JB Journals.ASM.org

Dual RpoH Sigma Factors and Transcriptional Plasticity in a Symbiotic Bacterium

Melanie J. Barnett,[a] Alycia N. Bittner,[b]* Carol J. Toman,[a] Valerie Oke,[b] and Sharon R. Long[a]

Department of Biology, Stanford University, Stanford, California, USA,[a] and Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, USA[b]

***Sinorhizobium meliloti* can live as a soil saprophyte and can engage in a nitrogen-fixing symbiosis with plant roots. To succeed in such diverse environments, the bacteria must continually adjust gene expression. Transcriptional plasticity in eubacteria is often mediated by alternative sigma (σ) factors interacting with core RNA polymerase. The *S. meliloti* genome encodes 14 of these alternative σ factors, including two putative RpoH ("heat shock") σ factors. We used custom Affymetrix symbiosis chips to characterize the global transcriptional response of *S. meliloti rpoH1*, *rpoH2*, and *rpoH1 rpoH2* mutants during heat shock and stationary-phase growth. Under these conditions, expression of over 300 genes is dependent on *rpoH1* and *rpoH2*. We mapped transcript start sites of 69 *rpoH*-dependent genes using 5′ RACE (5′ rapid amplification of cDNA ends), which allowed us to determine putative RpoH1-dependent, RpoH2-dependent, and dual-promoter (RpoH1- and RpoH2-dependent) consensus sequences that were each used to search the genome for other potential direct targets of RpoH. The inferred *S. meliloti* RpoH promoter consensus sequences share features of *Escherichia coli* RpoH promoters but lack extended −10 motifs.**

Sinorhizobium meliloti is a soil-dwelling alphaproteobacterium that forms nitrogen-fixing root nodules on plants, including *Medicago sativa* (alfalfa) and a model legume, *Medicago truncatula.* In the earliest stage of the symbiosis, the bacteria associate with plant roots and exchange chemical signals (13, 16). Subsequently, the actively dividing bacteria invade the root cortex and developing nodule via a plant-synthesized infection thread (28). Bacteria are released into the cytoplasm of nodule cells, where they terminally differentiate into nitrogen-fixing bacteroids that convert dinitrogen into ammonia, which the plant can use as a nitrogen source for growth (15, 20, 37, 67).

Given this complicated lifestyle, *S. meliloti* must have phenotypic flexibility to adapt to a series of differing environments: unpredictable soil conditions, plant defense mechanisms, plant signals, and the internal plant milieu. Abiotic stresses may include extremes of pH, salinity, nutrient availability, and temperature, as well as toxic metals, reactive molecules, and other deleterious compounds (57). Effective adaptation usually includes major changes in gene expression; determining what, when, and how these changes occur in *S. meliloti* will increase our understanding of soil dynamics and of symbiotic nitrogen fixation (2).

Eubacterial transcription is mediated by RNA polymerase (RNAP), and the sigma (σ) factor subunit is required for promoter recognition and transcription initiation. All eubacterial genomes encode an essential housekeeping σ factor and most have at least one alternative σ factor. Housekeeping σ factors recognize a large set of promoters, while alternative σ factors recognize smaller groups of promoters for genes with a shared function. In general, σ factors recognize promoter motifs located approximately −35 and −10 nucleotides (nt) upstream of the transcription start site (23). Within a given species, the sequence and spacing of these motifs differ, depending on which σ factor interacts with core RNAP. Since alternative σ factors compete with the housekeeping σ factor for RNAP core enzyme, large shifts in gene expression can occur by controlling expression, activity, and availability of alternative σ factors (49). A large number of alternative σ factors in a species appears to correlate with a diverse lifestyle (23).

The *S. meliloti* genome encodes 14 alternative sigma factors: RpoN, essential for the transcription of nitrogen fixation genes; 11 extracytoplasmic function-type (ECF-type) σ factors (RpoE1 to RpoE10 and FecI); and two RpoH/heat shock-type σ factors (RpoH1 and RpoH2 [19]). Multiple RpoH σ factors are common in alphaproteobacterial genomes (9, 22, 33, 43). In *S. meliloti*, RpoH1 and RpoH2 share 44% sequence identity and are 38% and 40% identical, respectively, to the *Escherichia coli* RpoH heat shock σ factor. In *E. coli*, the main role of RpoH is to maintain protein-folding homeostasis under high temperature and other conditions that denature proteins, as well as in normal conditions (24). *S. meliloti rpoH1* and *rpoH2* are partially functionally equivalent to *E. coli rpoH*, as introduction of either gene on a plasmid restores viability of an *E. coli rpoH* null mutant at 30°C, although not 37°C (47, 48).

Unlike wild-type *S. meliloti*, *rpoH1* mutants are severely impaired for growth at 37°C, fail to grow at 40°C, and are sensitive to acid pH, deoxycholate, sodium dodecyl sulfate, and crystal violet (10, 40, 47, 48). In addition, *rpoH1* mutants have severe symbiotic defects: they initiate nodule formation, invade plant roots, and are released into plant cells but show poor colonization and survival in nodule cells and do not fix nitrogen (40, 47). In contrast to *rpoH1* mutation, *rpoH2* mutation has little effect on *S. meliloti* growth, stress adaptation, or symbiosis (40, 47, 48). However, the *rpoH1 rpoH2* double mutant has a more severe symbiotic phenotype than an *rpoH1* mutant: nodules are rarely formed, and those that do form contain bacteria carrying suppressor mutations (7).

S. meliloti rpoH1 and *rpoH2* are expressed differently under

Received 21 March 2012 Accepted 2 July 2012

Published ahead of print 6 July 2012

Address correspondence to Sharon R. Long, srl@stanford.edu.

* Present address: Alycia N. Bittner, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA.

Supplemental material for this article may be found at http://jb.asm.org/.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.00449-12

TABLE 1 RpoH-dependent promoters identified by RACE mapping

Gene set and unique identifier[a]	Gene	Description	Avg SLR[b]	Distance[c]	Sequence[d]
Gene set 1. RpoH1-dependent promoters in heat shock used to determine the RpoH1 promoter consensus sequence					
SMa1894	*msrB2*	Peptide methionine sulfoxide reductase	−2.9 H1, hs	26	ATTGGGAATCCTCAGGACCGCTCGCCATATTCAGGCA**A**
SMa1896	*msrA3*	Peptide methionine sulfoxide reductase	−2.4 H1, hs	NA	
SMb20708		Methylated-DNA-protein-Cys methyltransferase	−3.0 H1, hs	68	GTTGAATTGAAGGCCTGGGCGCACGATTCTCCATGA**A**
SMb20707	*cyaG2*	Adenylate cyclase	−2.7 H1, hs	NA	
SMb22023	*groES5*	60-kDa chaperonin	Probe set not on chip	96	CTTGAAATCCATTTTTAGCCCAACCAGATCATCCC**G**
SMb21566	*groEL5*	10-kDa chaperonin	−3.5 H1, hs	NA	
SMc00109		Transcription regulator	−1.1 H1, hs	70*	CTTGATCGACGGCGGATCCTGCCCATATAAAGCTCT**A**
SMc00117	*msrB1*	Peptide methionine sulfoxide reductase	−2.5 H1, hs	18/20	TTTGTGCCCGAGCGGATGCCATCCTATATATTCGCT**GCA**
SMc00467		Conserved hypothetical protein	−1.7 H1, hs	82	GTTGCGCTGACGGAAACATTTCCTATATCGCTCCA**G**
SMc00466		Conserved hypothetical protein	−1.5 H1, hs	NA	
SMc01142	*grpE*	Heat shock protein	−1.3 H1, hs	89	CTTGATTTTTCGGGCCCAAACATCGATATCGGGCTT**C**
SMc01224	*trxB*	Thioredoxin reductase	−3.0 H1, hs	66	CTTGAAAGTGCGCGCCGTGAATACCTAAAGTAAGGCA**A**
SMc01256	*sda*	L-Serine dehydratase	−2.1 H1, hs	106	ATAGGATCGCTTAGGGTTCGATCCTATATTAGGGA**A**
SMc01441	*hflK*	Membrane-bound protease	−1.3 H1, hs	65	GTTGAAAGCAATGCACATGATACCTATAACGGGTTC**A**
SMc01440	*hflC*	Membrane-bound protease	−1.3 H1, hs	NA	
SMc01465	*creA*	Conserved hypothetical protein	−4.4 H1, hs	20	CTTGCATTGCAGCATCCGCTGCCTATCTGAACGGT**G**
SMc01905	*lon*	ATP-dependent protease	−1.5 H1, hs	72	CTTGAAAAGGCCTCTCTTGCACTCCACTTTCATGTAC**G**
SMc02110	*clpS1*	Elongation factor TS	−1.4 H1, hs	52	CTTAAAATCCAAGAACGAACCATTAGATAAAGGCAA**G**
SMc02109	*clpA*	ATP-dependent protease	−1.5 H1, hs	NA	
SMc02380		Oxidoreductase	−2.9 H1, hs	23	CTTGTGCCTTTTGAGCATAGGGCCCATGTAGCTCCT**G**
SMc02720	*clpP2*	ATP-dependent protease	−1.4 H1, hs	24	ATTGTTTTCGCCGTCGAGTCGCATTATCTGGTTGGA**A**
SMc02882		Conserved hypothetical protein	−4.5 H1, hs	28	CTTGCCCCTGCCGGTCGCTCTGCCTACATGCCTTCC**G**
SMc02886		Conserved hypothetical protein	−2.4 H1, hs	23	ATTGCGCTTGCCCGCCCTTGCGCCTATCTCTTGCGC**A**
SMc02885	*msrA1*	Peptide methionine sulfoxide reductase	−2.2 H1, hs	NA	
SMc03152		Conserved hypothetical protein	−4.0 H1, hs	68	TTTGTATTTCCGTGGCAACGTTCCTATGTCTCAATC**G**
SMc03801		Thioredoxin	−1.9 H1, hs	41	CTTGAAAGTGACGGCCTGACATACGATCTTTGGCGC**G**
SMc04403	*dcp*	Peptidyl-dipeptidase	−2.8 H1, hs	53	CTTGACGGTCCGGCGTCGATCGACCACTTCAAGCAC**C**
Gene set 2. RpoH2-dependent promoters in stationary phase used to determine the RpoH2 promoter consensus sequence					
SMa1158		Conserved hypothetical protein, UspA-like	−1.6 H2, sp	34	ATTGCTGTTTTCGTATTTGGGCCTAATTTCTGTCGGT**A**
SMa1364		ABC transporter, periplasmic solute-binding protein	−2.4 H2, sp	61	CTCGACGTTTCCGCAGACAGAGCTATCTACCTCCTG**A**
SMa2301		Diguanylate cyclase	−4.1 H2, sp	0*	CTTGCCAGCAGGAGGTGCTCCCCTAGATGGGCAAGG**A**
SMa2349		Oxidoreductase, YagT-like	−3.8 H2, sp	187	GTAGTCACACGCGCTACCCGCCCTAACTCACTTGT**G**
SMa2351		Oxidoreductase, YagS-like	−2.7 H2, sp	NA	
SMa2353		Oxidoreductase, YagR-like	−2.5 H2, sp	NA	
SMc01354		Conserved hypothetical protein	−1.0 H2, sp	67	CTTTCCAGTGCGCTGGTCTGATCCATATGCTTGTCCT**A**
SMc01723		Conserved hypothetical protein	−3.9 H2, sp	39	CTACCCTGACATGAAAAGCGCTCTATCTCTGCTTC**G**
SMc01960		Oxidoreductase	−1.8 H2, sp	42	CTTTCCTTTTGCCGCGAGCCCGCTAGTTTTTC**G**
SMc02703		Conserved hypothetical protein	**−0.93 H2, sp**	58	CTTGTCAGCCGCCCCGCGGGGTCTAAATTGAAAAAC**A**
SMc04146		Phosphoketolase	−3.2 H2, sp	58	ATTGCCCTGGCGCCGCACCGCCATAGATTCTGCAGC**G**
SMc04181		Hypothetical protein	−1.4 H2, sp	59	ATGGTCACCGGGTCTACGAGACCTATGTCATTAAT**C**
SMc05020	*ecnA*	Entericidin A	−2.2 H2, sp	25	CTTGATTTCTCGCAGGCATGCATCATCTTTTCTTGG**A**
Gene set 3. RpoH1-dependent promoters in heat shock and RpoH2-dependent promoters in stationary phase used to determine the dual RpoH1/RpoH2 promoter consensus sequence					
SMa2061		Conserved hypothetical protein	−1.8 H1, hs; −3.0 H2, sp	26	CTCGAAATCATACCTGGACGCCCTAACTTCCATTCTC**G**
SMa2063		Sensor histidine kinase	−1.3 H1, hs; −2.8 H1H2, sp	NA	

