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Identification of the heat-shock sigma factor RpoH and a second RpoH-like protein in *Sinorhizobium meliloti*

Valerie Oke[1,3] Brenda G. Rushing,[1]† Emily J. Fisher,[3] Mohamad Moghadam-Tabrizi[1,2] and Sharon R. Long[1,2]

Author for correspondence: Valerie Oke. Tel: +1 412 624 4635. Fax: +1 412 624 4759. e-mail: voke@pitt.edu

[1,2] Department of Biological Sciences[1] and Howard Hughes Medical Institute[2], Stanford University, Stanford, CA 94305, USA

[3] Department of Biological Sciences, A527A Langley Hall, University of Pittsburgh, Pittsburgh, PA 15260, USA

Hybridization to a PCR product derived from conserved sigma-factor sequences led to the identification of two *Sinorhizobium meliloti* DNA segments that display significant sequence similarity to the family of *rpoH* genes encoding the σ^{32} (RpoH) heat-shock transcription factors. The first gene, *rpoH1*, complements an *Escherichia coli rpoH* mutation. Cells containing an *rpoH1* mutation are impaired in growth at 37 °C under free-living conditions and are defective in nitrogen fixation during symbiosis with alfalfa. A plasmid-borne *rpoH1–gusA* fusion increases in expression upon entry of the culture into the stationary phase of growth. The second gene, designated *rpoH2*, is 42% identical to the *S. meliloti rpoH1* gene. Cells containing an *rpoH2* mutation have no apparent phenotype under free-living conditions or during symbiosis with the host plant alfalfa. An *rpoH2–gusA* fusion increases in expression during the stationary phase of growth. The presence of two *rpoH*-like sequences in *S. meliloti* is reminiscent of the situation in *Bradyrhizobium japonicum*, which has three *rpoH* genes.

Keywords: sigma-32, transcription factor, symbiosis, stress

INTRODUCTION

Sigma subunits of RNA polymerase are responsible for directing specific transcription initiation. The primary sigma factor is mainly used for transcription of housekeeping genes that are required for cell viability, as well as for expression of genes that are sufficiently regulated by other methods such as activating proteins or repressors. Since the sigma subunit associates with core RNA polymerase only during initiation and is then released during elongation of the transcript, the use of specialized subunits is an effective method of transcriptional regulation. The usefulness of this strategy is apparent from the number of bacterial pathways that utilize secondary sigma factors: sporulation, flagellum biosynthesis, the stress response, stationary-phase growth/survival, and nitrogen fixation (Wösten, 1998).

A specialized sigma factor, σ^{32} (RpoH), has been characterized as a component of the heat-shock response in *Escherichia coli* (reviewed by Bukau, 1993; Georgopoulos *et al*., 1994; Yura, 1996). In response to a sudden increase in temperature or other stresses, the levels of σ^{32} rise transiently because of increased synthesis and protein stabilization. The induction of synthesis is mainly mediated by relief of translational repression due to a secondary structure in the mRNA (Morita *et al*., 1999; Nagai *et al*., 1991; Yuzawa *et al*., 1993), though the level of *rpoH* transcription also increases slightly (Erickson *et al*., 1987; Tilly *et al*., 1986). Stabilization occurs with the release of σ^{32} from a DnaK/DnaJ/GrpE chaperone complex as DnaK binds denatured proteins generated under stress conditions (Gamer *et al*., 1996). Free σ^{32} associates with core RNA polymerase to direct the transcription of genes encoding heat-shock proteins, which include molecular chaperones, such as DnaK, and proteases, such as Clp (Gross, 1996). Alternative mechanisms for the response to heat shock can be found in other bacteria. For example, in *Bacillus subtilis*, multiple mechanisms for heat induction have been characterized, including negative regulation of some genes via the action of a repressor at an inverted repeat (called the CIRCE element:

† **Present address:** Department of Biology, Trinity University, 715 Stadium Drive, San Antonio, TX 78212, USA.

The GenBank accession numbers for the sequences reported in this paper are AF128845 (*rpoH1*) and AF149031 (*rpoH2*).

controlling inverted repeat of chaperone expression) and control of other genes by the alternative stress sigma factor, σ^B (Hecker *et al*., 1996).

The nitrogen-fixing symbionts *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and their relatives exist both as soil saprophytes (subject to the conditions of the soil environment) and as symbionts that carry out a series of coordinated behaviours with a host plant. Numerous genes involved in symbiosis have been identified, and regulatory circuits have been partly elucidated. The free-living state of *Rhizobium* and related bacteria has not been extensively characterized, but both metabolism and regulation in response to environmental change are likely to be important as factors in bacterial survival and in strain competitiveness. Sigma factors, as components of the regulatory apparatus that guides general and specific bacterial behaviour, are of interest in *Rhizobium* and allied symbiotic bacteria. *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* (previously *Rhizobium meliloti*) have been the major targets of molecular and biochemical study.

In *Br. japonicum*, the gene for the housekeeping sigma factor SigA (Beck *et al*., 1997), and two copies of the gene for RpoN (NtrA) (Kullik *et al*., 1991), have been identified. RpoN directs expression of nitrogen-fixation (*nif* and *fix*) genes, and the two *rpoN* loci are able to replace each other for symbiotic nitrogen fixation. However, their expression is regulated differently: one is regulated by oxygen tension, and the other is subject to negative autoregulation. In addition, *Br. japonicum* contains three genes encoding σ^{32}-type heat-shock sigma factors (Narberhaus *et al*., 1996, 1997). These *rpoH* loci also show differential regulation and only one can fully function in an *E. coli rpoH* mutant. Although RpoH controls the expression of some heat-inducible genes in *Br. japonicum*, others are controlled, as in *B. subtilis*, by negative regulation using CIRCE or ROSE (repression of heat-shock expression) *cis*-acting elements (Babst *et al*., 1996; Narberhaus *et al*., 1998a). The presence of several copies of genes for particular alternative sigma factors suggests that rhizobia may contain multigene sigma families in order to respond more specifically to changes faced in either their symbiotic or free-living state.

In *S. meliloti*, the genes for the SigA housekeeping sigma factor and for the single RpoN sigma factor for nitrogen-fixation gene expression have been characterized (Ronson *et al*., 1987; Rushing & Long, 1995). A previous attempt to isolate an *rpoH* homologue from *S. meliloti* by complementation of an *E. coli rpoH* amber mutant yielded the gene *suhR*, whose product has no homology to sigma factors and was postulated to function by stabilizing or increasing translation of σ^{32} (Bent & Signer, 1990). We report, here, the identification, characterization, and initial expression studies of two genes, *rpoH1* and *rpoH2*, encoding alternative *rpoH*-like sigma factors from *S. meliloti*. These genes were also recently identified by Ono *et al*. (2001).

