Dual Control of *Sinorhizobium meliloti* RpoE2 Sigma Factor Activity by Two PhyR-Type Two-Component Response Regulators[▽][†]

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RpoE2 is an extracytoplasmic function (ECF) sigma factor involved in the general stress response of *Sinorhizobium meliloti*, the nitrogen-fixing symbiont of the legume plant alfalfa. RpoE2 orthologues are widely found among alphaproteobacteria, where they play various roles in stress resistance and/or host colonization. In this paper, we report a genetic and biochemical investigation of the mechanisms of signal transduction leading to *S. meliloti* RpoE2 activation in response to stress. We showed that RpoE2 activity is negatively controlled by two paralogous anti-sigma factors, RsiA1 (SMc01505) and RsiA2 (SMc04884), and that RpoE2 activation by stress requires two redundant paralogous PhyR-type response regulators, RsiB1 (SMc01504) and RsiB2 (SMc00794). RsiB1 and RsiB2 do not act at the level of *rpoE2* transcription but instead interact with the anti-sigma factors, and we therefore propose that they act as anti-anti-sigma factors to relieve RpoE2 inhibition in response to stress. This model closely resembles a recently proposed model of activation of RpoE2-like sigma factors in *Methylobacterium extorquens* and *Bradyrhizobium japonicum*, but the existence of two pairs of anti- and anti-anti-sigma factors in *S. meliloti* adds an unexpected level of complexity, which may allow the regulatory system to integrate multiple stimuli.

The capacity to sense and respond to environmental changes is essential for every living organism. In bacteria, a part of these responses occurs through modulation of initiation of gene transcription by changing the sigma factor associated with the core RNA polymerase. Sigma factors are dissociable subunits which provide the specificity of promoter recognition to RNA polymerase. Association of different sigma factors with the core enzyme makes it possible for the holoenzyme to recognize different promoters and express different sets of target genes. Sigma factors thus provide efficient mechanisms for simultaneous regulation of large numbers of genes (18). The so-called sigma 70 family of sigma factors includes primary sigma factors, which direct the transcription of housekeeping genes, as well as related alternative sigma factors which associate with the core RNA polymerase under various conditions, including stresses. The most abundant class of such alternative sigma factors is composed of structurally related proteins called *extra-cytoplasmic function* (ECF) sigma factors, as many of them control functions associated with various aspects of the cell surface or transport (20, 35).

How stress stimuli are sensed and transduced to ECF sigma factors has been the subject of numerous studies. It appears that most ECF sigma factors share the important property of specifically interacting with a protein called anti-sigma factor, which plays a pivotal role in the control of sigma factor activity. In the absence of stimulus, the ECF sigma factor is kept inactive by interaction with its cognate anti-sigma factor. In the presence of stimulus, the anti-sigma factor gets inactivated, either via a mechanism involving successive steps of proteolysis or through conformational changes of the protein (for reviews, see references 2, 20, and 35).

Sinorhizobium meliloti is a Gram-negative bacterium belonging to the alpha subclass of proteobacteria. This bacterium lives in the soil and can establish a symbiotic association with legume plants of the Medicago genera, including the cultivated lucerne alfalfa (*Medicago sativa*) and the model legume *Medicago truncatula* (for a recent review, see reference 25). Both in soil and *in planta*, *S. meliloti* is exposed to a variety of environmental stimuli, including numerous stresses (33). Interestingly, the *S. meliloti* genome encodes 11 ECF sigma factors which, among other regulatory systems, could provide effective ways for responding to these various stimuli (13, 35). Accordingly, we have shown that one of these sigma factors, RpoE2, is activated under various conditions, including heat shock, salt stress, and entry into stationary phase following nitrogen or carbon starvation (33), and is probably activated *in planta* as well (3; unpublished data). RpoE2 controls the transcription of a large regulon comprising at least 45 target genes, some of which encode stress-related functions, like *katC*, *rpoH2*, and *sodC* (10, 33). *S. meliloti rpoE2* mutants were recently described as more sensitive than the wild-type strain to desiccation (23) as well as to high H_2O_2 concentrations in stationary phase (10), confirming the involvement of RpoE2 in stress responses. Interestingly, this sigma factor is largely conserved among alphaproteobacteria (33), and RpoE2 orthologues have been involved in stress resistance and/or host colonization in several species, including *Brucella melitensis* (7), *Caulobacter crescentus* (1), and more recently *Rhizobium etli* (28) and *Bradyrhizobium japonicum* (17). RpoE2-like

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A

B

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GCTTCGGAACATTTCATTTTTCCGAGGCGTTATGGTGCAGGCG
     -35                        -10        +1
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AACTTTCAAGGGCGAAATTGCAACAATGAGATATACGACAGGG
                         M  R  Y  T  T  G
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GCAGGGCGGATGATCGGTAAAAGTCCGTCCTCCGACGATCCG ...
A  G  R  M  I  G  K  S  P  S  S  D  D  P  ...
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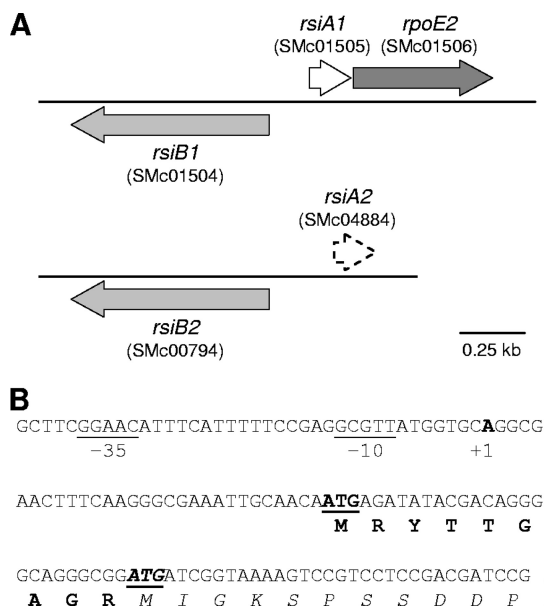
FIG. 1. (A) Schematic representation of the *S. meliloti* chromosomal regions analyzed in this study. Arrows represent open reading frames, and the dotted arrow indicates the previously unannotated *rsiA2* (SMc04884; chromosome coordinates 815835 to 816023). (B) DNA sequence of the 5' region of *rsiA1* (SMc01505), showing the previously annotated start codon (ATG in bold italic letters) and the first amino acids of the protein, as well as the 9-amino-acid N-terminal extension predicted from the present study (roman capital letters, with the new ATG start codon in bold). The position of the transcription start site mapped in the present study by 5'-RACE (see Materials and Methods) is indicated (+1), with the putative −35 and −10 promoter sequences recognized by RpoE2 underlined (33).

sigma factors can therefore be viewed as the long-searched regulators of the general stress response in alphaproteobacteria (33).

S. meliloti rpoE2 is transcribed as an operon with SMc01505 and divergently from SMc01504, which encodes a member of a novel family of atypical response regulators of two-component regulatory systems specifically found in alphaproteobacteria, exemplified by PhyR in *Methylobacterium extorquens* (Fig. 1) (14–16). Strikingly, this organization is well conserved among alphaproteobacteria, and regions encoding RpoE2 orthologues display a remarkable synteny (15, 33). This suggested to us a possible interplay between the corresponding proteins. Several additional observations indicated that SMc01505 and SMc01504 could play a role in RpoE2 regulation. First, we showed that SMc01505 is able to negatively regulate the activity of RpoE2, and we therefore proposed that this protein acts as an anti-sigma factor (33). However, the small size of the protein, the absence of transmembrane domains (a hallmark of a majority of anti-ECF sigma factors) (35), and the lack of similarity with known anti-sigma factors suggested that it could need unknown protein partners to both sense the stimuli and transduce the signals. Second, J. Vorholt's laboratory has reported the importance of PhyR, the *M. extorquens* SMc01504 orthologue, for stress response and phyllosphere colonization in this bacterium (16). Interestingly, 45% of the genes positively regulated by PhyR possess in their promoters the −10 and −35 boxes recognized by RpoE2 in *S. meliloti* (15, 33).

This suggested that PhyR could participate in the positive regulation of an unknown RpoE2-like ECF sigma factor in *M. extorquens*. This hypothesis was supported by the observation that the gene located next to *phyR* (called *nepR*) encodes a homologue of SMc01505, although no sigma factor-encoding gene was found in the neighborhood of these genes (15). We therefore investigated the role played by SMc01505 and SMc01504 in the mechanisms of signal transduction leading to RpoE2 activation in response to stress in *S. meliloti*. During the course of our work, Vorholt and colleagues reported that PhyR and NepR control the activity of the closest *M. extorquens* RpoE2 orthologue, called σ^{EcfG} (11). They proposed a partner-switching model in which PhyR, once phosphorylated in response to stress by a yet-unknown histidine kinase, becomes active as anti-anti-sigma and thus relieves inhibition of σ^{EcfG} by the NepR anti-sigma. In a subsequent study, Gourion and colleagues suggested a similar regulation for the *B. japonicum* RpoE2 orthologue (17).