SMb20117		Glycosyltransferase	−4.0 H1, hs; −2.9 H2, sp	70	CTTGAAGAGCACGGCGCATCGACTAGATCATAGATGTC**G**
SMb20302		Conserved hypothetical protein	−1.1 H1, hs; −5.4 H2, sp	32	CTTGCCTTTTGCTTTGCCTCCCATAGTTGAAATAGA**A**
SMb21683		Hypothetical protein	−1.6 H1, hs; −4.3 H2, sp	15*	CTTGCCGGCGGCCGGTCGGCTCCTATTCTTTATCCAT**G**
SMc00106		Conserved hypothetical protein	−2.7 H1, hs; −3.9 H2, sp	36	CTCGACGCGCCGCCTTGCGGTCCTATCTTACGTTATC**G**
SMc00367		Conserved hypothetical protein	−5.3 H1, hs; −3.0 H2, sp	83	ATCGCAATTTCGTTGCGATGTCCTACCTATGAGG**G**
SMc00952		Thioredoxin-like protein	−2.3 H1, hs; −1.4 H2, sp	25	ATTGAAAGATGTTTCCGGCCGGCCTATGTCGCAG**T**
SMc01280	*degP3*	Serine protease	−2.0 H1, hs; **−1.1 H2, sp**	24	TTTGCCTTTTTCCGTGCGAAATCCTATCTGAACGTA**G**
SMc01279		Replication-associated recombination protein	−1.3 H1, hs	NA	
SMc01759		Conserved hypothetical protein	−3.8 H1, hs; −1.9 H2, sp	48	CTTGATGCTCGCACGCGATCGCCTATCTATTTTTGC**G**
SMc01758	*groEL4*	60-kDa chaperonin	−3.4 H1, hs; −1.8 H2, sp	NA	
SMc03176		Conserved hypothetical protein	−1.5 H1, hs; −1.7 H2, sp	0	CTGGCAGATGCGCCGCCGCCGGCTATGTTTATCCGT**A**
SMc03177		ATP-dependent DNA ligase	−1.1 H1, hs	NA	
SMc03246		Phage-related integrase	−3.0 H1, hs; −1.1 H2, sp	47	CTTGGAAGTTTCAGCTTGGTTCCCTATTTTACCTGTT**G**
SMc03802		Peptidase	−2.6 H1, hs; −2.6 H2, sp	52	CTTGAGATTTCGAAGGGCCGCACCATTTTTTGGTC**C**
SMc03803		Conserved hypothetical protein	−2.3 H1, hs; −2.6 H2, sp	NA	
SMc03836		Arylesterase	−3.2 H1, hs; −2.0 H2, sp	44*	TTTGCCACGCGAAAGAGCCCTATATGAAAATTG**G**
SMc04202		Conserved hypothetical protein	−3.3 H1, hs; −1.3 H2, sp	220	CTTAAATTCCGGCACTTCCGGCTCTATATGGCGTTCT**A**
Gene set 4. Promoters of *rpoH*-dependent genes not included in any of the RpoH promoter consensus sequences					
SMa0136		Conserved hypothetical protein	−2.4 H2, sp	60	CTGGGCGGCGTCCAACACTGCCCTACCTACTTGCGA**A**
SMb20303		Hypothetical protein	−4.3 H1, hs; −1.2 H1, sp	38	CTGGATTCGAGCCGTATCCGCACCGATATTAACTC**A**
SMb21295		Heat shock protein, Hsp20 family	−4.6 H1, hs; −2.7 H1H2, sp	87*	CTTGAAACCCGGCTTCGAATGACTAAATCGGGTGC**G**
SMb21294		Heat shock protein, Hsp20 family	−5.3 H1, hs	NA	
SMb21296		Hypothetical protein	−5.5 H1, hs; −2.2 H1H2, sp	119	CTTGATTTACCTTTTCCGCTCCAATATCTGTTCAGC**G**
SMc00030		Hypothetical protein	−1.9 H2, sp	30	CTGTCCATCTGCTTCGCGTGCTCCACTTTCAGACTG**A**
SMc00031		Hypothetical protein	−1.3 H2, sp	NA	
SMc00048		Conserved hypothetical protein, Usp-like	−1.1 H2, sp	197	GTTGACAGTCGTCACGGCCGCGCTACCTTTAACGGT**G**
SMc00049		Conserved hypothetical protein	−1.2 H2, sp	NA	
SMc00814		Hypothetical protein	−1.9 H1, hs; −2.9 H2, sp	46	CCTGTCCATTTCTGTCAAGGCACTACTTCTCGCCT**G**
SMc00949		HspQ-like protein	−3.8 H1, hs; −1.6 H1, sp	17	CTTGATGTTCCATATGCGACAACCCAGATAACTGTCT**G**
SMc00969		Amidohydrolase	−1.1 H1, hs; −2.1 H2, sp	0	ATGGATTTTGACAGGCATGCGCACTAGTTTCGCCGG**A**
SMc00970		Exodeoxyribonuclease VII	−1.4 H2, sp	NA	
SMc01180		Conserved hypothetical protein	−4.7 H1, hs; −1.3 H1H2, sp	19	GTTGCCGCCCTGTGGAGAAGGCACCAACTGTGGGGTC**A**
SMc01329	*azoR*	FMN-dependent NADH-azoreductase[e]	−1.9 H1, hs	26	CTTATCGAAGGATTGTTTGCAGATCATATGTGATGC**A**
SMc02382		Conserved hypothetical protein	−3.9 H1, hs; −2.5 H1H2, sp	30	ATGGAAACCCCCGCGGCCGCTCTTATCTGAGAAGCT**G**
SMc02433	*clpB*	ATP-dependent protease	−3.3 H1, hs; −2.5 H1H2, sp	165	CTTTAATTCAGAAGTGCGCTGCCCCATATTTCGCTTC**G**
SMc02575	*hslV*	ATP-dependent protease	−4.0 H1, hs; −1.3 H1H2, sp	58	TTTGCAATCGCGCGGCTGCGACTTACATAGGCCTC**A**
SMc02576		GCN5-related *N*-acetyltransferase	−4.4 H1, hs	NA	
SMc02577	*hslU*	ATP-dependent protease	−4.3 H1, hs	NA	
SMc02769		Conserved hypothetical protein	−4.6 H1, hs; −3.2 H1, sp	44	CTTGCGTCGGGGCTGTGGGTTTCCCACATCGTTTTC**A**
SMc02857	*dnaK*	Chaperone	−1.1 H1, hs	104	CTTGCAGTCCGAAACGCCGGTCCTTATATACGCCGC**A**
SMc02863	*recF*	DNA repair protein	−2.2 H1, hs	14	CTTGCCTTGGCCGGGCTTTCATCCTACCTGACAGCAACGC**G**
SMc02900		Conserved hypothetical protein	−1.7 H1, hs; −1.1 H1, sp; −1.4 H2, sp	19/21	CTTAAATCTTAGGCAAATGCCTATTTAGCTCTC**GTA**
SMc03857	*ffh*	Signal recognition particle protein	−1.1 H1, hs; −2.0 H1H2, sp	40/41	CTTGTTTTCCGCGCGAAGGGACCCACGTTTCCGGA**CG**
SMc03858	*pheAa*	Chorismate mutase	−1.1 H1H2, sp	NA	
SMc03859	*rpsP*	30S ribosomal protein S16	−1.4 H1H2, sp	NA	

(Continued on following page)

TABLE 1 (Continued)

Gene set and unique identifier[a]	Gene	Description	Avg SLR[b]	Distance[c]	Sequence[d]
SMc04040	*ibpA*	Heat shock protein, Hsp20 family	−5.8 H1, hs; −2.0 H1, sp	97	CTTGAACTCGTGCGCGGGCATTCCCATGTCTTCTCC**G**
SMc04310		Hypothetical protein	−3.6 H1, hs	45	ATTCAAGTCGTCCCGGCAAGAGCCTATATCCGCGCC**A**
SMc04406		Conserved hypothetical protein	−4.3 H1, hs; −3.1 H1H2, sp	14	CTTGATTTTGCCGGCCGCGCCTTCACATCCATAAGGC**G**
SmelC456		*trans*-Encoded sRNA	−2.4 H1, hs	NA	ATTGAACCGACGCCCGTCGCGCACCAAATGTCGCA**A**
SmelC781		*trans*-Encoded sRNA	−2.5 H1, hs; −6.7 H2, sp	NA	GTTGACGAAATGACAGGGCGCCCTAGATGGAAGAAT**A**

[a] Identifier in italic type indicates that the gene is predicted to be in an operon with the gene listed directly above it.

