JOURNAL OF BACTERIOLOGY, July 2009, p. 4122–4132 Vol. 191, No. 13
0021-9193/09/$08.00+0 doi:10.1128/JB.01626-08

Role of the Extracytoplasmic Function Sigma Factor RpoE4 in Oxidative and Osmotic Stress Responses in *Rhizobium etli*▽†

Jaime M. Martínez-Salazar,[1]* Emmanuel Salazar,[2] Sergio Encarnación,[2] Miguel A. Ramírez-Romero,[3] and Javier Rivera[1]

Programa de Ingeniería Genómica,[1] Programa de Genómica Funcional de Procariotes,[2] and Programa de Genómica Evolutiva,[3] Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Apdo Postal 565-A, C. P. 62210, Cuernavaca, Morelos, Mexico

Received 15 November 2008/Accepted 13 April 2009

The aims of this study were to functionally characterize and analyze the transcriptional regulation and transcriptome of the *Rhizobium etli rpoE4* gene. An *R. etli rpoE4* mutant was sensitive to oxidative, saline, and osmotic stresses. Using transcriptional fusions, we determined that RpoE4 controls its own transcription and that it is negatively regulated by *rseF* (*r*egulator of *s*igma *rpoE4*; CH03274), which is cotranscribed with *rpoE4*. *rpoE4* expression was induced not only after oxidative, saline, and osmotic shocks, but also under microaerobic and stationary-phase growth conditions. The transcriptome analyses of an *rpoE4* mutant and an *rpoE4*-overexpressing strain revealed that the RpoE4 extracytoplasmic function sigma factor regulates about 98 genes; 50 of them have the *rpoE4* promoter motifs in the upstream regulatory regions. Interestingly, 16 of 38 genes upregulated in the *rpoE4*-overexpressing strain encode unknown putative cell envelope proteins. Other genes controlled by RpoE4 include *rpoH2*, CH00462, CH02434, CH03474, and *xthA1*, which encode proteins involved in the stress response (a heat shock sigma factor, a putative Mn-catalase, an alkylation DNA repair protein, pyridoxine phosphate oxidase, and exonuclease III, respectively), as well as several genes, such as CH01253, CH03555, and PF00247, encoding putative proteins involved in cell envelope biogenesis (a putative peptidoglycan binding protein, a cell wall degradation protein, and phospholipase D, respectively). These results suggest that *rpoE4* has a relevant function in cell envelope biogenesis and that it plays a role as a general regulator in the responses to several kinds of stress.

In eubacteria, gene expression is controlled at the transcriptional level by the combined actions of sigma factors, activators, and repressors. Sigma factors bind to core RNA polymerase ($\alpha_2\beta\beta'\omega$) and recognize specific promoters. The replacement of one sigma factor with another allows the controlled transcription of different genes. Gene expression in exponentially growing bacterial cells depends on a single sigma factor (the σ^{70} factor) aimed at transcribing housekeeping genes (8, 23). A variable number of alternative sigma factors coordinate the expression of genes required for defined growth conditions and/or responses to specific stimuli (23). Therefore, alternative sigma factors play relevant roles in responding and adapting to different kinds of stresses and environments.

Based on sequence similarities and conserved regions, sigma factors are grouped into two families: σ^{54} and σ^{70}. In general, bacterial cells have several members from the σ^{70} family and usually only one or two members from the σ^{54} family. Members of the diverse σ^{70} family have four conserved regions; the 2.4 and 4.2 subregions are significantly conserved and recognize the −10 and −35 promoter elements, respectively (8, 23, 32). Moreover, the σ^{70} family is divided into four phylogenetic groups (23, 26, 32): group 1, the primary sigma factors (σ^{70}-related factors); group 2, nonessential proteins highly similar to primary sigma factors (σ^{S}-related factors); group 3, secondary sigma factors (σ^{28}-related factors); and group 4, the extracytoplasmic function (ECF) subfamily (ECF σ, or σ^{E}-related, factors). Among these groups, the ECF σ subfamily is the largest and most diverse, and it is involved in a wide range of stress responses and environmental adaptation processes, such as alginate production, carotenoid biosynthesis, starvation responses, and resistance to high temperatures, reactive oxygen species, and antibiotics, etc. (3, 4, 5, 6, 7, 17, 26, 35, 44, 50).

The ECF σ subfamily comprises small proteins (24 to 50 kDa) that in most cases are cotranscribed with an inner membrane anti-σ protein. Anti-σ binds to the sigma factor, reducing the potential interaction with the RNA polymerase (2, 24, 26, 43, 44). After receiving a stimulus, the sigma factor is released and can bind to the promoter regions of specific genes, enabling transcription. Most ECF σ factors positively regulate their own transcription, while coexpression with an anti-σ factor results in the switching off of the ECF σ response (2, 24, 26). The best-characterized ECF σ factors are the homologs of σ^{E}, encoded by a gene essential in *Escherichia coli* (12, 43) but not in *Azotobacter vinelandii*, *Pseudomonas aeruginosa*, or *Salmonella enterica* serovar Typhimurium (6, 17, 35, 50). This protein regulates a large number of genes involved in both the biogenesis and stress responses of the cell envelope (2, 24, 43, 44). Under different conditions, it also controls the transcription of other sigma factors, such as those encoded by *rpoD*, *rpoH*, and *rpoN* (24, 43).

Free-living rhizobia are able to establish symbiotic relation-

* Corresponding author. Mailing address: Programa de Ingeniería Genómica, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Apdo Postal 565-A, C. P. 62210, Cuernavaca, Morelos, Mexico. Phone: 52 (777) 3175867. Fax: 52 (777) 3175581. E-mail: jaime@ccg.unam.mx.

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

▽ Published ahead of print on 17 April 2009.

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Description and/or genotype[a]	Reference or source
R. etli strains		
CE3	CFN42 derivative; Str^r Nal^r	40
CFNXE1Sp	CE3 derivative; *rpoE1*::*loxP* Sp	This work
CFNXE2Sp	CE3 derivative; *rpoE2*::*loxP* Sp	This work
CFNXE3Sp	CE3 derivative; *rpoE3*::*loxP* Sp	This work
CFNXE4Sp	CE3 derivative; *rpoE4*::*loxP* Sp	This work
CFNXTXSp	CE3 derivative; *tcrX*::*loxP* Sp	This work
CFNXΔ3274Sp	CE3 derivative with deletion/substitution ΔCH03274::*loxP* Sp allele	This work
CFNXΔ3274lox	CFNXΔ3274Sp derivative with *loxP* Sp interposon deletion; ΔCH03274::*loxP*	This work
E. coli DH5α	Nal^r; host for recombinant plasmids	Stratagene
Plasmids		
pBBMCS53	pBBR1MCS-5 derivative carrying a promoterless β-glucuronidase gene	20
pRK2013	Conjugation helper plasmid; Km^r	51
pFAJ1708	RK2 derivative carrying *nptII* promoter; Tc^r	14
pK18mobsacB	Km^r Sac^s; used for gene replacement	49
pGUSprpoH2	pBBMCS53 derivative carrying the promoter of the *rpoH2* gene	37
pJMS2	Plasmid harboring *loxP* Sp interposon	36
pJMS8	pRK7813 derivative harboring *cre* gene	38
pJMS24	pBBMCS53 derivative harboring 570 bp upstream of *R. etli* CH03274; *rpoE4-uidA* fusion vector	This work
pJMS25	pBBMCS53 derivative harboring 570 bp upstream of *R. etli* CH03274; *tcrX-uidA* fusion vector	This work
pJMS26	pK18mobsacB derivative harboring 1,701 bp of *R. etli rpoE1* region	This work
pJMS27	pK18mobsacB derivative harboring 1,878 bp of *R. etli rpoE2* region	This work
pJMS28	pK18mobsacB derivative harboring 1,584 bp of *R. etli rpoE3* region	This work
pJMS29	pK18mobsacB derivative harboring 1,804 bp of *R. etli tcrX-rpoE4* region	This work
pJMS30	pJMS26 derivative harboring *loxP Sp* interposon in EcoRV site located at codon 47 of *rpoE1*	This work
pJMS31	pJMS27 derivative harboring *loxP* Sp interposon in XhoI site located at codon 147 of *rpoE2*	This work
pJMS32	pJMS28 derivative harboring *loxP* Sp interposon in EcoRV site located at codon 89 of *rpoE3*	This work
pJMS33	pJMS29 derivative harboring *loxP* Sp interposon in ClaI site located at codon 90 of *rpoE4*	This work
pJMS34	pJMS29 derivative harboring *loxP* Sp interposon in NarI site located at bp 282 of *trcX*	This work
pJMS35	pK18mobsacB derivative harboring 2,517 bp of *R. etli rpoE4* region with CH03274 deletion	This work
pJMS36	pJMS35 derivative harboring the deletion/substitution ΔCH03274::*loxP* Sp allele	This work
pJMS37	pFAJ1708 derivative harboring 1,267 bp of *rpoE4*; *PntpII-rpoE4* expression vector	This work

[a] Nal^r, nalidixic acid resistant.

ships with roots of leguminous plants and to form nodules in which differentiated bacterial cells reduce atmospheric nitrogen to ammonia (19, 33). In the rhizosphere, but also inside the root nodule, rhizobia may suffer from different kinds of stress, e.g., oxidative stress during the infection process and oxygen limitation after nodule formation (11, 31, 46, 47, 52). In the free-living state, rhizobia face several kinds of stress, such as starvation and changes in osmolarity, pH, and temperature; also, oxidant species are generated as by-products of aerobic respiration or as products from other microorganisms (53).

Rhizobium etli is a gram-negative, free-living soil bacterium able to form nodules on roots of *Phaseolus vulgaris* and harbors one chromosome and six large plasmids (ranging from 184 to 642 kb) (21). Analysis of the *R. etli* genome revealed 23 sigma factor-encoding genes: 1 σ^{70} (*rpoD*) gene, a housekeeping gene; 2 σ^{54} (*rpoN*) genes, involved in symbiosis and nitrogen assimilation; 2 σ^{32} (*rpoH*) genes, involved in responses to oxidative and heat stresses; and 18 ECF σ factor genes. Interestingly, none of the encoded sigma factors seem to be homologous to the general stress sigma factor σ^S, while four of them show high levels of similarity to the *E. coli* σ^E factor (21).

In this study, we describe the characterization of a gene encoding an ECF σ factor in *R. etli*, *rpoE4* (CH03273), and evaluate its role under several biological and abiotic stress conditions. The results suggest that RpoE4 is an important general regulator involved in the responses to several stresses, as well as in cell envelope biogenesis.