METHODS

Strains, plasmids and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. Bacterial cultures were grown in LB or TY (tryptone yeast extract) rich media or M9 sucrose minimal medium supplemented with appropriate antibiotics, as follows: 50 μg ampicillin ml^{-1}, 5 μg gentamicin ml^{-1}, 25 μg kanamycin ml^{-1}, 50–200 μg neomycin ml^{-1}, 25 and 200 μg spectinomycin ml^{-1} (*E. coli* and *S. meliloti*, respectively), 500 μg streptomycin ml^{-1} and 2–10 μg tetracycline ml^{-1}. *E. coli* cells were grown at 37 °C, and *S. meliloti* cells were grown at 30 °C unless otherwise indicated. Plasmids were introduced into *S. meliloti* cells by triparental conjugation (Glazebrook & Walker, 1991) using the *E. coli* strain MT616 carrying the helper plasmid pRK600 (Finan *et al*., 1986). Alfalfa plants (*Medicago sativa* GT13R plus) were grown and inoculated with *S. meliloti* cells as described previously (Oke & Long, 1999).

Screening of λ libraries, and Southern blot conditions. The λ library used for this work contained *S. meliloti* Rm1021 genomic DNA digested with *Bgl*II and ligated into LambdaGEM-11 (Promega). The conditions used for screening the λ library and for Southern hybridization analysis have been described previously (Rushing & Long, 1995).

Isolation of the *rpoH1* gene. The production of a 146 bp PCR fragment (PCR1), corresponding to a portion of the *S. meliloti sigA* gene, has been described previously (Rushing & Long, 1995). The PCR1 fragment weakly hybridized with several plaques from a λ library of *S. meliloti* DNA, which suggests the presence of additional genes encoding sigma factors. We therefore obtained lysates of these λ isolates and performed a second round of PCR as described previously (Rushing & Long, 1995), except that 2 μl phage lysate was used as the template. We obtained a PCR product (PCR2) of 227 bp, corresponding to *rpoH1*, that was cloned as an *Xho*I–*Hin*dIII fragment in pBluescript SK+, creating pBGR24 (Fig. 1). Southern analysis with the PCR2 probe indicated that the *rpoH1* gene was located on a 4·8 kb *Bam*HI chromosomal DNA fragment and that this fragment was contained in the λ *Bgl*II clones from which PCR2 was derived. The 4·8 kb *Bam*HI fragment was therefore subcloned from λ in both orientations in pBluescript SK+, resulting in pBGR70a and pBGR70b.

Isolation of the *rpoH2* gene. Hybridization of PCR1 to the λ *Bgl*II library also identified two λ clones that shared a 1·2 kb *Bgl*II–*Xho*I DNA fragment. This fragment was cloned into pBluescript SK+, resulting in pBGR38a (Fig. 1). Sequence analysis indicated that the fragment contained the 3′ end of the *rpoH2* gene truncated at the *Bgl*II site. To obtain DNA containing the entire *rpoH2* ORF, we employed the technique of chromosome walking. A 1·4 kb *Sma*I–*Kpn*I fragment from pVO122 (Barnett *et al*., 2000), containing the *aadA* gene encoding spectinomycin resistance, was inserted into pBGR38a digested with *Xho*I and *Kpn*I to create pVO128, which was then introduced into the *S. meliloti* electroporatable strain MB501 by selecting for spectinomycin resistance. Since the plasmid is unable to replicate in *S. meliloti*, the transformants contained pVO128 integrated at the *rpoH2* locus by single-reciprocal recombination, as confirmed by Southern analysis. Chromosomal DNA from this strain was digested with *Bst*XI, blunted, ligated, and transformed into *E. coli* cells. One of the resulting transformants harboured a plasmid (pVO147) which contained a 2·3 kb fragment of chromosomal DNA extending from a *Bst*XI site upstream of *rpoH2* and ending at the *Xho*I site downstream of *rpoH2*. In addition, pVO147 contained a non-contiguous 120 bp *Bst*XI fragment which was deleted, resulting in pVO198.

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference/source
***E. coli* strain**		
KY1612	MC4100 Δ*rpoH30*::*kan zhf-50*::Tn*10* [λpF13-(*groE–lacZ*)]	Zhou *et al.* (1988)
***S. meliloti* strain**		
Rm1021	Wild-type, Sm^r	Meade *et al.* (1982)
MMT12	*rpoH1*::pMMT53, Sm^r Sp^r Gm^r	This study
VO3128	*rpoH1*::*aadA*, Sm^r Sp^r	This study
VO2148	*rpoH2*::pVO101(*rpoH2* disruption), Sm^r Tc^r	This study
VO2257	*rpoH2*::pVO194 (*rpoH2–gusA* transcriptional fusion), Sm^r Nm^r	This study
Plasmid		
pBluescript SK+	Cloning vector, Ap^r	Stratagene
pBR322	Cloning vector, Ap^r Tc^r	Bolivar *et al.* (1977)
pJQ200SK	Suicide vector containing the *sacB* gene allowing negative selection, Gm^r	Quandt & Hynes (1993)
pLAFR3	Wide-host-range cosmid vector, Tc^r	Staskawicz *et al.* (1987)
pUC119	Cloning vector, Ap^r	Vieira & Messing (1987)
pBGR24	227 bp PCR2 product of *rpoH1*, *Xho*I–*Hin*dIII insert in pBluescript SK+	This study
pBGR38a	1·2 kb *Bgl*II–*Xho*I fragment with 3′ end of *rpoH2* in pBluescript SK+	This study
pBGR47	4·2 kb *Sac*I fragment with 5′ end of *rpoH1* in pBluescript SK+	This study
pBGR70a	4·8 kb *Bam*HI fragment containing *rpoH1* in pBluescript SK+, *rpoH1* in the opposite direction to P_{lac}	This study
pBGR70b	Same insert as pBGR70a, reverse orientation	This study
pBGR72	1·4 kb *Sac*I–(partial digest)*Xho*I fragment containing the 5′ end of *rpoH1* in pUC119	This study
pBGR79	3·4 kb *Sal*I *gusA–aph* fragment from pGK19 in *Xho*I of pBGR72, *rpoH1–gusA* transcriptional fusion	This study
pBGR86	4·8 kb *Hin*dIII–(partial digest)*Eco*RI from pBGR79 in pLAFR3, *rpoH1–gusA* transcriptional fusion	This study
pBGR91	1·8 kb *Eag*I from pBGR70b in pBluescript KS+, *rpoH1* same orientation as P_{lac}	This study
pBGR97	4·8 kb *Bam*HI from pBGR70a in pLAFR3, *rpoH1* same orientation as P_{lac}	This study
pGK19	pUC1813 containing promoterless *gusA* and *aph* encoding neomycin resistance	Kalinowski & Long (1996)
pMMT52	pBGR70b containing a Sp^r cassette inserted into the *Sac*I site of *rpoH1*	This study
pMMT53	*rpoH1*::*aadA* from pMMT52 in pJQ200SK	This study
pVO101	471 bp *Bgl*II–*Sac*I internal *rpoH2* fragment in pBR322 at *Pst*I	This study
pVO128	pBGR38a containing *aadA* encoding spectinomycin resistance	This study
pVO147	2·3 kb fragment containing *rpoH2* in pBluescript SK+; also contains a non-contiguous 120 bp *Bst*XI fragment	This study
pVO155	Mobilizable vector for constructing *gusA* fusions, Ap^r Km/Nm^r	Oke & Long (1999)
pVO194	*rpoH2–gusA* transcriptional fusion in pVO155	This study
pVO198	2·3 kb fragment containing *rpoH2* in pBluescript SK+	This study

DNA manipulation and sequencing. Plasmid constructions in *E. coli*, and DNA isolation, were carried out essentially as described by Sambrook *et al.* (1989). The nucleotide sequence on both strands was determined with the dideoxy chain termination method using the Sequenase 2.0 kit (US Biochemicals), the IsoTherm DNA sequencing kit (Epicentre Technologies) and fluorescent sequencing with an Applied Biosystems model 373A or Prism 310 machine.