[b] Average SLR change of all pairwise comparisons for *rpoH* mutants compared to the wild type. A negative value means that expression was decreased in the mutant. Abbreviations: H1, *rpoH1* mutant; H2, *rpoH2* mutant; hs, heat shock; sp, stationary phase. SLR values for the double mutant (H1H2) are shown only when there was no change for a single mutant under that growth condition. SLR is expressed as the $\log_2$ ratio of the change, i.e., an SLR of 1 equals a 2-fold change. Values in bold type were slightly below our cutoff for SLR (SMc02703) or pairwise comparisons (SMc01280), but the *rpoH* dependence of the respective genes was confirmed by RACE mapping.

[c] Distance between the transcription start site (+1) and the first nucleotide of the translational start codon. An asterisk next to the value indicates that the value was adjusted because the annotated start codon is likely incorrect. NA, not applicable.

[d] Putative RpoH-dependent promoters determined by RACE mapping. The transcription start site is shown in bold type. Putative −35 and −10 motifs are underlined.

[e] FMN, flavin mononucleotide.

both free-living and symbiotic conditions. During free-living growth, *rpoH1* expression appears mostly constitutive (3, 12, 26, 36, 52), although its expression during exponential-phase growth is higher in minimal medium compared to rich TY medium (3). While its expression increased with the onset of stationary-phase growth in rich medium (47), other work showed either no change (52) or a decrease (8) during stationary-phase growth in minimal medium. *rpoH1* expression decreases during nitrogen starvation in a *relA*-dependent manner (31). *rpoH1* is expressed strongly in *M. sativa* (47) and *M. truncatula* (3) nodules. In contrast, *rpoH2* expression is detected only in minimal medium, not in rich medium, after growth to stationary phase (47). *rpoH2* expression is induced during heat shock in minimal medium (47), probably due to increased activity of RpoE2 (52); it also increases during osmotic stress (12). In *M. sativa* and *M. truncatula* nodules, *rpoH2* is expressed at low levels (3, 47).

In *E. coli*, RpoH function is regulated mainly at the level of translation, protein stability, and protein activity (24). In *S. meliloti*, nothing is known about posttranscriptional regulation of RpoH1 or RpoH2, but since *rpoH1* expression is largely constitutive, it is likely that posttranscriptional regulation plays a significant role.

To determine how these dual RpoH σ factors contribute to gene expression during *S. meliloti* stress adaptation, we identified putative RpoH1 and RpoH2 targets by global transcription profiling of *rpoH1*, *rpoH2*, and *rpoH1 rpoH2* mutants under conditions of heat shock or stationary-phase growth. We performed 5′ rapid amplification of cDNA ends (5′ RACE) mapping on selected target genes to identify possible RpoH promoter elements. Our work shows that RpoH1 and RpoH2 directly or indirectly control hundreds of *S. meliloti* genes and that the putative promoters of many of these genes have overlapping, yet distinct features.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. meliloti* strains used in this study were Rm1021 (wild type), VO3128 (*rpoH1*::*aadA*), AB3 (*rpoH2*::*aacCI*), and AB9 (*rpoH1*::*aadA rpoH2*::*aacCI*) (7, 35, 47). Bacterial cultures were grown at 30°C in LB/MC medium (21) or M9 minimal medium containing 0.2% sucrose, 0.5 μg ml^{-1} biotin, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, and 500 μg ml^{-1} streptomycin.

For heat shock, cells were grown overnight in LB/MC medium, diluted to an optical density at 595 nm (OD_{595}) of 0.05 the next day, and allowed to grow overnight. Growth of three replicates was staggered by 1 h. The cultures were diluted to OD_{595} of 0.05 in 65 ml LB/MC medium and allowed to grow to mid-exponential phase (OD_{595} of 0.5 to 0.7). Each wild-type culture was split so that 30 ml remained at 30°C for 15 min as a control, and 30 ml was heat shocked for 15 min at 42°C. The cells were harvested by mixing cultures with 1/10 volume of ice-cold stop solution (5% buffer-equilibrated phenol in ethanol) and centrifuging at 4°C. The supernatant was removed, and the cells were frozen in liquid nitrogen and stored at −80°C.

For the stationary-phase growth experiment, three replicates were started on the same day in identical medium. Overnight cultures were grown in LB/MC medium, diluted to an OD_{595} of 0.05 the next day, and allowed to grow overnight to ensure even growth. The cells (8 ml) were washed twice and diluted to an OD_{595} of 0.05 in 300 ml M9 minimal medium. Growth of the three replicates was staggered by 1.5 h. The cultures were incubated with shaking for 48 h, until late stationary phase. The cells were harvested in the same manner as described above for the heat shock experiments.

RNA purification, cDNA synthesis, labeling, and hybridization. Cell pellets were resuspended in 1 mg ml^{-1} lysozyme in Tris-EDTA (TE) and

buffer RLT (Qiagen RNeasy kit). Cells grown to stationary phase were additionally lysed by bead beating with 0.09-mm to 0.135-mm glass beads (Thomas Scientific) at 4°C with three 30-s pulses and a 1-min incubation on ice between each pulse. Total RNA was isolated as described previously (3), but with an RNeasy Midi kit. This protocol includes both on-column and off-column DNase digestions to remove all contaminating chromosomal DNA. The absence of chromosomal DNA was confirmed by PCR amplification using primers to an intergenic region, and RNA integrity was validated on a 1.2% agarose formaldehyde gel.

cDNA synthesis, labeling, and hybridization to Affymetrix symbiosis chips were performed as described previously (3), except for the stationary-phase experiment, where 12 μg (instead of 4 μg) of fragmented, labeled cDNA was hybridized to each chip.

Affymetrix data analysis. Design of the *S. meliloti*/*M. truncatula* dual-genome symbiosis chip was described previously (3); the *S. meliloti* sequences on the GeneChip array correspond to the original genome annotation reported in reference 19. The symbiosis chip also contains probe sets corresponding to *S. meliloti* intergenic regions (IGR) of ≥150 nt and to ~10,000 *M. truncatula* expressed sequence tag (EST) sequences. As with other array platforms, our symbiosis chip measures mRNA abundance, of which both transcription and mRNA turnover are components. For brevity in this report, we use the term "expression" to include the sum of all factors affecting mRNA abundance. Data were processed using GeneChip operating software and data mining tool (Affymetrix). The chips were scaled to a target signal intensity of 500 by using the global scaling option, and each experimental array was compared with a baseline array. Thus, an experiment with three control arrays and three experimental arrays yielded nine pairwise comparisons. We deemed an increase or decrease of average signal log ratio (SLR) of ≥0.96 to be significant if either eight or nine of the nine pairwise comparisons were evaluated by the software as significantly changed ($P \leq 0.05$).

Transcription start site determination. 5′ RACE (5′ rapid amplification of cDNA ends) was performed on a subset of *rpoH*-dependent genes essentially as described previously (45, 51) with minor modifications. Briefly, 14 μg of RNA isolated from heat-shocked or stationary-phase cells (the same RNA samples used for Affymetrix symbiosis chips) was treated with tobacco acid pyrophosphatase (TAP) (Epicentre Technologies), ligated to an RNA primer (5′-GAGGACUCGAGCUCAGUC-3′) with T4 RNA ligase (Epicentre Technologies), and reverse transcribed with SuperScript II or III reverse transcriptase (Invitrogen). For reverse transcription, a cocktail of gene-specific primers was used (see Table S1 in the supplemental material). cDNA was amplified using a primer specific to the 5′ end of each transcript (RACE primer [5′-GAGGACTCGAGCTCA GTC-3′]) and a gene-specific primer (Table S1). Amplified fragments were separated by polyacrylamide gel electrophoresis (7.5%) and stained with ethidium bromide or SYBR Gold (Invitrogen). Fragments that displayed decreased intensity in *rpoH* cells compared to wild-type cells were excised and subjected to a second round of PCR amplification. PCR samples were purified with USB ExoSAP-IT (Affymetrix) or QIAquick columns (Qiagen) and sequenced. Altogether, we determined the transcription start sites of 69 *rpoH*-dependent genes that were chosen to represent a range of gene expression fold changes, as well as genes orthologous to those in the *E. coli* RpoH regulon (45). All but two of these genes were significantly decreased in expression by Affymetrix GeneChip analysis, in one or more of the *rpoH* mutants. Values for SMc02703 and SMc01280 were slightly below our cutoff, but we confirmed their *rpoH* dependence by 5′ RACE mapping (Table 1).

Promoter consensus determination and *in silico* genome-wide predictions. To identify putative promoter consensus sequences specific for RpoH1 and/or RpoH2, sequences upstream of mapped transcription start sites of protein-coding genes were sorted into four gene sets (Table 1) based on their expression pattern: (gene set 1) 23 genes dependent only on *rpoH1*, only during heat shock; (gene set 2) 14 genes dependent only on *rpoH2*, only in stationary phase; (gene set 3) 16 dual-promoter genes dependent on *rpoH1* in heat shock and *rpoH2* in stationary phase; and (gene set 4) 16 genes that were *rpoH* dependent but with different expression patterns. Each of the first three sets was used as input for MEME (Multiple Em for Motif Elicitation) (1). Initially, we used 20-nt search windows for each motif (−35 and −10) and then decreased the window size in subsequent iterations to obtain the final putative RpoH-dependent promoter consensus sequence. Eight 5′ RACE sequences could not be fit to their respective consensus sequence; these may include genes whose expression is indirectly *rpoH* dependent (SMa0136, SMc00030, SMc00048, SMc00814, SMc00969, SMc01329, SMc02863, and SMc04310 in Table 1). Hence, the final three consensus sequences incorporate data from 45 mapped promoters, distributed among the three sets as follows: (gene set 1) 20 genes for the RpoH1 consensus; (gene set 2) 11 genes for the RpoH2 consensus; and (gene set 3) 14 genes for the dual-promoter consensus (Table 1).

To identify putative RpoH-dependent promoters in the *S. meliloti* genome, we used a matrix-based search method, RSA-tools-matrix-scan (59), to search upstream of each *S. meliloti* open reading frame (ORF) with position-specific scoring matrices designed for each of the three consensus sequence gene sets. To decrease noise and prevent bias in estimation of *P* values, we prohibited overlap with upstream coding regions. Only those putative promoters with both a positive weight score and a *P* value of $\leq 1 \times 10^{-4}$ were considered significant. After eliminating those genes whose expression was not decreased in one or more *rpoH* mutants in our Affymetrix experiments (i.e., those not in the group of "*rpoH*-dependent genes"), we obtained a set of 75 putative *rpoH*-dependent promoters representing 100 *rpoH*-dependent genes. Of the eight genes listed above whose RACE sequence could not be fit to a consensus, two were identified in the genome-wide search for putative RpoH1 promoters (SMc02863 and SMc04310).

