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Multiple *groESL* Operons Are Not Key Targets of RpoH1 and RpoH2 in *Sinorhizobium meliloti*

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Among the rhizobia that establish nitrogen-fixing nodules on the roots of host plants, many contain multiple copies of genes encoding the sigma factor RpoH and the chaperone GroEL/GroES. In *Sinorhizobium meliloti* there are two *rpoH* genes, four *groESL* operons, and one *groEL* gene. *rpoH1* mutants are defective for growth at high temperature and form ineffective nodules, *rpoH1 rpoH2* double mutants are unable to form nodules, and *groESL1* mutants form ineffective nodules. To explore the roles of RpoH1 and RpoH2, we identified mutants that suppress both the growth and nodulation defects. These mutants do not suppress the nitrogen fixation defect. This implies that the functions of RpoH1 during growth and RpoH1/RpoH2 during the initiation of symbiosis are similar but that there is a different function of RpoH1 needed later during symbiosis. We showed that, unlike in *Escherichia coli*, overexpression of *groESL* is not sufficient to bypass any of the RpoH defects. Under free-living conditions, we determined that RpoH2 does not control expression of the *groE* genes, and RpoH1 only controls expression of *groESL5*. Finally, we completed the series of *groE* mutants by constructing *groESL3* and *groEL4* mutants and demonstrated that they do not display symbiotic defects. Therefore, the only *groESL* operon required by itself for symbiosis is *groESL1*. Taken together, these results suggest that GroEL/GroES production alone cannot explain the requirements for RpoH1 and RpoH2 in *S. meliloti* and that there must be other crucial targets.

Sinorhizobium meliloti can be found as a free-living bacterium residing in the soil or as a nitrogen-fixing symbiont residing in nodules on the roots of leguminous host plants, such as alfalfa. The *S. meliloti* genome contains 14 genes for sigma factors (14), which are subunits of RNA polymerase that direct transcription initiation by recognizing promoters. Two of these genes, *rpoH1* and *rpoH2*, encode members of the RpoH family of secondary sigma factors. RpoH (σ^{32}) was originally identified in *Escherichia coli* as a sigma factor that responds to heat shock. In response to a sudden increase in temperature or other stresses, the levels of RpoH rise transiently, inducing transcription of a subset of genes encoding heat shock proteins (HSPs). HSPs include chaperones involved in protein folding, such as GroEL/GroES and DnaK/DnaJ/GrpE, and proteases, such as FtsH and Lon (48). Although RpoH and the HSPs were identified as part of the heat shock response, these proteins are present at low temperature and play important roles in cellular processes under nonstress conditions, such that the *rpoH* gene in *E. coli* is essential above 20°C (49). The requirement for RpoH in *E. coli* can largely be explained as a requirement for expression of the *groESL* operon, because overexpression of *groESL* is sufficient to suppress the temperature-sensitive growth defect of the *rpoH* mutant from 20 to 40°C (25).

Although the *E. coli* genome only contains one *rpoH* gene and one *groESL* operon, other bacterial genomes contain multiple copies of these genes. In particular, many *Rhizobium* species have multiple *rpoH* and *groESL* genes, and mutations in some of them result in symbiotic defects. In *S. meliloti*, in addition to the two *rpoH* genes (35, 36), there are four *groESL* operons (14, 33, 34, 40) and one *groEL* gene (7). *rpoH1* and *groESL1* mutants are unable to fix nitrogen (Fix$^-$) (33, 35, 36), and *rpoH1 rpoH2* double mutants are unable to form nodules (Nod$^-$) (36). In *Bradyrhizobium japonicum*, there are three *rpoH* genes (30, 31), five *groESL* operons (10), and two *groEL* genes (23). $groESL3_{Bj}$ is regulated with nitrogen fixation genes (10), and a $groESL3_{Bj}$ $groESL4_{Bj}$ double mutant is unable to fix nitrogen (11). *Rhizobium* sp. strain TAL1145 has at least one *rpoH* gene, and the *rpoH* mutant exhibits reduced nodulation, resulting in stunted plant growth (24). *Rhizobium leguminosarum* has at least three *groESL* operons (39, 44), and *Mesorhizobium loti* has two *rpoH* genes and five *groESL* operons (22, 24). Interestingly, the genome of the closely related plant pathogen *Agrobacterium tumefaciens*, also a member of the *Rhizobiaceae*, only contains single copies of these genes (46). The reason for multiple *rpoH* and *groESL* genes in these plant endosymbionts is unclear. Are the genes regulated differentially but encode proteins with similar functions, or do they encode proteins with specialized functions?

The two *rpoH* genes in *S. meliloti* were identified as members of the *rpoH* family by sequence analysis and by the ability to complement an *E. coli rpoH* mutation (35, 36). Under free-living conditions, *rpoH1* mutants exhibit a slight growth defect at the optimum growth temperature (30°C) and a severe defect at higher temperatures (35). During symbiosis, *rpoH1* mutant cells invade the nodule and differentiate into bacteroids but undergo early senescence (28), resulting in a Fix$^-$ phenotype (35, 36). *rpoH2* mutants have no phenotype under free-living or symbiotic conditions (35, 36). However, Ono et al. (36) discovered that an *rpoH1 rpoH2* double mutant is unable to form nodules.

Transcriptional reporter fusions to *rpoH1* and *rpoH2* have

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shown that *rpoH1* is transcribed during stationary phase in Luria-Bertani (LB) rich medium and M9 minimal medium and that *rpoH2* is transcribed during stationary phase only in M9 medium. During symbiosis, *rpoH1* is strongly expressed throughout the nodule, whereas *rpoH2* is not expressed in the nodule, except for low levels at the tip and variable punctate spots at other locations (35). The phenotypes and expression data suggest that *rpoH1* and *rpoH2* have distinct but overlapping functions.

The presence of a family of four to five *groEL* genes in *S. meliloti* was initially discovered by Southern blot analysis (40). Additional work by other groups and subsequent sequencing of the *S. meliloti* genome has led to a final count of four *groESL* operons and one *groEL* gene (7, 14, 33, 34, 40). The names of the *groE* genes used in this paper are those given in the genome annotation, although *groES5* was not annotated (14).

A connection between GroEL/GroES and symbiosis was uncovered when *groESL1* was identified in a genetic screen for *S. meliloti* genes required for full induction of *nod* genes, which are required for formation of a bacterial signal that initiates nodule formation by host plants (33). The *groESL1* mutation affects the activities of several related transcription factors (NodD1, NodD3, and SyrM) that activate gene expression of *nod* genes, and GroEL copurifies with NodD1 and NodD3 (12, 33). In vitro work has demonstrated that the NodD proteins are substrates for GroEL/GroES, resulting in modulation of the DNA binding activity (47). *groESL1*, *groESL2*, and *groESL5* mutants have been studied. *groESL1* mutants have a slight growth defect, are delayed for nodulation, and form Fix^- nodules (33). A *groESL2* mutant displays neither a growth nor a symbiotic defect, but the *groESL1 groESL2* double mutant is not viable (32). The *groESL5* mutant has no symbiotic defect (28). GroES1/GroEL1 and GroES2/GroEL2 are the most similar to each other (97% identical for GroES and 99% identical for GroEL), whereas GroES3/GroEL3 is the most dissimilar from any other *S. meliloti* homolog (75 to 78% identical for GroES and 72 to 74% identical for GroEL).

