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ORIGINAL PAPER

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Sinorhizobium meliloti $RpoH_1$ is required for effective nitrogen-fixing symbiosis with alfalfa

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Abstract *Sinorhizobium meliloti* is a root-nodulating, nitrogen-fixing bacterium. An *S. meliloti* strain that is mutant for the *rpoH*$_1$ gene, which encodes a σ^{32}-like protein, elicits the formation of ineffective nodules on the host plant alfalfa. We characterized the *rpoH*$_1$ mutant for phenotypes related to symbiosis. Alfalfa nodules formed by the *rpoH*$_1$ mutant exhibited greatly reduced levels of acetylene reduction activity compared to the wild-type nodules. Whereas intracellular colonization by rhizobia was observed in a zone just below the apical meristem, we found ultrastructural abnormalities and signs of degeneration of bacteroids within many host cells in the proximally adjacent zone. In the proximal part of the nodule, only a few nodule cells contained bacteroids. In contrast, the *rpoH*$_1$ mutant showed normal induction of nitrogen fixation gene expression in microaerobic culture. These results suggest that the *rpoH*$_1$ mutation causes early senescence of bacteroids during the endosymbiotic process, but does not affect the invasion process or the synthesis of the nitrogenase machinery. The *rpoH*$_1$ mutant exhibited increased sensitivity to various agents and to acid pH, suggesting that $RpoH_1$ is required to protect the bacterial cell against environmental stresses encountered within the host. Since $RpoH_1$ was previously reported to be required for the synthesis of some heat shock proteins (Hsps), we examined the transcription of several genes for Hsp homologs. We found that transcription of *groESL*$_5$, *lon*, and *clpB* after heat shock was $RpoH_1$-dependent, and conserved nucleotide sequences were found in the –35 and –10 regions upstream of the transcription start sites of these genes. Although *groESL*$_5$ expression is almost completely dependent on $RpoH_1$, we found that a *groESL*$_5$ mutant strain is still capable of normal symbiotic nitrogen fixation on alfalfa.

Keywords *Sinorhizobium meliloti* · Sigma factor · Symbiosis · Nitrogen fixation · Heat shock protein

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Introduction

Rhizobia form a group that includes members of the α-subclass and β-subclass (Moulin et al. (2001) of Proteobacteria, which establish root-nodulating, nitrogen-fixing, symbioses with leguminous plants. Effective symbiosis requires the coordinated development of each symbiont: it is triggered by a molecular dialogue between the partners, which is mediated by flavonoid compounds exuded from host plant roots and lipochitooligosaccharides, called Nod factors, produced by rhizobia in response to the specific flavonoids. The Nod factors induce symbiosis-specific development of the host plants, including root hair curling and cortical cell division, leading to infection by rhizobia and the formation of nodule primordia, respectively. In the case of *Sinorhizobium meliloti*, a symbiont of alfalfa, the rhizobia penetrate a root through an infection thread, a tubular structure that initiates with invagination of a root-hair cell wall, to the inner cortex, and are released and enclosed in peribacteroid membranes in the host cell cytoplasm. Bacteroids, the rhizobial cells inside a host cell, undergo propagation and develop into nitrogen-fixing endosymbionts that are elongated and sometimes pleomorphic. Such an endosymbiotic process seems to be a prerequisite for symbiotic nitrogen fixation, because free-living *S. meliloti* exhibit no nitrogen fixation activity under any of the conditions tested so far (for a review, see Brewin 1998).

Sigma factors of bacterial RNA polymerases confer upon the core enzymes the ability to recognize promoters, and multiple sigma factors in a single organism, each with different promoter selectivity, often play a key

role in the regulation of gene expression. In the case of rhizobia, fourteen putative sigma factor genes have been identified by genome sequence analysis of *S. meliloti* (Galibert et al. 2001), and they are expected to be among the important regulators of the endosymbiotic process, during which extensive changes in gene expression occur (Ampe et al. 2003).

Of these sigma factors, σ^{32} (the *rpoH* gene product) plays a central role in the transcription of heat-shock genes, such as those encoding molecular chaperones and proteases in *Escherichia coli* (Gross 1996). *S. meliloti* has two genes that code for σ^{32}-like proteins: $rpoH_1$ and $rpoH_2$ (Ono et al. 2001; Oke et al. 2001). The presence of more than one copy of the *rpoH* homolog seems to be characteristic of rhizobia, since *Bradyrhizobium japonicum* has three genes and *Mesorhizobium loti* has two genes for σ^{32}-like proteins (Narberhaus et al. 1996, 1997; Kaneko et al. 2000). We previously reported that the two RpoH homologs are responsible for heat-induced synthesis of several proteins in *S. meliloti*, as is the case for *E. coli* σ^{32}. Moreover, in symbiosis with alfalfa, the $rpoH_1$ mutant exhibits a nitrogen fixation defect (Fix^- phenotype), and the $rpoH_1$ $rpoH_2$ double mutant exhibits a nodule formation defect (Nod^- phenotype). On the other hand, no apparent phenotype was found for the $rpoH_2$ single mutant either in culture and in symbiotic situations (Ono et al. 2001; Oke et al. 2001). The $rpoH_1$ gene is expressed within a symbiotic nodule, while $rpoH_2$ is not induced to any significant extent (Oke et al. 2001). However, the regulatory system by which $RpoH_1$ and $RpoH_2$ enable the formation of an effective nitrogen-fixing symbiosis has remained elusive.

In the present study, we characterized the phenotypes of *S. meliloti* $rpoH_1$ mutants from the perspective of symbiotic nitrogen fixation. The results we obtained show that the $rpoH_1$ mutation causes early senescence of bacteroids during the symbiotic process and reduced tolerance to environmental stress in the culture, while the mutation does not affect the expression of nitrogen fixation genes. We also examined the transcription of genes encoding several heat shock protein (Hsp) homologs and found $rpoH_1$-dependent transcripts for three. These analyses revealed conserved –35 and –10 sequences located upstream of the $rpoH_1$-dependent transcription start sites that resemble the consensus sequences recognized by the σ^{32} protein of *Escherichia coli*.

Materials and methods

Strains, plasmids, growth media, and plant methods

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) medium (Miller 1992), and *S. meliloti* was grown in LB/MC medium (LB supplemented with 2.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$) or LB as indicated (Glazebrook and Walker 1991). Antibiotics were used as previously described (Ono et al. 2001). Alfalfa (*Medicago sativa* cv. Du Puits) was germinated, inoculated, and grown on Jensen's nitrogen-free medium as described by Leigh et al. (1985).