MATERIALS AND METHODS

Bacterial strains and microbiological methods. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37°C in Luria-Bertani medium (45). *R. etli* strains were grown at 25°C in either PY medium (40) or minimal medium (MM) (20). Microaerobic (1% O_2) conditions were generated as described previously (20). Antibiotics were added at the following final concentrations (in micrograms per milliliter): gentamicin, 5; carbenicillin, 100; nalidixic acid, 20; streptomycin, 100; spectinomycin, 100; kanamycin, 30; and tetracycline, 5. For positive selection of the *sacB* gene, sucrose was used at concentrations ranging from 7.5 to 10% (wt/vol). To determine the survival rates in the presence of H_2O_2, *R. etli* strains were grown in PY medium at 25°C. Aliquots of cultures with optical densities at 600 nm (OD_{600}) of ~0.3 were taken and incubated with different concentrations of H_2O_2 (Sigma) for 45 min at 25°C. After treatment, samples were diluted in 10 mM $MgSO_4$–20 mM Tween 40 and plated onto PY medium. To evaluate sensitivity to sucrose, NaCl, and methyl viologen (Paraquat; Sigma), overnight cultures (OD_{600}, ~1.0) were diluted and plated onto PY medium containing sucrose at 15%, NaCl at 80 mM, or methyl viologen at 40 μM.

Recombinant-DNA procedures. Genomic DNA was isolated using the GenomicPrep cell and tissue DNA isolation kit according to the instructions of the manufacturer (Amersham Biosciences). Plasmid DNA was isolated by an alkaline-sodium dodecyl sulfate lysis method, and $CaCl_2$-treated *E. coli* cells were transformed with the DNA; other general DNA methods were carried out according to standard protocols (45). Restriction enzymes and T4 DNA ligase were used as specified by the manufacturer (Invitrogen). *Pfu* DNA polymerase (Altaenzymes) was used for PCRs. The conjugative mobilization of plasmids

from *E. coli* to *R. etli* was done by triparental mating, using the pRK2013 plasmid as a helper (51).

Plasmid and *R. etli* mutant construction. The sequences of the oligonucleotides (Unidad de Biosíntesis, Instituto de Biotecnología/UNAM, Mexico) used for *R. etli* gene amplifications are available in the supplemental material (see Table S1). Primers tcrX-lw and ch3274wb were both used to obtain a PCR product of 570 bp from the region upstream of ATG in *rseF* (CH03274; 246 bp of a noncoding region and 324 bp of the *trcX* gene) from *R. etli* CE3, and the product was cloned in both orientations into the EcoRV site of pBBMCS53 (20) to create pJMS24 (carrying an *rpoE4-uidA* fusion) and pJMS25 (carrying a *tcrX-uidA* fusion).

The 1.70-kb *R. etli rpoE1*, 1.87-kb *R. etli rpoE2*, 1.58-kb *R. etli rpoE3*, and 1.80-kb *R. etli tcrX-rpoE4* regions were amplified from strain CE3 by PCR and cloned into the appropriate sites in pK18mobsac (49) to give pJMS26, pJMS27, pJMS28, and pJMS29, respectively. pJMS30 is a pJMS26 derivative with a 2.3-kb fill-out ClaI-digested *loxP* spectinomycin resistance (Sp) interposon (36) inserted into the EcoRV site located at codon 47 of *rpoE1*. pJMS31 is a pJMS27 derivative with a 2.3-kb SalI-digested *loxP* Sp interposon inserted into the XhoI site located at codon 147 of *rpoE2*. pJMS32 is a pJMS28 derivative with a 2.3-kb fill-out ClaI-digested *loxP* Sp interposon inserted into the EcoRV site located at codon 89 of *rpoE3*. pJMS33 is a pJMS29 derivative with a 2.3-kb ClaI-digested *loxP* Sp interposon inserted into the ClaI site located at codon 90 of *rpoE4*. pJMS34 is a pJMS29 derivative with a 2.3-kb ClaI-digested *loxP* Sp interposon inserted into the NarI site located at codon 94 of *tcrX*.

The primer pairs RechE4uh-ch3274wb and ch3274E4ub-RerpoE4we were used to amplify fragments containing the *tcrX* (1,257-bp) and *rpoE4* (1,253-bp) genes, respectively, from strain CE3 by PCR. Both fragments were digested with BamHI, ligated with T4 DNA ligase, and used as templates to amplify a fragment with the RechE4uh and RerpoE4we primers. The 2,510-bp amplification product, which contained a deletion of the *rseF* (CH03274) gene, was cloned into the HindIII/EcoRI site of pK18mobsacB to give pJMS35. The pJMS36 plasmid is a pJMS35 derivative with a 2.3-kb BamHI-digested *loxP* Sp interposon inserted into the BamHI site located 33 bp upstream of *rpoE4*. This construct has *rseF* deleted by replacement with the *loxP* Sp interposon (ΔCH03274::*loxP* Sp).

The 1,267-bp BamHI-EcoRI PCR product generated by using ch3274ub and RerpoE4we primers was cloned into the BamHI/EcoRI sites of pFAJ1708 to produce pJMS37; this construct has the *rpoE4* gene under the control of the *nptII* promoter.

The *R. etli rpoE1*, *rpoE2*, *rpoE3*, *rpoE4*, *trcX*, and *rseF* mutants were obtained by the replacement of the wild-type allele with the *rpoE1*::*loxP* Sp (pJMS30), *rpoE2*::*loxP* Sp (pJMS31), *rpoE3*::*loxP* Sp (pJMS32), *rpoE4*::*loxP* Sp (pJMS33), *tcrX*::*loxP* Sp (pJMS34), and ΔCH03274::*loxP* Sp (pJMS35) alleles, respectively. For this purpose, the corresponding plasmid was mobilized from *E. coli* to *R. etli* by triparental mating, the single recombinants (exhibiting a streptomycin-resistant [Str^r], Sp^r, kanamycin-resistant [Km^r] phenotype) obtained by plasmid cointegration were plated onto PY medium with sucrose at 7.5 or 10% (wt/vol), and double recombinants were screened by detection of the Sp^r Km^s sucrose-resistant (Sac^r) phenotype.

To generate a nonpolar *rseF* mutation, the *loxP* Sp interposon was excised from the CFNX3274Sp strain by using the *loxP*-specific Cre recombinase expressed by the pJMS8 plasmid (38). Losses of the Sp^r marker and pJMS8 were screened by detection of the Sp^s, tetracycline-sensitive (Tc^s) phenotype.

Microarray hybridizations and analysis. For microarrays, 70-mer oligonucleotides representing all (6,034) predicted *R. etli* open reading frames (ORFs) were designed by E. Salazar et al. (submitted for publication), synthesized by MWG-Biotech (Ebersberg, Germany), and spotted in duplicate onto superamide-coated slides (25 by 75 mm; TeleChem International, Inc.) by means of a high-speed robot at the microarray facility at the Instituto de Fisiología Celular/UNAM. Total RNAs from exponential-phase cultures grown in MM were obtained using the RNeasy minikit (Qiagen). The RNA was reverse transcribed and labeled using the CyScribe first-strand cDNA labeling kit according to the instructions of the manufacturer. Briefly, the RNA concentration was determined by measuring the absorbance at 260 nm, and RNA integrity was evaluated by electrophoresis on 1.3% agarose. Samples of 10 μg of RNA were reverse transcribed and differentially labeled with Cy3-dCTP and Cy5-dCTP by using the CyScribe first-strand cDNA labeling kit (Amersham Biosciences). Pairs of Cy3- and Cy5-labeled cDNA samples were mixed and hybridized to the array as described by Hegde et al. (25). After being washed, the array was scanned with a Scan Array Lite microarray scanner (Perkin-Elmer, Boston, MA) using a 10-μm pixel size. Microarray spot detection was carried out, mean signals and mean local background intensities were determined, and image segmentation and signal quantification were performed by using the Array-Pro Analyzer 4.0 software (Media Cybernetics, L.P). Microarray data analysis was performed with GenArise software, developed in the computing unit at the Instituto de Fisología Celular/UNAM (http://www.ifc.unam.mx/genarise/). This software identifies the genes expressed differentially by calculating an intensity-dependent *Z*-score. It uses a sliding-window algorithm to calculate the mean and standard deviation within the window surrounding each data point and defines a *Z*-score, where *Z* represents the number of standard deviations of a data point from the mean, as follows: $Z_i = [R_i \cdot \text{mean}(R)]/\text{sd}(R)$, where Z_i is the *Z*-score for an individual element, mean(R) is the mean $\log_2$ ratio of the duplicate of the same element, R_i is the $\log_2$ ratio for the individual element, and sd(R) is the standard deviation of the $\log_2$ ratio. With these criteria, the elements with *Z*-scores of >2 or <-2 were considered to be significantly differentially expressed. The DNA microarray experiments were performed three times with the RNAs isolated from independent cultures.

Consensus promoter sequences were obtained from consensus matrices generated and selected by using WCONSENSUS v5c (28). Briefly, sequences upstream (bp −350 to +10 relative to the translation start site) from the genes identified in the microarray analysis were used as input for CONSENSUS to identify the motifs. Sequences containing promoters were aligned using SeqView version 1.0.1 (ftp://gimr.garvan.unsw.edu.au/pub/) and displayed using WebLogo version 2.8.2 (10). The best matrix was selected to deduce the consensus sequence. The prediction of other promoters was done using the genome-scale gene pattern program from the Regulatory Sequence Analysis Tools website (http://embnet.ccg.unam.mx/rsa-tools/) (54).

To carry out semiquantitative reverse transcriptase PCR (RT-PCR) experiments, the RNA was incubated with 1 U μg^{-1} RNase-free DNase I according to the instructions of the manufacturer (Fermentas Life Sciences) and the absence of DNA contamination was confirmed by PCR. Amplifications were performed with the Thermoscript RT-PCR system (Invitrogen) and with a reduced number of cycles, in order to avoid the plateau of the DNA amplification reaction. The relative quantification of gene expression was done using the CH02950 gene as an endogenous control. The PCR products were separated by agarose gel electrophoresis, and the intensities of the products were quantified by using the Gel Logic 100 imaging system and molecular imaging software (Kodak).

Nodulation and nitrogen fixation determination. *P. vulgaris* cv. Negro Jamapa seed surfaces were sterilized, and the seeds were germinated on sterile trays containing sterile vermiculite. Three-day-old seedlings were transferred into 1-liter plastic pots with sterile vermiculite and inoculated with 1 ml of overnight culture (in PY medium). Plants were kept in a culture room at 25°C under a 12-h light/dark cycle and watered with a nitrogen-free nutrient solution (16). Acetylene reduction assays for nitrogenase activity were carried out as described by Girard et al. (20).