Here we present the results of our parallel investigations on the mechanisms of RpoE2 regulation in *S. meliloti*. We first showed that SMc01505 interacts with RpoE2, confirming that it is an anti-sigma factor, which we renamed RsiA1. We also observed that RpoE2 activity is positively regulated by SMc01504, which we renamed RsiB1, and showed that this protein behaves as a response regulator, which in its phosphorylated form interacts with the anti-sigma factor RsiA1. These data therefore suggest that RsiB1 acts as an anti-anti-sigma factor in response to stress, a model in line with Vorholt's work. But, strikingly, we found that RpoE2 is also regulated by RsiA2 and RsiB2, two paralogues of RsiA1 and RsiB1 with similar activities. The finding that RpoE2 activity can be controlled by two pairs of anti- and anti-anti-sigma factors suggests an unanticipated complexity of its regulation mechanism in *S. meliloti*.

MATERIALS AND METHODS

Strains and growth conditions. The strains and plasmids used in this study are listed in Table 1 (also see Table S1 in the supplemental material). *Escherichia coli* strains were propagated in Luria-Bertani (LB) medium. *S. meliloti* strains were constructed and propagated in LB medium supplemented with 2.5 mM CaCl$_2$ and 2.5 mM MgSO$_4$ (LBMC medium) or TY medium (5 g · liter^{-1} tryptone, 3 g · liter^{-1} yeast extract) supplemented with 6 mM CaCl$_2$. For stress response assays, *S. meliloti* strains were grown in Vincent minimal medium (VMM) (7.35 mM KH$_2$PO$_4$, 5.74 mM K$_2$HPO$_4$, 1 mM MgSO$_4$, 18.7 mM NH$_4$Cl, 10 mM Na$_2$ succinate, 456 μM CaCl$_2$, 35 μM FeCl$_3$, 4 μM biotine, 48.5 μM H$_3$BO$_3$, 10 μM MnSO$_4$, 1 μM ZnSO$_4$, 0.5 μM CuSO$_4$, 0.27 μM CoCl$_2$, 0.5 μM NaMoO$_4$; pH = 7) at 28°C or 40°C, as indicated. Antibiotics, when required, were added at the following concentrations: streptomycin (Sm), 100 to 300 μg · ml^{-1}; tetracycline (Tc), 5 to 10 μg · ml^{-1}; gentamicin (Gm), 40 μg · ml^{-1}; hygromycin (Hyg), 40 μg · ml^{-1}; trimethoprim (Tmp), 12.5 to 25 μg · ml^{-1} (*S. meliloti*) or 50 μg · ml^{-1} (*E. coli*); ampicillin (Amp), 100 μg · ml^{-1}; carbenicillin (Car), 50 μg · ml^{-1}; and kanamycin (Kan), 50 μg · ml^{-1}.

Saccharomyces cerevisiae strains were grown at 28°C either in yeast extract-peptone-dextrose (YPD) rich medium or in synthetic defined (SD) minimal medium (with 2% glucose as a carbon source) and were transformed as described by the manufacturer (yeast protocols handbook, Clontech Laboratories, Mountain View, CA). Generally, cotransformations of plasmid pairs (~500 ng each) were performed, and cotransformants were selected on solid SD medium lacking tryptophan and leucine. For two-hybrid interaction assays, growth of two to six independent cotransformants were tested on SD medium lacking tryptophan, leucine, and histidine and with or without adenine, as shown in Table 2. Plasmids pGBKT7-53, pGADT7-T, and pGBKT7-Lam were used as controls in each experiment according to the manufacturer's recommendations (Clontech Laboratories).

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Sinorhizobium meliloti strains		
Rm1021	Wild-type strain (Sm^r)	29
CBT208	Rm1021 *rpoE2::hph* (Hyg^r)	33
CBT390	Rm1021 Δ*rsiB1* (*smc01504*)	This work
CBT392	Rm1021 Δ*rsiB2* (*smc00794*)	This work
CBT430	Rm1021 Δ*rsiB1* (*smc01504*) Δ*rsiB2* (*smc00794*)	This work
CBT557	Rm1021 Δ*rsiA2* (*smc04884*)	This work
Escherichia coli DH5α	*supE44* Δ*lacU169* (φ80d*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
Saccharomyces cerevisiae AH109	*MAT**a** trp1-901 leu2-3 leu112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3 GAL2_{UAS}-GAL2_{TATA}-ADE2 URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ*	Clontech Laboratories
Plasmids		
pMLBAD	Expression vector, inducible by arabinose (Tmp^r)	26
pSCRhaB2	Expression vector, inducible by rhamnose (Tmp^r)	4
pMP220	Promoter probe vector, IncP1 (Tet^r)	34
pMP220-885	pMP220-P_{SMc00885}-*lacZ* (Tet^r)	A.-M. Garnerone
pRK2013	Helper plasmid for triparental matings (Kan^r)	9
pJQ200mp19	Gene replacement vector (Gm^r)	32
pLS6.32	pCZ750 P_{rsiA1-rpoE2}-*lacZ* (Tet^r)	33
pGEM-T	Cloning vector (Amp^r)	Promega
pGBKT7	Yeast Gal4 DNA BD fusion vector (Kan^r)	Clontech Laboratories
pGADT7	Yeast Gal4 AD fusion vector (Amp^r)	Clontech Laboratories
pGBKT7-53	Matchmaker two-hybrid system 3, control vector	Clontech Laboratories
pGADT7-T	Matchmaker two-hybrid system 3, control vector	Clontech Laboratories
pGBKT7-Lam	Matchmaker two-hybrid system 3, control vector	Clontech Laboratories
pLS16-1	pSCRhaB2 + *rpoE2*	This work
pGEMT-x	pGEM-T + gene or region x^a	This work
pMLBAD-x	pMLBAD + gene x^a	This work
pGBKT7-x	pGBKT7 + gene x^a	This work
pGADT7-x	pGADT7 + gene x^a	This work
pLS38-9	pJQ200mp19 derivative for *rsiB1* deletion	This work
pLS37-9	pJQ200mp19 derivative for *rsiB2* deletion	This work
pCBT121	pJQ200mp19 derivative for *rsiA2* deletion	This work

^a x, name of the cloned gene or DNA region; see Table S1 in the supplemental material.

Strain and plasmid constructions. All plasmid constructions were performed using *E. coli* DH5α. DNA sequences of oligonucleotide primers used for PCR amplifications are available in Table S2 in the supplemental material. Absence of mutations in all constructs was checked by DNA sequencing.

pMP220-885 was constructed by A.-M. Garnerone by cloning the promoter region of SMc00885 (amplified by PCR, using oligonucleotides 885R and 885L

TABLE 2. Detection of protein-protein interactions in a yeast two-hybrid assay

Fusion protein expressed from pGBKT7	Interaction^a with fusion protein expressed from pGADT7					
	RsiA1	RsiA2	RpoE2	RsiB1	RsiB2	None
RsiA1	−	−	++	++	+	−
RsiA2	−	−	++	++	+	−
RpoE2	++	+	−	−	−	−
RsiB1	++	+	−	−	−	−
RsiB2	++	++	−	−	−	−
None	−	−	−	−	−	−

^a Shown is the ability (+) or not (−) of the AH109 strain containing the indicated pGADT7 and pGBKT7 derivatives to grow on plates of minimal medium lacking tryptophan, leucine, and histidine. ++ indicates that the strain was also able to grow on minimal medium lacking tryptophan, leucine, histidine, and adenine (higher stringency of the interaction test).

as primers and genomic DNA of Rm1021 as a template) in pGEM-T and then subcloning into pMP220 as an SphI-PstI restriction fragment.