Microarray data accession number. The Affymetrix data described in this article are available in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) (14) and are accessible through GEO Series accession number GSE36186.

RESULTS

The *S. meliloti* transcriptome changes dramatically in response to heat shock. To identify *rpoH*-dependent genes, we used custom Affymetrix symbiosis chips (3) for transcription profiling of wild-type *S. meliloti* Rm1021 cells and strains with mutations in *rpoH1* (strain VO3128), *rpoH2* (strain AB3), and *rpoH1 rpoH2* (strain AB9). Since *rpoH1* mutants are deficient in heat shock response, we compared these strains under heat shock conditions. Exponential-phase cultures grown in LB/MC medium were subjected to heat shock for 15 min at 42°C (Materials and Methods). We chose this early time point to favor discovery of direct RpoH1 targets and because reverse transcription-quantitative PCR (RT-qPCR) experiments showed maximal induction of a known RpoH1 target, *groESL5*, at 15 min (data not shown). Wild-type *S. meliloti* cells cultured for 15 min at 30°C served as a normal temperature control. We performed three biological replicates for each condition and defined changes in mRNA abundance of more than 2-fold as significant.

Although we were interested in *rpoH1*- or *rpoH2*-dependent genes, we started with a comparison of wild-type heat shock-treated *S. meliloti* cells to those cultured at 30°C to look at the full transcriptional response to heat shock. Expression of 997 genes increased and expression of 1,015 genes decreased; these changes represent about 40% of the genome (see Data Set S1 in the supplemental material). As expected, many of the genes whose expression increased with heat shock have annotated functions in adaptation to heat and other stress responses. Expression of genes encoding enzymes for exopolysaccharide synthesis was also increased, as observed in other *S. meliloti* stress responses (26). The

S. meliloti genome contains three replicons: a 3.65-Mb chromosome and two megaplasmids (1.35 and 1.68 Mb) that carry genes necessary for symbiosis. Expression of most genes involved in replication and conjugal transfer of the megaplasmids (*traA1CDG*, *traA2*, *repABC-1*, *repABC-2*, *virB1* to *virB5*, *virB7*, and *virB9*) increased with heat shock. Genes with decreased expression in heat shock encode components involved in housekeeping, motility, chemotaxis, and pilus biogenesis functions. Expression of biosynthesis genes decreased, especially those for amino acid and nucleotide biosynthesis. An apparent decrease in expression during heat shock of the *fixNOQP1* operon was due to higher than normal expression of this operon in the wild-type, normal-temperature control (data not shown). A few other genes previously shown to be induced with microoxia were similarly affected during this experimental trial, suggesting that it is an artifact. Genes whose expression increased with heat shock were more likely to lack a predicted function than those whose expression decreased (472 versus 306). Expression of numerous genes encoding regulators changed during heat shock, including that of seven sigma factors: *rpoE2*, *rpoE5*, *rpoE9*, *rpoH2*, and *rpoN* expression increased, while *rpoE1* and *rpoE4* expression decreased. In sum, our results show that 15 min of heat shock results in extensive transcriptional remodeling.

RpoH1 plays a more significant role than RpoH2 in heat shock. We saw clear differences when wild-type *S. meliloti* and each of the three *rpoH* mutant strains were subjected to heat shock. When the *rpoH1* mutant was compared to the wild type, 593 genes showed altered expression (see Data Set S1 in the supplemental material) of which 282 decreased and 311 increased. The genes whose expression decreased in the *rpoH1* mutant (i.e., the set of *rpoH1*-dependent genes) likely include both direct targets and downstream, indirect targets. More than one-third of the genes whose expression was *rpoH1* dependent during heat shock were not themselves significantly induced in the wild type with heat shock, and about a dozen of these genes, including *rpoE1*, showed reduced expression in the wild type. These genes may represent genes whose expression was already *rpoH1* dependent at 30°C, as well as genes whose expression was induced by heat shock but not above the fold change cutoff in our experiments. Because not all *rpoH1*-dependent genes were induced by heat in the wild type, we distinguished a subset of *rpoH1*-dependent, heat shock-induced genes. Figure 1 shows a Venn diagram of these relationships as they relate to a previous study of the role of *rpoH1* in acid shock (described below). Among the genes that are *rpoH1* dependent and heat shock induced, annotated functions in stress response, chaperoning, macromolecular degradation, and fatty acid biosynthesis are overrepresented compared to the genome as a whole, whereas genes predicted to be involved in small molecule metabolism, including transport functions, are underrepresented (Data Set S1). At least 10% of *S. meliloti rpoH1*-dependent genes are orthologous to members of the *E. coli* RpoH regulon; for example, *clpA*, *clpB*, *clpP*, *clpX*, *creA*, *dnaK*, *dnaJ*, *ftsH*, *groESL*, *grpE*, *hflC*, *hflK*, *hslU*, *hslV*, *htpG*, *htpX*, and *lon* (45, 63, 65, 69).

Eight percent of *rpoH1*-dependent genes were previously reported to be induced during osmotic stress (12), suggesting that *rpoH1* may play a role in osmotic stress resistance. It was also reported that *rpoH1* is critical for response to acidic pH stress (10; see below). Although the *S. meliloti* global response to oxidative stress response has not been characterized, we found that about 5% of our *rpoH1*-dependent genes were orthologous to oxidative

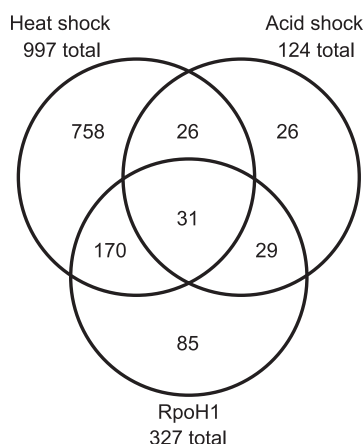

FIG 1 Intersection of *rpoH1*-dependent genes with heat shock- and acid shock-induced genes. Genes induced by heat shock were identified in this study, and genes induced by acid shock were determined by de Lucena et al. (10). Seventeen genes whose expression was significantly decreased in the *rpoH1 rpoH2* double mutant during heat shock and whose expression was also decreased in the *rpoH1* mutant but with an SLR of more than −0.96 are also shown in this diagram. Genes whose expression was *rpoH1* dependent during acid shock but that are not themselves induced by acid shock in the wild type were not identified by de Lucena et al.; therefore, this diagram shows only 85 genes that were *rpoH1* dependent in heat shock, but not themselves induced by heat or acid shock. Not shown on the diagram are 12 genes whose expression increased with both heat and acid shock treatments but that were *rpoH1* dependent in only one of these treatments: 4 of these genes were *rpoH1* dependent during heat shock, and 8 were *rpoH1* dependent during acid shock.

stress-induced genes of other bacteria: for example, methionine sulfoxide reductase (*msrB1*, *msrB2*, *msrA1*, and *msrA3*), thioredoxin (*trxA*), thioredoxin reductase (*trxB*), glutaredoxin (*grxC*), superoxide dismutase (*sodB*), glutathione *S*-transferase (*gst7* and *gst11*), and glutathione reductase (*gor*).

In contrast to the *rpoH1* mutant, no genes showed decreased expression in the *rpoH2* mutant compared to the wild type (see Data Set S1 in the supplemental material). In addition, a comparison of the *rpoH1 rpoH2* double mutant to the wild type gave essentially the same result as for the *rpoH1* mutant. Moreover, directly comparing the *rpoH1* strain to the *rpoH1 rpoH2* strain failed to detect any differences in expression. Thus, the impact of *rpoH2* on gene expression during the early heat shock response in rich medium appears negligible, whereas *rpoH1* clearly plays a major role.

The *rpoH1*-dependent and *rpoE2*-dependent gene sets show little overlap. *S. meliloti* RpoE2 mediates a major transcriptional response to general stress, and *rpoE2* expression increases in response to various stresses (4, 52, 56). However, *rpoE2* mutants show few phenotypic differences from the wild type and are symbiotically normal (17, 18, 52). Sauviac et al. performed transcriptome analysis of the *S. meliloti* heat shock response in the wild type and an *rpoE2* mutant as part of their study on the general stress response (52). Although they used slightly different conditions to study heat shock, we still found that >75% of the changes they saw were represented in our heat shock data set. We also saw 4-fold-more (2,012 versus 451) expression changes, perhaps due to differences in growth and heat shock conditions or to increased detection sensitivity of our Affymetrix symbiosis chips.

We compared our *rpoH1*-dependent gene set to their *rpoE2*-dependent (by microarray and *in silico* prediction) gene set (52).

Sixty-five of their 89 *rpoE2*-dependent genes were induced in our wild-type heat shock experiment, but we found that only five of these 65 were *rpoH1* dependent and one has already been shown to be only partially *rpoE2* dependent (SMb21456 [52]). Thus, RpoH1 and RpoE2 largely activate distinct sets of targets in response to heat shock.

Many genes that were *rpoH1* dependent in our study were also *rpoH1* dependent during acid shock. de Lucena et al. discovered that an *rpoH1* mutant was severely impaired for growth in acidic medium (pH 5.75) (10). Their transcription profiling of wild-type and *rpoH1* mutant cells over a 1-h time course revealed that 68 of 124 genes whose expression was induced by acid shock also showed decreased expression in the *rpoH1* mutant at one or more time points (10). Consolidating their results with our data, we conclude that 31 of these 68 genes showed *rpoH1*-dependent induction in both heat and acid shock and 37 (29 plus 8) genes showed *rpoH1* dependence in acid shock only (Fig. 1). Table S2 in the supplemental material lists *rpoH*-dependent, acid-induced genes, grouped by their *rpoH1* expression pattern. We identified 259 genes whose expression was *rpoH1* dependent in heat shock, but not acid shock: 174 (170 plus 4) of these were significantly induced by heat shock in the wild type, and 85 were not (Fig. 1). There may be additional genes whose expression is *rpoH1* dependent during acid shock but which are not themselves induced by acid shock in the wild type; however, these were not identified by de Lucena (10). In summary, these comparisons demonstrated an overlapping transcriptional response to heat shock and acid shock in *S. meliloti.*

RpoH2 plays a larger role than RpoH1 during late stationary-phase growth. Previous work showed that expression of both *rpoH1* and *rpoH2* increases during stationary phase (47). To define the contributions of *rpoH1* and *rpoH2* in stationary phase, we compared transcription profiles of wild-type *S. meliloti* Rm1021 and strains with mutations in *rpoH1* (VO3128), *rpoH2* (AB3), and *rpoH1 rpoH2* (AB9) grown to late stationary phase in minimal medium with sucrose as the carbon source and ammonium as the nitrogen source (Materials and Methods). We chose minimal medium because previous studies reported extremely low *rpoH2* expression in rich medium (3, 47). Since cells from late stationary-phase cultures had a low mRNA yield, we hybridized three times more cDNA than usual to each Affymetrix symbiosis chip to compensate (Materials and Methods).