Mitsui et al. (28) tested whether RpoH1 or RpoH2 controls expression of the *groESL* genes in *S. meliloti* during heat shock. *groESL5* was the only *groESL* operon whose transcription was controlled by RpoH1, and none of the genes were controlled by RpoH2. However, this work did not explore regulation during stationary phase and within the nodule, other conditions where we know that *rpoH1* and *rpoH2* are expressed (35).

Given that *groESL* is a crucial target of RpoH in *E. coli* and that *groESL1*, *rpoH1*, and *rpoH1 rpoH2 S. meliloti* mutants have symbiotic phenotypes, we hypothesized that *groESL* might also be a key target of RpoH in *S. meliloti*. However, in this paper we use suppressor mutant analysis and overexpression experiments to demonstrate that the relationships between RpoH and GroEL/GroES are different in the two organisms. Specifically, our results suggest that GroEL/GroES production is not sufficient to bypass the requirements for RpoH1 or RpoH1/RpoH2 during growth and symbiosis and that there must be other crucial targets. In addition, we show that only *groESL5* is controlled by RpoH1 during free-living growth and stationary phase at 30°C, which agrees with results obtained by Mitsui et al. during growth and heat shock (28). Finally, we demonstrate that *groESL3* and *groEL4* mutants are able to nodulate and fix nitrogen like wild-type cells.

MATERIALS AND 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 medium, LB medium supplemented with $MgSO_4$ and $CaCl_2$ (LB/MC medium) (17), or M9 minimal medium containing 0.2% sucrose, 0.5 μg biotin ml^{-1}, 1 mM $MgSO_4$, and 0.25 mM $CaCl_2$. Antibiotics were added to the media as follows: 100 μg ampicillin ml^{-1}, 25 μg gentamicin ml^{-1}, 5 to 50 μg hygromycin ml^{-1}, 25 μg kanamycin ml^{-1}, 50 or 200 μg neomycin ml^{-1}, 50 or 200 μg spectinomycin ml^{-1}, 500 μg streptomycin ml^{-1}, and 2 or 10 μg tetracycline ml^{-1}. *S. meliloti* cells were grown at 30°C unless otherwise indicated. Plasmids were introduced into *S. meliloti* cells by triparental conjugation (17). Chromosomally located constructs were moved between *S. meliloti* strains by generalized transduction using N3 phage (26). Although *rpoH2*::*aacC1* containing strain BY294 (36) was constructed in the Rm1021 background, we transferred the mutation by transduction into our own Rm1021 strain, creating AB3, to ensure isogenicity.

Plant assays. Alfalfa plants (*Medicago sativa* GT13R plus) were grown on nitrogen-free buffered nodulation medium and inoculated with *S. meliloti* cells as previously described (34). Plant height, leaf color, and nodule color were scored at 6 weeks postinoculation to determine the status of nitrogen fixation. Inoculation with Fix^+ bacteria results in tall, green plants with pink nodules. Inoculation with Fix^- bacteria results in stunted, chlorotic plants with white nodules. Bacteria were isolated from nodules by surface sterilizing nodules in 20% Clorox bleach for 5 min, washing two times with water and one time with LB medium, crushing with forceps, and then streaking on LB medium.

Western blot analysis. To obtain samples for Western blot analysis, cells were grown overnight at 30°C in LB/MC medium with streptomycin, diluted back to an optical density at 595 nm (OD_{595}) of 0.1, grown to mid-log phase ($0.6 \leq OD_{595} \leq 0.8$), harvested, and stored at −80°C. Cells were resuspended in 1× phosphate-buffered saline at 0.1 ml per OD_{595} unit. The cells were disrupted by sonication, and the resulting extracts were combined with 2× Laemmli sample buffer. Equal volumes of extract were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Blots were probed with rabbit polyclonal antibodies to the *E. coli* proteins at the following dilutions: anti-GroEL (Stressgen) at 1:5,000, anti-DnaK (gift from J. Brodsky) at 1:5,000 or anti-DnaK (Upstate Biotechnology) at 1:2,500, and anti-DnaJ (Stressgen) at 1:1,250. Blots were then probed with a 1:15,000 dilution of anti-rabbit horseradish peroxidase-conjugated secondary antibody, developed with enhanced chemiluminescence reagents (Pierce), and imaged using a Fujifilm LAS-3000 imaging system.

To quantify relative protein levels, band density was determined by ImageGauge software (Fuji). Protein concentration of cell lysates was determined by bicinchoninic acid protein assay (Pierce), and band intensities were then normalized to protein concentration.

Statistical analysis. Significance of differences in bacterial growth levels and protein levels was determined by using both the Student's *t* test and the Wilcoxon rank sum test, which does not assume a normal distribution. Although the *P* values varied, differences were significant with both tests unless otherwise noted.

Construction of plasmids for overexpression of *groESL1* and *groESL3*. To place *groESL1* under the control of the *E. coli lac* promoter, a 2.1-kb DNA fragment that extends from 68 bp upstream of the *groES1* start codon to 30 bp downstream of the *groEL1* stop codon was amplified using primers that generate ApaI and XbaI restriction sites. The fragment was inserted into ApaI-SpeI-digested pMB403 (3), a broad-host-range vector that contains the *lac* promoter, creating pAB1 (P_{lac}-*groESL1*). To place *groESL3* under the control of the *lac* promoter, a 2.2-kb fragment that extends from 78 bp upstream of the *groES3* start codon to 85 bp downstream of the *groEL3* stop codon was amplified with primers generating ApaI and XbaI restriction sites. The fragment was inserted into ApaI-SpeI-digested pMB403, creating pAB2 (P_{lac}-*groESL3*).

To place *groESL1* and *groESL3* under the control of the *Salmonella enterica* serovar Typhimurium *trp* promoter, the *lac* promoter was removed from pAB1 and pAB2 and replaced with a fragment containing 141 bp of *S. enterica* serovar Typhimurium DNA containing the *trp* promoter. First, a 220-bp EcoRV-Acc65I fragment containing P_{trp} was isolated from pVO131. To delete the *lac* promoter, pAB1 and pAB2 were digested with NsiI, blunted with T4 DNA polymerase, and digested with Acc65I. The P_{trp} fragment was then inserted into pAB1 to create pAB7 (P_{trp}-*groESL1*) and into pAB2 to create pAB8 (P_{trp}-*groESL3*).

The expression plasmids were introduced into wild-type (Rm1021), *groEL1*::Tn*5* (B4T1), *rpoH1*::*aadA* (VO3128), and *rpoH1*::*aadA rpoH2*::pVO101 (VO3148) strains by triparental conjugation.