The different open reading frames (ORFs) under study were totally or partially amplified by PCR, using Rm1021 genomic DNA as a template and oligonucleotides listed in Table S2 in the supplemental material, and cloned in plasmid pGEM-T as described in Table S1 in the supplemental material. Generally, inserts were subcloned from the resulting plasmids into pMLBAD as EcoRI-SmaI restriction fragments and into NdeI-SalI of pGBKT7 and NdeI-XhoI of pGADT7 as NdeI-SalI fragments. Plasmid pMLBAD-*rsiB2* was constructed by subcloning in pMLBAD an EcoRI-SphI fragment from plasmid pGEMT-*rsiB2*. Plasmids pMLBAD-*rsiA1_{55}* and pMLBAD-*rpoE2* were constructed by subcloning in pMLBAD the EcoRI-XbaI fragments from pGEMT-*rsiA1_{55}* and pGEMT-*rpoE2*, respectively. Plasmids pGADT7-*rpoE2* and pGBKT7-*rpoE2* were constructed as follows. The NdeI-XbaI fragment from plasmid pGEMT-*rpoE2* (NdeI/XbaI) was first subcloned in pSCRhaB2, giving plasmid pLS16-1, and then the insert was transferred in destination vectors as an NdeI-SalI fragment, as described above.

For the construction of pMLBAD-*rsiB1*-D191A, part of the *rsiB1* gene was amplified by PCR using OCB668 and OCB785, which generates the corresponding nucleotide substitution GAC (Asp) → GCG (Ala), and cloned in pGEM-T to give pLS102-7. An internal ClaI-XhoI fragment of wild-type *rsiB1* in pLS19-7 was exchanged with the same mutated fragment from pLS102-7, thus generating pGEMT-*rsiB1*-D191A. The mutated gene was then finally transferred in pMLBAD as an EcoRI-XmaI fragment.

pMLBAD derivatives expressing tagged versions of the proteins were constructed as follows. *rpoE2*-HA was generated by PCR analysis using OCB874 and

OCB876, which adds the hemagglutinin (HA) tag-coding sequence at the 5′ end of the gene, and was cloned in pGEM-T. The insert was then subcloned into EcoRI-SmaI-cut pMLBAD as a BamHI (filled-in)-EcoRI fragment. *rsiA1*-StrepII was generated by PCR amplification using OCB670 and OCB830, which adds the Strep-II tag-coding sequence at the 3′ end of the gene, and was cloned in pGEM-T. The insert was then subcloned into EcoRI-XmaI-cut pMLBAD. *rsiB1*-c-Myc was generated by PCR using oligonucleotides OCB668 and OCB829, which adds the c-Myc tag-coding sequence at the 3′ end of the gene, and was cloned in pGEM-T. The insert was then subcloned as a BamHI (filled-in)-EcoRI fragment into EcoRI-SmaI-cut pMLBAD. To construct pMLBAD-*rsiB1*-D191A-c-Myc, an internal ClaI-XhoI fragment of *rsiB1* in pGEMT-*rsiB1*-c-Myc was exchanged with the same fragment from *rsiB1*(D191A) prepared from pLS102-7, giving pLS123-1. The insert was then subcloned in pMLBAD as described above for *rsiB1*-c-Myc.

For deletions of chromosomal genes, plasmids derived from pJQ200mp19 were constructed (pLS38-9, pLS37-9, and pCBT121) containing 350- to 400-bp regions flanking the gene to be deleted (*rsiB1*, *rsiB2*, and *rsiA2*, respectively). These flanking regions were first produced by PCR amplification using Rm1021 genomic DNA as a template and oligonucleotides listed in Table S2 in the supplemental material as primers and were individually cloned in pGEM-T, as indicated in Table S1. These regions were subsequently juxtaposed into SalI-SacI-cut pJQ200mp19 as SacI-BamHI and BamHI-SalI fragments.

Plasmids were introduced in *S. meliloti* strain Rm1021 by triparental mating using pRK2013 as a helper and subsequent selection for antibiotic resistance. For the construction of deletion mutants, genomic insertion by single-crossover recombination of the pJQ200mp19 derivatives was selected by Gm resistance. The resulting strains were then propagated in the absence of an antibiotic, and cells having lost the plasmid by a second recombination event were selected by plating on LBMC medium in the presence of 5% sucrose (Suc). Gm^s Suc^r colonies were then screened by PCR analysis for the loss of the region to be deleted, using as primers the oligonucleotides OCB694-OCB715, OCB700-OCB701, and OCB694-OCB695 for the deletion of *rsiA2*, *rsiB1*, and *rsiB2*, respectively.

Estimation of the activity of tagged proteins. Activity of RsiA1-Strep was verified by measuring the ability of pMLBAD-RsiA1-Strep to negatively regulate RpoE2 activity, as described for RsiA1 in Results. Activity of RsiB1-c-Myc was verified by measuring the capacity of pMLBAD-RsiB1-c-Myc to complement the RpoE2-dependent response in an Δ*rsiB1* Δ*rsiB2* mutant, as described for RsiB1 in Results. Activity of RpoE2-HA was verified by measuring the capacity of pMLBAD-RpoE2-HA to induce the expression of the P$_{SMc00885}$-*lacZ* fusion in *E. coli*. In every case, the activity of the tagged protein was equivalent to that of its wild-type counterpart tested in a parallel control experiment.

Expression of proteins from pMLBAD. To estimate the level of gene expression from pMLBAD in *S. meliloti*, we measured using quantitative reverse transcription-PCR (qRT-PCR) the amount of *rsiB1* transcripts as described in reference 33 in Rm1021 cells carrying either pMLBAD-*rsiB1* or the empty pMLBAD vector, both in the absence and in the presence of arabinose (n = 2). In noninducing conditions, the level of *rsiB1* transcription was already ~6-fold higher with pMLBAD-*rsiB1* compared to that in the strain containing the empty vector, which suggests a basal level of transcription in the plasmid, even in the absence of inducer. After incubation in the presence of 2% arabinose for 2 h, this level further increased to ~70-fold. This shows that *rsiB1* is transcribed at a higher-than-normal level in strains containing pMLBAD-*rsiB1*. Increase of expression upon arabinose addition was confirmed at the protein level by Western blotting, using the pMLBAD derivative expressing the c-Myc-tagged version of RsiB1.

Measurement of RpoE2 activity. To measure RpoE2 activity in *S. meliloti*, the following procedure was generally used. Overnight precultures (5 to 10 ml) of strains carrying the reporter plasmids pMP220-885 or pLS6.32 were diluted to an optical density at 600 nm (OD$_{600}$) of 0.1 in 10 ml of fresh VMM and grown for ~6 to 8 h. Cultures were then diluted once more in 20 to 25 ml in order to reach an OD$_{600}$ of ~0.1 to 0.2 the day after. After overnight growth, cultures were divided into two flasks; one was kept at 28°C, and the other was shifted to 40°C. After 1 h, 100 μl of culture was collected, frozen in liquid nitrogen, and stored at −20°C. The culture at 28°C was kept for an additional 24 h (stationary phase sample), and 100 μl of culture was collected as described above. Alternatively, for strains carrying pMLBAD derivatives, arabinose was first added to the overnight culture at a final concentration of 0.2% or 2%, and cultures were allowed to grow for an additional 1 or 2 h, respectively, before being divided in two halves as described above. β-Galactosidase assays were performed on the thawed samples as described previously (30).

5′-RACE mapping of the *rsiA1* transcription start site. To map the transcription start site of *rsiA1*, we performed a rapid amplification of cDNA 5′ ends (5′-RACE) using a protocol derived from reference 37. Total RNA was prepared from Rm1021 cells grown at either 28°C or 40°C as described in reference 33, and 2 μg of RNA was used for reverse transcription for 1 h at 42°C in the presence of Superscript II reverse transcriptase (Invitrogen) and with random hexamers as primers. As a control, the same reaction was performed without the addition of enzyme. Then, RNA templates were degraded with RNase H, and cDNAs were purified on MicroSpin S-400 high-resolution columns (GE Healthcare). 3′ ends of cDNAs were ligated with the anchor oligonucleotide DT88 (37) by overnight incubation at 18°C in the presence of T4 RNA ligase (Promega). PCR analyses were performed on aliquots of the ligation mixtures using DT89 and the *rsiA1*-specific primer OCB540, and amplification products were analyzed by agarose gel electrophoresis. A single DNA fragment of ~220 bp was obtained but only in the sample derived from the wild-type strain cultivated at 40°C and treated with reverse transcriptase. The PCR product was cloned in pGEM-T, and its sequence was determined using universal primers.