Altogether, we identified 79 genes whose expression decreased and 12 genes whose expression increased in stationary phase in at least one of the mutant strains (see Data Set S1 in the supplemental material). Over half (44 genes) were dependent only on *rpoH2*, whereas 9 genes were dependent only on *rpoH1* (see Fig. S1 in the supplemental material). We were unable to determine *rpoH1* versus *rpoH2* dependence for 25 genes that showed decreased expression in the *rpoH1 rpoH2* double mutant, but not in either single mutant. Just one gene, SMc02900, showed decreased expression in all three mutants. Forty-five of the 79 genes whose expression appeared *rpoH* dependent in stationary phase also demonstrated *rpoH1* dependence during heat shock (see above).

About half of *rpoH2*-dependent genes lack a predicted function. Genes with predicted functions include some plausibly involved in stress responses: *ecnA*, orthologous to an *E. coli* antitoxin gene whose expression in *E coli* is induced by osmotic stress in stationary phase (6); orthologs of *E. coli yagTSR*, a broad-spectrum, molybdopterin cytosine dinucleotide cofactor-containing aldehyde dehydrogenase involved in detoxification (44); *xseB*, predicted to encode a DNA repair enzyme; and SMa1158, which encodes a universal stress protein that is induced in *S. meliloti* upon exposure to low oxygen and nitric oxide (5, 36). Forty-three percent of our *rpoH2*-dependent genes were induced, and none repressed, during osmotic stress in a previous study (12), suggesting that RpoH2 is important for response to osmotic stress.

Since *rpoH2* expression is induced in stationary phase, we compared our list of *rpoH2* genes with those reported in two recent studies to be induced in stationary phase (either carbon limitation serving to trigger stationary-phase onset while cell densities remained low [OD_{600} of 0.25] [52] or in cells harvested at higher cell densities [OD_{600} of 1.2] [8]). About one-third of our *rpoH2*-dependent genes overlapped with the combined stationary-phase data but represented less than 1% of those data. This suggests that either few stationary-phase-induced genes are *rpoH2* dependent or that our experimental conditions failed to detect additional *rpoH2*-dependent genes.

Expression of several *S. meliloti* sRNAs may be RpoH dependent. Recent studies have identified and characterized potential small, noncoding RNAs (sRNAs) in *S. meliloti*, most of which are encoded in intergenic regions (IGR) (11, 53, 61, 62). Since our Affymetrix symbiosis chip can detect mRNA hybridization to IGR of ≥150 nt (3), we examined our data for IGR whose corresponding hybridization signal appeared *rpoH* dependent. The vast majority appear to represent 5′ and 3′ untranslated regions (UTR) of *rpoH*-dependent coding mRNAs. In addition, two IGR likely detect mRNAs containing small ORFs that were not annotated in the original genome sequencing effort (SMc05011 and SMc05020 [see Table S3 in the supplemental material]). We identified eight IGR (Table S3) unlikely to represent *rpoH*-dependent UTR on the basis of the expression pattern of the adjacent genes; half of these overlap with sRNAs previously identified in a genome-wide survey (53). Of these potential *rpoH*-dependent sRNAs, the *trans*-encoded sRNA SmelC781 is intriguing, because it is adjacent to, and divergently transcribed from, *rpoH2*: it overlaps the predicted RpoE2-dependent promoter of *rpoH2*. SmelC781 expression was previously found to be induced by heat, pH, salt, and oxidative stress (53). In that study, the SmelC781 promoter (GTTGAC-N_{15}-CCTAGAT) was characterized as RpoD-like (53), but we note its similarity to our RpoH promoter consensus sequences (see below). Another sRNA candidate, SmelC456, is located upstream of *dksA*, a gene known to be important for environmental adaptation (31, 55), and also has a promoter (ATTGAA-N_{16}-ACCA AAT) similar to our RpoH promoter consensus sequences. We mapped the transcription start sites of both SmelC781 and SmelC456 and confirmed that their expression was indeed *rpoH* dependent (Fig. S2). SmelB130 and SmelC487 may be indirectly RpoH regulated, as we did not identify a consensus match for these. Thus, our work identifies IGR with *rpoH*-dependent expression, some of which are candidates for small regulatory RNAs involved in stress responses, and which will be objects of future research.

RpoH-dependent promoters share conserved features. As a first step in determining direct transcriptional targets of RpoH1 and RpoH2, we selected *rpoH1*- and *rpoH2*-dependent genes for 5′ RACE mapping (Materials and Methods). We used RNA from heat-shocked wild-type, *rpoH1*, *rpoH2*, and *rpoH1 rpoH2* cells for 5′ RACE, which also served as a qualitative confirmation of our Affymetrix data. For positive controls, we used *groESL5*, *lon*, and

clpB, which have previously been shown to be dependent on *rpoH1* (40). As expected, RACE products for these control genes were less abundant in the *rpoH1* and *rpoH1 rpoH2* mutants, but not the *rpoH2* mutant (data not shown). Moreover, the *groESL5*, *lon*, and *clpB* transcription start sites inferred from our RACE mapping (Table 1) match those previously determined by primer extension (40). Expression of *groEL4* was previously reported to be *rpoH* independent (7, 40), but our Affymetrix results, which we confirmed by RACE mapping, show that its expression is dependent on *rpoH1* in heat shock and *rpoH2* in stationary phase and that it is transcribed from a promoter upstream of the adjacent gene, SMc01759, encoding a protein of unknown function (see Fig. S2A in the supplemental material). Expression of *dnaK* was also previously reported to be *rpoH* independent, although those data appear to demonstrate partial *rpoH1* dependence (Fig. 3d of reference 40). Our Affymetrix results showed a 2-fold decrease in *dnaK* expression in the *rpoH1* mutant and double mutants compared to the wild type. In our RACE mapping, we observed a single, intensely staining band corresponding to the *dnaK* transcript, but were unable to confirm its *rpoH1* dependence (Fig. S2A). We did not attempt to determine whether other sigma factors act at this promoter, but note that in *E. coli* RpoH and RpoD initiate transcription at the same *dnaK* promoter *in vitro* (63).

We searched for consensus motifs upstream of our putative mapped transcription start sites using MEME as described in Materials and Methods. To identify differences in RpoH1- versus RpoH2-dependent versus dual (RpoH1- and RpoH2-dependent) promoters of protein-coding genes, we constructed three different consensus sequences (Fig. 2). The RpoH1-specific consensus sequence (CTTGAA-N_{15-16}-CCTATAT) comprises promoters of 20 genes dependent only on *rpoH1* in heat shock (Table 1). The RpoH2-specific consensus sequence (CTTGCC-N_{15-16}-CCTATCT) comprises promoters of 11 genes dependent only on *rpoH2* during stationary-phase growth (Table 1). Finally, the dual-promoter consensus sequence (CTTGAA-N_{15-16}-CCTATCT) comprises 14 promoters dependent on *rpoH1* in heat shock and on *rpoH2* in stationary phase (Table 1). The remaining RACE-mapped promoters that did not belong to any of the aforementioned groups (16 genes) or that could not be fit to their respective consensus sequences (8 genes) are also listed in Table 1. The vast majority of RpoH-dependent promoters have spacing of 15 or 16 nt between the end of the −35 motif and the start of the −10 motif. The RpoH1 consensus promoters were biased toward 16-nt spacers (70% of promoters), whereas the RpoH2 consensus promoters favored 15-nt spacers (91% of promoters). Promoters responsive to both sigma factors showed a slight preference for 15-nt spacers (64% of promoters).

Our RpoH1 consensus sequence closely matches the consensus sequence previously determined from three *S. meliloti rpoH1*-dependent promoters (CTTGAA-N_{17}-CCANAT determined from the *groES5*, *lon*, and *clpB* promoters [40]). All three of our consensus sequences share some similarity to the *E. coli* RpoH-dependent promoter consensus (TTGAAA-N_{13-14}-CCCCATAT) identified by Nonaka et al. (45), with the RpoH1 consensus sequence showing the most similarity. Discriminator length (distance between the end of the −10 motif and the mapped start site) for most *S. meliloti rpoH*-dependent promoters was seven or eight nucleotides, as is the case in *E. coli* (45). We searched 30-nt windows upstream of the −35 motif of our mapped *S. meliloti* promoters for sequences similar to the A/T-rich "UP element" enhancer found upstream of ~40% of *E. coli* RpoH-dependent promoters (45). While about one-third of our *S. meliloti* promoters had A/T-rich stretches, we could not identify a consensus sequence among them (data not shown). Similarly, we failed to identify an extended −10 region such as that found in *E. coli* RpoH promoters. The spacing between the −35 and −10 motifs is identical between *E. coli* and *S. meliloti* once the different motif lengths are taken into account. Our RpoH-dependent promoter motifs closely match RpoH-dependent promoters from other alphaproteobacterial species, such as *Rhodobacter sphaeroides* (46), *Caulobacter crescentus* (34), *Agrobacterium tumefaciens* (41), and *Bradyrhizobium japonicum* (39).

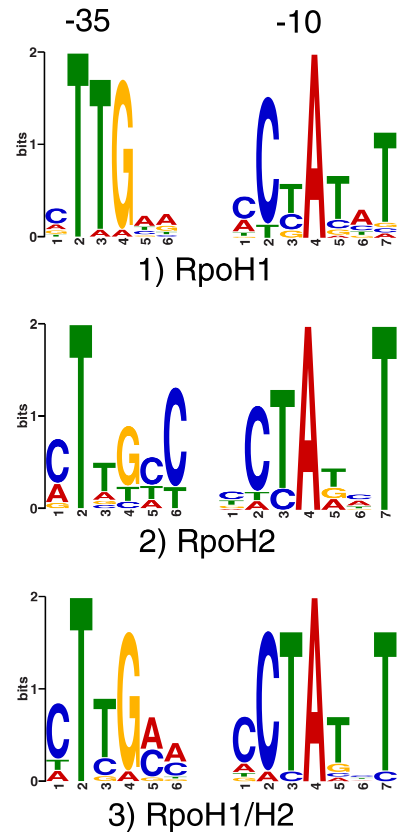

FIG 2 *S. meliloti rpoH*-dependent promoter consensus motifs. The sequence logos for three gene sets are shown. Gene set 1 shows RpoH1-specific promoter consensus motifs (20 genes). Gene set 2 shows RpoH2-specific promoter consensus motifs (11 genes). Gene set 3 shows dual-promoter (RpoH1 and RpoH2) consensus motifs (14 genes). Colored sequence logos for gene sets 1, 2, and 3 of Table 1 were generated using MEME as described in Materials and Methods. The total information content of each logo is given in bits; the height of each nucleotide in the logo represents the positional probability of that nucleotide multiplied by the information content of the logo. −35 consensus motifs are displayed on the left side of the figure, and −10 consensus motifs are displayed on the right side of the figure. Nonconserved spacer regions of 15 to 16 nt between the motifs are not shown.