β-Glucuronidase activity measurements. Exponential cultures of strains harboring the *uidA* transcriptional fusions were grown on MM (to an OD_{600} of ~0.3) and incubated with H_2O_2 at 1 mM, NaCl at 100 mM, or sucrose at 10% (wt/vol) for 45 min. Bacterial cultures containing the pJMS24, pJMS25, or pGUSprpoH2 plasmid were grown in MM under aerobic or microaerobic conditions, and samples at exponential growth phase (OD_{600}, ~0.3) and stationary growth phase (OD_{600}, ~0.8) were collected. Quantitative β-glucuronidase assays were performed with 4-nitrophenyl-β-D-glucuronide as the substrate as described previously (20). Nodules were isolated and stained for β-glucuronidase activity as described previously (42).

RESULTS

Phenotypes associated with *rpoE* homologs in *R. etli*. The four *R. etli* RpoE factors are phylogenetically related to *E. coli* RpoE and have the 2.1, 2.4, 4.1, and 4.2 subregions, typical domains of the ECF σ factors, conserved (see Fig. S1 in the supplemental material). In addition, *rpoE1*, *rpoE2*, and *rpoE3* are present downstream of, and oriented in the same transcriptional direction as, an ORF encoding a product with anti-σ domain-transmembrane, or COG5662, domains, common in proteins that perform as anti-σ factors (9). The fourth homolog, *rpoE4*, is present upstream of, and oriented in same transcriptional direction as, the ORF CH03274 (*rseF*). Upstream of *rseF*, transcribed in the opposite direction, is ORF CH03275 (*tcrX*) (see Fig. S1 in the supplemental material). Genes similar to *rseF* were identified in *Sinorhizobium meliloti* as ORF SMc01505, a negative regulator of *S. melitoli rpoE2*

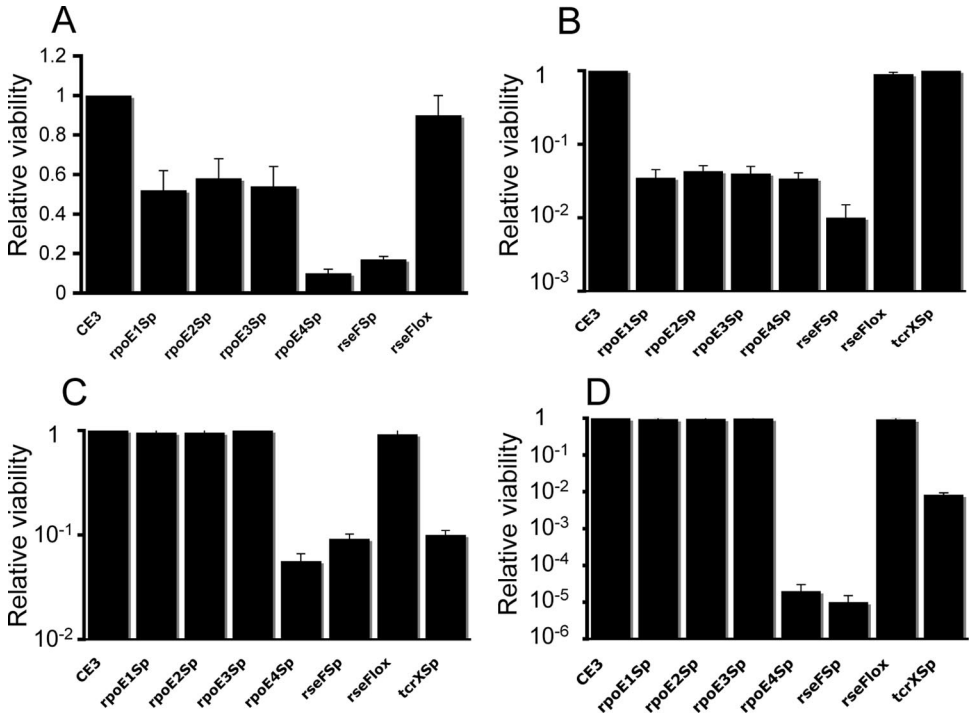


FIG. 1. Roles of the *tcrX* and *rpoE* genes of *R. etli* in responses to oxidative and osmotic stresses. The viabilities of the CFNXE1Sp (*rpoE1* Sp), CFNXE2Sp (*rpoE2* Sp), CFNXE3Sp (*rpoE3* Sp), CFNXE4Sp (*rpoE4* Sp), CFNXΔ3274Sp (*rseF* Sp), CFNXΔ3274lox (*rseF lox*), and CFNXTXSp (*tcrX* Sp) strains relative to that of wild-type strain CE3 in the presence of H_2O_2 (A), methyl viologen (B), NaCl (C), and sucrose (D) are expressed as the surviving fractions of the mutant strain populations divided by the surviving fraction of the CE3 population. The surviving fraction was calculated as the number of viable cells after treatment with or in the presence of the compound, divided by the number of viable cells in the absence of stress. The surviving fractions of the CE3 populations in the presence of H_2O_2, methyl viologen, NaCl, and sucrose were 0.02, 0.92, 0.93, and 0.93, respectively. The data presented are the averages of results from at least three independent experiments. For H_2O_2 treatment, exponential-phase cultures (OD_{600}, ~0.3) were incubated with 5 mM H_2O_2 for 45 min. For the other treatments, the bacteria were grown in PY medium containing methyl viologen (40 μM), NaCl (80 mM), or sucrose (15%, wt/vol).

(homologous to *R. etli rpoE4*) (48), and in *Caulobacter crescentus* as ORF CC3476, which has activity similar to that of the *S. meliloti* ORF and is present upstream of the σ^T gene (homologous to *R. etli rpoE4*) (4). On the other hand, TcrX is highly similar to *Methylobacterium extorquens* PhyR and includes regions homologous to RpoE-like (COG1595) and CheY-like receiver sensor (COG0784) domains. In *M. extorquens*, PhyR regulates a large number of genes involved in responses to different stresses (22).

In order to evaluate the roles of the four putative *R. etli rpoE* homologs, we constructed individual interruptions with the *loxP* Sp interposon in each one of them (see Materials and Methods). The mutant strains had growth rates, colony morphologies, and levels of viability similar to those of the parental strain at 25°C. They were also able to establish symbiotic interactions with *P. vulgaris* roots, and 28 days after inoculation, the nodules could perform nitrogen fixation at levels similar to the wild-type strain (data not shown). Additionally, none of the mutations affected the sensitivities of the carrier strains to heat shock (20 min at 42 or 55°C in exponential or stationary phase) compared with that of the parental strain (data not shown).

Since ECF σ factors are involved in the responses to environmental stresses, we evaluated whether any of the *R. etli rpoE* homologs were implicated in the responses to different challenges: oxidative, saline, and osmotic. The oxidative challenge was exerted by exposure of the cells to the strong oxidant H_2O_2 or to the superoxide generator methyl viologen. The *rpoE1*::Sp, *rpoE2*::Sp, and *rpoE3*::Sp mutants displayed the same degrees of tolerance toward sodium chloride (80 mM) and sucrose (15%) as the wild-type strain, but they were sensitive to oxidative agents, with approximately 50% decreases in viability after H_2O_2 shock (exposure to 5 mM H_2O_2 for 45 min during exponential phase) and 23-fold decreases in viability in the presence of methyl viologen (40 μM) compared to the viability of the parental strain (Fig. 1). In contrast, the *rpoE4*::Sp mutant was significantly more vulnerable than the wild type to all challenges, exhibiting 10-fold-higher sensitivity to H_2O_2, 24-fold-higher sensitivity to methyl viologen, 17-fold-higher sensitivity to NaCl, and 50,000-fold-higher sensitivity to sucrose (Fig. 1). These results indicate that *rpoE1*, *rpoE2*, and *rpoE3* participate in the oxidative stress responses elicited by methyl viologen and H_2O_2 and that *rpoE4* may be involved in more general responses to different kinds of environmental challenges, implying an important and general role. With these results and with the aim of pinpointing other elements involved in the regulatory network comprising the *rpoE4* ECF σ factor, we decided to explore the regulation of *rpoE4*, as well as identify the genes regulated by its product.

Different mutants were constructed with the aim of identifying the physiological roles of *rseF* (*r*egulator of *s*igma *rpo*E*4*;

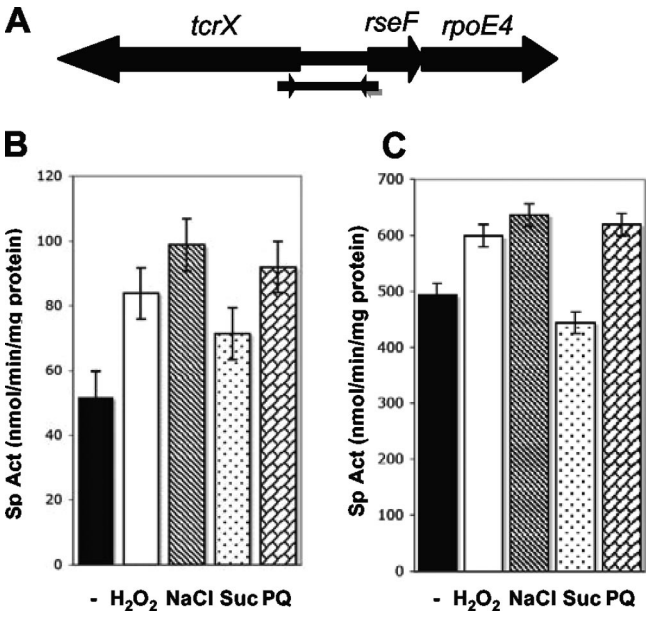


FIG. 2. Physical organization of *R. etli rseF-rpoE4* and *tcrX* genes and expression under different stress conditions. (A) Genomic organization of the *rseF-rpoE4* and *tcrX* region. The fragment cloned upstream of *uidA* into pBBMCS53 is indicated by the lower diagram. (B and C) Levels of expression of the *rpoE4-uidA* (B) and *trcX-uidA* (C) transcriptional fusions under oxidative, saline, or osmotic stress. Exponential-phase cultures (OD_{600}, ~0.3) were grown on MM, and CE3/pJMS24 and CE3/pJMS25 strains were incubated with 1 mM H_2O_2, 100 mM NaCl, 10% (wt/vol) sucrose (Suc), or 40 μM methyl viologen (PQ) for 45 min. Specific activity (Sp Act) was determined by using β-glucuronidase activity. The data shown are the averages of results from at least three independent experiments, and the vertical bars represent the standard deviations. −, no stress treatment.