Sequence analysis. Sequence assembly was performed using the University of Wisconsin Genetics Computer Group program (Devereux *et al.*, 1984) or SEQUENCHER 3.0 (Gene Codes Corporation). Database searches were performed through the NCBI Web page by using BLAST 2.0 (Altschul *et al.*, 1997).

Construction of an *rpoH1* null mutation. To disrupt the *rpoH1* gene, a 1·4 kb *Xho*I–*Eco*RI fragment from pVO121 (Barnett *et al.*, 2000), containing the *aadA* gene encoding spectinomycin resistance, was inserted into the *Sac*I site of the *rpoH1* ORF in pBGR70b, creating pMMT52. The *rpoH1*::*aadA* construct was removed from pMMT52 as a *Bam*HI fragment and was inserted into pJQ200SK digested with *Bam*HI, creating pMMT53. This plasmid contains the *sacB* gene from *B. subtilis*, allowing negative selection in Gram-negative bacteria when grown on sucrose (Gay *et al.*,

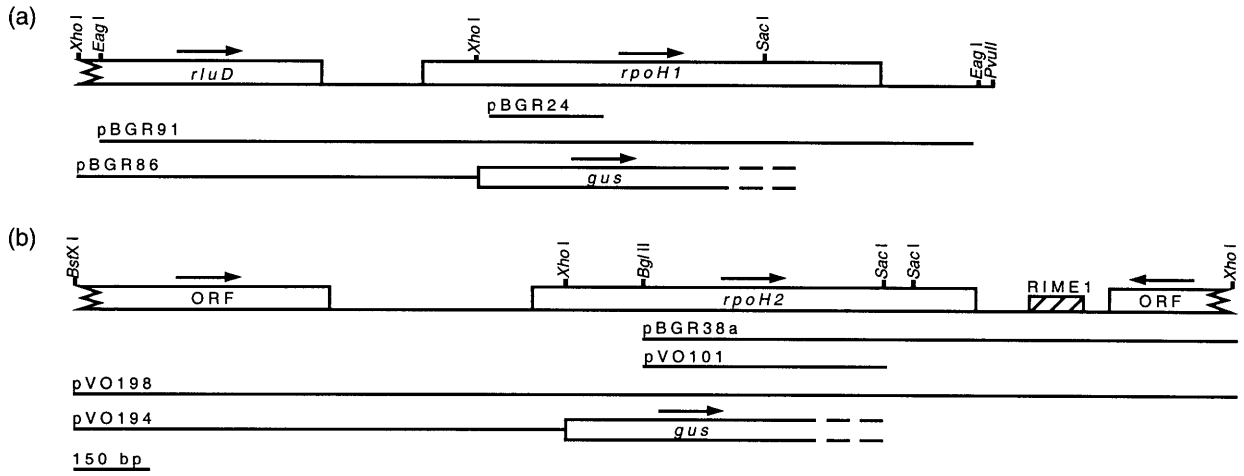

Fig. 1. Map of the *S. meliloti* Rm1021 *rpoH1* and *rpoH2* regions and plasmid constructs. (a) Map of the 1·8 kb *Xho*I–*Pvu*II fragment containing *rpoH1* and part of an ORF similar to *rluD*. (b) Map of the 2285 bp sequenced fragment containing *rpoH2* and two partial ORFs. The repetitive DNA element RIME1 is indicated by a hatched box. DNA contained in plasmid constructs is indicated below each map and includes the junction points for the *gusA* fusions.

1985; Quandt & Hynes, 1993). To construct a strain carrying an *rpoH1* mutation in the chromosome, we first introduced pMMT53 into the wild-type strain Rm1021 by triparental mating followed by selection for spectinomycin resistance; this produced MMT12 (*rpoH1*::pMMT53). Integration of the plasmid by single-reciprocal recombination generated a disrupted copy of *rpoH1* (*rpoH1*::*aadA*) in tandem with a full-length copy of *rpoH1*. If the plasmid is removed from the chromosome by homologous recombination, a single copy of either *rpoH1*::*aadA* or the wild-type *rpoH1* gene will remain in the chromosome, depending on the point of crossover. To select for cells containing only *rpoH1*::*aadA* in the chromosome, MMT12 was grown on LB medium containing 5% sucrose (to select for the loss of pMMT53) and spectinomycin (to select for the retention of *rpoH1*::*aadA*), resulting in VO3128 (*rpoH1*::*aadA*). All strains were confirmed by Southern analysis.

Construction of an *rpoH2* null mutation. To construct a disruption of *rpoH2*, a 475 bp *Bgl*II–*Sac*I DNA fragment internal to the *rpoH2* ORF was removed from pBGR38a and ligated into pBR322 digested with *Pst*I, creating plasmid pVO101 (Fig. 1). pVO101 was introduced into the wild-type strain Rm1021 by triparental mating followed by selection for tetracycline resistance. Since the plasmid is unable to replicate in *S. meliloti*, the resulting strain, VO2148, contained pVO101 integrated by single-reciprocal recombination at the *rpoH2* locus, which was confirmed by Southern analysis. Integration of the plasmid generates two partial copies of the *rpoH2* gene. The first copy is under the control of the *rpoH2* promoter and is deleted for 63 codons at the 3′ end of the gene encoding region 4.2, which is involved in recognition of the −35 region of promoters (Lonetto *et al.*, 1992). The second copy, which is probably not expressed because of the lack of a promoter, has a deletion of 67 codons at the 5′ end of the gene encoding region 2.1 and part of region 2.2 (Lonetto *et al.*, 1992). Thus, we expect no functional RpoH2 protein to be produced in this strain.

Construction of an *rpoH1–gusA* fusion. To construct a transcriptional *rpoH1–gusA* fusion, we started with pBGR47, which contains the 5′ end of the *rpoH1* ORF, ending at the *Sac*I site. To make use of the *Xho*I site located in the *rpoH1*-coding region 84 bp from the start codon as the point of fusion to the reporter gene *gusA* (*uidA*) encoding β-glucuronidase, we first had to destroy an upstream *Xho*I site. We accomplished this by digesting pBGR47 to completion with *Sac*I and then partially with *Xho*I. The 1·4 kb *Xho*I–*Sac*I fragment that contains 690 bp of upstream DNA and the start of *rpoH1* with the desired internal *Xho*I site was then ligated into *Sal*I–*Sac*I-digested pUC119. The resulting plasmid, pBGR72, was digested with *Xho*I, and a 3·4 kb *Sal*I DNA fragment containing a promoterless *gusA* gene and the *aph* gene encoding neomycin resistance from pGK19 was inserted, creating pBGR79 (*rpoH1–gusA*). pBGR79 was digested fully with *Hin*dIII and partially with *Eco*RI to recover the 4·8 kb *rpoH1–gusA* fusion for insertion into the broad-host-range plasmid pLAFR3, creating pBGR86 (Fig. 1).