Acetylene reduction assay

Acetylene gas (3.5 ml) was injected into stoppered 35-ml bottles containing alfalfa roots from 25-day-old plants that had been inoculated with various *S. meliloti* strains. The bottles were incubated at 25°C for 10, 30, or 60 min before analysis. A 1-ml sample from each bottle was analyzed for the presence of ethylene using a Shimadzu GC-18A gas chromatograph equipped with a Porapak R column (mesh size, 80/100; internal diameter, 2.2 mm; length, 2 m; Shimadzu) and a flame ionization detector. The flow rate of nitrogen carrier gas was set at 60 ml/min. The injector temperature was 100°C, and the column temperature was 40°C. Under these conditions, ethylene typically eluted after 1.4 min. The amount of ethylene evolved per plant was calculated. No acetylene was detected in roots from plants that had been inoculated with water alone.

Microscopy

Nineteen-day old nodules were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at 4°C for light microscopy (LM), or were fixed in 2.75% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) at room temperature for 2 h and then in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 1 h for electron microscopy (EM). For LM, the samples were

Table 1 Strains and plasmids used

Strain/plasmid	Genotype/relevant characteristics	Source/reference
Sinorhizobium meliloti strains		
Rm1021	Wild-type, Sm^r	Meade et al. (1982)
HY658G	Rm1021 $rpoH_1$:: *aacC1*	Ono et al. (2001)
HY658 N	Rm1021 $rpoH_1$:: *aphII*	Ono et al. (2001)
BY294	Rm1021 $rpoH_2$:: *aacC1*	Ono et al. (2001)
RmHM9	Rm1021 $rpoH_1$:: *aphII*, $rpoH_2$:: *aacC1*	Ono et al. (2001)
Rm8501	Rm1021 *lac*	Glazebrook and Walker (1991)
Rm1681	Rm1021 *rpoN*:: Tn*5*	Ronson et al. (1987)
Rm1491	Rm1021 *nifH*:: Tn*5*	Zimmerman et al. (1983)
NI001	Rm1021 $groESL_5$:: *aacC1*	This work
Plasmids		
pMB210	pGD926 derivative, *nifH-lacZ* fusion	Better et al. (1985)
pGMI931	pIJ1363 derivative, *fixN-lacZ* fusion	David et al. (1988)
pAM49	pGD926 derivative, P *nifA-lacZ* fusion	This work
pMS266	Gentamicin resistance gene cassette	Becker et al. (1995)
pGD926	Tc^r *lacZ* fusion vector, RK2 *ori*	Ditta et al. (1985)

dehydrated in a graded ethanol series and embedded in LR White resin. The histological organization of nodules was observed using 1-μm sections, stained with toluidine blue. For EM, the samples were dehydrated in a graded ethanol series, followed by propylene oxide treatment, and embedded in Spurr's resin. Ultrathin (90 nm) sections were cut, stained with 2% uranyl acetate and 2% lead citrate at room temperature for 5 min each, and examined with a JEM-100SX electron microscope (JEOL).

DNA manipulations and genetic techniques

Recombinant DNA techniques were performed according to standard protocols (Sambrook and Russell 2001). Generalized transduction of genetic markers with *Φ*M12, and conjugative transfer of plasmid into *S. meliloti* strains by triparental matings using pRK600 were performed as described by Glazebrook and Walker (1991).

Assay for microaerobic induction of *nif* and *fix* expression

The *nifA* - *lacZ* reporter plasmid pAM49 was constructed by inserting a 310-bp DNA fragment of the region between –209 and +101 relative to the *nifA* start codon into pGD926 (Ditta at al. 1985). Each of the plasmids pMB210 (Better et al. 1985), pGMI931 (David et al. 1988) and pAM49, carrying the *nifH* - *lacZ*, *fixN* - *lacZ* and *nifA* - *lacZ* fusions, respectively, was conjugated into Rm8501 (Lac^-) and Rm8501-derived strains harboring *rpoH*$_1$:: *aacC1* or *rpoN*::Tn*5*, which was transduced from HY658G or Rm1681 (Ronson et al. 1987). The strains were grown aerobically to an optical density at 660 nm (OD_{660}) of 0.5 in LB/MC medium at 25°C, and then samples were taken for the *β*-galactosidase assay. For microaerobic culture, 2 ml of culture grown aerobically to an OD_{660} of 0.5 was added to 4 ml of fresh LB/MC medium in a 125-ml bottle. The bottle was stoppered and flushed for 10 min with nitrogen gas before readmitting oxygen gas to a final concentration of 2%. The bottle was incubated at 25°C with shaking for 6 h; then the OD_{660} was measured, and a sample was taken. *β*-Galactosidase activity was measured according to the method described by Miller (1992).

Sensitivity assays

Sensitivity to sodium deoxycholate (DOC) was scored by monitoring growth at 30°C on LB agar plates containing DOC at concentrations of 0, 0.1, 0.2, 0.3, 0.5 or 1.0% (w/v). Paper disk and gradient plate assays were performed as described by Ferguson et al. (2002), starting with *S. meliloti* strains that had grown to an OD_{660} of 0.2. For the sodium dodecyl sulfate (SDS) and crystal violet (CV) assays, 100 μl of culture fluid was added to 3 ml of LB soft agar (6.5 g agar/l), which was then poured onto the surface of an LB plate and allowed to solidify. A paper disk (6-mm diameter; Advantec Toyo) was placed in the center of the plate and 5 μl of 10% (w/v) SDS or 0.4% (w/v) CV was spotted onto it. For the ethanol and acid pH assays, gradient plates containing 100 ml of LB agar (50 ml per layer) were prepared in square (9.7×13.7 cm) petri dishes. For the ethanol assay, the top layer contained 8% (v/v) ethanol; for the acid pH assay, the top layer contained 35 mM 2-(*N* -morpholino)ethanesulfonic acid (MES) (pH 5.5) and the bottom layer contained 35 mM MES (pH 7.0). A 30-μl aliquot of culture fluid was streaked evenly across the plates (9.7 cm in length), and the plates were incubated for 48 h at 30°C. For paper disk or gradient plate assays, zones of bacterial growth inhibition were measured, and the results from six replicates were averaged.

RNA preparation

RNA was prepared from *S. meliloti* cultures grown to an OD_{660} of 0.5 in LB/MC medium at 25°C or shifted to 37°C for 10 or 30 min. A 30-ml culture sample was rapidly chilled on ice and centrifuged. The bacterial pellet was homogenized by vortexing in 5 ml of TRIzol reagent (Invitrogen), incubated at 60°C for 1 min, and cooled to room temperature. Phase separation and RNA precipitation were performed according to the manufacturer's instructions. The RNA sample was further purified using an RNeasy Midi kit (Qiagen).