In vitro pull-down assays. *E. coli* strains carrying pMLBAD derivatives expressing the protein of interest were grown exponentially at 37°C in 25 ml LB medium supplemented with Tmp until an OD$_{600}$ of ~0.4 to 0.5, and then 2% arabinose was added and the cells were further grown for 2 h (OD$_{600}$ of ~2). After centrifugation, pellets were resuspended in 1 ml lysis buffer (Bugbuster MasterMix; Novagen) supplemented with protease inhibitors (complete mini EDTA-free; Roche, Mannheim, Germany) and incubated for 30 min at room temperature under agitation. Lysates were then centrifuged (20,000 × g) for 20 min at 4°C, and supernatants were kept on ice until used. To prepare the RsiA1-Strep columns, 1 ml supernatant of *E. coli* cells expressing RsiA1-Strep was mixed with a 200-μl slurry of Strep-Tactin Macroprep (IBA, Göttingen, Germany) and incubated for 30 min at room temperature on an end-over-end rotation wheel. As a negative control, the supernatant from *E. coli* cells carrying the empty pMLBAD vector was used. The slurry was transferred on a minicolumn (732-6204; Bio-Rad Laboratories, Hercules, CA), the flowthrough was collected, and the resin was washed five times with 200 μl W buffer (100 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM EDTA).

To test the interaction of RpoE2-HA or RsiB1-Nterm with RsiA1, 1 ml supernatant was loaded on RsiA1-Strep columns. The flowthrough was collected and loaded again on the columns, which were finally washed five times with 200 μl W buffer.

To test the interaction of RsiB1-c-Myc, RsiB2, or RsiB1-D191A-c-Myc with RsiA1, the supernatants were first preincubated for 10 min at room temperature in the presence of 10 mM MgCl$_2$ with or without 100 mM acetyl phosphate. Before sample loading, columns were first equilibrated by two additional washes with WM buffer (W buffer without EDTA, supplemented with 10 mM MgCl$_2$) containing or not 100 mM acetyl-phosphate, respectively. The treated supernatants were then loaded on the columns as described above, the flowthrough was collected, and the columns were washed five times with the same buffers (WM with or without acetyl-phosphate).

In all cases, RsiA1-Strep and its interacting partners were eluted six times with 100 μl E buffer (W buffer supplemented with 10 mM desthiobiotine). Six fractions were thus collected. For all assays, supernatants, flowthrough, and elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by either staining with Sypro Ruby (Bio-Rad Laboratories, Hercules, CA) or, when applicable, Western blot analysis. In this case, proteins were electrotransferred to nitrocellulose membranes, and immunodetection was performed using peroxidase-conjugated monoclonal antibodies (rat anti-HA-peroxidase, clone 3F10, or mouse anti-c-Myc-peroxidase, clone 9E10; Roche, Mannheim, Germany) as described by the manufacturer.

RESULTS

RsiA1 and RsiA2 are two negative regulators of RpoE2. Throughout this study, RpoE2 activity was monitored mainly using pMP220-885, a reporter plasmid carrying a transcriptional *lacZ* fusion to the promoter of SMc00885. This promoter was chosen as it was one of the most highly induced RpoE2 targets following a heat shock or upon artificial RpoE2 overexpression in microarrays or qRT-PCR experiments (33; unpublished data). Transcription from this promoter following a heat shock or entry in stationary phase is strictly dependent on RpoE2 (Fig. 2) (33). SMc00885 is a direct RpoE2 target since (i) the typical sequences recognized by RpoE2 are present in its promoter region (33) and (ii) the *lacZ* fusion carried by pMP220-885 is activated in the heterologous host *E.*
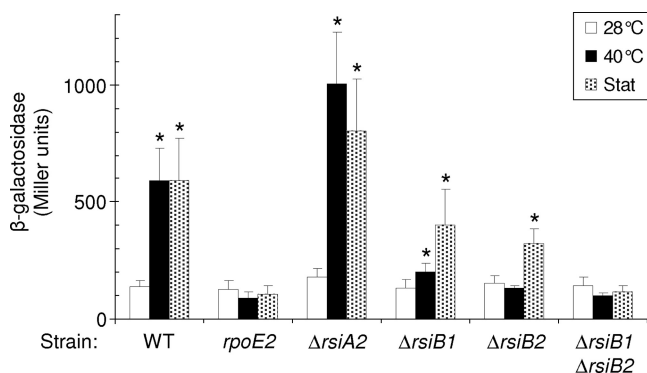
FIG. 2. Induction of RpoE2-dependent transcriptional responses in various genetic backgrounds. The transcription level of the $P_{SMc00885}$-*lacZ* fusion carried on plasmid pMP220-885, used as a reporter of RpoE2 activity, was measured in the *S. meliloti* strain Rm1021 (WT), CBT208 (*rpoE2*), CBT557 (Δ*rsiA2*), CBT390 (Δ*rsiB1*), CBT392 (Δ*rsiB2*), or CBT430 (Δ*rsiB1* Δ*rsiB2*), as indicated below the graph. β-Galactosidase activity was measured using aliquots of cultures grown to exponential phase at 28°C (white bars), after 1 h at 40°C (black bars), or to stationary phase at 28°C (dotted bars). Average values and standard deviations of results from at least three independent biological experiments are shown. Stars indicate significant induction relative to the log phase control at 28°C (Student's *t* test; $P < 0.05$).

FIG. 3. RsiA1and RsiA2 are negative regulators of RpoE2. The transcription level of the $P_{SMc00885}$-*lacZ* fusion carried on plasmid pMP220-885 was measured in the wild-type *S. meliloti* strain Rm1021 carrying either the empty vector pMLBAD(−) or pMLBAD derivatives expressing RsiA1$_{55}$, RsiA1$_{64}$, or RsiA2, as indicated below the graph. Cultures were grown to exponential phase in the absence (white bars) or in the presence of either 0.2% (gray bars) or 2% (black bars) arabinose for 2 h and then incubated for a further 1 h at either 28°C or 40°C before β-galactosidase activity was measured. Results are expressed as the ratio of activities measured at 40°C *versus* 28°C. Average values and standard deviations of results from at least three independent biological experiments are shown. Stars indicate that the induction level is significantly lower relative to that of the control without inducer (Student's *t* test; $P < 0.05$). Note that the basal levels of expression of the fusion at 28°C were not significantly changed by the strain background or the presence of arabinose (Student's *t* test; $P < 0.05$).

coli following artificial overexpression of RpoE2 (unpublished data).

In our previous report (33), we described SMc01505 as encoding a negative regulator of RpoE2. We therefore renamed this gene *rsiA1* (for *r*egulator of *si*gma). During the course of the present study, we discovered that RsiA1 was likely incorrectly annotated, since the fully active protein is longer than previously predicted. This was demonstrated by putting either the annotated ORF (55 codons; RsiA1$_{55}$) or an alternative ORF starting from an ATG codon located 9 codons upstream (RsiA1$_{64}$) (Fig. 1B) under the control of the arabinose-inducible P_{BAD} promoter of plasmid pMLBAD. The resulting constructs were introduced in *S. meliloti* Rm1021 together with the reporter plasmid pMP220-885. Expression of the $P_{SMc00885}$-*lacZ* fusion was induced 4- to 5-fold upon a heat shock in the presence of the empty vector pMLBAD, in the absence or presence of 0.2 or 2% arabinose (Fig. 3). In the presence of the plasmid expressing RsiA1$_{55}$, addition of 0.2 or 2% arabinose led to a lower induction of the fusion (3.6- and 1.8-fold, respectively), but in the presence of the plasmid expressing RsiA1$_{64}$, the fusion was no longer detectably inducible upon addition of as little as 0.2% arabinose (Fig. 3). These data therefore suggest that the longer version of RsiA1 is more efficient at negatively regulating RpoE2 activity and probably corresponds to the actual, fully active form of the protein. This new annotation is still consistent with the position of the transcription start site of *rsiA1*, which we mapped 30 nucleotides upstream from the new start codon using a 5′-RACE procedure (Fig. 1B and see Materials and Methods). It is also in agreement with the mean length of RsiA1 orthologues in other alphaproteobacteria (>60 amino acids) (data not shown). In the rest of the text, RsiA1 will refer to the longer version of the protein.

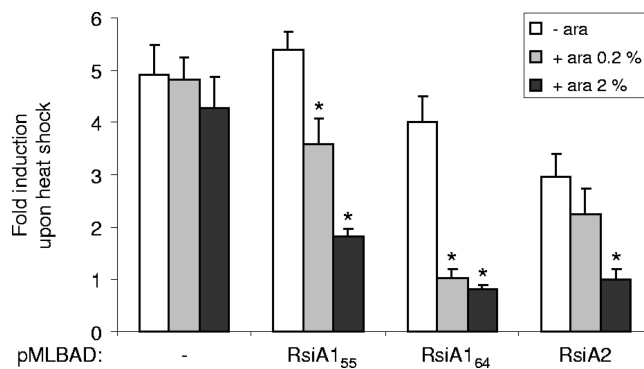Although no *rsiA1* orthologue was present in the *S. meliloti*

genome, the small size of *rsiA1* prompted us to search possible ones that may have been missed during genome annotation. Surprisingly, using a tblastn procedure, we found a new putative chromosomal ORF encoding a 62-amino-acid protein, 39% identical to RsiA1, which we named RsiA2 (Fig. 1A and see Fig. S1A in the supplemental material). To know whether RsiA2 could, like RsiA1, act as a negative regulator of RpoE2, we expressed it from pMLBAD in an *S. meliloti* strain carrying the reporter plasmid pMP220-885. The *lacZ* fusion was heat induced only ∼3-fold in the absence of arabinose (Fig. 3), presumably because of the background expression of the protein from the plasmid. This induction was no longer detectable in the presence of 2% arabinose (Fig. 3). These data therefore show that RsiA2, like RsiA1, can act as a negative regulator of RpoE2.