CH03274) and *trcX*, genes located in the *rpoE4* region (see Fig. 2A). The mutations included two deletions/substitutions, expressed in the mutant strains CFNXΔ3274Sp and CFNXΔ 3274lox (carrying polar and nonpolar mutations in relation to *rpoE4*, respectively). A third mutant (the CFNXTXSp strain) harbored a *tcrX* (CH03275) gene inactivated by the insertion of a *loxP* Sp interposon. The *rseF*::*loxP* Sp polar insertion mutant exhibited degrees of sensitivity to all environmental challenges similar to those observed in the *rpoE4* mutant. In contrast, the sensitivities of the *rseF*::*loxP* nonpolar insertion mutant were similar to those of the wild-type strain (Fig. 1), indicating that *rseF* and *rpoE4* form an operon. The methyl viologen sensitivity caused by the inactivation of *trcX* was similar to that of the parental strain. Interestingly, the *trcX* inactivation mutant was significantly more sensitive than the wild-type strain to exposure to sucrose and NaCl, suggesting a role for *trcX* in the response to osmotic stress (Fig. 1).

Expression profile of *R. etli rpoE4*. To evaluate *rseF-rpoE4* and *tcrX* expression under different free-living conditions, we cloned the 570-bp region upstream of *rseF* into pBBMCS53 in both orientations relative to the *uidA* gene (see Materials and Methods) to create *rpoE4-uidA* (pJMS24) and *tcrX-uidA* (pJMS25) transcriptional fusions (Fig. 2A). In the wild-type strain CE3, the *rpoE4* promoter was induced 1.6-fold by H_2O_2, 1.9-fold by NaCl, 1.4-fold by sucrose, and 1.8-fold by methyl viologen (Fig. 2B) while the *tcrX* promoter was induced 1.2-fold by H_2O_2, 1.3-fold by NaCl, and 1.2-fold by methyl viologen but was not induced by sucrose (Fig. 2C). In addition, induction of the *rpoE4* promoter (2.2- and 2.9-fold) in the stationary phase under aerobic and microaerobic conditions, respectively, was observed (Fig. 3A). In contrast, the levels of *tcrX-uidA* expression under the different conditions were similar and independent of the oxygen concentration, with a slight induction (1.4-fold) only in stationary phase under microaerobic conditions (Fig. 3B).

In other alphaproteobacteria, such as *Brucella melitensis*, *C. crescentus*, and *S. meliloti*, *rpoE4* homologs regulate the expression of *rpoH* (4, 13, 48). In order to elucidate whether the same cascade is present in *R. etli*, the expression of an *rpoH2-uidA* fusion was evaluated. With the aim of characterizing the genetic regulation of *rpoE4*, *tcrX*, and *rpoH2*, transcriptional fusions comprising these genes were conjugated into two differ-

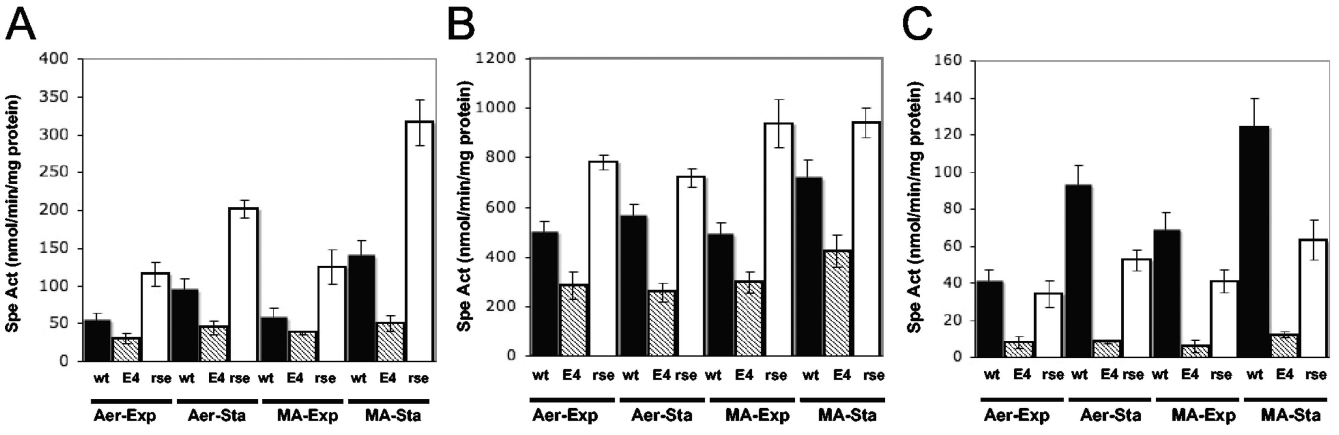


FIG. 3. The *R. etli rpoE4* gene is autoregulated and controls the expression of the *rpoE4*, *trcX*, and *rpoH2* genes under different conditions. The *rpoE4-uidA* (A), *trcX-uidA* (B), and *rpoH2-uidA* (C) transcriptional fusions were expressed in the wild-type (wt), *rpoE4*::Sp (E4), and Δ*rseF*::*lox* (rse) genetic backgrounds. The strains containing pJMS24, pJMS25, or pGUSprpoH2 were grown in MM under aerobic (Aer) or microaerobic (MA) conditions, and samples were collected at exponential (Exp; OD_{600}, ~0.3) and stationary (Sta; OD_{600}, ~0.8) growth phases. Specific activity (Sp Act) was determined by using β-glucuronidase activity. The data shown are the averages of results from at least three independent experiments, and the vertical bars represent the standard deviations.

ent mutant backgrounds: those of the CFNXE4Sp strain (lacking *rpoE4*) and the CFNXΔ3274lox strain (carrying a nonpolar *rseF* interruption). Under all tested conditions, the three constructs presented reduced expression (35 to 65%) in the CFNXE4Sp mutant background relative to wild-type levels, with the decline of *rpoH2* expression being the most dramatic (Fig. 3). These results indicate that RpoE4 regulates the expression of the transcriptional regulators *tcrX* and *rpoH2*, which are involved in the responses to heat, oxidative, saline, and osmotic stresses. Moreover, *rpoE4* expression, which was induced by microaerobic and stationary growth conditions, was autoregulated. The basal activity in the *rpoE4* mutant suggests that RpoE4, along with another sigma factor(s), transcribes the *tcrX* gene under aerobic and microaerobic conditions; also, *tcrX* induction under microaerobic conditions seems to be independent of RpoE4. In clear contrast, the expression of the three fusions in the CFNXΔ3274lox mutant background was enhanced under all testing conditions. Moreover, the expression of *rpoE4*, *tcrX*, and *rpoH2* in *rpoE4* and *rseF* mutant backgrounds was not affected by oxidative or osmotic stress under the same growth conditions (data not shown). These results suggest that *rseF*, either directly or indirectly, is a negative regulator of the *rseF-rpoE4* operon and also of *rpoH2*.

Identification of genes regulated by the *rpoE4* sigma factor. With the aim of identifying the *R. etli* genes regulated by RpoE4, transcriptome analyses of the *rpoE4* mutant and the *rpoE4*-overexpressing strain (CE3/pJMS37) were performed. Total RNAs from exponential-phase cultures, grown on MM without treatment, were obtained and used in microarray experiments. A total of 64 genes in the *rpoE4* mutant were downregulated with respect to those in the wild type (Z-score < -2); 48 of them were located on the chromosome, and the other 16 were carried in large plasmids (mainly pSym and pCFN42e) (Table 2). Thirteen of the genes code for unknown proteins, although some of these proteins present domains, such as the PCR barrel domain (CH00479 and CH00851) and the MutT/nudix domain (CH00806 and CH01407), similar to those of proteins involved in the stress response. Moreover, putative zinc and iron transport proteins (CH02683, CH02712, and CH04056) may also be implicated in the stress response. Fifteen genes, including *agaL1*, *atpH*, *ggt2*, and *aglE*, encoding proteins potentially involved in metabolism and 13 genes encoding products, including PheA, RpmG, and RpsC, etc., involved in protein biosynthesis were downregulated in the *rpoE4* mutant, suggesting differences in metabolism between the *rpoE4* mutant and the wild type. Five *rpoE4*-regulated genes, three (pE00108, pE00148, and pE00373) located in the pCFN42e plasmid and two (CH01253 and CH03555) on the chromosome, encode proteins with a putative role in cell wall biogenesis, while four genes encode unknown proteins with domains associated with the cell envelope. Interestingly, eight genes encode proteins involved in gene regulation, as components of signal transduction systems (PF00269, PE00176, CH02204, and *tcrX*) and as transcriptional regulators (CH00371, CH3529, CH04025, and *rpoH2*).

To overexpress the *rpoE4* sigma factor, the gene from strain CE3 was amplified by PCR and cloned under the control of the *ntpII* promoter in pFAJ1708 (14), generating plasmid pJMS37 (see Materials and Methods). Total RNAs from CE3/pJMS37 and CE3/pFAJ1708 in exponential-phase cultures grown in MM were obtained and used in the microarray experiments. Thirty-eight genes in the *rpoE4*-overexpressing strain were upregulated (Z-score > 2) with respect to those in the wild type; 28 of them are located on the chromosome, while 10 are in large plasmids (Table 3). Six *rpoE4*-regulated genes may be involved in stress responses, CH02434 and *xthA1* in DNA repair and CH00462 and CH03474 in the response to oxidative stress, while CH00965 and CH01802 encode CsbD-like proteins. Three genes encode proteins potentially implicated in metabolism, and two encode proteins possibly involved in gene regulation. Curiously, a large number (25 of 38) of the *rpoE4*-upregulated genes encode proteins with unknown functions, and some of the genes (15 of 38) encode proteins without domains or motifs. Interestingly, 16 of the unknown proteins were predicted (using the PSORT program) to be associated with the cell envelope. These data suggest that *rpoE4* regulates the transcription of the genes related to stress responses, as well as those related to cell envelope biogenesis.