Construction of an *rpoH2–gusA* fusion. To construct an *rpoH2–gusA* transcriptional fusion, both pVO147 (containing *rpoH2*) and pVO155 (containing a promoterless copy of *gusA* and the *aph* gene encoding neomycin resistance to allow selection in *S. meliloti*; Oke & Long, 1999) were digested with *Xho*I, filled in with Klenow, and then digested with *Bsa*I (which cuts in the *bla* gene of the vector backbones). The fragments were ligated together, resulting in pVO194. The plasmid essentially contains the *Bst*XI–*Xho*I fragment of *rpoH2* in front of *gusA* in pVO155 such that the Shine–Dalgarno sequence for *gusA* is maintained and *rpoH2* and *gusA* are in different reading frames (Fig. 1). pVO194 was introduced into the chromosome of Rm1021 by single-reciprocal recombination, as confirmed by Southern analysis, creating strain VO2257.

Assay of β-glucuronidase activity. Cells collected for β-glucuronidase assays were either assayed immediately or placed on ice or frozen at −80 °C until assayed for activity. β-Glucuronidase activity was determined as described previously (Swanson *et al.*, 1993), except that in some cases the cells were permeabilized with lysozyme (200 μg ml^{-1}; 37 °C for 10 min).

Nodules were sectioned and stained for β-glucuronidase activity as described previously (Swanson *et al*., 1993).

RESULTS

Isolation of *rpoH1* from *S. meliloti*

In previous work isolating the *sigA* gene encoding the primary sigma factor from *S. meliloti* (Rushing & Long, 1995), we observed that a PCR fragment of *sigA* corresponding to conserved region 2 of sigma factors hybridized to multiple bands on a Southern blot and hybridized weakly to some plaques in λ libraries of *S. meliloti* genomic DNA (data not shown), suggesting that the probe was detecting other *S. meliloti* DNA fragments with similarity to genes encoding sigma factors. We isolated a hybridizing 4·8 kb *Bam*HI fragment from the λ library, as described in Methods, and determined the nucleotide sequence of a 1·8 kb *Xho*I–*Pvu*II region (Fig. 1). We identified an ORF with significant similarity to the σ^{70} class of sigma factors. The predicted protein contains 301 amino acids and has a calculated size of 34·5 kDa. Sequence comparison using the database showed that the protein is most similar to the RpoH family of heat-shock sigma factors, and then to a related family of developmental sigma factors (SigB and SigC) from *Myxococcus xanthus*. Because of the functional studies discussed below, we named this gene *rpoH1*. As was found with SigA (Rushing & Long, 1995), RpoH1 from *S. meliloti* is most similar to that of the closely related species *Agrobacterium tumefaciens* (86% identity, 93% similarity); the next most similar protein is $RpoH_2$ from *Br. japonicum* (71% identity, 83% similarity). The *S. meliloti* protein sequence includes the conserved, nine-residue 'RpoH box' that is present in other RpoH proteins (Nakahigashi *et al*., 1995). An alignment of the *S. meliloti* RpoH1 protein with heat-shock sigma factors from other proteobacteria has been published recently by Ono *et al*. (2001).

Approximately 200 bp upstream of the *rpoH1* locus in *S. meliloti* we identified part of an ORF with similarity to a gene known as *rluD* (*sfhB*) (42% identity) from *E. coli* which encodes an enzyme responsible for the insertion of pseudouridine residues in 23S rRNA (Raychaudhuri *et al*., 1998). *rluD* was identified as a suppressor of a temperature-sensitive mutant of *ftsH* (cited in Myler *et al*., 1994), a gene encoding a protease that regulates the levels of σ^{32} in *E. coli* (Herman *et al*., 1995; Tomoyasu *et al*., 1995). *rluD* in *E. coli* is located upstream of *clpB*, a σ^{32}-regulated gene encoding a protease (Kitagawa *et al*., 1991; Squires *et al*., 1991). A similar ORF is also located upstream of the *Br. japonicum* $rpoH_2$ gene (Narberhaus *et al*., 1997).

Southern analysis indicated that the *rpoH1* DNA is not located on either of the *S. meliloti* megaplasmids (data not shown). Therefore, the gene is presumably located on the main chromosome. This result has been confirmed by the presence of the gene in the chromosome sequence data from the genome project (Galibert *et al*., 2001).

Isolation of a second gene in *S. meliloti* encoding an RpoH homologue

Hybridization of the DNA fragment from *sigA* to the λ *Bgl*II library also identified two λ clones that shared a common 1·2 kb DNA fragment which did not correspond to either *sigA* or *rpoH1* (Rushing, 1995). Sequencing of the fragment revealed a partial ORF, and we isolated a 2·3 kb DNA fragment containing the intact gene by integrating a plasmid into the chromosome and then retrieving the plasmid along with flanking DNA, as described in Methods. Sequence analysis demonstrated that the DNA fragment (Fig. 1) consists of a partial ORF encoding a protein with homology to a hypothetical protein in *Mycobacterium tuberculosis* and the CarD transcription factor in *Myx. xanthus* (Nicolas *et al*., 1996), a full-length ORF encoding the protein with homology to the sigma factors, a repetitive DNA element of the *Rhizobiaceae* (Østerås *et al*., 1995), and a partial ORF in the opposite direction encoding a putative protein with no homology to proteins in the database. As with *rpoH1*, Southern analysis indicated that this region of DNA is not located on either of the *S. meliloti* megaplasmids (data not shown) and is therefore located on the chromosome, as confirmed by the genome sequencing project (Galibert *et al*., 2001)

The predicted protein encoded by the intact ORF is 288 amino acids long and has homology, along its length, to the σ^{70} class of sigma factors. We have named the gene *rpoH2* because the predicted protein product shares greatest homology (~ 43% identity, 63% similarity) with the heat-shock σ^{32} factors from the α-proteobacteria, then with the related family of developmental sigma factors – SigB and SigC – from *Myx. xanthus* and the σ^{32} factors from the γ-proteobacteria. The *rpoH1* and *rpoH2* nomenclature is consistent with the designation of these genes in the annotation of the *S. meliloti* genome (Galibert *et al*., 2001). Ono *et al*. (2001) show an alignment of RpoH2 with RpoH1 of *S. meliloti* and other RpoH proteins. The RpoH2 protein has one mismatch [QKALFFNLR] within the signature RpoH box [Q(R/K)(K/R)LFFNLR] (Nakahigashi *et al*., 1995). RpoH2 is no more related to the *S. meliloti* RpoH1 protein (42% identity, 64% similarity) than to the RpoH proteins from other α-proteobacteria.