RNase protection assay

An antisense RNA probe was synthesized by in vitro transcription of the DNA sequence covering an upstream and 5′-terminal coding region of the gene to be tested. The region between +119 and –231 in *groES*$_1$, +107 and –215 in *groES*$_2$, +98 and –201 in *groES*$_3$, +147 and –184 in *groEL*$_4$, +110 and –190 in *groES*$_5$, +92 and –266 in *dnaK*, +233 and –101 in *clpA*, +95 and –259 in *clpB*, and +110 and –396 in *lon* (positions are given relative to the start codon of each gene) was amplified by PCR and cloned in pTZ18R or pTZ19R (Pharmacia) to allow transcription from the T7 promoter flanking the polylinker on the vector. All of the cloned fragments were sequenced to ensure that errors had not been introduced during PCR. The DNA template was linearized with a restriction enzyme, transcribed and then treated with DNase using the Riboprobe system-T7 (Promega) and incorporating [α-^{32}P]CTP (800 Ci/mmol; NEN). The transcription product was mixed with 10 μg of *S. meliloti* RNA, and hybridization was carried out at 50°C for 16 h in 80% formamide containing 40 mM PIPES (pH 6.8), 1 mM EDTA (pH 8.0), and 0.4 M NaCl. The sample was treated with RNase A and RNase T_1, and then processed by the method described by Sambrook and Russell (2001). The RNA samples were electrophoresed on 4% polyacrylamide/8% urea gels together with RNA size markers, which were synthesized by in vitro transcription of the Perfect RNA Marker Template Mix (0.1–1 kb; Novagen) and the aforementioned pTZ18R/19R derivatives linearized with various restriction enzymes. Autoradiography was carried out with the imaging analyzer FLA-2000 (Fuji Photo Film).

Mapping of the 5′ ends of transcripts by primer extension

Primers (25 nt in length) were designed to be complementary to the sequences of mRNAs at the locations between –21 and –45 in *groES*$_5$, +22 and –3 in *lon*, –71 and –95 in *clpB*, and –58 and –82 in *dnaK* (relative to the respective start codons). Each primer was end-labeled with [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham Biosciences) using T4 polynucleotide kinase (Toyobo). Then 40 fmol of a labeled primer was annealed to 10 μg of *S. meliloti* RNA, and the primer extension reaction was carried out using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) and the method described by Sambrook and Russell (2001). The samples were electrophoresed on 6% polyacrylamide/8% urea gels together with sequencing reaction products, which were prepared using the same labeled primers and their respective plasmid DNA templates and Sequencing PRO for dCTP Label (Toyobo). Autoradiography was carried out as described above.

Generation of an *S. meliloti groESL*$_5$ mutant

A 4.4-kb *Bgl* II fragment containing the upstream region and 5′-terminal portion of *groES*$_5$, a 1.0-kb *Bam* HI fragment of the gentamicin resistance (Gm^R) cassette from pMS266 (Becker et al. 1995), and a 9.0-kb *Bam* HI-*Kpn*I fragment containing the 3′-terminal portion and downstream region of *groEL*$_5$ were cloned into pK18mob (Schäfer et al. 1994) to yield the plasmid pKGRHZC, which carries the *groESL*$_5$ DNA with 770 bp of the *groES*$_5$- and *groEL*$_5$-coding region replaced by the Gm^R cassette. pKGRHZC was conjugated into the strain Rm1021, and gentamicin-resistant, neomycin-sensitive clones were selected. The Gm^R marker was transduced again into Rm1021 to yield the mutant strain NI001.

Correct genome replacement was confirmed by Southern hybridization using a mixture of a 2.2-kb *Eco* RV-*Eco*RI fragment and a 0.37-kb *Bam* HI-*Eco*RV fragment as the probe and digesting genomic DNAs with *Eco* RV. We observed a 2.8-kb band in Rm1021 and two bands of 1.7 and 1.3 kb in NI001. In the NI001 DNA, the hybridized 3-kb region was cut into two bands because there is an *Eco* RV site in the Gm^R cassette (data not shown).

Results

Symbiotic phenotypes of the $rpoH_1$ mutant

Alfalfa root nodules elicited by $rpoH_1$ mutants are white and remain small, and the plants are stunted and chlorotic on nitrogen-free medium (Ono et al. 2001; Oke et al. 2001). We compared the acetylene reduction activity of nodules induced by the $rpoH_1$ mutant with those of nodules elicited by the wild type and a known *nif* mutant. Nodules elicited by the strains Rm1021 (wild type) and HY658G (the $rpoH_1$ mutant) showed activities of 513 ± 232 (mean ± standard error) and 1.8 ± 1.4 nmol C_2H_4 evolved per h per plant, respectively (data derived from sixteen replicate experiments). No activity (< 0.1 nmol C_2H_4 evolved) was detected in the nodules elicited by Rm1491, the *nifH* mutant. The severe defect in acetylene reduction activity observed for the $rpoH_1$ mutant was considered to be the basis for the Fix^- phenotype, although the mutant did retain a very low but significant level of activity.

To analyze the effect of the $rpoH_1$ mutation on the symbiotic process, we examined the structure of nodules formed by infection of alfalfa with the $rpoH_1$ mutant. At 19 days after inoculation with HY658G, nodules were no longer than 0.8 mm, in contrast to the elongated nodules (no less than 2.3 mm in length) formed by Rm1021 over the same period. Nodules of the same age formed by Rm1491, which we included to serve as a reference for Fix^- nodules, varied in size between those of $rpoH_1$ and wild-type nodules. In accordance with the difference in nodule shape and size, the overall appearance of an $rpoH_1$ nodule in section was quite different from that of the wild-type one (Fig. 1). In sectioned $rpoH_1$ nodules, bacterial colonization was observed in nodule cells containing large vacuoles in a zone just below the atrophied apical meristem, indicating that the mutation does not block rhizobial invasion and release into host cells, and has little effect, if any, on the efficiency of invasion. However, the numbers of intracellular bacteria decreased in the proximally adjacent zone, and in the part of the nodule further proximal, only a few cells contained bacteria (Fig. 1B). We observed in the electron microscope that such intracellular bacteria in the proximal portion of the nodule were elongated and indistinguishable from the bacteroids observed in a wild-type nodule (data not shown). Such intracellular bacteria might account for the low level of acetylene reduction activity observed for $rpoH_1$ nodules as mentioned above. In *nifH* nodules of 2 mm in length, intracellular colonization by the bacteria was maintained in a more extended area than in the case of an $rpoH_1$ nodule (Fig. 1C), indicating that the $rpoH_1$ mutation affect the symbiotic process more severely than the *nifH* mutation.

