobacteria described above have a single RpoH homologue, while *M. xanthus* and *A. dehalogenans* have three RpoH homologues. In addition, RpoH-dependent promoters were predicted to be present in several genes in anaerobic metal-reducing δ-Proteobacteria (Rodionov et al., 2004). Thus, it appears likely that most of the δ-Proteobacteria have RpoH-dependent heat-shock response transcription as found in *G. sulfurreducens*.

HrcA homologues were identified in other δ-Proteobacteria including *G. metallireducens*, *D. vulgaris*, *M. xanthus* and *A. dehalogenans*, whereas they are absent from *B. bacteriovorus* and *S. aciditrophicus* (WWW.ncbi.nlm.nih.gov/Genomes). In addition, the CIRCE consensus sequence was predicted to be located in several genes from anaerobic metal-reducing δ-Proteobacteria (Rodionov et al., 2004). Thus, the HrcA/CIRCE negative regulatory system may also be involved in transcription of heat-shock genes in δ-Proteobacteria.

Based on the genomic analyses described above, it appears likely that transcription mechanisms during heat-shock response are diverse in δ-Proteobacteria. Anaerobic metal-reducing bacteria utilize both RpoH as an activator and HrcA as a repressor for transcription of heat-shock genes. A developmental myxobacterium, *M. xanthus*, seems to contain only the HrcA/CIRCE system. Bacteriolytic and syntrophic microorganisms, *B. bacteriovorus* and *S. aciditrophicus*, respectively, appear to possess only RpoH-dependent transcription.

Apparent homologues of other repressors known to be involved in bacterial heat-shock response transcription such as CtsR, HspR and RheA are absent from *G. sulfurreducens*. However, it is possible that a regulatory system unidentified in other bacteria is present in *G. sulfurreducens* as well as other δ-Proteobacteria. For instance, *M. xanthus* utilizes the HsAB two-component system to activate *lonD* expression upon heat shock (Ueki & Inouye, 2002).

**Conclusions**

The results demonstrate that the heat-shock sigma factor, RpoH, is essential for adaptation to a higher temperature in *G. sulfurreducens*. Furthermore, it is most likely that the HrcA/CIRCE repression system is also involved in heat-shock response transcription in *G. sulfurreducens*. Taken together with the genomic information, the mechanisms of heat-shock response transcription appear to be diversified in the Gram-negative δ-Proteobacteria. Temperature is one of important environmental factors that influence microbial activities in the subsurface. This study will serve as a foundation for further characterization of *Geobacter* species in adaptation to different temperatures, which should allow optimization of conditions for applications of *Geobacter* species to bioremediation and electricity production.

**ACKNOWLEDGEMENTS**

This work was supported by the Genomics : GTL program of the Office of Science (BER), US Department of Energy, Grant no. DE-FC02-20ER63446. We thank L. DiDonato for critical reading of the manuscript.

**REFERENCES**


Edited by: M. Hecker