Function of *S. meliloti rpoH*-like genes in *E. coli*

As a direct test of whether *S. meliloti* RpoH1 and RpoH2 can function as heat-shock sigma factors, we investigated whether the *S. meliloti rpoH1* or *rpoH2* genes could complement the temperature-sensitive phenotype of an *E. coli* strain, KY1612, carrying an *rpoH* deletion (Zhou *et al*., 1988). Although KY1612 originally was unable to grow at temperatures above 20 °C (Zhou *et al*., 1988), several laboratories, including ours, have found the strain to be capable of growing at room temperature; thus, 23 °C is the permissive temperature and 37 °C is the restrictive temperature. We

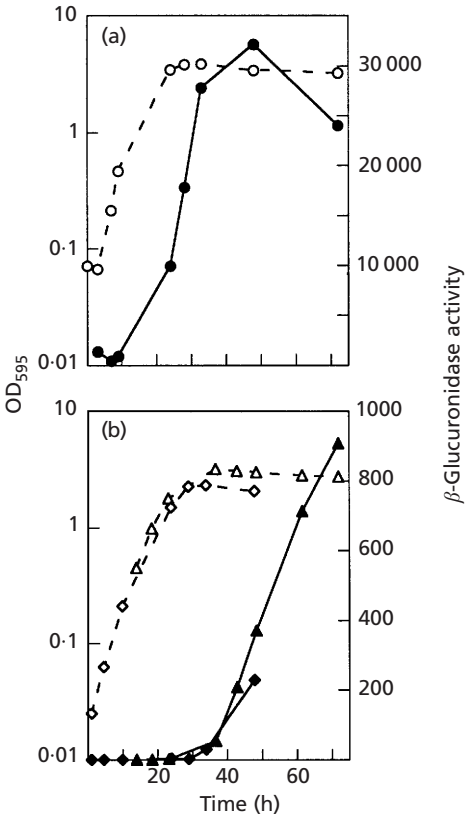

Fig. 2. Expression of *rpoH1* and *rpoH2* as monitored via β-glucuronidase activity. Growth (OD_{595}), open symbols; β-glucuronidase activity in (nmol min^{-1} per OD_{595} unit) × 1000, filled symbols. (a) Expression of an *rpoH1–gusA* transcriptional fusion during growth and the stationary phase of Rm1021/pBGR86 cells in LB medium. (b) Expression of an *rpoH2–gusA* transcriptional fusion during growth and the stationary phase of VO2257 cells in M9 minimal medium. Expression was monitored in two staggered cultures to cover the full time period from 20–40 h when the fusion is induced (diamonds and triangles). Each panel shows the data from one representative experiment.

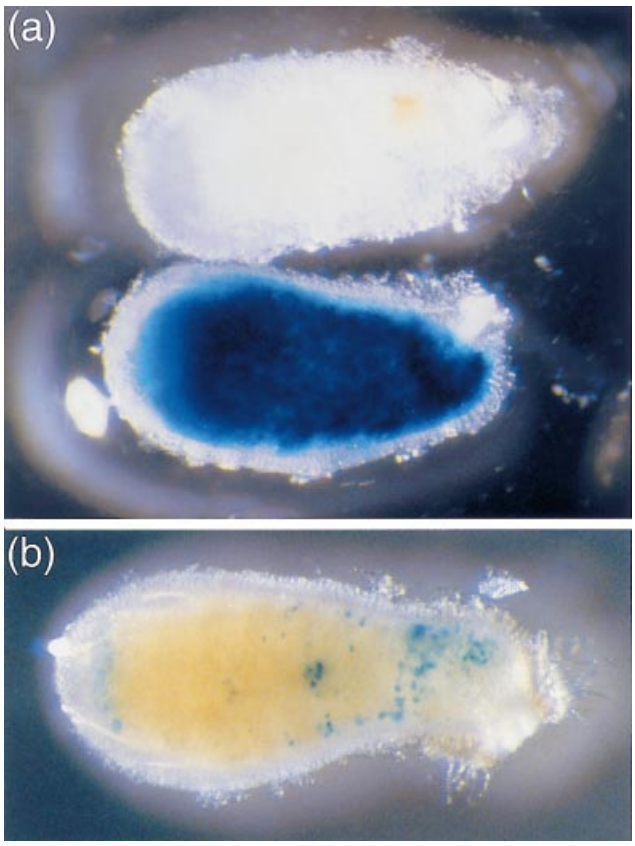

Fig. 3. Expression of *rpoH1* and *rpoH2* within the symbiotic root nodule, as monitored via β-glucuronidase activity. In (a), the top nodule contains Rm1021/pBGR103 (negative control, *gusA* in opposite orientation to *rpoH1*) cells, and the bottom nodule contains Rm1021/pBGR86 (*rpoH1–gusA*) cells. In (b), the nodule contains VO2257 (*rpoH2–gusA*) cells. Nodules were harvested 24 d (a) or 31 d (b) after inoculation, hand-sectioned, then stained with X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) to determine the β-glucuronidase activity. The tip of the nodule is on the left and the root-proximal portion is on the right.

introduced pBGR91, containing the *S. meliloti rpoH1* gene downstream of the *lac* promoter, or pVO198, containing *rpoH2* downstream of the *lac* promoter, into KY1612 cells. Transformation with either the parent vector or pVO198 (*rpoH2*) resulted in 50–600 times more colonies at 23 °C than at 37 °C. In contrast, transformation with pBGR91 (*rpoH1*) resulted in equal numbers of colonies at 23 °C and at 37 °C. Therefore, the *S. meliloti rpoH1* gene is capable of producing a protein that can functionally replace the heat-shock σ^{32} in *E. coli*. Western analysis of extracts from KY1612 cells carrying pBGR91 or pVO198, using antibody to *E. coli* σ^{32}, detected *S. meliloti* RpoH1 but not RpoH2 (data not shown). Recently, Ono *et al.* (2001) demonstrated, with a different construct, that the *S. meliloti rpoH2* gene can complement the *E. coli rpoH* mutant. Therefore, it is most likely that the *S. meliloti rpoH2* gene in our construct is not expressed in *E. coli* (for unknown reasons).

Expression of the *rpoH1–gusA* fusion

To characterize *rpoH1* in more detail, we determined the pattern of *rpoH1* gene expression during free-living growth and under symbiotic conditions by using a plasmid-borne transcriptional *rpoH1–gusA* fusion. We followed the expression of the *rpoH1–gusA* fusion during growth of Rm1021/pBGR86 cells in rich medium. The fusion was expressed during growth, and the β-glucuronidase activity markedly increased as the cells entered stationary phase (Fig. 2a).