We compared the ultrastructure of wild-type and $rpoH_1$ nodules in a transverse sections taken one-third of the distance from the apex of the nodule. In a wild-type nodule, numerous elongate bacteroids displaying cytoplasmic heterogeneity with electron-dense and electron-translucent areas were seen to occupy most of the cytoplasm of nodule cells, as reported previously (Hirsch et al. 1983; Vasse et al. 1990) (Fig. 2A). In the same cells, a few small amyloplasts were found near the cell periphery (not shown in Fig. 2). In contrast, some $rpoH_1$ nodule cells contained intracellular bacteria with abnormal shapes (Fig. 2B), while others contained elongated bacteria similar to wild-type bacteroids (data not shown). Figure 2B shows numerous round and bloated bacteria with patchy cytoplasm; a few bacteria were compact and electron-dense, representing degenerating bacteria. Strikingly, many large amyloplasts were found lining the periphery of the same host cells (not shown in the Figure), a feature which is also characteristic for various Fix^- mutants (Hirsch et al. 1983; Hirsch and Smith 1987; Vasse et al. 1990). Moreover, in more proximal sections of $rpoH_1$ nodules we frequently found signs of cytoplasmic disorganization or cell collapse, a feature similar to that described for nodules induced by a lipopolysaccharide-defective mutant (Perotto et al. 1994) (data not shown). Collectively, the results strongly suggest that the $rpoH_1$ mutation causes senescence of bacteroids and, as a consequence, host cells die at an early stage in the endosymbiotic process.

Expression of nitrogen fixation genes in the $rpoH_1$ mutant

Expression of nitrogen fixation genes is controlled in *S. meliloti* primarily by the oxygen tension (for a review, see Fischer 1994), though nitrogen availability also affects expression levels, as previously demonstrated by Oláh et al. (2001). Under low oxygen tension, the oxygen sensor FixL autophosphorylates, and transfers phosphate to its cognate response regulator FixJ, which in turn is activated and induces transcription of the *nifA* and *fixK* genes. NifA, in conjunction with RpoN, then directs transcription of nitrogen fixation genes, such as *nifHDKE* and *fixABCX* operons. Likewise, FixK induces the transcription of other nitrogen fixation genes, such as the *fixNOQP* and *fixGHIS* operons. We tested whether $RpoH_1$ is involved in this regulatory cascade for *nif* / *fix* expression. To do this, we introduced plasmids carrying *nifH-lacZ*, *fixN-lacZ* , and *nifA-lacZ* fusion genes into Lac^- derivatives of *S. meliloti*, and examined *β*-galactosidase (LacZ) activity under both aerobic and microaerobic culture conditions (Table 2). In Rm8501, a Lac^- derivative of the wild-type strain Rm1021, LacZ activities associated with the three reporters were

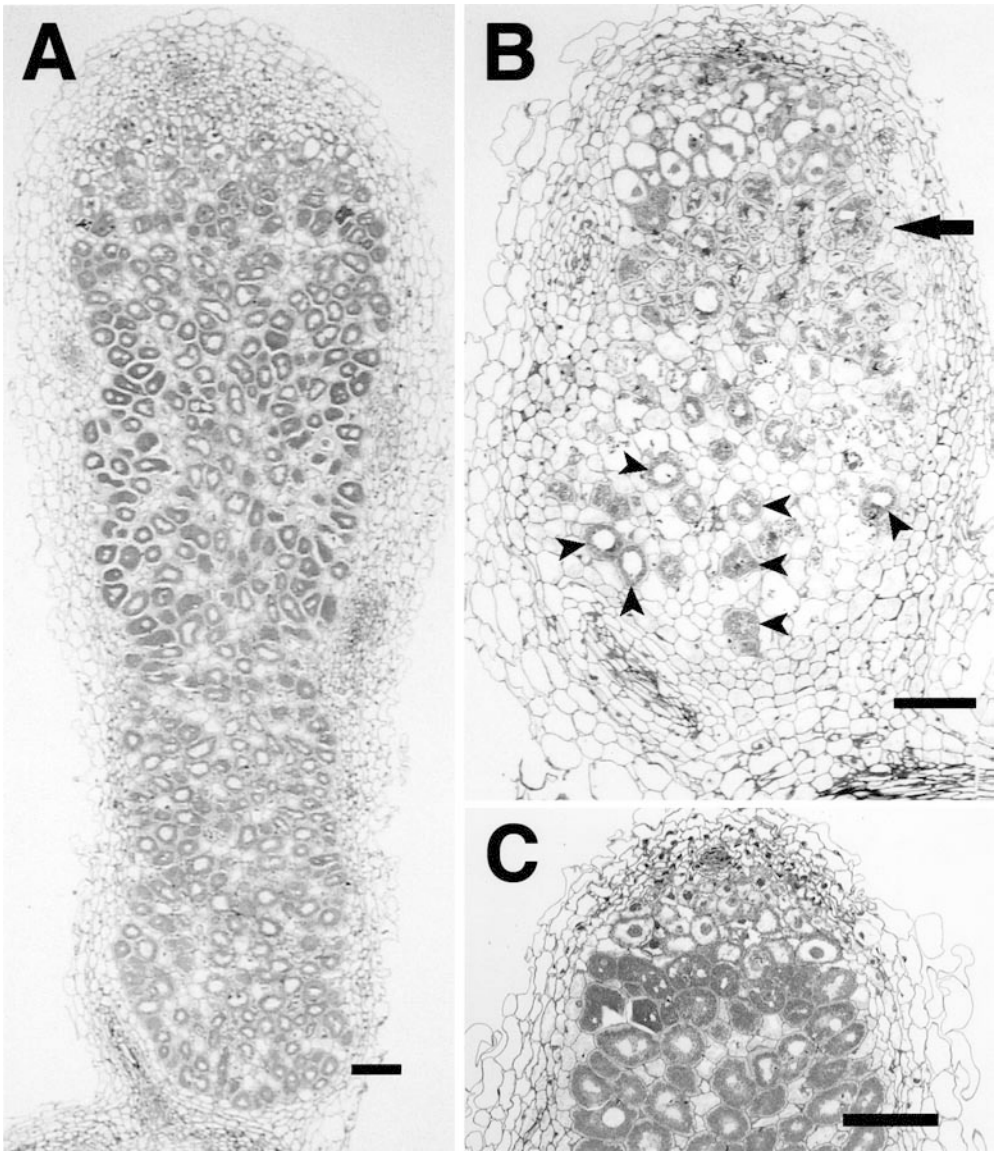

Fig. 1A–C Longitudinal sections of nodules formed by the strains Rm1021 (wild type) (**A**), HY658G (*rpoH*$_1$ mutant) (**B**), and Rm1491 (*nifH* mutant) (**C**). Scale bars: 100 μm. The *arrow* in **B** points to the zone where intracellular bacteria begin to disintegrate. The *arrowheads* indicate proximal nodule cells colonized by bacteria