As with *E. coli*, the *S. meliloti* RpoH and RpoD −35 and −10 consensus sequences are similar: the *S. meliloti* consensus sequence determined from 24 mapped putative RpoD-dependent promoters (32) is CTTGAC-N_{17-18}-CTATAT, although we note that the −10 motif of the mapped RpoD-dependent promoters is much more variable than that of our mapped RpoH-dependent promoters (data not shown). Taking into account the differences

in consensus motif lengths, an RpoD-dependent promoter with 17-nt spacing would have the same effective spacing as an RpoH-dependent promoter with 16-nt spacing. These similarities present challenges in predicting whether an *S. meliloti* promoter is RpoD or RpoH dependent; in fact, at least two promoters (*grpE* and *ibpA*) predicted to be RpoD dependent by MacLellan et al. (32) are probably RpoH dependent based on our array and 5′ RACE mapping data. Likewise, we found two candidate RpoH-dependent promoters in our genome-wide search (see below) that were previously predicted to be RpoD dependent (SMb20361 and SMc00043) (32).

To identify additional RpoH-dependent promoter candidates, we used each of the consensus sequences (Fig. 2) to search the *S. meliloti* genome as described in Materials and Methods. This analysis predicted 75 candidate promoters, representing 100 genes that showed *rpoH1*- and/or *rpoH2*-dependent decreases in gene expression (see Table S4 in the supplemental material). Consensus sequence matches did not strictly correlate with expression data. For example, of the 60 transcripts in Table S4 that were identified as *rpoH1* dependent during heat shock in our Affymetrix analysis, 46 were detected as significant using the RpoH1-specific promoter consensus as input; however, 29 of these were also detected with at least one of the other consensus sequences. This was not unexpected due to the similarity of the three consensus sequences (Fig. 2).

DISCUSSION

In this study, we used transcription profiling to assess the roles of dual RpoH sigma factors in *S. meliloti* under two different stress conditions: heat shock and late stationary-phase growth. We identified over 300 ORFs and putative sRNAs whose expression is *rpoH* dependent. We mapped the transcription start sites of ~20% of these genes by 5′ RACE and identified likely RpoH-dependent promoters. From these mapped promoters, we developed three different consensus sequences that we used to search the genome. On the basis of the results of this analysis, we predict that up to half of the *rpoH*-dependent genes are direct targets of RpoH1 and/or RpoH2.

In wild-type *S. meliloti*, ~20% of genes show increased expression in heat shock compared to normal temperature. However, expression of only a quarter of these genes is dependent on either RpoH1 (this study) or the general stress response sigma factor, RpoE2 (52). Thus, it is possible that other alternative sigma factors play a role in the *S. meliloti* heat shock response. In support of this hypothesis, we found that, in addition to *rpoE2*, expression of *rpoE5*, *rpoH2*, *rpoE9*, and *rpoN* increased during heat shock. Moreover, it was previously reported that *rpoE5* and *rpoH2* are targets of RpoE2 (52).

Beyond the use of sigma factors, previous research suggests that regulatory mechanisms such as CIRCE/HrcA and RNA thermometers are important in the alphaproteobacterial response to heat shock (42, 64). HrcA repressor binding to a *cis*-linked CIRCE element mediates negative regulation of bacterial heat shock genes (42). For instance, in *S. meliloti*, expression of *groESL1* and *groESL2* is not RpoH dependent but may be regulated by HrcA because upstream CIRCE elements are present (7). In *Bradyrhizobium japonicum*, *hrcA* expression itself is RpoH dependent (38). Although *S. meliloti hrcA* expression increased ~1.4-fold during heat shock, its expression was RpoH independent in our experiments (see Data Set S1 in the supplemental material). Posttranscriptional regulation of heat shock gene expression is mediated by elements such as RNA thermometers, possibly via high-temperature-induced unmasking of the Shine-Dalgarno sequence required for translation (64). A given gene may have multiple regulatory inputs; for example, two RpoH targets identified in our study, which encode small heat shock proteins (SMb21295 and *ibpA*), were previously predicted to possess upstream RNA thermometer elements (64). We infer that regulation of the *S. meliloti* heat shock response is complex.

The overall question of what are the roles of the two RpoHs in *S. meliloti* remains. All alphaproteobacterial genomes sequenced thus far contain at least one predicted *rpoH* gene; most contain two, and some contain more. Previous work on *S. meliloti* suggested largely distinct, yet partly overlapping roles for each RpoH (40, 47, 48). Transcription of *rpoH1* occurs constitutively during exponential-phase growth in rich medium and increases upon entry into stationary phase (47), but nothing is known about how RpoH1 activity is regulated. Transcription of *rpoH2* was reported to be entirely dependent on RpoE2, suggesting that RpoH2 acts in the general stress response circuit (52). In addition, we observed increased *rpoH2* expression in the *rpoH2* mutant and double mutants in both heat shock and stationary-phase growth. This raises the possibility that RpoH2 either directly or indirectly regulates its own expression. An important caveat is that the insertion mutation in the middle of the *rpoH2* gene might alter the stability of the resulting *rpoH2* transcript or its detection by our Affymetrix chip. Hybridization data corresponding to the region upstream of *rpoH2* shows that, while abundance of the putative *rpoH2* 5′ untranslated region increased in heat shock as expected, it was unchanged in the *rpoH2* mutant and double mutants. Thus, additional experiments are necessary to determine whether RpoH2 negatively regulates its own expression.

With respect to the heat shock response, we show that *rpoH1* appears to be the main player in the early transcriptional response to heat shock in rich medium, with no detectable contribution from *rpoH2*. On the other hand, with respect to late stationary-phase growth in minimal medium, *rpoH2* appears to play a larger role than *rpoH1*. To reconcile our findings that *rpoH2* expression increases during heat shock, yet *rpoH2* itself appears to play a minimal role in heat shock gene expression, we suggest that RpoH2 was not sufficiently active to initiate transcription at RpoH2-dependent promoters after 15 min of heat shock in rich medium. We speculate that higher levels of *rpoH2* transcripts and/or appropriate posttranscriptional regulation are required for full RpoH2 activity and that this serves to fine-tune the response depending on the particular stressor(s) encountered. Similarly, even though we found that *rpoH1* mRNA is abundant and that RpoH1 contributes to transcription during late stationary-phase growth, RpoH1 may not be active enough to affect the same magnitude of change in gene expression as it does during heat shock.

We note that the apparent differences between *rpoH1* and *rpoH2* contributions to gene expression in heat shock versus late stationary-phase growth may be due to other factors. For example, cells were grown in rich medium for the heat shock experiment and in minimal medium for the late stationary-phase growth experiment. Osmoprotective compounds such as glycine betaine and proline are more abundant in rich (LB) medium than in minimal medium (27, 68). Therefore, if RpoH2 responds more readily to osmolality than it does to heat, it could explain the minimal contribution of *rpoH2* to heat shock in rich medium. Indeed, we

find that >40% of *rpoH2*-dependent genes were previously shown to be induced during osmotic stress (12).

Our work points to an additional component in the *S. meliloti rpoH1* and *rpoH2* regulatory circuits: small, noncoding RNAs (sRNAs). We identified eight different intergenic regions with differential expression in the wild type versus one or more *rpoH* mutants (see Table S3 in the supplemental material). We used 5′ RACE mapping to confirm the transcription start sites and *rpoH* dependence for two of these sRNAs, SmelC781 and SmelC456 (Fig. S2). Both of these were classified as *trans*-encoded sRNAs in a genome-wide survey (53). Most *trans*-encoded sRNAs interact with their target mRNAs via short, imperfect base pairing and may control gene expression by a variety of mechanisms, including activation or repression of translation, degradation, or stabilization of mRNA and modification of protein activity (58). Because of the short, imperfect nature of sRNA-mRNA interactions, it is difficult to identify potential targets using bioinformatic approaches alone (58). On the basis of the *rpoH1*- and *rpoH2*-dependent expression of SmelC781 and its proximity to *rpoH2*, we speculate that SmelC781 affects expression of unknown targets involved in response to heat shock, stationary-phase growth, and other stresses. The SmelC781 transcript maps to nt 3939507 to 3539650 of the chromosome (53), overlapping the predicted RpoE2-binding site of the *rpoH2* promoter (52). Thus, even if SmelC781 does not affect posttranscriptional regulation of *rpoH2*, its transcription may interfere with that of *rpoH2*. The putative *trans*-encoded SmelC456 sRNA lies immediately upstream of *dksA*; therefore, it is tempting to speculate that SmelC456 might regulate DksA expression or function. In *E. coli*, the transcription factor DksA regulates transcription initiation in concert with the nucleotide (p)ppGpp, which is produced by the RelA synthetase during stress (49). Recent studies show that (p)ppGpp allows many alternative sigma factors, including *E. coli* RpoH, to compete more effectively for RNAP binding (49). In *S. meliloti*, *relA* is required for root nodule formation (66), and *dksA* appears to be required for most *relA*-dependent gene regulation (31). Interestingly, *rpoH1* expression showed a *relA*-dependent, *dksA*-independent decrease during nitrogen starvation (31), a mechanism that could enhance RNAP binding to other stress response sigma factors, such as RpoE2. The role, if any, of Smel456 in the *S. meliloti relA*, *dksA*, and *rpoH* regulatory circuits remains to be elucidated.