In order to validate the results of the microarray experiments, semiquantitative RT-PCR was performed with six selected genes identified as putative members of the *rpoE4* sigmulon. For this analysis, the gene expression levels in the CE3/pFAJ1708 and CE36/pJMS37 strains in the absence of treatment were compared. Increases of 1.99-, 2.15-, 1.85-, 2.05-, 2.43-, and 2.59-fold in the expression of the genes CH00268, CH00479, CH01335, CH01732, CH02453, and *tcrX*, respectively, in the *rpoE4*-overexpressing strain with respect to the expression in the parental strain were observed (see Fig. S2 in the supplemental material). In the *rpoE4* mutant, a 4.28-fold reduction in the expression of the *tcrX* gene with respect to that in the wild type was observed, while the genes CH00479, CH01732, CH00268, CH01335, and CH02453 were not expressed in the *rpoE4* mutant (after 35 cycles, RT-PCR products were not observed; data not shown). These results validate the findings of our microarray experiments, indicating that the genes are directly or indirectly regulated by *rpoE4*.

A search for an *rpoE4* consensus promoter in the regions from −350 to +10 relative to the translation start sites of the *rpoE4*-regulated genes identified by transcriptome analysis was performed with both the *rpoE4* Sp mutant and the *rpoE4*-overexpressing strain. WCONSENSUS v5s (28) was used to search for a conserved motif. The sequence GGAAC-N16/17-CGTT was found in 50 of 98 genes; 19 of these 50 genes were identified in the *rpoE4* mutant, and 35 were identified in the *rpoE4*-overexpressing strain (Fig. 4).

DISCUSSION

Several sigma factor genes are present in the alphaproteobacterial genomes; most of them belong to the ECF σ subfamily. Twenty-three sigma factors are found in *R. etli* (20): 1 σ^{70} factor (encoded by *rpoD*), 2 σ^{54} factors (encoded by *rpoN*), 2 σ^{32} factors (encoded by *rpoH*), and 18 ECF σ factors. Intriguingly, four ECF σ factors are homologous to the *E. coli* σ^{E} factor, while no homolog of the general stress sigma factor σ^{S} is found (21). In this study, we describe the role, regulation, and sigmulon of the RpoE4 sigma factor in *R. etli*.

Our initial approach was to generate mutations in the *R. etli rpoE* genes. The inactivation of *rpoE1*, *rpoE2*, and *rpoE3* resulted in only slight sensitivity to oxidative stress caused by

TABLE 2. Genes downregulated in the *rpoE4*::Sp mutant with respect to those in the wild type

Gene[a]	*Z*-score[b]	Gene name and/or description of gene product[c]	Function(s)
RHE_CH00131	−2.79	*pheA*; prephenate dehydratase	Phe biosynthesis
RHE_CH00180*	−2.05	Putative inner membrane protein	Cell envelope protein with unknown function
RHE_CH00265*	−2.57	Hypothetical conserved protein	Unknown
RHE_CH00371	−2.94	HTH_XRE transcriptional regulator protein	Gene regulation
RHE_CH00479*	−2.79	Protein with conserved PCR barrel domain	Unknown
RHE_CH00494	−2.64	Aldo/keto dehydrogenase protein	Metabolism
RHE_CH00696	−2.39	*aglE*; alpha-glucoside ABC transporter	Metabolism
RHE_CH00725*	−2.59	Putative nucleotidyltransferase protein	Unknown
RHE_CH00806*	−2.04	NTP pyrophosphohydrolase, MutT/nudix family	Stress response
RHE_CH00821*	−2.07	Histidyl-tRNA synthetase protein	Protein biosynthesis
RHE_CH00851*	−2.12	PCR barrel domain membrane protein	Cell envelope protein with unknown function
RHE_CH00900	−2.37	Hypothetical protein	Unknown
RHE_CH00926	−2.11	Hypothetical protein	Unknown
RHE_CH00996	−2.86	Putative dehalogenase-like hydrolase protein	Unknown
RHE_CH01253	−2.08	Putative peptidoglycan binding protein	Cell wall degradation
RHE_CH01407	−2.12	NTP pyrophosphohydrolase protein, MutT/nudix family	Stress response
RHE_CH01620	−2.11	*birA*; biotin-acetyl-CoA carboxylase ligase	Coenzyme biosynthesis
RHE_CH01634	−2.29	*rpmG*; 50S ribosomal protein L33	Protein biosynthesis
RHE_CH01663	−2.86	*rplK*; 50S ribosomal protein L11	Protein biosynthesis
RHE_CH01670	−2.15	*rpsL*; 30S ribosomal protein S12	Protein biosynthesis
RHE_CH01677	−2.24	*rplW*; 50S ribosomal protein L23	Protein biosynthesis
RHE_CH01681	−2.71	*rpsC*; 30S ribosomal protein S3	Protein biosynthesis
RHE_CH01682	−3.26	*rplP*; 50S ribosomal protein L16	Protein biosynthesis
RHE_CH01683	−2.6	*rpmC*; 50S ribosomal protein L29	Protein biosynthesis
RHE_CH01684	−2.19	*rpsQ*; 30S ribosomal protein S17	Protein biosynthesis
RHE_CH01685	−2.21	*rplN*; 50S ribosomal protein L14	Protein biosynthesis
RHE_CH01688	−2.07	*rpsN*; 30S ribosomal protein S14	Protein biosynthesis
RHE_CH01690	−2.24	*rplF*; 50S ribosomal protein L6	Protein biosynthesis
RHE_CH01715*	−2.12	Carboxymuconolactone decarboxylase family protein	Metabolism
RHE_CH01976*	−2.34	Nucleotide binding protein with TIR-like domain	Unknown
RHE_CH02172*	−2.86	Conserved inner membrane protein	Cell envelope protein with unknown function
RHE_CH02204*	−2.33	Putative signal transduction protein with CBS domain	Gene regulation
RHE_CH02683	−2.44	Putative ferrichrome ABC transporter	Stress response
RHE_CH02712*	−2.86	Zinc uptake ABC transporter	Stress response
RHE_CH02862	−2.19	6-Phosphofructokinase protein	Metabolism
RHE_CH03273	−3.55	*rpoE4*; ECF σ factor protein	Gene regulation
RHE_CH03274*	−3.74	*rseF*; negative regulator of *rpoE4*	Gene regulation
RHE_CH03275*	−4.89	*tcrX*; two-component response regulator protein	Gene regulation
RHE_CH03453*	−2.71	Hypothetical conserved protein	Unknown
RHE_CH03529	−2.5	HTH homodimeric repressor	Gene regulation
RHE_CH03536	−2.04	*ggt2*; gamma-glutamyltranspeptidase protein	Metabolism
RHE_CH03555*	−3.33	LysM domain-containing 5′-nucleotidase/2′,3′-cyclic phosphodiesterase	Cell wall degradation
RHE_CH03873*	−2.04	*atpH*; ATP synthase delta subunit	Energy production
RHE_CH03925	−2.4	Short-chain dehydrogenase/reductase	Metabolism, stress response
RHE_CH03966	−2.19	Probable aldo/keto oxidoreductase protein	Metabolism
RHE_CH04025	−2.67	Transcriptional regulator, CarD-TRCF family	Gene regulation
RHE_CH04026*	−2.8	*rpoH2*; heat shock sigma factor	Gene regulation
RHE_CH04056	−2.17	*sfuA*; Fe(III) ABC transporter	Stress response
RHE_PC00212	−2.59	Probable C_4-dicarboxylate permease	Metabolism
RHE_PD00100	−2.63	Insertion sequence transposase	
RHE_PD00238	−2.46	Insertion sequence transposase	
RHE_PD00265	−2.15	Hypothetical protein	Unknown
RHE_PD00295	−2.45	*fixO*; cytochrome *c* oxidase	Energy production
RHE_PE00016	−2.11	Sugar transporter family protein	Metabolism
RHE_PE00052	−2.8	Putative alpha/beta-hydrolase family protein	Metabolism
RHE_PE00089	−2.56	*agaL1*; alpha-galactosidase	Metabolism
RHE_PE00099	−2.67	Sugar ABC transporter protein	Metabolism
RHE_PE00108	−2.83	PDZ_CTP periplasm protease	Cell envelope biogenesis
RHE_PE00148	−2.31	Pilus assembly Flp-like protein	Cell envelope biogenesis
RHE_PE00163	−4.79	Conserved outer membrane protein	Cell envelope protein with unknown function
RHE_PE00176	−2.75	Two-component response sensor regulator	Gene regulation
RHE_PE00373	−3.3	Fasciclin domain protein	Cell envelope biogenesis
RHE_PF00264	−2.62	Two-component sensor histidine	Gene regulation
RHE_PF00478*	−2.15	Hypothetical protein	Unknown

[a] RHE_CH, RHE_PD, RHE_PE, and RHE_PF indicate genes located on the chromosome and in pCFN42d (pSym), pCFN42e, and pCFN42f, respectively. * denotes genes with RpoE4 promoter motifs.

[b] Values are based on the $\log_2$ ratios of hybridization signals. Only genes with *Z*-scores of ≤ -2 are listed. The results shown are the averages of results from independent experiments.

[c] NTP, nucleoside triphosphate; acetyl-CoA, acetyl coenzyme A; TIR, Toll-interleukin-1 receptor; CBS, cystathionine-beta-synthase; HTH, helix-turn-helix; TRCF, transcription-repair coupling factor.

TABLE 3. Genes upregulated in the *rpoE4*-overexpressing strain with respect to those in the wild type