enhanced upon shifting the cells from aerobiosis to microaerobiosis. Similarly, LacZ activities for all three reporters were also enhanced in the *rpoH*$_1$ mutant, although *nifH-lacZ* expression seemed to be affected slightly. In the *rpoN* mutant, included for comparison, LacZ activity was not induced from the *nifH-lacZ* reporter as expected, while the other reporters showed normal induction. These results indicate that the *rpoH*$_1$ mutation does not affect the expression of the major nitrogen fixation genes controlled by oxygen tension through the function of FixJ, NifA, or FixK.

Effect of the *rpoH*$_1$ mutation on sensitivity to environmental stress

The endosymbiotic process could be affected by the ability of rhizobial cells to protect themselves against environmental stresses encountered within the host. It is possible that the early senescence suggested above for the *rpoH*$_1$ mutant is caused by increased sensitivity to such stress. The fact that the *S. meliloti bacA* mutant, which is also deficient in the endosymbiotic process, is less tolerant to various agents and acid pH in the culture medium (Ferguson et al. 2002) prompted us to quantify the sensitivity of the *rpoH*$_1$ mutant. The *rpoH*$_1$ mutants (HY658G and HY658N) did not grow on LB plates containing 0.1% (w/v) sodium deoxycholate (DOC) after incubation for 3 days. In contrast, the same concentration did not affect the growth of Rm1021; the growth of Rm1021 was slowed by 0.2–0.5% (w/v) DOC and completely inhibited by 1% (w/v) DOC. We also examined the sensitivity to sodium dodecyl sulfate (SDS) and the hydrophobic dye crystal violet (CV) by using an LB plate containing a paper disk supplemented with each agent (see Materials and methods). HY658G exhibited more severe growth inhibition than Rm1021: diameters (in cm) of growth inhibition zones with

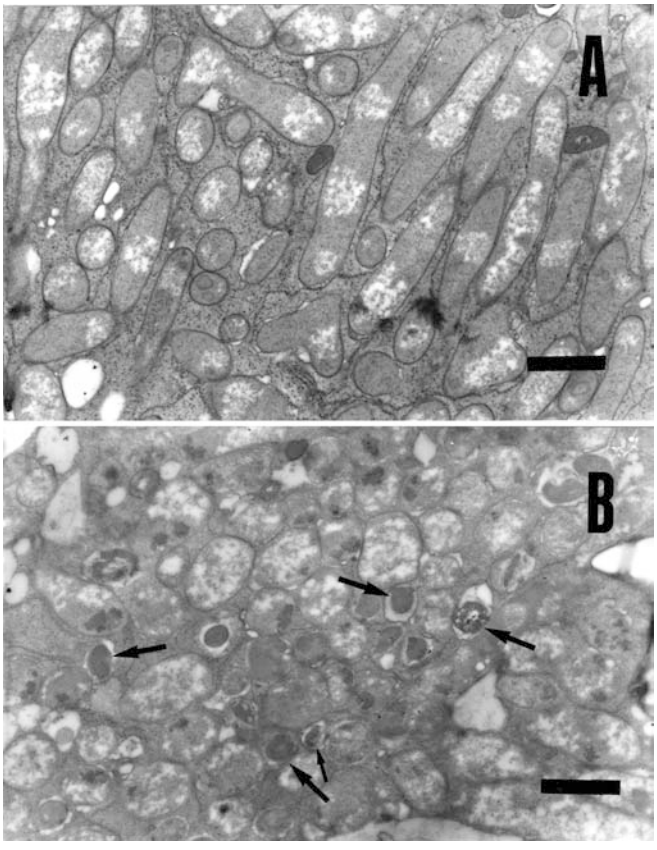

Fig. 2A, B Electron micrographs showing intracellular bacteria from nodule cells infected with the strains Rm1021 (**A**) and HY658G (**B**), respectively. Magnification: 5000×; scale bars: 2 μm. Some bacteroids have degenerated in the *rpoH*$_1$ nodule (*arrows*)

Rm1021 and HY658G were 1.8 ± 0.1 and 2.3 ± 0.2, respectively, for SDS, and 3.1 ± 0.0 and 3.4 ± 0.1, respectively, for CV. We also observed that HY658G was more sensitive to acid pH and ethanol than Rm1021 by plate assay employing a gradient of either pH (pH 7 to pH 5.5) or ethanol concentration (0–8%; v/v). Whereas Rm1021 grew well almost throughout this pH range, HY658G did not grow in the acidic portion (43 ± 5%) of the pH gradient. Growth inhibition was observed at lower ethanol concentrations (at 90 ± 8% along the gradient; 0% ethanol = 100% gradient) with HY658G than in the case of Rm1021 cells (68 ± 2%).

Transcription of heat shock protein genes in the *rpoH*$_1$ mutant

We previously reported that heat-inducible synthesis of proteins with apparent molecular masses ranging between 60 and 100 kDa was greatly reduced in the *rpoH*$_1$ mutant (Ono et al. 2001). We wished to extend these results by measuring the expression profiles of transcripts from several Hsp homologs found in the *S. meliloti* genome (Galibert et al. 2001), such as *groESL*$_1$, *groESL*$_2$, *groESL*$_3$, *groEL*$_4$, *groESL*$_5$, *dnaK*, *clpA*, *clpB* and *lon*, the predicted products of which have relatively large molecular masses. The expression level of each gene was measured under conditions of growth at 25°C and following a shift to 37°C. The ribonuclease protection method was used because it can discriminate among transcripts of the five *groE* (*S*) *L* and two *clpA*/*clpB* genes, which cross-hybridized to one another on genomic Southern blots (data not shown).