Construction of *groEL-gus* fusions. The *groEL-gus* fusions were constructed using recombinational cloning as described in House et al. (20). This method is a modification of Invitrogen's Gateway Technology, such that transfer of DNA

TABLE 1. Strains

Strain	Relevant characteristic(s)	Reference
AB3	*rpoH2*::*aacCI*	This study
AB4	Wild type/pAB1 (P_{lac}-*groESL1*)	This study
AB9	*rpoH1*::*aadA rpoH2*::*aacCI*	This study
AB16	Wild type/pAB2 (P_{lac}-*groESL3*)	This study
AB35	*rpoH1*::*aadA rpoH2*::pVO101 NDS-3	This study
AB36	*rpoH1*::*aadA rpoH2*::pVO101 NDS-4	This study
AB37	*rpoH1*::*aadA rpoH2*::pVO101 NDS-5	This study
AB38	*rpoH1*::*aadA rpoH2*::pVO101 NDS-6	This study
AB39	*rpoH1*::*aadA rpoH2*::pVO101 NDS-7	This study
AB40	*rpoH1*::*aadA rpoH2*::pVO101 NDS-8	This study
AB41	*rpoH1*::*aadA rpoH2*::pVO101 NDS-9	This study
AB42	*rpoH1*::*aadA rpoH2*::pVO101 NDS-10	This study
AB43	*rpoH1*::*aadA rpoH2*::pVO101 NDS-11	This study
AB44	*rpoH1*::*aadA rpoH2*::pVO101 NDS-12	This study
AB92	Wild type/pAB7 (P_{trp}-*groESL1*)	This study
AB103	Wild type/pAB8 (P_{trp}-*groESL3*)	This study
AB129	*groEL2*::pAB10 (*groEL2-gfp-gus* transcriptional fusion, *groEL2* not disrupted)	This study
AB140	*groEL1*::pAB11 (*groEL1-gfp-gus* transcriptional fusion, *groEL1* not disrupted)	This study
AB145	*groEL3*::pAB12 (*groEL3-gfp-gus* transcriptional fusion, *groEL3* not disrupted)	This study
AB147	*groEL4*::pAB13 (*groEL4-gfp-gus* transcriptional fusion, *groEL4* not disrupted)	This study
AB150	*groEL5*::pAB14 (*groEL5-gfp-gus* transcriptional fusion, *groEL5* not disrupted)	This study
AF14	*groESL3*Δ::*tet*	This study
AR12	*rpoH1*::*aadA rpoH2*::pVO194 (*rpoH2-gus* transcriptional fusion, *rpoH2* not disrupted)	This study
AR13	*rpoH1*::*aadA*/pBGR86 (*rpoH1-gus* transcriptional fusion)	This study
AR14	*rpoH2*::*aacCI*/pBGR86 (*rpoH1-gus* transcriptional fusion)	This study
AR15	*rpoH1*::*aadA rpoH2*::*aacCI*/pBGR86 (*rpoH1-gus* transcriptional fusion)	This study
B4T1	*groEL1*::Tn*5*	33
BY294	*rpoH2*::*aacCI*	36
Rm1021	Wild type	27
VO2012	Wild type/pBGR86 (*rpoH1-gus* transcriptional fusion)	35
VO2148	*rpoH2*::pVO101 (*rpoH2* disruption)	35
VO2257	*rpoH2*::pVO194 (*rpoH2-gus* transcriptional fusion, *rpoH2* not disrupted)	35
VO3128	*rpoH1*::*aadA*	35
VO3148	*rpoH1*::*aadA rpoH2*::pVO101	This study
VO3149	*rpoH1*::*aadA rpoH2*::pVO101	This study
VO3150	*rpoH1*::*aadA rpoH2*::pVO101 NDS-1	This study
VO3151	*rpoH1*::*aadA rpoH2*::pVO101 NDS-2	This study
VO3165	*rpoH1*::*aadA* GDS-1	This study
VO3166	*rpoH1*::*aadA rpoH2*::pVO101 GDS-2	This study
VO3170	*rpoH1*::*aadA rpoH2*::pVO101 GDS-1	This study
VO3193	*groEL4*Δ	This study

from an entry vector to a destination vector by the λ recombination system is performed in vivo via a pentaparental mating. In brief, each *groEL* open reading frame (ORF) was transferred from an entry vector (pESmc00913, pESma0744, pESma0124, pESmc01758, and pESmb21566) (41) to the destination vector pMK2030 (B. K. Schroeder, B. L. House, M. W. Mortimer, and M. L. Kahn, unpublished data) during a pentaparental mating using the helper plasmid pRK2013 (9) and the λ integrase- and excisionase-expressing plasmid pXINT129 (37). This destination vector is a suicide vector that contains *attR* recombination sites upstream of promoterless *gfp* and *gus* genes to allow the formation of transcriptional fusions. Each *groEL-gfp-gus*-containing plasmid was moved into Rm1021 by triparental mating and integrated at the respective *groEL* gene by single reciprocal recombination, resulting in a P_{groE}-*groES-groEL-gfp-gus* construct. The resulting strains AB140 (*groESL1-gfp-gus*), AB129 (*groESL2-gfp-gus*), AB145 (*groESL3-gfp-gus*), AB147 (*groEL4-gfp-gus*), and AB150 (*groESL5-gfp-gus*) were confirmed by Southern analysis. The fusions were transferred into *rpoH1*::*aadA* (VO3128), *rpoH2*::*aacCI* (AB3), and *rpoH1*::*aadA rpoH2*::*aacCI* (AB9) mutant backgrounds by transduction.

Assay of β-glucuronidase activity. Cells were collected for β-glucuronidase (GUS) assays at the indicated times and frozen at −80°C until assayed for activity. The cells were permeabilized with lysozyme (200 μg ml^{-1}, 37°C for 10 min), and β-glucuronidase activity was assayed using *p*-nitrophenyl-β-D-glucuronide as described previously (21). GUS activity is expressed in nanomoles per minute per OD_{595} unit × 1,000.

Construction of *groESL3* and *groEL4* null mutants. To disrupt the *groESL3* operon, a 3.3-kb DNA fragment containing *groESL3* and flanking DNA was amplified from chromosomal DNA by PCR and inserted into pCR-Blunt II-TOPO using the Zero Blunt TOPO Cloning kit (Invitrogen). The fragment was removed using XbaI and XhoI restriction sites generated by the primers and cloned into pBluescript II KS(−) (Stratagene), resulting in pAF2. A 1.6-kb fragment containing the *tet* gene encoding tetracycline resistance was amplified from pBR322 DNA and inserted into pAF2 digested with HindIII and blunted with Klenow, deleting 1,606 bp of *groESL3*. The resulting plasmid, pAF3, contains 161 bp of the 5′ end of *groES3*, the *tet* gene in the same orientation as *groESL3*, and 233 bp of the 3′ end of *groEL3*. The *groESL3*Δ::*tet* construct was removed from pAF3 as an XbaI-XhoI fragment and inserted into pJQ200SK, creating pAF4. This plasmid contains the *sacB* gene from *Bacillus subtilis*, allowing negative selection in gram-negative bacteria when grown on sucrose (15, 38). To construct a strain carrying the *groESL3* deletion in the chromosome, pAF4 was introduced into Rm1021 by triparental mating. A double recombination event was selected by plating on medium containing tetracycline and 5% sucrose, followed by screening for gentamicin sensitivity to confirm the absence of pJQ200SK. The resulting strain, AF14 (*groESL3*Δ::*tet*), was confirmed by Southern analysis.