To examine possible heat shock control, we tested the effect of a temperature shift from 30 °C to 40 °C on expression of *rpoH1–gusA*. We saw no increase in β-glucuronidase activity (data not shown). Expression of the *rpoH1–gusA* fusion thus did not appear to be induced by heat shock. However, any post-transcriptional control of RpoH1, as is seen in *E. coli* (Erickson *et al.*, 1987; Straus *et al.*, 1987; Tilly *et al.*,

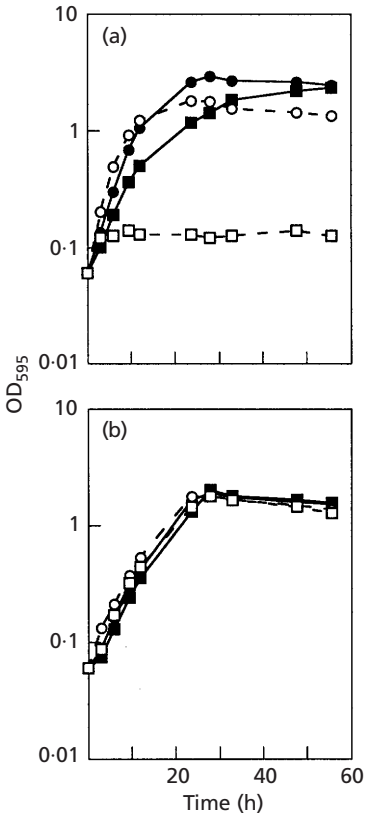

Fig. 4. Comparison of the growth of wild-type cells with the growth of *rpoH1* and *rpoH2* mutant cells, as measured using OD_{595}. (a) Growth of Rm1021 (circles) and VO3128 (*rpoH1*::*aadA*) (squares) in LB medium at 30 °C (filled symbols) and 37 °C (open symbols). (b) Growth of Rm1021 (circles) and VO2148 (*rpoH2*::pVO101) (squares) in M9 medium at 30 °C (filled symbols) and 34 °C (open symbols). Each panel shows the data from one representative experiment.

1989), would not be observed in these experiments since the *rpoH1–gusA* construct is a transcriptional fusion.

To test whether *rpoH1–gusA* is expressed during symbiosis within the nodule, we inoculated alfalfa plants with bacteria containing the plasmid-borne fusion, and stained the resulting nodules for β-glucuronidase activity. The *rpoH1–gusA* fusion was expressed throughout the nodule, whereas a negative control fusion in which *gusA* is in the reverse orientation from *rpoH1* showed little or no expression (Fig. 3a).

Disruption of the *rpoH1* gene and characterization of the mutant

To explore the function of *rpoH1*, we constructed a disruption of the gene by inserting a spectinomycin-resistance gene cassette into the ORF (see Methods). Cells containing the *rpoH1* mutation were viable at 30 °C, the optimal growing temperature for *S. meliloti*, but we noticed that the cells grew more slowly than the wild-type. To determine the growth properties and to test for a role for *rpoH1* in the heat-shock response, we monitored the growth of the *rpoH1* mutant cells and wild-type cells in LB medium at 30 and 37 °C (Fig. 4a). The growth of the mutant cells was modestly impaired at 30 °C, taking longer to plateau, but growth was severely impaired at 37 °C. Since *rpoH1–gusA* is expressed during stationary phase, we tested whether the mutant cells survived in stationary phase by measuring their ability to form colonies immediately after reaching stationary phase in LB medium and after being maintained at 30 °C for an additional week. Cells containing the *rpoH1* mutation had the same survival rate as wild-type cells (data not shown).

Given that *rpoH1* was expressed within the nodule, we tested whether the *rpoH1* mutant was affected in the symbiotic interaction with the host plant alfalfa. The *rpoH1* mutant cells were capable of eliciting the formation of nodules on alfalfa plants. The nodules were white in colour and the plants were stunted and chlorotic, indicating that the bacteria were defective in nitrogen fixation (Fix^-).

Expression of the *rpoH2–gusA* fusion

We determined the pattern of gene expression of a transcriptional chromosomal *rpoH2–gusA* fusion under free-living and symbiotic conditions. The fusion was not expressed during growth or stationary phase in LB medium, but was expressed in M9 minimal medium after the cells reached the stationary phase of growth (Fig. 2b).

The transcriptional *rpoH2–gusA* fusion was modestly induced (fivefold by 10 h) by a shift to 40 °C during growth in M9 minimal medium (data not shown); however, induction was correlated with the cessation of cell growth in response to the heat treatment, and therefore might not be specific to heat shock.

To test if *rpoH2–gusA* was expressed during symbiosis, we inoculated plants with cells containing the fusion and stained the resulting nodules for β-glucuronidase activity. The transcriptional *rpoH2–gusA* fusion was expressed at a low level within the nodules. Although there was some variability in the staining pattern, a typical example is shown in Fig. 3(b): staining occurs at the tip of the nodule and then there is punctate staining at other locations. No expression was observed within the central region of the nodule (where the bacterial cells fix nitrogen). The basis of the pattern of expression is unknown but could reflect the response of bacterial cells to different microenvironments within the nodule.

Disruption of the *rpoH2* gene and characterization of the mutant

We disrupted the *rpoH2* gene by integrating a plasmid containing a fragment internal to the ORF (see Methods). Cells containing the *rpoH2* mutation were viable at 30 °C, the optimal growing temperature for *S. meliloti*. We compared the growth and survival rates of wild-type and mutant cells at different temperatures.

There was no difference in growth between mutant and wild-type cells at 30 °C or at 37 °C in TY rich medium (data not shown), and there was no difference at 30 °C or 34 °C in M9 minimal medium (Fig. 4b) (the higher temperatures approaching the upper limit for growth in the respective medium). In addition, *rpoH2* mutant cells had the same survival rate (as measured by colony formation) as wild-type cells immediately after reaching stationary phase and with continued incubation for 1 week (data not shown). To test if *rpoH2* might play a role during symbiosis, we tested the mutant cells for the ability to nodulate alfalfa successfully. *rpoH2* was not required for nodulation or nitrogen fixation.

DISCUSSION

We have identified two genes, *rpoH1* and *rpoH2*, in *S. meliloti* that encode sigma factors similar to the heat-shock transcription factor σ^{32} of *E. coli*, using DNA hybridization analysis. Analysis of the complete sequence of the *S. meliloti* genome indicates that only two *rpoH*-like sequences are present (Galibert *et al.*, 2001).

Our studies show that *rpoH1* and *rpoH2* are not functionally equivalent. Only *rpoH1* is required for growth in liquid at 37 °C and for successful symbiosis with alfalfa and the two genes are expressed differentially during growth in culture and during symbiosis. Additional support for the idea that these genes are not functionally equivalent comes from work of Ono *et al.* (2001). These authors compared the pattern of protein synthesis in wild-type and *rpoH1* and/or *rpoH2 S. meliloti* mutants after a temperature upshift. Nine putative heat-shock proteins were identified in wild-type cells. An *rpoH1* mutant affected the production of a subset of these heat-shock proteins, whereas an *rpoH2* mutant did not affect production of any of the heat-shock proteins. However, an *rpoH1 rpoH2* double mutant showed further reduction of two heat-shock proteins from the levels seen with *rpoH1* alone. In addition, Ono *et al.* (2001) demonstrated that the *rpoH1 rpoH2* double mutant is unable to nodulate alfalfa. Therefore, the combination of the results from this paper and from Ono *et al.* (2001) suggests that *rpoH1* and *rpoH2* have distinct but overlapping functions. To understand the different roles of these proteins in *S. meliloti*, it will be revealing to analyse the responses of RpoH1 and RpoH2 during heat shock, and to identify and analyse promoters that are dependent on RpoH1 and/or RpoH2.