The results showed that RpoH$_1$ is involved in the transcription of *groESL*$_5$, *clpB* and *lon* (Fig. 3). The RpoH$_1$-dependent transcripts were detected after the temperature upshift, but not in the culture grown at 25°C. Remarkably, expression of *groESL*$_5$ was almost entirely dependent on RpoH$_1$ (Fig. 3a). For *lon*, multiple transcripts were found, only one of which is RpoH$_1$-dependent and heat-inducible (Fig. 3b). For *clpB*, a signal detected at 25°C was enhanced after the temperature shift, and this was partially dependent on RpoH$_1$ (Fig. 3c).

In contrast, the *rpoH*$_1$ mutation did not affect the expression patterns of *groESL*$_1$, *groESL*$_2$, *groESL*$_3$, *groEL*$_4$, *dnaK* or *clpA* (Fig. 3d and e; data not shown for *groESL*$_2$, *groESL*$_3$, *groEL*$_4$, and *clpA*). Transcription of *groESL*$_1$ was slightly increased after the temperature shift, irrespective of the presence of the *rpoH*$_1$ mutation. There is an inverted repeat sequence upstream of *groESL*$_1$ that is highly similar to one found in *Agrobacterium tumefaciens groESL*, suggesting the involvement of a HrcA regulator, which represses the expression of some heat-shock genes under non-stress conditions (Segal and Ron 1996; Nakahigashi et al. 1999). No appreciable effect of the *rpoH*$_2$ mutation was observed on the expression of any of the genes examined (Fig. 3).

Table 2 Assay for microaerobic induction of nitrogen fixation gene expression

Relevant genotypes of the strains used	Reporter plasmid[a]					
	pMB210 (*nifH-lacZ*)		pAM49 (*nifA-lacZ*)		pGMI931 (*fixN-lacZ*)	
	Aerobiosis	Microaerobiosis	Aerobiosis	Microaerobiosis	Aerobiosis	Microaerobiosis
Wild type	71 ± 9	291 ± 106	111 ± 18	425 ± 65	17 ± 9	611 ± 221
rpoH$_1$:: *aacC1*	89 ± 13	64 ± 5	100 ± 22	465 ± 111	15 ± 4	604 ± 245
rpoN:: Tn*5*	68 ± 11	173 ± 82	88 ± 20	435 ± 126	15 ± 3	552 ± 136

[a]The β-galactosidase activity expressed from the *lacZ* gene on the indicated reporter plasmid is given in Miller units. Each value shown is the mean (± standard error) of more than five replicated experiments

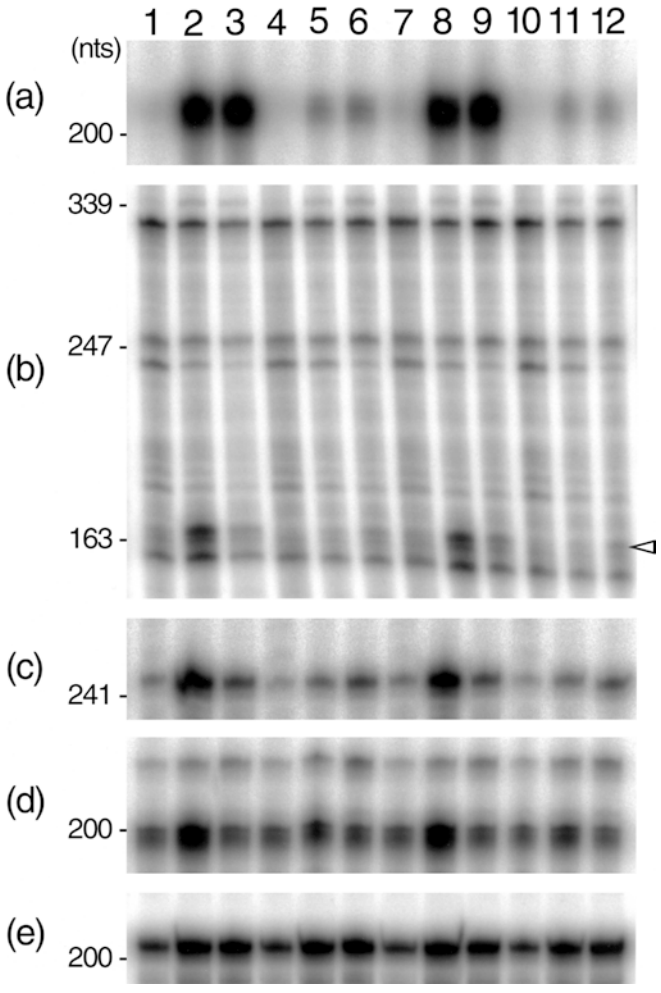

Fig. 3a–e Transcriptional analysis of the Hsp gene homologs $groESL_5$ (**a**), *lon* (**b**), *clpB* (**c**), *dnaK* (**d**), and $groES_1$ (**e**). Cultures of the strains Rm1021 (wild type; lanes 1–3), HY658N ($rpoH_1$ mutant; lanes 4–6), BY294 ($rpoH_2$ mutant; lanes 7–9), and RmHM9 ($rpoH_1$ $rpoH_2$ double mutant; lanes 10–12) were grown at 25°C and then shifted to 37°C. Samples were taken before the shift (lanes 1, 4, 7 and 10), and 10 min (lanes 2, 5, 8 and 11) and 30 min (lanes 3, 6, 9 and 12) after the shift. RNA was extracted from each sample, and 10-μg aliquots of each total RNA were subjected to an RNase protection assay. The products were resolved by polyacrylamide gel electrophoresis. A clear effect of the $rpoH_1$ mutation was observed on the transcription of $groES_5$, *lon* and *clpB* (indicated by the *open arrowhead* for *lon*). Results for *dnaK* and $groES_1$, whose expression is largely $RpoH_1$-independent, are shown for comparison. The positions of RNA size markers (sizes are given in nt) are indicated on the *left*. For *lon*, *clpB* and *dnaK*, a signal whose size is close to the size covered by the probe used for each gene was detected, irrespective of the presence of $rpoH_1$ and/or $rpoH_2$ mutations (data not shown)

We next performed primer extension analysis for $groESL_5$, *lon*, *clpB* and *dnaK*. The 5′ ends of the $RpoH_1$-dependent transcripts of $groESL_5$ and *lon* were mapped at 95 and 73 nt, respectively, upstream of their respective start codons (Fig. 4a, b). The transcript detected for *clpB* in the ribonuclease protection assay (see Fig. 3c) was resolved into two by primer extension analysis: one $RpoH_1$-dependent transcript initiates 166 nt upstream of the start codon, while the other is an $RpoH_1$-independent transcript (Fig. 4c). Alignment of DNA sequences upstream of the $RpoH_1$-dependent transcription initiation sites revealed shared sequences in the –35 and –10 regions, with a spacer of the same length (Fig. 5). In contrast, distinct sequences were found in the upstream, especially in the –10 regions, of $groESL_1$ and *dnaK*, which may account for the fact that their transcription is not dependent on $RpoH_1$.