To disrupt the *groEL4* gene, we used a recombinational method (20). The ORFs flanking *groEL4*, SMc01757 and SMc01759, were transferred from entry plasmids pESmc01757 and pESmc01759 (41) to destination vectors pMK2016

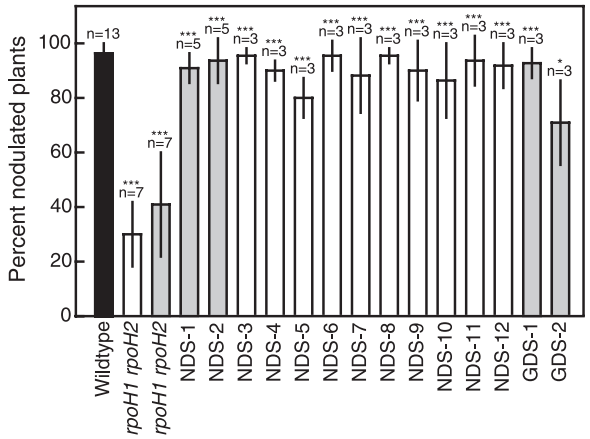

FIG. 1. Nodulation by suppressor mutant strains. Alfalfa plants were inoculated with control and suppressor mutant strains, and the percentage of nodulated plants was determined after at least 3 weeks of incubation. The graph depicts the average percentage of nodulation over the indicated number of experiments, and error bars represent the sample standard deviation. At least 50 plants in total were inoculated with each bacterial strain. White bars indicate strains derived from the *rpoH1 rpoH2* mutant strain VO3148, and gray bars indicate strains derived from the *rpoH1 rpoH2* mutant strain VO3149. Significance was determined using the Student's *t* test. ***, $P < 0.001$; *, $P < 0.05$. The strains from left to right were Rm1021, VO3148-VO3151, AB35-AB44, VO3170, and VO3166.

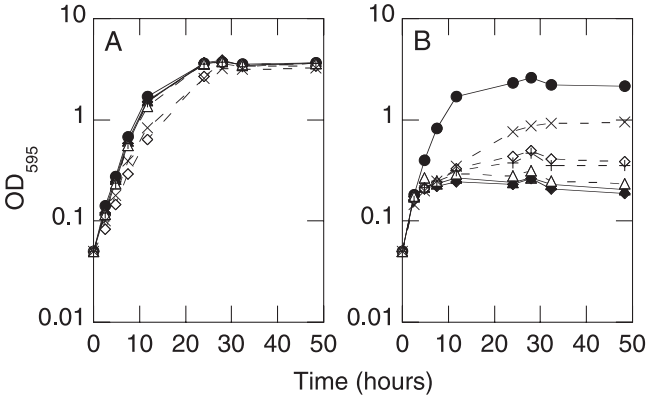

FIG. 2. Comparison of the growth of suppressor mutant cells with wild-type and *rpoH1 rpoH2* double mutant cells, as measured using OD_{595}. Cells were grown in LB/MC medium plus streptomycin at 30°C (A) or 40°C (B). The control strains are Rm1021 (wild type; filled circles), VO3148 (*rpoH1 rpoH2*; filled triangles), and VO3149 (*rpoH1 rpoH2*; filled diamonds), and the suppressor mutant strains are VO3150 (NDS-1; open diamonds), AB35 (NDS-3; open triangles), VO3170 (GDS-1; crosses), and VO3166 (GDS-2; plus signs). The panels show representative data from one of four experiments.

and pMK2017, respectively, by using lambda recombination in vivo as described above. The resulting plasmids were sequentially introduced into Rm1021 by triparental mating and integrated by single reciprocal recombination, creating KB113 (SMc01757::pKB101 SMc01759::pKB100), as confirmed by Southern and PCR analyses. The plasmids contain FLP recombinase target sequences oriented such that expression of FLP recombinase results in deletion of the region between the two ORFs, leaving a single FLP recombinase target sequence. pBH474, which expresses FLP recombinase, was introduced into KB113 by triparental mating, and the cells were grown without selection for pKB100 and pKB101. Multiple colonies were screened for the loss of the drug markers associated with pKB100 and pKB101, indicating a deletion event. pBH474, which contains the *sacB* gene which is lethal in the presence of sucrose, was removed by streaking the cells on plates containing 5% sucrose and then screening for loss of the drug resistance marker. The deletion in the resulting strain VO3193 (*groEL4*Δ) was confirmed by PCR analysis. Since the adjacent ORFs are oriented with the stop codons proximal to *groEL4*, the deletion removes *groEL4* and the adjacent intergenic region but leaves SMc01759 and SMc01757 intact.

RESULTS

Suppression of the *rpoH1* and *rpoH1 rpoH2* mutant defects. The *rpoH1 rpoH2* double mutant RmHM9 was reported to be Nod^- (36). Using our *rpoH1* and *rpoH2* mutant alleles (35), we generated two isolates of an *rpoH1 rpoH2* double mutant (VO3148 and VO3149). When we inoculated *Medicago sativa* GT13R plus alfalfa plants under our growth conditions with these strains, as well as RmHM9, we found that the double mutants varied greatly in the ability to nodulate plants from experiment to experiment (average of 36% nodulated plants with a range from 10 to 78%) (Fig. 1). There were two possible explanations for the variability: either the *rpoH1 rpoH2* phenotype is leaky, or the nodules contain suppressor mutants. To distinguish between these possibilities, we isolated bacteria from 12 nodules elicited by the *rpoH1 rpoH2* mutants in two independent experiments and confirmed that both mutations were still present (data not shown). We used these strains to inoculate alfalfa and found that they were similar to the wild type in nodulation efficiency (Fig. 1) although still defective in nitrogen fixation (data not shown). Therefore, the nodules are due to suppressor mutants, which we call NDS for "nodulation defect suppressor."

Cells containing an *rpoH1* mutation grow more slowly than wild-type cells in LB medium at 30°C (35). Because we were concerned about the generation of suppressor mutations, we looked for conditions in which the *rpoH1* mutant cells would grow as well as the wild-type cells. We switched to LB medium supplemented with $MgSO_4$ and $CaCl_2$ (LB/MC) (17). As shown in Fig. 2, *rpoH1 rpoH2* mutant cells grow like wild-type cells at 30°C in LB/MC but display a severe growth defect at 40°C.