We have previously shown that deletion of *rsiA1* is lethal, presumably because of the toxic effect of the subsequent RpoE2 overexpression resulting from uncontrolled autoactivation of RpoE2 (33). In contrast, we succeeded in deleting *rsiA2* without affecting strain viability. Nevertheless, in the resulting Δ*rsiA2* strain, the $P_{SMc00885}$-*lacZ* reporter fusion was upregulated by heat stress at a level slightly but significantly higher than that in the wild-type strain (Student's *t* test; *P* value < 0.05) (Fig. 2). These results therefore confirm that *rsiA2* encodes another negative regulator of RpoE2.

RsiA1 and RsiA2 behave as anti-sigma factors of RpoE2. RsiA1 and RsiA2 may exert their inhibitory action on RpoE2 either at the transcriptional level, i.e., as repressors of *rpoE2* transcription, or at a posttranscriptional level, i.e., as anti-sigma factors. We have shown using microarray and qRT-PCR analyses that induction of RpoE2 expression from plasmid pMLBAD

leads to upregulation of the whole RpoE2 regulon in the absence of any external stress (unpublished data). High constitutive over-expression of RpoE2 from plasmid pBBR1MCS5 was even observed to be toxic (33). In contrast, coexpression of RsiA1 with RpoE2 relieved this toxicity (33), suggesting that RpoE2 was less active under these conditions. Accordingly, qRT-PCR analysis revealed no or little induction of RpoE2 targets in this strain, even following a heat shock, although the *rpoE2* transcript level was 200-fold higher than that in the wild-type strain (data not shown). Although these observations do not completely rule out a possible action of RsiA1 at the transcriptional level, they strongly suggest that RsiA1 acts mainly posttranscriptionally to inhibit RpoE2 activity.

We therefore tested whether RsiA1 and RsiA2 could act as anti-sigma factors. A common feature of anti-sigma factors is that they directly interact with their cognate sigma factors, thus inhibiting their interaction with the core RNA polymerase. To test whether RsiA1 and RsiA2 physically interact with RpoE2, we first used a yeast GAL4-based two-hybrid system. The *rsiA1*, *rsiA2*, and *rpoE2* ORFs were cloned into pGBKT7 and pGADT7 to generate protein fusions to the GAL4-DNA binding and activating domains, respectively. The constructs were introduced in the yeast strain AH109 by transformation, and interactions were assayed by testing the ability of the resulting strains to grow on SD minimal medium in the absence of histidine and adenine. As summarized in Table 2, these analyses revealed that RpoE2 is able to interact with RsiA1 or RsiA2 in yeasts, regardless of which of the two interacting proteins was fused to the activating or binding domains of Gal4. These interactions are specific, as none of the tested proteins interacted with the free activating or DNA binding domains expressed from empty vectors, nor did they interact with several other proteins fused to these domains (Table 2).

To validate these interactions, we performed *in vitro* tag-based pull-down assays. For this, pMLBAD derivatives expressing tagged versions of the RsiA1 and RpoE2 proteins were constructed. RpoE2 was tagged at its N-terminal end with the HA epitope, while RsiA1 was tagged at its C-terminal end with the Strep-tag II peptide. That these tagged proteins were active *in vivo* at levels equivalent to their wild-type counterparts was first verified (see Materials and Methods). To test protein-protein interactions, a lysate of *E. coli* cells expressing RsiA1-Strep was used to bind RsiA1 on a Strep-Tactin column. In a control experiment, the RsiA1-Strep-containing lysate was replaced with a lysate of empty vector-carrying *E. coli* cells. The capacity of RpoE2 to interact with RsiA1 was then tested by loading on these columns a lysate of *E. coli* cells expressing RpoE2-HA. As shown in Fig. 4A, RpoE2-HA was retained on the RsiA1-Strep column, whereas it was not on the control column. These observations therefore validate the results of the two-hybrid assay.

In conclusion, our data therefore strongly suggest that RsiA1 and RsiA2 act as anti-sigma factors of RpoE2.

RsiB1 and RsiB2 are redundant and essential for upregulation of RpoE2-dependent genes under stress conditions. Interestingly, *rsiA1* and *rsiA2* are located upstream and transcribed divergently from the SMc01504 and SMc00794 ORFs (Fig. 1A), which encode homologous proteins with putative regulatory functions (see Fig. S1B in the supplemental material and see Introduction) (11, 14–16). SMc01504 and
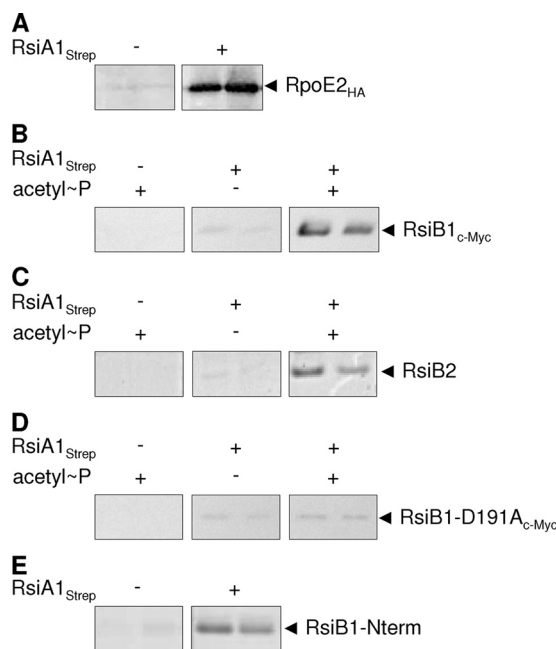

FIG. 4. Protein-protein interactions assessed by *in vitro* pull-down assays. RpoE2-HA (A), RsiB1-c-Myc (B), RsiB2 (C), RsiB1-D191A-c-Myc (D), or RsiB1-Nterm (E) was assayed for interaction with RsiA1 by loading corresponding *E. coli* cell lysates on a Strep-Tactin column bound or not with RsiA1-Strep (as indicated) and eluting with des-thiobiotin as described in Materials and Methods. In panels B, C, and D, the tested lysates were preincubated or not with 100 mM acetyl-phosphate (acetyl~P) as indicated. Elution fractions were separated by SDS-PAGE, and only the two fractions containing the largest amounts of proteins are shown (the same fractions are shown for all panels in a given experiment). Proteins were revealed either by Sypro Ruby staining (C and E) or by Western blotting using anti-HA or anti-c-Myc antibodies (A, B, and D). Every experiment was repeated at least twice independently.

SMc00794 were therefore renamed *rsiB1* and *rsiB2*, respectively.