Intriguingly, we noticed a difference in induction kinetics between the $RpoH_1$-dependent transcripts of $groESL_5$ and *lon*. Elevated expression of $groESL_5$ continued at 10 and 30 min after temperature upshift, while that of *lon* returned to the preshift level 30 min after upshift (Fig. 3a, b). This result suggests the differential involvement of another factor in $RpoH_1$-dependent transcription in the heat shock response.

The function of $RpoH_1$-regulated genes in symbiosis

The symbiotic defect of the $rpoH_1$ mutant suggests that it results from failure to express an $RpoH_1$-dependent gene. Hence mutation of this gene might be expected to lead to a similar symbiotic defect. We tested whether $groESL_5$ is such a gene, since transcription of $groESL_5$ is largely $RpoH_1$-dependent, while expression of the other *groESL* genes is not. A $groESL_5$ mutant strain was constructed by replacing a 766-bp segment of the Rm1021 $groELS_5$ gene with a gentamicin-resistance gene cassette (see Materials and methods). The resulting strain, NI001, which is deleted for the 3′-terminal 22% of $groES_5$ and the 5′-terminal 40% of $groEL_5$, was then tested for the symbiotic phenotype. Alfalfa plants inoculated with NI001 grew well, indeed indistinguishably from those inoculated with Rm1021, on nitrogen-free medium, and cylindrical pink nodules were formed on the roots. This result indicates that the $groESL_5$ mutant is Fix^+.

Discussion

We have shown in this report that nodules elicited on alfalfa by the $rpoH_1$ mutant exhibit a greatly reduced level of acetylene reduction activity, which is compatible with the Fix^- phenotype described previously (Ono et al. 2001; Oke et al. 2001). During symbiosis with legumes, wild-type rhizobia undergo specialized development into nitrogen-fixing bacteroids within a symbiosome in a host cell; in the case of *S. meliloti*, this characterized by a striking morphological change involving extensive elongation and pleomorphism (Vasse et al. 1990) (Fig. 2A). In $rpoH_1$ nodules, we found host cells that were colonized by abnormally shaped, non-elongated bacteroids, and degenerating bacteroids were present in the same cells (Fig. 2B). This is suggested to be a consequence of early senescence of bacteroids after invasion of nodule cells. Rhizobial Fix^- mutants generally induce small, white nodules, and several *nif* / *fix* mutations have

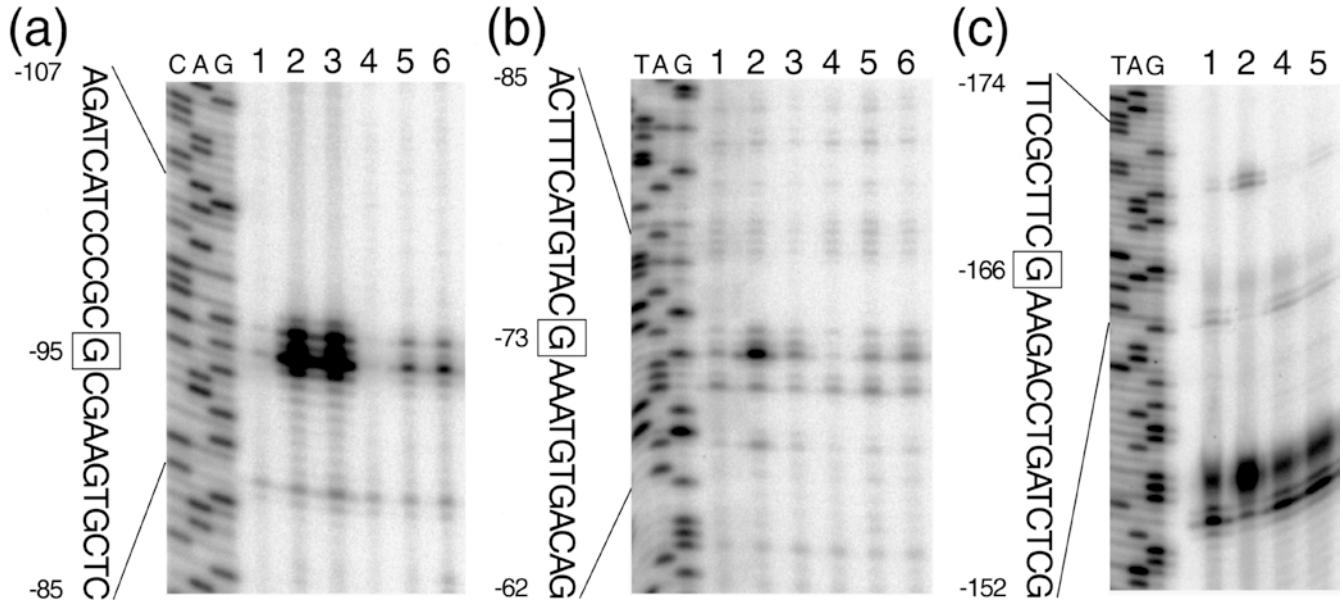

Fig. 4a–c Determination of the start sites of $RpoH_1$-dependent transcripts of $groES_5$ (**a**), *lon* (**b**) and *clpB* (**c**). Aliquots (10 μg) of total RNA extracted from Rm1021 and HY658N (the same RNA samples used for the RNase protection assay) were subjected to primer extension mapping. The extension products were electrophoresed together with the corresponding sequence ladders derived from the same primers (three lanes are shown in each case). Lanes are numbered as in Fig. 3. Sequences numbered relative to the initiation codon of each gene and the transcription start site (*boxed*) are indicated on the *left*

	-35		-10
$groES_5$	ggggcCtCTTGAAatccatttttagcccaaCCAgATcatcccgcg		
lon	gccgtacCTTGAAaaggcctctcttgcactCCActTtcatgtacg		
clpB	tgccgCtCTTtAAttcagaagtgcgctgccCCAtATttcgcttcg		
Consensus	cnCTTgAA	(17)	CCAnaT
E.coli σ^{32}	CnCTTGAA	(13-17)	CCCCATnT
$groES_1$	gcctatcCTTGActcatttcacggccagccttAtcTcggctggcg		
dnaK	agaatgcCTTGcAgtccgaaacgccggtccttAtATacgccgca		