To determine if the *rpoH1 rpoH2* nodulation suppressor mutations also suppressed the growth defect, we compared the growth of two independent suppressors strains (NDS-1 and NDS-3) to the *rpoH1 rpoH2* parent strains (VO3149 and VO3148, respectively) at 30°C and 40°C in LB/MC. As shown in Fig. 2, NDS-1 grows slightly more poorly than the wild type at 30°C, whereas NDS-3 is indistinguishable. At 40°C neither NDS-1 nor NDS-3 cells grow as well as wild-type cells, but NDS-1 cells do grow better than the *rpoH1 rpoH2* parent strain. To determine if the difference was significant, we compared the amount of growth as measured by OD_{595} at 48 h and performed the Student's *t* test. The OD_{595} of NDS-1 at 48 h was significantly higher than that of the double mutant parent ($P < 0.05$), whereas NDS-3 was not significantly different.

In *E. coli*, suppressors of the *rpoH* growth defect are readily obtained by plating *rpoH* mutant cells at 30 to 40°C (25). By streaking for single colonies, we found that wild-type *S. meliloti* cells form colonies on LB/MC plates at 42°C, whereas cells containing an *rpoH1* mutation do not (data not shown). Therefore, to select for suppressor mutants we plated *rpoH1* and *rpoH1 rpoH2* mutant cells at high density at 42°C and obtained colonies. Many of the mutations were not stable, such that the

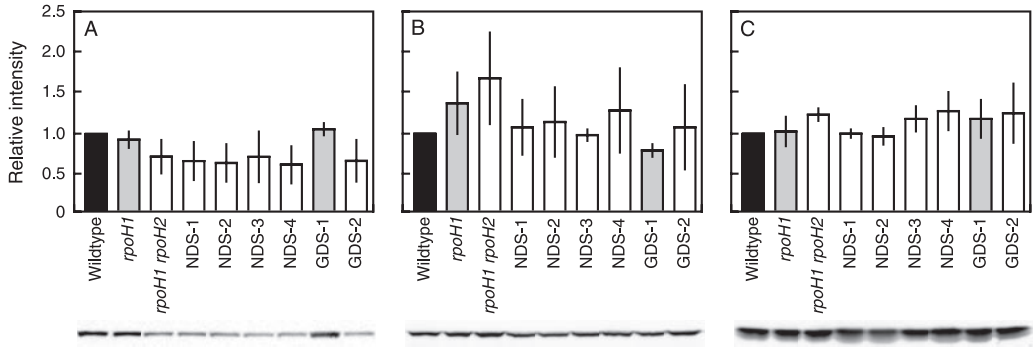

FIG. 3. Western analysis of heat shock proteins in suppressor mutant strains. Cells were grown to mid-log phase in LB/MC medium plus streptomycin at 30°C. Equal numbers of cells as measured by OD_{595} were resuspended in buffer and sonicated. Equal volumes of cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting using primary antibodies generated to the following *E. coli* proteins: GroEL (A), DnaK (B), and DnaJ (C). A representative immunoblot is shown. Graphs depict the mean band intensity normalized to protein concentration and relative to the wild-type signal, with error bars representing the sample standard deviation ($n = 3$). Gray bars indicate strains with the *rpoH1* mutant background, and white bars indicate strains with the *rpoH1 rpoH2* double mutant background. The strains from left to right were Rm1021, VO3128, VO3148, VO3150, VO3151, AB35, AB36, VO3165, and VO3166.

ability to grow at 42°C was lost upon streaking for single colonies at 30°C or 42°C. However, by selecting for growth at 42°C multiple times, we obtained two independent, stable suppressor mutants that we called GDS-1 (*rpoH1* background) and GDS-2 (*rpoH1 rpoH2* background) for "growth defect suppressor." To facilitate characterization of GDS-1 for suppression of the growth defect as well as the nodulation defect, we introduced the *rpoH2* mutation into the cells by generalized transduction so that all of the suppressor mutants were in the *rpoH1 rpoH2* background. To determine if these plate growth defect suppressor mutants also suppressed the growth defect in liquid medium, we grew the strains at 30°C and 40°C in LB/MC. The suppressor mutant cells grew better than the *rpoH1 rpoH2* mutant cells at 40°C but not as well as the wild type (Fig. 2). We compared the amount of growth as measured by OD_{595} at 48 h and performed the Student's *t* test. The OD_{595} at 48 h was significantly higher than that of the *rpoH1 rpoH2* parent strain for both GDS-1 ($P < 0.001$) and GDS-2 ($P < 0.05$).

To determine whether the growth defect suppressor mutations also suppress the nodulation and nitrogen fixation defects, we inoculated alfalfa plants with GDS-1 and GDS-2. The growth defect suppressor mutants nodulated alfalfa plants at levels significantly higher than those of the parent strains (Fig. 1), indicating suppression of the nodulation defect. However, the strains were still unable to fix nitrogen (data not shown).

In *E. coli*, an *rpoH* mutant cannot grow above 20°C (49). Suppressor mutants selected at 30°C to 40°C display increased expression of the *groESL* operon, and suppressor mutants selected at 42°C display increased expression of both *groESL* and *dnaK*. The increased transcription and subsequent synthesis of these HSPs in the suppressor mutants is independent of heat shock, such that high levels are observed at 30°C, unlike in wild-type cells (25). To test whether our NDS or GDS suppressor mutants function by a similar mechanism, we grew cells to mid-log phase at 30°C and performed Western blot analysis for GroEL and the DnaK/DnaJ chaperone complex using polyclonal antibodies to the *E. coli* proteins (Fig. 3). Each antibody recognized a major band of the appropriate molecular weight in *S. meliloti* cell extracts. In the case of GroEL, we know that the polyclonal antibody recognizes the *S. meliloti* GroEL1, GroEL2, and GroEL5 proteins (A. N. Bittner and V. Oke, unpublished results). It is likely that the antibody also recognizes GroEL3 and GroEL4, since all of the *S. meliloti* GroEL proteins are 57 to 62% identical to *E. coli* GroEL. Although the level of total GroEL appears lower in the *rpoH1 rpoH2* mutant than in the wild type and the level of DnaK appears higher in the *rpoH1* and *rpoH1 rpoH2* mutants than in the wild type, the differences were not significant by the Student's *t* test and were just significant using the Wilcoxon rank sum test ($P = 0.0496$).

Analysis of the suppressor mutants shows that GroEL, DnaK, and DnaJ protein levels were not significantly higher in the mutants compared to those of the *rpoH1* and the *rpoH1 rpoH2* parent strains by the Student's *t* test and the Wilcoxon rank sum test. However, it is possible that a small increase of one particular GroEL protein is masked by GroEL1, since *groESL1* is expressed at the highest levels (Fig. 4). In the case of DnaJ, there was a significant decrease in protein levels relative to the *rpoH1 rpoH2* double mutant in NDS-1 ($P < 0.01$) and NSD-2 ($P < 0.05$). Since the *S. meliloti* suppressor mutants do not exhibit the increased production of GroEL and DnaK seen in the *E. coli* suppressor mutants, the suppression appears to function by a different mechanism.

