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

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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 $\sigma^{32}$ (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, $\sigma^{32}$ (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 $\sigma^{32}$ 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 $\sigma^{32}$ from a DnaK/DnaJ/GrpE chaperone complex as DnaK binds denatured proteins generated under stress conditions (Gamer et al., 1996). Free $\sigma^{32}$ 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:

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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, σB (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 σB-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 CI/RCE 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 subR, whose product has no homology to sigma factors and was postulated to function by stabilizing or increasing translation of σB (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 BglII 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 plasmid lysis was used as the template. We obtained a PCR product (PCR2) of 227 bp, corresponding to rpoH1, that was cloned as an XbaI–HindIII fragment in pBluescript SK+ (Fig. 1). Southern analysis with the PCR2 probe indicated that the rpoH1 gene was located on a 4.8 kb BamHI chromosomal DNA fragment and that this fragment was contained in the λ BglII clones from which PCR2 was derived. The 4.8 kb BamHI 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 BglII library also identified two λ clones that shared a 1.2 kb BglII–XbaI 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 BglII site. To obtain DNA containing the entire rpoH2 ORF, we employed the technique of chromosomal walking. A 1.4 kb Smal–KpnI fragment from pVO122 (Barnett et al., 2000), containing the addA gene encoding spectinomycin resistance, was inserted into pBGR38a digested with Xhol and KpnI to create pVO128, which was then introduced into the S. meliloti electroporateable 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 BstXI, 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 BstXI site upstream of rpoH2 and ending at the Xhol site downstream of rpoH2. In addition, pVO147 contained a non-contiguous 120 bp BstXI fragment which was deleted, resulting in pVO198.
**Table 1. Strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td><strong>E. coli strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KY1612</td>
<td>MC4100 ΔrpoH30::kan zef-50::Tn10 [phF13-(groE-lacZ)]</td>
<td>Zhou et al. (1988)</td>
</tr>
<tr>
<td><strong>S. meliloti strain</strong></td>
<td></td>
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<tr>
<td>Rm1021</td>
<td>Wild-type, Sm'</td>
<td>Meade et al. (1982)</td>
</tr>
<tr>
<td>MMT12</td>
<td>rpoH1::pMMT53, Sm' Sp' Gm'</td>
<td>This study</td>
</tr>
<tr>
<td>VO3128</td>
<td>rpoH1::aadA, Sm' Sp'</td>
<td>This study</td>
</tr>
<tr>
<td>VO2148</td>
<td>rpoH2::pVO101 (rpoH2 disruption), Sm' Te'</td>
<td>This study</td>
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<tr>
<td>VO2257</td>
<td>rpoH2::pVO194 (rpoH2–gusA transcriptional fusion), Sm' Nm'</td>
<td>This study</td>
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<tr>
<td>Plasmid</td>
<td></td>
<td>Stratagene</td>
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<tr>
<td>pBluescript SK +</td>
<td>Cloning vector, Ap'</td>
<td></td>
</tr>
<tr>
<td>pBR322</td>
<td>Cloning vector, Ap' Te'</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pJQ200SK</td>
<td>Suicide vector containing the sacB gene allowing negative selection, Gm'</td>
<td>Quandt &amp; Hynes (1993)</td>
</tr>
<tr>
<td>pLAFR3</td>
<td>Wide-host-range cosmid vector, Te'</td>
<td>Tkackawicz et al. (1987)</td>
</tr>
<tr>
<td>pBGR24</td>
<td>227 bp PCR2 product of rpoH1, Xhol–HindIII insert in pBluescript SK +</td>
<td>This study</td>
</tr>
<tr>
<td>pBGR38a</td>
<td>1.2 kb BglII–Xhol fragment with 3' end of rpoH2 in pBluescript SK +</td>
<td>This study</td>
</tr>
<tr>
<td>pBGR47</td>
<td>4.2 kb SacI fragment with 3' end of rpoH1 in pBluescript SK +</td>
<td>This study</td>
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<tr>
<td>pBGR70a</td>
<td>4.8 kb BamHI fragment containing rpoH1 in pBluescript SK +, rpoH1 in the opposite direction to P_lac</td>
<td>This study</td>
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<tr>
<td>pBGR70b</td>
<td>Same insert as pBGR70a, reverse orientation</td>
<td>This study</td>
</tr>
<tr>
<td>pBGR72</td>
<td>1.4 kb SacI–(partial digest)Xhol fragment containing the 5' end of rpoH1 in pUC119</td>
<td>This study</td>
</tr>
<tr>
<td>pBGR79</td>
<td>3.4 kb SacI gusA–aph fragment from pGK19 in Xhol of pBGR72, rpoH1–gusA transcriptional fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pBGR86</td>
<td>4.8 kb HindIII–(partial digest)EcoRI from pBGR79 in pLAFR3, rpoH1–gusA transcriptional fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pBGR91</td>
<td>1.8 kb EagI from pBGR70b in pBluescript KS + , rpoH1 same orientation as P_lac</td>
<td>This study</td>
</tr>
<tr>
<td>pBGR97</td>
<td>4.8 kb BamHI from pBGR70a in pLAFR3, rpoH1 same orientation as P_lac encoding neomycin resistance</td>
<td>This study</td>
</tr>
<tr>
<td>pGK19</td>
<td>pUC1813 containing promoterless gusA and aph encoding neomycin resistance</td>
<td>Kalinowski &amp; Long (1996)</td>
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<td>pMMT52</td>
<td>pBGR70b containing a Sp' cassette inserted into the SacI site of rpoH1</td>
<td>This study</td>
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<tr>
<td>pMMT53</td>
<td>rpoH1::aadA from pMMT52 in pJQ200SK</td>
<td>This study</td>
</tr>
<tr>
<td>pVO101</td>
<td>471 bp BglII–SacI internal rpoH2 fragment in pBR322 at PstI</td>
<td>This study</td>
</tr>
<tr>
<td>pVO128</td>
<td>pBGR38a containing aadA encoding spectinomycin resistance</td>
<td>This study</td>
</tr>
<tr>
<td>pVO147</td>
<td>2.3 kb fragment containing rpoH2 in pBluescript SK +; also contains a non-contiguous 120 bp BstXI fragment</td>
<td>This study</td>
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<tr>
<td>pVO194</td>
<td>rpoH2–gusA transcriptional fusion in pVO155</td>
<td>This study</td>
</tr>
<tr>
<td>pVO198</td>
<td>2.3 kb fragment containing rpoH2 in pBluescript SK +</td>
<td>This study</td>
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</table>