To know whether these genes are involved in the regulation of RpoE2 activity, we deleted them from the *S. meliloti* chromosome. Whereas the P$_{SMc00885}$-*lacZ* fusion was upregulated ~5-fold after 1 h of incubation at 40°C in the wild-type strain (Fig. 2), it appeared weakly induced at 40°C in the Δ*rsiB1* mutant and was not detectably induced in the Δ*rsiB2* mutant (Fig. 2 and 5A). We also measured β-galactosidase activity in stationary phase, another RpoE2-activating condition (33), and compared it to the activity measured in log phase. Strikingly, under these conditions, the reporter fusion was still significantly activated in both Δ*rsiB1* and Δ*rsiB2* single mutants (Fig. 2). These results therefore suggest that RsiB1 and RsiB2 share redundant or synergistic activities that positively regulate the RpoE2-dependent response. To test these possibilities, we constructed an Δ*rsiB1* Δ*rsiB2* double mutant. The P$_{SMc00885}$-*lacZ* fusion was no longer detectably inducible either by heat stress or in stationary phase in the Δ*rsiB1* Δ*rsiB2* double mutant, which thus behaved as an *rpoE2* mutant (Fig. 2 and 5A). Introduction of pMLBAD derivatives expressing either RsiB1 or RsiB2 in either the single or double *rsiB* mutant strains restored the ability to induce the fusion under heat stress in all cases (Fig. 5A). This confirms that the observed phenotypes
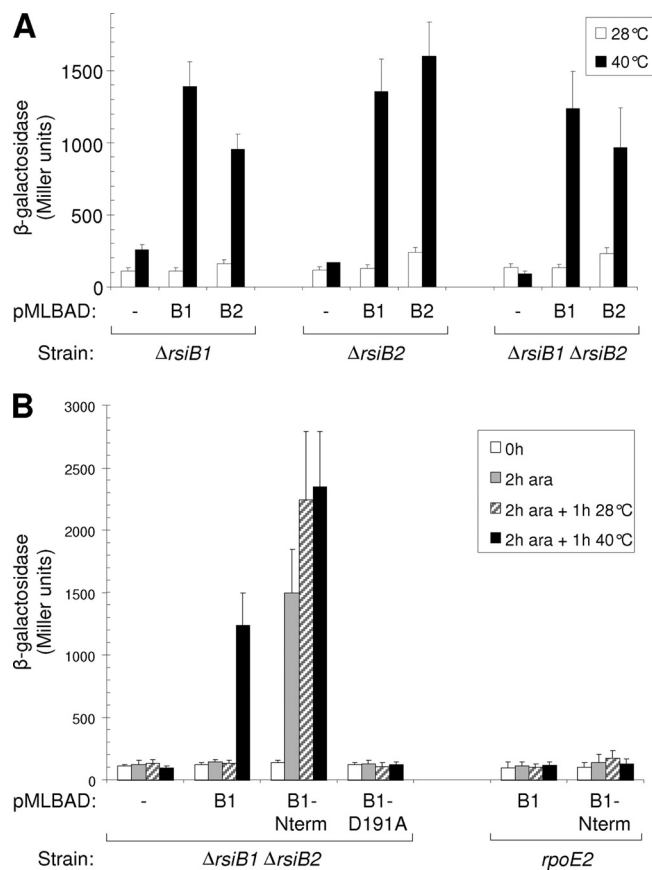
FIG. 5. RsiB1 and RsiB2 are positive regulators of RpoE2. The transcription level of the P$_{SMc00885}$-*lacZ* fusion carried on plasmid pMP220-885 was measured in the *S. meliloti* strains CBT390 (Δ*rsiB1*), CBT392 (Δ*rsiB2*), CBT430 (Δ*rsiB1* Δ*rsiB2*), or CBT208 (*rpoE2*) carrying either the empty vector pMLBAD(−) or pMLBAD derivatives expressing RsiB1 (B1), RsiB2 (B2), the N-terminal domain of RsiB1 (B1-Nterm), or the D191A mutant derivative of RsiB1 (RsiB1-D191A), as indicated below the graphs. (A) β-Galactosidase activity was measured on aliquots of cultures grown to exponential phase in the presence of 2% arabinose for 2 h and then incubated for a further 1 h at either 28°C (white bars) or 40°C (black bars). In every strain at 40°C, the presence of either pMLBAD-RsiB1 or pMLBAD-RsiB2 led to an activity significantly higher than did the empty vector in the same condition (Student's *t* test; *P* < 0.05). (B) β-Galactosidase activity was measured on aliquots of the cultures before (0 h) (white bars) and after incubation for 2 h in the presence of 2% arabinose (2 h ara) (gray bars), as well as after a further 1 h of incubation at either 28°C (2 h ara + 1 h 28°C) (hatched bars) or 40°C (2 h ara + 1 h 40°C) (black bars), as described in Materials and Methods. In each panel, average values and standard deviations of results from at least three independent biological experiments are shown. Only the high β-galactosidase activity levels resulting from expression of RsiB1 or RsiB1-Nterm in the Δ*rsiB1* Δ*rsiB2* strain were significantly different from those measured in the same strain containing the empty vector (Student's *t* test; *P* < 0.05).

were indeed due to the absence of these proteins and further suggests that RsiB1 and RsiB2 have redundant activities. Note that the fusion was expressed at levels ≥2-fold higher at 40°C in the complemented strains compared to that in the wild type (compare Fig. 5A and Fig. 2), an observation that we also made when expressing these proteins in the wild-type background and using various RpoE2 targets (data not shown). We

assume that this resulted from the higher-than-normal expression level of the RsiB proteins (see Materials and Methods). This observation, together with the fact that each genomic copy only partially complemented the absence of the other in the single *rsiB* mutants (Fig. 2), suggests to us that the amount of these proteins is normally slightly limiting for the RpoE2-dependent response in the wild-type strain.

Altogether, these data indicate that RsiB1 and RsiB2 share an activity which is essential for the RpoE2-dependent stress response.

RsiB1 and RsiB2 behave as response regulators, with an N-terminal RpoE2-activating domain under the control of the C-terminal phosphoreceiver domain. The domain structure of RsiB1 and RsiB2 suggests that these proteins are response regulators of two-component regulatory systems (11, 14–16). Indeed, their C-terminal moiety resembles typical phosphoreceiver domains (see Fig. S1C in the supplemental material), while their N-terminal domain is similar to ECF sigma factors (see Fig. S1D in the supplemental material). If these proteins are response regulators, their C-terminal "regulatory" domain may be phosphorylated by a cognate histidine kinase in response to stress, and this may result in activation of the N-terminal domain, assumed to carry the "effector" activity required for the RpoE2-dependent response.

To test the hypothesis that the N-terminal part of RsiB1 carries the effector activity, we expressed this domain (the first 148 amino acids of RsiB1) from pMLBAD in *S. meliloti* strains carrying the P$_{SMc00885}$-*lacZ* reporter fusion. Induction of expression of the N-terminal domain by addition of arabinose led to a strong increase of the transcription of the reporter fusion in both wild-type and Δ*rsiB1* Δ*rsiB2* strains, and interestingly this occurred even in the absence of stress, in contrast to what was described above for the full-length RsiB1 (Fig. 5B and data not shown). This upregulation was no longer observed in an *rpoE2* mutant (Fig. 5B), which suggests that it does not result from the replacement of RpoE2 with a putative sigma factor activity of the isolated N-terminal domain. These data therefore confirm the prediction that the N-terminal region of RsiB1 is the effector domain of the protein, required for upregulation of RpoE2 targets. These data also suggest that the activity of the N-terminal domain is negatively controlled by the C-terminal domain in the absence of stress, an inhibition that would be relieved under stress conditions, possibly as a consequence of phosphorylation of the C-terminal phosphoreceiver domain by a so-far-unknown histidine kinase(s).

That the C-terminal part of these proteins could be a phosphoreceiver domain was suggested by the good conservation of the residues involved in the formation of the active site, including the putatively phosphorylated aspartate at position 191 (see Fig. S1C in the supplemental material) (5). To confirm this prediction, we expressed from pMLBAD a mutant version of RsiB1 whose conserved aspartate residue has been substituted for an alanine (RsiB1-D191A). In contrast to RsiB1, expression of RsiB1-D191A in the Δ*rsiB1* Δ*rsiB2* double mutant did not complement the lack of induction of the P$_{SMc00885}$-*lacZ* reporter fusion under stress conditions (Fig. 5B). To exclude the possibility that this was due to a lower expression or stability of the mutated protein, we constructed plasmids expressing c-Myc-tagged versions of either RsiB1 or RsiB1-D191A. These proteins behaved similarly to their untagged

counterparts and were produced at equivalent levels in *S. meliloti*, as verified by Western blot analysis using anti-c-Myc antibodies (see Materials and Methods; data not shown). These data therefore suggest that the inhibition of the N-terminal effector domain of the mutant RsiB1-D191A protein can no longer be relieved by stress. This presumably results from the inability of the C-terminal domain to be phosphorylated on the conserved aspartate residue, although we cannot formally exclude that the D191A mutation indirectly affects the protein function. Nevertheless, additional data obtained *in vitro* further validate this hypothesis (see below).

Altogether, these results suggest that RsiB1 could be a response regulator. Given the activities shared by RsiB1 and RsiB2 (see above and *in vitro* data below), the high homology between these proteins at the amino acid level (69% identity, 81% similarity) (see Fig. S1B in the supplemental material) and the presence of anti-sigma-encoding genes next to both *rsiB1* and *rsiB2*, we assume that this conclusion can probably be extended to RsiB2.