In other alphaproteobacteria possessing dual *rpoH* genes, there is a division of labor. Notably, in the closely related species, *Rhizobium etli*, the *rpoH1* and *rpoH2* orthologs contributed to the oxidative stress response, while *rpoH2* appeared to be the main player in the response to osmotic stress (33). *R. etli rpoH1* mutants also have symbiotic defects similar to those of *S. meliloti rpoH1* mutants (33). In *Rhodobacter sphaeroides*, *rpoH1* is the main σ factor for responding to heat shock and *rpoH2* controls the response to singlet oxygen stress (22, 46). In the intracellular pathogen *Brucella melitensis*, *rpoH2* (orthologous to *S. meliloti rpoH1*) is important for response to heat shock, cold shock, and hydrogen peroxide, expression of virulence factors, and invasion and survival in mammalian cells, while *rpoH1* may be important for full virulence (9). It appears that for *S. meliloti* and other alphaproteobacteria, multiple *rpoH* genes provide flexibility in adapting to diverse environments.

We are especially interested in understanding why *S. meliloti rpoH* mutants are symbiotically impaired. de Lucena et al. suggested that decreased resistance to acid pH or oxidative stress might explain the nonfixing phenotype of *rpoH1* mutants (10). Adding support to this hypothesis is our prediction that at least 5% of *rpoH1*-dependent genes are involved in response to oxidative stress. Direct studies of RpoH function during symbiosis are difficult given that *rpoH1 rpoH2* double mutants do not form nodules and *rpoH1* mutants senesce soon after their release into plant cells (7, 40). Comparative studies can provide some insights: a comparison of *rpoH*-dependent genes from this work and de Lucena et al. (10) to other published data (3, 5, 8) reveals that 14% of *rpoH*-dependent genes are induced in nodule bacteria. Most of these genes lack a predicted function, but others are predicted to encode transcriptional regulators, small heat shock proteins, glutaredoxin, an outer membrane protein, and proteins involved in DNA repair, protein turnover, stress response, and small molecule metabolism. It is therefore possible that the primary function of RpoH1 and RpoH2 during symbiosis is to transcribe stress response genes either to repair cellular damage or to produce new proteins during symbiosis. However, the RpoH sigma factors may also be required for symbiosis-specific functions. While the RpoH regulons in several pathogens overlap with the *E. coli* RpoH regulon, they also include targets not found in *E. coli*, some of which might be involved in pathogenesis (25, 54). Here we have identified many RpoH-dependent genes that do not have obvious *E. coli* homologs; these genes could play a role in symbiosis. Targeted study of individual *rpoH*-dependent, nodule-induced genes may help elucidate the role of RpoH in the *S. meliloti* symbiosis and be generalizable to other alphaproteobacteria that invade eukaryotic hosts.

The ability to recognize a broader range of promoters may be an important feature of *S. meliloti* RpoH1 and RpoH2. Our RpoH promoter consensus sequences are similar to RpoH promoters previously identified in *S. meliloti* and other alphaproteobacteria (34, 39–41, 46) and to the −35 and −10 core hexamer motifs of the *E. coli* RpoH promoter consensus sequence (45). However, we found that *S. meliloti* RpoH promoters lack the two upstream (CC) nucleotides that make up the extended −10 motif of *E. coli* RpoH promoters (45). This is an expected finding given that, like all known alphaproteobacterial RpoH proteins, *S. meliloti* RpoH lacks the conserved K130 residue that recognizes the extended −10 region in *E. coli* (29). Further, it was demonstrated that RpoH from the alphaproteobacterium *Caulobacter crescentus* recognizes *E. coli* promoters that lack the extended −10 motif (29). It has been postulated that by increasing promoter stringency, the extended −10 motif allows for an efficient response to a restricted set of promoters and that lack of an extended motif may allow alphaproteobacterial RpoH to transcribe larger sets of genes involved in a variety of processes (30). This premise is supported by previous work showing that, in addition to heat shock, *S. meliloti rpoH1* is required for response to acid shock and survival in root nodules (10, 40, 47) and by our current study defining a large set of *rpoH*-dependent genes.

In *E. coli*, A/T-rich UP elements located upstream of the −35 motif interact with the α subunits of RNAP to increase promoter strength (29, 50). Although about one-third of our mapped RpoH-dependent promoters contained A/T-rich regions upstream of the −35 motif, we could not define a consensus sequence among them using MEME (data not shown). However, recent work modeling full-length *E. coli* RpoE promoters demonstrated that UP elements do not show position-specific sequence conservation; instead, the number of overlapping 3-nt A and T

tracts in the region from −64 to −35 is the best predictor of UP element strength (50). Several of our mapped promoters contain overlapping A and T tracts; however, functional tests are necessary to determine whether the *E. coli* UP element model is applicable to *S. meliloti* RpoH promoters.

That RpoH1 and RpoH2 have partly overlapping, yet distinct roles is supported by our consensus promoter analyses. The consensus sequences for RpoH1, RpoH2, and dual (RpoH1 and RpoH2) promoters share similar core motif features, −35 TTG and −10 CTANNT (Fig. 2). However, we found that the TTG consensus motif was more strongly conserved in RpoH1 promoters than in RpoH2 promoters or dual promoters (Fig. 2). In addition, unlike the RpoH1 and dual-promoter consensus sequences, the −35 motif of the RpoH2 consensus sequence is biased toward terminal C nucleotides (CTTGAA versus CTTGCC). This is similar to the situation in *Rhodobacter sphaeroides*, where a consensus sequence of seven −35 motifs specific for RpoH2 (CTTGCC) was distinguishable from a consensus sequence of eight −35 motifs (CTTGAN) recognized by both RpoH1 and RpoH2 (46). The −10 motif also shows differences among our three consensus sequences, with the third position T being more conserved in RpoH2 and dual promoters than in RpoH1 promoters (Fig. 2). Perhaps the most striking difference between the three sets of *S. meliloti* promoters is their spacer length distributions. Most RpoH1-specific promoters have 16-nt spacers (70% of total), whereas most RpoH2-specific promoters have 15-nt spacers (91% of total). Spacer length of dual promoters is more evenly distributed (15 nt, 64%; 16 nt, 29%). If this trend is generalizable to the full regulon of *S. meliloti* RpoH target genes, it could have large effects on promoter selectivity. For example, in *E. coli*, the core −35 and −10 motifs of RpoD and RpoS promoters are extremely similar, yet the corresponding RpoD and RpoS RNAP holoenzymes have distinct regulons and roles *in vivo* (60). In this instance, a major factor in RpoD versus RpoS promoter selectivity is spacer length: RpoD holoenzyme strongly prefers promoters with 17-nt spacing, while RpoS recognizes promoters that deviate 1 or 2 nucleotides from this spacing length (60). Thus, our work suggests that differences in core promoter motifs and spacer lengths play a role in RpoH1 and RpoH2 promoter selectivity.

In sum, it is likely that for species with genes encoding multiple RpoHs, promoter selectivity is controlled in various ways from expression of the *rpoH* genes themselves to RpoH selectivity for specific promoters. Our work here provides a foundation for further study of RpoH-RNAP-promoter interactions, regulation of *rpoH* expression, control of RpoH activity, and characterization of RpoH target genes, some of which may be important for symbiosis.

ACKNOWLEDGMENTS

M.J.B. was supported by the Hoover Circle fund and National Institutes of Health grant GM093628 to S.R.L. A.N.B. was supported by an Andrew Mellon Predoctoral Fellowship from the University of Pittsburgh and NRI Competitive Grants Program/USDA award 2001-35319-10902 to V.O.

We thank Robert Fisher and Claus Lang for critically reading the manuscript, are grateful to Jamil Afza and Glen Barnett for assistance with data extraction and software, and appreciate the constructive suggestions of the anonymous reviewers.

REFERENCES

1. **Bailey TL, Williams N, Misleh C, Li WW.** 2006. MEME: discovering and analyzing DNA and protein sequence motifs. Nucleic Acids Res. **34**: W369–W373.
2. **Barnett MJ, Fisher RF.** 2006. Global gene expression in the rhizobial-legume symbiosis. Symbiosis **42**:1–24.
3. **Barnett MJ, Toman CJ, Fisher RF, Long SR.** 2004. A dual-genome Symbiosis Chip for coordinate study of signal exchange and development in a prokaryote-host interaction. Proc. Natl. Acad. Sci. U. S. A. **101**: 16636–16641.
4. **Bastiat B, Sauviac L, Bruand C.** 2010. Dual control of *Sinorhizobium meliloti* RpoE2 sigma factor activity by two PhyR-type two-component response regulators. J. Bacteriol. **192**:2255–2265.
5. **Becker A, et al.** 2004. Global changes in gene expression in *Sinorhizobium meliloti* 1021 under microoxic and symbiotic conditions. Mol. Plant Microbe Interact. **17**:292–303.
6. **Bishop RE, Leskiw BK, Hodges RS, Kay CM, Weiner JH.** 1998. The entericidin locus of *Escherichia coli* and its implications for programmed bacterial cell death. J. Mol. Biol. **280**:583–596.
7. **Bittner AN, Oke V.** 2006. Multiple *groESL* operons are not key targets of RpoH1 and RpoH2 in *Sinorhizobium meliloti*. J. Bacteriol. **188**:3507–3515.
8. **Capela D, Filipe C, Bobik C, Batut J, Bruand C.** 2006. *Sinorhizobium meliloti* differentiation during symbiosis with alfalfa: a transcriptomic dissection. Mol. Plant Microbe Interact. **19**:363–372.
9. **Delory M, Hallez R, Letesson JJ, De Bolle X.** 2006. An RpoH-like heat shock sigma factor is involved in stress response and virulence in *Brucella melitensis* 16M. J. Bacteriol. **188**:7707–7710.
10. **de Lucena DK, Pühler A, Weidner S.** 2010. The role of sigma factor RpoH1 in the pH stress response of *Sinorhizobium meliloti*. BMC Microbiol. **10**:265. doi:10.1186/1471-2180-10-265.
11. **del Val C, Rivas E, Torres-Quesada O, Toro N, Jiménez-Zurdo JI.** 2007. Identification of differentially expressed small non-coding RNAs in the legume endosymbiont *Sinorhizobium meliloti* by comparative genomics. Mol. Microbiol. **66**:1080–1091.
12. **Dóminguez-Ferreras A, et al.** 2006. Transcriptome profiling reveals the importance of plasmid pSymB for osmoadaptation of *Sinorhizobium meliloti*. J. Bacteriol. **188**:7617–7625.
13. **Downie JA.** 2010. The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. FEMS Microbiol. Rev. **34**:150–170.
14. **Edgar R, Domrachev M, Lash AE.** 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. **30**:207–210.
15. **Fischer HM.** 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58**:352–386.
16. **Fisher RF, Long SR.** 1992. Rhizobium-plant signal exchange. Nature **357**:655–660.
17. **Flechard M, et al.** 2010. RpoE2 of *Sinorhizobium meliloti* is necessary for trehalose synthesis and growth in hyperosmotic media. Microbiology **156**: 1708–1718.
18. **Flechard M, Fontenelle C, Trautwetter A, Ermel G, Blanco C.** 2009. *Sinorhizobium meliloti rpoE2* is necessary for H_2O_2 stress resistance during the stationary growth phase. FEMS Microbiol. Lett. **290**:25–31.
19. **Galibert F, et al.** 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science **293**:668–672.
20. **Gibson KE, Kobayashi H, Walker GC.** 2008. Molecular determinants of a symbiotic chronic infection. Annu. Rev. Genet. **42**:413–441.
21. **Glazebrook J, Walker GC.** 1991. Genetic techniques in *Rhizobium meliloti*. Methods Enzymol. **204**:398–418.
22. **Green HA, Donohue TJ.** 2006. Activity of *Rhodobacter sphaeroides* $RpoH_{II}$, a second member of the heat shock sigma factor family. J. Bacteriol. **188**:5712–5721.
23. **Gruber TM, Gross CA.** 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. Annu. Rev. Microbiol. **57**:441–466.
24. **Guisbert E, Yura T, Rhodius VA, Gross CA.** 2008. Convergence of molecular, modeling, and systems approaches for an understanding of the *Escherichia coli* heat shock response. Microbiol. Mol. Biol. Rev. **72**:545–554.
25. **Gunesekere IC, et al.** 2006. Comparison of the RpoH-dependent regulon and general stress response in *Neisseria gonorrhoeae*. J. Bacteriol. **188**: 4769–4776.
26. **Hellweg C, Pühler A, Weidner S.** 2009. The time course of the transcrip-