**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 SEQUENCER 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 Xhol–EcoRI fragment from pVO121 (Barnett *et al.*, 2000), containing the aadA gene encoding spectinomycin resistance, was inserted into the SacI site of the rpoH1 ORF in pBGR70b, creating pMMT52. The rpoH1::aadA construct was removed from pMMT52 as a BamHI fragment and was inserted into pJQ200SK digested with BamHI, 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.*, 1997).
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 BglII–SacI DNA fragment internal to the rpoH2 ORF was removed from pBGR38a and ligated into pBR322 digested with PstI, 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 SacI site. To make use of the XhoI 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 XhoI site. We accomplished this by digesting pBGR47 to completion with SacI and then partially with XhoI. The 1.4 kb XhoI–SacI fragment that contains 690 bp of upstream DNA and the start of rpoH1 with the desired internal XhoI site was then ligated into SalI–SacI-digested pUC119. The resulting plasmid, pBGR72, was digested with XhoI, and a 3.4 kb SalI 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 HindIII and partially with EcoRI 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 XhoI, filled in with Klengon, and then digested with BsaI (which cuts in the bla gene of the vector backbones). The fragments were ligated together, resulting in pVO194. The plasmid essentially contains the BstXI–XhoI 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 BamHI fragment from the λ library, as described in Methods, and determined the nucleotide sequence of a 1.8 kb Xho–PvuII 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 RpoH2 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 rld (sfbB) (42% identity) from E. coli which encodes an enzyme responsible for the insertion of pseudouridine residues in 23S rRNA (Raychaudhuri et al., 1998). rld was identified as a suppressor of a temperature-sensitive mutant of ftsH (cited in Myler et al., 1994), a gene encoding a protease that regualtes the levels of σ^32 in E. coli (Herman et al., 1995; Tomoyasu et al., 1995). rld 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 rpoH2 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 BglII 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 (Osterás 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 x-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 [QKALFFNLK] within the signature RpoH box [QRK/K/R/LFFNLK] (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 x-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
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 σ32 in *E. coli*. Western analysis of extracts from KY1612 cells carrying pBGR91 or pVO198, using antibody to *E. coli* σ32, 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.,...
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 rpoH2–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

Fig. 4. Comparison of the growth of wild-type cells with the growth of rpoH1 and rpoH2 mutant cells, as measured using Od595. (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 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.

Sinorhizobium meliloti heat-shock RpoH homologues
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 σ32 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). BjRpoH2 in Br. japonicum is capable of replacing σ32 in E. coli at 37 °C, and BjRpoH1 and BjRpoH3 can replace σ32 at lower temperatures. Like rpoH1 and rpoH2 in S. meliloti, BjRpoH1 and BjRpoH3 in Br. japonicum are dispensable for growth. In contrast, a disruption of BjrpoH2 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 σ32 (Nagai et al., 1994), or, more recently, in the binding of σ32 to core RNA polymerase (Arsene et al., 1999; Joo et al., 1998). Interestingly, as is the case for RpoH2 in S. meliloti, BjRpoH1 and BjRpoH3 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 BjrpoH2 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 BjRpoH2 provides a basal level of RpoH-directed gene expression during normal growth, while BjRpoH1 is responsible for the induction of genes after heat shock. The possible functions of BjRpoH3, 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 σ32 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 σ32 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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