RsiB1 and RsiB2 act as anti-anti-sigma factors to relieve inhibition of RpoE2. Formally, RsiB1 and RsiB2 could be needed upstream from RpoE2 action, i.e., for its activation by stress, or they could be required for the RpoE2 target promoters to be active, i.e., as transcriptional activators. To discriminate between these hypotheses, we tested whether RpoE2 target promoters are functional in the absence of RsiB1 and RsiB2. For this, we made RpoE2 activity independent of stress by putting the *rpoE2* gene under the control of the P_{BAD} promoter of pMLBAD. Indeed, microarray and qRT-PCR analyses have shown that in the wild-type strain, induction of RpoE2 expression from this plasmid by addition of arabinose leads to the upregulation of the whole RpoE2 regulon in the absence of any external stress (unpublished data). In agreement with this observation, the $P_{SMc00885}$-*lacZ* fusion was induced 6.2-fold (± 1.05) upon addition of arabinose in the wild-type strain carrying pMLBAD-*rpoE2*. In the $\Delta rsiB1$ $\Delta rsiB2$ double mutant strain, a similar induction (5.5-fold $\pm$ 0.52) was observed. Moreover, when tested in the heterologous host *E. coli*, i.e., a bacterial species lacking *rsiB1* and *rsiB2* orthologues, production of RpoE2 from the same plasmid made possible the induction of the plasmid-borne $P_{SMc00885}$-*lacZ* fusion (data not shown). These observations therefore suggest that RsiB1 and RsiB2 are not essential for RpoE2 activity *per se* and are rather required for its activation in response to stress at either transcriptional or posttranscriptional levels.

We previously observed that the *rpoE2* operon is transcriptionally upregulated in response to stress (33). This transcriptional activation, however, was found to be strictly dependent on RpoE2 (33), which indicates that RpoE2 activation does not result primarily from transcriptional upregulation of *rpoE2*. To definitely exclude the possibility that RsiB1 and RsiB2 act by increasing the level of *rpoE2* transcription, we tested the effect of these proteins on the transcription of a *lacZ* fusion to the promoter of the *rsiA1-rpoE2* operon, carried on plasmid pLS6.32 (33). In an *rpoE2* mutant background, expression of these proteins did not lead to any induction of the fusion (data not shown). This therefore shows that RsiB1 and RsiB2 do not exert their positive regulatory action at the level of *rpoE2* transcription but rather posttranscriptionally.

Since RsiB1 and RsiB2 act on RpoE2 at a posttranscrip-

tional level, we first tested whether they could interact with the sigma factor. However, we could not detect any direct interaction between these proteins and RpoE2 in the yeast two-hybrid assay (Table 2). In contrast, we observed that RsiB1 and RsiB2 are both able to interact with RsiA1 or RsiA2 in yeasts (Table 2). This suggested that RsiB1 and RsiB2 do not act directly on RpoE2 but rather through interaction with its anti-sigma factors.

To validate these observations, we performed *in vitro* pull-down assays. To test the ability of RsiB1 to interact with RsiA1, a lysate of *E. coli* cells expressing RsiB1-c-Myc from pMLBAD was loaded on an RsiA1-Strep column. Since *in vivo* experiments have suggested that RsiB1 is active when phosphorylated (see above), the RsiB1-c-Myc-containing lysate was preincubated in the presence or absence of the phospho-donor acetyl phosphate before loading on the column. As shown in Fig. 4B, RsiB1-c-Myc was efficiently retained on the RsiA1-Strep column when preincubated with acetyl phosphate, whereas it was not in the absence of acetyl phosphate nor on the control RsiA1-free column. Similar results were obtained with RsiB2 (Fig. 4C). In contrast, the mutant protein RsiB1-D191A was not efficiently retained on the RsiA1-Strep column, even in the presence of acetyl phosphate (Fig. 4D). These results therefore suggest that in the presence of acetyl phosphate, RsiB1 and RsiB2 are phosphorylated, presumably on the conserved aspartate residues of their C-terminal domain, which makes them able to interact with RsiA1.

Finally, as the N-terminal region of RsiB1 is the domain involved in RpoE2 activation (see above), we tested its ability to interact with RsiA1. RsiB1-Nterm was efficiently retained on the RsiA1-Strep column in the absence of acetyl phosphate (Fig. 4E), which suggests that the interaction between RsiA1 and RsiB1 occurs through the N-terminal effector domain of RsiB1 and that this interaction is normally inhibited by the nonphosphorylated C-terminal domain.

Altogether, these data suggest that the phosphorylation of the C-terminal domain of RsiB1 (and presumably RsiB2) in response to stress activates the ability of their N-terminal domain to interact with RsiA1 (and presumably RsiA2). An exciting hypothesis is that this interaction is able to relieve the inhibition of the sigma factor by its anti-sigma factors: RsiB1 and RsiB2 would therefore act as anti-anti-sigma factors.

DISCUSSION

The main purpose of this work was to investigate the mechanisms of signal transduction leading to activation of the *S. meliloti* RpoE2 ECF sigma factor in response to stress. We showed that RpoE2 is negatively regulated by two anti-sigma factors (the paralogues RsiA1 and RsiA2, products of SMc01505 and SMc04884, respectively) and positively regulated by two putative response regulators (the paralogues RsiB1 and RsiB2, products of SMc01504 and SMc00794, respectively) which share an activity essential for activation of RpoE2 under stress conditions. Interestingly, these response regulators do not act at the level of *rpoE2* transcription but could instead function as anti-anti-sigma factors to relieve RpoE2 inhibition by the anti-sigma factors. As further discussed below, these findings are in line with recent reports on the mechanisms of regulation of σ^{EcfG} sigma factors by NepR

anti-sigma and PhyR anti-anti-sigma in *M. extorquens* and *B. japonicum* (11, 17). Nevertheless, the present work reveals an additional complexity of the regulatory system because of the presence of two pairs of anti- and anti-anti-sigma factors in *S. meliloti*.

We had previously shown that *rsiA1* (SMc01505), located upstream from *rpoE2* and transcribed in an operon with it, encodes a negative regulator of RpoE2 (33). In the present study, we found that the annotated ORF, encoding a 55-amino-acid protein, is actually less active *in vivo* than a protein starting 9 codons upstream, suggesting a wrong original annotation. This finding is in agreement with the length of RsiA1 homologues predicted in other alphaproteobacterial genomes (i.e., >60 amino acids) (data not shown). In addition, we found that the *S. meliloti* chromosome encodes a paralogue of RsiA1 (39% identity, 62 amino acids) and demonstrated that this protein also acts as a negative regulator of RpoE2. The corresponding gene, located between SMc00794 and SMc00795 (coordinates 815835 to 816023 on the chromosome sequence AL591688) had not been annotated previously, and we gave it the ORF number SMc04884, with a gene name *rsiA2*. Interestingly, putative RNA transcripts of *rsiA2* were recently identified in a high-throughput sequence analysis of the transcriptome of exponentially growing *S. meliloti* cells (27). New annotations and gene names proposed in this study will be included in the *S. meliloti* website (http://sequence.toulouse.inra.fr/S.meliloti).

We observed here that both RsiA1 and RsiA2 are able to interact with RpoE2. Together with the observation that they are negative regulators of RpoE2, these results strongly indicate that RsiA1 and RsiA2 act as anti-sigma factors with respect to RpoE2. In both *M. extorquens* and *B. japonicum*, a protein called NepR, displaying homology with RsiA1/A2 (28% identity, 59% similarity on a central 29-amino-acid window), was recently shown in each bacterial species to interact with an ECF sigma factor homologous to RpoE2 called σ^{EcfG} and, in *M. extorquens*, to negatively control the σ^{EcfG} regulon (11, 17).

Anti-sigma factors are a common way for negatively regulating the activity of ECF sigma factors, although they are generally unique, specific for a single sigma factor, and usually encoded in the same operon as their cognate sigma factor (20). Surprisingly, we found in *S. meliloti* two anti-sigma factors able to regulate the same sigma factor. Only one of them (RsiA1) is coexpressed with RpoE2 in an operon whose transcription is itself under RpoE2 control (33). Regulating the expression of an anti-sigma by the sigma factor itself is of particular importance in order to tightly control the sigma factor activity and thus avoid excessive accumulation of active sigma factor. Accordingly, we previously observed that inactivation of *rsiA1* is lethal, presumably because of the toxicity of the resulting RpoE2 overexpression (33). The second anti-sigma (RsiA2) is probably not as important as RsiA1 for the regulation of RpoE2 activity, since we could delete the *rsiA2* gene without detectably affecting either the basal level of RpoE2 activity or the strain viability. Nevertheless, RsiA2 seems to contribute to RpoE2 inhibition since we detected a higher RpoE2 activity at 40°C in the *rsiA2* deletion strain than in the wild-type strain. In contrast to RsiA1, expression of RsiA2 is probably not under RpoE2 control, as suggested by our previous studies of the

RpoE2 regulon (33; unpublished data), by the absence of the −10 and −35 boxes recognized by RpoE2 upstream from the gene (data not shown) and by the detection of transcripts of this gene in cells growing exponentially in the absence of stress (27). Moreover, the lack of regulation by RpoE2 may explain the inability of RsiA2 to complement the lethal effect of RpoE2 accumulation in the absence of RsiA1 (33).