Gene[a]	*Z*-score[b]	Gene name and/or description of gene product	Function(s)
RHE_CH00180*	3.48	Putative inner membrane protein	Cell envelope protein with unknown function
RHE_CH00268*	3.85	Conserved hypothetical protein	Unknown
RHE_CH00462*	3.43	Putative Mn-catalase	Oxidative stress response
RHE_CH00479*	3.93	PCR barrel domain protein	Unknown
RHE_CH00965*	3.48	CsbD-like protein, stress inducible	Stress response
RHE_CH01004*	2.90	Putative inner membrane protein	Cell envelope protein with unknown function
RHE_CH01231*	3.25	Conserved inner membrane protein	Cell envelope protein with unknown function
RHE_CH01335*	3.07	Attachment or secretion protein	Cell envelope protein with unknown function
RHE_CH01537*	4.2	Hypothetical periplasm protein	Cell envelope protein with unknown function
RHE_CH01731*	3.00	*xthA1*; exonuclease III	DNA repair
RHE_CH01732*	3.57	Conserved inner membrane protein	Cell envelope protein with unknown function
RHE_CH01778*	3.94	Conserved outer membrane protein	Cell envelope protein with unknown function
RHE_CH01802*	3.15	CsbD-like protein, stress inducible	Stress response
RHE_CH02152	3.23	Hypothetical inner membrane protein	Cell envelope protein with unknown function
RHE_CH02153	3.36	Conserved cytoplasm protein	Unknown
RHE_CH02434*	2.19	Putative DNA alkylation repair protein	DNA repair
RHE_CH02435*	2.85	Putative signal transduction protein with CBS[c] domain	Gene regulation
RHE_CH02503*	3.65	Hypothetical conserved protein	Unknown
RHE_CH02573*	3.03	Conserved inner membrane protein	Cell envelope protein with unknown function
RHE_CH02632*	4.01	Hypothetical protein	Unknown
RHE_CH02635*	3.47	Putative inner membrane protein	Cell envelope protein with unknown function
RHE_CH02742	2.4	Beta subunit of coenzyme F420 dehydrogenase	Energy production
RHE_CH02743*	3.75	Hypothetical conserved protein	Unknown
RHE_CH03254*	3.77	Conserved inner membrane protein	Cell envelope protein with unknown function
RHE_CH03273*	2.95	*rpoE4*; ECF σ factor	Gene regulation
RHE_CH03274*	1.9	*rseF*; negative regulator of *rpoE4*	Gene regulation
RHE_CH03404*	2.11	Conserved inner membrane protein	Cell envelope protein with unknown function
RHE_CH03474*	3.20	Putative pyridoxine-phosphate oxidase	B_6 biosynthesis, stress response
RHE_PB00004*	2.40	Hypothetical protein	Unknown
RHE_PB00039*	2.83	Putative inner membrane protein	Cell envelope protein with unknown function
RHE_PB00040*	3.87	Conserved periplasm protein	Cell envelope protein with unknown function
RHE_PE00299*	3.48	Short-chain dehydrogenase/reductase family protein	Metabolism, stress response
RHE_PF00044*	4.34	Hypothetical outer membrane protein	Cell envelope protein with unknown function
RHE_PF00051*	2.32	Hypothetical conserved protein	Unknown
RHE_PF00085*	2.04	Hypothetical inner membrane protein	Cell envelope protein with unknown function
RHE_PF00247*	3.33	Phospholipase D, PLDc family	Lipid biosynthesis, cell envelope biogenesis
RHE_PF00261*	4.15	Conserved cytoplasm protein	Unknown
RHE_PF00277*	2.60	Putative alpha-amilase	Metabolism

[a] RHE_CH, RHE_PB, RHE_PE, and RHE_PF indicate genes located on the chromosome and in pCFN42b, pCFN42e, and pCFN42f, respectively. * denotes genes with RpoE4 promoter motifs.

[b] Values are based on the $\log_2$ ratios of hybridization signals. Only genes with *Z*-scores of ≥2 are listed. The results shown are the averages of results from independent experiments.

[c] CBS, cystathionine-beta-synthase.

methyl viologen and H_2O_2, while the disruption of *rpoE4* resulted in sensitivities to agents that cause saline, osmotic, and oxidative stresses. To date, few studies of the roles of ECF σ factors in alphaproteobacteria have been described. The most closely related homolog of *R. etli rpoE4*, *rpoE2* in *S. meliloti*, is not associated with any phenotype (48), while in *C. crescentus*, the inactivation of σ^T provokes sensitivities to osmotic and oxidative stresses (4). Also in *C. crescentus*, σ^F is involved in the oxidative response of stationary-phase cultures (3).

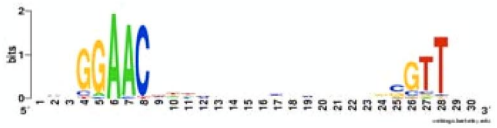
In several free-living alphaproteobacteria, the *rpoE4* region is highly syntenic (4, 48; http://mbgd.genome.ad.jp/), suggesting important roles for the *tcrX*, *rseF*, and *rpoE4* genes. We studied the roles of these genes, as well as their transcriptional regulation. Using the ΔCH03274::*loxP* Sp allele, we generated a mutant with a phenotype similar to the one shown by the *rpoE4* Sp strain; this finding implies that the mutation has a polar effect relative to *rpoE4* and that CH03274 and *rpoE4* form an operon. In addition, the deletion of the *loxP* Sp interposon by using the *loxP*-specific Cre recombinase generates a nonpolar mutation, and the resulting strain displays a phenotype similar to that of the parent strain. In the closely related species *S. meliloti*, the homologous genes SMc01505 and SMc01506 also form an operon, but SMc01505 cannot be disrupted (48). With respect to *trcX*, the associated phenotype indicates that this gene is involved in the responses to saline and osmotic stresses but not in the response to oxidative stress. In *M. extorquens* AM1, the *phyR* gene (highly homologous to *trcX*) is involved in the responses to heat shock, desiccation, and oxidative, UV, ethanol, and osmotic stresses, as well as in phyllosphere colonization (22). In addition, the phosphorylated PhyR form interacts with the anti-sigma factor NepR and therefore acts as anti-anti-sigma factor (18). Due to the high level of similarity between PhyR and TcrX, more studies are needed to clarify whether TcrX acts as a transcriptional regulator, as a sigma factor, or as an anti-anti-sigma factor for *rseF*.

Our results demonstrate that *R. etli rpoE4* regulates its own transcription and that it is negatively regulated by *rseF*. They also demonstrate that the gene is inducible by saline and os-

A

```
RHE_CH00180          cccGGAACcttccaga cggccaccCGTTtt-N52-atg
RHE_CH00265          ggaGGAACtcgatcag ctcggtgaatcTaa-N157-atg
RHE_CH00268          atcGGAACaaattgcg cgatcttcgcTTcg-N48-atg
RHE_CH00462          cggGGAACattcctgc gataggtcgGTTag-N29-atg
RHE_CH00479          ccgGGAACtttgagtc cgccgtgaCGTTga-N42-atg
RHE_CH00725          gctGGAcCtttccgcg gcttgacagGcTgc-N51-atg
RHE_CH00806          tatGAAACacgcaaaa acaaatcgCGTTcg-N91-atg
RHE_CH00821          ggatGAACtttcgatg cggcttgcCGgTca-N41-atg
RHE_CH00851          accGGAACcaatcggc gcaccactCaTTtt-N76-atg
RHE_CH00965          gcgGGAACgaataggc gcgagtgaCGTTcc-N48-atg
RHE_CH01004          acaGGAAtgagggaga atgaggagaGTggc-N28-atg
RHE_CH01231          gccGGAACaacctcgccgtccctggCGTTcg-N22-atg
RHE_CH01335          tctcGAACtctttcaa cggccggaCGTTct-N53-atg
RHE_CH01537          tgcGGAACtatgccgt atggccggCGTTgc-N34-atg
RHE_CH01715          tcccGAACtgaaggtg caggattgCtgTcc-N44-atg
RHE_CH01731          aagtGAACcaaagaac gcggcgtgCcTccg-N33-atg
RHE_CH01732          tctGGAACttctttgc tcgtcatcgcTTtg-N29-atg
RHE_CH01778          atcGGAACaatgaatc ctgcaagtgGTTac-N40-atg
RHE_CH01802          atcGGAACccttatcg ctcctctcCGTTac-N28-atg
RHE_CH01976          gcgGGAcCatattgca tgcacaccgGTTtc-N59-atg
RHE_CH02172          ggcGGAACctttcgggccgccatttCGTTga-N29-atg
RHE_CH02204          tgcGGAACcgagggga acggagaagGaTct-N108-atg
RHE_CH02434          ggctGAACtgatcgcgcatctcgaaCGcTgc-N24-atg
RHE_CH02435          gcaGGAACgtttccga atggtgcgCGTTca-N48-atg
RHE_CH02503          cttGGAACcaactccg ctccccatCGTTgg-N36-atg
RHE_CH02573          agaGAAACttattttc tcggtccgCGTTat-N33-atg
RHE_CH02632          catGAAACttctcgtc ccggatcggcTTct-N27-atg
RHE_CH02635          acaaGAACcggaatac tccccactCcTaat-N152-atg
RHE_CH02712          catcGAAttttccgaaagcgctgttgcaTcc-N73-atg
RHE_CH02743          ttgGGAACggaactgg agttgatcgGTTag-N31-atg
RHE_CH03254          gcgGGAACctcgattt gacgacatCGTTag-N31-atg
RHE_CH03274(rseF)    gcaGGAACtttttttg tcgcgccgCaTTat-N140-atg
RHE_CH03274(rseF)    agtGGAACggtcggtgtcggtggttgaTTtc-N44-atg
RHE_CH03275(tcrX)    tccGGAACttttttac caaggcagCGTTaa-N46-atg
RHE_CH03404          ttcGGAACctatcacc gtttgaggtGTTgt-N27-atg
RHE_CH03453          tatGGAACtattcttt cgatcatgCGTTtg-N39-atg
RHE_CH03474          gcgGcAACggtgatttcgcgcgcaagcTTgg-N175-atg
RHE_CH03555          gatGGAACatctgctcgctgggaaacCGccc-N62-atg
RHE_CH03873          tgccGAACagaattcatgtgaaaggCGTatt-N35-atg
RHE_CH04026(rpoH2)   attGGAACtttttccg cgctggcgCGTTca-N29-atg
RHE_CH04026(rpoH2)   ttcaGAACcccattcagtccggacagCGaTg-N60-atg
RHE_PB00004          tggGGAACttagatca gccgaattgGTTga-N21-atg
RHE_PB00039          gtccGAACaacaggagtgccggaggCGTTga-N27-atg
RHE_pB00040          acgGGAACtctgcgcc tgtccgcaaGTTtg-N24-atg
RHE_PE00299          gtgGGAACaaccagcc gcagcctgaGTTtg-N22-atg
RHE_PF00044          tgcGGAACaatgacgt cgtgacgagGTTgt-N40-atg
RHE_pF00051          tcgGGAACatccgacc catgcgagaGTTga-N31-atg
RHE_PF00085          tcgGcAACtccctcaatgatcatggtcTTtc-N58-atg
RHE_pF00247          tcgGGAACccgcgaaa gttccgggCGTTtg-N26-atg
RHE_PF00261          cccGGAACctccatct gcctggcgtGTTtt-N25-atg
RHE_PF00277          atgGGAACaacctccg tcaaaagaaGTTtg-N28-atg
RHE_PF00478          acgGcAACttccagcg ctggggtggGcTgt-N122-atg
```

B



C

```
R. etli rpoE4                GGAAC–N16/17-  cGTT
S. meliloti rpoE2            GGAAC–N16/17-  CGTTt
C. crescentus sigT           GGAAC–N16/17-  CGTTg
M. extorquens AM1 phyR       gGAAC–N16/17-  CGTT
M. tuberculosis sigH       c/gGGAAC –N17 -  cGTTg
E. coli rpoE                 ggAACt-N15/16- gGTC
S. coelicolor sigR           gGGAAT–N17 - g/cGTTg/c
R. sphaeroides rpoE           TGATCc-N15 -  cCGTA
B. subtilis sigW             tGAAAC-N16/17-  CGTA
P. aeruginosa algU             gAACTT–N16/17-TCtgA
```