Overexpression of *groESL1* or *groESL3* does not suppress the mutant phenotypes. In *E. coli*, expression of *groESL* from a multicopy plasmid is sufficient to suppress the temperature-sensitive phenotype of the *rpoH* mutant (25). In *S. meliloti*, we know that GroEL/GroES affects NodD activity and that *groESL1* mutants form Fix^- nodules. We hypothesized that the defects observed for the *S. meliloti rpoH1* single mutant and *rpoH1 rpoH2* double mutant might be suppressed by overexpression of *groESL*. Therefore, we created constructs to express *groESL1* and *groESL3* independently of any possible RpoH control. We chose *groESL1* because it is highly expressed (33) (Fig. 4) and the mutant displays a Fix^- phenotype (33), and we chose *groESL3* because it is the most divergent of the five *groESL* operons. We chose the *E. coli lac* promoter and the *S. enterica* serovar Typhimurium *trp* promoter because both act constitutively in *S. meliloti*, they have been successfully used to overexpress other genes in *S. meliloti* (4, 8, 12, 13), and

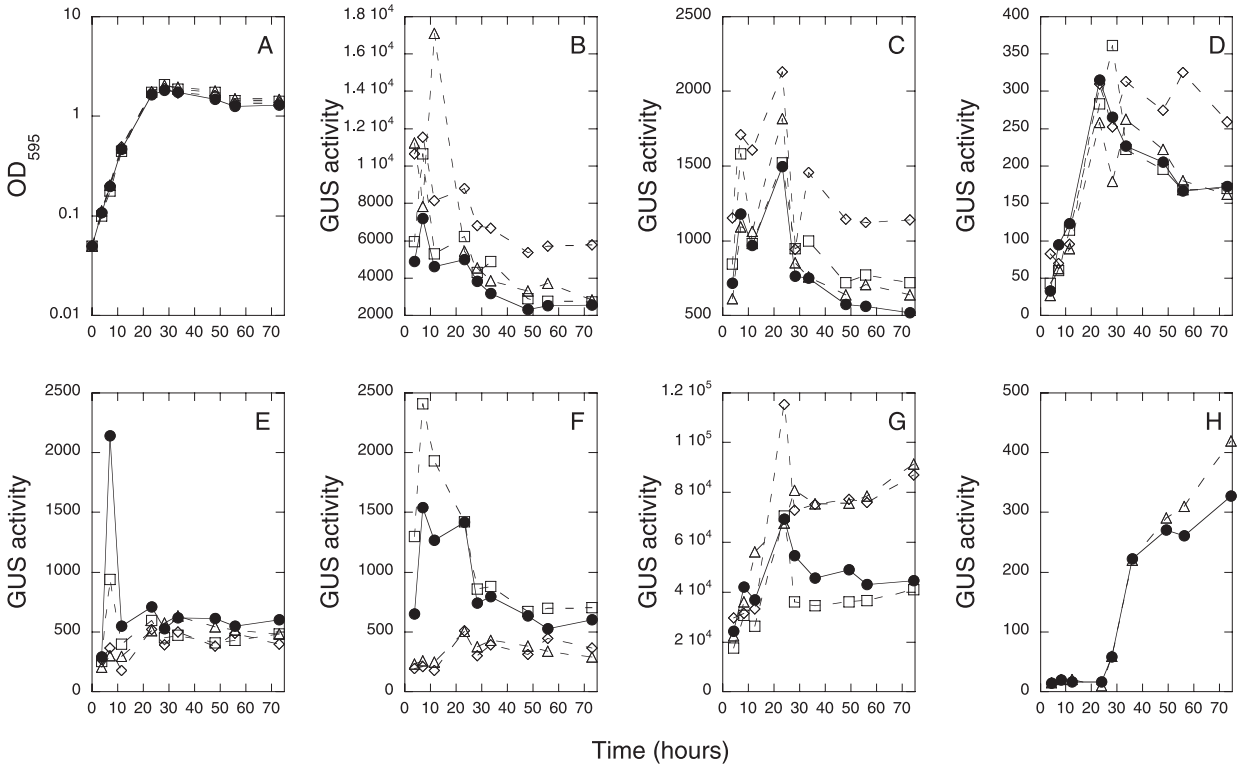

FIG. 4. *groESL* and *rpoH* expression in *rpoH* mutant cells grown in M9 sucrose minimal medium. Growth as measured by OD_{595} and gene expression as monitored by β-glucuronidase (GUS) activity were determined in wild-type (filled circles), *rpoH1* (open triangles), *rpoH2* (open squares), and *rpoH1 rpoH2* (open diamonds) backgrounds. (A) Representative growth curve of cells containing the *groEL1-gus* fusion. All of the strains in the experiment showed indistinguishable growth patterns. (B to H) GUS activity of cells containing *groEL1-gus* (B), *groEL2-gus* (C), *groEL3-gus* (D), *groEL4-gus* (E), *groEL5-gus* (F), *rpoH1-gus* (G), and *rpoH2-gus* (H). Each panel shows the data from one representative experiment.

expression from these promoters on a multicopy plasmid is stronger than expression from the endogenous *groESL1* and *groESL3* promoters (data not shown). Each construct was introduced separately into wild-type, *groEL1*, *rpoH1*, and *rpoH1 rpoH2* cells.

To determine whether expression of *groESL1* or *groESL3* bypasses the symbiotic phenotypes of the *rpoH1* and *rpoH1 rpoH2* mutants, we inoculated alfalfa seedlings with wild-type and mutant bacteria containing the expression constructs. As shown in Table 2, none of the constructs altered nodulation or nitrogen fixation in the wild-type strain. Both *groESL1* constructs were able to complement the Fix$^-$ phenotype of the *groEL1* mutant. Therefore, these constructs produce active GroEL1 protein. Complementation required the *lac* promoter (data not shown), confirming that the *groESL1* fragment does not contain the endogenous promoter. Neither *groESL1* construct was able to suppress the Fix$^-$ phenotype of the *rpoH1* mutant (Table 2). In terms of the nodulation defect of the *rpoH1 rpoH2* double mutant, P_{lac}-*groESL1* did not suppress the defect but P_{trp}-*groESL1* elicited an increase in the number of nodulated plants. However, the nodulation defect was still apparent in the low number of nodules per nodulated plant, which was similar to the *rpoH1 rpoH2* mutant. Therefore, overexpression of *groESL1* is only able to partially bypass the Nod$^-$ phenotype of the *rpoH1 rpoH2* mutant and has no effect on the Fix$^-$ phenotype of the *rpoH1* mutant.