We also showed that *rsiB1* and *rsiB2*, transcribed divergently from *rsiA1* and *rsiA2*, respectively, encode redundant positive regulators of RpoE2, essential for its activation by stress. From their amino acid sequences and domain organizations, RsiB1 and RsiB2 were predicted to belong to the PhyR family of response regulators of two-component regulatory systems (14–16). The structure of these putative response regulators is atypical in that (i) their predicted regulatory phosphoreceiver domain is located in the C terminus of the proteins rather than in the N terminus, as usually observed in most response regulators, and (ii) the N-terminal domain, assumed to be the effector domain of the regulator, presents similarities with ECF sigma factors. Our results strengthen the prediction that these proteins function as response regulators. Indeed, the isolated N-terminal domain of RsiB1 is able to activate RpoE2 in the absence of stress, whereas the full-length protein is able to do so under stress conditions only. This suggests that the C-terminal domain of these proteins normally inhibits the activity of the N-terminal domain and that this inhibition is relieved in the presence of stress. Such a relief of inhibition of the effector domain by the phosphoreceiver domain is one of the possible mechanisms proposed for stress activation of several response regulators (see, for example, references 6, 8, 24, and 36). That the C-terminal part of these proteins behaves as a phosphoreceiver domain *in vivo* was suggested by the inability of an RsiB1 derivative, mutated in the conserved phosphorylated aspartate residue (D191), to activate RpoE2 in response to stress. Presumably, the aspartate residue of RsiB1 and RsiB2 is phosphorylated by a cognate histidine kinase in response to stress. Surprisingly, RsiB1 and RsiB2 do not function as transcription regulators but instead interact with the anti-sigma factors RsiA1 and RsiA2. Interestingly, the behavior of RsiB1 and RsiB2 *in vitro* correlates with *in vivo* observations: (i) these proteins are able to efficiently interact with the anti-sigma factor *in vitro* only when phosphorylated, a phosphorylation that is supposed to occur on the D191 residue of the phosphoreceiver domain in RsiB1, and (ii) interaction of RsiB1 with the anti-sigma factor *in vitro* occurs via its N-terminal effector domain. We assume that the putative ECF-like structure of RsiB1/B2 in their N-terminal domain is responsible for their interaction with the anti-sigma factors and that they could possibly compete with the sigma factor for interacting with the anti-sigma factors, thus acting as anti-anti-sigma factors.

Similarly, in two recent reports, the *M. extorquens* and *B. japonicum* PhyR response regulators, orthologous to RsiB1/B2, were shown to positively regulate the same genes as the sigma factor σ^{EcfG} and to interact, in their phosphorylated form, with the NepR anti-sigma factors *in vitro* (11, 17). The authors therefore proposed a model of sigma factor mimicry for the regulation of the σ^{EcfG} sigma factors by PhyR. We reached similar conclusions using *S. meliloti*, suggesting that this new regulatory mechanism is widely conserved for activat-
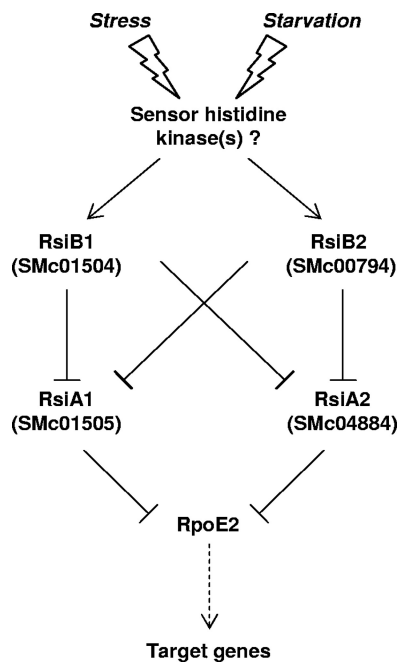
FIG. 6. Model for the activation of *S. meliloti* RpoE2 in response to stress. In unstressed bacteria, RpoE2 is maintained in an inactive form by interaction with its anti-sigma factors RsiA1 and RsiA2. Under stress or starvation conditions, one or several yet-unknown histidine kinase(s) sense the stimuli and phosphorylate the C-terminal domain of RsiB1 and/or RsiB2. This results in the activation of the N-terminal "effector" domains of these proteins which become available to contact the anti-sigma factors RsiA1 and RsiA2 and relieve their inhibiting effect on RpoE2. Sharp and flat arrowheads represent positive and negative regulatory actions, respectively.

ing RpoE2-like sigma factors among alphaproteobacteria. Nevertheless, an originality of *S. meliloti* is the presence of two pairs of anti- and anti-anti-RpoE2 sigma factors. Examination of sequence databases indicates that to date, two other sequenced alphaproteobacterial genomes could also encode two pairs of such regulators, namely, *S. medicae* and *Rhizobium* sp. strain NGR234, two rhizobial species closely related to *S. meliloti*. The *S. meliloti rsiB2-rsiA2* and *M. extorquens phyR-nepR* regions are very similar in that they both encode proteins acting in *trans* on a distant sigma factor. In contrast, in other alphaproteobacteria where an *rsiB/phyR* orthologue is present, both *rpoE2*/σ^EcfG and *rsiA/nepR* homologues were generally found next to it (15, 33, 35). Although the significance of two pairs of RpoE2 regulators in *S. meliloti* is so far unknown, an interesting hypothesis could be that the two anti-anti-sigma factors are phosphorylated by different histidine kinases, which may be able to sense different stimuli. While our data revealed similar involvements of RsiB1 and RsiB2 for RpoE2 activation in response to the stress and starvation conditions tested (Fig. 2), we cannot exclude that they respond differentially to other, so-far-untested conditions.

We therefore propose the following model (Fig. 6): in normal, unstressed bacteria, RpoE2 is maintained in an inactive form by interaction with its anti-sigma factors RsiA1 and RsiA2. Under stress or starvation conditions, one or several yet-unknown histidine kinases sense the stimuli, autophosphorylate, and transfer their phosphate to the C-terminal domain of

RsiB1 and/or RsiB2. This results in the activation of the N-terminal "effector" domains of these proteins which become available to contact the anti-sigma factors RsiA1 and RsiA2 and relieve their inhibiting effect on RpoE2.

A similar partner-switching mechanism of regulation was already described for *Bacillus subtilis* sigma factors involved in the control of sporulation (σ^F) and general stress response (σ^B). In both cases, the sigma factor is kept inactive by interaction with an anti-sigma. This inhibition is relieved by interaction of the anti-sigma with an anti-anti-sigma activated in response to a stimulus. This mechanism is regulated by the phosphorylation status of the anti-anti-sigma, itself controlled by several phosphatases whose activity is regulated by different stress conditions (for reviews, see references 19 and 38). However, σ^F and σ^B are not ECF sigma factors. More recently, the response to blue light of the *Myxococcus xanthus* ECF sigma factor CarQ was proposed to be regulated by an anti-sigma (CarR) and an anti-anti-sigma (CarF) (12). However, CarF is not a two-component response regulator. An ECF sigma factor regulated by a two-component system was already described for *S. coelicolor* σ^E, but in this case, the response regulator CseB acts directly as an activator of σ^E transcription in response to stimuli sensed by the CseB histidine kinase (22, 31). Several two-component systems have also been involved in the regulation of the non-ECF sigma factor σ^S in *E. coli* but mostly at the transcriptional level, with the exception of the response regulator (RssB) which directly binds σ^S and targets it to proteolytic degradation (21).

In summary, *S. meliloti* RpoE2 and related σ factors from alphaproteobacteria, like the *M. extorquens* and *B. japonicum* σ^EcfG, are to our knowledge the only ECF sigma factors whose mechanism of stimulus perception/signal transduction involves both anti-sigma factors and two-component regulatory systems. An originality of *S. meliloti* in this respect is the existence of two pairs of anti-sigma factors/response regulators, which suggests an unexpected complexity of the regulatory mechanism. The next step of this work will be obviously to identify the "second" component(s) of these systems, i.e., the putative histidine kinase(s) involved in stimulus perception, and to analyze in depth the functioning of this new signaling network.

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