FIG. 4. *R. etli rpoE4* motifs located in the regulatory regions of *rpoE4*-regulated genes. (A) Alignment of nucleotide sequences of upstream regions of *rpoE4*-regulated genes (motifs are shown in boldface, uppercase letters and are underlined); (B) WebLogo of the *rpoE4* consensus motifs; (C) alignment of *R. etli rpoE4* consensus motifs with promoter sequences of *S. meliloti rpoE2*, *C. crescentus sigT*, *M. extorquens* AM1 *phyR*, *M. tuberculosis sigH*, *E. coli rpoE*, *S. coelicolor sigR*, *R. sphaeroides rpoE*, *B. subtilis sigW*, and *P. aeruginosa algU* (4, 5, 17, 22, 34, 39, 41, 48).

motic and oxidative stresses, as well as microaerobic and stationary-phase growth conditions, but not heat shock. Moreover, at 21 days after inoculation, nodules formed by CE3/pJMS24 (carrying the *rpoE4-uidA* fusion) exhibited significant β-glucuronidase activity (data not shown), indicating that *rpoE4* is expressed during the symbiotic process and may have some role during symbiosis (see above). Similar results for *rpoE2* in *S. meliloti* (48) and the σ^T gene in *C. crescentus* (4) (homologous to *R. etli rpoE4*) have been reported previously. Furthermore, the products of *rseF* homologs in *S. meliloti* (48) and *C. crescentus* (4), as well as in other alphaproteobacteria, do not have transmembrane or anti-σ domains, suggesting a new regulation mechanism for the ECF σ factor.

In several bacteria, RpoE-like factors control the expression of other sigma factors under different conditions. RpoE regulates the expression of *rpoN*, *rpoH*, and *rpoD* in *E. coli* (2, 43, 44), σ^T controls σ^U and σ^R expression in *C. crescentus* (4), $rpoH_{II}$ in *Rhodobacter sphaeroides* is RpoE dependent (5), and the *rpoE5* and *rpoH2* genes in *S. meliloti* are RpoE2 dependent (48). We found that the expression of *rpoH2* in *R. etli* was RpoE4 dependent under aerobic and microaerobic conditions. This pattern was clearly observed in the *rpoE4* mutant, but not in the *rseF*::*lox* mutant and in the *rpoE4*-overexpressing strain (transcriptome data), suggesting that a second regulator is needed for transcription or for mRNA stability. Similar results for *E. coli rpoS* transcription, which is cyclic AMP-catabolite activator protein dependent, were reported previously (27); moreover, the promoter sequence recognized by RpoE4 is present in the upstream regions of *rpoH1* and ORF PF00052 (encoding an ECF σ factor) (see Table S2 in the supplemental material). In addition, in *R. etli*, *rpoH1* is involved mainly in oxidative and heat shock responses while *rpoH2* is involved in osmotic tolerance and oxidative stress. Both genes are also involved in the senescence of nodules in the symbiotic processes (37). These data suggest that *rpoE4* plays, directly or indirectly, a relevant role in survival under free-living conditions and possibly in the senescence of nodules as a master regulator of *rpoH1* and *rpoH2* (37).

The *rpoE4* sigmulon was identified using the *rpoE4* Sp mutant and the *rpoE4*-overexpressing strain under no-stress conditions; these analyses revealed that RpoE4 controls at least 98 genes, 50 of them containing a conserved motif in the upstream regulatory regions. Interestingly, the putative promoter consensus was identified in 35 of the 38 genes differentially expressed in the *rpoE4*-overexpressing strain but in only 19 of the 64 genes differentially expressed in the *rpoE4* Sp mutant. This result may be explained by the possibility that RpoE4 controls other regulators, i.e., *rpoH2* is repressed in the mutant, and therefore, more than one regulon may be affected. On the other hand, in the overexpressing strain, the large amount of RpoE4 RNA polymerase formed may transcribe principally *rpoE4*-dependent promoters. The putative consensus sequence identified here resembles the sequences of promoters recognized by the ECF σ factors, such as those of *E. coli rpoE*, *S.*

meliloti rpoE2, the *C. crescentus* σ^T gene, *M. extorquens phyR*, *Mycobacterium tuberculosis sigH*, *Bacillus subtilis sigW*, *P. aeruginosa algU*, *R. sphaeroides rpoE*, and *Streptomyces coelicolor sigR* (4, 5, 7, 17, 22, 26, 34, 39, 41, 43, 48) (Fig. 4). Among the genes regulated by *rpoE4* were (i) genes such as CH00371, CH02204, CH03529, CH04025, PE00176, PF00264, *tcrX*, and *rpoH2* that are involved in gene regulation and encode components of putative two-component systems, transcriptional regulators, or sigma factors; (ii) genes such as *rpoH2*, CH00462, CH02434, CH03474, *xthA1*, CH02712, and *sfuA* that are involved in stress responses (encoding a sigma factor, a putative Mn-catalase, an alkylation DNA repair protein, pyridoxine phosphate oxidase, exonuclease III, and Zn and Fe efflux transporters, respectively) and the CH00695 and CH01802 genes, whose products are homologous to CsbD-like proteins and are possibly involved in the general stress response (1); and (iii) genes such as CH01253, CH03555, and PF00247 (encoding peptidoglycan binding and cell wall degradation proteins and phospholipase D, respectively) with putative roles in cell envelope biogenesis. Intriguingly, a large number of genes that encode proteins with unknown functions that bear membrane domains were induced in the *rpoE4*-overexpressing strain; the gene products are possibly structural proteins from the cell envelope.

The genes regulated by the *rpoE2* sigma factor and by σ^T in two alphaproteobacteria, *S. meliloti* and *C. crescentus*, respectively, have been described previously (4, 48). In both cases, the ECF σ factor acts as a general regulator in response to several stress conditions and controls a large number of genes, consistent with our results. However, there are several differences among the sigmulons in *S. meliloti*, *C. crescentus*, and *R. etli*. In *S. meliloti*, a large number of genes regulated by *rpoE2* are located in pSymB (48) and some of them are also involved in the osmoadaptation response (15), while no plasmids are present in *C. crescentus* (4). In *R. etli*, a large number of genes controlled by *rpoE4* are located on the chromosome and about 25% are located in plasmids; these distributions suggest that the chromosome has an important role in the general stress response, although the large plasmids may also participate. In addition, using the bidirectional best fit, we found that only a few genes are shared among the sigmulons: *rpoE4*, *rseF*, *tcrX*, CH00965 (*csbD*-like gene), CH01802 (encoding a PCR barrel protein), and CH02435 (gene regulator) in *R. etli*, corresponding to SmrpoE2, SMc01505, SMc01504, SMa2071, SMb21330, and SMc21441 in *S. meliloti* and to CC3475, CC3476, CC3477, CC0938, CC0532, and CC2626 in *C. crescentus*, respectively. In addition, between the *R. etli* RpoE4 and *S. meliloti* RpoE2 sigmulons, some genes are shared: *rpoH2*, PF00247 (encoding phospholipase D), CH00268 (encoding a conserved protein), CH00851 (encoding a PCR barrel protein), CH01335 (encoding a membrane protein), CH02172 (encoding a membrane protein), CH03254 (encoding a membrane protein), and CH03453 (encoding a conserved protein) in *R. etli*, corresponding to SMc03873, SMb20094, SMc00371, SMc00885, SMc20879, SMc00063, SMb20454, and SMb21454 in *S. meliloti*, respectively. On the other hand, in *S. enterica* and *E. coli*, the *csbD*-like homolog is stress induced (1) and regulated by *rpoS* (55, 56). Furthermore, in *E. coli* the CH00268 homolog, encoding the YciF protein of unknown function, is *rpoS* dependent (29, 30). These results, along with the gene expression data, suggest that *rpoE4* plays an important role in *R. etli* during the stationary growth phase.

In conclusion, *rpoE4* in *R. etli* is an important general regulator that is involved in the responses to saline and osmotic and oxidative stresses, and it also has a relevant role in cell envelope biogenesis. Moreover, a bioinformatics analysis of the *R. etli* genome using the Regulatory Sequence Analysis Tools (http://embnet.ccg.unam.mx/rsa-tools/; 54) suggests that RpoE4 may regulate an additional approximately 140 genes (see Table S2 in the supplemental material).

ACKNOWLEDGMENTS

We thank Laura Cervantes and José Espíritu for their skillful technical support and B. Valderrama for helpful comments on the manuscript.

Partial financial support was from grant no. IN220307 and IN201006 from PAPIIT-DGAPA Universidad Nacional Autónoma de México.