The presence of two *rpoH*-like sequences in *S. meliloti* is reminiscent of the situation in *Br. japonicum* (another nitrogen-fixing, root-nodule symbiont of legumes), which has three *rpoH* genes (Narberhaus *et al.*, 1996, 1997). $BjRpoH_2$ in *Br. japonicum* is capable of replacing σ^{32} in *E. coli* at 37 °C, and $BjRpoH_1$ and $BjRpoH_3$ can replace σ^{32} at lower temperatures. Like *rpoH1* and *rpoH2* in *S. meliloti*, $BjrpoH_1$ and $BjrpoH_3$ in *Br. japonicum* are dispensable for growth. In contrast, a disruption of $BjrpoH_2$ in *Br. japonicum* was not obtained at either 18 °C or 30 °C.

A conserved, nine-residue sequence called the 'RpoH box' has been identified in members of the RpoH family (Nakahigashi *et al.*, 1995). The RpoH box is contained within a region that has been suggested to be involved in the DnaK/DnaJ-mediated control of the translation and stability of σ^{32} (Nagai *et al.*, 1994), or, more recently, in the binding of σ^{32} to core RNA polymerase (Arsène *et al.*, 1999; Joo *et al.*, 1998). Interestingly, as is the case for RpoH2 in *S. meliloti*, $BjRpoH_1$ and $BjRpoH_3$ each have a single mismatch in the RpoH box, albeit at a residue other than that of the mismatch in RpoH2. The other *rpoH* sequences in the database that encode confirmed RpoH-acting proteins (usually by complementation of an *E. coli rpoH* mutant) contain a perfect match for the conserved sequence. Therefore, the altered RpoH box may have functional significance for the action of these proteins in organisms with multiple *rpoH* genes.

The different functions of the three *rpoH*-like genes in *Br. japonicum* are unclear (Narberhaus *et al.*, 1997, 1998b). The *Br. japonicum* $BjrpoH_2$ cannot be disrupted under standard laboratory conditions. On the basis of data regarding differences in the affinity of each protein for the *groESL1* and *dnaK* promoters and differences in the induction of the *rpoH* genes by heat shock, a model (Narberhaus *et al.*, 1997) has been presented in which $BjRpoH_2$ provides a basal level of RpoH-directed gene expression during normal growth, while $BjRpoH_1$ is responsible for the induction of genes after heat shock. The possible functions of $BjRpoH_3$, however, remain unknown. Further work characterizing the regulon of genes specifically controlled by each RpoH protein in *S. meliloti* and *Br. japonicum* might help to determine why these soil bacteria, which are also endosymbionts of plants, make use of a family of *rpoH* genes.

In *S. meliloti*, both *rpoH1–gusA* and *rpoH2–gusA* fusions are induced during the stationary phase of growth: *rpoH1–gusA* is expressed during exponential phase, and expression then increases late in exponential phase/early in stationary phase in rich medium; *rpoH2–gusA* expression, however, increases later in stationary phase in minimal medium. A link between starvation and other stress responses, including heat shock, has been observed in many bacterial species. Entry of cells of several species, including *Rhizobium leguminosarum* bv. *phaseoli*, into stationary phase leads to multiple stress resistances, including protection against pH, heat, oxidants, and osmotic shock (Thorne & Williams, 1997). In *E. coli*, levels of σ^{32} increase during stationary phase, and the protein is required for the induction of several heat-shock proteins that are induced during starvation (Jenkins *et al.*, 1991). However, although an *E. coli rpoH* mutant is impaired for survival at high temperatures, starved cells are more thermotolerant than growing cells; this implies that σ^{32} is not required for the thermal cross-protection provided by starvation (Jenkins *et al.*, 1991). Since *rpoH1* and *rpoH2* in *S. meliloti* are induced during stationary phase, we specu-

late that the proteins they encode could play roles in the general stress tolerance that develops in starved cells.

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REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zheng, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

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*. *J Bacteriol* **181**, 3552–3561.

Babst, M., Hennecke, H. & Fischer, H.-M. (1996). Two different mechanisms are involved in the heat-shock regulation of chaperonin gene expression in *Bradyrhizobium japonicum*. *Mol Microbiol* **19**, 827–839.

Barnett, M. J., Oke, V. & Long, S. R. (2000). New genetic tools for use in the *Rhizobiaceae* and other bacteria. *BioTechniques* **29**, 240–245.

Beck, C., Marty, R., Kläusli, S., Hennecke, H. & Göttfert, M. (1997). Dissection of the transcription machinery for housekeeping genes of *Bradyrhizobium japonicum*. *J Bacteriol* **179**, 364–369.

Bent, A. F. & Signer, E. R. (1990). *Rhizobium meliloti suhR* suppresses the phenotype of an *Escherichia coli* RNA polymerase σ^{32} mutant. *J Bacteriol* **172**, 3559–3568.

Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–113.

Bukau, B. (1993). Regulation of the *Escherichia coli* heat-shock response. *Mol Microbiol* **9**, 671–680.

Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.

Erickson, J. W., Vaughn, V., Walter, W. A., Neidhardt, F. C. & Gross, C. A. (1987). Regulation of the promoters and transcripts of *rpoH*, the *Escherichia coli* heat shock regulatory gene. *Genes Dev* **1**, 419–432.

Finan, T. M., Kunkel, B., De Vos, G. F. & Signer, E. R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol* **167**, 66–72.

Galibert, F., Finan, T. M., Long, S. R. & 53 other authors (2001). The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* **293**, 668–672.

Gamer, J., Multhaup, G., Tomoyasu, T., McCarty, J. S., Rüdiger, S., Schönfeld, H.-J., Schirra, C., Bujard, H. & Bukau, B. (1996). A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates the activity of the *Escherichia coli* heat shock transcription factor σ^{32}. *EMBO J* **15**, 607–617.

Gay, P., Le Coq, D., Steinmetz, M., Berkelman, T. & Kado, C. I. (1985). Positive selection procedure for entrapment of insertion sequence elements in Gram-negative bacteria. *J Bacteriol* **164**, 918–921.

Georgopoulos, C., Liberek, K., Zylicz, M. & Ang, D. (1994). Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response. In *The Biology of Heat Shock Proteins and Molecular Chaperones*, pp. 202–249. Edited by R. I. Morimoto, A. Tissières & C. Georgopoulos. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Glazebrook, J. & Walker, G. C. (1991). Genetic techniques in *Rhizobium meliloti*. *Methods Enzymol* **204**, 398–418.

Gross, C. A. (1996). Function and regulation of the heat shock proteins. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 1382–1399. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.

Hecker, M., Schumann, W. & Völker, U. (1996). Heat-shock and general stress response in *Bacillus subtilis*. *Mol Microbiol* **19**, 417–428.

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. *Proc Natl Acad Sci U S A* **92**, 3516–3520.