tomic response of *Sinorhizobium meliloti* 1021 following a shift to acidic pH. BMC Microbiol. **9**:37. doi:10.1186/1471-2180-9-37.
27. **Hengge-Aronis R, Klein W, Lange R, Rimmele M, Boos W.** 1991. Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary-phase thermotolerance in *Escherichia coli.* J. Bacteriol. **173**:7918–7924.
28. **Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC.** 2007. How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. Nat. Rev. Microbiol. **5**:619–633.
29. **Koo BM, Rhodius VA, Campbell EA, Gross CA.** 2009. Dissection of recognition determinants of *Escherichia coli* σ^{32} suggests a composite −10 region with an 'extended −10′ motif and a core −10 element. Mol. Microbiol. **72**:815–829.
30. **Koo BM, Rhodius VA, Nonaka G, deHaseth PL, Gross CA.** 2009. Reduced capacity of alternative σs to melt promoters ensures stringent promoter recognition. Genes Dev. **23**:2426–2436.
31. **Krol E, Becker A.** 2011. ppGpp in *Sinorhizobium meliloti*: biosynthesis in response to sudden nutritional downshifts and modulation of the transcriptome. Mol. Microbiol. **81**:1233–1254.
32. **MacLellan SR, MacLean AM, Finan TM.** 2006. Promoter prediction in the rhizobia. Microbiology **152**:1751–1763.
33. **Martínez-Salazar JM, et al.** 2009. The *Rhizobium etli* RpoH1 and RpoH2 sigma factors are involved in different stress responses. Microbiology **155**: 386–397.
34. **McGrath PT, et al.** 2007. High-throughput identification of transcription start sites, conserved promoter motifs and predicted regulons. Nat. Biotechnol. **25**:584–592.
35. **Meade HM, Long SR, Ruvkun GB, Brown SE, Ausubel FM.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149**:114–122.
36. **Meilhoc E, Cam Y, Skapski A, Bruand C.** 2010. The response to nitric oxide of the nitrogen-fixing symbiont *Sinorhizobium meliloti.* Mol. Plant Microbe Interact. **23**:748–759.
37. **Mergaert P, et al.** 2006. Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*-legume symbiosis. Proc. Natl. Acad. Sci. U. S. A. **103**:5230–5235.
38. **Minder AC, Fischer HM, Hennecke H, Narberhaus F.** 2000. Role of HrcA and CIRCE in the heat shock regulatory network of *Bradyrhizobium japonicum.* J. Bacteriol. **182**:14–22.
39. **Minder AC, Narberhaus F, Babst M, Hennecke H, Fischer HM.** 1997. The *dnaKJ* operon belongs to the σ^{32}-dependent class of heat shock genes in *Bradyrhizobium japonicum.* Mol. Gen. Genet. **254**:195–206.
40. **Mitsui H, Sato T, Sato Y, Ito N, Minamisawa K.** 2004. *Sinorhizobium meliloti* RpoH1 is required for effective nitrogen-fixing symbiosis with alfalfa. Mol. Genet. Genomics **271**:416–425.
41. **Nakahigashi K, Ron EZ, Yanagi H, Yura T.** 1999. Differential and independent roles of a σ^{32} homolog (RpoH) and an HrcA repressor in the heat shock response of *Agrobacterium tumefaciens.* J. Bacteriol. **181**:7509–7515.
42. **Narberhaus F.** 1999. Negative regulation of bacterial heat shock genes. Mol. Microbiol. **31**:1–8.
43. **Narberhaus F, Krummenacher P, Fischer HM, Hennecke H.** 1997. Three disparately regulated genes for σ^{32}-like transcription factors in *Bradyrhizobium japonicum.* Mol. Microbiol. **24**:93–104.
44. **Neumann M, et al.** 2009. A periplasmic aldehyde oxidoreductase represents the first molybdopterin cytosine dinucleotide cofactor containing molybdo-flavoenzyme from *Escherichia coli.* FEBS J. **276**:2762–2774.
45. **Nonaka G, Blankschien M, Herman C, Gross CA, Rhodius VA.** 2006. Regulon and promoter analysis of the *E. coli* heat-shock factor, σ^{32}, reveals a multifaceted cellular response to heat stress. Genes Dev. **20**:1776–1789.
46. **Nuss AM, Glaeser J, Berghoff BA, Klug G.** 2010. Overlapping alternative sigma factor regulons in the response to singlet oxygen in *Rhodobacter sphaeroides.* J. Bacteriol. **192**:2613–2623.
47. **Oke V, Rushing BG, Fisher EJ, Moghadam-Tabrizi M, Long SR.** 2001. Identification of the heat-shock sigma factor RpoH and a second RpoH-like protein in *Sinorhizobium meliloti.* Microbiology **147**:2399–2408.
48. **Ono Y, Mitsui H, Sato T, Minamisawa K.** 2001. Two RpoH homologs responsible for the expression of heat shock protein genes in *Sinorhizobium meliloti.* Mol. Gen. Genet. **264**:902–912.
49. **Österberg S, Del Peso-Santos T, Shingler V.** 2011. Regulation of alternative sigma factor use. Annu. Rev. Microbiol. **65**:37–55.
50. **Rhodius VA, Mutalik VK, Gross CA.** 2012. Predicting the strength of UP-elements and full-length *E. coli* σ^E promoters. Nucleic Acids Res. **40**: 2907–2924.
51. **Rhodius VA, Suh WC, Nonaka G, West J, Gross CA.** 2006. Conserved and variable functions of the σ^E stress response in related genomes. PLoS Biol. **4**:e2. doi:10.1371/journal.pbio.0040002.
52. **Sauviac L, Philippe H, Phok K, Bruand C.** 2007. An extracytoplasmic function sigma factor acts as a general stress response regulator in *Sinorhizobium meliloti.* J. Bacteriol. **189**:4204–4216.
53. **Schlüter JP, et al.** 2010. A genome-wide survey of sRNAs in the symbiotic nitrogen-fixing alpha-proteobacterium *Sinorhizobium meliloti.* BMC Genomics **11**:245. doi:10.1186/1471-2164-11-245.
54. **Slamti L, Livny J, Waldor MK.** 2007. Global gene expression and phenotypic analysis of a *Vibrio cholerae rpoH* deletion mutant. J. Bacteriol. **189**:351–362.
55. **Srivatsan A, Wang JD.** 2008. Control of bacterial transcription, translation and replication by (p)ppGpp. Curr. Opin. Microbiol. **11**:100–105.
56. **Staroń A, et al.** 2009. The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family. Mol. Microbiol. **74**:557–581.
57. **Storz G, Hengge R (ed).** 2011. Bacterial stress responses, 2nd ed. ASM Press, Washington, DC.
58. **Storz G, Vogel J, Wassarman KM.** 2011. Regulation by small RNAs in bacteria: expanding frontiers. Mol. Cell **43**:880–891.
59. **Turatsinze JV, Thomas-Chollier M, Defrance M, van Helden J.** 2008. Using RSAT to scan genome sequences for transcription factor binding sites and cis-regulatory modules. Nat. Protoc. **3**:1578–1588.
60. **Typas A, Becker G, Hengge R.** 2007. The molecular basis of selective promoter activation by the σ^S subunit of RNA polymerase. Mol. Microbiol. **63**:1296–1306.
61. **Ulvé VM, Sevin EW, Cheron A, Barloy-Hubler F.** 2007. Identification of chromosomal alpha-proteobacterial small RNAs by comparative genome analysis and detection in *Sinorhizobium meliloti* strain 1021. BMC Genomics **8**:467. doi:10.1186/1471-2164-8-467.
62. **Valverde C, et al.** 2008. Prediction of *Sinorhizobium meliloti* sRNA genes and experimental detection in strain 2011. BMC Genomics **9**:416. doi: 10.1186/1471-2164-9-416.
63. **Wade JT, et al.** 2006. Extensive functional overlap between sigma factors in *Escherichia coli.* Nat. Struct. Mol. Biol. **13**:806–814.
64. **Waldminghaus T, Fippinger A, Alfsmann J, Narberhaus F.** 2005. RNA thermometers are common in α- and γ-proteobacteria. Biol. Chem. **386**: 1279–1286.
65. **Waldminghaus T, Skarstad K.** 2010. ChIP on Chip: surprising results are often artifacts. BMC Genomics **11**:414. doi:10.1186/1471-2164-11-414.
66. **Wells DH, Long SR.** 2002. The *Sinorhizobium meliloti* stringent response affects multiple aspects of symbiosis. Mol. Microbiol. **43**:1115–1127.
67. **White J, Prell J, James EK, Poole P.** 2007. Nutrient sharing between symbionts. Plant Physiol. **144**:604–614.
68. **Wood JM.** 2007. Bacterial osmosensing transporters. Methods Enzymol. **428**:77–107.
69. **Zhao K, Liu M, Burgess RR.** 2005. The global transcriptional response of *Escherichia coli* to induced σ^{32} protein involves σ^{32} regulon activation followed by inactivation and degradation of σ^{32} *in vivo.* J. Biol. Chem. **280**:17758–17768.