REFERENCES

1. **Akbar, S., S. Lee, S. Boylan, and C. Price.** 1999. Two genes from *Bacillus subtilis* under the sole control of the general stress transcription factor sigma B. Microbiology **145:**1069–1078.
2. **Alba, B. M., and C. A. Gross.** 2004. Regulation of the *Escherichia coli* σ^E-dependent envelope stress response. Mol. Microbiol. **52:**613–619.
3. **Alvarez-Martinez, C. E., R. L. Baldini, and S. L. Gomes.** 2006. A *Caulobacter crescentus* extracytoplasmic function sigma factor mediating the response to oxidative stress in stationary phase. J. Bacteriol. **188:**1835–1846.
4. **Alvarez-Martinez, C. E., R. F. Lourenço, R. L. Baldini, M. T. Laub, and S. L. Gomes.** 2007. The ECF sigma factor σ^T is involved in osmotic and oxidative stress responses in *Caulobacter crescentus*. Mol. Microbiol. **66:**1240–1255.
5. **Anthony, J., K. L. Warczak, and T. J. Donohue.** 2005. A transcriptional response to singlet oxygen, a toxic byproduct of photosynthesis. Proc. Natl. Acad. Sci. USA **102:**6502–6507.
6. **Bang, I.-S., J. G. Frye, M. McClelland, J. Velayudhan, and F. C. Fang.** 2005. Alternative sigma factor interactions in *Salmonella*: σ^E and σ^H promote antioxidant defences by enhancing σ^S levels. Mol. Microbiol. **56:**811–823.
7. **Braatsch, S., O. V. Moskvin, G. Klug, and M. Gomelsky.** 2004. Responses of the *Rhodobacter sphaeroides* transcriptome to blue light under semiaerobic conditions. J. Bacteriol. **186:**7726–7735.
8. **Browning, F. B., and S. J. Busby.** 2004. The regulation of bacterial transcription initiation. Nat. Rev. Microbiol. **2:**1–9.
9. **Campbell, E. A., R. Greenwell, J. R. Anthony, S. Wang, L. Lim, K. Das, H. J. Sofia, T. J. Donohue, and S. A. Darst.** 2007. A conserved structural module regulates transcriptional responses to diverse stress signals in Bacteria. Mol. Cell **27:**793–805.
10. **Crooks G.E., G. Hon, J. M. Chandonia, and S. E. Brenner.** 2004. WebLogo: a sequence logo generator. Genome Res. **14:**1188–1190.
11. **Davis, B. W., and G. C. Walker.** 2007. Identification of novel *Sinorhizobium meliloti* mutants compromised for oxidative stress protection and symbiosis. J. Bacteriol. **189:**2110–2113.
12. **De Las Peñas, A., L. Connolly, and C. A. Gross.** 1997. σ^E is an essential sigma factor in *Escherichia coli*. J. Bacteriol. **179:**6862–6864.
13. **Delory, M., R. Hallez, J. J. Letesson, and X. De Bolle.** 2006. An RpoH-like heat shock sigma factor is involved in stress response and virulence in *Brucella melitensis* 16M. J. Bacteriol. **188:**7707–7710.
14. **Dombrecht, B., J. Vanderleyden, and J. Michiels.** 2001. Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in gram-negative bacteria. Mol. Plant-Microbe Interact. **14:**426–430.
15. **Domínguez-Ferreras, A., R. Pérez-Arnedo, A. Becker, J. Olivares, M. J. Soto, and J. Sanjuán.** 2006. Transcriptome profiling reveals the importance of plasmid pSymB for osmoadaptation of *Sinorhizobium meliloti*. J. Bacteriol. **188:**7617–7625.
16. **Fahraeus, G.** 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. **16:**374–381.
17. **Firoved, A. M., J. C. Boucher, and V. Deretic.** 2002. Global genomic analysis of AlgU (σ^E)-dependent promoters (sigmulon) in *Pseudomonas aeruginosa* and implications for inflammatory processes in cystic fibrosis. J. Bacteriol. **184:**1057–1064.
18. **Francez-Charlot, A., J. Frunzke, C. Reichen, J. Z. Ebneter, B. Gourion, and J. A. Vorholt.** 2009. Sigma factor mimicry involved in regulation of general stress response. Proc. Natl. Acad. Sci. USA **106:**3467–3472.
19. **Gage, D. J.** 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol. Mol. Biol. Rev. **68:**280–300.
20. **Girard, L., S. Brown, A. Dávalos, O. López, M. Soberón, and D. Romero.** 2000. Differential regulation of *fixN*-reiterated genes in *Rhizobium etli* by a novel *fixL-fixK* cascade. Mol. Plant-Microbe Interact. **13:**1283–1292.

21. **González, V., R. I. Santamaría, P. Bustos, I. Hernández-González, A. Medrano-Soto, G. Moreno-Hagelsieb, S. Chandra-Janga, M. A. Ramírez, V. Jiménez-Jacinto, J. Collado-Vides, and G. Dávila.** 2005. The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons. Proc. Natl. Acad. Sci. USA **103:**3834–3839.
22. **Gourion, B., A. Francez-Charlot, and J. A. Vorholt.** 2008. PhyR is involved in the general stress response of *Methylobacterium extorquens* AM1. J. Bacteriol. **190:**1027–1035.
23. **Gruber, T. M., and C. A. Gross.** 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. Annu. Rev. Microbiol. **57:**441–466.
24. **Hayden, J. D., and S. E. Ades.** 2008. The extracytoplasmic stress factor, σ^E, is required to maintain cell envelope integrity in *Escherichia coli*. PLoS ONE **3:**e1573.
25. **Hegde, P., R. Qi, K. Abernathy, C. Gay, S. Dharap, R. Gaspard, J. E. Hughes, E. Snesrud, N. Lee, and J. Quackenbush.** 2000. A concise guide to cDNA microarray analysis. BioTechniques **29:**548–562.
26. **Helmann, J. D.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv. Microb. Physiol. **46:**47–110.
27. **Hengge-Aronis, R.** 2002. Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. Microbiol. Mol. Biol. Rev. **66:**373–395.
28. **Hertz, G. Z., and G. D. Stormo.** 1999. Identifying DNA and protein patterns with statistically significant alignments of multiple sequences. Bioinformatics **15:**563–577.
29. **Hindupur, A., D. Liu, Y. Zhao, H. D. Bellamy, M. A. White, and R. O. Fox.** 2006. The crystal structure of the *E. coli* stress protein YciF. Protein Sci. **15:**2605–2611.
30. **Ibanez-Ruiz, M., V. Robbe-Saule, D. Hermant, S. Labrude, and F. Nore.** 2000. Identification of RpoS (σ^S)-regulated genes in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **182:**5749–5756.
31. **Jamet, A., S. Sigaud, G. Van de Sype, A. Puppo, and D. Herouart.** 2003. Expression of the bacterial catalase genes during *Sinorhizobium meliloti-Medicago sativa* symbiosis and their crucial role during the infection process. Mol. Plant-Microbe Interact. **16:**217–225.
32. **Lonetto, M., M. Gribskov, and C. A. Gross.** 1992. The sigma 70 family: sequence conservation and evolutionary relationships. J. Bacteriol. **174:**3843–3849.
33. **Long, S. R.** 2001. Genes and signals in the rhizobium-legume symbiosis. Plant Physiol. **125:**69–72.
34. **Manganelli, R., M. I. Voskuil, G. K. Schoolnik, E. Dubnau, M. Gomez, and I. Smith.** 2002. Role of the extracytoplasmic-function σ factor σ^H in *Mycobacterium tuberculosis* global gene expression. Mol. Microbiol. **45:**365–374.
35. **Martínez-Salazar, J. M., S. Moreno, R. Najera, J. C. Boucher, G. Espín, G. Soberón-Chávez, and V. Deretic.** 1996. Characterization of the genes coding for the putative sigma factor AlgU and its regulators MucA, MucB, MucC, and MucD in *Azotobacter vinelandii* and evaluation of their role in alginate biosynthesis. J. Bacteriol. **178:**1800–1808.
36. **Martinez-Salazar, J. M., and D. Romero.** 2000. Role of the *ruvB* gene in homologous and homeologous recombination in *Rhizobium etli*. Gene **243:**125–131.
37. **Martínez-Salazar, J. M., M. Sandoval-Calderón, X. Guo, S. Castillo-Ramírez, A. Reyes, M. G. Loza, J. Rivera, X. Alvarado-Affantranger, F. Sánchez, V. González, G. Dávila, and M. A. Ramírez-Romero.** 2009. The *Rhizobium etli* RpoH1 and RpoH2 sigma factors are involved in different stress responses. Microbiology **155:**386–397.
38. **Martínez-Salazar, J. M., J. Zuñiga-Castillo, and D. Romero.** 2009. Differential roles of proteins involved in migration of Holliday junctions on recombination and tolerance to DNA damaging agents in *Rhizobium etli*. Gene **432:**26–32.
39. **Mascher, T., A.-B. Hachmann, and J. D. Helmann.** 2007. Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function factors. J. Bacteriol. **189:**6919–6927.
40. **Noel, K. D., A. Sánchez, L. Fernández, J. Leemans, and M. A. Cevallos.** 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn*5* insertions. J. Bacteriol. **158:**148–155.
41. **Paget, M. S., V. Molle, G. Cohen, Y. Aharonowitz, and M. J. Buttner.** 2001. Defining the disulphide stress response in *Streptomyces coelicolor* A3(2): identification of the sigma R regulon. Mol. Microbiol. **42:**1007–1020.
42. **Pichon, M., E. P. Journet, A. Dedieu, F. de Billy, G. Truchet, and D. G. Barker.** 1992. *Rhizobium meliloti* elicits transient expression of the early nodulin gene ENOD12 in the differentiating root epidermis of transgenic alfalfa. Plant Cell **4:**1199–1211.
43. **Rhodius, V. A., W. Suh, G. Nonaka, J. West, and C. A. Gross.** 2006. Conserved and variable functions of the σ^E stress response in related genomes. PLoS Biol. **4:**43–59.
44. **Rowley, G., M. Spector, J. Kormanec, and M. Roberts.** 2006. Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. Nat. Rev. Microbiol. **4:**383–394.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
46. **Santos, R., D. Herouart, A. Puppo, and D. Touati.** 2000. Critical protective role of bacterial superoxide dismutase in rhizobium-legume symbiosis. Mol. Microbiol. **38:**750–759.
47. **Santos, R., D. Herouart, S. Sigaud, D. Touati, and A. Puppo.** 2001. Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. Mol. Plant-Microbe Interact. **14:**86–89.
48. **Sauviac, L., H. Philippe, K. Phok, and C. Bruand.** 2007. An extracytoplasmic function sigma factor acts as a general stress response regulator in *Sinorhizobium meliloti*. J. Bacteriol. **189:**4204–4216.
49. **Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Puhler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
50. **Schurr, M. J., H. Yu, J. M. Martinez-Salazar, J. C. Boucher, and V. Deretic.** 1996. Control of AlgU, a member of the σ^E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J. Bacteriol. **178:**4997–5004.
51. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Biotechnology **1:**784–791.
52. **Soupène, E., M. Foussard, P. Boistard, G. Truchet, and J. Batut.** 1995. Oxygen as a key developmental regulator of *Rhizobium meliloti* N2-fixation gene expression within the alfalfa root nodule. Proc. Natl. Acad. Sci. USA **92:**3759–3763.
53. **Thorne, S. H., and H. D. Williams.** 1997. Adaptation to nutrient starvation in *Rhizobium leguminosarum* bv. phaseoli: analysis of survival, stress resistance, and changes in macromolecular synthesis during entry to and exit from stationary phase. J. Bacteriol. **179:**6894–6901.
54. **van Helden, J.** 2003. Regulatory sequence analysis tools. Nucleic Acids Res. **31:**3593–3596.
55. **Weber, A., S. A. Kogl, and K. Jung.** 2006. Time-dependent proteome alterations under osmotic stress during aerobic and anaerobic growth in *Escherichia coli*. J. Bacteriol. **188:**7165–7175.
56. **Yamashino, T., M. Kakeda, C. Ueguchi, and T. Mizuno.** 1994. An analogue of the DnaJ molecular chaperone whose expression is controlled by σ^S during the stationary phase and phosphate starvation in *Escherichia coli*. Mol. Microbiol. **13:**475–483.