Jenkins, D. E., Auger, E. A. & Matin, A. (1991). Role of RpoH, a heat shock regulator protein, in *Escherichia coli* carbon starvation protein synthesis and survival. *J Bacteriol* **173**, 1992–1996.

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

Kalinowski, G. & Long, S. R. (1996). Deletion analysis of the 5′ untranslated region of the *Rhizobium meliloti nodF* gene. *Mol Plant–Microbe Interact* **9**, 869–873.

Kitagawa, M., Wada, C., Yoshioka, S. & Yura, T. (1991). Expression of ClpB, an analog of the ATP-dependent protease regulatory subunit in *Escherichia coli*, is controlled by a heat shock σ factor (σ^{32}). *J Bacteriol* **173**, 4247–4253.

Kullik, I., Fritsche, S., Knobel, H., Sanjuan, J., Hennecke, H. & Fischer, H.-M. (1991). *Bradyrhizobium japonicum* has two differentially regulated, functional homologs of the σ^{54} gene (*rpoN*). *J Bacteriol* **173**, 1125–1138.

Lonetto, M., Gribskov, M. & Gross, C. A. (1992). The σ^{70} family: sequence conservation and evolutionary relationships. *J Bacteriol* **174**, 3843–3849.

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

Morita, M. T., Tanaka, Y., Kodama, T. S., Kyogoku, Y., Yanagi, H. & Yura, T. (1999). Translational induction of heat shock transcription factor σ^{32}: evidence for a built-in RNA thermosensor. *Genes Dev* **13**, 655–665.

Myler, P. J., Venkatarman, G. M., Lodes, M. J. & Stuart, K. D. (1994). A frequently amplified region in *Leishmania* contains a gene frequently conserved in prokaryotes and eukaryotes. *Gene* **148**, 187–193.

Nagai, H., Yuzawa, H. & Yura, T. (1991). Interplay of two cis-acting mRNA regions in translational control of σ^{32} synthesis during the heat shock response of *Escherichia coli*. *Proc Natl Acad Sci U S A* **88**, 10515–10519.

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*. *Proc Natl Acad Sci U S A* **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., Weiglhofer, W., Fisher, H.-M. & Hennecke, H. (1996). The *Bradyrhizobium japonicum* $rpoH_1$ gene encoding a σ^{32}-like protein is part of a unique heat shock gene cluster together with $groESL_1$ and three small heat shock genes. *J Bacteriol* **178**, 5337–5346.

Narberhaus, F., Krummenacher, P., Fischer, H.-M. & Hennecke, H. (1997). Three disparately regulated genes for σ^{32}-like transcription factors in *Bradyrhizobium japonicum*. *Mol Microbiol* **24**, 93–104.

Narberhaus, F., Kaser, R., Nocker, A. & Hennecke, H. (1998a). A novel DNA element that controls bacterial heat shock gene expression. *Mol Microbiol* **28**, 315–323.

Narberhaus, F., Kowarik, M., Beck, C. & Hennecke, H. (1998b). Promoter selectivity of the *Bradyrhizobium japonicum* transcription factors in vivo and in vitro. *J Bacteriol* **180**, 2395–2401.

Nicolas, F. J., Cayuela, M., Martínez-Argudo, I. M. & Ruiz-Vazquez, R. M. (1996). High mobility group I(Y)-like DNA-binding domains on a bacterial transcription factor. *Proc Natl Acad Sci U S A* **93**, 6881–6885.

Oke, V. & Long, S. R. (1999). Bacterial genes induced within the nodule during the *Rhizobium*–legume symbiosis. *Mol Microbiol* **32**, 837–850.

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

Østerås, M., Stanley, J. & Finan, T. M. (1995). Identification of *Rhizobium*-specific intergenic mosaic elements within an essential two-component regulatory system of *Rhizobium* species. *J Bacteriol* **177**, 5485–5494.

Quandt, J. & Hynes, M. F. (1993). Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. *Gene* **127**, 15–21.

Raychaudhuri, S., Conrad, J., Hall, B. G. & Ofengand, J. (1998). A pseudouridine synthase required for the formation of two universally conserved pseudouridines in ribosomal RNA is essential for normal growth of *Escherichia coli*. *RNA* **4**, 1407–1417.

Ronson, C. W., Nixon, B. T., Albright, L. M. & Ausubel, F. M. (1987). *Rhizobium meliloti ntrA* (*rpoN*) gene is required for diverse metabolic functions. *J Bacteriol* **169**, 2424–2431.

Rushing, B. G. (1995). *Transcription factors in Rhizobium meliloti: characterization of the positive regulator NodD3 and two sigma subunits, SigA and SigB*. PhD thesis, Stanford University.

Rushing, B. G. & Long, S. R. (1995). Cloning and characterization of the *sigA* gene encoding the major sigma subunit of *Rhizobium meliloti*. *J Bacteriol* **177**, 6952–6957.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Squires, C. L., Pedersen, S., Ross, B. M. & Squires, C. (1991). ClpB is the *Escherichia coli* heat shock protein F84.1. *J Bacteriol* **173**, 4254–4262.

Staskawicz, B., Dahlbeck, D., Keen, N. & Napoli, C. (1987). Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J Bacteriol* **169**, 5789–5794.

Straus, D. B., Walter, W. A. & Gross, C. A. (1987). The heat shock response of *E. coli* is regulated by changes in the concentration of σ^{32}. *Nature* **329**, 348–351.

Swanson, J. A., Mulligan, J. T. & Long, S. R. (1993). Regulation of *syrM* and *nodD3* in *Rhizobium meliloti*. *Genetics* **134**, 435–444.

Thorne, S. H. & Williams, H. D. (1997). Adaptation to nutrient starvation in *Rhizobium leguminosarum* bv. phaseoli: analysis of survival, stress resistance, and changes in macromolecular synthesis during entry to and exit from stationary phase. *J Bacteriol* **179**, 6894–6901.

Tilly, K., Erickson, J., Sharma, S. & Georgopoulos, C. (1986). Heat shock regulatory gene *rpoH* mRNA level increases after heat shock in *Escherichia coli*. *J Bacteriol* **168**, 1155–1158.

Tilly, K., Spence, J. & Georgopoulos, C. (1989). Modulation of stability of the *Escherichia coli* heat shock regulatory factor σ^{32}. *J Bacteriol* **171**, 1585–1589.

Tomoyasu, T., Gamer, J., Bukau, B. & 9 other authors (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.

Vieira, J. & Messing, J. (1987). Production of single-stranded plasmid DNA. *Methods Enzymol* **153**, 3–11.

Wösten, M. M. S. M. (1998). Eubacterial sigma-factors. *FEMS Microbiol Rev* **22**, 127–150.

Yura, T. (1996). Regulation and conservation of the heat-shock transcription factor σ^{32}. *Genes Cells* **1**, 277–284.

Yuzawa, H., Nagai, H., Mori, H. & Yura, T. (1993). Heat induction of σ^{32} synthesis mediated by mRNA secondary structure: a primary step of the heat shock response in *Escherichia coli*. *Nucleic Acids Res* **21**, 5449–5455.

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

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