Neither the P_{lac}-*groESL3* nor the P_{trp}-*groESL3* construct sup-

TABLE 2. Symbiotic phenotypes of strains overexpressing *groESL*

Strain/construct	% Plants nodulated[a]	No. of nodules/ nodulated plant[b]	Fixation status
Wild type	98 ± 4	3.9 ± 1.9	+
Wild type/P_{lac}-*groESL1*	98 ± 3	3.8 ± 1.6	+
Wild type/P_{trp}-*groESL1*	98 ± 3	3.8 ± 2.1	+
Wild type/P_{lac}-*groESL3*	98 ± 3	4.0 ± 1.9	+
Wild type/P_{trp}-*groESL3*	100 ± 0	3.6 ± 2.0	+
groEL1	98 ± 3	3.7 ± 2.4	−
groEL1/P_{lac}-*groESL1*	98 ± 3	3.6 ± 1.8	+
groEL1/P_{trp}-*groESL1*	97 ± 6	3.6 ± 1.8	+
groEL1/P_{lac}-*groESL3*	90 ± 14	3.2 ± 1.8	−
groEL1/P_{trp}-*groESL3*	95 ± 6	3.4 ± 2.0	−
rpoH1	96 ± 4	3.7 ± 2.2	−
rpoH1/P_{lac}-*groESL1*	90 ± 4	3.7 ± 2.3	−
rpoH1/P_{trp}-*groESL1*	100 ± 0	3.7 ± 2.0	−
rpoH1/P_{lac}-*groESL3*	97 ± 6	3.8 ± 2.2	−
rpoH1/P_{trp}-*groESL3*	100 ± 0	3.1 ± 1.8	−
rpoH1 rpoH2	38 ± 15	1.8 ± 1.2	−
rpoH1 rpoH2/P_{lac}-*groESL1*	35 ± 13	1.4 ± 0.8	−
rpoH1 rpoH2/P_{trp}-*groESL1*	74 ± 23	1.8 ± 1.0	−
rpoH1 rpoH2/P_{lac}-*groESL3*	31 ± 30	1.3 ± 0.5	−
rpoH1 rpoH2/P_{trp}-*groESL3*	47 ± 15	1.7 ± 1.1	−

[a] Shown are averages and standard deviations ($n \geq 3$).
[b] Shown are averages and standard deviations of total nodulated plants from all experiments ($n \geq 3$).

pressed the symbiotic phenotypes of the *groEL1*, *rpoH1*, and *rpoH1 rpoH2* mutants. Since a *groESL3* mutant has no phenotype (see below), we could not do a complementation test to prove that our *groESL3* constructs were producing active protein. Therefore, to determine whether the *groESL3* constructs were functional, we first sequenced the P_{lac}-*groESL3* construct and confirmed that no mutations were introduced during amplification of *groESL3*. We then used site-directed mutagenesis to insert codons generating a hexahistidine tag at the carboxy terminus of GroEL3. Subsequent detection using the SuperSignal West HisProbe kit (Pierce) showed that the P_{lac}-*groESL3* construct produced protein (data not shown). Therefore, our *groESL3* constructs probably produce active proteins. Thus, the results suggest that *groEL3* is not interchangeable with *groEL1* and that *groESL3* does not bypass the symbiotic phenotypes of the *rpoH1* and *rpoH1 rpoH2* mutants.

In addition to the symbiotic phenotype, the *rpoH1* mutant displays a high-temperature growth defect (Fig. 2). Given that overexpression of *groESL* bypasses the growth defect of the *rpoH* mutant in *E. coli* (25), we tested whether our *groESL1* or *groESL3* constructs could bypass the growth defect of the *rpoH1* mutant. There was no significant increase in growth of cells grown in LB/MC at 30°C or 40°C when the constructs were present (data not shown).

Control of *groESL* and *rpoH* gene expression by RpoH1 and RpoH2 under free-living conditions. To test if RpoH1 or RpoH2 controls expression of the various *groESL* genes, we constructed a matched set of chromosomal *groEL-gus* transcriptional fusions by recombinational cloning as described in Materials and Methods. We compared expression of the *groEL-gus* fusions in wild-type, *rpoH1*, *rpoH2*, and *rpoH1 rpoH2* cells during growth in M9 sucrose medium (Fig. 4). All five *groEL-gus* fusions generated GUS activity above background levels, with *groEL1* being the most highly expressed. Neither *rpoH1* nor *rpoH2* was required for expression of *groEL1*, *groEL2*, *groEL3*, or *groEL4*. However, *rpoH1* was required for full expression of *groEL5*. Similar results were obtained with the *rpoH1* mutant grown in LB/MC medium, although we did additionally observe a slight decrease in *groEL3* and *groEL4* expression (data not shown).

Although *rpoH1* is expressed within root nodules, we have been unable to test directly whether RpoH1 controls expression of the *groEL* genes during symbiosis, because *rpoH1* mutant cells undergo early senescence within the nodule (28). Therefore, it is possible that RpoH1 directs transcription of a different subset of these genes within the host plant.

We also tested whether RpoH1 or RpoH2 is autoregulatory or controls expression of the other. *rpoH1-gus* expression was not dependent on *rpoH1* or *rpoH2*, and *rpoH2-gus* expression was not dependent on *rpoH1* (Fig. 4). In the case of *rpoH1-gus*, deletion of *rpoH1* allows increased expression, perhaps reflecting reduced competition for core RNA polymerase by other sigma factors. We were unable to determine whether RpoH2 was autoregulatory, because our *rpoH2-gus* fusion, which does not disrupt *rpoH2*, is located at the *rpoH2* locus.

Mitsui et al. (28) defined a consensus sequence for *rpoH1*-dependent promoters in *S. meliloti* (cnCTTgAA-n17-CCAnaT) based on the *rpoH1*-dependent promoters of *groES5*, *lon*, and *clpB*. We used the program dna-pattern (43) to look for the consensus sequence upstream of the five *groE* loci, *rpoH1*, and *rpoH2*. The sequence is only upstream of *groESL5*, which is consistent with the experimental data.

***groESL3* and *groEL4* are not required for successful symbiosis.** During symbiosis, *groEL1* is required for nitrogen fixation (33), but *groEL2* (32) and *groESL5* (28) are not required for either nodulation or nitrogen fixation. To determine if the other *groESL* genes are necessary for symbiosis, we constructed deletions of *groESL3* and *groEL4* as described in Materials and Methods. Both *groESL3* and *groEL4* mutant cells were capable of eliciting Fix^+ nodules at wild-type levels on alfalfa.

DISCUSSION

A simple hypothesis to explain why RpoH1 is required for growth at high temperature and RpoH1 and RpoH2 are required for nodulation and nitrogen fixation during the *S. meliloti*-alfalfa symbiosis is that the transcription factors are required for the expression of one or more of the *groESL* operons and that production of GroEL/GroES is the crucial function. This hypothesis is based on two observations. First, in *E. coli groESL* is a key target of RpoH. This has been concluded because mutants that suppress the growth defect of *rpoH* overexpress *groESL*, and expression of *groESL* from a multicopy plasmid is sufficient to allow *rpoH* mutant cells to grow up to 40°C (25). Second, in *S. meliloti*, *groESL1* mutants are delayed in nodulation and form Fix^- nodules (33). However, several lines of evidence suggest that this hypothesis is not correct. First, suppressor mutants of the high-temperature growth defect and the nodulation defect do not exhibit increased production of total GroEL protein. Second, overexpression of *groESL1* or *groESL3* from constitutive promoters does not bypass the defects of the *rpoH* mutants. Third, at least under free-living conditions (heat shock in Mitsui et al. [28] and rich and minimal media in this study), RpoH2 does not control any of the *groESL* genes and RpoH1 only controls expression of *groESL5*. However, *groESL5* is not required for nodulation or nitrogen fixation (28). Therefore, *groESL5* cannot be a single key target. We conclude that the system is unlike *E. coli*, which is not surprising given the greater complexity, and that there must be other crucial targets of RpoH1 and RpoH2.