Fig. 5 Upstream DNA sequences aligned with respect to the start sites of the $rpoH_1$-dependent transcripts of $groES_5$, *lon* and *clpB*. The *E. coli* σ^{32} promoter consensus was described by Gross (1996). Nucleotides that occur in at least two of the three and that match the *E. coli* consensus are indicated in *upper case*. The consensus for *S. meliloti* $RpoH_1$ promoters was deduced from sequences of the –35 and –10 regions. The sequences upstream of the $groES_1$ and *dnaK* genes (which are expressed in an $rpoH_1$-independent manner) are included for comparison. The transcription start sites which we determined for $groES_5$, *lon*, *clpB* and *dnaK* are *underlined*. The sequence of $groES_1$ was placed so as to maximize alignment because the start site was not determined

been reported to cause early senescence in such ineffective nodules, possibly due to an epistatic effect of nitrogen fixation deficiency (Hirsch et al. 1983; Hirsch and Smith 1987; Vasse et al. 1990). Because the $rpoH_1$ mutation has a more severe effect than the *nifH* mutation (Fig. 1B and C), senescence in an $rpoH_1$ nodule should occur at an earlier stage in the symbiotic process, before bacteroids normally begin to express *nif*/*fix* genes. We infer that the early senescence leads to the apparent Fix^- phenotype, and that the $rpoH_1$ mutation does not directly affect nitrogen fixation activity. This inference is supported by two other observations: (1) the $rpoH_1$ mutant showed considerable induction of expression of *nifH*, *fixN*, and *nifA* under microaerobic conditions, and (2) the $rpoH_1$ nodules still sustained a low level of acetylene reduction activity, whereas no activity is detectable in the *nifH* nodules. The $rpoH_1$ mutation is unlikely to result in a defect in bacteroid development per se, because a few proximal nodule cells contained numerous elongate bacteroids similar in morphology to the wild-type ones, which appear to have escaped early senescence.

In agreement with this last suggestion, the $rpoH_1$ mutant shows increased sensitivity to acid pH, detergents, hydrophobic dye and ethanol, suggesting that it lacks the stress tolerance needed to survive within a host plant cell. Rhizobia will encounter various environmental stresses in the host, sometimes caused by plant defense mechanisms. One such stress may be the acidic pH, ranging between 5.5 and 6.0, found within a symbiosome, which has been proposed to be acidified by transport of protons or ionized dicarboxylic acids across peribacteroid membranes (Udvardi and Kahn 1992; Perez-Galdona and Kahn 1994).

Altered sensitivity to detergents and hydrophobic dyes is known to be a good indicator of a change in the bacterial cell envelope, especially in the lipopolysaccharide (LPS) (Gustafsson et al. 1973; Lagares et al. 1992). Increased sensitivity to the conditions employed in this study was previously reported for the *lpsB* and *bacA* mutants of *S. meliloti* (Campbell et al. 2002; Ferguson et al. 2002). The *lpsB* gene encodes a glycosyltransferase, and the mutant has an alteration in the carbohydrate core of the LPS. The *bacA* gene encodes an inner-membrane transporter, and the mutant is characterized by an altered distribution of the fatty acids in the LPS. Both these mutants are defective in the endosymbiotic process, as seen for the $rpoH_1$ mutant from the ultra-

structural study. These facts suggest that the reduced stress tolerance and/or the symbiotic deficiency of the *rpoH*$_1$ mutant are caused by a change in the LPS or another component of the cell envelope.

We previously reported that RpoH$_1$ is required for the heat-inducible synthesis of several proteins (Ono et al. 2001). By examining the transcription of nine genes encoding Hsp homologs, we found that the expression of *groESL*$_5$ is almost entirely dependent on RpoH$_1$. The other eight genes tested, *groESL*$_1$, *groESL*$_2$, *groESL*$_3$, *groEL*$_4$, *dnaK*, *clpA*, *clpB* and *lon*, showed significant expression in the *rpoH*$_1$ mutants, with *clpB* and *lon* showing some dependence on RpoH$_1$ (Fig. 3). These results could explain the difference in the effect of *rpoH* mutations on temperature sensitivity between *S. meliloti* and *E. coli*: in *S. meliloti* the *rpoH*$_1$ *rpoH*$_2$ double mutation affects the temperature sensitivity of growth only slightly, whereas in *E. coli* an *rpoH* mutation has a severe effect on growth (Ono et al. 2001). The difference in temperature sensitivity could be explained by assuming that sufficient amounts of the chaperones GroEL/GroES and DnaK/DnaJ are synthesized in the *rpoH*$_1$ and/or *rpoH* $_2$ mutants of *S. meliloti*, but not in the *rpoH* mutant of *E. coli* (Kusukawa and Yura 1988).

The sequences upstream of the three *rpoH*$_1$-dependent transcription start sites revealed a putative promoter consensus in the –35 and –10 regions, the sequence of which is distinct from those of RpoH$_1$-independent genes (Fig. 5). The similarity of this consensus to the *E. coli* σ^{32} consensus suggests a direct involvement of RpoH$_1$ in transcription of the three genes. The putative RpoH$_1$ consensus differs from the heat-shock promoter consensus of *A. tumefaciens*, a phylogenetically close relative of *S. meliloti* that possesses a single *rpoH* gene (Nakahigashi et al. 1999). The difference may be attributable to differences in the relative contributions of RpoH$_1$ and RpoH to the expression of heat-inducible promoters; in an *A. tumefaciens rpoH* mutant, reduced but significant induction of expression from the *groE* and *dnaK* promoters was observed upon temperature upshift (Nakahigashi et al. 1999).

The Fix$^-$ phenotype of the *rpoH*$_1$ mutant could be attributable to the inactivation of a gene that is essential for symbiosis and requires RpoH$_1$ for its expression. To test this possibility, we examined the symbiotic phenotype of the *groESL*$_5$ mutant. The mutant was found to show a Fix$^+$ phenotype, indicating that *groESL*$_5$ is not such a gene. In addition, we infer that the symbiotic defect of the *rpoH*$_1$ mutant cannot be ascribed to the loss of RpoH$_1$-dependent transcription of *lon* and *clpB*, because of the small contribution of RpoH$_1$ to the expression of these genes. In fact, a *clpB* insertion mutant was found to be Fix$^+$ (data not shown), while a *lon* mutant of a different *S. meliloti* strain was reported to be Fix$^-$ (Summers et al. 2000). To understand the reason for the Fix$^-$ phenotype, we have to search for another gene that is regulated by RpoH$_1$. Alternatively, we have to consider the possibility that the symbiotic defect is caused by reductions in the expression levels of several genes in the *rpoH*$_1$ mutant.

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