What genes might be under the control of RpoH1 and RpoH2? We envision two scenarios that could be true for either protein. First, the requirements for RpoH could solely be due to the need for properly folded proteins. The requirement during symbiosis may reflect the need to fold specific proteins induced during symbiosis and/or to respond to an increase in unfolded proteins due to stress within the nodule. The regulon would, therefore, be similar to that in *E. coli*. Second, although RpoH may direct expression of the classic HSPs, the requirement may reflect expression of other genes, perhaps specific to *Rhizobium*. For example, *rpoH2* in *Rhizobium* sp. strain TAL1145 regulates genes for exopolysaccharide synthesis, which is required for effective nodulation (24). Mitsui et al. (28) determined whether RpoH1 and RpoH2 control expression of nine HSP homologs in *S. meliloti* (*groESL1* through *groESL5*, *dnaK*, *clpA*, *clpB*, and *lon*) during heat shock. RpoH1 controlled expression of *groESL5* and partially controlled expression of *clpB* and *lon*. In contrast, RpoH2 did not control expression of any of these genes. Therefore, the

regulon of RpoH1 at least overlaps with the regulon of RpoH in *E. coli*, but genes under the control of RpoH2 are currently unknown. Microarray experiments to determine the regulons of RpoH1 and RpoH2 in *S. meliloti* should be illuminating.

Three different phenotypes are associated with *rpoH1* and *rpoH2* in *S. meliloti*. The *rpoH1* mutant has a growth defect at high temperature and forms ineffective nodules on plants (35, 36), and the *rpoH1 rpoH2* double mutant is unable to nodulate (36). Our suppressor mutant analysis suggests that the requirements for RpoH are not the same for all of the phenotypes. We have isolated spontaneous suppressor mutants based on the ability to grow at high temperature (bypassing RpoH1) or to nodulate (bypassing RpoH1 and RpoH2). Interestingly, regardless of how they were initially isolated, most of these mutants are able to suppress both the growth and nodulation defects. In contrast, none of our suppressor mutants are able to suppress the nitrogen fixation defect. This implies that the functions of RpoH1 during growth and RpoH1/RpoH2 during the early stages of symbiosis are similar but that there is a different or additional function of RpoH1 needed later during symbiosis. We do not know what has been altered in these suppressor mutants, although we have shown that the GroEL and DnaK/DnaJ chaperones are not overproduced. Analysis of the differences between the suppressor mutants and the parent strains should provide clues about the roles of RpoH1 and RpoH2 during free-living growth and symbiosis.

Although we cannot explain the requirements for RpoH1 and RpoH2 during symbiosis as a requirement for expression of *groESL*, the presence of multiple *groESL* genes and the connections to symbiosis make this gene family particularly interesting in the *Rhizobiaceae*. All of the nodule-forming rhizobia that have been fully sequenced (*S. meliloti*, *B. japonicum*, and *M. loti*), as well as *R. leguminosarum*, contain multiple *groESL* genes. Although many single and double *groESL* mutants do not have symbiotic defects, some mutants do (11, 28, 32, 39). In *S. meliloti*, *groESL1* mutants form nodules late and the nodules are Fix^- (33), and in *B. japonicum* a *groESL3 groESL4* double mutant is unable to fix nitrogen (11). What roles do these genes play in symbiosis? In *S. meliloti*, genetic and biochemical studies have demonstrated that two key regulatory proteins necessary for early gene expression during symbiosis, NodD1 and NodD3, are substrates of GroEL/GroES (33, 47). In addition, later during symbiosis, GroEL/GroES may help to form active nitrogenase. In *B. japonicum*, the level of nitrogenase subunits in the $groESL3_{Bj}$ mutant is greatly decreased, although transcription of the genes is unaffected (11). In the free-living bacterium *Klebsiella pneumoniae*, GroEL regulates nitrogen fixation, possibly as a result of direct interactions with the regulatory protein NifA and nitrogenase subunits (18, 19). Finally, the GroEL/GroES chaperone complex may help to fold other proteins that are newly produced as the cells adapt and differentiate within the plant host.

Currently there is no clear reason why multiple *groESL* genes are present in these genomes. One possibility is that the genes are simply regulated differentially, providing GroES and GroEL under different conditions. Evidence for differential gene expression has been obtained in *S. meliloti* (28), *B. japonicum* (1, 10), and *R. leguminosarum* (39). Specifically in *S. meliloti*, only *groESL1* and *groESL5* are induced by heat shock (28), only *groESL5* is controlled by RpoH1 (28 and this study), and only *groESL1* and *groESL2* are preceded by a CIRCE (controlling inverted repeat of chaperone expression) element that may indicate regulation by the HrcA repressor, which is used to regulate heat-inducible genes in some bacteria (29). An additional possibility is that the encoded chaperones have different ranges of substrates. Although the GroEL/GroES complex can assist in the folding of a wide variety of proteins, it cannot function universally. Directed evolution studies have demonstrated that small numbers of amino acid changes in GroES and GroEL can lead to shifts in the spectrum of substrates (45). Therefore, multiple *groESL* genes may allow the cell to fold a wider variety of proteins. As an extreme example, bacteriophage T4 encodes a protein of little sequence similarity to GroES that nevertheless substitutes for the host GroES, generating a new chaperone complex that can fold the major capsid protein (2, 42). In *R. leguminosarum*, the three GroEL proteins have different in vitro properties, including the ability to refold a specific denatured substrate (16). We found that *groESL3* is not interchangeable with *groESL1*, which would be consistent with different substrate specificities, whereas *groESL2* is interchangeable with *groESL1* (33), suggesting at least overlapping substrate specificities for that pair. As an added complexity, heteromeric complexes as well as homomeric complexes might be made, which would dramatically increase the number of different types of GroEL/GroES chaperones within the cells.

With the construction of the *groESL3* and *groEL4* mutations described in this paper, we now have mutations in all of the *groESL* operons in *S. meliloti*. Although the only single mutant that has a symbiotic defect is *groESL1* (28, 32, 33, and this study), we know that all of the genes are expressed within the nodule (5, 6, 32, and A. N. Bittner and V. Oke, unpublished). In addition, previous work has shown that *S. meliloti* cells need either *groESL1* or *groESL2* in order to be viable (32). It will be interesting to determine if the two possible quadruple mutants can be constructed and, if so, whether they are proficient in symbiosis.

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